

RESPONSE OF ONE-CARBON BIOMARKERS IN MATERNAL AND CORD BLOOD TO
FOLIC ACID DOSE DURING PREGNANCY

by

JENNIFER MARIE FLEMING

(Under the Direction of Hea Jin Park)

ABSTRACT

This study is a randomized controlled intervention trial investigating the response of unmetabolized folic acid and biomarkers of folate and one-carbon metabolism in maternal and cord blood to 400 μ g/d or 800 μ g/d folic acid (FA) supplementation during gestation. FA dose did not influence maternal folate status, while cord blood from the 800 μ g/d FA group contained higher concentrations of serum folate than cord blood from the 400 μ g/d group. FA dose affected unmetabolized folic acid concentrations in maternal but not cord blood, suggesting the presence of a protective mechanism in the placenta. Cord blood plasma choline concentrations from the 800 μ g/d FA group were higher than from the 400 μ g/d FA group, suggesting that a higher dose of maternal FA may spare choline in cord blood to be utilized for other fetal functions. These results indicate that FA dose during pregnancy has distinct effects on folate and one-carbon biomarkers in maternal and cord blood.

INDEX WORDS: Pregnancy, Folate, Folic acid, Unmetabolized folic acid, Oxidized folic acid, Serum folate, RBC folate, Choline, Cord blood, Folic acid supplementation

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TABLE OF CONTENTS

	Page
ACKNOWLEDGEMENTS	iv
LIST OF TABLES	vii
LIST OF FIGURES	ix
CHAPTER	
1 INTRODUCTION	1
References	7
2 LITERATURE REVIEW	9
Folate chemistry and metabolism	9
Folate bioavailability	10
Folate in pregnancy	11
Folate fortification	12
Folate recommendations and intake	13
Folic acid supplementation	14
Oxidized folic acid	14
Concerns with excess folic acid intake	16
Role of the placenta	18
Analysis of oxidized folic acid	19
Summary	20
References	21

3	RESPONSE OF ONE-CARBON BIOMARKERS TO FOLIC ACID DOSE	
	DURING PREGNANCY IN MATERNAL AND CORD BLOOD.....	26
	Abstract.....	27
	Introduction.....	28
	Methods.....	30
	Results.....	36
	Discussion.....	41
	Conclusion	48
	Acknowledgements.....	48
	Results tables and figures.....	49
	References.....	64
4	SUMMARY AND CONCLUSIONS	67
	References.....	72
	APPENDICES	74
	A RECRUITMENT LETTER (ENGLISH).....	75
	B RECRUITMENT LETTER (SPANISH)	77
	C CONSENT FORM (ENGLISH)	79
	D CONSENT FORM (SPANISH).....	86
	E HEALTH BEHAVIOR QUESTIONNAIRE (ENGLISH)	93
	F HEALTH BEHAVIOR QUESTIONNAIRE (SPANISH)	97
	G THREE DAY DIETARY RECALL FORM (ENGLISH).....	101
	H THREE DAY DIETARY RECALL FORM (SPANISH)	106
	I ASA-24 INSTRUCTIONS.....	111

J	BLOOD SAMPLE COLLECTION FORM.....	113
K	INITIAL CHECKLIST	115
L	MONTHLY CHECKLIST	117
M	28 WEEK CHECKLIST	119
N	36 WEEK CHECKLIST	121
O	DELIVERY CHECKLIST.....	123

LIST OF TABLES

	Page
Table 1: Demographic and clinical characteristics of mothers.....	51
Table 2: Clinical characteristics of infants.....	52
Table 3: Mixed effects models for serum folate.....	54
Table 4: Mixed effects models for 5MTHF.....	55
Table 5: Mixed effects models for RBC folate.....	56
Table 6: Mixed effects models for folic acid.....	58
Table 7: Mixed effects models for choline.....	60
Table 8: Mixed effects models for betaine.....	61
Table 9: Mixed effects models for DMG.....	62
Table 10: Mixed effects models for TMAO.....	63

LIST OF FIGURES

	Page
Figure 1: Study timeline.....	49
Figure 2: Participant flow	50
Figure 3: Serum folate, RBC folate, and 5MTHF concentration in maternal and cord blood in response to 400 or 800 µg/d folic acid supplementation during pregnancy	53
Figure 4: Unmetabolized folic acid concentration in maternal and cord blood in response to 400 or 800 µg/d folic acid supplementation during pregnancy	57
Figure 5: Choline, betaine, DMG and TMAO concentration in maternal and cord blood in response to 400 or 800 µg/d folic acid supplementation during pregnancy	59

CHAPTER 1

INTRODUCTION

Folate is a water soluble B vitamin that exists as naturally occurring folate found in foods such as leafy green vegetables, legumes, beans, and orange juice. It also exists as the synthetic form, folic acid, which is the form found in fortified foods and dietary supplements. Naturally occurring folate is in the reduced form while folic acid is the fully oxidized form. The majority of dietary folates are hydrolyzed in the gut before being transported across the intestinal mucosa (Zhao et al 2009). The circulating form of folate in plasma is primarily 5-methyl-tetrahydrofolate (THF). Once THF is delivered by the blood to non-hepatic tissues, it must be metabolized to polyglutamate derivatives in order to be retained by the cells.

THF carries out metabolism in the cytoplasm, mitochondria, and nucleus. Within these compartments, THF polyglutamates function as coenzymes which transfer one-carbons by accepting and donating them; these reactions are known as folate-mediated one carbon metabolism (Stover 2010). Folate-dependent pathways function interdependently in separate intracellular compartments. Major functions of these folate pathways include de novo synthesis of purines, thymidylate, and the remethylation of homocysteine to methionine.

Because of folate's key role in one-carbon metabolism needed for DNA and protein synthesis, the body's requirement for folate increases when there is an increase in cellular division (Tamura and Picciano 2006). During pregnancy, there is a dramatic increase in cellular reproduction associated with the growth of both maternal and fetal tissues, and folate needs are therefore increased during this period of rapid cell proliferation. Failing to consume adequate

amounts of folate from the diet and supplements can have serious negative consequences, including developmental disorders such as neural tube defects (NTDs, Cunningham et al 1993).

Because of the established preventative effects of folate against NTDs, in 1996 the Food and Drug Administration mandated folic acid fortification of all enriched cereal grain products by January 1, 1998 (FDA 1996). Folic acid fortification in the United States has increased overall dietary intake of folic acid, increased blood folate concentrations, and reduced the prevalence of NTD-affected births (Williams et al 2002). Additionally, NHANES data from pre- and post-fortification indicate an overall increase of 7.5 ng/mL in median serum folate concentrations after fortification (Pfeiffer et al 2007). The mean concentration of red blood cell (RBC) folate increased from 165.5 ng/mL to 260.4 ng/mL with fortification, a 94.9 ng/mL difference (Dietrich et al 2005).

To reduce risk for NTDs, the recommendation for women capable of becoming pregnant is to consume 400 µg of folic acid in addition to normal dietary intake. To meet increased demands during pregnancy, the RDA (Recommended Daily Allowance) for pregnant women is to consume 600 µg DFE per day. 1 µg DFE is equal to 1 µg of food folate or approximately 0.6 µg of folic acid as folic acid has a greater bioavailability than food folate (Institute of Medicine 1998). Folic acid supplementation is often encouraged for pregnant women in order to ensure that they are consuming adequate folate. Although the recommendation for pregnant women is to consume 400 µg of folic acid per day, the folic acid content of most over the counter prenatal supplements is at least twice that, ranging from 800-1000 µg or higher. It is estimated that 75% of pregnant women use a folic acid containing supplement, and pregnant women who supplement have a median intake of 846 µg of folic acid per day (Kauwell et al 2010).

Folic acid from fortified foods and supplements is the synthetic form of folate and is in an oxidized state. It must be reduced in order to function as a coenzyme throughout the body (Smith et al 2008). Oxidized folic acid, also referred to as unmetabolized or nonmetabolized folic acid, is folic acid that has not been reduced to the active form. At high levels of intake the body's ability to reduce synthetic folic acid to the reduced form may be exceeded, leading to detectable circulating levels of oxidized folic acid (Kelly et al 1997). Data from the 2007-2008 NHANES study found detectable concentrations of oxidized folic acid present in >95% of all samples, regardless of demographics, fasting status, or supplement use, although taking a folic acid containing supplement was a significant predictor of having elevated oxidized folic acid concentrations (Pfeiffer et al 2015). Additionally, oxidized folic acid is detectable in many cord blood samples, although research in this area is limited (Obeid et al 2010; Sweeney et al 2005).

With the presence of circulating oxidized folic acid observed in almost all of the United States population, there is rising concern that there are potential harmful, but not yet fully understood, consequences associated with excess intakes of folic acid. Maternal nutrition during gestation has been shown to play a role in determining long term metabolism of the offspring, a phenomenon called fetal programming (Chmurzysnka 2010). Because of folate's function as a one-carbon donor and its involvement in DNA methylation reactions, maternal folate intake has the potential to affect long term outcomes in the offspring including insulin resistance, fat accumulation, glycemic control, and cardiovascular health. Negative outcomes from high folic acid supplementation have been shown in an animal model, including excess weight gain, impaired glycemic control, and decreased DNA methylation (Hoyo et al 2011); however, the current research on long-term outcomes of folic acid supplementation and the presence of

oxidized folic acid in humans is mixed, and there is a need for more, larger-scale studies in order to draw any definitive conclusions.

As pregnant women consume nutrients including folic acid, these nutrients are transferred to the fetus via the placenta. The placental barrier that limits and regulates nutrient transfer is called the syncytiotrophoblast; it contains two polarized plasma membranes, one that is maternal-oriented and one that is fetal-oriented. These membranes contain nutrient transporters which can be regulated by placental, fetal and maternal signals; however, the mechanisms by which these placental nutrient transporters are regulated is not fully understood (Lager and Powell 2012).

Studies in both humans and animals indicate the possibility of a protective mechanism that prevents the fetus from receiving toxic amounts of certain vitamins, even in the presence of high maternal intakes and serum concentrations. Human studies have found that the expression of placental receptors for certain vitamins was related to neonatal concentrations of the nutrient but not to maternal concentrations (Young et al 2014). Animal studies have also indicated the possibility of a regulatory mechanism in the placenta, where the embryo of dams that took in excessive amounts of a certain vitamin had the same or lower concentrations as embryos of dams receiving an adequate amount that was over ten times less (Costabile et al 2014). Previous studies using lower doses of folic acid have not found differences in cord blood concentrations of unmetabolized folic acid from mothers who supplement than those whose mothers did not (Obeid et al 2010; Pfeiffer et al 2015). Research in this area however is limited and thus far inconclusive

The overall purpose of this study is fill in knowledge gaps through the investigation of whether folic acid dose during pregnancy correlates with circulating concentrations of oxidized

folic acid in pregnant women, and whether this is translated to the infant. The research question asks if there is a difference in maternal concentrations and cord blood concentrations of oxidized folic acid in women exposed to either 400 µg or 800 µg per day of folic acid throughout pregnancy. The first specific aim is to assess the differences in maternal oxidized folic acid concentrations in women exposed to 400 µg or 800 µg of folic acid. It is hypothesized that maternal concentrations will be higher in the group exposed to the higher dose of folic acid (800 µg). Although a previous study comparing no folic acid supplementation and a 400 µg per day dose found no differences in maternal concentrations, a recent analysis of NHANES data from 2007-2008 found that folic acid supplement users had higher concentrations of oxidized folic acid than non-users (Obeid et al 2010; Pfeiffer et al 2015). Because of the higher doses of folic acid used in this study, the current study is expected to indicate this difference as well. The second specific aim is to assess differences in cord blood oxidized folic acid status between infants exposed to 400 µg or 800 µg of folic acid per day throughout pregnancy. It is hypothesized that there will be no difference in cord blood concentrations. This is based on a previous study which found no difference in cord blood oxidized folic acid status between infants whose mothers did and did not supplement during pregnancy (Obeid et al 2010). Additionally, studies that have investigated the transport of other nutrients and vitamins to the fetus have found that maternal intake and serum concentrations are not always correlated to fetal concentrations, indicating the possibility of a protective mechanism present in the placenta (Young et al 2014; Costabile et al 2014). The third specific aim is to investigate differences in other one-carbon metabolites involved in the folate cycle in both maternal and cord blood exposed to 400 µg or 800 µg of folic acid per day throughout pregnancy. This study is significant because the identification of a correlation between the amount of folic acid supplemented and

concentrations of oxidized folic acid in pregnant women and their infants will contribute to the evidence base for establishing future folic acid recommendations.

Following this chapter, Chapter 2 is a review of the literature on folate and unmetabolized folic acid. Topics covered in the literature review include folate chemistry and metabolism, folate bioavailability, folate in pregnancy, folate fortification, folate recommendations and intake, folic acid supplementation, oxidized folic acid, concerns with excess folic acid, the role of the placenta, and analysis of oxidized folic acid. Chapter 3 is a manuscript on the response of unmetabolized folic acid and other folate and one-carbon metabolism biomarkers in mothers and cord blood to folic acid supplementation dose during pregnancy. Chapter 4 contains a summary of this thesis and conclusions about the study.

