DNA METHYLATION CHANGES IN WHOLE BLOOD AND CD16+ NEUTROPHILS IN RESPONSE TO CHRONIC FOLIC ACID SUPPLEMENTATION IN WOMEN OF CHILD-BEARING AGE

by

DEANNA SHADE

(Under the Direction of Dorothy B. Hausman)

ABSTRACT

The aim of this study was to determine if DNA methylation in a single leukocyte type would respond differently than whole blood (WB) in response to folic acid (FA) supplementation. This study was performed in normal weight women (18-35 y; n = 12), with blood samples taken before and after 8 weeks of supplementation with 800 μg/day of FA. DNA methylation patterns from WB and CD16+ neutrophils (CD16) were measured across 485,000 CpG sites. We identified 7839 and 8317 sites that changed (P<0.05) in WB and CD16, respectively with decreases in methylation in 77.2% (WB) and 65.6% (CD16) of these sites. These results suggest that the genome-wide DNA methylation response to FA supplementation is different in WB and CD16. Future studies should measure DNA methylation response to FA supplementation in other leukocyte types to determine if a single cell type could act as a more reliable epigenetic reporter than WB.

INDEX WORDS: Women of child-bearing age, Folate, Folic acid, One-carbon metabolism, DNA methylation, Genome-wide, Whole blood, CD16+ neutrophil
DNA METHYLATION CHANGES IN WHOLE BLOOD AND CD16+ NEUTROPHILS IN RESPONSE TO CHRONIC FOLIC ACID SUPPLEMENTATION IN WOMEN OF CHILD-BEARING AGE

By

DEANNA SHADE
B.S., The University of Florida, 2012

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

MASTER OF SCIENCE

ATHENS, GEORGIA
2014
DNA METHYLATION CHANGES IN WHOLE BLOOD AND CD16+ NEUTROPHILS IN RESPONSE TO CHRONIC FOLIC ACID SUPPLEMENTATION IN WOMEN OF CHILD-BEARING AGE

by

DEANNA SHADE

Major Professor: Dorothy Hausman
Committee: Lynn Bailey
Richard Meagher

Electronic Version Approved:

Julie Coffield
Interim Dean of the Graduate School
The University of Georgia
August 2014
ACKNOWLEDGEMENTS

I would like to thank each of the members of my committee and the folate research group for their dedication to the success of both myself as a student and this research project.

Dr. Hausman, thank you for providing me with a nurturing and well-rounded learning environment. I appreciate each of the challenges and goals you set for me, but most of all, I appreciate the tremendous support you provided these past two years. It is second to none!

Dr. Bailey, thank you for being an inspiration to me. Your passion for research and leading others will continue to motivate me in pursuing my dreams – whatever they may be.

Dr. Meagher, your appetite for learning has taught me not to hold back from opening new doors and that it is possible to be profoundly self-confident yet remarkably patient and humble.

Dr. Hea Jin Park, thank you for jumping right in to our project last year. I am so thankful to have had your input and encouragement throughout the study and especially during the writing process.

Finally, I would like to thank each of the students that helped with this project: Natalie Hohos, Courtney Alvis, Meagan Patterson, and Arielle Weekley. We could not have done it without you!
TABLE OF CONTENTS

ACKNOWLEDGEMENTS ........................................................................................................... iv

LIST OF TABLES ....................................................................................................................... viii

CHAPTER

1 INTRODUCTION ................................................................................................................1

References..................................................................................................................................5

2 LITERATURE REVIEW ......................................................................................................7

Folate chemistry and one-carbon metabolism ......................................................................7

Genetic variation....................................................................................................................8

Folate bioavailability ..........................................................................................................9

Folate recommendations and intake ..................................................................................10

Folate fortification in the US food supply ..........................................................................11

Folate status and birth defect risk .....................................................................................12

DNA methylation.................................................................................................................13
DNA METHYLATION CHANGES IN WHOLE BLOOD AND CD16+ NEUTROPHILS IN RESPONSE TO CHRONIC FOLIC ACID SUPPLEMENTATION IN WOMEN OF CHILD-BEARING AGE

Abstract

Introduction

Materials and methods

Results

Discussion

Conclusion

Results tables

References
### References

References .................................................................................................................................. 53

### APPENDICES

APPENDICES .................................................................................................................................. 54

<table>
<thead>
<tr>
<th>Appendix</th>
<th>Title</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>STUDY FLYER</td>
<td>55</td>
</tr>
<tr>
<td>B</td>
<td>TELEPHONE SCRIPT</td>
<td>57</td>
</tr>
<tr>
<td>C</td>
<td>TELEPHONE QUESTIONNAIRE</td>
<td>61</td>
</tr>
<tr>
<td>D</td>
<td>CONSENT FORM: SCREENING</td>
<td>66</td>
</tr>
<tr>
<td>E</td>
<td>CONSENT FORM: INTERVENTION</td>
<td>72</td>
</tr>
<tr>
<td>F</td>
<td>OVERALL STUDY INSTRUCTIONS</td>
<td>80</td>
</tr>
<tr>
<td>G</td>
<td>FOLATE FOOD TABLE</td>
<td>82</td>
</tr>
<tr>
<td>H</td>
<td>ASA24 INSTRUCTIONS</td>
<td>84</td>
</tr>
<tr>
<td>I</td>
<td>PARTICIPANT CHARACTERISTICS</td>
<td>86</td>
</tr>
</tbody>
</table>
LIST OF TABLES

Table 1: Demographic and clinical characteristics of study subjects ............................................38
Table 2: Number and pattern of changes in DNA methylation in whole blood (WB) and CD16+ neutrophils (CD16) after folic acid supplementation (p<0.05) ......................................................39
Table 3: Genes corresponding to associated CpG sites found in both whole blood (WB) and CD16+ neutrophils (CD16) and pattern of differential methylation changes .................................................40
Table 4: Gene ontology results for genes associated with folic acid supplementation in whole blood ..............................................................................................................................................41
Table 5: Gene ontology results for genes associated with folic acid supplementation in CD16+ neutrophils ......................................................................................................................................43
CHAPTER 1

INTRODUCTION

Folate is an essential water-soluble vitamin found naturally in food and synthetically as folic acid in fortified foods and supplements. It is well established that periconceptional intake of folic acid reduces the risk for neural tube defects (NTDs) (Berry et al 1999). Therefore women capable of becoming pregnant are recommended to consume 400 µg of folic acid per day (from supplements and/or fortified foods) in addition to normal dietary intake (IOM 1998). In 1996, the FDA mandated that enriched cereal grain products be fortified with 140 µg of folic acid per 100g of flour (FDA 1996) to ensure women consumed adequate folic acid. Since folic acid fortification in the US food supply was fully implemented in 1998, the prevalence of low serum folate (<10 nmol/L) concentrations has decreased from 24% to ≤1% (Pfeiffer et al 2012). Consequently, there has been a 19-31% reduction in the overall prevalence of NTDs (Honein et al 2001, Williams et al 2002). The biological mechanism by which folate contributes to NTD risk reduction is unknown; however, an understanding of the role of epigenetic mechanisms, such as DNA methylation, in the etiology of NTDs is emerging both from the study of human and mouse models (Greene et al 2011).

DNA methylation involves the covalent addition of a methyl group (CH₃) in position 5 of a cytosine (C) when this nucleotide occurs next to a guanine (G) forming a CpG site. This methyl group is enzymatically transferred from S-adenosylmethionine (SAM), a cofactor in folate metabolism, by DNA methyltransferase (DNMT) to form 5-methylcytosine (5mC) in genomic
Together with other epigenetic mechanisms, DNA methylation regulates expression of genes, a mechanism that is critical for normal development, differentiation, and homeostasis (Deaton and Bird 2011).

Since folate is a key source of the one carbon group used to methylate DNA, it is important to understand the relationship between folate status and DNA methylation. Controlled intervention studies have demonstrated that changes in folate status may be associated with variation in global DNA methylation, however, according to a recent review by Crider et al (2012), research findings on DNA methylation and folate status vary widely. For example, controlled feeding trials involving folate depletion and repletion have measured global DNA methylation response in whole blood and have yielded mixed results. Axume et al (2007) reported that DNA methylation levels did not vary significantly upon folate depletion or repletion except in subjects with MTHFR 677TT genotype where methylation decreased (p<0.05) following repletion whereas Shelnutt et al (2004) reported increases in DNA methylation only in MTHFR677 TT subjects upon repletion. Additionally in a folic acid supplementation study by Crider et al (2011), global DNA methylation decreased by 14% after 1 month and 3 months after supplement withdrawal methylation was found to decrease an additional 23%; however these changes were found only in coagulated blood samples, and no changes in mean methylation levels were found in uncoagulated blood samples.

DNA methylation studies in human subjects are commonly conducted in whole blood, on the presumption that DNA containing white blood cells are epigenetically reprogrammed in response to changes in nutrients, toxins, and various signals in the serum. However, whole blood contains a mixed population of seven major white blood cells (WBC) derived from two cell lineages. Granulocytes (60% of WBC), include neutrophils and eosinophils, while peripheral
blood mononuclear cells (PBMCs; 40% of WBC), include T cells, B cells, and NK cells and monocytes. It is suggested that the use of whole blood in DNA methylation analysis limits the specificity and significance of results, because specific differences in methylation of any one leukocyte type are averaged out when they are mixed together with the methylation differences of the six other cell types (Adalsteinsson et al 2012; Renius et al 2012). Therefore, analysis of single cell types may provide more valuable data than whole blood for identifying changes in DNA methylation response.

CD16+ neutrophils were selected for this study since the folate receptors on neutrophils have been well characterized (Ross et al 1999; Hao et al 2003; Hoier-Madsen et al 2008) and because neutrophils are the most abundant leukocyte in the blood (60% of all white blood cells). Cluster of differentiation marker 16 (CD16) is an antigen on the surface of most neutrophils. It is recognized by anti-CD16 antibodies, and these antibodies can be used to affinity tag or immunoaffinity purify CD16+ cells.

This study is significant because single cell DNA methylation analysis has the potential to strengthen the argument for folate’s impact on genetic regulation by generating more robust epigenetic data than ever before. In addition, this is the first study to use a genome-wide approach for measuring DNA methylation as a biomarker of folate status in response to folic acid supplementation in both whole blood and CD16+ neutrophils.

The research question asks if genome-wide DNA methylation is related to a change in folate status and whether it is different in CD16 neutrophils than in whole blood. The hypothesis is that genome-wide DNA methylation is related to change in folate status and the response is different in CD16+ neutrophils than in whole blood. The first specific aim of the study was to
determine the genome-wide DNA methylation response to folic acid supplementation. It is hypothesized that variations in serum folate concentration will be associated with differential DNA methylation, because of the role of this nutrient in one-carbon metabolism. The second specific aim was to determine if DNA methylation in a single cell type responds differently than whole blood in response to folic acid supplementation. It is hypothesized that single cell types will identify more changes in DNA methylation than whole blood in response to folate supplementation, because cell heterogeneity may have the potential to confound DNA methylation measurements.

Following the introduction, this thesis will contain additional chapters. Chapter 2 is a review of the literature on folate and DNA methylation. Topics covered on folate include its chemistry, metabolism, bioavailability, recommendations and intake, food supply fortification, and birth defect risk. In addition, topics related to DNA methylation include its mechanisms, folate’s biological role, single cell types as epigenetic reporters, CD16+ neutrophil as a biomarker of folate status, and the microarray for genome-wide analysis. Chapter 3 is a manuscript on the DNA methylation changes in whole blood and CD16+ neutrophils in response to chronic folic acid supplementation in women of child-bearing age that will be submitted to the Plos One journal. This chapter includes an introduction, the methods, results, and discussion. Finally, Chapter 4 is a conclusion that summarizes the findings of this study and presents general conclusions and suggestions for future research.
References


Axume J, Smith SS, Pogribny IP, Moriarty DsJ, Caudill MA. Global leukocyte DNA methylation is similar in African American and Caucasian women under conditions of controlled folate intake. Epigenetics 2007;2(1):66-68.


Folate chemistry and one-carbon metabolism

Folate is the generic name for the water soluble B vitamin with structural features including a pteridine bicyclic ring system, \textit{p}-aminobenzoic acid, and one or more glutamic acid residues. Folate refers to the naturally occurring reduced polyglutamate form whereas folic acid refers to the synthetic fully oxidized monoglutamate form.

Folic acid metabolism involves the reduction of the pyrazine ring of the pterin moiety to the coenzymatically active tetrahydrofolate (THF) form, the elongation of the glutamate chain by the addition of glutamate residues, and the acquisition and oxidation or reduction of one-carbon units at the N-5 or N-10 positions (Stipanuk and Caudill 2013).

Under normal dietary conditions, absorbed folate is metabolized in the intestine or liver to 5-methyltetrahydrofolate (5-methylTHF). 5-methylTHF is the primary folate constituent taken up by non-hepatic tissues where it must be polyglutamated for cellular retention. Folate pathways function interdependently in three cellular compartments including the cytoplasm, nucleus, and mitochondria (Fox and Stover 2008).

Inside the cell, THF polyglutamate coenzymes accept and donate one-carbons in a network of reactions known as folate-mediated one carbon metabolism (Stover 2009). Major pathways of folate-mediated one-carbon metabolism include de novo purine biosynthesis, de
novo thymidylate biosynthesis, and the remethylation cycle to form methionine. In this remethylation cycle, vitamin B12 (cobalamin) dependent methionine synthase (MS or MTR) transfers the methyl group (CH$_3$) from 5-methylTHF to homocysteine to form methionine, an essential amino acid. Methionine can then be adenosylated to form S-adenosylmethionine (SAM), a cofactor and methyl group donor for numerous methylation reactions including the methylation of DNA, RNA, and other small molecules (Stover 2009). Folate deficiency has been associated with increased homocysteine concentrations (Kang et al 1987), because folate is a limiting factor in this remethylation cycle.

**Genetic variation**

Genetic variation within genes involved in folate metabolism has the potential to alter development of diseases such as NTDs, cardiovascular disease, cancer, and dementia (Christensen and Rozen 2009). Variation is commonly studied in the form of genetic polymorphisms, which can be defined as variants present in greater than 1% of alleles in a population. Several genetic polymorphisms related to folate metabolism have been identified. These include but are not limited to MTHFR 677C→T, MTHFR 1298A→C, MTR2756A→G, MTRR66A→G, MTHFD1 1958G→A, SHMT1 1420C→T, and RFC1 80A→G. Due to their potential impact on population health, polymorphisms have been the subject of thorough investigation in the past 15 years with the majority of publications on variants in the MTHFR gene (Christensen and Rozen 2009).

Methylenetetrahydrofolate reductase (MTHFR), a fundamental enzyme in the folate metabolic pathway, catalyzes the irreversible reduction of methylene-THF to methyl-THF. MTHFR activity is negatively affected by a common genetic variant in the gene that codes for
MTHFR. The C→T single nucleotide polymorphism at base pair 677 causes an alanine to valine substitution that impairs stability and activity of MTHFR. Enzymatic activity has been shown to decrease by approximately 30% in heterozygotes (CT) and 60% in homozygotes (TT) (Weisberg 1998; Frosst 1995). This mutation is significantly associated with low plasma folate and high plasma homocysteine and is considered a risk factor for both NTDs (Vollset and Botto 2005) and cardiovascular disease (Wald et al 2002). The MTHFR677C→T polymorphism affects a large proportion of the US population with an estimated frequency of about 12% for the homozygous (TT) genotype (Botto and Yang 2000). The prevalence of MTHFR 677TT genotype has been reported to vary widely according region/ethnic group. For example, the frequency varies between 10-14% in North American whites and 6-18% in European whites. Additionally, frequencies are very high in Hispanics at 18-21% and in northern China at 18% but relatively low in African and African American Black populations at less than 2% (Leclerc et al. 2005). Adequate folate concentrations have been shown to modify the effect of MTHFR677C→T on NTD risk (Christensen et al. 1999). Therefore it is important to maintain optimal folate status in individuals with genetic variations in order to reduce potential negative consequences.

