# FOLATE STATUS AND MILK FOLATE CONCENTRATION IN LACTATING WOMEN

by

Karen Ruggio Amanna

Thesis submitted to the Faculty of the

Virginia Polytechnic Institute and State University

in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

in

HUMAN NUTRITION, FOODS, AND EXERCISE

APPROVED:

Elizabeth Thomas

Elizabeth Thomas, Chair

Denise Brochetti

Denise Brochetti

Eleanor Schlenker

Eleanor Schlenker

December 1996

Blacksburg, Virginia

Key words: Ferritin, Folate, Iron, Lactation, Milk

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Karen Ruggio Amanna

Elizabeth A. Thomas, Chairperson

Department of Human Nutrition, Foods, and Exercise

(ABSTRACT)

Lactating women have an increased requirement for folate which contributes to their risk for suboptimal folate status. Although milk folate secretion appears to be maintained independent of folate intake and maternal folate status, studies with animal species have demonstrated a relationship between iron deficiency and impaired milk folate secretion. Objectives of this study were to monitor the folate status of lactating women and to examine the relationship among folate intake, dietary iron, folate status, iron status and milk folate. Seven-day dietary records, milk samples, and blood samples were collected monthly for four months from five lactating women. Dietary iron and folate was analyzed. Milk folate, serum ferritin, serum folate, and red blood cell (rbc) folate concentrations were measured. Mean folate and iron intakes were 495 $\pm$ 105 µg/d and 24 $\pm$ 4 mg/d, respectively. All women had

normal rbc folate and serum ferritin values during the study. Milk folate increased (p=.06) from 35 $\pm$ 10 μg/L in month one to 69 $\pm$ 30 μg/L in month three. Dietary and rbc folate were not significantly correlated with milk folate. There was a significant positive correlation between milk folate and serum folate (r = .48, p= .04) and between milk folate and iron intake (r=.63, p=.003). Results indicate that the folate intake in this population of lactating women was sufficient to maintain adequate folate stores. Results also suggest a relationship between iron intake and milk folate. Research is needed to determine dietary requirements during lactation and to investigate the relationship between dietary iron and milk folate.

# ACKNOWLEDGMENTS

I would like to express my appreciation to Dr. Elizabeth Thomas for her guidance, support, encouragement, and most of all for her enthusiasm for this research which was so inspirational to me. I would also like to thank my committee members Dr. Denise Brochetti and Dr. Eleanor Schlenker for their assistance with this thesis as well as other aspects of my graduate program. Special thanks are in order for Janet Rinehart for all the times she drove to subjects' homes, for performing blood collections, for all her work with the folate assay, for her eagerness to provide assistance, and for her overall technical expertise. I also would like to thank the women that participated in this study for their dedication and their children for sharing their mothers' time with me. I would like to thank my fellow graduate students for their friendship and empathy. Sincere thanks are extended to my mother and father Theresa and James Ruggio; to my brothers David, Michael, and Steven; and to my sister Debie for providing a strong supportive family network that has been critical to my success. And finally, words fail to express my gratitude to my husband Ashwin E. Amanna for his unconditional love, patience, commitment, encouragement, praise, and valuable words of wisdom.

# TABLE OF CONTENTS

# LIST OF TABLES

# LIST OF FIGURES

# CHAPTER I

# INTRODUCTION

Folate compounds function as coenzymes for one-carbon transfer reactions. Folate-requiring one-carbon transfer reactions are essential for the biosynthesis of purine nucleotides and of deoxythymidylic acid, required for DNA and RNA synthesis (Wagner, 1995). Therefore, a deficiency of folate results in impaired cell division and alterations in protein synthesis.

During periods of rapid growth and anabolic activity, such as in regnancy and lactation, the requirement for folate is increased (Bailey, 1995). Adequate folate intake during pregnancy and lactation is necessary to support enhanced DNA, RNA, and amino acid biosynthesis, hematopoiesis, fetal growth and milk production. Because of the increased requirement for folate, pregnant and lactating women are particularly susceptible to folate deficiency (O'Connor, 1994).

Folate deficiency has been reported to be rare among breastfed infants in the United States (Tamura, 1980). However, maternal folate status may be inadequate despite sufficient milk folate secretion to maintain adequate folate status of the infant (Picciano, 1995). Breast milk concentrations are often

maintained at the expense of maternal folate reserves (Picciano, 1995; Tamura et al., 1980).

Maternal folate intake during the periconceptual period has been shown to be inversely correlated with the risk of having a baby with a neural tube defect (NTD) (Wald, 1993). The United States Public Health Service has made the recommendation that all women of childbearing age consume 0.4 mg folic acid per day to reduce their risk of having a pregnancy affected with spina bifida and other NTDs (Rush, 1994). However, the actual requirement for folate in an individual women would be affected by her current folate status. If a women is depleted of folate stores by the end of lactation, she may be at risk of having a NTD baby if she does not replete her stores before her subsequent pregnancy. Winkvist et al. (1992) defined maternal depletion as a condition that should be evaluated over a reproductive cycle, characterized by a negative change in maternal nutritional status which most likely occurs among women with marginally inadequate food intake. Adequate folate intake is essential to replete folate stores during lactation and to prevent depletion in subsequent pregnancies.

The folate requirement of lactating women has been determined by measuring milk folate secretion of mothers of healthy infants added to the requirement for nonpregnant, nonlactating women (Picciano, 1995). The folate nutritional status of the mother has been considered to be adequate if

her nursing infant is growing well (Picciano, 1995). The actual health outcomes of lactating women are usually not considered. The current RDA for folate in lactating women (280 μg/d) is based on limited research and recent evidence suggests that the recommended allowance may underestimate requirements. A decline in folate status has been reported in nonpregnant, nonlactating subjects consuming the current RDA (180 μg/d) for folate (O'Keefe et al., 1995; Sauberlich et al., 1987). Furthermore, the reported values for milk folate secretion have varied considerably (O'Connor, 1994). Therefore, it is unclear what the actual need for folate is to sustain lactation and maintain adequate maternal folate status.

Although milk folate secretion does not appear to be affected by maternal folate status, studies with animal species suggest that dietary factors other than folate may influence milk folate content (O'Connor, 1991). Depressed milk folate secretion was an early manifestation of iron deficiency in rats (O'Connor et al., 1989). In humans, iron and folate deficiencies are reported to exist simultaneously; however, the effect of iron deficiency on milk folate secretion in lactating women has not been investigated.

## Objectives

1. To monitor the folate status of lactating women over the period from parturition through four months postpartum.
2. To examine the relationship between maternal folate status and dietary folate intake during lactation.
3. To examine the relationship between maternal folate status and milk folate concentration.
4. To examine the relationship between maternal folate status and dietary iron intake.
5. To investigate the relationship between maternal iron status and milk folate concentration.

## Hypotheses

The following null hypotheses were tested with a sample population of lactating women from parturition through four months postpartum:

Ho 1: There is no change in red blood cell folate concentrations.

Ho 2: There is no change in serum folate concentrations.

Ho 3: There is no change in milk folate concentrations.

Ho 4: There is no correlation between dietary folate intake and red blood cell folate concentrations.

Ho 5: There is no correlation between dietary folate intake and serum folate concentrations.

Ho 6: There is no correlation between dietary folate intake and milk folate concentrations.

Ho 7: There is no correlation between red blood cell folate and milk folate concentrations.

Ho 8: There is no correlation between serum folate and milk folate concentrations.

Ho 9: There is no correlation between serum ferritin and milk folate concentrations.

Ho 10: There is no correlation between dietary iron intake and milk folate concentrations.

# CHAPTER II

# REVIEW OF THE LITERATURE

## Metabolic Functions of Folate

Pterolyglutamic acid is the base molecule for the group of compounds referred to as folates (Herbert and Das, 1994). Most dietary folates are in the form of the polyglutamate derivatives, 5-methyl tetrahydrofolate and 10-formyltetrahydrofolate with glutamic acid residues on side chains (Shane, 1995). Prior to absorption, polyglutamates are hydrolyzed to pteroyl monoglutamate forms by glutamyl hydrolase (conjugase) in the intestinal mucosa.

Folate compounds serve as coenzymes which are required for several reactions involving transfer of one-carbon units (Wagner, 1995). These reactions include 1) pyrimidine nucleotide biosynthesis (methylation of deoxyuridylic acid to thymidylic acid); 2) de novo purine synthesis (formylation of glycinamide ribonucleotide and 5-amino-4-imidazole carboxamide ribonucleotide); 3) amino acid conversions including the interconversion of serine and glycine, catabolism of histidine to glutamic

acid, and conversion of homocysteine to methionine; 4) methylation of small amounts of transfer RNA; and 5) generation of formate into the formate pool (Herbert and Das, 1994).

When methionine intake is insufficient, folate is required to provide methyl groups for many S-adenosylmethionine (SAM)-mediated methylation reactions. Methyl transferase enzymes transfer the methyl groups of SAM to a variety of compounds including proteins, lipids, DNA, and RNA (Scott et al., 1994). Methylation reactions are also important for gene expression (Wagner, 1995).

## Manifestations of Folate Deficiency

Folate deficiency results in impaired nucleic acid synthesis, impaired amino acid synthesis, and defective cell division (Wagner, 1995). One manifestation of defective cell division is megaloblastic anemia which is characterized by large, abnormal, nucleated cells which accumulate in the bone marrow. In addition to deficiency of erythrocytes, there are decreased numbers of white cells and platelets. Research indicates that folate deficiency leads to defective cell division as the result of impaired formation of thymidylic acid, which is required for DNA synthesis (Wickrenmasinghe and Hoffbrand, 1980).

The dietary supply of methyl groups from choline and methionine is usually insufficient to supply the needs of the body (Wagner, 1995); therefore, de novo synthesis of methyl groups, which requires folate, is needed. Folate deficiency may lead to impaired SAM-mediated methylation reactions (Wagner, 1995). Defective methylation may potentially result in alterations in gene expression and impaired synthesis of certain proteins and other compounds which are formed by SAM-mediated reactions.

Folate antagonists, such as methotrexate and sulfasalazine, have been used as chemotherapeutic agents (Priest and Bunni, 1995) as well as in the treatment of rheumatoid arthritis and other nonneoplastic diseases (Morgan and Baggott, 1995). Although folate antagonists have been shown to have beneficial effects after the onset of certain forms of cancer, folate deficiency has been associated with increased risk of colon and cervical cancer development (Blount and Ames, 1995). Although the mechanism is not known, it appears that folate has a protective effect against carcinogenesis (Butterworth, 1991). In a recent review of research, Blount and Ames (1995) reported that folate deficiency causes uracil misincorporation and significant increases in chromosome breaks. Chromosomal breakage and decreased DNA methylation results in epithelial tissues which are at increased risk of neoplastic transformation (Butterworth, 1993). Folate supplementation of folate deficient subjects significantly reduced chromosome breakage (Everson

et al., 1988) and decreased preneoplastic markers in the cervix (Butterworth et al., 1992) and colon (Butterworth, 1991).

Several studies have suggested that folate deficiency is involved in the pathogeneis of NTDs (Scott et al., 1995). In the developing embryo, the neural tube is formed by fusion of paired folds of the neural groove, followed by formation of the caudal part of the spinal cord (Elwood et al., 1992b). Closure of the neural tube occurs between 24 and 28 days postconception (Scott et al., 1995). Incomplete closure of the spinal cord results in spina bifida. Anencephaly is a lethal condition where there is incomplete closure of the skull. In North America and Europe the incidence of NTDs ranges from 0.6 to 3.7 cases per 1000 live births (Christensen and Rosenblatt, 1995).

Although the mechanisms by which folate prevents NTDs is unknown, studies have indicated that periconceptional folate supplementation reduces the occurrence and reoccurrence of NTDs (Czeizal and Dudas, 1992; MRC, 1991). There appears to be an inherited alteration in folate metabolism which predisposes a woman to NTDs (Christensen and Rosenblatt, 1995). The most recent evidence indicates that impaired methionine synthase activity may be the primary cause of NTDs (Scott et al., 1995). It has been suggested that higher amounts of folate protect against NTDs by overcoming the metabolic defect (Christensen and Rosenblatt, 1995).

The British Medical Research Council (MRC, 1991) conducted a randomized double-blind prevention trial from 1983 to 1991. Results of this study indicated that among women who previously had a pregnancy with a NTD and who were subsequently supplemented with 360 μg of folate per day prior to conception, the risk of recurrence of NTDs was reduced by 72% compared to women who did not take folate supplements. Czeizal and Dudas (1991) conducted a randomized trial of multivitamin supplementation with 800 μg of folate among 4,682 women who did not have a previous NTD pregnancy. Of the 2,052 women consuming the multivitamin supplement there were no cases of NTDs reported compared to six cases reported among the 2,104 women in the placebo group.

## Requirements and Recommended Intakes for Adult Women

The two approaches that have been used to establish the RDA for folate include 1) determination of minimum folate requirements taking into consideration bioavailability, individual variation, and need for adequate stores; and 2) evaluation of usual intakes of food folate by persons in good folate status (NRC, 1989). The current RDA for folate is 180 μg/d for adult non-pregnant and non-lactating females (NRC, 1989). This estimate was partially based on average folate intakes in the United States and Canada of

3 μg/kg body weight (149 μg/d). This amount of folate, with an estimated 50% bioavailability, was believed to be sufficient to maintain adequate circulating concentrations and liver stores of folate in the majority of the population.

Three studies have estimated folate requirements and allowances for adult non-pregnant women utilizing different methods (O'Keefe et al., 1995; McPartlin et al., 1993; Sauberlich et al., 1987). Sauberlich et al. (1987) estimated folate requirements of adult non-pregnant women in a depletion-repletion study. After a 28-day folate depletion period, 10 healthy adult females received increasing supplements of folate. Plasma levels fell 60% by the end of the 28-day depletion period and continued to fall until 200 μg of daily food folate was consumed. A daily folate intake of 300 μg for three weeks resulted in a rise in plasma folate levels; however, erythrocyte folate levels continued to fall. The researchers concluded that a daily intake of 200 - 250 μg of dietary folate is required for adult women and 300 μg/d provides for storage.

McPartlin et al. (1993) estimated folate requirements for non-pregnant women based on excretion of the urinary folate catabolites, ρ-amino-benzoyl glutamate and ρ-actamidobenzoyl glutamate. The estimated folate requirement for non-pregnant women was 280 μg/d based on 50% bioavailability of dietary folate.

O'Keefe et al. (1995) conducted a 70-day metabolic study with non-pregnant women to determine folate status in response to daily folate intake of 200, 300, and 400 μg. Over a 10 week period, serum and erythrocyte folate decreased 40% and 20%, respectively, in the group consuming 200 μg/d compared to a 27% and 7% respective increase in the group consuming 400 μg/d. In addition, plasma homocysteine levels were significantly higher in the group consuming 200 μg/d compared to the groups consuming 300 μg/d or 400 μg/d. Based on these data, the investigators concluded that 200 μg/d of dietary folate is inadequate to maintain folate status in non-pregnant women and that 400 μg/d may reflect actual requirements.

Pregnancy is associated with an increased requirement for folate due to expansion of blood volume and increasing folate demands of the growing fetus and placenta (Bailey, 1995). In addition, urinary folate excretion increases during pregnancy (Fleming et al., 1972). Fleming et al. (1972) found that during pregnancy, glomerular filtration rate increases about 60% accompanied by less efficient renal tubular absorption of nutrients.

Chanarin et al. (1968) observed a rapid decline in blood folate values in unsupplemented pregnant women in the United Kingdom and then estimated the amount of supplemental folate required to prevent this fall. Addition of 100 μg/d of synthetic folate in addition to the normal diet of the

women resulted in a rise in red blood cell folate concentrations during pregnancy. The normal food folate intake of these women was not reported; however, a subsequent study reported average dietary folate intakes in the United Kingdom to be 190 μg/d (Bates et al., 1982). Colman (1982) estimated that 300 μg/d increased blood folate levels in pregnant women of poor folate status. Based on these types of studies, the current RDA for pregnant women was set at 400 μg/d.

Lactation is also associated with an increased requirement for folate due to milk folate secretion. Folate requirements during lactation have been estimated by adding the amount of dietary folate needed to replace milk folate secretion to the amount recommended for non-pregnant, non lactating women (Picciano, 1995). The current RDAs for folate for lactating women in the first six months and second six months are 280 μg/d and 260 μg/d, respectively. This estimation was based on an estimated typical milk folate secretion of 50 μg/L with daily milk production of 750 ml during the first six months and 600 ml in the second six months (NRC, 1989).