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CHAPTER 2

LITERATURE REVIEW

Folate chemistry and metabolism

Folate is a water soluble B vitamin that exists as naturally occurring folate found in food as well as the synthetic form, folic acid. Folates are made up of a pteridine bicyclic ring system, *p*-Aminobenzoic acid, and one or more glutamate residues (Shane 2010). Naturally occurring folate is in the reduced polyglutamate form while folic acid is the fully oxidized monoglutamate form.

In order to be in its bioactive form, folic acid must be reduced. The pyrazine ring of the pterin moiety is reduced to the tetrahydro form, which is the coenzymatically active form. Additionally, the glutamate chain is elongated by the addition of glutamate residues and one-carbon units are acquired and either oxidized or reduced at the N-5 and/or N-10 positions.

The majority of dietary folates are polyglutamate derivatives which are hydrolyzed in the gut to monoglutamate forms before being transported across the intestinal mucosa (Zhao et al 2009). The circulating forms of folate in plasma are pteroylmonoglutamates, primarily 5-methyl-tetrahydrofolate (THF). Once THF is delivered by the blood to non-hepatic tissues, it must be metabolized to polyglutamate derivatives in order to be retained by the cells.

THF carries out metabolism in the cytoplasm, mitochondria, and nucleus. Within these compartments, THF polyglutamates function as coenzymes which transfer one-carbons by accepting and donating them from the N-5 and/or N-10 position; these reactions are known as

folate-mediated one carbon metabolism (Stover 2010). Folate-dependent pathways function interdependently in separate intracellular compartments. Major functions of these folate pathways include de novo synthesis of purines, thymidylate, and the remethylation of homocysteine to methionine. Methionine, an essential amino acid, is the precursor of S-adenosylmethionine (SAM), a cofactor and methyl donor for a number of methylation reactions including the methylation of DNA, RNA, and other molecules (Stover 2010).

Folate shares its methylation pathway with choline. Choline is oxidized to betaine, which along with folate can also donate methyl groups used to remethylate homocysteine to methionine (Jacob et al 1999). Choline is used to methylate this reaction when folate availability is limited, causing choline concentrations to be depleted as well (Kim et al 1994). These compounds are all therefore interdependent.

Folate Bioavailability

Folic acid is more resistant to degradation, more stable, and more bioavailable than folate from food (Coleman et al 1987). Bioavailability refers to the proportion of an ingested nutrient that is absorbed and becomes available for metabolic processes or storage (McNulty and Pentiva 2009). Many factors can influence the bioavailability of food folate, including intestinal hydrolysis of polyglutamyl folates, the extent of conjugation of food folate sources, folate absorption in the small and large intestine, and the food that the folate is contained in.

According to the Institute of Medicine, folic acid is more bioavailable than food folate by a factor of 1.7 (Institute of Medicine 1998). In order to account for differences in bioavailability, the dietary reference intake (DRI) uses the unit of dietary folate equivalents (DFE). DFE is the quantity of natural food folate plus 1.7 times the quantity of folic acid in the diet.

Folate in pregnancy

Because of folate's key role in one-carbon metabolism needed for DNA and protein synthesis, the body's requirement for folate increases when there is an increase in cellular division (Tamura and Picciano 2006). During pregnancy, there is a dramatic increase in cellular reproduction associated with the growth of both maternal and fetal tissues, and folate needs are therefore increased during this period of rapid cell proliferation (Cunningham et al 1993). Along with tissue growth, there is also a marked increase in blood volume during pregnancy in order to supply the developing fetus (Tamura and Picciano 2006). Failing to consume adequate amounts of folate from the diet and supplements can have serious negative consequences, including developmental disorders such as neural tube defects (NTDs, Cunningham et al 1993).

NTDs occur when the neural tube fails to close during the first stages of development. The two most common forms of NTDs are spina bifida and anencephaly, wherein the neural tube does not fully close at either the base of the spinal cord or at the brain, respectively (Mitchell 2005). According to birth certificates prior to 1998, the prevalence of NTDs in the United States was approximately 37.8 instances per 100,000 live births (Honein et al 2001). There are a variety of factors that have been identified as increasing the risk for NTDs; these include low blood concentrations of folate as well as obesity and the methylenetetrahydrofolate reductase (MTHFR) 677C→T polymorphism (McMahon et al 2013; Davis et al 2005). MTHFR is an enzyme in the folate metabolic pathway that catalyzes the reduction of methylene-tetrahydrofolate to methyl-tetrahydrofolate. When the C→T polymorphism is present in the gene which codes for MTHFR, its stability and activity are impaired (Weisberg et al 1998). Many studies have shown that women with higher concentrations of blood folate have considerably

lower incidence of NTDs than those with lower concentrations (MRC Vitamin Study Research Group 1991).

Folate fortification

Fortification, which is the process of adding micronutrients to particular foods, serves as a measure to increase the intake of those micronutrients among a population in order to prevent deficiencies and minimize their associated health risks (WHO 2006; Samaniego-Vaesken 2012). Fortification has been used in many countries throughout the world and has proven to be a cost-effective method of improving nutrition status and reducing the incidence of deficiency-related complications in populations (Mannar and Sankar 2004).

By the early 1990s it had been established that consuming adequate amounts of folic acid periconceptually greatly reduces the risk for having a child with an NTD, and in 1991-92 the Centers for Disease Control and Prevention (CDC) and United States Public Health Service (USPHS) issued the first recommendations for folic acid intake for women of childbearing age – to consume 400 µg /d of folic acid (CDC 1991; CDC 1992). In 1996, the Food and Drug Administration mandated folic acid fortification of all enriched cereal grain products by January 1, 1998 in order to increase folic acid intake in women capable of becoming pregnant and assist in the prevention of NTDs (FDA 1996). Accordingly, enriched cereal grain products, including flour, rice, breads, rolls, pasta, corn grits, corn meal, macaroni, and noodles processed in the US are fortified with 140 µg of folic acid per 100g of flour.

Folic acid fortification in the United States has increased overall dietary intake of folic acid by 100-150 µg/day, increased blood folate concentrations, and reduced the prevalence of NTD-affected births (Berry et al 2010). When compared to NTD prevalence pre-fortification

(1995-1996), there was a 26% decrease in all NTDs and a 31% reduction in spina bifida post-fortification (Williams et al 2002). Along with a reduction in NTD prevalence, fortification increased blood folate concentrations in the population. NHANES data from pre- and post-fortification indicate an overall increase of 7.5 ng/mL in median serum folate concentrations after fortification (Pfeiffer et al 2007). The mean concentration of red blood cell (RBC) folate increased from 165.5 ng/mL to 260.4 ng/mL with fortification, a 94.9 ng/mL difference (Dietrich et al 2005).

Folate recommendations and intake

For women ages 19-50, the RDA (Recommended Daily Allowance) for folate is 400 µg per day DFE; 1 µg DFE is equal to 1 µg of food folate or approximately 0.6 µg of folic acid (Institute of Medicine 1998). To reduce risk for NTDs, the recommendation for women capable of becoming pregnant is to consume 400 µg of folic acid in addition to normal dietary intake. To meet increased demands during pregnancy, the RDA for pregnant women is to consume 600 µg DFE per day

According to The National Health and Nutrition Examination Survey (NHANES) data from 2003-2006, women aged 19-30 consumed a mean total folate of 645 µg DFEs and those 31-50 consumed 714 µg DFEs (Bailey et al 2010). 17% of 19-30 year olds and 14.5% of 31-50 year olds were consuming below the EAR (Estimated Average Requirement) of total folate. The median usual folic acid intake per day for women ages 15-44 years old was 245 µg, with the highest intakes among 25-34 year old women, and 23.8% of women ages 15-44 were consuming at least 400 µg of folic acid per day as recommended (Tinker et al 2010). Those taking a

supplement containing folic acid had a median intake of 502 µg folic acid per day, while those who were not taking a supplement averaged only 163 µg per day.

Folic acid supplementation

NHANES data indicates that 35% of adults in the United States ages 19 years and older use a supplement that contains folic acid, and median total folic acid intake among supplement users is at least 2.5 times higher than in non-users (Bailey et al 2010). Additionally, it is estimated that 75% of pregnant women use a folic acid containing supplement, and pregnant women who supplement have a median intake of 846 µg of folic acid per day as compared to 216 µg in those who do not supplement (Kauwell et al 2010).

The Tolerable Upper Intake Level (UL) for adults ages 19 and older, including pregnant women, is 1000 µg of folic acid per day (Institute of Medicine 1998). An estimated 2-3% of 19-50 year olds and 5% of 51-70 year olds have intakes of folic acid above the UL, with the majority of their folic acid coming from supplements (Bailey et al 2010). Although the recommendation for pregnant women is to consume 400 µg of folic acid per day, the folic acid content of most over the counter prenatal supplements is at least twice that, ranging from 800-1000 µg or higher.

Oxidized folic acid

Folic acid is the synthetic form of folate and is in an oxidized state; therefore, it must be reduced in order to function as a coenzyme throughout the body (Smith et al 2008). Oxidized folic acid, also referred to as unmetabolized or nonmetabolized folic acid, is folic acid that has not been reduced to the active form.

Several epidemiological studies conducted post-fortification report detectable circulating concentrations of oxidized folic acid. Among men and women ages 29-86 from the Framingham Offspring Cohort that did not take B vitamin supplements, 74.7% had detectable oxidized folic acid after food fortification, and 80.7% of those who did supplement with B vitamins had detectable amounts (Kalmbach et al 2008). NHANES data from 2001-2002 indicate 40% of adults 60 years of age and older had oxidized folic acid present in their serum after a fast (Bailey et al 2010). Oxidized folic acid concentrations in serum were also reported from the 2007-2008 NHANES study with oxidized folic acid found in >95% of all samples regardless of demographics, fasting status, or supplement use (Pfeiffer et al 2015). Additionally, 33% of the samples had oxidized folic acid concentrations >1 nmol/L, 14% had >2 nmol/L, and 7% had >5 nmol/L. With each 200 µg increase in total folic acid intake per day, individuals were 1.5 times more likely to have oxidized folic acid concentrations >1 nmol/L. Taking a folic acid containing supplement was also a significant predictor of having elevated oxidized folic acid concentrations.

In a study of 87 pregnant women, 38 had detectable concentrations (≥ 0.20 nmol/L) of oxidized folic acid in their serum (Obeid et al 2010). In the same study, among 29 cord blood samples 55% had detectable amounts of oxidized folic acid present. In another study, oxidized folic acid was present in the cord blood from all 11 births, and in a follow up at 4 days after birth 7 out of 9 samples indicated circulating oxidized folic acid (Sweeney et al 2005). Although the implications of circulating oxidized folic acid are not fully understood, population studies as well as studies specifically on pregnant women indicate its prevalence.

Concerns with excess folic acid intake

With the presence of oxidized folic acid observed in almost all of the United States population, there is rising concern that there are potential harmful, but not yet fully understood, consequences associated with excess intakes of folic acid. Possible negative outcomes for both the general population as well as the offspring of women with high amounts are being explored.

Maternal nutrition during gestation has been shown to play a role in determining metabolism of the offspring, a phenomenon called fetal programming (Chmurzysnka 2010). Because of folate's function as a one-carbon donor and its involvement in DNA methylation reactions, maternal folate intake has the potential to affect long-term outcomes in the offspring including insulin resistance, fat accumulation, glycemic control, and cardiovascular health. Keating et al. explored this concept using an animal model (Keating et al 2015). Female Sprague-Dawley rats were fed either a diet with adequate folic acid for pregnancy (2 mg FA/kg of diet) or a high folic acid diet (40 mg FA/kg of diet) throughout mating, pregnancy, and lactation. The mothers fed the high folic acid diet had increased weight gain and food intake throughout the 13-month study. Female offspring of the high folic acid supplemented dams were found to have increased birth weight, heart weight, weight gain, and food intake, as well as disturbed feeding patterns and impaired glycemic control; these outcomes were not seen in male offspring. In addition, female offspring of the high folic acid dams had higher insulin and lower adiponectin levels than the control group while male offspring had higher levels of leptin. When metabolic syndrome was induced in the offspring, female offspring of the high folic acid dams had increased fat mass. In humans, the offspring of women who supplemented with folic acid were found to have decreased DNA methylation at two regions in which aberrant methylation is associated with deregulation of Insulin-like Growth Factor 2 (IGF2) and increased susceptibility

to chronic disease; this effect was more pronounced in male offspring than female (Hoyo et al 2011). These studies give compelling evidence that high maternal folic acid intake may have sex-specific fetal programming effects.

In a prospective cohort study done in Australia, folic acid supplementation in late pregnancy was associated with an increased risk of asthma in offspring at 3.5 years as well as persistent asthma (Whitrow et al 2009). The Norwegian Mother and Child Cohort Study found that children of women who supplemented with folic acid during pregnancy were at higher risk for wheeze and lower respiratory tract infections compared to those whose mothers did not supplement (Haberg et al 2009). Additionally, another study showed that infants exposed to $>500 \mu\text{g}$ of folic acid/day as a supplement in utero were more likely to develop eczema than those exposed to $<200 \mu\text{g/day}$ (Dunstan et al 2012).