**Folate Bioavailability**

Natural food folates are a mixture of the reduced forms with a variable number of glutamate residues. The synthetic form, folic acid is a monoglutamate and is only found in the diet due to fortification and supplementation. Folic acid is more stable, resistant to degradation, and more bioavailable than food folate (Coleman et al 1975). Bioavailability can be defined as the proportion of an ingested nutrient that is absorbed and becomes available for metabolic processes or storage (McNulty and Pentiva 2009). The extent to which folic acid is more bioavailable than food folate is still controversial. Results of controlled long-term feeding trials
vary greatly, ranging between 30% (Hannon-Fletcher et al 2004) and 98% (Brouwer et al 1999) relative bioavailability of food folate to folic acid. These variations are often a result of diversity in biomarkers and the type of folate-containing food being provided. Factors that influence the bioavailability of food folates include: intestinal hydrolysis of polyglutamyl folates, the extent of conjugation of food folate sources, folate absorption in the small and large intestine, the food matrix and others (McNulty and Pentiva 2009).

The Institute of Medicine Panel on Folate, Other B Vitamins, and Choline (IOM 1998) reported that the bioavailability of folic acid is greater than that of natural food by a factor of 1.7. Therefore, to adjust for differences in folate bioavailability, the dietary reference intake (DRI) uses the unit dietary folate equivalents (DFE) where DFE is defined as the quantity of natural food folate plus 1.7 times the quantity of folic acid in the diet.

**Folate recommendations and intake**

According to the Institute of Medicine (IOM 1998) the recommended daily allowance (RDA) across the lifecycle varies from 150-300 µg DFE for children 1-13 year olds to 400 µg DFE for adult men and women. Women capable of becoming pregnant are recommended to consume 400 µg of folic acid per day (from supplements and/or fortified foods) in addition to normal dietary intake. The RDA for pregnant women is 600 µg DFE to cover accelerated demands placed on the supply of folate for DNA synthesis and the RDA for lactating women is 500 µg DFE to cover the amount of folate secreted in milk.

There is no evidence of adverse effects of high intakes of food folate; however, folic acid can mask the hematological response normally caused by B12 deficiency. Accordingly, the IOM
1998 set the tolerable upper intake level (UL) at 1000 μg DFE per day from fortified foods or supplements.

The National Health and Nutrition Examination Survey (NHANES) data from 2003–2006 provides a large amount of information about total folate intake in the US. Bailey et al (2010) showed that median (50%) total folate intake (in μgDFEs) was 574 μg for women and 709 μg for men age 31-50. Additionally 17% of 19–30 year olds and 15% of 31–50 year olds did not meet the estimated average requirement (EAR). Both total folate and folic acid intakes have been reported to be the highest for those aged ≥ 50 y and 5% exceed the UL. In addition, approximately 53% of the US population uses dietary supplements and 34.5% uses dietary supplements that contained folic acid (Bailey et al 2010).

Folate fortification in the US food supply

Fortification is usually regarded as “the deliberate addition of one or more micronutrients to particular foods, so as to increase the intake of these micronutrient(s) in order to correct or prevent a demonstrated deficiency and provide a health benefit” (WHO 2006). This method has been used around the world as a nutrition intervention strategy that is cost effective and able to reach large numbers of the population in order to improve nutrition status (Mannar and Sankar 2004).

In the early 1990s it became widely accepted that adequate periconceptional consumption of folic acid reduces the risk for NTDs. In an effort to ensure that women consumed adequate folic acid, the Food and Drug Administration mandated folic acid fortification of all enriched cereal grain products in 1996, making the United States a pioneer in folic acid fortification. The mandate states that enriched cereal grain products including flour, rice, breads, rolls and buns,
pasta, corn grits, corn meal, farina, and noodle products should be fortified with 140 µg of folic acid per 100 g of flour (FDA 1996). Fortification was fully implemented by January 1998.

Folic acid fortification policies have effectively increased overall dietary intake of folic acid, increased blood folate concentrations, and reduced the prevalence rate of NTDs. Studies have shown that intake of folic acid has increased since fortification, however data sources vary widely on the estimated amounts (Berry et al 2010). A comparison of NHANES data from 1988-1994 with those from 1999-2004, reveal an overall increase of 7.5 ng/mL in median serum folate concentration after fortification (Pfeiffer et al 2007). The mandatory fortification resulted in substantial reductions in the prevalence of NTDs by 19-31% (Honein et al 2001, Williams et al 2002).

**Folate status and birth defect risk**

NTDs occur when the neural tube fails to close early in embryonic development. NTDs are believed to be the result of both genetic and environmental influences (Detrait et al 2005), however due to the complexity of human embryological development identifying the specific causal factors has proven difficult. Some known risk factors for NTDs include socioeconomic status, MTHFR677C→T polymorphism, low blood concentrations of folate and B12, and obesity (Botto et al 1999). Two of the most common and severe forms of NTDs are spina bifida and anencephaly. Infants born with anencephaly die shortly after birth, whereas infants with spina bifida survive with severe, lifelong disabilities. The prevalence of NTDs in the US was estimated to be 37.8 per 100,000 live births according to birth certificate reports prior to 1998 (Honein et al 2001).
It is well established that periconceptional intake of folic acid reduces the risk for NTDs (Berry et al 1999). Since folic acid fortification in the US food supply began in 1998, the prevalence of low serum (<10 nmol/L) folate concentrations has decreased from 24% to <1% (Pfeiffer et al 2012). Concomitantly, there has been a 19-31% reduction in the overall prevalence of NTDs (Honein et al 2001, Williams et al 2002). The biological mechanism by which folic acid contributes to NTD risk reduction is unknown; however, an epigenetic explanation has gained considerable attention. The role of epigenetic mechanisms, such as DNA methylation, in the etiology of NTDs is emerging both from the study of humans and mouse models (Greene et al 2011).

DNA methylation

Epigenetics is the study of heritable changes in phenotype or gene expression that do not involve changes in DNA sequence (Portella and Esteller 2010). Epigenetic regulation in mammals occurs through various mechanisms including DNA methylation, post-translational modification of histones and nucleosome positioning. According to a review by Portella and Esteller (2010), DNA methylation is the most widely studied epigenetic modification in humans. DNA methylation involves the covalent addition of a methyl group (CH₃) in position 5 of a cytosine (C) when this nucleotide occurs next to a guanine (G) forming a CpG site. This methyl group is enzymatically transferred from S-adenosylmethionine (SAM), a cofactor in folate metabolism, by DNA methyltransferase (DNMT) to form 5-methylcytosine (5-MC) in genomic DNA. A CpG site can be methylated, unmethylated or hemi-methylated depending on the chromosomal region, cell type, developmental stage, alleles and parent-of-origin (Reinius 2012).
There are around 28 million CpG sites in the human genome, however certain gene regions have approximately 10-fold higher frequency of the CpG dinucleotide. These regions are referred to as CpG islands and are on average 1000 base pairs (bp) long and show frequent absence of DNA methylation (Deaton and Bird 2011). Together with other epigenetic mechanisms, DNA methylation functions as a switch that turns relevant genes on and off, a mechanism that is crucial in development, differentiation and homeostasis (Deaton and Bird 2011). There are various mechanisms by which DNA methylation can inhibit gene expression. These include but are not limited to, indirect inhibition by the recruitment of methyl-CpG-binding domain proteins and direct inhibition of transcription by precluding the recruitment of DNA binding proteins from their target sites (Portella and Esteller 2010).

Folate status and DNA methylation

Folate, a methyl group donor in DNA methylation, is believed to play a role in epigenetic regulation; however, according to a recent review research findings on DNA methylation and folate status vary widely (Crider et al 2012). Controlled feeding trials (7 wk depletion, 7 wk repletion) that measure global DNA methylation have yielded mixed results. Shelnutt et al (2004), measured genomic DNA methylation in whole blood from women (age 20-30 years; N=41). Results suggested that following depletion, global methylation tended to decrease, however this finding was not significant (p<0.08). Upon repletion, significant increases ($P = 0.03$) were found only in subjects with the MTHFR677 TT genotype. Additionally, Axume et al (2007), measured global DNA methylation following folic acid depletion/repletion in woman (age 18-45 years; N=43). The authors reported that DNA methylation levels did not vary significantly upon folate depletion or repletion except in subjects with MTHFR677TT genotype
where methylation decreased (p<0.05) following repletion. It has been suggested that methylation activity may decrease when MTHFR activity is reduced because of decreased SAM, a known inhibitor of DNA methyltransferases (James 2005).

An additional study by Friso et al (2002) evaluated the impact of the MTHFR 677 polymorphism on DNA methylation combined with the influence of folate status. Using 105 TT (mutation) subjects and 187 CC (wild-type) subjects, the authors reported that TT genotypes had a lower level of genomic DNA methylation compared to those with the CC genotype (P < 0.0001). Additionally, when analyzed according to folate status, only the TT subjects with low levels of folate accounted for the decreased DNA methylation (P < 0.0001). These results suggest that the MTHFR 677 polymorphism effects DNA methylation through an interaction with folate status.

Additionally, a 6 month supplemental intervention trial in woman (30.4 ± 4 years) using 100 μg, 400 μg, or 4000 μg folic acid/day produced a 14% decrease in global DNA methylation after 1 month of folic acid supplementation. Methylation was found to decrease an additional 23%, 3 months after supplement withdrawal. These changes were found only in coagulated blood samples, and no changes in mean methylation levels were found in uncoagulated blood samples (Crider et al 2011). Other researchers have also reported decreases in DNA methylation following folate supplementation in the liver and brain of rodent models (Finnel et al 2002, Sie et al 2013). Finnell et al (2002) argued that decreased methylation may be due to folate’s competitive inhibition of glycine hydroxymethyltransferase (GMT) which limits the activity of the homocysteine remethylation cycle needed to regenerate S-adenosylmethionine (SAM), which is the methyl donor for DNA methyltransferases. Additionally, Sie et al (2013), reasoned that hypomethylation may be partly explained by down regulation of DNA methyltransferase
(DNMT), the enzyme regulating transfer of methyl groups from SAM to DNA or by preferential movement of methyl groups to the nucleotide synthesis pathway over the methylation pathway. These hypotheses highlight the complexity of folate’s biochemical role in epigenetic control and the need for more research in this area.

**Single cell types as epigenetic reporters**

Epigenetic studies are commonly conducted in a mixed population of white blood cells, which can confound the outcome of DNA methylation measurements. Reinius et al (2012) examined the DNA methylation profiles for 7 purified white blood cell types in 6 men and reported that single cell types differed from whole blood by up to 125,713 (26%) methylation sites indicating that “whole blood methylation results might be unintelligible.” In Adalsteinsson et al (2012), the DNA methylation in 4 genes with known inter-individual variation was measured in whole blood and compared to two blood cell fractions, peripheral blood mononuclear cells (PBMC) and polymorphonuclear cells (PMN). Cell heterogeneity explained up to 40% of the inter-individual variation in whole blood DNA methylation levels in the hematopoietically expressed homeobox (HHEX) CpG island. Jeffries et al (2011) compared the genome-wide DNA methylation of CD4+ T cells from 12 lupus patients to 12 controls and found differences for 341 CG sites. Thus, there is evidence that single cell types may provide more valuable data than whole blood for identifying DNA methylation changes.

**CD16+ neutrophils**

In addition to macrophages and natural killer cells, neutrophils express the CD16+ antigen that is known to be a component of low affinity Fc receptor, FcγRIII. Depending on the cell type, this antigen has been shown to function by mediating phagocytosis and antibody-dependent cell-
mediated cytotoxicity (Janeway et al 2001). Neutrophils express folate receptors (Hoier-Madsen et al 2008) and are the most abundant leukocyte in the blood, making up about 60% of all white blood cells. Genome-wide DNA methylation in 7 leukocyte types has been reported to differ from that in whole blood by 6-26%, with neutrophils differing the least (6%) (Reinius et al 2012). Additionally, it has been reported that granulocytes, which include neutrophils and eosinophils, have a very low fraction of methylated sites (5.2%), while peripheral blood mononuclear cells (PBMCs), which include T, B, and NK cells have a very high fraction of these sites significantly methylated (72.5%) (Reinius et al 2012). Additionally, neutrophils are known to have a relatively short circulating half-life of hours to a few days before migrating into tissue (Daniels et al 1979, Summers et al 2010) which may limit their response to serum status.

**Microarray for genome-wide analysis**

The Infinium Human Methylation450 BeadChip Array allows researchers to interrogate > 485,000 methylation sites throughout the human genome. It covers 99% of RefSeq genes with an average of 17 CpG sites per gene region distributed across the promoter, 5'UTR, first exon, gene body, and 3'UTR. Additionally, the array has a 12-sample per array format and requires only 500 ng-1000 ng of DNA input per sample. This tool is important for studying genome-wide epigenetic patterns associated with disease and offers a high-throughput, low cost alternative to more comprehensive sequencing-based methodologies (Pan et al 2012).

This array has been validated using 4 human cancer cell lines where both the Infinium HumanMethylation450k BeadChip and pyro-sequencing techniques were compared. It was reported that β-values represent a very good measure of the methylation status for the majority of CpG sites (Roessler et al 2012). Additionally, it has been reported that Infinium 450K data can
be vastly improved by careful processing to correct for known limitations and batch effects (Pan et al 2012).

Summary

A review of the literature has yielded considerable research knowledge on folate and DNA methylation. Areas of interest relevant to folate included its chemistry, metabolism, bioavailability, recommendations and intake, food supply fortification, and birth defect risk. Areas of interest relevant to DNA methylation included its mechanisms, folate’s role, single cell types as epigenetic reporters, CD16+ neutrophil as a biomarker of folate status, and the microarray for genome-wide analysis. Several gaps in the research knowledge have been identified and include the biological processes by which folic acid supplementation contribute to NTD risk deduction, how changes in folate status affect DNA methylation patterns in single white blood cells types, and the possible implications of DNA hypomethylation as a result of high folate intake.
References

Axume J, Smith SS, Pogribny IP, Moriarty DsJ, Caudill MA. Global leukocyte DNA
methylation is similar in African American and Caucasian women under conditions of controlled

Bailey RL, Dodd KW, Gahche JJ, Dwyer JT, McDowell MA, et al. Total folate and folic acid
2010;91:231-237.


Berry RJ, Mulinare J, Hamner HC. Folic acid fortification: Neural tube defect risk reduction – a

Botto LD, Moore CA, Khoury MJ, Erickson JD. Neural-tube defects. New England Journal of

Botto L, Yang Q. 5,10-Methylenetetrahydrofolate reductase gene variants and congenital

Brouwer IA, van Dusseldorp M, West CE, Meyboom S, Thomas CM, et al. Dietary folate from
vegetables and citrus fruit decreases plasma homocysteine concentrations in humans in a dietary

Christensen KE, Arbour L, Tran P, Leclerc D, Sabbaghian N, et al. Genetic polymorphisms in
methylenetetrahydrofolate reductase and methionine synthase, folate levels in red blood cells,

Christensen KE, and Rozen R. Genetic variation: Effect on folate metabolism and health. 2nd.