## Maternal Folate Intake and Folate Status During Lactation

There are limited dietary data and nutritional status information available on lactating women (Picciano, 1995). Dietary intakes of 59 lactating

women, 114 pregnant women, and 1,952 other women in the United States were reported in the United States Department of Agriculture (USDA) 1985 and 1986 Continuing Surveys of Food Intakes by Individuals (CSFII) (Borrud et al., 1993). The CSFII provided estimated dietary folate intakes based on 24-hour dietary recalls. The mean folate intake of the lactating women was 299 μg/d compared to 263 μg/d for pregnant women and 209 μg/d for other women. Maternal folate status was not measured in the CSFII.

Sneed et al. (1981) estimated folate intakes of seven nonsupplemented low income women from 4-day diet records during the first and sixth week postpartum. Mean folate intakes of these women were 290 and 340 μg/day, respectively. In another study (Thomas et al., 1980) of six lactating women described as well nourished, the estimated folate intake from 4-day diet records was 194 μg/day. The women in both of these studies showed no signs of folate depletion using serum folate concentrations as the criterion of folate status (Sneed et al., 1981; Thomas et al., 1980). Keizer et al. (1995) examined dietary intake and folate status of 14 lactating adolescents who were supplemented with 0.8-1 mg/day of folate during pregnancy and were provided with supplements containing 300 μg folate to consume daily throughout lactation. Dietary intake, serum, and red blood cells were analyzed for folate at 4, 8, and 12 weeks postpartum. Mean folate intake including the supplements, adjusted for supplement compliance, was

450 µg/day. Red blood cell and serum folate levels remained normal throughout the study period.

Other studies with lactating women have reported signs of folate depletion. Studies have also indicated that the folate status of a lactating woman is influenced by nutritional intake and folate status during pregnancy (Ek, 1983; Qvist et al., 1986; Salmenpera et al., 1986). Salmenpera et al. (1986) found that 5% of lactating women had low serum folate levels (< 3 µg/L) despite supplementation with 100 µg/day throughout lactation. These researchers also found that declining folate status was more frequent among lactating women who did not take folate supplements during pregnancy. Qvist et al. (1986) reported low red blood cell folate levels among otherwise healthy unsupplemented lactating women. Ek (1983) found a decline in red blood cell folate during the first two months postpartum with an increase in the subsequent ten months. In a study of 87 Navajo women, 9% of the women had low serum folate levels at term, and 23% had low serum folate levels after one month of lactation (Butte et al., 1981). Smith et al. (1983) examined folate status of lactating women who were supplemented throughout pregnancy. Serum and red blood cell levels remained within the normal range; however, red blood cell folate levels decreased significantly from 6-12 weeks postpartum. Total dietary folate intake was not assessed in these studies.

Parity and time between pregnancies significantly affects the folate status of non-pregnant women (Martinez, 1980; Colman et al., 1975). Martinez (1980) observed that age of the youngest child and interval between the last two children were significantly correlated with red blood cell folate levels. Colman et al. (1975) found among a group of low income women, 49% that had been pregnant or lactated within the previous 6-12 months were folate deficient compared to 27% that had not been pregnant or lactated for > 2 years.

Dietary data from 24-hour recalls collected in the Second and Third National Health and Nutritional Examination Surveys (NHANES II and III) provided information on nutrient intakes of nonpregnant, nonlactating women in the United States. In the 1976-1980 NHANES II (Subar, 1989), the mean folate intake for women of 207 μg/d was similar to the folate intake reported for nonlactating women in the CSFII (Borrud et al., 1993). Estimates of plasma and red blood cell folate data in a subsample of 10% of adults in the NHANES II suggested that 15% of women aged 20-44 years had low plasma folate values (< 3.0 μg/L) and 13% had low red blood cell folate values (<140μg/L) (Senti et al., 1984). The mean folate intakes for women age 12-49 reported in the 1988-1991 NHANES III (Alaimo et al., 1994) were 220-237 μg/d.

Overall these studies suggest that there are variations in folate status of lactating women. Although a decreases in folate levels during lactation have been observed; well nourished women, supplemented with folate during pregnancy, generally appear to maintain adequate folate stores (Sneed et al., 1981, Thomas et al., 1980). However, some studies have demonstrated low folate levels in women that were supplemented (Salamenpera et al., 1986) and unsupplemented (Qvist et al., 1986) during lactation. Dietary folate intake data among lactating women are limited. Few studies have assessed both dietary intake and biochemical measures of folate status (O'Connor, 1994). Therefore, dietary folate requirements to sustain adequate folate levels during lactation are unknown.

## Food Sources of Folate

Foods with the highest content of folate per unit dry weight include liver, fortified cereals, fresh green vegetables, and dried beans (Subar et al., 1989). Data from the NHANES II survey indicated that orange juice, white breads, dried beans, green salad, and ready-to-eat breakfast cereals are the major food sources of folate in the US diet, contributing 37% of total folate intake (Subar et al., 1989). Orange juice ranked number one as the major source, contributing 9.7% of total dietary folate. Table 1 shows the major contributors of food folate to the US diet from the NHANES II.

**TABLE 1**
**Major Contributors of Folate in the US Diet According to NHANES II Data**

| Food Item | Percent Total Folate (%) | Folate per 100g (μg) | Folate per usual serving (μg) |
|---|---|---|---|
| Orange Juice | 9.70 | 41 | 43 |
| White bread, rolls, crackers | 8.61 | 35 | 9 |
| Pinto, navy, and other dried beans | 7.08 | 275 | 112 |
| Green salad | 6.85 | 100 | 84 |
| Cold cereals (not superfortified) | 4.96 | 275 | 112 |
| Eggs | 4.63 | 43 | 31 |
| Liver | 3.07 | 428 | 383 |
| Superfortified cereal | 3.06 | 991 | 242 |
| Whole milk, whole-milk beverages | 2.90 | 5 | 12 |
| Bran and granola cereals | 2.48 | 236 | 58 |
| Whole-wheat breads | 2.43 | 55 | 16 |
| Corn | 1.89 | 23 | 19 |
| Spaghetti with tomato sauce | 1.51 | 14 | 24 |
| Tomatoes, tomato juice | 1.31 | 10 | 21 |
| Beef steaks, roasts | 1.19 | 12 | 10 |
| Peanuts, peanut butter | 1.18 | 92 | 25 |

Subar et al. (1989)

Folate is highly susceptible to destruction by heat, oxidation, and ultraviolet light. During food preparation, processing, and storage, as much as 50 - 95% of the folate content of food may be destroyed (Herbert and Das,

1994). Diets containing thoroughly cooked foods are likely to be low in folate (Elwood et al., 1992a).

## Human Milk Folate Content

The folate content of milk was underestimated by early investigators for several reasons. Ascorbate was not added to milk samples to prevent folate oxidation, folate hydrolase treatment was not used to cleave long-chain pterolpolyglutamates, samples were not heat treated to release folate from binding proteins, or test organisms were used that did not utilize all folate forms in the samples (Picciano, 1995).

More recent investigators have applied improved techniques to analyze folate; however, considerable variation exists in reported folate values of human milk. Recently, O'Connor (1994) reviewed human milk folate values reported by investigators that employed a microbiological assay with *L. casei* as the test organism. Reported values for milk folate, presented in Table 2, ranged from 20 to 142 μg/L.

Variation may be due to differences in genetics, nutritional status, stage of lactation, protocols for folate assays, and sampling and storage techniques. Studies have indicated that folate content in milk increases as lactation progresses (Cooperman et al., 1982; Tamura et al., 1980; Udipi et al., 1987), the folate content in milk increases throughout the day (Udipi et al., 1987), and

folate is higher in hind-milk versus foremilk (Brown et al., 1986, Udipi et al., 1987). Freezer storage of milk samples for three months at -20°C showed a decrease in folate (Bank et al., 1985).

**TABLE 2**
**Reported Milk Folate Values using *L. casei* as the Test Organism**

| Investigators | Milk folate content (μg /L)* |
|---|---|
| Tamura et al. (1980) | 142 ± 48 |
| Thomas et al. (1980) | 50 ± 4 |
| Sneed et al. (1981) | 43 ± 5 |
| Cooperman et al. (1982) | 26 ± 4.42 |
| Ek (1983) | 69 - 135 ± 6 - 27 |
| Smith et al. (1983) | 45 - 104 ± 27 - 53 |
| Eitenmiller et al. (1984) | 49 ± 19 |
| Bank et al. (1985) | 50 ± 10 |
| Udipi et al. (1987) | 22 - 127 ± 4 - 28 |
| Brown et al. (1986) | 85 ± 38 |
| O'Connor et al (1991) | 101 ± 12 |

* mean ± S.D.

## Maternal Folate Status and Milk Folate Content

In well nourished women it appears that there is no correlation between maternal serum and milk folate levels before or after maternal folate

supplementation (Keizer et al., 1995; Salmenpera et al., 1986; Smith et al., 1983; Tamura et al., 1980). Smith et al. (1983) reported that even as levels of maternal serum and red blood cell folate levels decreased, folate levels in human milk increased as lactation progressed. Tamura et al. (1980) reported no significant increase in milk folate levels of well nourished lactating women after four weeks of supplementation with 1 mg/day of folate. Kirksey (1986) observed that folate concentrations in milk of well-nourished mothers were not significantly changed by short-term supplementation with 100 to 800 mg/day.

Milk folate concentrations have been observed to increase following supplementation in malnourished women. Milk folate levels significantly increased in women suffering from megaloblastic anemia after folate supplementation of 100 to 200 mg/d for four days (Metz et al., 1970). Serum folate levels, however, remained low after supplementation indicating preference for maintenance of milk folate levels over serum folate. It appears that human milk folate content is maintained at the expense of maternal reserves (Picciano, 1990; Smith et al., 1983). Sneed et al. (1981) reported significant increases in milk folate levels of malnourished women following folate supplementation with 800 mg/d for six weeks.

## Iron and Milk Folate

Iron deficiency and folate deficiency frequently occur simultaneously. It has often been the assumption that these two deficiencies occurred independent of each other due to concurrent dietary inadequacies (O'Connor, 1991). Chanarin et al. (1965) were the first to show a relationship between iron and folate during reproduction. Pregnant women who were iron deficient were more likely to be folate deficient than pregnant iron sufficient women.

More recently, results from controlled animal studies have provided evidence to suggest that iron deficiency may result in impaired folate utilization. Studies with rats have shown that in lactation, decreased milk folate secretion was an early manifestation of maternal iron deficiency (O'Connor et al., 1987; O'Connor et al., 1989a; O'Connor et al., 1989b). O'Connor et al. (1987) examined the relationship between iron deficiency and mammary gland uptake of folate and folate secretion into milk of nursing rat dams. The rate of pteroylmonoglutamic acid incorporation into milk and the amount of milk folate in iron depleted rats was significantly lower than in the iron sufficient controls despite provision of folate at twice the RDA of folate for rats (1mg/kg). On day 17 of lactation, milk collected from moderately and severely iron deficient rat dams was 35% and 46%, respectively, lower in folate than the milk collected from iron sufficient rats.

Mean incorporation of injected pteroylmonoglutamic acid into milk of severely iron-deficient rat dams was 33% lower than iron-sufficient controls.

Another study using rats (O'Connor et al., 1989a) was conducted to determine the phase of lactation in which maternal iron deficiency resulted in a depression of milk folate secretion and to what extent the quantity of folate delivered to nursing rat pups was affected. Free and total milk folate activities increased in iron sufficient rats as lactation progressed from day 7 to day 17. However, in the iron deficient rats, the mean total folate concentration in milk was significantly less (42%) on day 17 compared to day 7 of lactation. By day 17 of lactation, the total milk folate content of the iron deficient rats was 81% lower compared to the iron sufficient controls. The mean folate intake by the iron deficient rat pups was 72% lower than the iron sufficient rat pups on day 17 of lactation.

In rat dams, the amount of iron added to the maternal diet had no effect on indices of maternal folate status with the exception of plasma folate and milk folate secretion (O'Connor et al., 1990). In the iron sufficient rats, the amount of folate added to the maternal diet was correlated to milk folate concentration; however, in the iron deficient rats there was no correlation. Addition of supplemental folate to the diets of iron-deficient rats did not improve milk folate levels.

Iron deficient rat dams were found to secrete milk with a reduced percentage of long chain polyglutamates (O'Connor et al., 1989a). Milk collected from the iron sufficient rats contained 54% long chain folypolyglutamates compared to 33% from the iron deficient rats on day 17 of lactation. During iron sufficiency, folate incorporation into milk from plasma occurs against a steep concentration gradient in humans (Tamura, 1980) and in rats (O'Connor et al., 1989a). In iron sufficient rats the concentration of milk folate was nine times greater than plasma folate on day 17 of lactation (O'Connor et al., 1989a). In contrast, the iron deficient rats had similar plasma and milk folate concentrations.

Metabolism of folate to folypolyglutamates is important for intracellular storage and concentration of folates (Shane, 1990). Folypolyglutamate synthetase and methionine synthetase catalyze the reactions in which glutamate residues are added to folate. Impaired polyglutamation, secondary to iron deficiency, may reduce milk folate; however, decreased milk folate secretion in rats was not explained by depressed mammary tissue folypolyglutamate synthetase and methionine synthetase enzymes in an in vitro system (O'Connor et al., 1989b). Similar results were found in an in vivo system (O'Connor et al., 1991). Folypolyglutamate synthetase and methionine synthetase were not effected by iron deficiency despite reduced milk folate concentrations.

## Assessment of Folate Status

A fall in serum folate levels usually provides the first sign of declining folate status. Serum folate levels below 3 μg/L suggests negative folate balance (Bailey, 1990). Serum levels between 3 μg/L and 5 μg/L suggests a marginal risk of deficiency. Serum folate levels provide an indication of folate balance at the time serum samples are drawn (Herbert, 1987). However, low serum folate levels may not provide evidence for tissue folate depletion. After acute alcohol administration, serum folate levels may be depressed without evidence of other signs of deficiency (Lindenbaum and Allen, 1995). In some patients diagnosed with megaloblastic anemia due to folate deficiency, serum folate levels were only slightly decreased or normal (Savage, 1994). Investigators have frequently found an overlap between serum folate values of normal subjects and folate deficient subjects (Lindenbaum and Allen, 1995). Low serial serum folates over a period more than a month, however, may indicate that stores are low (Herbert, 1987). Therefore, serum folate should be used with other diagnostic tests to determine folate status.

Folate depletion is characterized by a fall in erythrocyte folate below 140 μg/L (Herbert and Das, 1994). Erythrocyte folate concentration is a more accurate measurement of tissue folate stores (Lindenbaum and Allen, 1995).

Herbert (1962) found that reduction of erythrocyte folate levels paralleled a reduction in liver folate stores. Erythrocyte folate provides a direct measurement of tissue folate concentrations in cells produced by the bone marrow (Lindenbaum and Allen, 1995). Hoffbrand et al. (1966) showed that red blood cell folate levels correlated more strongly with the presence of megaloblastic erythropoiesis than serum folate levels. However, since red blood cells have a relatively long life span (120 days), the erythrocyte folate concentration may not reflect current folate status. The concentrations in the red blood cells are determined by folate status when they were formed (O'Connor, 1994).

Homocysteine concentration provides a functional indicator of folate status. Serum homocysteine levels are increased in folate deficiency due to a decrease in methionine synthase activity which requires methyltetrahydrofolate as a substrate in the conversion of homocysteine to methionine (Stabler et al., 1988). Elevated homocysteine levels were found in 90% of patients with megaloblastic anemia due to folate deficiency (Savage et al., 1994). Stabler et al. (1988) reported elevated serum homocysteine levels in 18 of 19 patients with folate deficiency.

Increased homocysteine levels may also be an indication of cobalamin deficiency. Cobalamin is required as a cofactor for conversion of homocysteine to methionine. Therefore, a deficiency of cobalamin may also

result in elevated homocysteine levels. Savage et al. (1994) found elevated homocysteine levels in 96% of patients with diagnosed cobalamin deficiency.

Forminoglutamic acid (FIGLU), a product of histidine breakdown, is utilized as a substrate in the reaction in which tetrahydrofolate is converted to 10-formyltetrahydrofolate. In folate deficiency, FIGLU accumulates and appears in the urine (Wagner, 1995). Urinary FIGLU is no longer used as an indicator of folate status due to problems with specificity and sensitivity (Lindenbaum and Allen, 1995).