The relationship between folate and cancer remains unclear, but it has been suggested that folate plays a dual role in cancer by potentially suppressing tumor development and progression if taken prior to initiation but enhancing tumor growth if taken afterward (Smith et al 2008). One study done in female rats found that both maternal and post-weaning folic acid supplementation increased risk of mammary adenocarcinomas in the offspring (Ly et al 2010). In a large cohort study of post-menopausal women, supplemental folic acid intake $\geq 400 \mu\text{g/day}$ was significantly associated with risk for developing breast cancer, whereas food folate was not (Stolzenberg-Solomon et al 2006). The relationship between folic acid and colorectal cancer (CRC) has been closely studied as both the US and Canada had reversals of the downward trend of incidence concurrent with the mandate of folic acid fortification (Kim 2004). However, research in this area remains inconclusive as some studies indicate a possible protective effect of

folic acid on CRC while others indicate it may contribute to the progression of CRC (Mason et al 2007; Cole et al 2007).

Another study done in postmenopausal women found that there was an inverse relationship between the presence of oxidized folic acid in plasma and natural killer cell cytotoxicity, which is an index of immune function (Troen et al 2006). This relationship became more pronounced with higher oxidized folic acid concentrations.

The current research on long-term outcomes of folic acid supplementation and the presence of oxidized folic acid is mixed, and there is a need for more, larger-scale studies in order to draw any definitive conclusions. However, based on the current evidence base suggesting the possibility of a variety of deleterious consequences, it is worth considering that high levels of folic acid supplementation may not be completely harmless.

Role of the Placenta

As pregnant women consume nutrients including folic acid, these nutrients are transferred to the fetus via the placenta. The placental barrier that limits and regulates nutrient transfer is called the syncytiotrophoblast. It contains two polarized plasma membranes, one that is maternal-oriented and one that is fetal-oriented. These membranes contain nutrient transporters which can be regulated by placental, fetal and maternal signals. The mechanisms by which these placental proteins are regulated is not fully understood (Lager and Powell 2012).

Studies have been done in both humans and animals that suggest the presence of a mechanism that prevents the fetus from receiving toxic amounts of certain vitamins, regardless of maternal concentrations. One study in humans found that the expression of placental receptors for certain vitamins was related to neonatal concentrations of the nutrient but not maternal

concentrations (Young et al 2014). Animal studies have also indicated the possibility of a regulatory mechanism in the placenta. One study found that the embryos of dams that consumed excessive amounts of Vitamin D had the same or lower concentrations of Vitamin D as embryos from dams that took in an adequate amount that was over ten times less (Costabile et al 2014). In a study done in human placetas, two placental cell lines and placenta tissue explants were exposed to increasing amounts of folic acid. They found that the primary indicators of placental health were compromised during conditions of folate deificiency but not with excess folic acid (Ahmed et al 2016). To our knowledge, however, research is limited in respect to this mechanism in regards to unmetabolized folic acid.

Analysis of oxidized folic acid

Liquid Chromatography-Mass Spectrometry and Liquid Chromatography-Tandem Mass Spectrometry (LC-MS/MS) stable isotope dilution methods are a validated measure for quantifying folate forms in serum, including 5-methyltetrahydrofolate, oxidized folic acid, and 5-formyltetrahydrofolic acid (Pfeiffer et al 2004). This methodology is more sensitive than previous methods used to determine serum folate concentrations, requiring only 275 μ L of serum to quantify all three folate forms.

The limit of detection for oxidized folic acid using LC-MS/MS is 0.07 nmol/L, allowing for accurate detection even in individuals who are folate deficient (West et al 2012). When compared with previous methods used to measure folate, stable-isotope-dilution LC-MS/MS is highly specific and can accurately determine three folate forms. This methodology has been used to quantify oxidized folic acid concentrations in previous studies, including serum measurements in pregnant women and cord blood (Obeid et al 2010; West et al 2012).

Summary

A review of the current literature has revealed a large amount of research and knowledge concerning folate and oxidized folic acid. Areas of research relevant to folate include chemistry and metabolism, bioavailability, folate in pregnancy, fortification, recommendations and intake. Relevant research on oxidized folic acid includes folic acid supplementation, prevalence of oxidized folic acid in serum, potential negative outcomes of circulating oxidized folic acid, the role of the placenta in nutrient transfer, and the analysis of oxidized folic acid concentrations. Gaps in the current research include the effect of supplemental folic acid dose on oxidized folic acid concentrations in mothers and their offspring as well as its effects on other one-carbon biomarkers.

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CHAPTER 3
RESPONSE OF ONE-CARBON BIOMARKERS IN MATERNAL AND CORD BLOOD
TO FOLIC ACID DOSE DURING PREGNANCY

1

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Abstract

The folate RDA for pregnant women is 600 $\mu\text{g}/\text{d}$ Dietary Folate Equivalents, which is equivalent to approximately 400 μg folic acid. Many prenatal supplements contain much higher doses of folic acid. The body's ability to reduce synthetic folic acid to the metabolically active form may be exceeded with high levels of supplementation. The objective of this double-blinded randomized controlled intervention trial was to determine changes in unmetabolized folic acid and other biomarkers of folate and one-carbon metabolism in maternal and cord blood in response to a folic acid dose commonly found in prenatal supplements (800 $\mu\text{g}/\text{d}$) compared to the dose equivalent to the RDA (400 $\mu\text{g}/\text{d}$). Healthy pregnant women were randomized and provided supplements from their first prenatal visit (<12 weeks gestation) through delivery. A repeated measures analysis of variance revealed that there was a significant group supplemental dose effect ($P = 0.0225$) on serum unmetabolized folic acid concentration in mothers but no difference in cord blood unmetabolized folic acid concentrations between groups. Mixed effects analysis found a significant overall effect of pre-pregnancy BMI ($P = 0.0360$) and length of previous folic acid supplementation ($P = 0.0281$) on serum folate concentrations. No treatment effect was seen in RBC folate concentrations. Choline concentrations were higher in cord blood from the 800 $\mu\text{g}/\text{d}$ group compared to the 400 $\mu\text{g}/\text{d}$ group, but there was no group effect in maternal choline concentrations. These results indicate that folic acid dose during pregnancy does affect certain folate and one-carbon biomarkers, and these effects are not consistent between maternal and cord blood. Potential long-term effects on both mothers and offspring of these results are unknown and merit further investigation.

Introduction

Folate is a water soluble B vitamin that exists as naturally occurring folate found in foods such as leafy green vegetables, legumes, beans, and orange juice. It also exists as the synthetic form, folic acid, which is the form found in fortified foods and dietary supplements. Naturally occurring folate is in the reduced form while folic acid is the fully oxidized form (Zhao et al 2009). Folate metabolites function as coenzymes which transfer one-carbons by accepting and donating them; these reactions are known as folate-mediated one-carbon metabolism (Stover 2010). Because of folate's key role in one-carbon metabolism needed for DNA and protein synthesis, the body's requirement for folate increases when there is an increase in cellular division (Tamura and Picciano 2006). During pregnancy, there is a dramatic increase in cellular reproduction associated with the growth of both maternal and fetal tissues, and folate needs are therefore increased during this period of rapid cell proliferation (Cunningham et al 1993). Failing to consume adequate amounts of folate from the diet and supplements can have serious negative consequences, including developmental disorders such as neural tube defects (NTDs, Cunningham et al 1993).

To reduce risk for NTDs, the recommendation for women capable of becoming pregnant is to consume 400 µg of folic acid in addition to normal dietary intake (CDC 1992). To meet increased demands during pregnancy, the RDA (Recommended Daily Allowance) for pregnant women is to consume 600 µg DFE per day. 1 µg DFE is equal to 1 µg of food folate or approximately 0.6 µg of folic acid as folic acid has a greater bioavailability than food folate (Institute of Medicine 1998). Folic acid supplementation is often encouraged for pregnant women in order to ensure that they are consuming adequate folate. Although the recommendation for pregnant women is to consume 400 µg of folic acid per day, the folic acid

content of most over the counter prenatal supplements is at least twice that, ranging from 800-1000 µg or higher. It is estimated that 75% of pregnant women use a folic acid containing supplement, and pregnant women who supplement have a median intake of 846 µg of folic acid per day (Kauwell et al 2010).

Folic acid from fortified foods and supplements is the synthetic form of folate and is in an oxidized state. It must be reduced in order to function as a coenzyme throughout the body (Smith et al 2008). Oxidized folic acid, also referred to as unmetabolized or nonmetabolized folic acid, is folic acid that has not been reduced to the active form. At high levels of intake the body's ability to reduce synthetic folic acid to the reduced form may be exceeded, leading to detectable circulating levels of unmetabolized folic acid (Kelly et al 1997). Unmetabolized folic acid has been found to be present in the majority of the US population, regardless of demographics, fasting status, or supplement use (Pfeiffer et al 2015). Additionally, unmetabolized folic acid is now present in many cord blood samples, although research in this area is limited (Obeid et al 2010; Sweeney et al 2005).

With the presence of circulating oxidized folic acid observed in almost all of the United States population, there is rising concern that there are potential harmful, but not yet fully understood, consequences associated with excess intakes of folic acid (Hoyo et al 2011). As pregnant women consume nutrients including folic acid, these nutrients are transferred to the fetus via the placenta (Lager and Powell 2012). Studies in both humans and animals indicate the possibility of a protective mechanism that prevents the fetus from receiving toxic amounts of certain vitamins, even in the presence of high maternal intakes and serum concentrations (Young et al 2014; Costabile et al 2014). Previous studies using lower doses of folic acid have not found differences in cord blood concentrations of unmetabolized folic acid from mothers who

supplement than those whose mothers did not (Obeid et al 2010; Pfeiffer et al 2015). Research in this area however is limited and thus far inconclusive.

The aim of this study is fill in knowledge gaps by investigating whether there are differences in circulating concentrations of unmetabolized folic acid in pregnant women consuming the recommended dose of folic acid (400 μ /d) as compared with the dose commonly found in over-the-counter prenatal supplements (800 μ /d), and whether this is translated to the infant. Additionally we sought to determine the effect of folic acid supplementation on other folate and one-carbon biomarkers in maternal and cord blood. Based on previous research findings it was hypothesized that maternal concentrations would be higher in the group exposed to the higher dose of folic acid (Obeid et al 2010; Pfeiffer et al 2015) while cord blood concentrations would not differ between groups (Obeid et al 2010; Pentieva et al 2016). We also hypothesized that there would be differences in other one-carbon metabolites between groups.

Methods

Participants

Participants were recruited by midwives from Athens Regional Midwifery Clinic (ARMC) at their initial prenatal visit (<12 weeks gestation) by midwives. The midwife recruiters attended several in-service training sessions which covered folate and its role in pregnancy, the study purpose and design, participant inclusion and exclusion criteria, and their role in the study. They also completed the Collaborative Institutional Training Initiative (CITI) on human subjects. Inclusion criteria included: healthy, aged 18-40 year old pregnant women with a singleton pregnancy and willingness to comply with study protocols. Exclusion criteria were a pre-existing chronic condition including anemia, diabetes and hypertension, smokers, use of prescription

drugs, complications associated with pregnancy such as pre-eclampsia and gestational diabetes, and carrying more than one fetus. Participants were not allowed to take any prenatal supplements other than those provided by the research team for the study. The midwives obtained written informed consent from all eligible and willing women before being enrolled in the study. All methods and procedures were approved by the University of Georgia Institutional Review Board on Human Subjects (STUDY00000506) and the Athens Regional Medical Center Institutional Review Board before the study began. The study was registered at ClinicalTrials.gov (NCT02124642).

Intervention and supplementation

This study was performed as a double-blinded randomized controlled intervention trial (Figure 1). Participants were randomized into two treatment groups upon enrollment, one providing 400 µg of folic acid and the other 800 µg of folic acid daily throughout pregnancy until delivery. All participants were provided three tablet/capsules daily including a multivitamin/multimineral tablet (One-A-Day® Women's, Bayer's Healthcare, USA) that contained 400 µg of folic acid along with other vitamins and minerals that are required for pregnant women, a capsule containing 200 mg docosahexaenoic acid (DHA) (Life's DHA™ 200 mg vegetarian capsules, DSM Nutritional Products North America, Parsippany, NJ), and the specially formulated capsule containing 10 mg of iron and either 0 or 400 µg of folic acid, providing a total of either 400 or 800 µg of folic acid per day. Because the multivitamin/multimineral tablet (One-A-Day® Women's) contained 18 mg of iron, 10 mg of iron was added to bring the total iron from supplements to the recommended amount of about 27 mg per day. Specially formulated capsules contained either 0 or 400 µg of folic acid because the

multivitamin/multimineral contained 400 µg of folic acid, totaling either 400 or 800 µg depending on treatment group. The treatment capsules were compounded by Westlab Pharmacy (Gainesville, Florida) and were tested by third-party laboratories for folic acid (Analytical Research Laboratories, Oklahoma City, OK) and iron (Covance, Madison, WI) content. This supplement regimen provided the participants with vitamin and mineral amounts that are recommended for a healthy pregnancy by the Institute of Medicine. Participants were instructed to take all three supplements daily at around the same time each day. Supplements were provided at study enrollment and exchanged monthly at each follow-up prenatal visit throughout pregnancy.