Coleman N, Green R, Metz J. Prevention of deficiency by food fortification. II Absorption of

Crider KS, Quinlivan EP, Berry RJ, Hao L, Li Z, et al. Genomic DNA methylation changes in
response to folic acid supplementation in a population-based intervention study among women of

Crider KS, Yang TP, Berry RJ, Bailey LB. Folate and DNA methylation: A review of molecular

Daniels V, Wheater R, and Burkitt H. Functional Histology: A text and colour atlas. In:
Functional Histology: A text and colour atlas, Editor, eds. 1979 (Edinburgh: Churchill
Livinstone).


Meagher RB and Müssar KJ. The influence of DNA sequence on epigenome-induced pathologies. Epigenetics Chromatin 2012;5(11):1-19


CHAPTER 3

DNA METHYLATION CHANGES IN WHOLE BLOOD AND CD16+ NEUTROPHILS IN RESPONSE TO CHRONIC FOLIC ACID SUPPLEMENTATION IN WOMEN OF CHILD-BEARING AGE

\[1\]

\[1\]Shade DC, Park HJ, Hausman DB, Hohos N, Meagher RB, Kauwell GPA, Lewis RD, Smith AK, Bailey LB. To be submitted to *Plos One*. 
Abstract

Folate, a water-soluble vitamin, is a key source of one-carbon groups for DNA methylation, but studies of the DNA methylation response to supplemental folic acid (FA) show inconsistent results. These studies are commonly conducted using whole blood (WB), which contains a mixed population of white blood cells (leukocytes) that have been shown to confound results. We hypothesize that a single white cell type may provide more valuable data than WB for identifying DNA methylation response to FA supplementation. Therefore, the aim of this study was to determine if DNA methylation in a single dominant white blood cell type, neutrophils, would respond differently than WB in response to FA supplementation. This study was performed in normal weight (BMI 18.5 – 24.9 kg/m²) women (18 – 35 y; n = 12), with blood samples taken before and after 8 weeks of supplementation with 800 μg/day of FA. DNA methylation patterns from WB and isolated CD16+ neutrophils (CD16) were measured across 485,000 CpG sites. For each CpG site, we assessed the proportion of DNA methylation that changed over the 8 week supplementation and identified 7839 and 8317 CpG sites that changed (P<0.05) in WB and CD16, respectively. DNA methylation decreased in 77.2% (WB) and 65.6% (CD16) of these sites, respectively. While the overall changes in methylation were consistent for 58.2% of CpG sites, only 188 of the sites that changed in response to FA supplementation (p<0.05) changed in the same direction in both the WB and CD16 samples. These results suggest that the genome-wide DNA methylation response to FA supplementation is different in WB and CD16 and that a single WBC type may function as a more reliable epigenetic reporter than WB. Future studies should measure DNA methylation response to FA supplementation in other white blood cell types.
Introduction

Folate is an essential water-soluble vitamin found naturally in food and synthetically in fortified foods and supplements. It is well established that periconceptional intake of folic acid reduces the risk for neural tube defects (NTDs) (Berry et al 1999). Since folic acid fortification in the US food supply began in 1998, the prevalence of low serum (<10 nmol/L) folate concentrations has decreased from 24% to ≤1% (Pfeiffer et al 2012). Concomitantly, there has been a 19-31% reduction in the overall prevalence of NTDs (Honein et al 2001, Williams et al 2002). The biological mechanism by which improved folate status may contribute to NTD risk reduction is unknown; however, and understanding of the role of epigenetic mechanisms, such as DNA methylation, in the etiology of NTDs is emerging both from the study of human and mouse models (Greene et al 2011).

DNA methylation involves the covalent addition of a methyl group (CH$_3$) in position 5 of cytosine. In adults, this primarily occurs in the context of a cytosine-guanine dinucleotide, commonly referred to as a CpG site. Together with other epigenetic mechanisms, DNA methylation regulates expression of genes, a mechanism that is critical for normal development, differentiation, and homeostasis (Deaton and Bird 2011).

Since folate is a key source of the one carbon group used to methylate DNA, it is important to understand the relationship between folate status and DNA methylation. Controlled intervention studies have demonstrated that changes in folate status may be associated with variation in global DNA methylation however, according to a recent review (Crider et al 2012), research findings on DNA methylation and folate status vary widely. For example, controlled feeding trials involving folate depletion and repletion have measured global DNA methylation response in whole blood and have yielded mixed results. Shelnutt et al (2004) reported increases
in DNA methylation only in MTHFR677 TT subjects upon repletion while Axume et al (2007) reported that DNA methylation levels did not vary significantly upon folate depletion or repletion. Additionally in a folic acid supplementation study, global DNA methylation decreased by 14% after 1 month, and 3 months after supplement withdrawal methylation was found to decrease an additional 23%. These changes were found only in coagulated blood samples, and no changes in mean methylation levels were found in uncoagulated blood samples (Crider et al 2011).

DNA methylation studies in human subjects are commonly conducted in whole blood, on the presumption that DNA containing white cells are epigenetically reprogrammed in response to changes in nutrients, toxins, and various signals in the serum. However, whole blood contains a mixed population of seven major white blood cells. It is suggested that the use of whole blood DNA methylation analysis limits the specificity and significance of results, because specific differences in methylation of any one leukocyte type are averaged out when they are mixed together with the methylation differences of the six other cell types (Adalsteinsson et al 2012; Reinius et al 2012). Therefore, analysis of single cell types may provide more valuable data than whole blood for identifying changes in DNA methylation response.

The folate receptors on neutrophils have been well characterized (Ross et al 1999; Hao et al 2003; Hoier-Madsen et al 2008) and neutrophils are the most abundant leukocyte in the blood (60% of all white blood cells), suggesting that they might be an ideal cell type to monitor folate status in the blood. Cluster of Differentiation marker 16 (CD16) is an antigen on the surface of most neutrophils. It is recognized by anti-CD16 antibodies, and these antibodies can be used to affinity tag or immunoaffinity purify CD16+ cells.
The aim of this study was to measure the genome-wide DNA methylation response to folic acid supplementation and to determine if a single cell type could serve as a more reliable epigenetic reporter of DNA methylation changes. This is the first study to use a genome-wide approach for measuring DNA methylation as a biomarker of folate status in response to folic acid supplementation in both whole blood and CD16+ neutrophils.

Materials and Methods

Participants

Twelve healthy normal weight (BMI 18.5-24.9 kg/m$^2$) adult female subjects (18-35 years old) were recruited and successfully screened for this study. Inclusion criteria were no pregnancy within the past year, no use of dietary supplements within the past 30 days, no chronic disease or prescription drug use other than oral contraceptives, no smoking or heavy alcohol consumption, and no weight change greater than 10% in the past 6 months. Initially 50 women who met inclusion criteria were screened and only subjects with serum folate <60 nmol/L and the CC or CT $MTHFR$ 677 (rs1801133) genotype (sequenced using real-time PCR by the Georgia Genomics Facility in Athens, GA) were eligible. To limit genetic variation, only individuals self-identified as Caucasians were selected. The University of Georgia Institutional Review Board approved this protocol and all subjects provided written informed consent, after being made cognizant as to the design of the study. This study was registered at ClinicalTrials.gov (#NCT01841658).

Intervention and vitamin supplement protocol

Participants were provided with folic acid supplements (Douglas Laboratories, Pittsburgh, PA, USA) and were instructed to take 800 µg daily for 8 weeks. The folic acid
content of these supplements was verified at a third-party laboratory (Covance, Princeton, NJ). The supplements were packaged as individual 4-week supplies and compliance was determined by return pill count. Additionally, participants were provided with a list of high folate containing foods to avoid throughout the intervention in an attempt to reduce day-to-day and between subject variations in dietary folate intake.

Participant height and weight were obtained at baseline and 8 weeks using standard protocols and used for calculation of BMI (kg/m\(^2\)). Percent body fat was determined by dual-energy X-ray absorptiometry (DXA) (Hologic Discovery A, Hologic Inc., Waltham, MA). Participants completed three sets of Automated Self-Administered 24-hour dietary recall (ASA24) (Subar et al 2012), for three non-consecutive days, including one weekend day during baseline, at weeks 4 and 8.

**Blood collection, cell isolation, and DNA extraction**

Blood samples were collected, after an overnight fast, at baseline and after 8 weeks of supplementation. Serum folate concentrations were determined by microbiological assay based on the growth of the folate auxitroph *Lactobacillus rhamnosus* (Tamura 1990, Horne and Patterson 1988). The inter- and intra- assay coefficients of variation were 7.7\% and 5.5\% respectively. Aliquots of whole blood from each time point were stored on ice and processed within 4 hours: a 2 ml sample for whole blood leukocyte extraction and, a 10 ml sample for neutrophil isolation. Neutrophil populations were purified from an enriched leukocyte population by positive selection using CD16 antibodies (Sc-19620 Santa Cruz Biotechnology, USA) coupled to Protein G paramagnetic Dynabeads (#10004D Life Technologies, USA). Isolated cells were stored at -80°C until genomic DNA extraction using a DNeasy Kit (QIAGEN, Germany).
**Bisulfite treatment and DNA methylation measurement**

Forty-eight samples of genomic DNA (1 μg) were bisulfite-converted using Zymo EZ DNA Methylation-Gold kit (Zymo Research, USA) and analyzed using the Infinium HumanMethylation450 BeadChip (Illumina, San Diego, CA) according to the manufacturer’s instructions (Pan et al 2012, Roessler et al 2012). The HumanMethylation450 BeadChip was used to interrogate >485,000 independent CpG sites throughout the genome with 99% coverage of RefSeq Genes. Samples with probe detection call rates < 90% were excluded, as were those with an average intensity value of either < 50% of the experiment-wide sample mean or < 2,000 arbitrary units. Data points with detection p-values > 0.001 were set to missing. Estimated DNA methylation proportions or β-values were then computed for each CpG site and sampled as the ratio of methylated signal to total (methylated + unmethylated) signal. Quality control parameters were implemented using CpGassoc, (Barfield et al 2012). Based on this, 1500 individual probes (less than 0.4%) were excluded from the analysis. No individual subject samples were excluded.

**Statistical Methods**

SAS version 9.1 (Cary, NC) was used to perform a paired samples t-test to compare participant characteristics between baseline and week 8. MethLAB (Kilaru et al 2012) was used to test for association with folic acid supplementation via linear regressions that modeled β-values as the outcome and folic acid supplementation as the independent variable. For each CpG site, the proportion of DNA methylation that changed over the 8 week intervention was evaluated by fitting a separate linear model. Additionally, we compared the overall direction of t-statistics for comparable analyses of whole blood and CD16+ neutrophils using a chi-square test.
Gene Ontology analysis was performed using GeneCodis (Nogales-Cadenas et al 2009, Carmona-Saez et al 2007) for the genes corresponding to CpG sites that showed evidence of differential methylation (p<0.005) after supplementation. This program matches gene lists with common biological processes to determine if any specific biological processes were enriched in the data set. Significance (p<0.05) was determined by a hypergeometric p-value that is obtained through FDR correction.

Results

A total of twelve normal weight women participated in this study. The women served as their own controls and did not differ significantly in average age, height, weight, and BMI from baseline to week 8 of the intervention (Table 1). Overall, subjects were compliant and met 96.6% (772.6 ± 23.9 μg/d) of the daily folic acid supplement goal. Dietary folate intake decreased by 21% (p<0.021) from baseline (459.9 ± 99.5 μg/d) to week 8 (364.7 ± 116.16 μg/d) of the intervention likely due to the requested avoidance of high folate foods. The overall increase in folate intake throughout the 8 week period resulted in an 86% (p<0.001) increase in mean serum folate from baseline (38.4 ± 8.7nmol/L) to week 8 (71.4 ± 10.5nmol/L).

Overall, the directions of DNA methylation changes were more consistent in WB and CD16+ neutrophils than expected by chance (58.2% of sites; p<.0001). In whole blood (WB), DNA methylation of 7839 CpG sites (1.6%) in 4670 genes changed following folic acid supplementation (p<0.05; Table 2). In CD16+ neutrophils (CD16), DNA methylation in 8317 CpG sites (1.7%) in 4975 genes changed following supplementation. However, of the CpG sites that changed following supplementation, only 235 changed in both the WB and CD16 samples and 188 of these sites changed in the same direction. The majority of CpG sites that changed following supplementation decreased in methylation for WB (77.2%) and CD16 (65.6%).
To provide biological context to these findings, we evaluated the genes containing CpG sites whose methylation levels changed the most robustly (p<0.005) following folic acid supplementation and determined enrichment for specific biological processes of these genes using gene ontology. The most robust methylation changes were confined to 292 genes in WB and 367 genes in CD16; however CpG sites in only 11 of these genes responded in both WB and CD16 (Table 3). Further differences were observed in the direction of change in CpG site TargetID for all of these genes except ATF6B (activating transcription factor 6 beta) whose methylation decreased in both WB and C16. Gene ontology analysis revealed that 21 biological processes were enriched in WB (Table 4), and 24 were enriched in CD16 (Table 5). Of the 21 biological processes enriched in WB, 3 were related specifically to development including multicellular organismal development (GO:0007275), brain development (GO:0007420), and cell differentiation (GO:0030154) while others were involved in more general processes such as transport (GO:0006810) and signal transduction (GO:0007165). Of the 24 biological processes enriched in CD16, 2 were related specifically to development including brain development (GO:0007420) and lateral ventricle development (GO:0021670) while others were related to various processes such as protein localization (GO:0045176 and GO:0045184) and wound healing (GO:0042060). Additionally, several biological processes were found to overlap between WB and CD16 including actin cytoskeleton organization (GO:0030036), axon guidance (GO:0007411) and brain development (GO:0007420). The gene myosin, heavy chain 10, non-muscle (MYH10) was the only gene in common between these 3 biological processes.

**Discussion**

As a methyl donor, folate is known to play a role in DNA methylation however previous studies on global DNA methylation response to changes in folate status have yielded inconsistent
results. Based on controlled human feeding studies, researchers have reported decreased methylation following folate depletion (Rampersaud et al 2000), increased methylation following folate repletion (Shelnutt et al 2004), and no response to folate depletion/repletion (Axume et al 2007) while folic acid supplementation studies have revealed decreased DNA methylation in coagulated blood samples but no response in uncoagulated samples (Crider et al 2011). The majority of these studies have measured DNA methylation response in whole blood, which consists of multiple cell types whose DNA methylation patterns are widely distributed. The use of whole blood when evaluating DNA methylation response has the potential to average out the effect of folate status. Therefore, this study investigated, for the first time, the use of a single cell type as a novel reporter for DNA methylation changes in response to folic acid supplementation.

When comparing DNA methylation changes in CD16+ neutrophils to whole blood, we observed that the genome-wide response to folic acid supplementation is different.