The deoxyuridine (dU) suppression test measures the ability of added deoxyuridine to suppress the incorporation of titrated tritium labeled thymidine into DNA by bone marrow cells (Lindenbaum and Allen, 1995). In folate deficiency, function of thymidylate synthase is impaired resulting in decreased methylation of the deoxyuridine and more incorporation of the labeled thymidine (Chanarin, 1986). The dU suppression test is not widely used due to expensive and cumbersome procedures involved (Lindenbaum and Allen, 1995).

There are limitations with specificity and sensitivity of each of these diagnostic tests for folate deficiency. Therefore, a combination of tests are recommended for assessment of folate status (Lindenbaum and Allen, 1995). Currently, serum and erythrocyte folate levels are widely used in folate status assessment (Lindenbaum and Allen, 1995). However, results of an

investigation conducted by the Centers for Disease Control, of the reproducibility of serum and red blood cell folate measurements of pooled samples by twenty laboratories, indicated up to 5 - 10 fold between-method differences in concentrations (Gunter et al., 1996). Recent improvements in analytical techniques have resulted in increased use of homocysteine for diagnosis of folate deficiency (Green, 1995).

# CHAPTER III

# MATERIALS AND METHODS

## Subjects

Five pregnant women were recruited from the Blacksburg community for participation in the four month study. Local obstetricians and lactation groups at Montgomery Regional Hospital were contacted as sources of subjects. Women were accepted for inclusion in this study if they were planning to breast feed for at least six months. Women were excluded if they were smokers or currently taking any antifolate medications such as methotrexate.

The protocol for this study was approved by the Institutional Review Board of Virginia Polytechnic Institute and State University (Appendix A). All subjects were informed of the procedures, right to withdraw, risks, and benefits of the study both verbally and via an informed consent form (Appendix B). A medical history (Appendix C) and food frequency questionnaire (Appendix D) were completed by all participants prior to enrollment in the study. The food frequency questionnaire was adapted from the National Cancer Institute's Health Habits and Diet Qestionnaire (Block,

1987). Following parturition, all subjects were administered a post-delivery questionnaire to obtain information regarding the delivery including complications and treatment and to obtain information about planned use of oral contraceptives (Appendix E). Results of this study were kept strictly confidential. All information and samples were coded with subject numbers.

Benefits to the subjects included dietary counseling and provision of nutritional status information obtained from biochemical and dietary analysis. Researchers maintained continual personal interaction with subjects to ensure optimal compliance with the study protocol.

## Study Protocol

The overall study protocol is illustrated in Figure 1. Each subject was enrolled in the study for a 14 week period. At an initial interview, conducted during the last month of pregnancy, subjects received a verbal and written overview of procedures, completed a medical history and food frequency questionnaire, and signed a consent form for participation. Subjects were provided with multivitamin/multimineral supplements to take once a day for the duration of the study. Nutrient intake was recorded for a seven day period once a month for four months. Milk and blood samples were collected once a month for four months. Milk samples were analyzed for folate.

Blood samples were analyzed for serum ferritin, serum folate, and erythrocyte folate.

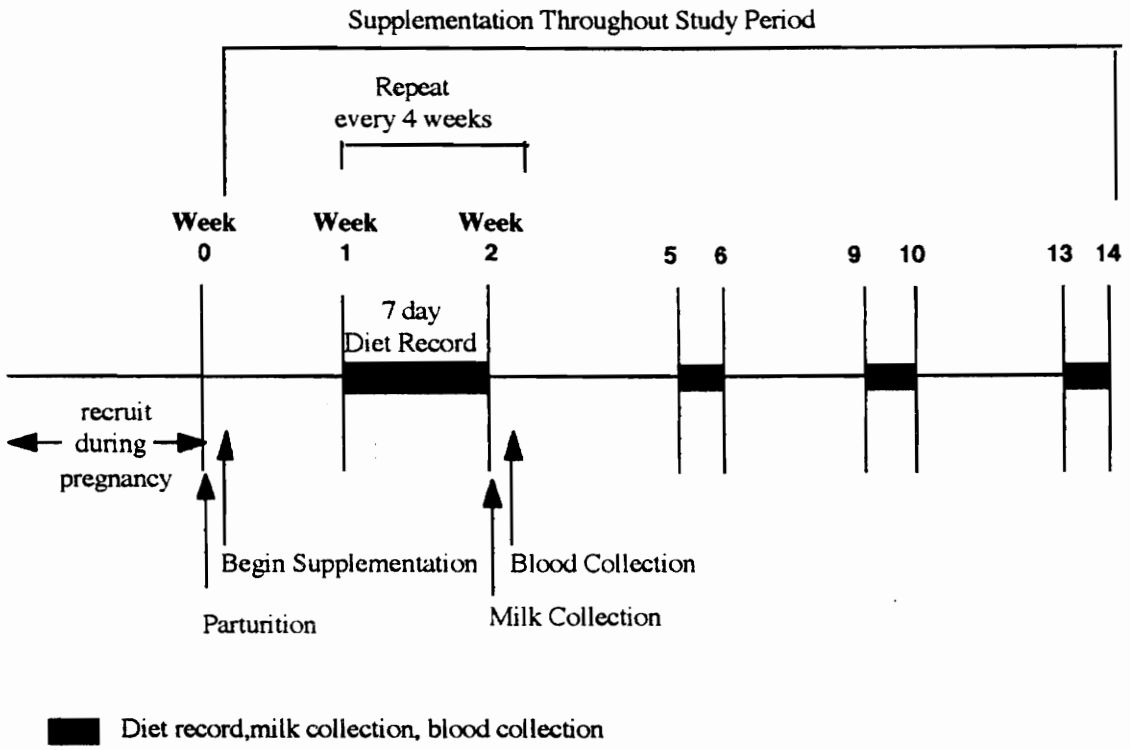


**Figure 1. Sample Study Protocol**

## Supplementation

Immediately following parturition, subjects discontinued the use of all supplements except those provided by the researchers. Each subject was provided with multivitamin/multimineral supplements (Century Senior, Kmart Corporation, Troy MI) prior to giving birth and began taking the supplements on the first day postpartum. A supply of supplements was

provided after the first screening interview that lasted until the second sample collection. After the second sample collection, an additional one month supply was provided each month for the remainder of the study.

The vitamin and mineral content, according to the label, and the percent RDA for lactating women (0- 6 months) provided by the supplement are shown in Table 3.

## Sample Collection and Processing

Milk samples were collected beginning within the first three weeks postpartum then once a month for four months. Milk collection dates were indicated on calendars provided to each subject at the beginning of the study. A sample calendar for one subject is included in Appendix F.

The procedure for milk collection was adapted from several studies (O'Connor et al., 1991; Tamura et al., 1980; Udipi et al., 1987). Subjects collected approximately 10 ml of foremilk from the first milk expressed after letdown using a breast pump sterilized by boiling water. Because of the wide variation in milk folate concentrations over a 24-h period, all samples were collected between 1:00 PM and 2:50 PM. The folate content between 1:00 PM and 2:50 PM appears to represent average milk folate concentration over a 24-h period (Udipi et al., 1987).

After collection, milk samples were immediately transferred to foil covered plastic tubes containing ascorbic acid (~1 mg/ml), mixed well, and aliquoted into microtubes. The milk samples were then frozen in liquid nitrogen and transported to Wallace Hall on ice where they were frozen at -80°C until assayed.

Ten-hour fasting blood samples were drawn the day following each milk sample collection. Blood sample collection dates were indicated on the calendars provided to each subject (Appendix F). For a complete blood count (CBC) analysis, 3 ml of the collected whole blood was sent to Carilion Consolidated Laboratories (Roanoke, VA).

For serum folate and serum ferritin analysis, approximately 6 ml of fasting venous blood was drawn from each subject in trace element free tubes. These samples were allowed to coagulate at room temperature. Serum was separated by centrifugation (IEC Model Centra MP4R Centrifuge, Damon/IEC Division, International Equipment Co., Needham Heights, MA) at 2500 X g for 15 min. Serum was aliquoted to microtubes for ferritin analysis and to microtubes with ascorbate (~1mg/ml) for folate analysis. An additional 6 ml of blood was collected in trace element free tubes containing sodium heparin for whole blood folate analysis. For whole blood folate, 500ml aliquot of whole blood was transferred to tubes containing 5 mg ascorbate and 4.5 ml

autoclaved de-ionized water. Serum and whole blood lysate samples were frozen at -20°C.

**TABLE 3.**
**Supplement[a] Vitamin and Mineral Content**

| Vitamin/Mineral | Amount per Tablet | % RDA for Lactating Women (1st 6 months) |
|---|---|---|
| Vitamin A | 6000 IU | 140 |
| Vitamin C | 60 µg | 63 |
| Vitamin D | 400 IU | 100 |
| Vitamin E | 45 IU | 125 |
| Vitamin K | 10 µg | 15 |
| Thiamin | 1.5 mg | 94 |
| Riboflavin | 1.7 mg | 94 |
| Niacin | 20 mg | 100 |
| Vitamin B6 | 3 mg | 143 |
| Folate | 200 µg | 71 |
| Vitamin B12 | 25 µg | 962 |
| Biotin | 30 µg | N/A |
| Pantothenic Acid | 10 mg | N/A |
| Calcium | 200 mg | 17 |
| Iron | 9 mg | 60 |
| Phosphorus | 48 mg | 4 |
| Iodine | 150 µg | 75 |
| Magnesium | 100 mg | 28 |
| Zinc | 15 mg | 100 |
| Selenium | 20 µg | 27 |
| Copper | 2 mg | N/A |
| Manganese | 2.5 mg | N/A |
| Chromium | 100 µg | N/A |
| Molybdenum | 25 µg | N/A |
| Chloride | 72 mg | N/A |
| Potassium | 80 mg | N/A |
| Nickel | 5 µg | N/A |
| Silicon | 10 µg | N/A |
| Vanadium | 10 µg | N/A |
| Boron | 150 µg | N/A |

N/A - No RDA Established
a. Century Senior, Kmart Corporation, Troy, MI

## Biochemical Analysis

The procedure for preparation of milk samples for analysis was adapted from the method described by Tamura et al. (1980). Prior to folate analysis, milk samples were thawed for 5 min at 37°C and diluted 1:2 with phosphate buffer (pH 6.3). Samples were then autoclaved for 5 min at 121°C. Milk samples were further diluted to obtain a final dilution of 1:4 with phosphate buffer containing 1 mg/ml of ascorbic acid and mixed with partially purified folate conjugase. The mixture of conjugase and diluted milk sample was incubated for five hours at 37°C. The conjugase was prepared by forming a suspension of desiccated chicken pancreas (Difco, Detroit, MI) in potassium phosphate buffer. The suspension was centrifuged for 10 min at 5000 rpm (IEC Centrifuge, Damon/IEC Division, International Equipment Co., Needham Heights, MA). The supernatant was transferred to dialysis tubing, sealed, placed into a beaker which contained autoclaved potassium phosphate buffer and charcoal, and dialyzed for 24 h at 4°C.

A microbiological assay with *Lactobacillus casei* (ATCC 7469) (Tamura, 1990) was used to determine milk folate, serum folate, and whole blood folate. A suspension of the assay organism was prepared from freeze dried cultures of *L. casei* and folic acid assay maintenance medium (Difco Laboratories,

Detroit, MI). A 96-well microplate assay procedure was used (Tamura, 1990). Serum, whole blood, and folate conjugase-treated milk samples were diluted with a potassium phosphate buffer (pH 6.3) containing 1 mg/ml ascorbic acid. Samples were adjusted to a volume of 150 µl with the same buffer. Six serial dilutions of samples and standards were made. Medium containing a suspension of the assay organism was added to all wells. The wells were incubated at 37°C for 24 h. The contents of each well was suspended by repeated aspiration and flushing several times until the bacterial suspension became homogeneous. Bacterial growth was measured by reading the turbidity of each well at 650nm with a microplate reader (CERES 900 HDi, Bio-Tek Instruments, Highland Park, VT) interfaced with a personal computer. To check interassay reproducibility, folate levels of pooled samples were measured each time the assay was performed.

Erythrocyte folate was calculated as follows (Hoffbrand et al., 1966):

$$\text{RBC folate} = \frac{\text{(whole blood folate)} - \text{(serum folate X (1-hct/100))}}{\text{hct/100}}$$

Serum ferritin was measured by an enzyme linked immunoassay method (ELISA) as specified by the manufacturer (Dako Corp, Carpinteria,

CA). Antibodies and standards were purchased from Dako Corporation (Carpinteria, CA). A dilution of Dako rabbit anti-human ferritin (A133) in a sodium phosphate coating buffer (pH 6.3) was prepared, added to each well, then incubated at 4 °C for 12 hrs. Wells then were washed with sodium phosphate buffer. Standards, serum samples, and controls were added to appropriate wells and incubated for an additional 12 h at 4°C. After incubation, the plates were washed with sodium phosphate buffer. A dilution of Dako peroxidase conjugated antibody (P145) and coating buffer was prepared, added to each well, then incubated at room temperature for 2 h. After incubation, plates were washed with sodium phosphate buffer. For color development, OPD tablets were dissolved in 12 ml citric acid buffer (pH 5.0) in a foil covered polypropylene tube. After 15 min, 5 µl of hydrogen peroxide was added to the OPD and citric acid buffer solution to check for contamination. If no color change occurred, 100 µl was added to each well. The plates were incubated for a final time at 37°C for 20 min. Color development was measured at 490 nm with a CERES 900 HDi, Bio-Tek Instruments (Highland Park, VT) microplate reader interfaced with a personal computer. To check interassay reproducibility, ferritin levels of pooled samples were measured each time the assay was performed.

## Dietary Analysis

At the initial screening, subjects completed a food frequency questionnaire (Block, 1987) to provide an estimate of the dietary intake during pregnancy (Appendix D). The food frequency questionnaire provided estimated daily, weekly, monthly, and yearly food consumption patterns. To obtain dietary information throughout the study, seven-day diet records were completed once per month for four months. Dietary record forms are provided in Appendix G. Subjects were porvided with verbal and written instructions for completion of diet records. Diet records began one week prior to each blood sample collection with the last day recorded on the day of milk sample collection. The date at which each diet record was to begin was indicated on calendars provided (Appendix F). Nutrient intake was analyzed using the Nutritionist III 7.2 computer program (N-squared Computing, The Hearst Corporation, San Bruno, CA).

## Statistical Analysis

Two-factor analysis of variance (ANOVA) for repeated measures was performed to evaluate changes in serum folate, red blood cell folate, and milk folate over the period of parturition through four months postpartum. The two factors were time postpartum and subject. Simple linear regression analysis was performed to evaluate relationships between indices of folate

status and dietary folate and dietary iron intake and between serum folate, red blood cell folate, serum ferritin, dietary folate, dietary iron and milk folate concentration. Statistical analysis was performed using Excel computer program version 5.0 (Microsoft Corporation). A probability level of 5% was chosen as the level of statistical significance. Statistical consulting was provided by the Statistics department of Virginia Tech, Blacksburg, Virginia.

# CHAPTER IV

# RESULTS

## Subjects

The subjects included five women with a mean age of 32, range 30-35 yr, and mean parity of 2.4, range 1 to 5 (Table 4). All subjects enrolled in the study completed the four month protocol. At the time of enrollment in the study, all subjects reported that they were in good general health. The subjects reported taking daily prenatal supplements during pregnancy containing 0.4 mg folate per supplement. Results of the post-delivery questionnaire indicated that all subjects had vaginal deliveries in which labor was not induced. None of the subjects received blood transfusions nor iron injections. One subject reported experiencing excessive bleeding during delivery. This subject reported recieving an injection of Pitocin to stop the bleeding. Another subject reported receiving an injection of Pitocin to aleviate cramping. One subject reported receiving cefuroxime treatment after delivery for a Streptococcus infection. The type of Streptococcus infection was not reported. Use of this antibiotic was discontinued approximately 20 hours prior to the first milk collection and 40 hours prior to the first blood collection. None of the subjects used oral contraceptives during the study.

All subjects exclusively breastfed their infants throughout the study. Contact was maintained with the subjects to ensure compliance with recording of dietary intake, consumption of supplements, and fasting prior to blood collections. In the anonymous post-study questionnaire (Appendix G), all subjects reported that they recorded their dietary intake completely and honestly. Four of the five subjects reported consuming the multivitamin/multimineral supplements daily throughout the study. One subject reported consuming the supplement an average of five days per week.