Demographic and health information

Maternal demographic and anthropometric data including age, race, weight and height at baseline and throughout pregnancy were acquired from medical records, and BMI was calculated using the Centers for Disease Control BMI calculator website (http://www.cdc.gov/healthyweight/assessing/bmi/adult_bmi/english_bmi_calculator/bmi_calculator.html). Pre-pregnancy weight was per self-report. Medical records were also used to collect health information including previous pregnancies, medical history, and prescription and non-prescription drug use. Use of participant health information was authorized by signature of consent and protected by the Health Insurance Portability and Accountability Act (HIPAA). Additionally participants answered a health-behavior questionnaire over the phone after enrollment including information on pre-pregnancy supplementation, consumption of highly fortified foods, smoking and alcohol history, physical activity before and during pregnancy, and amount of time spent outdoors.

Researchers also maintained contact with participants throughout the study via e-mail, telephone, and text message to provide information and answer questions as needed.

Infant information was also obtained through medical records, including date and mode of delivery, gestational age, gender, anthropometric measurements, and Apgar score, which is a subjective test used to assess clinical infant status at one and five minutes after birth.

Dietary Assessment

At study enrollment, participants were provided with a diet recall packet which they were instructed to complete around 24 weeks gestation. They were then provided with another packet that they were instructed to complete around 32 weeks gestation. Each set of recalls included three non-consecutive days of 24-hour recalls, two week days and one weekend day. The recall followed the format of the Automated Self-Administered 24-hour dietary recall (ASA24) system (<http://riskfactor.cancer.gov/tools/instruments/asa24>). Researchers called participants on the telephone to remind them when it was time to complete the two recalls, and participants were given the option to fill out the paper version and mail it in a pre-addressed and stamped envelope to the researchers or to log in to the ASA24 system and complete the recall online. For paper versions, a trained researcher called participants once the recall was received and used questions and probes from the ASA24 program to ensure thorough recall information was obtained and then entered the recall data into the ASA24 system. Nutrient analysis obtained within the ASA24 system is based on the Food and Nutrient Database for Dietary Surveys as described by Subar et al (2012).

Compliance

Participants were contacted every two weeks via e-mail, text message or phone call by researchers in order to provide information on how to complete dietary recalls, obtain health behavior information, and answer any questions participants may have. Additionally, participants were reminded to return their pillboxes at their next prenatal visit and pick up their next box of supplements, which encouraged compliance to the study protocol. Compliance was measured by counting pills that were returned.

Blood sample collection

Non-fasting venous blood was collected at the midwifery clinic by trained ARMC staff at the patient's initial visit, 28 weeks gestation, and 36 weeks gestation. Both maternal and cord blood were obtained at delivery at Athens Regional Medical Center by nurses at the Labor and Delivery Unit. Cord blood was collected via the umbilical vein. Blood was collected in EDTA-coated tubes and serum separator tubes which were wrapped in foil, stored on ice, and processed within two hours of collection. Serum was allowed to clot for 30 minutes at room temperature, then was centrifuged for 15 minutes at 1200 x g. 1 mL of serum was removed and combined with 71.4 μL of 7% ascorbic acid solution, then divided into two 500 μL aliquots for serum folate analysis. A 100 μL sample of whole blood was added to 1.0 mL of 1% ascorbic acid, wrapped in foil to protect from light, and mixed on a rotating platform for 30 minutes. This was then divided into two 500 μL aliquots for RBC folate analysis. 10 mg of ascorbic acid was added to 1 mL of serum, then divided into two 500 μL aliquots for folic acid analysis. Complete blood count (CBC) samples were placed in biohazard sample bags and picked up by LapCorp. This analysis was performed immediately. Samples for RBC folate, serum folate, folic acid, and other

one-carbon metabolite samples for analysis were stored at -80°C prior to shipping on dry ice to analytical labs.

Blood sample analysis

MTHFR 677C→T (rs18001133) genotype was determined after purifying PCR products (QUIquick PCR Purification kit) and sequencing DNA templates with an Applied Biosystems Automated 3730 DNA analyzer (Applied Biosystems) by the Georgia Genomics Facility (Athens, GA). Serum and red blood cell folate concentrations were determined by microbiological assay using *Lactobacillus rhamnosus*. (Horne and Patterson 1988, Tamura 1990). The inter- and intra-assay coefficients of variation were 7.7% and 6.7% for serum folate, respectively, and 5.1% and 3.3% for RBC folate, respectively. Oxidized folic acid in the serum was analyzed using LC-MS/MS stable isotope-dilution methods as described by West et al (2012). The LC-MS/MS system was a TSQ Quantum mass spectrometer (Thermo) with refrigerated Accela autosampler (Thermo) and Accela pump with degrasser (Thermo). Plasma choline, betaine, dimethylglycerine (DMG), and trimethylamine *N*-oxide (TMAO) measurements were taken using LC-MS/MS stable isotope dilution methods as described by Yan et al (2012).

Statistics

Differences between two folic acid supplementation groups at each time point were assessed by unpaired *t*-tests (continuous dependent variables) and differences in values of maternal and cord blood pairs were assessed by paired *t*-tests using GraphPad Prism version 5 (La Jolla, CA). Repeated measures analyses were done using SAS version 9.3 (Cary, NC). Blood concentration response to folic acid supplementation was 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. The level of statistical significance was defined at $P < 0.05$.

Results

Participant Characteristics

Fifty-one pregnant women were recruited for the study at their initial prenatal visit. Participant flow is summarized in Figure 2. Out of the 51 pregnant women, 23 women discontinued the study during the intervention period. Reasons for discontinuing included morning sickness ($n = 8$), taking other prenatal supplements ($n = 2$), moving to another prenatal clinic ($n = 2$), non-singleton pregnancy ($n = 1$), failure to comply with study protocols ($n = 1$), miscarriage ($n = 1$), and 8 women dropped for unknown reasons. 28 participants completed the intervention and were retained through delivery with 92.1% compliance. Among the 28 participants who completed the study, 16 participants had been randomized to take 400 $\mu\text{g}/\text{d}$ of folic acid and 12 had been randomized to take 800 $\mu\text{g}/\text{d}$ of folic acid. Clinical and demographic characteristics of these participants are summarized in Table 1. There was no difference in clinical measures at baseline or delivery between groups, however the 400 $\mu\text{g}/\text{d}$ group had higher pre-pregnancy BMI based on self-report. Women in this group also had higher body weight at baseline but there was no significant difference in BMI between groups at baseline.

Eight women from each group (16 total) reported taking a supplement containing folic acid prior to study baseline. In the 400 µg/d group, 25% of mothers and 15.4% of infants had the TT polymorphism for the MTHFR genotype, which indicates higher risk for folate deficiency. In the 800 µg/day group, 8.3% of mothers and 8.3% of infants had the TT polymorphism. Mothers with the TT genotype were unrelated to infants with the same genotype. There were no differences in average intake of total calories between the 400 and 800 µg/d groups (1971.9 ± 201.6 kcal vs 2329.3 ± 322.4 kcal, respectively), food folate (220.4 ± 28.3 µg vs 279.1 ± 29.0 µg), food folic acid (167.0 ± 19.6 µg vs 245.9 ± 29.0 µg), dietary folate equivalents (504.3 ± 52.0 µg vs 697.0 ± 102.1 µg), or average intake of vitamin B12 (5.2 ± 0.6 µg vs 5.8 ± 1.6 µg), protein (81.5 ± 8.3 g vs 95.0 ± 12.5 g), iron (14.7 ± 1.1 mg vs 18.5 ± 3.2 mg) or fiber (19.3 ± 3.8 g vs 22.1 ± 2.5 g) between groups during the intervention period ($P > 0.05$).

Infant outcomes are summarized in Table 2. There were no differences between groups except for one minute Apgar score, where the 400 µg/d group was significantly higher ($P = 0.0411$). This difference disappeared at five minutes and all Apgar scores at both time points fell within the normal range.

Effect of folic acid dose during pregnancy on folate biomarkers in maternal and cord blood

Serum folate, plasma 5MTHF and RBC folate responses were measured to investigate the effect of folic acid dose during pregnancy on folate status. The responses of serum folate and plasma 5MTHF to folic acid dose were similar. A repeated measures analysis of variance of serum folate and plasma 5MTHF concentrations identified no significant group or group*time effects but did indicate a significant time effect. Women in the 800 µg/d group tended to have higher concentrations of serum folate compared to the 400 µg/d group at baseline and 36 weeks,

but this difference disappeared at delivery as shown in Figure 3a and 3c. Mixed effects models were utilized to investigate potential confounding factors including maternal MTHFR genotype, pre-pregnancy BMI, length of folic acid supplementation before enrolling in the study and gestational age at baseline. Pre-pregnancy BMI had a significant overall effect on serum folate ($P = 0.0360$) and plasma 5MTHF ($P = 0.0419$). After adjusting for pre-pregnancy BMI, the group effect became significant ($P=0.0407$ and $P = 0.0497$ for serum folate and plasma 5MTHF, respectively) (Table 3 and Table 4). The time effect on serum folate and plasma 5MTHF disappeared after adjusting for pre-pregnancy BMI. Length of supplementation before enrolling in the study and gestational age at baseline also had an overall effect on serum folate and plasma 5MTHF. After adjusting for length of folic acid supplementation before enrolling in the study, the time effect found for serum folate and plasma 5MTHF became non-significant. Because serum folate reflects short-term folate status, concentrations may have been affected by previous folic acid supplementation. Participants who did not previously supplement with folic acid tended to have an increase in serum folate and plasma 5MTHF over time while participants who did report previously supplementing did not demonstrate this increase during pregnancy likely because of their higher baseline folate status. The cord blood in the 800 $\mu\text{g}/\text{d}$ group also had significantly higher serum folate than cord blood in the 400 $\mu\text{g}/\text{d}$ group ($P = 0.037$, Figure 3b). Cord blood in the 800 $\mu\text{g}/\text{d}$ group had significantly higher concentrations of serum folate and plasma 5MTHF than mothers in the same group (Figure 5d), but there was no difference in maternal and cord concentrations in the 400 $\mu\text{g}/\text{day}$ group. Moreover, serum folate and plasma 5MTHF were highly correlated both in maternal ($P < 0.0001$, $r = 0.9958$ at baseline and $P < 0.0001$, $r = 0.9649$ at delivery) and cord ($P < 0.0001$, $r = 0.7992$) blood.

Responses of RBC folate concentration to folic acid dose during pregnancy were measured to represent long-term folate status. RBC folate concentrations were increased during gestation in both groups but no group effect was observed by a repeated measures analysis of variance (Figure 3e). None of the variables considered for the mixed effect model analysis including maternal MTHFR, pre-pregnancy BMI, length of folic acid supplementation before enrolling in study or gestational age at baseline had an overall effect on RBC folate and the time effect observed on RBC folate remained significant after adjusting for these variables as shown in Table 5. Additionally, folic acid dose during pregnancy did not affect RBC folate concentration in maternal or cord blood (Figure 3f).

Effect of folic acid dose during pregnancy on unmetabolized folic acid in maternal and cord blood

Serum unmetabolized folic acid in maternal and cord blood was measured in response to folic acid dose during pregnancy. Detectable amounts of folic acid were present in all maternal and cord blood samples (limit of detection was 0.075 ng/mL). There was a significant group effect ($P = 0.0225$) on serum folic acid (Figure 4a), indicating that women with 800 $\mu\text{g}/\text{d}$ folic acid supplementation during pregnancy maintained higher serum folic acid concentrations compared to those with 400 $\mu\text{g}/\text{d}$ folic acid supplementation. Accordingly, at delivery the 800 $\mu\text{g}/\text{d}$ group of mothers had significantly higher concentrations than the 400 $\mu\text{g}/\text{d}$ group ($P = 0.006$, Figure 4b). However, there was no difference in serum folic acid in cord blood between two groups. This indicates that folic acid dose affects concentrations of unmetabolized folic acid in mothers but not in infants, suggesting the presence of a mechanism that maintains consistent fetal concentrations. Moreover, cord blood serum folic acid was not correlated to cord blood

serum folate or RBC folate ($P > 0.05$). In addition, cord blood serum folic acid was not correlated to maternal serum folate or RBC folate at delivery or maternal serum folic acid at delivery ($P > 0.05$), suggesting that unmetabolized folic acid concentration is not closely related to concentrations of other folate biomarkers.