CD16+ neutrophils where chosen as an epigenetic reporter in this study because they express folate receptors (Hoier-Madsen et al 2008) and are the most abundant leukocyte in the blood, making up about 60% of all white blood cells. Compared to whole blood, DNA methylation has been shown to differ the least in neutrophils (6%) compared to other blood leukocytes (9-26%) (Reinius et al 2012). Additionally, it has been reported that granulocytes, which include neutrophils and eosinophils, have a very low fraction of methylated sites (5.2%), while peripheral blood mononuclear cells (PBMCs), which include T cells, B cells, and NK cells have a very high fraction of these sites significantly methylated (72.5%) (Reinius et al 2012). Therefore, the high levels of methylation of PBMCs that make up 40% of the leukocyte population may negate the methylation data from neutrophils in whole blood samples, skewing the data away from the dominant cell types, granulocytes, to that of PBMCs. Therefore it is
hypothesized that when compared to whole blood, neutrophils will offer statistical improvements for measuring DNA methylation response to folic acid supplementation.

When comparing genome-wide DNA methylation response to folic acid supplementation in whole blood to CD16+ neutrophils, we observed that 7839 (WB) and 8317 (CD16) of the 484,039 CpG sites measured were associated with supplementation, however only about 3% of associated sites were found in both whole blood and CD16+ neutrophils. Even as the major white blood cell type, neutrophils still yielded very different results than whole blood after folic acid supplementation. Our data suggest that the source of DNA is a critical component in the interpretation of DNA methylation patterns from whole blood and that a single cell type may act as a novel epigenetic reporter of folate status.

Since folate is a key source of the one carbon group used to methylate DNA, an overall increase in DNA methylation was anticipated however we observed that the majority of associated CpG sites decreased in methylation in both whole blood (77.2%) and CD16+ neutrophils (65.6%) after 8 weeks of folic acid supplementation. Several previous folic acid supplementation studies in human and animal models have also observed a decrease in DNA methylation. Crider et al (2011) reported a 14% decrease in global DNA methylation after 1 month of folic acid supplementation and methylation was found to decrease an additional 23%, 3 months after supplement withdrawal. Interestingly, these changes were found only in coagulated blood samples, and no changes in mean methylation levels were found in uncoagulated blood samples. In contrast, neither WB nor CD16+ neutrophils samples in our study were allowed to coagulate yet we still found that the majority of CpG sites associated with folic acid supplementation decreased in methylation. Differing whole blood results between our study and the Crider study may be attributed to differences in measurement, where our study measured
genome-wide gene-specific methylation and Crider et al measured global DNA methylation. It is possible that, by using a genome-wide gene specific approach, our study was able to detect decreases in DNA methylation even in uncoagulated whole blood samples.

In rodent models, a decrease in DNA methylation in the liver and brain following folic acid supplementation has been reported (Finnell et al 2002, Sie et al 2013). It has been suggested that decreased methylation in response to folic acid supplementation may be partly explained by preferential movement of methyl groups to the thymidylate and purine synthesis pathways over the remethylation pathway. For example, Finnell et al (2002) suggested that decreased methylation may be due to folate’s competitive inhibition of glycine hydroxymethyltransferase (GMT) which limits the activity of the homocysteine remethylation cycle needed to regenerate S-adenosylmethionine (SAM), the methyl donor for DNA methyltransferases. In addition, Sie et al (2013) reasoned that hypomethylation may be attributed to upregulation of dihydrofolate reductase in certain situations that may increase thymidylate synthase activity at the expense of methylation reactions or to excess dihydrofolate which inhibits MTHFR and reduces availability of 5-methyltetrahydrofolate needed for the remethylation cycle. In addition, we hypothesize that decreased methylation may be the result of the higher concentrations of SAM, which have been associated with higher total folate concentrations (Reed et al 2006). SAM functions as an allosteric inhibitor of MTHFR (Yamada et al 2005) and decreased activity of MTHFR has been known to reduce DNA methylation (Friso et al 2002) while modestly increasing thymidine or purine synthesis (Reed et al 2006). These hypotheses highlight the complexity of folate’s biochemical role in epigenetic control and the need for more research in this area.

Gene ontology analysis indicated 21 unique biological processes that were enriched in whole blood (WB) and 24 unique biological processes that were enriched in CD16+ neutrophils
(CD16). Although several of these biological processes were related to development in WB (multicellular organismal development, brain development, and cell differentiation) and in CD16 (brain development and lateral ventricle development), none were found to be typically associated with folate metabolism. Overall, the associated biological processes varied greatly between WB and CD16, with only 3 biological processes found to be associated with both WB and CD16. These include actin cytoskeleton organization (GO:0030036), axon guidance (GO:0007411) and brain development (GO:0007420). Additionally, only one gene (MYH10-myosin, heavy chain 10, non-muscle) was found in common between the 3 overlapping biological processes. These results suggest that the biological processes indicated for the genes which contained the most robustly associated CpG sites were very different in CD16 and WB. Therefore, these data provide additional evidence that CD16+ neutrophils have a different response to folic acid supplementation than WB.

Our study was strengthened by several factors. Overall mean folate intake did not vary greatly in subjects, due to high compliance of folic acid supplement intake and limited day-to-day and between subject variations in dietary folate intake due to requested avoidance of high-folate foods throughout the intervention. Additionally, the dose of folic acid is physiologically relevant, since it is recommended that women of child-bearing age consume 400 µg folic acid per day from fortified foods and/or supplements in addition to consuming folate in a varied diet (National Research Council 1998) and many pregnant women have been reported to use multivitamin supplements containing a mean of 817 µg/d folic acid per day (Branum et al 2013). There were also several limitations of this study. First, our study subject’s mean baseline serum concentrations (40.8 ± 8.8nmol/L) were well above that which is considered to be deficient (<10 nmol/L) (Selhub et al 2008) and we may have observed a more significant response if baseline
values had been lower. Second, a potential disadvantage of using neutrophils as epigenetic reporters might be their relatively short circulating half-life of hours to a few days before migrating into tissue (Summers et al 2010, Daniels et al 1979). This could result in DNA methylation patterns that reflect their bone marrow origin rather than their blood environment. Third, this study had a small sample size and needs to be repeated in a larger population to allow detection of more robust effects on site-specific DNA methylation.

Conclusion

In summary, we observed that the genome-wide DNA methylation response to an 8-week folic acid supplement intervention was different in whole blood and CD16+ neutrophils. This result provides additional evidence that single cell types may confound the outcome of DNA methylation measurements using the mix of white blood cells found in whole blood. In order to determine if a single cell type could act as a novel biomarker of folate status, future studies should measure DNA methylation response to folic acid supplementation in other white blood cell types.

Acknowledgements

Support for this project was provided by Georgia Experimental Agricultural Station, HATCH #GEO00706 and #GEO00707 and by the University of Georgia through the Obesity Initiative and the Office of Vice President for Research.
Table 1. Demographic and clinical characteristics of study subjects\textsuperscript{a}.

<table>
<thead>
<tr>
<th></th>
<th>Baseline (n=12)</th>
<th>Week 8 (n=12)</th>
<th>p-value\textsuperscript{c}</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>27.2 ± 5.2</td>
<td>27.3 ± 5.4</td>
<td>0.166</td>
</tr>
<tr>
<td>Height (cm)</td>
<td>165.5 ± 6.0</td>
<td>165.8 ± 5.9</td>
<td>0.064</td>
</tr>
<tr>
<td>Weight (kg)</td>
<td>59.3 ± 6.5</td>
<td>60.0 ± 6.4</td>
<td>0.188</td>
</tr>
<tr>
<td>BMI (kg/m\textsuperscript{2})</td>
<td>21.6 ± 1.4</td>
<td>21.8 ± 1.2</td>
<td>0.364</td>
</tr>
<tr>
<td>Body fat (%)</td>
<td>29.3 ± 5.5</td>
<td>29.5 ± 5.7</td>
<td>0.522</td>
</tr>
<tr>
<td>Serum folate (nmol/L)</td>
<td>38.4 ± 8.7</td>
<td>71.4 ± 10.5</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Dietary folate intake\textsuperscript{b} (μg/d)</td>
<td>459.9 ± 99.5</td>
<td>364.7 ± 116.6</td>
<td>0.021</td>
</tr>
</tbody>
</table>

\textsuperscript{a}Means ± s.d. \textsuperscript{b}Measured as dietary folate equivalents (DFE) \textsuperscript{c}Paired two-tailed t-test.
Table 2. Number and pattern of changes in DNA methylation in whole blood (WB) and CD16+ neutrophils (CD16) after folic acid supplementation (p<0.05).

<table>
<thead>
<tr>
<th></th>
<th>WB</th>
<th>CD16</th>
<th>WB &amp; CD16</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of CpG sites with a significant change</td>
<td>7839</td>
<td>8317</td>
<td>235</td>
</tr>
<tr>
<td>Number of sites with increased methylation (%)</td>
<td>1789 (22.8)</td>
<td>2857 (34.4)</td>
<td>18 (7.7)</td>
</tr>
<tr>
<td>Number of sites with decreased methylation (%)</td>
<td>6050 (77.2)</td>
<td>5460 (65.6)</td>
<td>170 (72.3)</td>
</tr>
<tr>
<td>Number of sites with inconsistent change in methylation (%)</td>
<td>-</td>
<td>-</td>
<td>47 (20.0)</td>
</tr>
</tbody>
</table>

Total number of CpG sites in analysis: 484,039
Table 3. Genes corresponding to associated CpG sites found in both whole blood (WB) and CD16+ neutrophils (CD16) and pattern of differential methylation changes.

<table>
<thead>
<tr>
<th>Gene Name</th>
<th>CpG TargetID</th>
<th>Pattern</th>
<th>CpG TargetID</th>
<th>Pattern</th>
</tr>
</thead>
<tbody>
<tr>
<td>ASAP2</td>
<td>cg01259423</td>
<td>↓</td>
<td>cg21821308</td>
<td>↑</td>
</tr>
<tr>
<td>ATF6B</td>
<td>cg18979423</td>
<td>↓</td>
<td>cg18979423</td>
<td>↓</td>
</tr>
<tr>
<td>CCHCR1</td>
<td>cg02652369</td>
<td>↓</td>
<td>cg02451928</td>
<td>↓</td>
</tr>
<tr>
<td>CMIP</td>
<td>cg01033642, cg04714088</td>
<td>↓↓</td>
<td>cg04759112</td>
<td>↓</td>
</tr>
<tr>
<td>ESAM</td>
<td>cg03119844</td>
<td>↑</td>
<td>cg19477600</td>
<td>↓</td>
</tr>
<tr>
<td>LRP5</td>
<td>cg01822532, cg17198658</td>
<td>↓↓</td>
<td>cg25044834</td>
<td>↓</td>
</tr>
<tr>
<td>MGMT</td>
<td>cg03639152</td>
<td>↑</td>
<td>cg09858782</td>
<td>↑</td>
</tr>
<tr>
<td>MIA3</td>
<td>cg08133669</td>
<td>↓</td>
<td>cg19587105</td>
<td>↑</td>
</tr>
<tr>
<td>MYH10</td>
<td>cg20420603</td>
<td>↓</td>
<td>cg05211642</td>
<td>↑</td>
</tr>
<tr>
<td>TMCO4</td>
<td>cg22626525</td>
<td>↓</td>
<td>cg00576736</td>
<td>↓</td>
</tr>
<tr>
<td>TSC2</td>
<td>cg08249385</td>
<td>↓</td>
<td>cg08043994</td>
<td>↓</td>
</tr>
</tbody>
</table>

A total of 292 genes in WB and 367 genes in CD16 contained CpG sites whose methylation levels had the most robust response (p < 0.005) to FA supplementation; ↑ represents increased methylation; ↓ represents decreased methylation; ATF6B (Activating transcription factor β) CpG site is the same in WB and CD16 (Target ID: cg18979423).
Table 4. Gene ontology results for genes associated with folic acid supplementation in whole blood.

<table>
<thead>
<tr>
<th>Annotations</th>
<th>GO term</th>
<th>Corrected p value*</th>
<th>Genes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Multicellular organismal development</td>
<td>GO:0007275</td>
<td>2.5 x 10^{-4}</td>
<td>PDLIM7, HOXB3, DDX25, RFX6, LRP5, BMP8A, CDX4, ZBTB7A, NRL, PAQR5, CUX1, MINK1, RBM19, ARVCF, TBCB, MESP1, SLIT1, WNT2B, PAX4, OBSCN, CCHCR1, KIAA1217, KDR, SPATA20, AXIN1</td>
</tr>
<tr>
<td>Brain development</td>
<td>GO:0007420</td>
<td>0.0010</td>
<td>BCR, MYH10, SLC6A11, FZD9, GABRA5, SLIT1, SYNJ2, AFF2, TCF7L1, IRS2</td>
</tr>
<tr>
<td>Nerve growth factor receptor signaling pathway</td>
<td>GO:0048011</td>
<td>0.0025</td>
<td>ADCY6, NR4A1, PLCG1, RPS6KA2, OBSCN, TSC2, MCF2L, ARHGEF2, AP2A1, IRS2</td>
</tr>
<tr>
<td>Actin cytoskeleton organization</td>
<td>GO:0030036</td>
<td>0.0031</td>
<td>BCR, NF2, PDLIM7, MYH10, CYTH2, DAAM1, EPB4I, NISCH</td>
</tr>
<tr>
<td>Axon guidance</td>
<td>GO:0007411</td>
<td>0.0070</td>
<td>PSPN, FEZ2, MYH10, ABLIM2, SPTBN2, PLCG1, RPS6KA2, ITGA5, MYH11, SLIT1, AP2A1</td>
</tr>
<tr>
<td>Transport</td>
<td>GO:0006810</td>
<td>0.0071</td>
<td>P2RX1, NDUFB7, ABCG5, SLC15A1, DDX25, SLC20A2, SLC29A1, LCN10, SLC25A37, PITPNM2, AQP8, CUX1, GJC1, GABRA5, GJA5, RBP3</td>
</tr>
<tr>
<td>Positive regulation of catalytic activity</td>
<td>GO:0043085</td>
<td>0.0097</td>
<td>NOXA1, MMP15, ASAP2, CTSA, PPP1R12B</td>
</tr>
<tr>
<td>Signal transduction</td>
<td>GO:0007165</td>
<td>0.010</td>
<td>P2RX1, BCR, FEZ2, CHRNA2, MCC, MAP3K10, BAIAp2, PPP1R12B, NR4A1, PLCG1, RPS6KA2, GABRA5, ATF6B, PKI1, TSC2, TNFRSF10C, MTA1, FCN3, TRAF2, AXIN1, RASA2, IRS2, ODZ4</td>
</tr>
<tr>
<td>Cell differentiation</td>
<td>GO:0030154</td>
<td>0.011</td>
<td>PDLIM7, DDX25, BMP8A, ZBTB7A, PAQR5, KRT6A, TBCB, SLIT1, PAX4, OBSCN, CCHCR1, LRRCA8A, TRAPPC9, SPATA20</td>
</tr>
<tr>
<td>Biological Process</td>
<td>GO ID</td>
<td>p Value</td>
<td>Genes</td>
</tr>
<tr>
<td>--------------------</td>
<td>-------</td>
<td>---------</td>
<td>-------</td>
</tr>
<tr>
<td>Negative regulation of cell migration</td>
<td>GO:0030336</td>
<td>0.012</td>
<td>BCR, NF2, MIA3, NISCH, MUC2</td>
</tr>
<tr>
<td>Positive regulation of GTPase activity</td>
<td>GO:0043547</td>
<td>0.012</td>
<td>BCR, BNIP2, TBCD, TSC2, AXIN1</td>
</tr>
<tr>
<td>‘de novo’ posttranslational protein folding</td>
<td>GO:0051084</td>
<td>0.013</td>
<td>PFDN6, TBCD, TBCB, ENTPD5</td>
</tr>
<tr>
<td>Canonical Wnt receptor signaling pathway</td>
<td>GO:0060070</td>
<td>0.014</td>
<td>LRP5, FZD9, WNT2B, TCF7L1, AXIN1</td>
</tr>
<tr>
<td>Skeletal muscle myosin thick filament assembly</td>
<td>GO:0030241</td>
<td>0.014</td>
<td>TCAP, MYH11</td>
</tr>
<tr>
<td>Wnt receptor signaling pathway</td>
<td>GO:0016055</td>
<td>0.016</td>
<td>MCC, GRK5, LRP5, LZTS2, WNT2B, TCF7L1</td>
</tr>
<tr>
<td>Regulation of ARF protein signal transduction</td>
<td>GO:0032012</td>
<td>0.016</td>
<td>CYTH2, CYTH3, IQSEC1</td>
</tr>
<tr>
<td>Smooth muscle contraction</td>
<td>GO:0006939</td>
<td>0.016</td>
<td>MYL7, NMUR1, MYH11</td>
</tr>
<tr>
<td>Regulation of Rho protein signal transduction</td>
<td>GO:0035023</td>
<td>0.017</td>
<td>BCR, OBSCN, MCF2L, ARHGEF19, ARHGEF2</td>
</tr>
<tr>
<td>Apoptotic process</td>
<td>GO:006915</td>
<td>0.019</td>
<td>BNIP2, PHF17, OBSCN, NISCH, MCF2L, PPP3CC, CYFIP2, TPX2, TNFRSF10C, TCHP, MUC2, ARHGEF2, TRAF2, AXIN1</td>
</tr>
<tr>
<td>Fibroblast growth factor receptor signaling pathway</td>
<td>GO:0008543</td>
<td>0.019</td>
<td>NR4A1, PLCG1, FGFR3, TSC2, IRS2</td>
</tr>
<tr>
<td>Insulin receptor signaling pathway</td>
<td>GO:0008286</td>
<td>0.041</td>
<td>BAIAP2, ATP6V1G2, FGFR3, ATP6V1H, TSC2, IRS2</td>
</tr>
</tbody>
</table>

*p values indicate the significance between the number of genes differentially methylated in this analysis and the total number of genes in each annotated biological process.*
Table 5. Gene ontology results for genes associated with folic acid supplementation in CD16+ neutrophils.