**Table 4**
**Subject Characteristics**

| Subject | Age | Parity |
|---|---|---|
| 1 | 32 | 2 |
| 2 | 34 | 2 |
| 3 | 30 | 5 |
| 4 | 30 | 2 |
| 5 | 35 | 1 |
| Mean $\pm$ SD | 32 $\pm$ 2 | 2 $\pm$ 2 |

## Folate and Iron Intake

All subjects had dietary intakes of iron and folate which exceeded the current recommended levels for lactating women. Dietary intake data are

provided in Table 5. Mean folate and iron intakes, including supplements, were 495 ± 105 µg/d and 24 ± 4 mg/d, respectively.

**Table 5**
**Mean Folate and Iron Intakes For Four Months Postpartum**

| Month | Folate[a] µg/d Mean (Range) n = 5 | Iron[b] mg/d Mean (Range) n = 5 |
|---|---|---|
| 1 | 553 (361 - 665) | 22 (17 - 26) |
| 2 | 484 (371 - 616) | 24 (19 - 30) |
| 3 | 445 (390 - 510) | 25 (18 - 35) |
| 4 | 498 (379 - 691) | 24 (20 - 27) |
| Mean ± SD | 495 ± 105 | 24 ± 4 |

a. Includes daily supplement with 200 µg folate

b. Includes daily supplement with 9 mg iron

## Red Blood Cell and Serum Folate

Red blood cell folate levels are shown in Table 6 and are illustrated for each subject in Figures 2 to 6 and for all subjects combined in Figure 7. Red blood cell folate concentrations were within the normal range (>140 µg/L) for all subjects throughout the study. Mean red blood cell folate levels increased

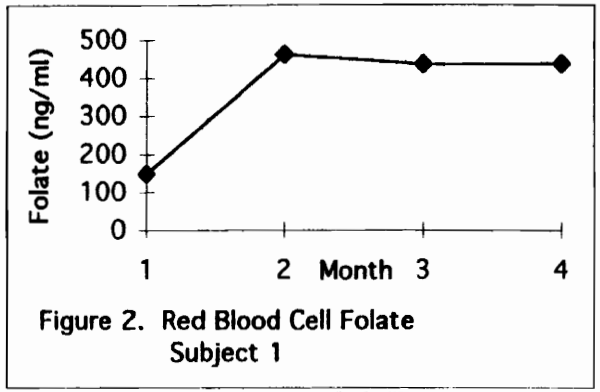


Figure 2. Red Blood Cell Folate
Subject 1

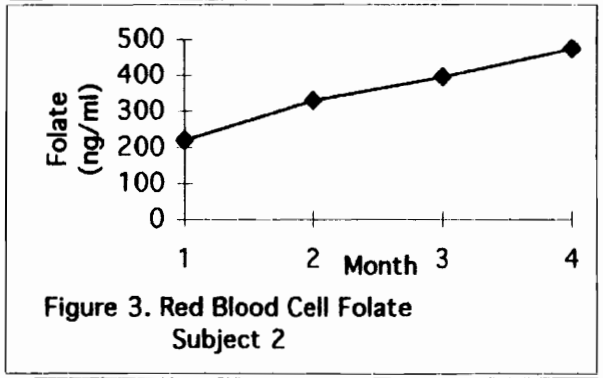


Figure 3. Red Blood Cell Folate
Subject 2

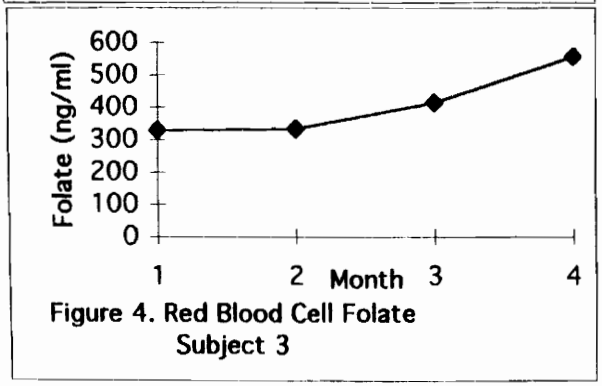


Figure 4. Red Blood Cell Folate
Subject 3

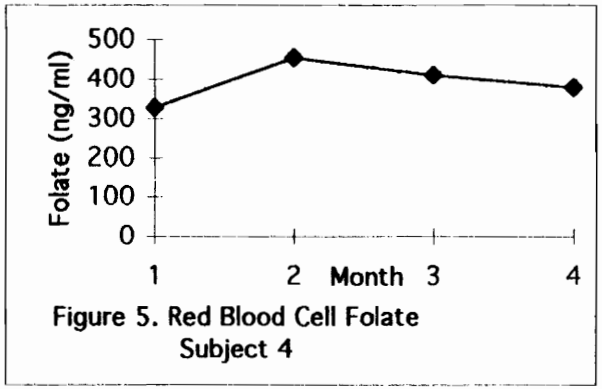


Figure 5. Red Blood Cell Folate
Subject 4

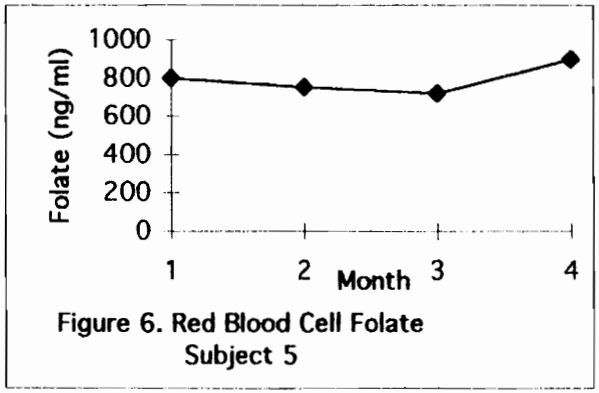


Figure 6. Red Blood Cell Folate
Subject 5

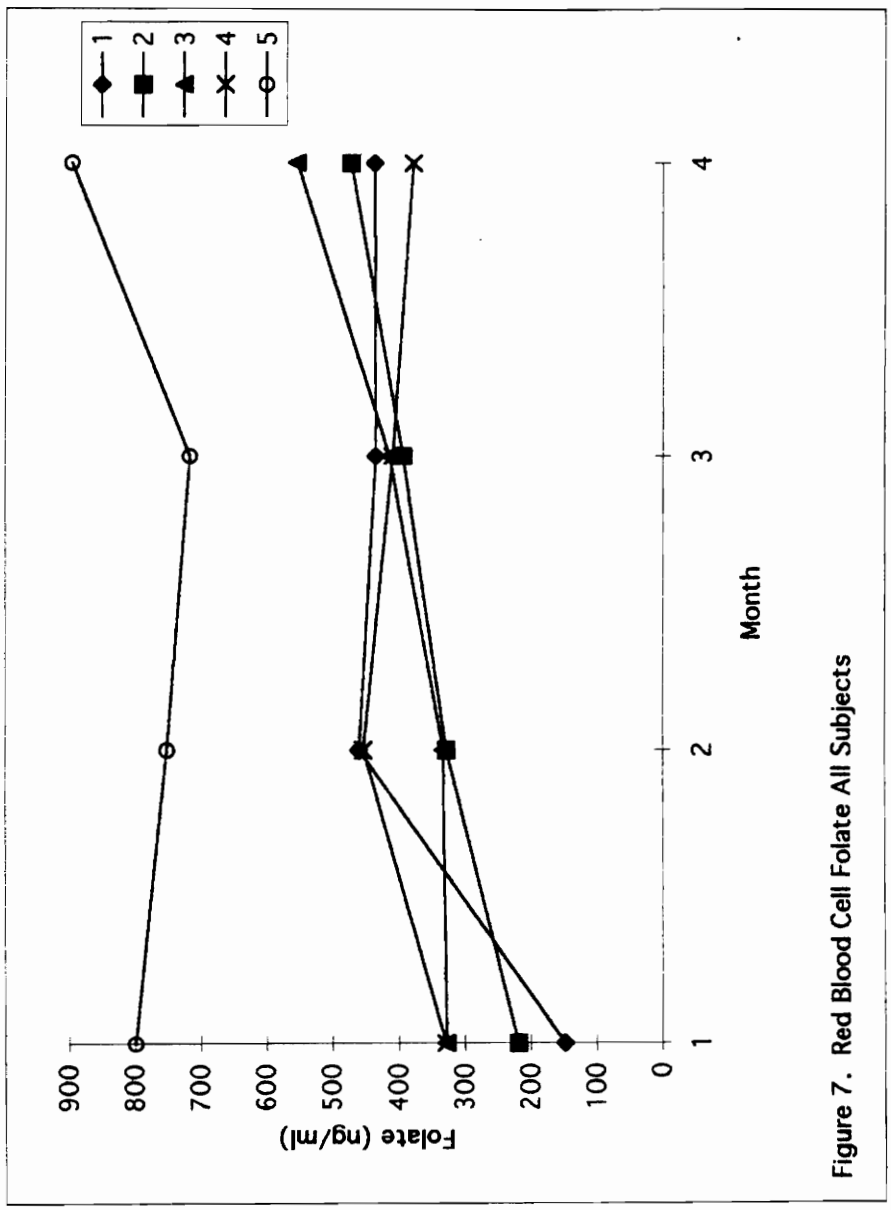


Figure 7. Red Blood Cell Folate All Subjects

significantly ($p = .02$) from $364 \pm 255$ µg/L in month one to $548 \pm 205$ µg/L in month four. Dietary folate was not significantly correlated to red blood cell folate ($r = .36$, $p = .13$).

**Table 6**
**Red Blood Cell Folate Concentration**

| | Folate (µg/L) | | | |
|---|---|---|---|---|
| | Month | | | |
| Subject | 1 | 2 | 3 | 4 |
| 1 | 147.5 | 460.3 | 435.2 | 437.0 |
| 2 | 218.3 | 328.3 | 393.6 | 473.1 |
| 3 | 327.1 | 333.1 | 414.1 | 555.0 |
| 4 | 327.8 | 454.1 | 409.1 | 378.3 |
| 5 | 799.3 | 751.9 | 717.5 | 896.9 |
| Mean ± SD | 364.0 ± 255.1 | 465.6 ± 172.1 | 473.9 ± 137.0 | 548.1 ± 205.3 |

Serum folate levels are shown in Table 7 and are illustrated for each subject in Figures 8 to 12 and for all subjects combined in Figure 13. In the first sample collected after parturition, one subject had a serum folate level in the deficient range (<3 µg/L) and two other subjects had serum folate levels in the marginal range (3-5 µg/L). Mean serum folate levels significantly increased ($p < .001$) from $5 \pm 2$ µg/L in month one to $7 \pm 2$ µg/L in month two. Serum folate levels for all subjects combined did not significantly

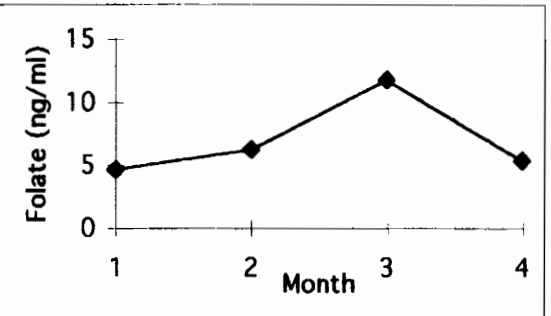


Figure 8. Serum Folate Subject 1

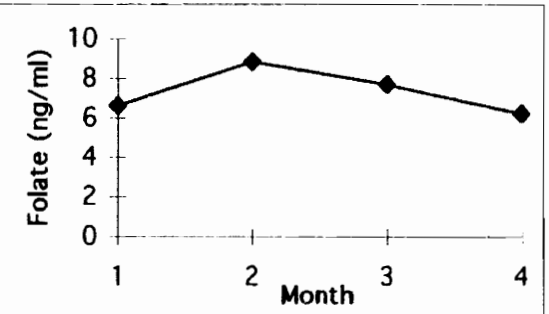


Figure 9. Serum Folate Subject 2

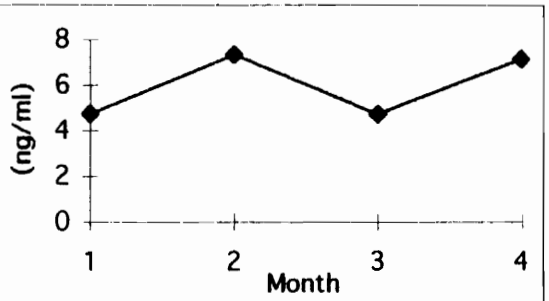


Figure 10. Serum Folate Subject 3

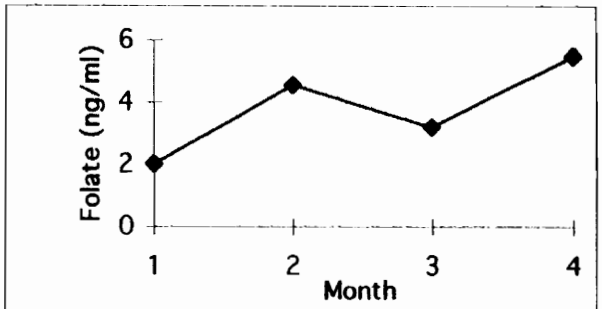


Figure 11. Serum Folate Subject 4

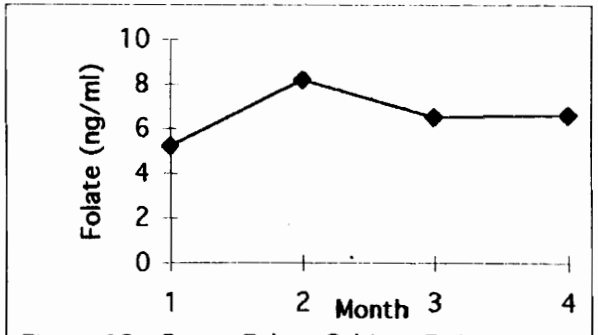


Figure 12. Serum Folate Subject 5

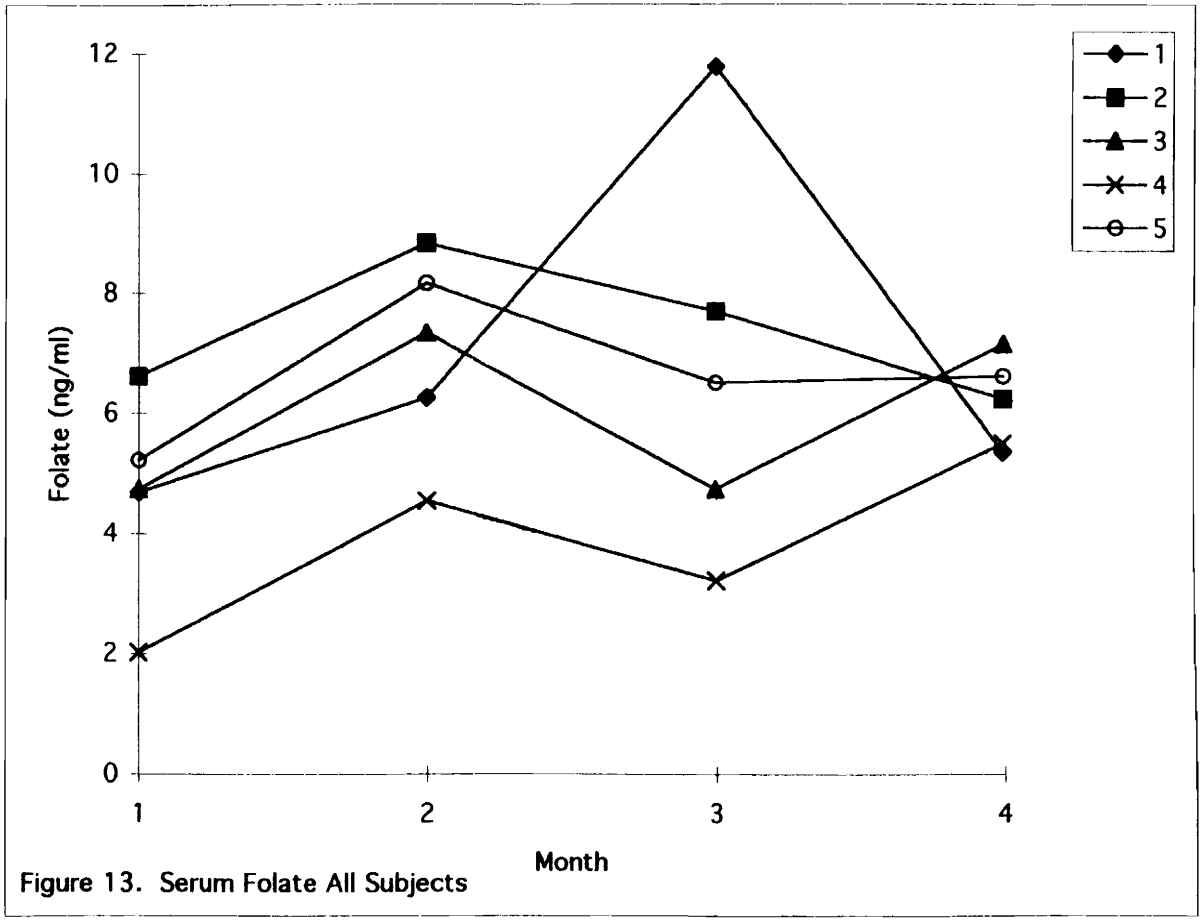


Figure 13. Serum Folate All Subjects

change from month two to month four. Dietary folate was not significantly correlated with serum folate ($r = .24$, $p = .33$).