Effect of folic acid dose during pregnancy on choline and choline metabolites in maternal and cord blood

To explore the effect of folic acid dose on one-carbon metabolism in addition to folate status, plasma choline and its metabolites betaine, DMG and TMAO were measured as shown in Figure 3. There was no group or group*time effect on plasma choline (Figure 3a) or choline metabolites (Figure 5c, 5e, and 5g) and only a time effect was observed on plasma choline, betaine and DMG. Plasma choline was increased throughout pregnancy in both groups and betaine and DMG were decreased throughout pregnancy in both groups. Variables tested by a mixed effects model had no overall effect on plasma choline (Table 7), betaine (Table 8), DMG (Table 9, except for pre-pregnancy BMI) and TMAO (Table 10, except for length of supplementation). The time effect observed in plasma choline, betaine and DMG remained significant after adjusting for MTHFR genotype, pre-pregnancy BMI, previous folic acid supplementation, and gestational age. Cord blood plasma choline concentrations were higher in the 800 $\mu\text{g}/\text{d}$ group than in the 400 $\mu\text{g}/\text{d}$ group ($P = 0.0031$), suggesting that maternal folic acid dose during pregnancy may affect cord blood choline metabolism although it does not affect maternal plasma choline at delivery. Cord blood concentrations of choline, betaine, and DMG were significantly higher than blood concentrations in their mothers ($P < 0.05$), which is consistent with previous studies (Visentin et al 2015; Yan et al 2012).

Discussion

The current study was performed to determine changes in unmetabolized folic acid concentrations and one-carbon biomarkers in pregnant women and their infants in response to folic acid supplementation of either 400 $\mu\text{g}/\text{d}$ (equivalent to the current RDA) or 800 $\mu\text{g}/\text{d}$ (the amount commonly found in over-the-counter prenatal supplements) throughout pregnancy. To our knowledge, this is the first randomized controlled intervention trial comparing folate biomarkers in response to these two supplementation protocols throughout pregnancy and in cord blood. Results indicated that pregnant women who took 800 $\mu\text{g}/\text{d}$ folic acid throughout the pregnancy had higher concentrations of unmetabolized folic acid than women in the 400 $\mu\text{g}/\text{d}$ group at delivery. Conversely, there was no difference in unmetabolized folic acid concentrations between the two groups of cord blood, suggesting the presence of a placental mechanism maintaining fetal concentrations. In addition, choline concentrations were higher in cord blood from the 800 $\mu\text{g}/\text{d}$ group, suggesting that higher folate availability could have a sparing effect on cord blood choline.

Folic acid is the synthetic form of folate and is in an oxidized state. It must be reduced to the active form in order to carry out its functions throughout the body (Smith et al 2008). Unmetabolized folic acid, also called oxidized folic acid, is folic acid that has not yet been reduced to the active form in body by enzymes due to saturation of the reducing enzyme, dihydrofolate reductase (DHFR, Bailey and Ayling 2009). In the current study, unmetabolized folic acid was detectable in all maternal samples and cord blood samples. Previous studies conducted in pregnant women and cord blood (Obeid et al 2010; Petntieva et al 2016) reported a higher prevalence of undetectable amount of unmetabolized folic acid in the maternal and cord blood samples following 400 $\mu\text{g}/\text{d}$ folic acid supplementation. Obeid et al. detected

unmetabolized folic acid only in 43.6% of their pregnant participants and 54.2% of cord blood samples (Obeid et al 2010). A more recent study (Pentieva et al 2016) also found that 33- 42% of maternal blood during pregnancy and only 20% of cord blood samples contained a detectable amount of unmetabolized folic acid. The difference in results may be due the fact that the limit of detection (LOD) of the current study was 0.075 ng/mL (equivalent to 0.17 nmol/L) while those of other two studies were 0.20 nmol/L and 0.27 nmol/L, respectively. Differences in limit of detection were likely due to differences in values of signal-to-noise ratio in the studies, where the current study defined the LOD for a signal-to-noise ratio of 3 and the previously mentioned studies defined the LOD as a signal-to-noise ratio ≥ 5 . All three studies used the same method of liquid chromatography-tandem mass spectrometry (LC-MS/MS) for analysis of unmetabolized folic acid in samples. In addition, the previous two studies were performed in European countries where folic acid fortification is voluntary but not mandatory. Although Obeid et al (2010) did not report food folate or folic acid intake, Pentieva et al (2016) reported lower intakes than the current study of food folate (182-186 $\mu\text{g}/\text{d}$ vs 220-279 $\mu\text{g}/\text{d}$, respectively), food folic acid (102-112 $\mu\text{g}/\text{d}$ vs 167-246 $\mu\text{g}/\text{d}$), and dietary folate equivalents (356-376 $\mu\text{g}/\text{d}$ vs 504-697 $\mu\text{g}/\text{d}$).

The effect of a dose of folic acid higher than that which is recommended during pregnancy on unmetabolized folic acid status has not been fully understood. Previous studies (Pentieva et al 2016; Obeid et al 2010) compared the recommended dose of folic acid supplementation to placebo. Pentieva et al (2016) found that 400 $\mu\text{g}/\text{d}$ of folic acid during pregnancy increased the detection rate of maternal unmetabolized folic acid without detecting differences in concentrations between groups. Contrarily, Obeid et al (2010) found no differences in the number of cord or maternal samples with detectable unmetabolized folic acid between groups. In our current study, we compared 400 μg and 800 μg of folic acid doses and

found that 800 µg of folic acid increased maternal unmetabolized folic acid concentration throughout pregnancy. Due to the lack of placebo group in our study, the result may not be compared to the previous findings. However, our finding suggests that 800 µg of folic acid may exceed the capacity of DHFR to reduce dietary folic acid that results in increase in unmetabolized folic acid concentration. Although the function of unmetabolized folic acid in blood is unclear, concerns have been raised by several investigators of adverse health outcomes including cancer, tumor development, and reduced immune function related to high concentrations of unmetabolized folic acid (Stolzenberg-Solomon et al 2006; Smith et al 2008; Troen et al 2006). More research is needed to investigate the possibility of negative long-term outcomes related to high intakes of folic acid during pregnancy.

Despite the increase in maternal unmetabolized folic acid concentration by 800 µg/d of folic acid supplementation during pregnancy compared to a 400 µg/d of folic acid dose, the concentrations in cord blood were not different between groups in the current study. Similarly, in previous studies comparing 400 µg/d of folic acid to placebo there were also no differences found between cord blood groups in percentage of cord blood samples with detectable unmetabolized folic acid or in folic acid concentrations (Obeid et al 2010; Pentieva et al 2016). This is suggestive that the placenta may play a protective role in maintaining consistent amounts of folic acid in the fetus. Membranes within the placenta contain folate transporters which are regulated by signals from the mother, fetus and placenta (Lager and Powell 2012). Although there has been some investigation of the transporter for folate, only few studies have reported transport of unmetabolized folic acid (Henderson et al 1995). Studies in both human and animals have also suggested that even in the presence of high maternal intakes of certain nutrients, there is a mechanism in place that keeps fetal concentrations stable. Expression of placental receptors

for certain nutrients in humans is regulated by neonatal concentrations but not maternal concentrations (Young et al 2014). This indicates that it is possible for certain placental nutrient transporters to be signaled to limit transport of nutrients after fetal concentrations reach a certain level. In animals, embryos of dams who consumed very high amounts of vitamin D had the same or lower concentrations as embryos of dams who took in an adequate amount that was over ten times less (Costabile et al 2014). Our findings in the current study support that a similar mechanism may be in place regulating placental receptors for unmetabolized folic acid and maintaining stable amounts of unmetabolized folic acid in the fetus.

Serum folate concentration and RBC folate concentration reflect folate status in the body. Serum folate concentration reflects short-term folate status and is highly affected by recent folate intake and supplementation. Therefore, it is ideal to take this measure from samples obtained while fasted (Shane 2011). In our current study, we observed a higher serum folate concentration in the 800 $\mu\text{g}/\text{d}$ folic acid group at baseline but this may be due to previous folic acid supplementation or folate from the diet before blood draw. In our study the blood draws were conducted at the participants' prenatal visits for which fasting is not required. Additionally, data collected in our health behavior questionnaire administered shortly after enrolling in the study indicated that half of participants (50%) supplemented with folic acid prior to enrolling in the study. Regardless of fasting status at the time of sampling, folic acid doses during pregnancy did not affect serum folate or 5MTHF concentration in mothers during pregnancy and at delivery, while a higher dose of folic acid supplementation increased cord blood serum folate concentrations.

Although there was no group effect on maternal serum folate response to folic acid dose during pregnancy, pre-pregnancy BMI did have an overall effect on this biomarker. After

adjusting for pre-pregnancy BMI, the group effect became significant for serum folate response. This suggests that pre-pregnancy BMI plays an important role on maternal serum folate status during pregnancy. Consistent with our findings, Shin et al (2016) reported women with lower pre-pregnancy BMI had higher serum folate concentrations than their obese counterparts. Similarly, Han et al (2011) reported lower serum folate concentrations in pregnant women who fell into the obese category pre-pregnancy than those who did not. The negative correlation between serum folate concentration and BMI is also consistent with correlations observed in non-pregnant women of childbearing age, wherein NHANES data from 1988-1994 and 1999-2000 indicated that increased BMI in this population was associated with lower serum folate levels (Mojtabai 2004). Although the mechanism of this disparity is not yet fully understood, this indicates the potential need for overweight and obese women to take higher doses of folic acid in order to achieve serum folate levels equal to normal weight women.

Choline is a vitamin that, along with folate, is involved in one-carbon metabolism and functions as a methyl donor. Though there were no differences in maternal choline concentrations during gestation in our study, cord blood samples in the 800 $\mu\text{g}/\text{d}$ group had higher concentrations of choline than those in the 400 $\mu\text{g}/\text{d}$ group. Visentin et al (2015) found that maternal free choline concentration was not a significant predictor of fetal free choline, which results from the current study support. Based on these results, maternal folic acid intake and maternal folate status may play a role in signaling fetal uptake of choline. Some animal studies have also indicated that high folate availability in the body can spare choline because they both function as methyl donors (Schwahn et al 2004). Another study conducted in mice found that folate deficiency led to a decrease in choline concentrations but a dramatic increase in the reductase that converts choline to betaine, the compound that donates methyl groups (Chew

et al 2011), which likely occurred in order to compensate for the low amounts of folate available. Consistent with these findings, in the current study where folate intake was sufficient in both groups choline concentrations increased throughout pregnancy while betaine concentrations decreased because this compensatory action was not needed. Higher folic acid supplementation during pregnancy may affect fetal choline availability by sparing choline from being used as a one-carbon donor, thus allowing choline to carry out other functions in the body including formation of acetylcholine, a neurotransmitter necessary for proper brain development (Zeisel and Caudill 2010).

Maternal nutrition during gestation plays a critical role in offspring metabolism, a phenomenon called fetal programming (Chmurzysnka 2010). Because of folate's involvement in DNA methylation, maternal folate intake could affect infant health outcomes later in life including cardiovascular health, fat accumulation, insulin resistance and glycemic control. In our study, there were no differences in clinical characteristics of infants between the two groups except for one minute Apgar score. However, this difference in one minute Apgar score is not clinically relevant because all infants in both groups had Apgar scores within the normal range both at one and five minutes. Because the current study only followed up until birth, there is still the potential for differences in long-term health outcomes between the two groups of infants, even if infant characteristics were not different at birth. Keating et al (2015) found that Sprague-Dawley rats fed a high folic acid diet had female offspring with higher weight gain, food intake, and impaired glycemic control as compared to offspring of mothers fed a diet with adequate folic acid. In humans, offspring of women who supplemented with folic acid had decreased DNA methylation associated with deregulation of IGF2 and increased risk of chronic disease compared to offspring of women who did not supplement (Hoyo et al 2011). These studies indicate the

need for more long-term studies to investigate potential health consequences of maternal folic acid intake not evident at birth.

This study has several strengths. First, it is a randomized controlled intervention trial which allows results to be unbiased. This study also is the only one to our knowledge that investigated outcomes in response to these two specific folic acid doses throughout pregnancy, the RDA and the dose commonly found in prenatal supplements. It is also the first study that we are aware of comparing two doses of folic acid supplementation and unmetabolized folic acid in maternal and cord blood in a country with mandatory folic acid fortification of enriched wheat products. We also measured folate biomarkers along with biomarkers of choline metabolism in order to better understand the relationship between these two compounds. The current study is not without limitations. As mentioned previously, both women who did and did not supplement with folic acid prior to study baseline were included, which may have had an effect on our results. Ideally a follow up study would control for previous supplementation. Blood samples were also non-fasting. Additionally we did not observe the mechanisms for the results presented, so explanations of our results are speculative. Finally because this trial was only conducted through delivery, we do not know if there were any long-term effects in mothers or infants as a result of these two treatments.

Conclusion

In summary, supplementation with 800 µg of folic acid per day throughout pregnancy did not affect serum folate or RBC folate concentrations but did increase unmetabolized folic acid concentrations in mothers compared to a 400 µg per day dose. Conversely, the higher dose of folic acid supplementation during pregnancy increased serum folate but did not affect serum

unmetabolized folic acid concentrations in cord blood. Although there was no group difference with choline concentrations in maternal samples, cord blood samples from the 800 µg/d group had higher choline concentrations. Taken together, folic acid dose during pregnancy had a distinct influence on folate biomarkers in maternal and cord blood. Potential mechanisms for these disparities may include regulatory roles of placenta and investigation is needed to better understand how the placenta regulates one-carbon biomarkers between maternal and cord blood. Further study is also warranted to investigate long-term effects of increased unmetabolized folic acid in mothers and increased serum folate and choline in infants in response to folic acid doses contained in over-the counter prenatal vitamins..