<table>
<thead>
<tr>
<th>Annotations</th>
<th>GO term</th>
<th>Corrected p value*</th>
<th>Genes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Axon guidance</td>
<td>GO:0007411</td>
<td>0.0013</td>
<td>MAPK1, NFASC, MYH10, MAPK8IP3, TRPC6, PLXNC1, BDNF, CNTN4, MYL9, ABLIM1, DLG4, NRXN1, MET, COL4A2, RELN</td>
</tr>
<tr>
<td>B cell receptor signaling pathway</td>
<td>GO:0050853</td>
<td>0.0033</td>
<td>MAPK1, BTK, CD19, NFATC2, BCAR1</td>
</tr>
<tr>
<td>Lateral ventricle development</td>
<td>GO:0021670</td>
<td>0.024</td>
<td>RPGRIPL1, MYH10, CDK6</td>
</tr>
<tr>
<td>Apical protein localization</td>
<td>GO:0045176</td>
<td>0.026</td>
<td>SHROOM2, CELSR1, SHROOM3</td>
</tr>
<tr>
<td>Cellular pigment accumulation</td>
<td>GO:0043482</td>
<td>0.026</td>
<td>SHROOM2, SHROOM3</td>
</tr>
<tr>
<td>Sarcoplasmic reticulum calcium ion transport</td>
<td>GO:0070296</td>
<td>0.026</td>
<td>ATP2A2, RYR2</td>
</tr>
<tr>
<td>Protein autophosphorylation</td>
<td>GO:0046777</td>
<td>0.027</td>
<td>EIF2AK2, EIF2AK3, AAK1, EPHA4, SIK1, MET, CAMK2D, UHMK1</td>
</tr>
<tr>
<td>Neuron migration</td>
<td>GO:0001764</td>
<td>0.030</td>
<td>DCDC2, MYH10, CELSR1, PEX2, MET, RELN</td>
</tr>
<tr>
<td>Adult locomotory behavior</td>
<td>GO:0008344</td>
<td>0.034</td>
<td>PARK2, TSHR, PBX3, TBCE</td>
</tr>
<tr>
<td>Response to drug</td>
<td>GO:0042493</td>
<td>0.035</td>
<td>ABCA2, NFATC2, POR, BDNF, MGMT, TERF1, MET, RPH3AL, MAP2K6, NME1, PDGFA</td>
</tr>
<tr>
<td>Regulation of grooming behavior</td>
<td>GO:2000821</td>
<td>0.035</td>
<td>DLG4, NRXN1</td>
</tr>
<tr>
<td>Regulation of cardiac muscle contraction by regulation of the release of sequestered calcium ion</td>
<td>GO:0010881</td>
<td>0.035</td>
<td>RYR2, CAMK2D</td>
</tr>
<tr>
<td>Biological Process</td>
<td>GO: SID</td>
<td>p Value</td>
<td>Genes</td>
</tr>
<tr>
<td>-----------------------------------------------------------------------------------</td>
<td>-------------</td>
<td>---------</td>
<td>------------------------------------------------------------------------------------------------</td>
</tr>
<tr>
<td>Receptor localization to synapse</td>
<td>GO:0097120</td>
<td>0.035</td>
<td>DLG4, NRXN1</td>
</tr>
<tr>
<td>Actin cytoskeleton organization</td>
<td>GO:0030036</td>
<td>0.036</td>
<td>MYH10, ARHGAP26, ABR, SHROOM3, TESK2, BCAR1, PDGFA</td>
</tr>
<tr>
<td>Regulation of transcription, DNA-dependent</td>
<td>GO:0006355</td>
<td>0.037</td>
<td>C14orf43, SND1, CUX2, HOXC8, HSF2, RNF141, ZNF518B, EBF3, ZFAT, PER2, NYA, ZNF738, ARID3B, LIN54, FLI1, ZNF613, ZNF205, BTK, ZNF697, NFATC2, HDAC2, ATF6B, SAFB2, ELAVL2, GTF2B, TERF1, RYBP, KDM5B, ZMIZ2, VGLL4, MAP2K6, SOLH</td>
</tr>
<tr>
<td>Neuromuscular process controlling balance</td>
<td>GO:0050885</td>
<td>0.039</td>
<td>MYH10, ABR, DLG4, NRXN1</td>
</tr>
<tr>
<td>Brain development</td>
<td>GO:0007420</td>
<td>0.040</td>
<td>RPGRIP1L, SHROOM2, MYH10, ABR, CNTN4, BID, MET, RELN</td>
</tr>
<tr>
<td>Wound healing</td>
<td>GO:0042060</td>
<td>0.043</td>
<td>DSP, MIA3, CELSR1, SCARB1, PDGFA</td>
</tr>
<tr>
<td>Virus-infected cell apoptosis</td>
<td>GO:0006926</td>
<td>0.043</td>
<td>EIF2AK2, EIF2AK3</td>
</tr>
<tr>
<td>Carnitine metabolic process</td>
<td>GO:0009437</td>
<td>0.043</td>
<td>ALDH9A1, POR</td>
</tr>
<tr>
<td>Establishment of protein localization</td>
<td>GO:0045184</td>
<td>0.043</td>
<td>TSC2, DLG4, NRXN1</td>
</tr>
<tr>
<td>Cell adhesion</td>
<td>GO:0007155</td>
<td>0.049</td>
<td>NFASC, ATP2A2, FLRT1, CELSR1, PLXNC1, CNTN4, SCARB1, EPHA4, RADIL, NRXN1, NELL2, BCAN, BCAR1, LSAMP, RELN</td>
</tr>
<tr>
<td>Cilium assembly</td>
<td>GO:0042384</td>
<td>0.049</td>
<td>RPGRIP1L, KIF27, B9D1, WDR35</td>
</tr>
<tr>
<td>Blood coagulation</td>
<td>GO:0007596</td>
<td>0.050</td>
<td>CD59, MAPK1, EHD2, ATP2A2, ESAM, CALM2, HPS1, TRPC6, HDAC2, GNA11, PRKAR1B, BCAR1, PDGFA</td>
</tr>
</tbody>
</table>

p values indicate the significance between the number of genes differentially methylated in this analysis and the total number of genes in each annotated biological process.
References


Axume J, Smith SS, Pogribny IP, Moriarty DSJ, Caudill MA. Global leukocyte DNA methylation is similar in African American and Caucasian women under conditions of controlled folate intake. Epigenetics 2007;2(1):66-68.


CHAPTER 4

SUMMARY AND CONCLUSIONS

As a methyl donor, folate is known to play a role in DNA methylation however previous studies on global DNA methylation response to changes in folate status have yielded inconsistent results. Based on controlled human feeding studies, researchers have reported decreased methylation following folate depletion (Rampersaud et al 2000), increased methylation following folate repletion (Shelnutt et al 2004), and no response to folate depletion/repletion (Axume et al 2007) while folic acid supplementation studies have reported decreased DNA methylation in coagulated blood samples but no response in uncoagulated samples (Crider et al 2011). The majority of these studies have measured DNA methylation response in whole blood, which consists of multiple cell types whose DNA methylation patterns are widely distributed. The use of whole blood when evaluating DNA methylation response has the potential to average out the effect of folate status. Therefore, this study investigated, for the first time, the use of a single cell type as a novel reporter for DNA methylation changes in response to folic acid supplementation. When comparing DNA methylation in CD16+ neutrophils to whole blood, we observed that the genome-wide response to folic acid supplementation is remarkably different.

When comparing genome-wide DNA methylation response to folic acid supplementation in whole blood to CD16+ neutrophils, we observed that 7839 (WB) and 8317 (CD16) of the 484,039 CpG sites measured were associated with supplementation however only about 3% of associated sites were found in both whole blood and CD16+ neutrophils. We also observed that
the majority of associated CpG sites decreased in methylation in both whole blood (77.2%) and CD16+ neutrophils (65.6%) after treatment with folic acid supplementation. Even as the major white blood cell type, neutrophils still yielded very different results than whole blood after folic acid supplementation.

In addition, gene ontology analysis indicated 21 biological processes that were enriched in WB and 24 biological processes that were enriched in CD16. Although several of these biological processes were related to development in WB (multicellular organismal development, brain development, and cell differentiation) and in CD16 (brain development and lateral ventricle development), none were found to be typically associated with folate metabolism or neural tube development. Overall, the associated biological processes varied greatly between WB and CD16, with only 3 biological processes found to be associated with both WB and CD16. These include actin cytoskeleton organization, axon guidance and brain development. The findings from the gene ontology analysis should be replicated and explored further to determine importance of each biological processes and its impact on the health of both women and their infants.

In summary, this is the first study to use a genome-wide approach for measuring DNA methylation as a biomarker of folate status in WB and CD16+ neutrophils. Our group observed similarities between WB and CD16 response to folic acid supplementation with respect to decreasing DNA methylation and differences in DNA methylation response between WB and CD16 according to associated CpG sites, their representative genes, and their biological processes. This result provides additional evidence that single cell types may confound the outcome of DNA methylation measurements using the mix of white blood cells found in whole blood. Our data suggest that the source of DNA is a critical component in the interpretation of
DNA methylation patterns from whole blood and that a single cell type may act as a more informative indicator of response to folic acid supplementation.

In order to determine if a single cell type could act as a novel biomarker of folate status, future studies should aim to overcome the limitations of this study by: 1) performing the study in a population where lower serum folate concentrations are common, 2) using a larger population to allow for detection of more robust effects on site-specific DNA methylation and 3) measuring DNA methylation response to changes in folic acid supplementation in other white blood cell types.

The response to folic acid supplementation in studies conducted in the general US population may be limited since mandatory fortification of the food supply with folic acid has reduced the prevalence of low serum folate concentrations to ≤1% (Pfeiffer et al 2012). Our study subject’s mean baseline serum levels (40.8 ± 8.8nmol/L) were well above that which is considered to be deficient (<10 nmol/L) (Selhub et al 2008) and therefore methyl substrate was likely widely available and DNA methylation may have already been at an optimal level. Future studies should be implemented in a population where dietary intake of folate is low and folate deficiency is common. This type of intervention would provide a better understanding of the relationship between folate status and DNA methylation patterns.

In addition, this study needs to be repeated in a larger population to allow for detection of more robust effects on site-specific DNA methylation. This study had a sample size of just 12 women which likely prohibited us from finding the most highly associated CpG sites with true significance. Using the current data and calculations from Liu and Hwang (2007), an additional
power analysis should be performed to determine the number of added participants that would be needed for detection of more robust effects on site-specific DNA methylation.

Finally, DNA methylation response to folic acid supplementation should be measured in other white blood cell types, since the use of neutrophils as epigenetic reporters might have been limited by their relatively short circulating half-life of hours to a few days before migrating into tissue (Summers et al 2010, Daniels et al 1979). This could result in DNA methylation patterns that reflect their bone marrow origin rather than their blood environment.

The next white blood cell types whose DNA methylation should be measured in response to folic acid supplementation are CD4+ cells. These cells are peripheral blood mononuclear cells (PBMCs), and have been reported that to have a very high fraction of methylated CpG sites (72.5%) as compared to neutrophils which are polymorphonuclear cells (PMN) with a reported lower fraction of methylated sites (5.2%) (Reinius et al 2012). Additionally, folate receptor 4 is highly expressed in this cell type (Walker 2007). Finally, our research group already has the methods and equipment for paramagnetic bead isolation of these cells. A comparison among whole blood, CD16+ neutrophils and CD4+ T cells would provide additional understanding as to the confounding effect of cell type variation on determining DNA methylation response to folic acid supplementation. The CD4+ T cell may act as a more specific epigenetic reporter of DNA methylation response to folic acid supplementation and could have the potential to be used as a novel biomarker of folate status.

A larger study on the genome-wide DNA methylation response to folic acid supplementation in a population with lower initial folate status and measurement of DNA methylation response in CD4+ T cells would add to the evidence that single cell types respond
differently than whole blood in response to changes in folate status. In addition, CD4+ T cells have great potential to act as more specific epigenetic reporters than whole blood or neutrophils and could have the potential to be used as a novel biomarker of folate status in the future.
References

Axume J, Smith SS, Pogribny IP, Moriarty DsJ, Caudill MA. Global leukocyte DNA methylation is similar in African American and Caucasian women under conditions of controlled folate intake. Epigenetics 2007;2(1):66-68.


APPENDIX A

STUDY FLYER
Nutrition Research:
Folic Acid Supplementation

18 - 40 y Healthy, Non-Pregnant Females
Normal Weight (~115 - 160 lbs) or Obese (~200 – 300 lbs)

We Will:
• Measure body composition & bone density
• Provide a folic acid supplement
• Collect blood samples
• Request 4 in lab visits, an 11-hour commitment

You Get:
• $20 for screening
• $150 for completion of an eight week intervention study
• Free body composition analysis
• Free blood metabolic panel

Contact: The Folate Research Lab (Dr. Lynn Bailey, Director, 161 Dawson Hall)
706-542-7689 or dshade@uga.edu
APPENDIX B

TELEPHONE SCRIPT
Thank you for calling to find out more about our research study. My name is ________________________, and I am a _______________________ conducting this research under the direction of Dr. Lynn Bailey at the University of Georgia’s Department of Foods and Nutrition. The purpose of the study is to determine there is a difference in genetic markers of folate status after folic acid supplementation in women of childbearing age. Do you think you might be interested in participating in this study?