**Table 7**
**Serum Folate Concentration**

| | Folate (μg/L) | | | |
|---|---|---|---|---|
| | Month | | | |
| Subject | 1 | 2 | 3 | 4 |
| 1 | 4.7 | 6.3 | 11.8 | 5.3 |
| 2 | 6.6 | 8.8 | 7.7 | 6.2 |
| 3 | 4.7 | 7.3 | 4.7 | 7.2 |
| 4 | 2.0 | 4.6 | 3.2 | 5.5 |
| 5 | 5.2 | 8.2 | 6.5 | 6.6 |
| Mean ± SD | 4.7 ± 1.7 | 7.0 ± 1.7 | 6.8 ± 3.3 | 6.2 ± .8 |

## Serum Ferritin

Serum ferritin concentrations are shown in Table 8. Mean serum ferritin concentrations were within the normal range (10-130 ng/ml) for all subjects throughout the study. Two subjects, however, had low serum ferritin levels (<30 ng/ml) in month one. One subject had serum ferritin levels below 30 ng/ml throughout the study period. There was a positive significant correlation ($r = .63$, $p = .003$) between iron intake and serum ferritin concentrations.

**Table 8**
**Serum Ferritin**

| | Ferritin (ng/ml) Month | | | |
|---|---|---|---|---|
| Subject | 1 | 2 | 3 | 4 |
| 1 | 103.2 | 111.6 | 107.2 | 75.3 |
| 2 | 24.2 | 23.6 | 23.8 | 27.8 |
| 3 | 43.7 | 57.7 | 57.4 | 55.6 |
| 4 | 61.3 | 90.6 | 93.7 | 88.1 |
| 5 | 26.5 | 38.7 | 28.4 | 46.2 |
| Mean ± SD | 51.8 ± 32.4 | 64.4 ± 36.3 | 62.1 ± 37.6 | 58.6 ± 23.8 |

## Milk Folate

Milk folate concentrations for each subject one month through four months postpartum are shown in Table 9. The change in milk folate concentrations for each subject are illustrated in Figures 14 to 18 and for all subjects combined in Figure 19. All samples collected were foremilk excluding the milk sample from subject 4 month four. This subject reported, after completion of the study, that she fed her infant on the breast in which the milk was collected prior to sample collection. Studies have found that folate in hindmilk is greater than in foremilk (Smith et al., 1983; Udipi et al., 1986). Therefore, this sample was excluded from statistical analysis. Mean

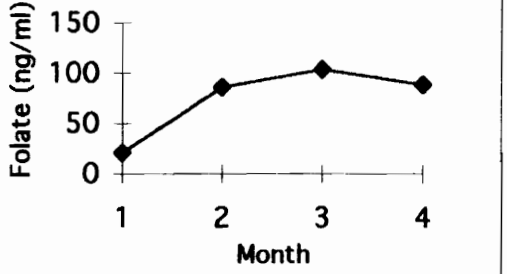


Figure 14. Milk Folate Subject 1

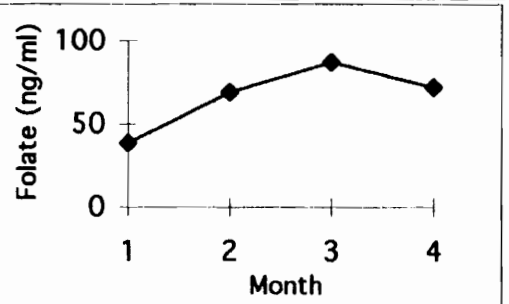


Figure 15. Milk Folate Subject 2

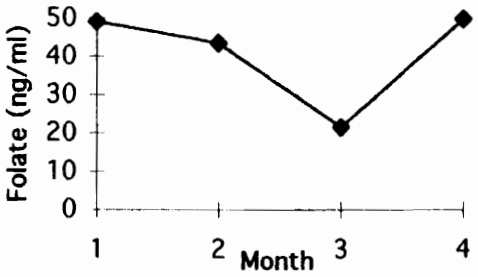


Figure 16. Milk Folate Subject 3

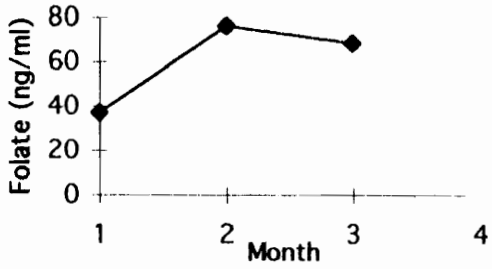


Figure 17. Milk Folate Subject 4

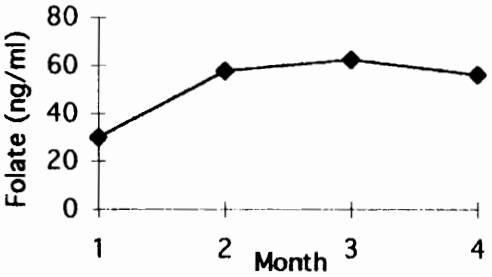


Figure 18. Milk Folate Subject 5

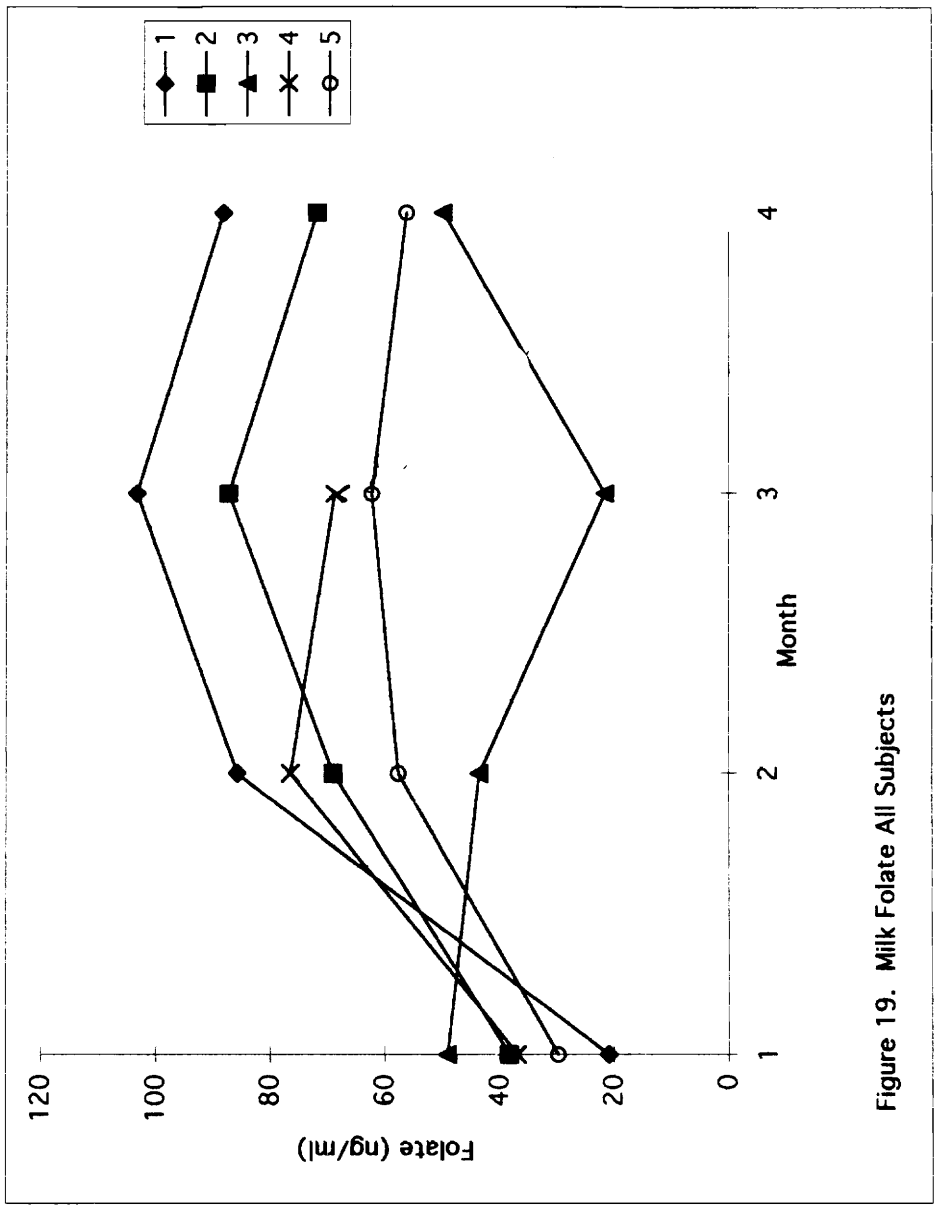


Figure 19. Milk Folate All Subjects

milk concentration for all subjects over the four month period was 59 μg/L, with a range of 21 μg/L to 103 μg/L. Mean milk folate concentrations increased ($p = .06$) from 35 ± 10 μg/L in month one to 69 ± 30 μg/L in month three. Mean milk folate concentrations for subjects 1, 2, and 5 increased significantly ( $p = .02$) from 30 μg/L in month one to 84 μg/L in month four. Milk folate did not significantly change between month three and month four for all subjects combined. Milk folate decreased ($p = .06$) in subjects 1,2, and 5 from 84 μg/L in month three to 72 μg/L in month four.

**Table 9**
**Milk Folate Concentrations**

| | Folate (μg/L) | | | |
|---|---|---|---|---|
| | Month | | | |
| Subject | 1 | 2 | 3 | 4 |
| 1 | 20.9 | 85.7 | 103.2 | 88.2 |
| 2 | 38.3 | 69.0 | 87.2 | 71.8 |
| 3 | 49.1 | 43.8 | 21.6 | 49.9 |
| 4 | 36.9 | 76.5 | 68.7 | N/A |
| 5 | 29.8 | 57.7 | 62.3 | 56 |
| Mean ± SD | 35.0 ± 10.4 | 66.5 ± 16.6 | 68.6 ± 30.1 | 66.5 ± 17.1 |

Correlations between milk folate concentrations and dietary intake of folate, dietary intake of iron, blood indices of folate status, and serum ferritin

are shown in Table 10. Dietary and red blood cell folate levels were not significantly correlated with milk folate. Milk folate levels of subjects 1, 4, and 5 increased significantly ($p < .05$) despite a decrease in folate intake. Serum folate was significantly correlated with milk folate ($r = .48$, $p = .04$). There was a significant positive correlation between dietary iron intake and milk folate concentration for month one through month four ($r = .57$, $p = .01$). Serum Ferritin, however, was not significantly correlated with milk folate ($r = .25$, $p = .30$).

**Table 10**
**Correlations Among Milk, Blood, and Dietary Levels of Iron and Folate**

| | Correlation | |
|---|---|---|
| Correlation Criteria | r | p |
| Folate | | |
| Milk Folate, Dietary Folate | -.14 | .57 |
| RBC Folate, Dietary Folate | .36 | .13 |
| Serum Folate, Dietary Folate | .24 | .33 |
| RBC Folate, Milk Folate | .10 | .70 |
| Serum Folate, Milk Folate | .48 | .04 |
| Iron | | |
| Milk Folate, Dietary Iron | .57 | .01 |
| RBC Folate, Dietary Iron | .06 | .81 |
| Serum Folate, Dietary Iron | .16 | .50 |
| Serum Ferritin, Dietary Iron | .63 | .003 |
| Serum Ferritin, Milk Folate | .25 | .30 |

# CHAPTER V

# DISCUSSION

## Dietary Folate Intake and Maternal Folate Status

Several researchers have suggested that maternal folate status declines throughout lactation (Qvist et al., 1986; Salmenpera et al., 1986; Smith et al., 1983). Lactating women may be particularly at risk for suboptimal folate status due to increased dietary requirements. In this study, red blood cell folate levels remained normal and increased throughout the study period. Dietary folate intake was generally not reported in previous studies which assessed folate status of lactating women. The women in this study, which were supplemented with 200 μg of folate daily, had folate intakes of $495 \pm 105$ μg/d. This level of intake is higher than that found in other studies which have reported dietary intakes of lactating women (Borrud et al., 1993; Thomas et al., 1980). The level of dietary folate in this study appeared to be adequate to maintain red blood cell folate levels within the normal range.

Dietary intake during pregnancy may have an impact on maternal folate status during lactation. Qvist et al. (1986) found that women not

consuming folate supplements during pregnancy experienced a decline in red blood cell folate levels during the subsequent lactation period. In this study, folate nutritional status was not assessed nor were dietary records kept during pregnancy, therefore the folate status of these women prior to delivery is not known. Food frequency questionnaires completed at the initial interview, which took place during the third trimester of pregnancy, indicated that these women frequently consumed foods with relatively high levels of folate. The women reported consuming vegetables such as broccoli, spinach, peas, and other vegetables with >30 μg/serving on a regular basis (≥ one time per day). These women also consumed other vegetables, such as potatoes and tomatoes, with >10 μg folate/serving on a regular basis. Three subjects reported consumption of orange juice, which contains 109 μg folate/average serving, ≥ three times per week. Overall, these women consumed approximately three or more servings of vegetables and three or more servings of fruit or fruit juice daily throughout pregnancy. In addition, all women reported consuming supplements containing folate during pregnancy. Levels of folate intake may have been high enough to prevent folate depletion during pregnancy.

The red blood cell folate levels for subject 5 appeared to be much higher than the mean red blood cell folate levels for all the subjects throughout the study. This subject had higher dietary intakes of folate (594 μg/d) compared to

the overall mean folate intake of all the subjects combined (495 μg/d). In addition, subject 5 had given birth to only one child compared to the other subjects which had two or more children. Martinez (1980) reported that parity affects the folate status of women. Subject 5 may have had a higher preconception folate status than the other women that had previous pregnancies.

Although red blood cell folate levels were above normal for all of the women throughout the study, serum folate levels were in the low or marginal range for three of the women during the first month postpartum. One woman (subject 4) had serum folate levels below 5 μg/L in the first three months postpartum despite dietary intakes above 400 μg/d. This subject also had red blood cell folate levels well above the normal range, however, red blood cell folate levels declined from 454 μg/L in month two to 378 μg/L in month four. This subject had given birth to two children within two years. This was in contrast to subject 1 and subject 2 that had given birth to a previous child > 2 years prior to the beginning of the study and subject 5 that had been previously pregnant. Subject 4 had given birth to five children with the fourth child being born < 2 years prior to the study. Subject 4 also had marginal folate levels in month one and month three; however, red blood cell folate levels of this subject increased throughout the study. The folate status of this subject prior to and during each of the five pregnancies is

unknown, however, it may have been adequate to prevent a fall in tissue folate stores during this study. This subject reported consuming prenatal supplements during and between previous pregnancies. Supplementation may have resulted in high levels of tissue folate at the beginning of each pregnancy.

Low serum folate levels usually provides the first sign of tissue folate depletion (Lindenbaum and Allen, 1995). However, all the women had serum folate levels above 5 μg/L by the fourth month and had normal red blood cell folate levels throughout the four months. Therefore, folate deficiency was unlikely among these women.

The low red blood cell folate levels observed in subject one month one (148 μg/L) may reflect marginal folate stores during pregnancy. Since folate levels were not assessed during pregnancy, the folate status of this subject prior to delivery is not known. This subject received antibiotic treatment after delivery which was discontinued the day before the first milk collection and two days prior to her blood sample collection. Given the usual dosage of 250 mg of cefuroxime with a half life of 1.2 hours (PDR, 1994), the amount of antibiotic present in the milk and blood was negligible. However, it cannot be ruled out that even small traces of the antibiotic may have interfered with the growth of *L. casei* . This may explain the low red blood cell folate level measured in this subject's samples during the first month. Elimination of the

milk and blood folate values for this subject would not have a significant effect on the overall findings of this study. All subjects had red blood cell folate levels within the normal range even with inclusion of the blood values for subject one. The significant relationships between milk folate and iron intake and serum folate would still exist. Milk folate levels significantly increased with or without the milk folate value from this subject.