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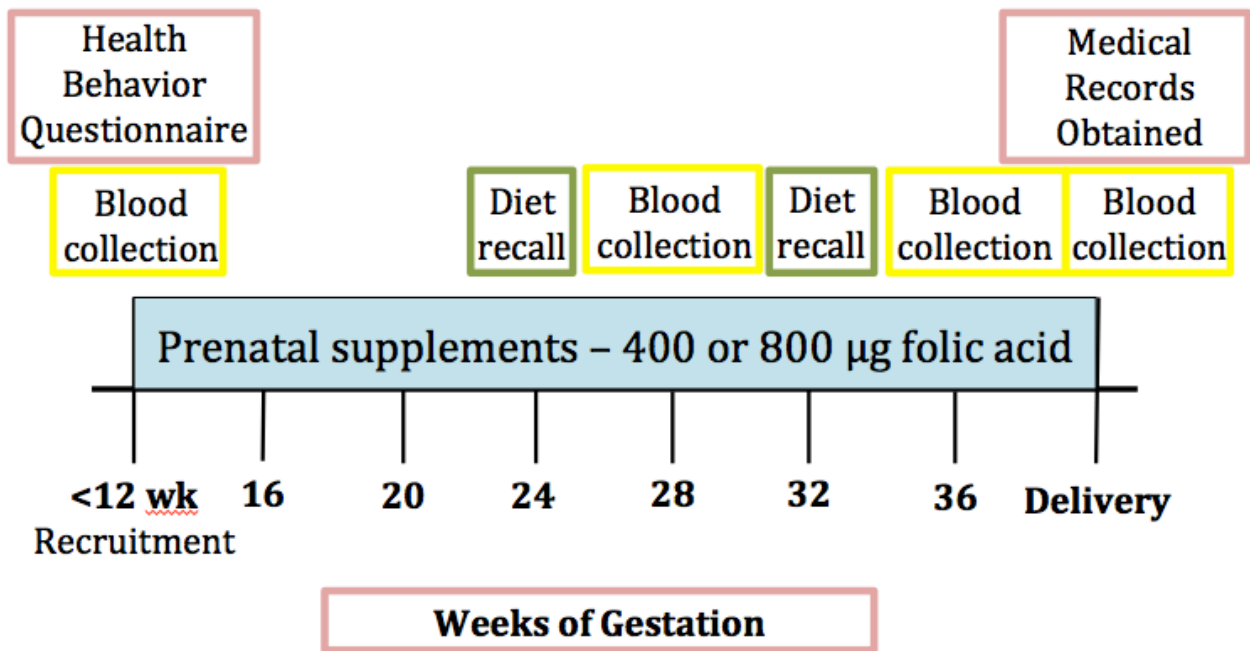


Figure 1. Study timeline

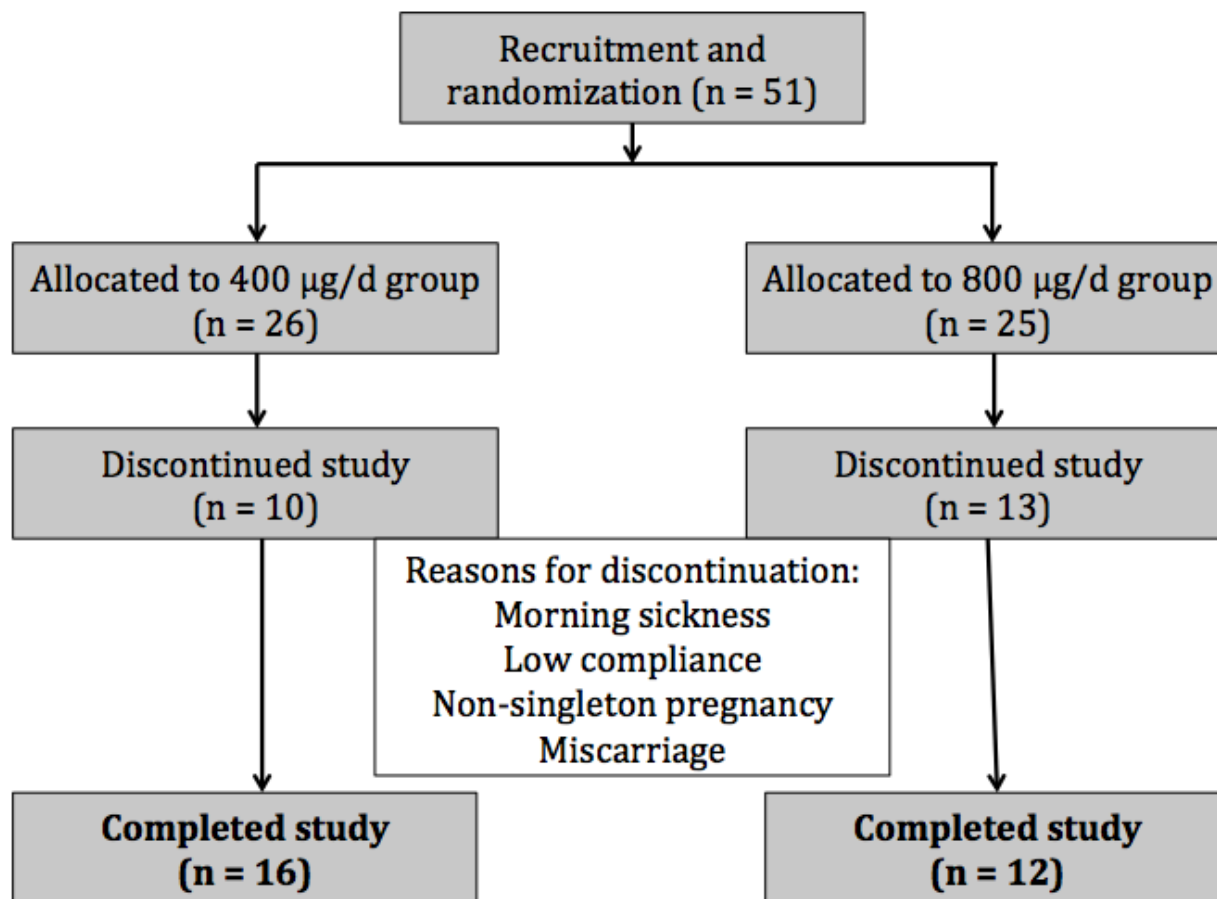


Figure 2. Participant flow

Table 1. Demographic and clinical characteristics of mothers^a

		Baseline		Delivery	
		400 µg/day (n = 16)	800 µg/day (n = 12)	400 µg/day (n = 16)	800 µg/day (n = 12)
Age (years)		28.3 ± 1.5	26.6 ± 1.2	-	-
Weight (kg)		75.1 ± 3.6	62.9 ± 3.1*	86.3 ± 3.0 ⁺	76.5 ± 2.3* ⁺
Height (cm)		166.0 ± 2.0	161.8 ± 1.9	-	-
BMI (kg/m²)		27.2 ± 1.2	24.1 ± 1.3	31.3 ± 0.9 ⁺	29.1 ± 0.9 ⁺
Gestational Age (weeks)		7.7 ± 0.3	7.1 ± 0.6	40.1 ± 0.2 ⁺	40.0 ± 0.4 ⁺
Pre-Pregnancy BMI (kg/m²)		27.0 ± 1.2	23.3 ± 1.3*	-	-
Hemoglobin (g/dL)		13.0 ± 0.2	12.9 ± 0.8	12.0 ± 0.4	12.5 ± 1.0
Hematocrit (%)		39.2 ± 0.7	39.4 ± 3.0	36.6 ± 1.2 ⁺	36.8 ± 1.95 ⁺
RBC (10E6/µL)		4.5 ± 0.1	4.3 ± 0.4	4.1 ± 0.1	4.1 ± 0.4
WBC (10E3/µL)		8.5 ± 2.6	8.0 ± 2.7	10.2 ± 3.0 ⁺	12.2 ± 3.4 ⁺
Previous FA Supplementation	Yes	n = 8 (50%)	n = 8 (66%)	-	-
	No	n = 5 (31%)	n = 2 (17%)	-	-
	Unknown	n = 3 (19%)	n = 2 (17%)	-	-
Race/Ethnicity	White	n = 7 (44%)	n = 7 (58%)	-	-
	Hispanic	n = 5 (31%)	n = 4 (33%)	-	-
	African American	n = 3 (18%)	n = 0 (0%)	-	-
	Other	n = 1 (7%)	n = 1 (9%)	-	-
MTHFR Genotype	CC	n = 9 (56%)	n = 8 (66%)	-	-
	TC	n = 3 (19%)	n = 3 (25%)	-	-
	TT	n = 4 (25%)	n = 1 (9%)	-	-

^aMean ± standard deviation, *Groups are significantly different (P < 0.05) at baseline or delivery by paired t-test, ⁺baseline and delivery values are significantly different (P < 0.05) using unpaired t-test.

Table 2: Clinical characteristics of infants^a

	400 µg/day (n = 16)	800 µg/day (n = 12)	
Length (cm)	51.3 ± 0.5	51.7 ± 0.5	
Weight (kg)	3.6 ± 0.1	3.5 ± 0.1	
Head Circumference (cm)	33.6 ± 1.4	33.9 ± 0.5	
Gestational Age at delivery	40.1 ± 0.2	40.0 ± 0.4	
Apgar – 1 min	8.3 ± 0.2	7.6 ± 0.3*	
Apgar – 5 min	8.8 ± 0.1	8.5 ± 0.4	
Hemoglobin (g/dL)	16.2 ± 1.8	14.5 ± 1.5	
Hematocrit (%)	48.4 ± 5.6	48.0 ± 7.4	
RBC (10E6/µL)	4.6 ± 0.6	4.3 ± 0.2	
WBC (10E3/µL)	12.6 ± 4.8	14.3 ± 4.6	
MTHFR Genotype	CC	n = 4 (25%)	n = 7 (58%)
	TC	n = 7 (44%)	n = 4 (33%)
	TT	n = 2 (13%)	n = 1 (9%)

^aMean ± standard deviation, *Groups are significantly different (p<0.05) by unpaired t-test.

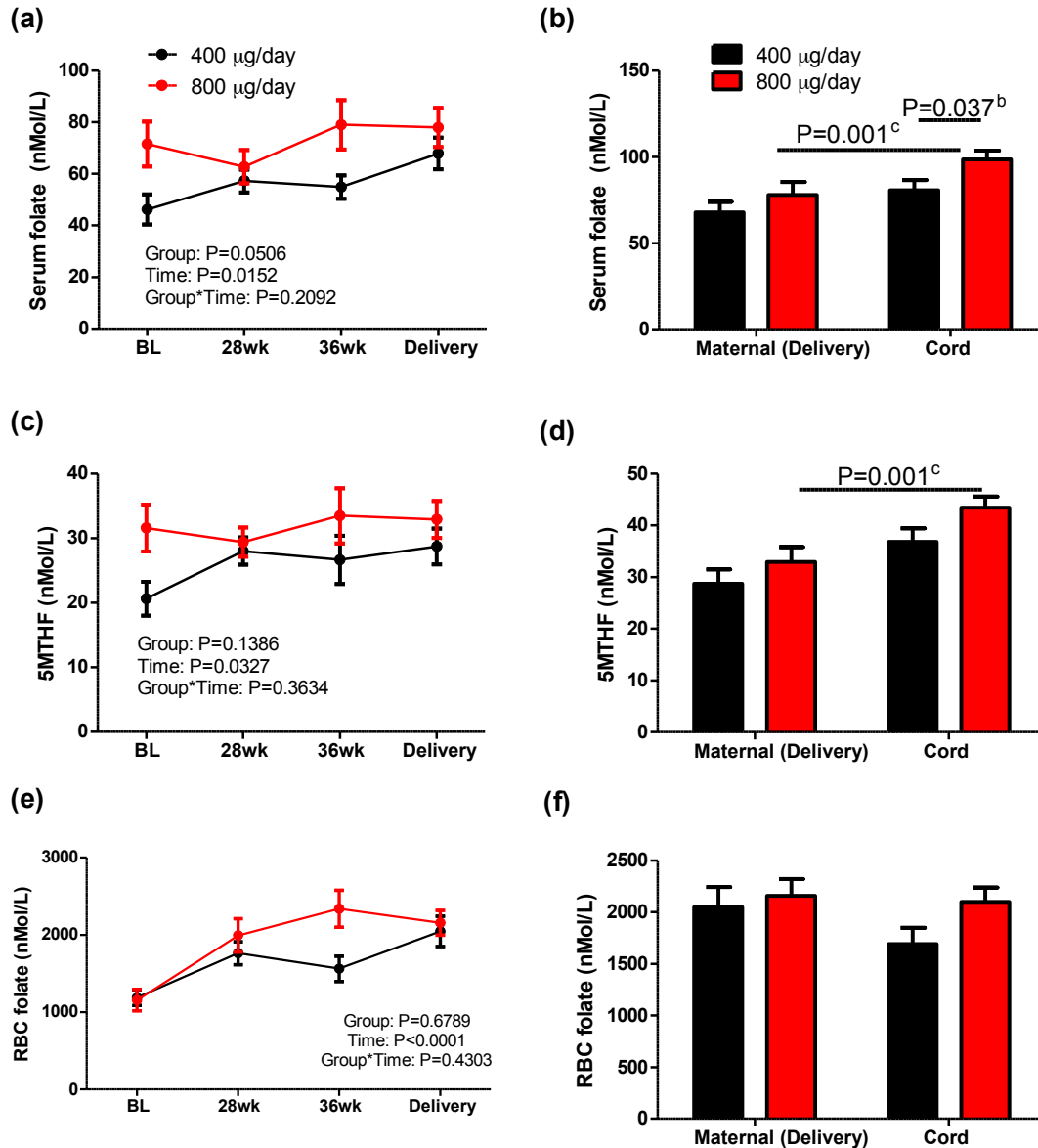


Figure 3. Serum folate (a and b), RBC (red blood cell) folate (c and d), and 5MTHF (5methyltetrahydrofolate, e and f) concentration in maternal and cord blood in response to 400 or 800 µg/d folic acid supplementation during pregnancy^a. ^aMean ± sem, ^btwo-tailed unpaired t-test between groups of infants, ^ctwo-tailed paired t-test between mother-infant pairs.