{If No}: Thank you very much for your time.

{If Yes}: OK. To qualify for the study, you must:

- Be female and 18-40 years of age
- Not be pregnant or have intentions of becoming pregnant in the next six months
- Be willing to come for one screening visit (2 hours) and three study visits (9 hours total)
  - The visits will be at the Department of Foods and Nutrition at the University of Georgia where you will have anthropometric measurements, body composition, and bone density testing done, and will be asked to complete a questionnaire. Each visit will also include one blood draw.

Do you match these qualifications?

{If No}: Thank you very much for your time. Goodbye.

We will be selecting subjects based on height and weight, specifically recruiting women in the normal and high weight ranges. Do you feel comfortable sharing height and weight information with us?

{If Yes}: Are you interested in participating in this study?

{If No}: Thank you very much for your time. Goodbye.

{If Yes}: Before enrolling people in this study, we need to ask you some questions to determine if you are eligible to participate. What I would like to do now is ask you a series of questions related to our research criteria for age, body size, and medical history. This should only take about 15 minutes of your time.

The study will be conducted in two phases. The screening phase will include one visit to our lab for collection of a small blood sample, completion of a diet recall and body composition measurement. The blood sample will be used for screening for current folate status and eligibility for the subsequent intervention study. The intervention study is an eight-week folic acid supplementation study. Three visits to our lab will be required, at baseline and after 4 and 8 weeks – with blood samples and body composition measurements taken at each visit.
{If Yes}: Are you still interested in participating in this study?

{If No}: Thank you very much for your time. Goodbye.

If you meet these selection criteria and are enrolled in the study, you will receive free body composition, diet, and blood panel and nutritional indicator information. (FYI: this information is ~ $950 value). Risks during the study may include discomfort from fasting (low blood sugar, which may cause trembling, clamminess, sweating, anxiety, hunger, confusion, dizziness, fainting) and small blood draws.. The bone and body composition scans will expose you to a minimal radiation dose, approximately 2 to 4% of the amount received from an adult chest X-ray. There is also a potential to earn up to $170 ($20 for screening; $150 for completion of an eight week intervention study) compensation for participation in the study.

I also wanted to assure you that all information I receive from you during this phone interview, including your name and other information that can identify you, will be strictly confidential and will be kept under lock and key. If you are determined to be ineligible for the study, the screening data collected over the telephone will be immediately destroyed. Your participation is voluntary; you can refuse to answer any questions, or stop this phone interview at any time without penalty or loss of benefits to which you are otherwise entitled.

There is a possibility that some of the questions you will be asked concerning diet, history of menstruation status, and medical history may make you uncomfortable or distressed; if so, please let me know. You don’t have to answer these questions if you don’t want to.

Do I have your permission to ask you these questions?

{If No}: Thank you very much for your time. Goodbye.

{If Yes}: I will now complete a short Telephone Screening Questionnaire with you that will further help to determine if you are eligible to participate. There are no direct benefits to you for participating in the screening. Do you have a few minutes to talk right now?

{If No schedule a time/date to place a return call}: ____________________________.

{If Yes}: Do you verbally agree to participate?

Prior to any testing or participation, a consent form will be mailed/ emailed to you outlining the testing procedures that will be used during the study. You will be instructed to sign this form prior to your appointment. However, if you misplace or do not bring the signed form upon your arrival to the laboratory, you will be given the opportunity to reread these forms and ask any questions that you may have about the study before signing the form. The researcher will then sign the consent form and will provide you with a copy of this form.

Do you have any questions?
Thank you. If you have any questions about this research project, please feel free to call Dr. Lynn Bailey at 706-542-4256. Questions or concerns about your rights as a research participant should be directed to Institutional Review Board, 629 Boyd GSRC, Athens, Georgia 30602-7411; telephone (706) 542-3199; email address irb@uga.edu.

{Provide researcher’s contact information at the end of the interaction.}

Dr. Lynn Bailey

280 Dawson Hall

University of Georgia

706-542-4256

folate@uga.edu

{Continue to telephone screen.}
APPENDIX C

TELEPHONE QUESTIONNAIRE
Date: ____________  Time: ___________  Screen completed by: _______________________

- Participants’ name: ________________________________
- Participants’ DOB: ______________
- Participants’ age: ______________
- Participants’ race: ______________
- Approximately, how tall are you? ______ ft ______ in
- Approximately, how much do you weigh? __________ lbs
  - Calculate BMI: ___________kg/m^2

- Have you lost or gained weight in the past 3 months? _____Yes _____No
  - If yes, how much? __________ lbs
    Significant weight loss or gain in the past 6 mo is ±10% initial body weight

- Did you have any other significant weight changes, either weight loss or weight gain, in the past? ________________________________________________________________  
  ________________________________________________________________

- Are you currently taking any medication? _____Yes _____No
  - If yes, what medication(s)? __________________________________________  
    ________________________________________________________________ (check approved and non-approved medication list)

- Do you have children? _____Yes _____No
  - If yes, how many? __________ Age(s) __________

- Are you currently taking any oral contraceptives (birth control)? _____Yes _____No
  If yes, what is the name and duration of oral contraceptive use? ______________________________
• How many cycles do you currently have?
   _______ ≥9 cycles/year _________ between 4-8 cycles/year _________ ≤3 cycles/year

• First date of last menstrual period (approx.) _______________

• Do you smoke? _______ Yes ______ No
• Are you currently taking any dietary supplements? _______ Yes _____ No
  o If yes, what kind of supplements and what doses? __________________________

• Do you regularly consume any of the following?
  o Breakfast cereal  ____Yes ____No  ______Brand ____How Often
  o Meal replacement drinks/bars  ____Yes ____No  ______Brand ____How Often
  o Energy drinks  ____Yes ____No  ______Brand ____How Often
  o Protein shakes  ____Yes ____No  ______Brand ____How Often
  o Snack bars  ____Yes ____No  ______Brand ____How Often

• Do you drink more than one serving of alcoholic beverages a day? _____Yes _____ No
  o Is yes, how often do you drink 2 or more alcoholic beverages a day? ________

• Have you ever been diagnosed with chronic disease (for ex., diabetes, epilepsy, cancer,
  hypertension, kidney disease, cardiovascular disease)? _____Yes _____ No  If yes, what
  medication(s)? ________________________________

• Have you ever had major surgery? _____________ Yes ______ No
  If yes, what surgery? ________________________________
Do you currently have, or have you recently had, an acute illness (for ex. Pneumonia, urinary tract infection, mononucleosis)? _____Yes _____No

Have you recently taken any, or are you currently taking, any form of antibiotics?

_______Yes______No

In this study, all participants must provide blood samples. Are you willing to do this?

___Yes _____No

The blood samples must be collected in the fasting state. This will require you to not eat anything after midnight the night before the testing days, and remain fasting until mid-morning on the days of testing. Are you willing to do this? ______Yes ______No

During the eight-week intervention study, all participants are required to abstain from binge-drinking or from consuming alcoholic beverages in excess. Are you willing to do this?

_______Yes ______No

During the eight-week intervention study, all participants are requested to avoid consuming dietary supplements and fortified ready-to-eat cereals. Are you willing to do this?

_______Yes ______No

Collect the following information:

Address: ______________________________________________________________

City: ___________________________ State _________ Zip: ______________________

Phone Number: __________________________ __Cell? ___Yes ___No

Email Address: __________________________
How would you prefer us to contact you? _______ Phone _______ Email

If selected to participate, what days during the week would you be available to come to the Department of Foods and Nutrition, University of Georgia, for testing? Testing is conducted in the morning and requires about 2-3 hours commitment per testing session.

M_______ T___________ W___________ Th _________ F___________ S___________

“This is the end of our telephone screening. We will review this and determine your eligibility for the study. We will get back to you within one week to let you know the status of your eligibility. Do you have any additional questions for me?”

Make sure the potential volunteer has contact numbers for future questions.
APPENDIX D

CONSENT FORM: SCREENING
I, _________________________________, agree to participate in the screening phase for a research study titled “Folate Supplementation in Women of Childbearing Age” conducted by Drs. Lynn Bailey, and Dorothy Hausman and Ms. Deanna Shade from the Department of Foods and Nutrition at the University of Georgia. Dr. Bailey can be reached at (706) 542-4256. I understand that my participation is voluntary. I can refuse to participate or stop taking part at any time without giving any reason, and without penalty or loss of benefits to which I am otherwise entitled. I understand that the researchers can terminate my participation without regard to my consent if I do not abide by the study protocols (such as arriving at the testing facility in a non-fasted state). If I decide to discontinue or withdraw from the study or if the investigator decides to terminate my participation without regard to my consent, the information/data collected from or about me up to the point of my withdrawal will be kept as part of the study and may continue to be analyzed, unless I ask to have information that can be identified as mine returned to me, removed from the research records, or destroyed.

The following points have been explained to me:

1) **Purpose:** The purpose of this screening phase is to determine the folate status and folate metabolism specific genotype of potential participants for a folic acid supplementation study. Individuals meeting these screening criteria would be eligible to participate in a subsequent eight week intervention study the purpose of which is to measure genetic markers of folate status in blood samples taken before, during and after folic acid supplementation in women of childbearing age.

2) **Duration:** My participation will involve:
   - a. 1 in-lab screening session of approximately 2 hours
   - b. Completion of two 24-hr dietary recalls, by logging into an online program from my home computer. Each recall should take 30 minutes or less to complete.

3) **Procedures:**
   As an eligible participant, based on the telephone pre-screening questionnaire, I will be invited to a testing session, which will take place in the Department of Foods and Nutrition at the University of Georgia. During the testing, the following will occur:
   
   - a. I will arrive in the testing facility at the scheduled time, following an overnight fast. Each aspect of the study will be explained to me during testing, including the option for me to withdraw from the study at any time.
   
   - b. A trained phlebotomist will insert a small needle into a vein in my arm and will draw approximately 9 mL (about 2 teaspoons) of blood from my arm. A portion of my blood will be sent for analysis of folate levels to the University of Florida in Gainesville, FL in the care of Dr. Gail Kauwell. The other portion of my blood will be sent for analysis of folate metabolism marker to the Department of Genetics at the University of Georgia in the care of Dr. Richard Meagher. The samples will be sent
with a participant number code and Drs. Kauwell and Meagher will not be given any information that would allow them to identify me. If my blood values meet eligibility criteria then I will proceed with the study. If my blood values do not meet the eligibility criteria no further participation in the study will be required.

c. Using an online computer program, I will be instructed in the use of the dietary recall program and will do a trial recall session to become familiar with its operation. Following the screening session, I will complete two additional 24-hr recalls for non-consecutive days, including one weekend day, by logging into the program from my home computer or from another computer to which I have access. Each recall should take 30 minutes or less to complete.

d. I will have measurements taken for height, weight, and waist circumference. If my height and weight meet the eligibility criteria, then I will proceed with the study. If my height, weight, and waist circumference do not meet the eligibility criteria I will be given a snack and no further participation in the study will be required.

e. I will be accompanied by a researcher to the UGA Bone and Body Composition Laboratory, Dawson Hall, Room 279. I will then complete a whole body scan on the Hologic DXA machine which includes additional measures of my body composition, including bone health indicators. This will require me to lie still approximately 10 minutes while the measurements are taken.

4) **Risks and Discomforts of Questionnaires:** The discomfort or stress that I may face during this research may be associated with the disclosure of information concerning my dietary intake and health history; however it is important to share this information so that my health and nutritional status can be evaluated correctly. All individually-identifiable information will be kept strictly confidential and my 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.

5) **Risks and Discomforts of Blood Draw:** One of the foreseen risks is discomfort during the blood draw. The risks of drawing blood from the arm include the unlikely possibilities of a small bruise or localized infection, bleeding, and fainting. These risks will be reduced in the following ways: blood will be drawn only by a qualified and experienced phlebotomist who will follow standard sterile techniques, who will observe me after the needle is withdrawn, and who will apply pressure to the blood-draw site. I understand that if a blood sample cannot be obtained after two attempts, no further attempts will be made.

6) **Risks and Discomforts of DXA:** I understand that another foreseen risk is exposure to a small amount of radiation during the bone scans or DXA. The DXA scans will expose me to a minimal radiation dose, far less than (approximately 2 to 4%) the amount received from an adult chest X-ray. In the event that information from any scan is lost or unstable, no additional scans will be performed. A copy of my DXA scans will be provided to me, but I understand that the
researchers are not medical doctors. The DXA results will be explained to me and may be clinically relevant, but for diagnosis and health questions, I should consult a qualified physician.

7) **Benefits:** The benefits I can expect from participation are the assessment of nutritional indicators (serum folate status), body composition (percentage of body fat and nonfat tissue), and diet (total calories and specific nutrients). All measurements are being used for research purposes only, not medical purposes. However, if abnormalities are found in any measure, I will be notified and referred to my health care professional.

This is the screening phase for a subsequent research study that should help reveal genes whose expression is potentially modifiable due to changes in folate intake and help delineate mechanisms accounting for the variability in response to folic acid supplementation. This may provide a basis for future, personalized folic acid recommendations to maximize beneficial effects of the vitamin.

8) **Incentive:** I will receive monetary compensation ($20) for participation in the testing session for this screening phase. In order to process payments for my participation following the testing session, the researcher(s) need to collect my name and mailing address on a separate payment form. This completed form will be sent to the Department of Foods and Nutrition business office and then to the UGA Business Office. The researchers have been informed that these offices will keep my information private, but may have to release my name and the amount of compensation paid to the IRS, if ever asked. The researchers connected with this study will go to great lengths to protect my private information and will keep this confidential in their locked files. However, they are not responsible once my name and mailing address leave their office/laboratory for payment processing.

9) **Confidentiality:** Every effort will be taken to protect my identity. No individually-identifiable information about me, or provided by me during the research, will be shared with others without my permission, except if necessary to protect my rights or welfare (for example, if I am injured and need emergency care), or if required by law. My telephone screening form, which contains information that can identify me, will be stored in locked filing cabinets. My participation results, which will include an assigned participant number and my consent form, will not be stored together. A separate list will be the only document linking my name and my participant number, and therefore 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 the DXA data files, diet assessment form and blood sample submission forms will only include my 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. In the unlikely event that there is a violation in confidentiality, a recent federal law the Genetic Information Nondiscrimination Act (GINA) will help protect me from health insurance or employment discrimination based on genetic information potentially obtained through this research.
This research involves the use of an on-line program for the recall and assessment of dietary intake information. Internet communications are insecure and there is a limit to the confidentiality that can be guaranteed due to the technology itself. However, once the materials are received by the researcher, standard confidentiality procedures will be employed. Further, I will use only my coded participant ID as identification in completing the on-line recall program and will not be asked to provide my name or other personally identifying information. I will not be identified in any report or publication of this study.

10) **Financial costs of this research:** There is no cost to me to participate in this research, however, I must provide my own transportation to and from the sessions, all of which are in on-campus facilities. Driving directions will be provided.

11) **Compensation in case of injury:** The researchers will exercise all reasonable care to protect me from harm as a result of my participation. In the event of an injury, as an immediate and direct result of my participation, the researchers’ sole responsibility is to arrange transportation for me to an appropriate facility if additional care is needed. In the event that I suffer a research-related injury, my medical expenses will be my responsibility or that of my third-party payer, although I am not precluded from seeking to collect compensation for injury related to malpractice, fault, or blame on the part of those involved in the research. In the event of a research-related injury, I agree to seek medical care first, and then contact Dr. Bailey at (706) 542-4256 (office); 352-359-3974 (cell).