From this study the level of dietary folate required to maintain normal serum and red blood cell folate cannot be determined. It is unclear whether dietary intake of folate without supplementation would have been sufficient to maintain normal levels. Mean dietary folate intakes without supplementation would have been 295 μg/d compared to the RDA of 280 μg/d. Studies conducted by O'Keefe et al. (1995) and Sauberlich et al. (1987) suggested that the RDA of 180 μg/d for non-pregnant, non-lactating women may be too low. The women in the present study may have experienced tissue folate depletion without supplementation.

## Milk Folate

The recommended additional dietary folate of 100 μg/d was based on an estimated average milk folate level of 50 μg/L, adjusted for 50% bioavailability, with secretion of between 750 and 850 ml of milk per day

(NRC, 1989). Studies have shown higher levels of breastmilk folate independent of the folate status of the lactating women. Ek (1983) reported folate levels of 69-135 μg/L from milk collected during the middle of feeding. Brown et al. (1986) collected both foremilk and hindmilk samples and reported mean milk folate levels of 85 μg/L. In this study the milk folate concentration from foremilk samples for these lactating women was 59 ± 24 μg/L. Several studies have shown that folate in foremilk is lower than in hindmilk (Brown et al., 1986; Smith et al., 1983). The total milk folate concentrations for the women in this study would most likely have been higher if hindmilk samples were included in analysis. The concentration of folate in the one hindmilk sample excluded from analysis was 190 μg/L. These results suggest that the average milk folate level of 50 μg/L used to determine additional folate requirements during lactation may be too low.

## Maternal Folate Status and Milk Folate

Neither dietary folate nor red blood cell folate were significantly correlated with milk folate concentration. Milk folate concentration increased from month one to month three despite a decline in folate intake during that same time period. Picciano (1995) noted that maternal dietary

intake is more highly correlated to milk folate content when maternal folate stores are low or absent.

Although overall red blood cell folate levels increased significantly during the study period, the levels fluctuated within individual subjects. These fluctuations in red blood cell folate concentrations were not correlated with fluctuations in milk folate concentrations. These findings were similar to other studies with well nourished women (Ek et al., 1983; Smith et al., 1983). Ek et al. (1983) and Smith et al. (1983) found that milk folate increased during the first three months of lactation independent of maternal folate status. In these studies the level of milk folate did not increase between three and four months, a finding which was also observed in this study. Other studies of women with depleted folate reserves have also observed an increase in milk folate independent of maternal folate status (Picciano, 1990). These studies suggest that there may be a regulatory mechanism involved in maintaining milk folate concentrations.

Unlike the findings from other studies (Ek et al., 1983; Smith et al., 1983), serum folate levels of the women in this study were significantly correlated with milk folate concentrations. Blood samples, in the study conducted by Ek et al. (1983), were collected after only a three to five-hour fast in contrast to the ten-hour fasting samples collected in this study. Recent dietary intake of folate may have had an effect on serum folate levels. The

three to five hour fast may not have been sufficient to prevent the effects of recent dietary intake on serum folate. Ek et al. (1983) did not indicate when milk samples were collected in relationship to blood collections. Smith et al. (1983) reported that milk and blood samples were collected within the same week, however, the interval between collections was not specified. In the present study, blood samples were drawn the following morning after milk sample collections. The timing of blood and milk sample collections may effect how these variables are correlated. Collection of both milk and blood samples within the same 24-h period may have allowed for detection of the significant correlation between serum folate and milk folate concentrations. This study demonstrates the importance of maintaining consistency with the interval between milk and blood sample collections. This study suggests that collection of samples within a 24-h period may be sufficient to show a relationship between serum and milk folate.

Serum folate concentration does not provide an accurate measurement of tissue folate stores; however, it does indicate short-term folate balance (O'Connor, 1994). Results from this study show that although there appears to be no relationship between tissue folate stores and milk folate secretion, there may be a significant correlation between short-term folate balance and milk folate. There may be a mechanism that regulates serum folate

concentration during lactation to provide for appropriate milk folate secretion.

## Iron and Milk Folate

Studies with rats have indicated that maternal iron deficiency was associated with impaired milk folate secretion. There was no indication of iron deficiency in these subjects during the study period. Serum ferritin, hemoglobin, hematocrit, and mean corpuscular volume values for all subjects in this study were within the normal range. Therefore, the correlation between iron deficiency and milk folate secretion could not be examined in this study. However, examination of dietary iron intake showed that there was a significant positive correlation between milk folate concentrations and iron intake. Iron intake was not significantly correlated with serum or red blood cell folate levels. Although the relationship between iron status and milk folate has been studied in animal species (O'Connor, 1991); the relationship between iron intake and milk folate secretion has not been previously examined in humans.

Iron deficient rats have been found to secrete reduced levels of milk folate (O'Connor et al., 1989). It is unclear, however, if differences in iron intake among iron sufficient rats would have an effect on milk folate. The concentration of milk folate is positively correlated with folate-binding

proteins which suggests that their secretion may be coordinated (Picciano, 1995). Secretion of other proteins involves synthesis, segregation, intracellular transport, concentration, intracellular storage, and exocytosis (O'Connor et al., 1989). The role of iron, if one exists, in this secretion process is not clear. Utley et al. (1985) found that iron is a component of methionine synthase extracted from purified placental tissue. Methionine synthase is involved in the production of tetrahydrofolate which is used as a substrate in polyglutamation catalyzed by folypolyglutamate synthetase. Polyglutamation is involved in intracellular storage and concentration of folates (Shane, 1995). O'Connor (1991), however, did not find a significant relationship between iron status and methionine synthase nor folypolyglutamate synthetase activity in swine. It is possible that iron may be involved in other aspects of the folate secretion process into milk. Research is needed to determine whether iron does play a role in milk folate secretion and to determine what the mechanism may be for alterations in milk folate concentrations with iron intake.

# CHAPTER VI

# CONCLUSIONS

Pregnant and lactating women are at risk for suboptimal folate status as the result of increased requirements to meet the nutrient demands of the fetus and nursing infant. Folate nutritional costs during lactation are determined in part by the amount of folate secreted into milk. Little is understood about the mechanism involved and factors associated with milk folate secretion. It appears that milk folate secretion is maintained independent of dietary folate intake and folate tissue stores. The objectives of this study were to monitor folate status of lactating women and to examine relationships between folate status, dietary intake, and milk folate concentrations.

The lactating women in this study had optimal folate stores which may have been the result of adequate nutrition during pregnancy and high levels of folate intake during lactation. Women in this study were supplemented with 200 μg/d of folate. It is not clear whether these women would have maintained adequate folate stores on diets without supplementation or if

they would have maintained adequate stores over an extended lactation period.

The presence of casein in milk has been suggested as a potential source of error in determination of milk folate concentrations using the microbiological method. Casein in milk may interfere with turbidimetric measurement of microbiological growth (Cooperman et al., 1982). Cooperman et al. (1982) used rennin precipitation of milk casein to obtain a clear solution. O'Connor et al. (1991), however, found no difference in folate concentrations between milk samples before or after treatment with rennin. Smith et al. (1983) noted that the smaller proportion of casein to whey in human milk (40:60) as compared with cow's milk (80:20) allows for use of the turbidimetric microbiological assay without rennin precipitation of casein.

A limitation of this study was that the subjects were fairly well educated and consumed high levels of dietary folate. Therefore, these women are not representative of the majority of lactating women. From this study it is unclear whether typical folate intakes of the population of lactating women as a whole are sufficient to maintain adequate folate stores during lactation. The level of dietary folate required during lactation remains unknown.

Measuring milk folate concentrations from samples collected at only one time during a 24-hr period may be another limitation of this study. Mean

milk folate levels were determined from concentrations in samples collected between 1:00 and 2:50 PM. The assumption was made that this level represented average milk folate concentrations over a 24-h period. Brown et al. (1986) reported that milk folate levels increased throughout the day. Udipi et al. (1987) found that milk folate levels in samples obtained between 1:00 and 2:50 PM were representative of mean folate concentrations in samples from all feedings during a 24-h period.

Another limitation of this study was that folate status during pregnancy was not assessed. Therefore, it is not clear what effect that maternal folate status during pregnancy may have had on folate status during lactation. It is also unclear whether the marginal serum folate levels observed in three of the subjects during the first month postpartum reflected folate balance prior to or after delivery.

This study was also limited by the length of the study period. Samples were collected for four months, however, all the women planned on continuing lactation for one year. Although tissue folate stores appeared to be well maintained during the study period, the changes in folate status of these women in subsequent months of lactation are not known.

Information is limited on the additional folate requirement needed to maintain adequate folate stores during lactation. Further research is needed to determine dietary requirements for folate and to establish whether folate

supplementation during lactation should be recommended. Studies are needed which assess both dietary intake and folate status of lactating women.

Generally, studies examining the changes in folate status during lactation have not considered folate status during pregnancy. Studies have shown that women unsupplemented during pregnancy are at greater risk for suboptimal folate nutrition during lactation (Salmenpera et al., 1986). Women with suboptimal folate nutritional status during pregnancy may be more likely to become folate depleted during lactation than women with optimal folate stores during pregnancy. Research is needed to address the impact of folate nutritional status during pregnancy on lactation.

Research is also limited on the effects of extended lactation on maternal folate status. It is not known whether prolonged lactation increases the risk of suboptimal folate status. Studies are needed to determine folate requirements to maintain nutrient stores during extended lactation periods.

Finally, research is needed to investigate the effect of folate status during lactation on subsequent pregnancy outcomes. Folate deficiency during pregnancy has been associated with increased risk of infants with low birth weight and neural tube defects (O'Connor, 1994). Periconceptual exposure to folate appears to have a protective effect against neural tube defects (Werler et al., 1993). It is important to consider the impact of lactation on preconception

folate nutritional status, particularly with women having short birth intervals.

In this investigation, milk folate secretion was maintained independent of dietary folate intake and red blood cell folate concentrations. This suggests that there may be other mechanisms involved in the maintenance of milk folate. An understanding of the factors associated with milk folate secretion may be important in beginning to comprehend the secretion process. In addition, iron intake and serum folate status were found to have a significant relationship to milk folate secretion. Iron may play a role in delivery to, or maintenance of, folate in the mammary tissue, and serum folate may be regulated to provide for adequate folate secretion.

Prior to this study the relationship between iron intake and milk folate secretion has not been studied in humans. More research is needed to verify a relationship between dietary iron intake and milk folate in humans and to determine what role iron may have in maintenance of milk folate concentrations. Overall, these results demonstrate the importance of considering nutrient interactions when determining mechanisms involved in maintaining vitamin concentrations in milk. Since folate concentrations in milk have been used to determine additional folate requirements, an understanding of the interactions between nutrients may aid in establishing recommendations for maternal dietary intakes during lactation.

# LITERATURE CITED


Alaimo K, McDowell MA, Briefel RR, Bischof AM, Caughman CR, Loria CM, Johnson CL. Dietary intake of vitamins, mineral, and fiber of persons ages 2 months and over in the United States: Third National Health and Nutrition Examination Survey, Phase I, 1988-91. Advance data from Vital and Health Statistics, 1994.

Bailey LB. The role of folate in human nutrition. Nutr Today 1990;25:12-19

Bailey LB. Folate requirements and dietary recommendations In: Bailey LB ed. Folate in Health and Disease. Marcel Decker, Inc., New York, NY. 1995:123-51.

Bailey LB. Evaluation of a new recommended dietary allowance for folate. J Am Diet Assoc 1992;92:468-71.

Bank MR, Kirksey A, West K, Giacoia G. Effect of storage time and temperature on folacin and vitamin C levels in term and preterm human milk. Am J Clin Nutr 1985;41:235-42.

Bates CJ, Black AE, Phillips DR, Wright AJ, Southgate DA. The discrepancy between normal folate intakes and the folate RDA. Human Nutr Appl Nutr 1982;36:422-9.

Block G. National Cancer Insittute Health Habits and Diet Questionnaire. Information Management Services, Bethesda, MD. 1987.

Blount BC, Ames BN. DNA damage in folate deficiency. Baill Clin Haematol 1995;8:461-78.

Borrud LG, Krebs-Smith SM, Friedman L, Guenther PM. Food and nutrient intakes of pregnant and lactating women in the United States. J Nutr Ed 1993;25:176-85.

Brown CM, Smith AM, Picciano MF. Forms of human milk folacin and variation in patterns. J Pediatr Gastroenterol Nutr 1986;5:278-82.

Butte NF, Calloway DH, Van Duzen JL. Nutritional assessment of pregnant and lactating Navajo women. Am J Clin Nutr 1981;34:2216-28.

Butterworth CE. Folate deficiency and cancer. In: Bendich A, Butterworth CE eds. Micronutrients in Health and Disease. Marcel Decker, Inc., New York, NY. 1991:165-85.

Chanarin I, Rothman D, Berry V. Iron deficiency and its relation to folic-acid status in pregnancy: results of a clinical trial. Br Med J 1965;1:480-5.

Chanarin I, Rothman D, Ward A, Perry J. Folate status and requirement in pregnancy. Br Med J 1968;2:390-4.

Christensen B, Rosenblatt DS. Effects of folate deficiency on embryonic development. Bail Clin Haematol 1996;8:617-37.

Colman N. Addition of folic acid to staple foods as a selective nutrition intervention strategy. Nutr Rev 1982;40:225-33.

Colman N, Barker EA, Barker M, Green R, Metz J. Prevention of neural tube deficency by food fortification. IV. Identification of target groups in addition to pregnant women in an adult rural population. Am J Clin Nutr 1975;28:471-6.

Cooperman JM, Dweck HS, Newman LJ, Garbarino C, Lopez R. The folate in human milk. Am J Clin Nutr 1982;36:576-80.

Czeizal AF, Dudas J. Prevention of the first occurrence of neural tube defects by periconceptional vitamin supplementation. N Engl J Med 1992;327:1832-5.

Ek J. Plasma, red cell, and breast milk folacin concentrations in lactating women. Am J Clin Nutr 1983;38:929-35.

Elwood M, Elwood H, Little J. Diet In: Elwood JM, Little J, and Elwood JH. Epidemiology and Control of Neural Tube Defects. Oxford University Press, New York, N.Y. 1992a:521-603.

Elwood M, Elwood H, Little J. Classification, anatomy, and embryology In: Elwood JM, Little J, and Elwood JH. Epidemiology and Control of Neural Tube Defects. Oxford University Press, New York, N.Y. 1992b:521-603.

Eitenmiller RR, Bryan WD, Khalsa IK, Feeley RM, Barnhart HM. Folate content of human milk during early lactational stages. Nutr Res. 1984;4:391-7.

Everson RB, Wehr CM, Erexson GL, MacGregor JT. Association of marginal folate depletion with increased chromosomal damage in vivo: demonstration by analysis of micronucleated erythrocytes. J Nat Cancer Inst 1988;80:525-9.

Fleming AF. Urinary excretion of folate in pregnancy. J Obstet Gyn 1972;79:916-20.

Green R. Metabolite assays in cobalamin and folate deficiency. Baill Clin Haematol 1995;8:533-66.

Gunter EW, Bowman BA, Caudil DB, Twite DB, Adams M. Results of a round robin for serum and red cell folate. FASEB J 1996;10:a40 (Abstract).

Herbert V. Experimental nutritional folate deficiency in man. Trans Assoc Am Physicians 1962;75:307-20.

Herbert V. Making sense of laboratory tests of folate status: Folate requirements to sustain normality. Am J Hematol. 1987;26:199-207.

Herbert V, Das KC. Folic acid and vitamin B12 In: Shils ME, Olson JA, Shike M eds. Modern Nutrition in Health and Disease. 8th ed. Lea and Febiger, Malvern, PA. 1994:402-25.

Hoffbrand AV, Newcombe BFA, Molin DL. Method of assay of red cell folate activity and the value of the assay as a test for folate deficiency. J Clin Path 1966;19:17-28.

Keizer SE, Gibson RS, O'Connor DL. Postpartum folic acid supplementation of adolescents: impact on maternal folate and zinc status and milk composition. Am J Clin Nutr 1995;62:377-84.