Table 3. Mixed effects models for serum folate^a

	Group		Time		Group*Time		Variable ^d		Variable*Time		Variable*Group	
	F	P	F	P	F	P	F	P	F	P	F	P
Repeat Measures Analysis	4.20	0.0506	4.94	0.0152	1.66	0.2092	-	-	-	-	-	-
MTHFR Genotype	0.58	0.4563	4.11	0.0303	1.17	0.3293	0.40	0.6767	2.04	0.1230	3.050	0.0680
Pre-pregnancy BMI (kg/m ²)	4.73	0.0407	2.94	0.0738	1.21	0.3163	3.88	0.0360	0.42	0.7958	2.39	0.1153
Length of supplementation (days) ^b	1.91	0.1867	0.18	0.8398	0.22	0.8036	4.00	0.0281	0.64	0.7005	1.34	0.2977
Gestational age (days) ^c	2.75	0.1103	3.77	0.0376	1.84	0.1813	10.22	0.0039	3.70	0.0399	3.48	0.0742

^aStatistically significant if P < 0.05. 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 model.

Table 4. Mixed effects models for 5MTHF^a

	Group		Time		Group*Time		Variable ^d		Variable*Time		Variable*Group	
	F	P	F	P	F	P	F	P	F	P	F	P
Repeat Measures Analysis	2.04	0.1386	3.52	0.0327	1.69	0.3634	-	-	-	-	-	-
MTHFR Genotype	0.62	0.4347	2.25	0.0572	1.27	0.2989	0.49	0.7125	2.36	0.1716	3.28	0.0710
Pre-pregnancy BMI (kg/m ²)	4.01	0.0497	3.14	0.0608	1.42	0.3947	3.99	0.0419	0.36	0.7262	2.40	0.1182
Length of supplementation (days) ^b	2.98	0.1489	0.21	0.8053	0.47	0.7515	3.29	0.0492	0.84	0.4336	2.18	0.3921
Gestational age (days) ^c	2.82	0.1496	3.79	0.0466	2.14	0.1503	11.39	0.0075	4.05	0.0411	3.02	0.0872

^aStatistically significant if $P < 0.05$. 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 model.

Table 5. Mixed effects models for RBC folate^a

	Group		Time		Group*Time		Variable ^d		Variable*Time		Variable*Group	
	F	P	F	P	F	P	F	P	F	P	F	P
Repeat Measures Analysis	0.18	0.6789	25.41	<0.0001	0.87	0.4303	-	-	-	-	-	-
MTHFR Genotype	0.02	0.8942	17.77	<0.0001	0.72	0.4996	0.24	0.7919	0.02	0.9991	0.04	0.9655
Pre-pregnancy BMI (kg/m ²)	0.37	0.5486	18.80	<0.0001	0.83	0.4501	1.07	0.3606	1.44	0.2545	0.49	0.6213
Length of supplementation (days) ^b	0.65	0.4310	22.79	<0.0001	0.04	0.9565	1.97	0.1613	3.27	0.0293	0.59	0.6340
Gestational age (days) ^c	0.14	0.7110	21.03	<0.0001	1.33	0.2821	2.07	0.1633	1.62	0.2179	0.17	0.6844

^aStatistically significant if P < 0.05. RBC, red blood cell; 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 model.

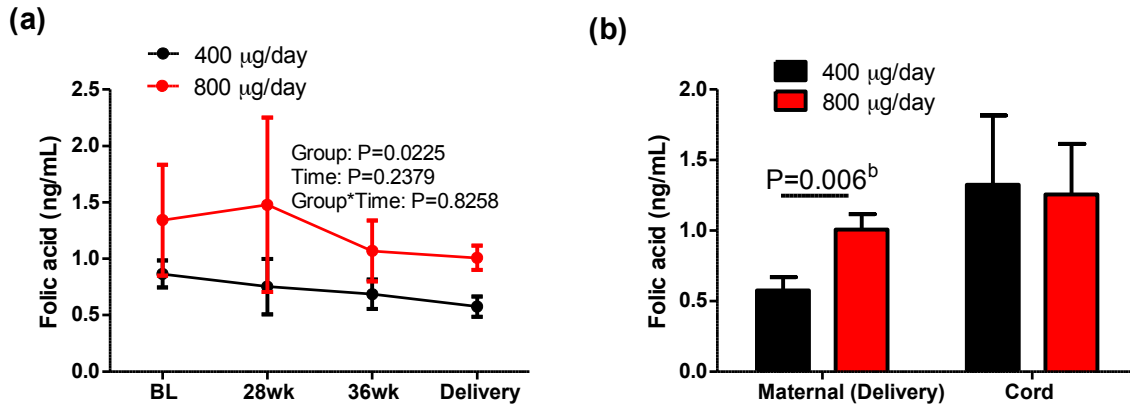


Figure 4. Unmetabolized folic acid concentration in maternal and cord blood in response to 400 or 800 µg/d folic acid supplementation during pregnancy^a. ^aMean ± sem, ^btwo-tailed unpaired t-test between groups of mothers

Table 6. Mixed effects models for unmetabolized folic acid^a

	Group		Time		Group*Time		Variable ^d		Variable*Time		Variable*Group	
	F	P	F	P	F	P	F	P	F	P	F	P
Repeat Measures Analysis	5.88	0.0225	1.52	0.2379	1.19	0.8258	-	-	-	-	-	-
MTHFR Genotype	3.00	0.0973	0.88	0.4307	0.14	0.8679	1.00	0.3853	0.11	0.9783	1.81	0.1872
Pre-pregnancy BMI (kg/m ²)	3.99	0.0582	0.67	0.5236	0.00	0.9963	1.80	0.1885	1.13	0.3678	3.12	0.0642
Length of supplementation (days) ^b	0.74	0.4032	0.54	0.5943	0.00	1.0000	1.38	0.2859	0.46	0.8273	3.24	0.0521
Gestational age (days) ^c	6.12	0.0209	1.19	0.3201	0.19	0.8261	2.29	0.1436	0.58	0.5679	0.09	0.7622

^aStatistically significant if $P < 0.05$. 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 model.

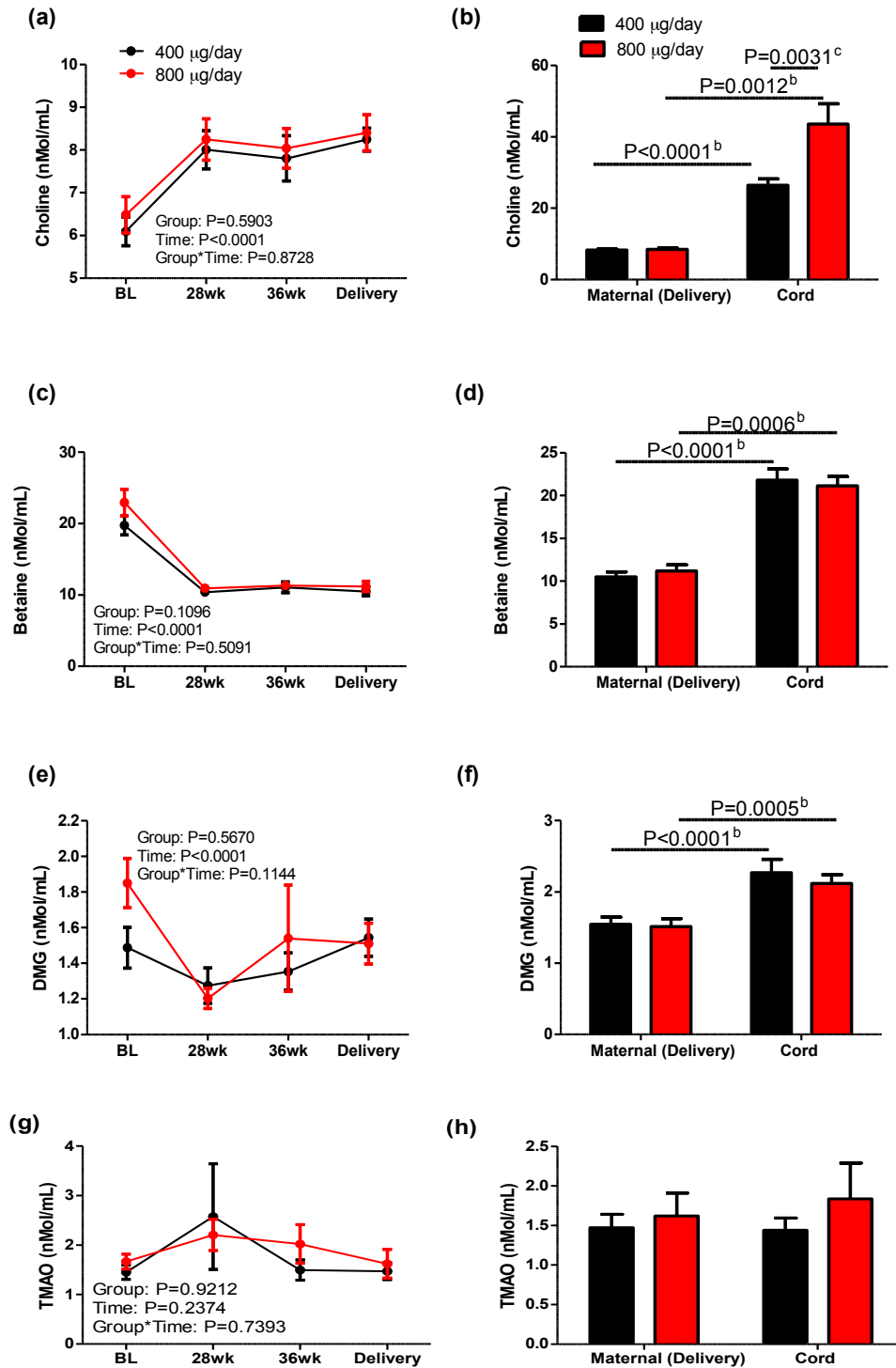


Figure 5. Choline (a and b), betaine (c and d), DMG (dimethylglycine, e and f) and TMAO (g and h) concentration in maternal and cord blood in response to 400 or 800 µg/d folic acid supplementation during pregnancy^a. ^aMean ± sem, ^btwo-tailed unpaired t-test between groups of mothers or groups of infants, ^ctwo-tailed paired t-test between mother-infant pairs.

Table 7. Mixed effects models for choline^a

	Group		Time		Group*Time		Variable ^d		Variable*Time		Variable*Group	
	F	P	F	P	F	P	F	P	F	P	F	P
Repeat Measures Analysis	0.30	0.5903	42.6	<0.0001	0.14	0.8728	-	-	-	-	-	-
MTHFR Genotype	0.47	0.4996	25.87	<0.0001	0.06	0.9430	0.95	0.4015	0.28	0.8886	3.50	0.0479
Pre-pregnancy BMI (kg/m ²)	3.12	0.0911	36.73	<0.0001	1.64	0.2174	3.16	0.0622	4.67	0.0070	0.22	0.8049
Length of supplementation (days) ^b	0.02	0.9021	11.13	0.0011	0.73	0.4995	0.08	0.9716	4.14	0.0119	0.35	0.7908
Gestational age (days) ^c	0.47	0.4997	35.81	<0.0001	0.15	0.8589	2.20	0.1510	1.79	0.1892	0.70	0.4097

^aStatistically significant if P < 0.05. 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 model.

Table 8. Mixed effects models for betaine^a

	Group		Time		Group*Time		Variable ^d		Variable*Time		Variable*Group	
	F	P	F	P	F	P	F	P	F	P	F	P
Repeat Measures Analysis	2.75	0.1096	42.77	<0.0001	0.69	0.5091	-	-	-	-	-	-
MTHFR Genotype	0.95	0.3406	30.85	<0.0001	0.81	0.4565	0.49	0.6216	0.88	0.4939	1.12	0.3449
Pre-pregnancy BMI (kg/m ²)	3.88	0.0616	36.69	<0.0001	0.69	0.5112	0.91	0.4179	0.79	0.5471	2.84	0.0801
Length of supplementation (days) ^b	0.06	0.8041	41.94	<0.0001	0.31	0.7404	1.38	0.2862	0.76	0.6991	0.49	0.6948
Gestational age (days) ^c	3.55	0.0716	44.99	<0.0001	0.83	0.4467	2.8	0.1075	4.86	0.0169	1.10	0.3045

^aStatistically significant if P < 0.05. 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 model.