12) **Permission for future contact:** By signing my initials here, ________, I agree to allow the investigators of this study to contact me in the future to request my participation in future studies. I understand that at that time, I may refuse any further participation with no negative consequences. The best way to locate me in the future is:

Address: ____________________________________________________________

Telephone Number(s): __________________________

Email: __________________________

13) **Additional risks for pregnant women:** Being a part of this study while pregnant may expose the unborn child to a yet undiscovered risk. Therefore, pregnant women or those who suspect they could be pregnant will be excluded from the study. By signing this form, females of childbearing potential are certifying to the best of their knowledge that they are not pregnant and agree to utilize adequate birth control methods during their participation in this study.

If I express any doubts regarding my pregnancy status, a pregnancy test will be provided to me, which I may complete in a private location prior to undergoing DXA scanning. If the pregnancy test is positive, I may maintain confidentiality by electing not to disclose any information to the research group, but I must voluntarily decline to take the DXA test. If I elect to notify the research group of the pregnancy, I will receive information about and referral to the UGA Health
Center Women’s Clinic or off-campus medical clinic. My refusal to take the pregnancy test will also be documented below.

I certify that I am not pregnant, or trying to become pregnant.

(Check one):  YES____  NO____

I was given the opportunity to complete a simple urine test for pregnancy.

(Check one):  YES____  NO____

I understand the risks described above, and refuse to take the pregnancy test.

(Check one):  YES____  NO____

I certify that I have not had frequent or high dose X-ray testing/treatment in the last year.

(Check one):  YES____  NO____

14) Questions: I have the opportunity to ask, and to have answered, any questions I may have about this research. If I have other questions, either now or during the course of the project, or if a research-related injury occurs, I may call Dr. Bailey at (706) 542-4256 (office); 352-359-3974 (cell).

I have read the information provided above. My questions have been answered to my satisfaction, and I voluntarily agree to participate in this study. After it is signed I understand that I will receive a copy of this consent form.

_________________________    _______________________   ___________
Dr. Lynn B. Bailey          Signature Date

Telephone: 706-542-4256

Email: folate@uga.edu

_________________________ _______________________ ___________
Name of Participant Signature            Date

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

Additional questions or problems regarding your rights as a research participant should be addressed to The Chairperson, Institutional Review Board, University of Georgia, 629 Boyd Graduate Studies Research Center, Athens, Georgia 30602-7411; Telephone (706) 542-3199; E-Mail Address IRB@uga.edu
APPENDIX E

CONSENT FORM: INTERVENTION
I, _________________________________, agree to participate in a research study titled "Folate Supplementation in Women of Childbearing Age " conducted by Drs. Lynn Bailey, Dorothy Hausman, Deanna Shade from the Department of Foods and Nutrition at the University of Georgia. Dr. Bailey can be reached at (706) 542-4256. I understand that my participation is voluntary. I can refuse to participate or stop taking part at any time without giving any reason, and without penalty or loss of benefits to which I am otherwise entitled. I understand that the researchers can terminate my participation without regard to my consent if I do not abide by the study protocols (such as unexplained absence from the testing facility, non-compliance [missing > 7 daily doses] in taking supplements or failure to report for four week testing, taking dietary supplements other than those provided by study investigators, eating fortified cereals, consuming excess alcohol, or becoming pregnant during the course of the study). If I decide to discontinue or withdraw from the study or if the investigator decides to terminate my participation without regard to my consent, the information/data collected from or about me up to the point of my withdrawal will be kept as part of the study and may continue to be analyzed, unless I ask to have information that can be identified as mine returned to me, removed from the research records, or destroyed.

The following points have been explained to me:

1) **Purpose:** The purpose of this study is to measure genetic markers of folate status in blood samples taken before, during and after folic acid supplementation in women of childbearing age. Anthropometric measurements (height, weight, and waist circumference), body fat percentage using the BOD POD instrument, bone health indicators by DXA, and my blood levels of nutritional status indicators will also be determined before, during and after taking the folic acid vitamin supplement for 8 weeks.

2) **Duration:** My participation will involve:
   a. Eight weeks of vitamin supplementation;
   b. 3 in-lab sessions of approximately 3 hours, and
   c. 2 sets of dietary recall computer sessions that I will complete at home, less than 3 hours each.

3) **Procedures:**
   As an eligible participant, I will be invited to testing sessions, which will take place in the Department of Foods and Nutrition at the University of Georgia. During the testing sessions (3-3 hour sessions) and intervention study, the following will occur:

   a. I will arrive in the testing facility at the scheduled time, following an overnight fast. Each aspect of the study will be explained to me during testing, including the option for me to withdraw from the study at any time. During testing, photographs may be taken, with my permission only, for subsequent use for research-related purposes such as presentations related to this research study.
b. A trained phlebotomist will insert a small needle into a vein in my arm and will draw approximately 24 mL (about 5 teaspoons) of blood from my arm to measure my hemoglobin, blood sugar, vitamins and other indicators of nutritional status. A portion of my blood will be sent for analysis of folate levels to University of Florida in Gainesville, FL in the care of Dr. Gail Kauwell. Other portions of my blood will be sent to the Department of Genetics at the University of Georgia in the care of Dr. Richard Meagher and to Dr. Marie Caudill at Cornell University for analysis of genetic markers of folate status. The samples will be sent with a participant number code and Drs. Kauwell, Meagher and Caudill will not be given any information that would allow them to identify me. For possible analysis in the future of additional folate-related metabolic indicators, a portion of the blood will be saved. Blood samples will be stored with a number code and therefore my personal information will not be associated to my sample. 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. This information will help the researchers advance their knowledge about the role of folate in women of childbearing age. Any unused portions of blood that is collected will be discarded per safe handling of hazardous material as defined by The University of Georgia Hazardous Material Safety protocol and discarded after 10 years following completion of the study. This procedure will be performed during a baseline visit and after four and eight weeks of folic acid supplementation (described below).

c. I will next complete the measurement of body composition (percent body fat and lean body mass) in the BOD POD machine. For this, I will change from my street clothes into my simple one or two piece bathing suit (which has no ruffles, hanging cords or other decorations) and will be required to wear a swim cap. I will be instructed to enter and sit comfortably in the BOD POD for body volume measurement. I will be asked to sit still and breath as normal as possible while seated in the testing chamber for the body volume measurements. Two body volume measurements will be conducted with each lasting approximately 50 seconds and the door to the testing chamber open between body volume measurements. If the two body volume measurements are consistent then I will be connected to a breathing tube in the test chamber with my nose plugged to measure my residual lung volume (thoracic gas volume [TGV]), otherwise a third body volume measurement will first be taken and the two closest measurements averaged for the estimation of body density. During the TGV measurement, I will be instructed to breath normally with a firm seal around the breathing tube in my month. Also, I will be made aware of a blockage in the tube and should exhale about 2-3 gentle puffs against the blockage. The BOD POD measurements should take approximately five minutes to complete. This procedure will be performed during a baseline visit and after four and eight weeks of folic acid supplementation (described below).

d. I will be accompanied by a researcher to the UGA Bone and Body Composition Laboratory, Dawson Hall, Room 279. I will then complete a whole body scan on the Hologic DXA machine which includes additional measures of my body composition, including bone health indicators. This will require me to lie still approximately 10 minutes while the measurements are taken. This procedure will be performed during the
baseline visit and after four and eight weeks of folic acid supplementation (described below).

e. I will participate in an eight week intervention study of 800 micrograms supplemental folic acid per day. The supplements will be packaged as individual eight week supplies and I will be instructed to consume 2 tablets (400 microgram each) per day. To insure that I remember to take my supplement, I will be instructed in the use of a compliance calendar and will receive frequent telephone and/or ‘text’ message reminders from project staff. I will be asked to return my pill bottle and any unused pills at the next visit. I will be asked to follow my typical diet, but to refrain from consuming dietary supplements, multivitamins, and foods of high folate or folic acid content such as fortified ready to eat cereal products, leafy greens and some fruits. I will be given a list of these foods prior to the start of the intervention period.

f. I will also be asked to complete three 24-hr dietary recalls, using an online computer program. During my first visit, I will be instructed in the use of the dietary recall program and will do a trial recall session to become familiar with its operation. During weeks 3 and 6 of the intervention study period, I will complete the 24-hr recall for three non-consecutive days, including one weekend day, by logging into the program from my home computer or from another computer to which I have access. Each recall set should take less than three hours to complete.

4) Risks and Discomforts of Questionnaires: The discomfort or stress that I may face during this research may be associated with the disclosure of information concerning my dietary intake and health history; however it is important to share this information so that my health and nutritional status can be evaluated correctly. All individually-identifiable information will be kept strictly confidential and my 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.

5) Risks and Discomforts of Blood Draw: One of the foreseen risks is discomfort during the blood draw. The risks of drawing blood from the arm include the unlikely possibilities of a small bruise or localized infection, bleeding, and fainting. These risks will be reduced in the following ways: blood will be drawn only by a qualified and experienced phlebotomist who will follow standard sterile techniques, who will observe me after the needle is withdrawn, and who will apply pressure to the blood-draw site. I understand that if a blood sample cannot be obtained after two attempts, no further attempts will be made.

6) Risks and Discomforts of the Vitamin Supplement: There are no known risks related to the consumption of the single dose of the vitamin folate. For this study, I will consume one dose of folate 800 mcg per day for 8 weeks. 800 micrograms is twice the amount currently recommended that all women capable of becoming pregnant consume each day from supplements, fortified foods, or both in addition to consuming food folate from a varied diet. This level of supplementation does not present a foreseeable risk. The overall safety of folic acid has been analyzed in detail by the Institute of Medicine’s Food and Nutrition Board who found
that there are no known adverse effects associated with the consumption of this or any level of folate in healthy subjects of my age group.

7) **Risks and Discomforts of Body Composition Measurement with the BOD POD:** For the body composition measurement, I will be required to change from my street clothes into my bathing suit. This may cause feelings of discomfort or self-consciousness for some individuals. Accordingly, upon request disposable paper gowns will be available for use as a cover-up while walking from the changing area to the BOD POD, but will need to be removed before entering the BOD POD. The BOD POD can also cause claustrophobia (“feeling “closed in”) in some participants, as it requires sitting in a small, enclosed chamber during the measurement. These feelings should be minimized by the presence of a large window in the unit, which will allow for constant visual contact with the researcher. The measurement period will also be kept as brief as possible, typically about 5 minutes. Nonetheless, if undue discomfort or stress occurs, I have the right to discontinue the test at any time. A copy of my BOD POD test results will be provided to me, but I understand that the researchers are not medical doctors. The BOD POD results will be explained to me and may be clinically relevant, but for diagnosis and health questions, I should consult a qualified physician.

8) **Risks and Discomforts of DXA:** I understand that another foreseen risk is exposure to a small amount of radiation during the bone scans or DXA. The DXA scans will expose me to a minimal radiation dose, far less than (approximately 2 to 4%) the amount received from an adult chest X-ray. In the event that information from any scan is lost or unstable, no additional scans will be performed. A copy of my DXA scans will be provided to me, but I understand that the researchers are not medical doctors. The DXA results will be explained to me and may be clinically relevant, but for diagnosis and health questions, I should consult a qualified physician.

9) **Benefits:** The benefits I can expect from participation are the assessment of blood panel and nutritional indicators (hemoglobin, glucose, serum folate), body composition (percentage of body fat and nonfat tissue), and diet (total calories and specific nutrients). All measurements are being used for research purposes only, not medical purposes. However, if abnormalities are found in any measure, I will be notified and referred to my health care professional.

Results of this research should help reveal genes whose expression is potentially modifiable due to changes in folate intake and help delineate mechanisms accounting for the variability in response to folic acid supplementation. This may provide a basis for future, personalized folic acid recommendations to maximize beneficial effects of the vitamin.

10) **Incentive:** I will receive monetary compensation ($150) for full participation and completion of three study days, 8 weeks of folic acid supplement, and 2 diet recall assessments. The payment(s) will be pro-rated and processed as follows: $25 paid after baseline testing; $60 paid after the four week testing session [for completion of the diet recall assessment at 3 weeks ($10); 4 week testing session ($25); and 4 weeks of intervention ($25)]; and $65 paid after the eight week testing session [for completion of the diet recall assessment at 6 weeks ($10); 8 week testing session ($25); and final 4 weeks of intervention ($30). In order to process payments for my participation following the testing session, the researcher(s) need to collect my name and
mailing address on a separate payment form. This completed form will be sent to the Department of Foods and Nutrition business office and then to the UGA Business Office. The researchers have been informed that these offices will keep my information private, but may have to release my name and the amount of compensation paid to the IRS, if ever asked. The researchers connected with this study will go to great lengths to protect my private information and will keep this confidential in their locked files. However, they are not responsible once my name and mailing address leave their office/laboratory for payment processing.

11) Confidentiality: Every effort will be taken to protect my identity. No individually-identifiable information about me, or provided by me during the research, will be shared with others without my permission, except if necessary to protect my rights or welfare (for example, if I am injured and need emergency care), or if required by law. My telephone screening form, which contains information that can identify me, will be stored in locked filing cabinets. My participation results, which will include an assigned participant number and my consent form, will not be stored together. A separate list will be the only document linking my name and my participant number, and therefore 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 the BOD POD and DXA data files, diet assessment form and blood sample submission forms will only include my 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 me from health insurance or employment discrimination based on genetic information potentially obtained through this research.

This research involves the use of an on-line program for the recall and assessment of dietary intake information. Internet communications are insecure and there is a limit to the confidentiality that can be guaranteed due to the technology itself. However, once the materials are received by the researcher, standard confidentiality procedures will be employed. Further, I will use only my coded participant ID as identification in completing the on-line recall program and will not be asked to provide my name or other personally identifying information. I will not be identified in any report or publication of this study.

12) Financial costs of this research: There is no cost to me to participate in this research, however, I must provide my own transportation to and from the sessions, all of which are in on-campus facilities. Driving directions will be provided.

13) Compensation in case of injury: The researchers will exercise all reasonable care to protect me from harm as a result of my participation. In the event of an injury, as an immediate and direct result of my participation, the researchers’ sole responsibility is to arrange transportation for me to an appropriate facility if additional care is needed. In the event that I suffer a research-related injury, my medical expenses will be my responsibility or that of my third-party payer, although I am not precluded from seeking to collect compensation for injury
related to malpractice, fault, or blame on the part of those involved in the research. In the event of a research-related injury, I agree to seek medical care first, and then contact Dr. Bailey at (706) 542-4256 (office); 352-359-3974 (cell).

14) Permission for future contact: By signing my initials here, _______, I agree to allow the investigators of this study to contact me in the future to request my participation in future studies. I understand that at that time, I may refuse any further participation with no negative consequences. The best way to locate me in the future is:

Address: __________________________________________________________

Telephone Number(s): _________________________

Email: __________________________

15) Permission for photograph-taking: I hereby give consent for photography and subsequent use of my image for research-related purposes, such as presentations and publications related to this UGA research study.

(Check one): YES____ NO____

16) Additional risks for pregnant women: Being a part of this study while pregnant may expose the unborn child to a yet undiscovered risk. Therefore, pregnant women or those who suspect they could be pregnant will be excluded from the study. By signing this form, females of childbearing potential are certifying to the best of their knowledge that they are not pregnant and agree to utilize adequate birth control methods during their participation in this study.