Kirksey A. Effects of vitamin supplementation on vitamin levels in human milk: vitamin B6, vitamin C and folacin In: Hamosh M, Goldman AS eds. Human Lactation 2: Maternal and Environment Factors. Plenum Press, New York, NY. 1986:339-48.

Lindenbaum J, Allen RH. Clinical spectrum and diagnosis of folate deficiency. In: Bailey LB ed. Folate in Health and Disease. Marcel Decker, Inc., New York, NY. 1995:43-73.

Matinez OB. Red cell folate values of a group of nonpregnant mothers. Can J Pub Health 1980;71:163-9.

McPartlin J, Halligan A, Scott JM, Darling M, Weir DG. Accelerated folate breakdown in pregnancy. Lancet 1993;341:148-9.

Metz J. Folate deficiency conditioned by lactation. Am J Clin Nutr 1970;23:843-7.

Morgan SL, Baggott JE. Folate antagonists in nonneoplastic disease: proposed mechanisms of efficacy and toxicity. In: Bailey LB ed. Folate in Health and Disease. Marcel Decker, Inc., New York, NY. 1995:405-33.

MRC Vitamin Study Research Group. Prevention of neural tube defects: results of the medical research council vitamin study. Lancet 1991;338:131-7.

National Research Council. Recommended Dietary Allowances. 10th ed. Washington DC: National Academy of Science. 1989.

O'Connor DL. Folate status during pregnancy and lactation In: Allen LA, King J, Lonnerdal B eds. Nutrient Regulation During Pregnancy, Lactation, and Infant Growth. New York: Plenum Press, 1994:157-72.

O'Connor DL. Interaction of folate during reproduction. Prog Food Nutr Sci 1991;15:231-54.

O'Connor DL, Moriarty P, Picciano MF. The impact of iron deficiency on the flux of folates within the mammary gland. Internat J Vit Res 1991;62:173-80.

O'Connor DL, Picciano MF, Roos MA, Easter RA. Iron and folate utilization in reproducing swine and their progeny. J Nutr 1989;119:1984-9.

O'Connor DL, Picciano MF, Sherman AR. Milk folate secretion and folate status of suckling pups during iron deficiency. Nutr Res 1989;9:307-18.

O'Connor DL, Picciano MF, Sherman AR, Burgert SL. Depressed folate incorporation into milk secondary to iron deficiency in the rat. J Nutr 1987;117:1715-20.

O'Connor DL, Picciano MF, Tamura T, Shane B. Impaired milk folate secretion is not corrected by supplemental folate during iron deficiency in rats. J Nutr 1990;120:499-506.

O'Connor DL, Tamura T, Picciano MF. Pteroylpolyglutamates in human milk. Am J Clin Nutr 1991;53:930-4.

O'Keefe CA, Bailey LB, Thomas EA, Hofler SA, Davis BA, Cerda JJ, Gregory JF. Controlled dietary folate affects folate status in nonpregnant women. J Nutr 1995;125:2717-25.

Pancharuniti N, Lewis CA, Sauberlich HE. Plasma homocysteine, folate, and vitamin B12 concentrations and risk for early onset coronary artery disease. Am J Clin Nutr 1994;59:940-8.

Physicians' Desk Reference (PDR) Edition 48. Medical Economics Data Production Company, Montvale, NJ. 1994:477.

Picciano MF. Folate nutrition in lactation. In: Bailey LB ed. Folate in Health and Disease. Marcel Decker, Inc., New York, NY. 1995:153-69.

Priest DG, Bunni MA. Folates and folate antagonists in cancer chemotherapy. In: Bailey LB ed. Folate in Health and Disease. Marcel Decker, Inc., New York, NY. 1995:379-403.

Qvist J, Abdulla M, Jagerstad M, Svensson S. Iron, zinc, and folate status during pregnancy and two months after delivery. Acta Obstet Gynecol Scand 1986;65:15-22.

Rush D. Periconceptual folate and neural tube defect. Am J Clin Nutr 1994;59:511S-6S.

Salmenpera L, Perheentupa J, Siimes MA. Folate nutrition is optimal in exclusively breast-fed infants but inadequate in some of their mothers and in formula-fed infants. J Pediatr Gastroenterol Nutr 1986; 5:283-9.

Sauberlich HE, Kretsch MJ, Skala JH, Johnson HL, Taylor PC. Folate requirement and metabolism in nonpregnant women. Am J Clin Nutr 1987;46:1016-28.

Savage DG, Lindenbaum J, Stabler SP, Allen RH. Sensitivity of serum methylmalonic acid and total homocysteine determinations for diagnosing cobalamin and folate deficiencies. Am J Med 1994;96:239-46.

Scott JM, Weir DG, Kirke PN. Folate and neural tube defects In: Bailey LB ed. Folate in Health and Disease. Marcel Decker, Inc., New York, NY. 1995:329-60.

Scott JM, Weir DG, Malloy A, McPartlin J, Daly L, Kirke P. Folic acid metabolism and mechanisms of neural tube defects In: Ciba Foundation Symposium 181 Neural Tube Defects. London: John Wiley, 1994:180-91.

Selhub J, Arnold R, Smith AM, Picciano MF. Milk folate binding protein (FBP): a secretory protein for folate? Nutr Res 1984;4:181-7.

Senti FR, Pilch SM, eds. Assessment of the foltae nutritional status of the U.S. population based on data collected in the second National Health and Nutrition Examination Survey, 1976-2980. Life Sciences Research Office, Federation of American Societies for Experimental Biology, Bethesda MD, 1984.

Shane B. Folate metabolism In: Picciano MF, Stokstad EFR, Gregory JF eds. Folic Acid Metabolism in Health and Disease. New York: Wiley-Liss, 1990:165-78.

Shane B. Folate chemistry and metabolism In: Bailey LB ed. Folate in Health and Disease. Marcel Decker, Inc., New York, NY. 1995:1-22.

Smith AM, Picciano MF, Deering RH. Folate supplementation during lactation: maternal folate status, human milk folate content, and their relationship to infant folate status. J Pediatr Gastoenterol Nutr 1983;2:622-8.

Smith AM, Picciano MF, Deering RH. Folate intake and blood concentrations of term infants. Am J Clin Nutr 1985;41:590-8.

Sneed SM, Zane C, Thomas MR. The effects of ascorbic acid, vitamin B6, vitamin B12, and folic acid supplementation on the breast milk of low socioeconomic lactating women. Am J Clin Nutr 1981;34:1338-46.

Stabler SP, Marcell PD, Podell ER, Allen RH, Savage DG, Lindenbaum J. Elevation of total and homocysteine in the serum of patients with cobalamin or folate deficiency detected by capillary gas chromatography-mass spectrometry. J Clin Invest. 1988;81:466-74.

Subar AF, Block G, James LD. Folate intake and food sources in the US population. Am J Clin Nutr 1989;50:508-16.

Tamura T. Microbiological assay of folates In: Picciano MF, Stokstad EFR, Gregory JF eds. Folic Acid Metabolism in Health and Disease. New York: Wiley-Liss, 1990:121-37.

Tamura T, Yoshimura Y, Arakawa T. Human milk folate and folate status in lactating mothers and their infants. Am J Clin Nutr 1980;33:193-7.

Thomas MR, Sneed SM, Wei C, Nail PA, Wilson M, Sprinkle EE. The effects of vitamin C, vitamin B-6, vitamin B-12, folic acid, riboflavin, and thiamin on the breastmilk and maternal status of well-nourished women at 6 months postpartum. Am J Clin Nutr 1980;33:2151-6.

Udipi SA, Kirksey A, Roepke JLB. Diurnal variations in folacin levels of human milk: use of a single sample to represent folacin concentration in milk during a 24-h period. Am J Clin Nutr 1987;45:770-9.

Utley CS, Marcell PD, Allen RH, Antony AC, Kolhouse JF. Isolation and characterization of methionine synthetase from human placenta. J Biol Chem 1985;260:13656-65.98

Wagner C. Biochemical role of folate in cellular metabolism. In: Bailey LB ed. Folate in Health and Disease. Marcel Decker, Inc., New York, NY. 1995:23-42.

Wickramasinghe RG, Hoffbrand AV. Reduced rate of replication fork movement in megaloblastic anemia. Biochem Biophys Acta 563:46-58.

Winkvist A, Rasmussen KM, Habicht JP. A new definition of maternal depletion syndrome. Am J Pub Health 1992;82:691-4.

# APPENDIX A

# APPROVAL FROM THE INSTITUTIONAL REVIEW BOARD

# APPENDIX B

# SUBJECTS INFORMED CONSENT

**VIRGINIA POLYTECHNIC INSTITUTE AND STATE UNIVERSITY**

**Informed Consent for Participants of Investigative Projects**

**Title of Project:** **Maternal Folate Status and Breast Milk Folate Concentration of Lactating Women**

**Investigators:** **Elizabeth A. Thomas, Ph.D., R.D. and Karen B. Ruggio**
**Human Nutrition and Foods Department**

## I. The Purpose of this Research

Maternal folate intake two months prior to conception through the first month of pregnancy has been inversely correlated with having a baby with neural tube birth defect (NTD). Few studies have been directed towards assessing the effect of lactation on the mother's folate status. The amount of folate required by lactating women that will result in appropriate folate stores at the completion of lactation is unknown. A major purpose of this study is to track the folate status of lactating women over a period of time, and correlate changes in folate status with dietary folate intake.

Animal studies have shown a relationship between iron deficiency and depressed milk folate secretion. In women, iron and folate deficiencies are reported to coexist. However, the effect of iron deficiency on milk folate content of lactating women has not been studied. A secondary purpose of this study is to investigate the relationship between iron status and folate content in the milk of lactating women.

## II. Procedures

Subjects will receive dietary counseling and be instructed to consume the Recommended Dietary Allowance of folate for lactating women (280 mg/day). Dietary records will be collected for seven days prior to each sample collection. Once per month for four months, subjects will collect approximately 10 ml (2 tsp) of breast milk in their home. Milk samples will be collected using a breast pump. The day after each milk sample collection, following a 10 hour fast, one 30 ml (2 Tbs.) venous blood sample will be collected in Wallace Hall on the Virginia Tech campus.

## III. Risks

Venous blood collection occasionally results in bruising. Some subjects may experience distress during blood draws. Every effort will be made to assure the comfort of the subject. No coercion will be used to persuade subjects that are reluctant to participate. Juice and a snack will be provided for the subjects following the blood

collections. There are no risks associated with breast milk collection or dietary intake collection.

## IV. Benefits of this Project

Benefits of participation include dietary counseling and the provision of nutritional status information and assessment based on the biochemical and dietary analysis. Additionally, the findings of this study will be the basis of future research aimed at identifying maternal folate requirements during lactation. No promise or guarantee of benefits have been made to encourage you to participate.

## V. Extent of Anonymity and Confidentiality

The results of this study will be kept strictly confidential. All information and samples will be coded with subject numbers. In any publication or presentation of the results of this study, subjects will be referred to by code. The principal investigator and graduate research assistant will be the only individuals that will have access to the data and codes.

## VI. Compensation

No compensation for subject participation is provided.

## VII. Freedom to Withdraw

Subjects are free to withdraw from the study at any time without penalty. Subjects are free not to answer any questions or respond to experimental situations that they choose without penalty.

## VIII. Approval of Research

This research project has been approved, as required, by the Institutional Review Board for Research Involving Human Subjects at Virginia Polytechnic Institute and State University.

## IX. Subjects Responsibilities

I voluntarily agree to participate in this study. I have the following responsibilities:

Provide a 10 ml of breast milk once per month for four months
Complete a seven day dietary record each month prior to milk sample collection
Eat and drink nothing except water for 10 hours prior to blood collection
Provide a 30 ml sample of venous blood per month for four months

## X. Subject's Permission

I have read and understand the Informed Consent and conditions of this project. I have had all my questions answered. I hereby acknowledge the above and give my voluntary consent for participation in this project.

If I participate, I may withdraw at any time without penalty. I agree to abide by the rules of this project.

_____ _____

Signature Date

Should I have any questions about this research or its conduct, I may contact:

| | |
|---|---|
| Karen Ruggio<br>Graduate Research Assistant | (work) 540-231-7708<br>(home) 540-552-2896 |
| Elizabeth Thomas, Ph.D., R.D.<br>Assistant Professor | (work) 540-231-8763<br>(home) 540-951-0807 |
| E.R. Stout<br>Chair, IRB Research Division | 540-231-9359 |

# APPENDIX C

# MEDICAL HISTORY QUESTIONNAIRE

## MEDICAL HISTORY QUESTIONNAIRE

Please complete the information on the following pages. Please ask if you have any questions regarding any information requested.

### SECTION I

Name

_____

Date of Birth_____ Age _____

Home Phone Number _____ Work Phone _____

Address _____

Physician _____

### SECTION II

Date of last physical examination _____

Do you have any allergies (Medications, Food, Other) _____ No _____ Yes (please list)

_____

Do you have any chronic or serious illnesses _____ No _____Yes (please list)

_____

Please list information about your last three hospitalizations

1. Type of operation _____
   Month and year hospitalized _____

2. Type of operation _____
   Month and year hospitalized _____

3. Type of operation _____
   Month and year hospitalized _____

## SECTION II

1. Are you taking any medications on a regular basis? _____ No _____Yes
   If Yes, please list all medications including both prescription and non-prescription drugs. _____

2. Did you take a vitamin and/or mineral supplement during your pregnancy?
   _____ No _____Yes
   If yes, please list the following:
   Brand name of supplements_____
   Dosage _____
   Consumption frequency _____

3. Are you currently taking a vitamin and/or mineral supplement ? ____No ___Yes
   If yes, please list the following:
   Brand name of supplements_____
   Dosage _____
   Consumption frequency _____

## SECTION III

## SMOKING HABITS

1. Have you ever smoked cigarettes, cigars, or a pipe ? _____ No _____ Yes
2. Do you presently smoke? _____ No _____ Yes
   If Yes, please indicate the number smoked per day.
   Cigarettes _____ per day
   Cigars _____ per day
   Pipefulls _____ per day
3. At what age did you begin smoking ? _____ years
4. If you have quit smoking, when did you quit ? (month and year) _____

## DRINKING HABITS

1. Did you drink alcoholic beverages prior to your pregnancy ? _____ No ____ Yes
   If Yes, approximately how many glasses on average of beer, wine, or highballs did you consume per week ? _____ glasses

2. Did you drink alcoholic beverages during your pregnancy ? _____ No ____ Yes
   If Yes, approximately how many glasses on average of beer, wine, or highballs did you consume per week ? _____ glasses

3. Are you currently or do you plan on drinking alcoholic beverages after your pregnancy ? _____ No ____ Yes
   If Yes, approximately how many glasses on average of beer, wine, or highballs do you plan consume per week ? _____ glasses

## PHYSICAL ACTIVITY

1. Do you exercise on a regular basis ? _____ No _____ Yes
   If Yes, what activities do you engage in on a regular basis? _____

   _____

2. How many hours an average do you exercise per week ? _____ hours

3. Is your occupation: (Please check response)
   Inactive (i.e. desk job) _____
   Light work (i.e. housework)_____
   Heavy work (i.e. lifting) _____

# APPENDIX D
# FOOD FREQUENCY QUESTIONNAIRE

## FOOD QUESTIONNAIRE

This section is about your *usual* eating habits. Thinking back over the past year, how often do you usually eat the foods listed on the next pages?

First, check (√) whether your usual serving size is small, medium or large.

Then, put a NUMBER in the most appropriate column to indicate how often you usually eat the food. If you never eat the food, check Rarely/Never (Nv). Please do not skip foods. And please be careful which column you put your answer in.