Table 9. Mixed effects models for DMG^a

	Group		Time		Group*Time		Variable ^d		Variable*Time		Variable*Group	
	F	P	F	P	F	P	F	P	F	P	F	P
Repeat Measures Analysis	0.34	0.5670	17.12	<0.0001	2.36	0.1144	-	-	-	-	-	-
MTHFR Genotype	0.01	0.9336	20.16	<0.0001	2.81	0.0817	0.88	0.4302	1.12	0.3724	4.01	0.0327
Pre-pregnancy BMI (kg/m ²)	6.78	0.0162	9.86	0.0009	4.38	0.0250	3.72	0.0404	1.22	0.3300	1.91	0.1712
Length of supplementation (days) ^b	1.62	0.2225	10.84	0.0012	1.80	0.1987	0.99	0.4239	1.62	0.2089	0.16	0.9227
Gestational age (days) ^c	0.86	0.3638	14.13	<0.0001	2.00	0.1568	2.25	0.1464	0.61	0.5522	2.20	0.1509

^aStatistically significant if P < 0.05. DMG, dimethylglycine; 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 model.

Table 10. Mixed effects models for TMAO^a

	Group		Time		Group*Time		Variable ^d		Variable*Time		Variable*Group	
	F	P	F	P	F	P	F	P	F	P	F	P
Repeat Measures Analysis	0.00	0.9938	0.83	0.4476	0.11	0.8967	-	-	-	-	-	-
MTHFR Genotype	0.07	0.8009	0.29	0.7645	0.26	0.7750	0.79	0.4650	0.55	0.7003	0.18	0.8360
Pre-pregnancy BMI (kg/m ²)	0.26	0.6144	0.23	0.7950	0.87	0.4350	0.73	0.4945	0.93	0.4676	1.96	0.1651
Length of supplementation (days) ^b	0.08	0.7798	0.50	0.6190	1.27	0.3118	3.60	0.0407	1.90	0.1512	6.49	0.0056
Gestational age (days) ^c	0.02	0.8808	1.09	0.3514	0.10	0.9051	0.48	0.4940	0.57	0.5734	2.08	0.1629

^aStatistically significant if P < 0.05. TMAO, trimethylamine N-oxide; 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 model.

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CHAPTER 4

SUMMARY AND CONCLUSIONS

Because of folate's key role in one-carbon metabolism needed for DNA and protein synthesis, the body's requirement for folate increases when there is an increase in cellular division such as pregnancy (Tamura and Picciano 2006). To meet increased demands during pregnancy, the RDA (Recommended Daily Allowance) for pregnant women is to consume 600 μg DFE per day. 1 μg DFE is equal to 1 μg of food folate or approximately 0.6 μg of folic acid as folic acid has a greater bioavailability than food folate (Institute of Medicine 1998). Although the recommendation for pregnant women is to consume 400 μg of folic acid per day, most over the counter prenatal supplements contain at least twice that amount, ranging from 800-1000 μg or higher. At high levels of intake the body's ability to reduce synthetic folic acid to the reduced, metabolically active form may be exceeded, leading to detectable circulating levels of unmetabolized folic acid (Kelly et al 1997). Additionally, as pregnant women consume nutrients including folic acid, these nutrients are transferred to the fetus via the placenta (Lager and Powell 2012). This is of interest because there is rising concern that there are potential harmful, but not yet fully understood, consequences associated with excess intakes of folic acid (Hoyo et al 2011). Therefore, the aim of this study was to fill in knowledge gaps by investigating whether there are differences in circulating concentrations of unmetabolized folic acid in pregnant women when comparing the recommended dose of folic acid (400 $\mu\text{g}/\text{d}$) to the dose commonly found in over-the-counter prenatal supplements (800 $\mu\text{g}/\text{d}$), and whether this is translated to the infant. Additionally we sought to determine folic acid supplementation dose effect on other biomarkers

of folate and one-carbon metabolism in mothers and cord blood. This study was conducted as a double-blinded randomized controlled intervention trial.

Fifty-one women were recruited from Athens Regional Midwifery Clinic and enrolled in the study. Twenty-three women discontinued the study during the intervention period for various reasons, leaving 28 participants who were retained throughout the study; sixteen women randomized to take 400 µg/d of folic acid and twelve women randomized to take 800 µg/d. Maternal blood samples were taken throughout pregnancy and cord blood samples were taken at delivery to measure one-carbon biomarkers and unmetabolized folic acid. Serum folate and plasma 5MTHF responses to folic acid dose were similar, with both having a significant time effect but no group effect. Pre-pregnancy BMI had a significant overall effect on serum folate and plasma 5MTHF concentrations, and after adjusting for pre-pregnancy BMI the group effect for both serum folate and plasma 5MTHF became significant. This suggests that pre-pregnancy BMI has an effect on folate status, which is supported by previous studies that found women who were obese prior to becoming pregnant had lower serum folate concentrations during pregnancy (Shin et al 2016; Han et al 2010). After adjusting for length of folic acid supplementation prior to study enrollment, the time effect found on serum folate became non-significant. This indicates that women who were already taking a folic acid supplement had an elevated folate status at baseline and did not see a dramatic change compared to those that did not previously supplement who saw an increased folate status over time. In cord blood, the 800 µg/d group had higher concentrations of serum folate than the 400 µg/d group, which may influence DNA synthesis and DNA methylation in development of the fetus.

RBC folate concentration is representative of long-term folate status and had a significant time effect but no group effect. There was also no difference in cord blood RBC folate

concentrations. Although RBC folate tended to be higher in the 800 µg/d group throughout pregnancy, this difference was no longer present at delivery. Based on previous research, it is possible that this is due to the 800 µg/d group reaching steady state concentrations sooner than the 400 µg/d group, which eventually reached similar steady state levels (Pietrzik et al 2007). No variables considered for the mixed effects model analysis including maternal MTHFR, pre-pregnancy BMI, length of folic acid supplementation before enrolling in the study or gestational age at baseline had an overall effect on RBC folate. Additionally, the time effect observed remained significant after adjusting for all of these variables.

Unmetabolized folic acid was found in detectable amounts in all maternal samples except one sample at delivery and in all cord blood samples, which is higher than previously reported percentages (Obeid et al 2010; Pentieva et al 2016). This is likely due to the lower limit of detection in the current study compared to those in the previous studies as well as differences in folic acid fortification laws in the countries where these studies took place. There was a significant group effect on unmetabolized folic acid where women that supplemented with 800 µg/d folic acid maintained higher serum folic acid concentrations than women who supplemented with 400 µg/d. However, there was no difference in serum folic acid between the two groups in cord blood. This suggests the presence of a placental mechanism that maintains consistent fetal concentrations of unmetabolized folic acid. Furthermore, cord blood folic acid was not correlated to maternal serum folate, RBC folate, or serum folic acid at delivery or to cord blood serum folate or RBC folate. This suggests that cord blood concentrations of unmetabolized folic acid are not closely related to other folate biomarkers.

When observing the effect of folic acid dose on one-carbon metabolism, choline, betaine, DMG and TMAO were measured. No group effect was found in any of these biomarkers for

maternal responses to folic acid dose. A time effect was observed for choline, betaine and DMG where choline increased throughout pregnancy while betaine and DMG decreased, and these time effects remained significant after adjusting for MTHFR genotype, pre-pregnancy BMI, previous folic acid supplementation, and gestational age. Cord blood choline concentrations were higher in the 800 µg/d group than the 400 µg/d group, suggesting that the higher dose of folic acid spared choline from being used as a one-carbon donor in cord blood, potentially allowing more choline to be used for other functions in the body.

To our knowledge, this is the first randomized controlled intervention trial to compare folate biomarkers and biomarkers of one-carbon metabolism between these two doses of folic acid supplementation throughout pregnancy and in cord blood. There is a need for future, larger-scale studies to be conducted in order to account for limitations in the current study including variable previous folic acid supplementation status and non-fasting collection of blood samples. Additionally, recruitment of a larger participant cohort would be beneficial in order to account for the high drop out rate typical of longer studies done in this population.

Eight participants in each supplementation group reporting supplementing with folic acid prior to study enrollment (50% in the 400 µg/day group, 67% in the 800 µg/day group). Because this variable had a significant overall effect on serum folate and the time effect for serum folate disappeared after adjusting for this previous supplementation, it is likely that this had an effect on baseline serum folate values. Although there was no effect of previous folic acid supplementation observed on any other folate biomarkers, future studies should control for this confounding variable by normalizing for baseline folate concentrations. This would provide a clearer picture of folate biomarker response to folic acid dose taken during the intervention period.

In order to reduce participant burden, blood samples were collected during routine blood draws at prenatal clinic visits so no additional needle sticks were required. This meant that samples were non-fasted as per ARMC protocol which may have affected several observed biomarkers including serum folate. In future studies participants should fast prior to blood sample collection in order to prevent serum folate concentrations from reflecting recent intake; another alternative could include a controlled feeding study.

Studies conducted in the future with a larger sample of participants controlling for previous folic acid supplementation and including fasting blood samples will provide helpful information on the dose response to the doses of folic acid used in the current study. The results from this innovative study include information on the effect of the dose of folic acid commonly found in over-the-counter prenatal supplements on unmetabolized folic acid concentrations in maternal and cord blood compared to the recommended dose. This could contribute to the evidence base that will determine future folate and folic acid intake recommendations for pregnant women.

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APPENDICES

APPENDIX A

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

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

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

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 cualesquiera pastillas 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 abstener 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 fólica 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 cualesquiera preguntas 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 coleccionar 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) _____ (Móbil)

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 cualquier 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

THREE DAY DIET RECALL: FOLIC ACID SUPPLEMENTATION IN PREGNANCY STUDY

ID# _____

DAY 1 DATE: _____

MON

TUE

WED

THURS

FRI

SAT

SUN

TIME	PLACE	MEAL OR SNACK	FOOD/BEVERAGE	HOW MUCH	FOOD ITEM DETAILS (BRAND, CONDIMENTS, ETC)
8 AM	Home	Breakfast	Wheat toast	1 slice	With butter and jam
1 PM	Wendy's	Lunch	Chicken sandwich	1	Grilled, with lettuce, tomato, mayo

**THREE DAY DIET RECALL:
FOLIC ACID SUPPLEMENTATION IN PREGNANCY STUDY**

ID# _____
DAY 2 DATE: _____

MON TUE WED THURS FRI SAT SUN

TIME	PLACE	MEAL OR SNACK	FOOD/BEVERAGE	HOW MUCH	FOOD ITEM DETAILS (BRAND, CONDIMENTS, ETC)

**THREE DAY DIET RECALL:
FOLIC ACID SUPPLEMENTATION IN PREGNANCY STUDY**

ID# _____
 DAY 3 DATE: _____

MON TUE WED THURS FRI SAT SUN

TIME	PLACE	MEAL OR SNACK	FOOD/BEVERAGE	HOW MUCH	FOOD ITEM DETAILS (BRAND, CONDIMENTS, ETC)

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

RECORDATORIO DE DIETA DE 3 DIAS:

ESTUDIO DE LA SUPLEMENTACIÓN DEL FOLATO DURANTE EL EMBARAZO

ID# _____

FECHA DE DIA 1: _____

LUN MAR MIE JUE VIE SAB DOM

TIEMPO	LUGAR	DESAYUNO, ALMUERZO, CENA, O BOCADILLO	COMIDA/BEBIDA	CANTIDAD	DETALLES (MARCA, CONDIMENTOS, ETC)
8 de la mañana	En casa	Desayuno	Pan integral tostado	1 pedazo	Con mantequilla
1 de la tarde	Wendy's	Almuerzo	Sandwich de pollo	1	A la parrilla con tomate, lechuga, y mayonesa

RECORDATORIO DE DIETA DE 3 DIAS:

ESTUDIO DE LA SUPLEMENTACIÓN DEL FOLATO DURANTE EL EMBARAZO

ID# _____

FECHA DE DIA 2: _____

LUN

MAR

MIE

JUE

VIE

SAB

DOM

TIEMPO	LUGAR	DESAYUNO, ALMUERZO, CENA, O BOCADILLO	COMIDA/BEBIDA	CANTIDAD	DETALLES (MARCA, CONDIMENTOS, ETC)

RECORDATORIO DE DIETA DE 3 DIAS:

ESTUDIO DE LA SUPLEMENTACIÓN DEL FOLATO DURANTE EL EMBARAZO

ID# _____

FECHA DE DÍA 3: _____

LUN MAR MIE JUE VIE SAB DOM

TIEMPO	LUGAR	DESAYUNO, ALMUERZO, CENA, O BOCADILLO	COMIDA/BEBIDA	CANTIDAD	DETALLES (MARCA, CONDIMENTOS, ETC)

APPENDIX I

ASA-24 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>

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 #101** _____ 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)

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)

THE END