If I express any doubts regarding my pregnancy status, a pregnancy test will be provided to me, which I may complete in a private location prior to undergoing DXA scanning. If the pregnancy test is positive, I may maintain confidentiality by electing not to disclose any information to the research group, but I must voluntarily decline to take the DXA test. If I elect to notify the research group of the pregnancy, I will receive information about and referral to the UGA Health Center Women’s Clinic or off-campus medical clinic. My refusal to take the pregnancy test will also be documented below.

I certify that I am not pregnant, or trying to become pregnant.

(Check one): YES____ NO____

I was given the opportunity to complete a simple urine test for pregnancy.

(Check one): YES____ NO____

I understand the risks described above, and refuse to take the pregnancy test.

(Check one): YES____ NO____
I certify that I have not had frequent or high dose X-ray testing/treatment in the last year.

(Check one):  YES_____  NO_____

17) Questions: I have the opportunity to ask, and to have answered, any questions I may have about this research. If I have other questions, either now or during the course of the project, or if a research-related injury occurs, I may call Dr. Bailey at (706) 542-4256 (office); 352-359-3974 (cell).

I have read the information provided above. My questions have been answered to my satisfaction, and I voluntarily agree to participate in this study. After it is signed I understand that I will receive a copy of this consent form.

Dr. Lynn B. Bailey
Telephone: 706-542-4256

Email: folate@uga.edu

_________________________________________  ___________________________  ___________
Name of Participant    Signature   Date

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

Additional questions or problems regarding your rights as a research participant should be addressed to The Chairperson, Institutional Review Board, University of Georgia, 629 Boyd Graduate Studies Research Center, Athens, Georgia 30602-7411; Telephone (706) 542-3199; E-Mail Address IRB@uga.edu
We are delighted to have you as a participant in our study. As you recall, the purpose of this study is to measure genetic markers of folate status in blood samples taken before, during and after folic acid supplementation in women of childbearing age. Blood levels of nutritional status indicators will also be determined before, during and after taking the folic acid vitamin supplement for 8 weeks.

**Folic acid supplement:**

- Dose - two 400 mcg folic acid tablets per day
- Tablets are as daily doses in the pillbox
  - Take the dose on the corresponding date
  - When you take a supplement, transfer the sticker inside the box to the calendar
- Please take the supplements the first thing in the morning without food except on the day that you come in for a blood draw- on those days please wait until after the blood draw to take the supplement.

**Folate foods list:**

- It is very important to avoid other foods that are high in folate during the study period
- A list of foods to avoid (red) and some acceptable alternatives (green) are on the list
- Items on the ‘avoid’ list in **bold** are especially high in folate and should not consumed

**Diet recall:**

- Please complete the ASA24 diet recall again during week 3 and week 7 of the study
  - Remember: each time we need info for three non-consecutive days including one weekend day
  - Log-in information: Website: [https://asa24.westat.com](https://asa24.westat.com)
    - Username: _____________   Password:____________________

**Follow-up visits:**

- Four-weeks -- Thursday June 27th (please bring pillbox and any unused pills)
- Eight weeks – Thursday July 27th (please bring pillbox and any unused pills)

Folate Research Lab contact information: 706-542-7689 or folate13@uga.edu
APPENDIX G

FOLATE FOOD TABLE
FOLIC ACID SUPPLEMENTATION STUDY - University of Georgia
706-542-7689, folate13@uga.edu

**AVOID**

Nutrition Facts for
Folic Acid >25% Daily Values/serving

Fortified Ready-to-eat breakfast cereal
Pinto beans, Black beans, Green peas,
Black-eyed peas, Lentils
Asparagus, Romaine Lettuce, Turnip greens,
Broccoli, Brussels sprout, Spinach
Orange juice, Oranges
Egg noodles, Macaroni, White rice,
Plain bagel, White bread, Flour tortilla, Roll,
Pretzels (hard), Saltine crackers,
Pancakes, Plain biscuit, cornmeal (enriched)
Sunflowerseeds, Peanuts
Liver (beef, chicken, turkey etc.)
Energy drinks (Redbull, VitaminWater etc.),
Snack bars (Clif bars etc.)

*High folate containing foods

**ALTERNATIVE**

Nutrition Facts for
Folic Acid <25% Daily Values/serving

Oatmeal (unenriched)
Baked beans, Snap green beans (canned)
Yellow corn, Iceberg lettuce, Carrot, Tomatoes
Apple juice, Apples, Grapes
Wheat pasta, Baked potato, Brown rice, Whole
wheat bagel, Whole wheat bread, Potato chips,
Whole wheat roll, Wheat thins, Pancakes made with
Wheat flour
Walnuts, Almond, Peanut butter
Ground beef or steak, Chicken, Pork, Tuna (canned)

**Low folate containing foods

FACS
COLLEGE OF FAMILY AND CONSUMER SCIENCES
DEPARTMENT OF FOODS AND NUTRITION
APPENDIX H

ASA24 INSTRUCTIONS
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’. During this in-lab screening visit, you will be instructed in the use of the ASA24™ and the students will assist you with the first recall session so that you can become familiar with the program. During the next week, you will need to complete 24-hr dietary recalls for two additional days (non-consecutive) including one weekend day, from your home or other location.

Log-in information (will also be set by email):

Website: https://asa24.westat.com
Username: ________________
Password: ________________

If you experience problems logging in or using the program please contact:
Deanna Shade: dshade@uga.edu
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 I

PARTICIPANT CHARACTERISTICS
### Participant Characteristics: Baseline

<table>
<thead>
<tr>
<th>Participant #</th>
<th>DOB</th>
<th>Age (y)</th>
<th>Ht (cm)</th>
<th>Wt (kg)</th>
<th>BMI</th>
<th>Body Fat (%)</th>
<th>Serum folate (nmol/L)</th>
<th>Folate intake DFE (mcg/d)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1004</td>
<td>7/1/1978</td>
<td>34</td>
<td>162.9</td>
<td>57.3</td>
<td>21.59</td>
<td>36.2</td>
<td>50.5</td>
<td>480.8</td>
</tr>
<tr>
<td>1008</td>
<td>8/13/1983</td>
<td>34</td>
<td>167.2</td>
<td>60.1</td>
<td>21.52</td>
<td>29.9</td>
<td>35.5</td>
<td>511.7</td>
</tr>
<tr>
<td>1012</td>
<td>8/30/1994</td>
<td>18</td>
<td>169.2</td>
<td>61.1</td>
<td>21.32</td>
<td>27</td>
<td>37.5</td>
<td>325.6</td>
</tr>
<tr>
<td>1017</td>
<td>9/2/1982</td>
<td>30</td>
<td>160.0</td>
<td>52.0</td>
<td>20.32</td>
<td>19.2</td>
<td>29.1</td>
<td>352.4</td>
</tr>
<tr>
<td>1018</td>
<td>10/30/1990</td>
<td>22</td>
<td>177.8</td>
<td>69.2</td>
<td>21.89</td>
<td>23.3</td>
<td>40.7</td>
<td>427.6</td>
</tr>
<tr>
<td>1026</td>
<td>5/14/1993</td>
<td>20</td>
<td>161.7</td>
<td>63.1</td>
<td>24.14</td>
<td>38.0</td>
<td>29.6</td>
<td>438.7</td>
</tr>
<tr>
<td>1029</td>
<td>6/1/1982</td>
<td>31</td>
<td>170.6</td>
<td>65.0</td>
<td>23.23</td>
<td>30.9</td>
<td>30.7</td>
<td>664.5</td>
</tr>
<tr>
<td>1030</td>
<td>10/19/1985</td>
<td>27</td>
<td>168.0</td>
<td>56.8</td>
<td>20.15</td>
<td>25.7</td>
<td>29.7</td>
<td>458.9</td>
</tr>
<tr>
<td>1031</td>
<td>10/8/1997</td>
<td>35</td>
<td>161.0</td>
<td>55.7</td>
<td>21.51</td>
<td>32.9</td>
<td>53.6</td>
<td>457.1</td>
</tr>
<tr>
<td>1038</td>
<td>2/28/1984</td>
<td>29</td>
<td>156.5</td>
<td>51.1</td>
<td>20.86</td>
<td>27.1</td>
<td>48.6</td>
<td>613.5</td>
</tr>
<tr>
<td>1039</td>
<td>7/31/1987</td>
<td>26</td>
<td>161.9</td>
<td>50.6</td>
<td>19.29</td>
<td>27.4</td>
<td>33.5</td>
<td>377.7</td>
</tr>
<tr>
<td>1040</td>
<td>4/6/1987</td>
<td>26</td>
<td>172.7</td>
<td>69.3</td>
<td>23.22</td>
<td>34.2</td>
<td>41.3</td>
<td>409.9</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>Mean</th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>DOB</td>
<td>27.2</td>
<td>165.5</td>
<td>59.3</td>
<td>21.6</td>
<td>29.3</td>
<td>38.4</td>
<td>459.9</td>
<td></td>
</tr>
<tr>
<td>SD</td>
<td>5.2</td>
<td>6.0</td>
<td>6.5</td>
<td>1.4</td>
<td>5.5</td>
<td>8.7</td>
<td>99.5</td>
<td></td>
</tr>
<tr>
<td>SE</td>
<td>1.4</td>
<td>1.7</td>
<td>1.8</td>
<td>0.4</td>
<td>1.5</td>
<td>2.4</td>
<td>28.7</td>
<td></td>
</tr>
</tbody>
</table>

### Participant Characteristics: Week 8

<table>
<thead>
<tr>
<th>Participant #</th>
<th>DOB</th>
<th>Age (y)</th>
<th>Ht (cm)</th>
<th>Wt (kg)</th>
<th>BMI</th>
<th>Body Fat (%)</th>
<th>Serum folate (nmol/L)</th>
<th>Folate intake DFE (mcg/d)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1004</td>
<td>7/1/1978</td>
<td>35</td>
<td>163.2</td>
<td>58.1</td>
<td>21.82</td>
<td>37.3</td>
<td>83.7</td>
<td>329.6</td>
</tr>
<tr>
<td>1008</td>
<td>8/13/1983</td>
<td>29</td>
<td>167.6</td>
<td>61.1</td>
<td>21.76</td>
<td>30.9</td>
<td>64.0</td>
<td>295.6</td>
</tr>
<tr>
<td>1012</td>
<td>8/30/1994</td>
<td>18</td>
<td>168.9</td>
<td>63.7</td>
<td>22.32</td>
<td>27.3</td>
<td>68.9</td>
<td>275.5</td>
</tr>
<tr>
<td>1017</td>
<td>9/2/1982</td>
<td>30</td>
<td>160.7</td>
<td>53.0</td>
<td>20.53</td>
<td>19.4</td>
<td>83.3</td>
<td>361.1</td>
</tr>
<tr>
<td>1018</td>
<td>10/30/1990</td>
<td>22</td>
<td>177.8</td>
<td>71.0</td>
<td>22.46</td>
<td>22.9</td>
<td>52.9</td>
<td>300.6</td>
</tr>
<tr>
<td>1026</td>
<td>5/14/1993</td>
<td>20</td>
<td>163.2</td>
<td>65.7</td>
<td>24.68</td>
<td>39.2</td>
<td>66.8</td>
<td>236.0</td>
</tr>
<tr>
<td>1029</td>
<td>6/1/1982</td>
<td>31</td>
<td>167.6</td>
<td>62.2</td>
<td>22.13</td>
<td>30.1</td>
<td>72.8</td>
<td>420.7</td>
</tr>
<tr>
<td>1030</td>
<td>10/19/1985</td>
<td>27</td>
<td>167.6</td>
<td>58.3</td>
<td>20.76</td>
<td>25.3</td>
<td>71.6</td>
<td>375.5</td>
</tr>
<tr>
<td>1031</td>
<td>10/8/1987</td>
<td>35</td>
<td>161.4</td>
<td>55.7</td>
<td>21.38</td>
<td>32.7</td>
<td>67.5</td>
<td>223.8</td>
</tr>
<tr>
<td>1038</td>
<td>2/28/1984</td>
<td>29</td>
<td>156.4</td>
<td>50.6</td>
<td>20.69</td>
<td>26.5</td>
<td>70.7</td>
<td>611.9</td>
</tr>
<tr>
<td>1039</td>
<td>7/31/1987</td>
<td>26</td>
<td>161.9</td>
<td>52.5</td>
<td>20.03</td>
<td>28.3</td>
<td>91.4</td>
<td>532.7</td>
</tr>
<tr>
<td>1040</td>
<td>4/6/1987</td>
<td>26</td>
<td>173.4</td>
<td>67.6</td>
<td>22.50</td>
<td>33.6</td>
<td>63.3</td>
<td>413.0</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>Mean</th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>DOB</td>
<td>27.3</td>
<td>165.8</td>
<td>60.0</td>
<td>21.8</td>
<td>29.5</td>
<td>71.4</td>
<td>364.7</td>
<td></td>
</tr>
<tr>
<td>SD</td>
<td>5.4</td>
<td>5.9</td>
<td>6.4</td>
<td>1.2</td>
<td>5.7</td>
<td>10.5</td>
<td>116.6</td>
<td></td>
</tr>
<tr>
<td>SE</td>
<td>1.5</td>
<td>1.6</td>
<td>1.8</td>
<td>0.3</td>
<td>1.6</td>
<td>2.9</td>
<td>32.3</td>
<td></td>
</tr>
</tbody>
</table>
### Participant Characteristics: Other

<table>
<thead>
<tr>
<th>Participant #</th>
<th>Week 4 Serum folate (nmol/L)</th>
<th># pills missed</th>
<th>Mean FA Supp Intake (mcg/d)</th>
<th>MTHFR genotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>1004</td>
<td>45.6</td>
<td>4</td>
<td>743</td>
<td>C/T</td>
</tr>
<tr>
<td>1008</td>
<td>55.3</td>
<td>5</td>
<td>729</td>
<td>C/C</td>
</tr>
<tr>
<td>1012</td>
<td>54.2</td>
<td>2</td>
<td>771</td>
<td>C/T</td>
</tr>
<tr>
<td>1017</td>
<td>40.8</td>
<td>2</td>
<td>771</td>
<td>C/T</td>
</tr>
<tr>
<td>1018</td>
<td>51.9</td>
<td>2</td>
<td>771</td>
<td>C/T</td>
</tr>
<tr>
<td>1026</td>
<td>74.6</td>
<td>0</td>
<td>800</td>
<td>C/T</td>
</tr>
<tr>
<td>1029</td>
<td>56.8</td>
<td>1</td>
<td>786</td>
<td>C/T</td>
</tr>
<tr>
<td>1030</td>
<td>52.9</td>
<td>4</td>
<td>743</td>
<td>C/C</td>
</tr>
<tr>
<td>1031</td>
<td>66.7</td>
<td>2</td>
<td>771</td>
<td>C/T</td>
</tr>
<tr>
<td>1038</td>
<td>54.8</td>
<td>1</td>
<td>786</td>
<td>C/C</td>
</tr>
<tr>
<td>1039</td>
<td>69.3</td>
<td>0</td>
<td>800</td>
<td>C/C</td>
</tr>
<tr>
<td>1040</td>
<td>37.1</td>
<td>0</td>
<td>800</td>
<td>C/C</td>
</tr>
</tbody>
</table>

Mean | 55.0 | 1.9 | 772.6 |
SD   | 11.1 | 1.7 | 23.9  |
SE   | 3.1  | 0.5 | 6.6   |
THE END