Please look at the example below. This person

1) eats an apple 4 times per week
2) has 1 egg once per day
3) has 1 large piece of fried chicken 2 times per month
4) has a small serving of sweet potatoes about 3 times per year
5) never eats liver

EXAMPLE:

| | Medium Serving | Serving Size | | | How Often? | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | S | M | L | Da | Wk | Mo | Yr | Nv |
| APPLES, APPLE SAUCE, PEARS | (1) or 1/2 Cup | | √ | | | 4 | | | |
| EGGS | 1 =Small 2 =Med. | | √ | | 1 | | | | |
| FRIED CHICKEN | 1 Lg. Piece | | | √ | | | 2 | | |
| SWEET POTATOES | 1/2 Cup | √ | | | | | | 3 | |
| LIVER | 4 oz. | | | | | | | | √ |

* Da = Day, Wk = Week, Mo = Month, Yr = Year, Nv = Rarely/Never

## FOOD FREQUENCY QUESTIONNAIRE

| | Medium Serving | Serving Size | | | How Often? | | | | |
|---|---|---|---|---|---|---|---|---|---|
| **FRUITS & JUICES** | | S | M | L | Da | Wk | Mo | Yr | Nv |
| EXAMPLE - APPLES, APPLESAUCE, PEARS | (1) or 1/2 Cup | | √ | | | 4 | | | |
| APPLES, APPLESAUCE, PEARS | (1) or 1/2 Cup | | | | | | | | |
| BANANAS | 1 Medium | | | | | | | | |
| PEACHES, APRICOTS (Canned, frozen or dried, whole year) | (1) or 1/2 Cup | | | | | | | | |
| PEACHES, APRICOTS, NECTARINES (Fresh, in season) | 1 Medium | | | | | | | | |
| CANTALOUPE (in season) | 1/4 Medium | | | | | | | | |
| WATERMELON (in season) | 1 Slice | | | | | | | | |
| STRAWBERRIES (Fresh, in season) | 1/2 Cup | | | | | | | | |
| ORANGES | 1 Medium | | | | | | | | |
| ORANGE JUICE or GRAPEFRUIT JUICE | 6 oz. Glass | | | | | | | | |
| GRAPEFRUIT | 1 Medium | | | | | | | | |
| TANG, START FRUIT DRINKS | 6 oz. Glass | | | | | | | | |
| OTHER FRUIT JUICE, FORTIFIED FRUIT DRINKS | 6 oz. Glass | | | | | | | | |
| ANY OTHER FRUIT, INCLUDING BERRIES, FRUIT COCKTAIL | 1/2 Cup | | | | | | | | |
| **VEGETABLES** | | S | M | L | Da | Wk | Mo | Yr | Nv |
| STRING BEANS, GREEN BEANS | 1/2 Cup | | | | | | | | |
| PEAS | 1/2 Cup | | | | | | | | |
| CHILI WITH BEANS | 3/4 Cup | | | | | | | | |
| OTHER BEANS SUCH AS BAKED BEANS, PINTOS, KIDNEY BEANS | 3/4 Cup | | | | | | | | |

| | Medium Serving | S | M | L | Da | Wk | Mo | Yr | Nv |
|---|---|---|---|---|---|---|---|---|---|
| CORN | 1/2 Cup | | | | | | | | |
| WINTER SQUASH, BAKED SQUASH | 1/2 Cup | | | | | | | | |
| TOMATOES, TOMATO JUICE | (1) or 6 oz. | | | | | | | | |
| RED CHILI SAUCE, TACO SAUCE, SALSA PICANTE | 2 Tbs. Sauce | | | | | | | | |
| BROCCOLI | 1/2 Cup | | | | | | | | |
| CAULIFLOWER OR BRUSSEL SPROUTS | 1/2 Cup | | | | | | | | |
| SPINACH (Raw) | 3/4 Cup | | | | | | | | |
| SPINACH (Cooked) | 1/2 Cup | | | | | | | | |
| MUSTARD GREENS, TURNIP GREENS, COLLARDS | 1/2 Cup | | | | | | | | |
| COLE SLAW, CABBAGE, SAUERKRAUT | 1/2 Cup | | | | | | | | |
| CARROTS, OR MIXED VEGETABLES CONTAINING CARROTS | 1/2 Cup | | | | | | | | |
| GREEN SALAD | 1 Med. Bowl | | | | | | | | |
| SALAD DRESSING, MAYONNAISE (Including on sandwiches) | 2 Tbs. | | | | | | | | |
| FRENCH FRIES AND FRIED POTATOES | 3/4 Cup | | | | | | | | |
| SWEET POTATOES, YAMS | 1/2 Cup | | | | | | | | |
| OTHER POTATOES, INCLUDING BOILED, BAKED, POTATO SALAD | (1) or 1/2 Cup | | | | | | | | |
| RICE | 3/4 Cup | | | | | | | | |
| ANY OTHER VEGETABLE, INCLUDING SUMMER SQUASH | 1/2 Cup | | | | | | | | |
| BUTTER, MARGARINE OR ANY OTHER FAT ON VEGETABLES | 2 Pats | | | | | | | | |
| | | | | | | | | | |

| MEAT, FISH, POULTRY & MIXED DISHES | | S | M | L | Da | Wk | Mo | Yr | Nv |
|---|---|---|---|---|---|---|---|---|---|
| HAMBURGERS, CHEESE BURGERS, MEAT LOAF | 1 Medium | | | | | | | | |
| BEEF - STEAKS, ROASTS | | | | | | | | | |
| BEEF STEW OR POT PIE WITH CARROTS, OTHER VEGETABLES | 1 Cup | | | | | | | | |
| LIVER, INCLUDING CHICKEN LIVERS | 4 oz. | | | | | | | | |
| PORK, INCLUDING CHOPS, ROASTS | 2 Chops or 4 oz. | | | | | | | | |
| FRIED CHICKEN | 2 Sm. or 1 Lg. Piece | | | | | | | | |
| CHICKEN OR TURKEY, ROASTED, STEWED, OR BROILED | 2 Sm. or 1 Lg. Piece | | | | | | | | |
| FRIED FISH, OR FISH SANDWICH | 4 oz. or 1 sand. | | | | | | | | |
| TUNA FISH, TUNA SALAD, TUNA CASSEROLE | 1/2 Cup | | | | | | | | |
| SHELL FISH (SHRIMP, LOBSTER, CRAB, OYSTERS, ETC.) | (5) 1/4 Cup or 3 oz. | | | | | | | | |
| OTHER FISH, BROILED, BAKED | 4 oz, | | | | | | | | |
| SPAGHETTI, LASAGNA, OTHER PASTA WITH TOMATO SAUCE | 1 Cup | | | | | | | | |
| PIZZA | 2 Slices | | | | | | | | |
| MIXED DISHES WITH CHEESE | 1 Cup | | | | | | | | |
| **LUNCH ITEMS** | | **S** | **M** | **L** | **Da** | **Wk** | **Mo** | **Yr** | **Nv** |
| LIVERWURST | 2 Slices | | | | | | | | |
| HOT DOGS | 2 Dogs | | | | | | | | |
| HAM, LUNCH MEATS | 2 Slices | | | | | | | | |
| VEGETABLE SOUP, VEGETABLE BEEF, MINESTRONE, TOMATO | 1 Med. Bowl | | | | | | | | |
| OTHER SOUPS | 1 Med. Bowl | | | | | | | | |

| BREADS/ SALTY SNACKS/ SPREADS | | S | M | L | Da | Wk | Mo | Yr | Nv |
|---|---|---|---|---|---|---|---|---|---|
| BISCUITS, MUFFINS, BURGER ROLLS (Including Fast Food) | 1 Med. Piece | | | | | | | | |
| WHITE BREAD, BAGEL, ETC., CRACKERS | 2 Slices, 3 Crackers | | | | | | | | |
| DARK BREAD, INCLUDING WHOLE WHEAT, RYE, PUMPERNICKEL | 2 Slices | | | | | | | | |
| CORN BREAD, CORN MUFFINS, CORN TORTILLAS | 1 Med. Piece | | | | | | | | |
| SALTY SNACKS (Such As Chips) | 2 handfuls | | | | | | | | |
| PEANUTS, PEANUT BUTTER | 2 Tbs. | | | | | | | | |
| BUTTER ON BREAD OR ROLLS | 2 Pats | | | | | | | | |
| GRAVIES MADE WITH MEAT DRIPPINGS, OR WHITE SAUCE | 2 Tbs. | | | | | | | | |
| **BREAKFAST FOODS** | | **S** | **M** | **L** | **Da** | **Wk** | **Mo** | **Yr** | **Nv** |
| HIGH FIBER, BRAN OR GRANOLA CEREALS, SHREDDED WHEAT | 1 Med. Bowl | | | | | | | | |
| HIGHLY FORTIFIED CEREALS, SUCH AS TOTAL OR PRODUCT 19 | 1 Med. Bowl | | | | | | | | |
| OTHER COLD CEREALS, SUCH AS CORN FLAKES | 1 Med. Bowl | | | | | | | | |
| COOKED CEREALS | 1 Med. Bowl | | | | | | | | |
| SUGAR ADDED TO CEREALS | 2 teaspoons | | | | | | | | |
| EGGS | 1 egg=S<br>2 egg=M | | | | | | | | |
| BACON | 2 Slices | | | | | | | | |
| SAUSAGE | 2 patties or links | | | | | | | | |
| **SWEETS** | | **S** | **M** | **L** | **Da** | **Wk** | **Mo** | **Yr** | **Nv** |
| ICE CREAM | 1 Scoop | | | | | | | | |
| DOUGHNUTS, COOKIES, CAKES, PASTRY | 1 Piece or 3 Cookies | | | | | | | | |

| | Serving Size | S | M | L | Da | Wk | Mo | Yr | Nv |
|---|---|---|---|---|---|---|---|---|---|
| PUMPKIN PIE | 1 Med Slice | | | | | | | | |
| OTHER PIES | 1 Med Slice | | | | | | | | |
| CHOCOLATE CANDY | Sm Bar 1oz. | | | | | | | | |
| OTHER CANDY, JELLY, HONEY, BROWN SUGAR | 3 Pieces or 1 Tbs. | | | | | | | | |
| **DAIRY PRODUCTS** | | **S** | **M** | **L** | **Da** | **Wk** | **Mo** | **Yr** | **Nv** |
| COTTAGE CHEESE | 1/2 Cup | | | | | | | | |
| OTHER CHEESES AND CHEESE SPREADS | 2 Slices or 2 oz. | | | | | | | | |
| FLAVORED YOGURT | 1 Cup | | | | | | | | |
| WHOLE MILK AND BEVERAGES WITH WHOLE MILK (Not including on cereal) | 8 oz. Glass | | | | | | | | |
| 2% MILK AND BEVS. WITH 2% MILK (Not including on cereal) | 8 oz. Glass | | | | | | | | |
| SKIM MILK, 1 % MILK OR BUTTERMILK (Not including on cereal) | 8 oz. Glass | | | | | | | | |
| **BEVERAGES** | | **S** | **M** | **L** | **Da** | **Wk** | **Mo** | **Yr** | **Nv** |
| REGULAR SOFT DRINKS | 12 oz. | | | | | | | | |
| DIET SOFT DRINKS | 12 oz. | | | | | | | | |
| BEER | 12 oz. | | | | | | | | |
| WINE | 1 Med. Glass | | | | | | | | |
| LIQUOR | 1 Shot | | | | | | | | |
| DECAF COFFEE | 1 Cup | | | | | | | | |
| REGULAR COFFEE | 1 Cup | | | | | | | | |
| TEA (Hot or iced) | 1 Cup | | | | | | | | |
| LEMON IN TEA | 1 Teaspoon | | | | | | | | |
| NON-DAIRY CREAMER IN COFFEE OR TEA | 1 Tbs. | | | | | | | | |
| MILK IN COFFEE OR TEA | 1 Tbs. | | | | | | | | |
| | | | | | | | | | |

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| CREAM (real) or HALF-AND-HALF IN COFFEE OR TEA | 1 Tbs. | | | | | | | | |
| SUGAR IN COFFEE OR TEA | 2 Teaspoon | | | | | | | | |
| ARTIFICIAL SWEETENER IN COFFEE OR TEA | 1 Packet | | | | | | | | |
| GLASSES OF WATER, NOT COUNTING IN COFFEE OR TEA | 8 oz. Glass | | | | | | | | |

Adapted from: Block, G. National Cancer Institute Health Habits and Diet Questionnaire. Information Management Services. Bethesda, MD. 1987.

# APPENDIX E

# POST-DELIVERY QUESTIONNAIRE

## Post Delivery Questionnaire

Name _____ Subject Number _____

Delivery Date _____

1. Was your birth ___ vaginal or ____ cesarean?
2. Was your labor induced? ____ No ____ Yes?
3. Did you have excessive bleeding? ____ No ____ Yes
4. Did you receive a blood transfusion? ____ No ____ Yes
5. Did you receive an iron injection? ____ No ____ Yes
6. Did you receive hormone injections? ____ No ____ Yes, Please explain_____
7. Did you have any postoperative infections? ____ No ____ Yes, Please explain_____
8. Did you receive antibiotics? ____ No ____ Yes, Please describe _____

   _____
9. Did you experience any other complications associated with your delivery? ___ No ____ Yes, Please explain _____

   _____
10. Are you taking oral contraceptives? ____ No _____ Yes
11. Do you plan on taking oral contraceptives during the next 4 months? ___ No ___ Yes, If yes when do plan on starting oral contraceptive use

    _____

# APPENIDIX F

# SAMPLE CALENDAR

# JUNE

| SUNDAY | MONDAY | TUESDAY | WEDNESDAY | THURSDAY | FRIDAY | SATURDAY |
|---|---|---|---|---|---|---|
| | | | | | | 1 |
| 2 | 3 | 4 | 5 | 6 | 7 | 8 |
| 9 | 10 | 11 | 12 | 13<br>**begin diet record** | 14<br>**diet record** | 15<br>**diet record** |
| 16<br>**diet record** | 17<br>**diet record** | 18<br>**diet record** | 19<br>**diet record**<br>**milk sample collection** | 20<br>**blood sample collection** | 21 | 22 |
| 23 30 | 24 | 25 | 26 | 27 | 28 | 29 |

# APPENDIX G

# FOOD RECORDS AND PROCEDURES

## INSTRUCTIONS FOR 7-DAY FOOD RECORDS

1. Include everything you eat for seven consecutive days, with each day beginning at 6:00 am and ending at 6:00 am the following day. This includes all meals, snacks, beverages, condiments, gravies, butter, medications, supplements, gum, and mints.

2. Record each food or beverage item immediately after you consume it. If you do not have the food record with you when you eat, please record the food and beverage items on a piece of paper, then copy them into the food record as soon as possible.

3. Describe all foods and beverages in as much detail as possible. For example, cooking method (steamed, fried, baked ...), canned, frozen, fresh, brand name, and sizes of food items (large, small, thin, thick ...).

4. List amounts eaten as precisely as possible. For example, 1/2 cup, 1 cup, 1 tablespoon, 4 ounces.

5. For mixed items such as spaghetti, list out all components of the item separately. For example, the spaghetti may be listed as follows: 1 1/2 cups thin spaghetti noodles, 1/2 cup Ragu meat sauce, and 1 tablespoon of Kraft Parmesan cheese.

**FOOD INTAKE RECORD - DAY 1 (EXAMPLE)**

NAME _____ DATE _____

| FOOD /BEVERAGE ITEM | DESCRIPTION (HOW PREPARED) | AMOUNT/ SIZE |
|---|---|---|
| **Wheat Bread** | **Toasted** | **1 slice** |
| **Margarine** | | **1 tablespoon** |
| **Orange Juice** | **From Concentrate** | **1 cup** |
| **Kellogg's Corn Flakes** | | **1 Med. Bowel** |
| **Milk, 2 %** | | **1/4 cup** |
| **Broccoli, fresh** | **Steamed** | **1/2 cup** |
| **Potato** | **Baked** | **Med.** |
| **Cheddar Cheese, Kraft Reg.** | | **3 oz.** |
| **Cookies, Choc. Chip** | **Home Made** | **3 cookies - med. size** |
| | | |
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**FOOD INTAKE RECORD - DAY 1**

NAME _____ DATE _____

| FOOD /BEVERAGE ITEM | DESCRIPTION (HOW PREPARED) | AMOUNT/ SIZE |
|---|---|---|
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# APPENDIX H

# POST-STUDY QUESTIONNAIRE RESPONSES

**Post-Study Subject Survey**

Please complete the following survey and return it by mail in the enclosed addressed envelope. This survey is anonymous and simply serves to assist me in analyzing the information you have provided and to help in assessment of the validity of the results obtained. Your name will not be associated with this information. Thank you again for participating in the lactation study.

---

1. Did you complete your diet records accurately and honestly?
   ✓ Yes _____ No If no, please provide a brief explanation. _____

2. Did you consume the supplements provided daily throughout the study period? ✓ Yes _____ No If no, approximately how many times per week on average did you take the supplement? _____ times per week

3. Please feel free to share any comments that you have regarding your participation in this study. I appreciated the feedback on my nutritional analyses.

**Post-Study Subject Survey**

Please complete the following survey and return it by mail in the enclosed addressed envelope. This survey is anonymous and simply serves to assist me in analyzing the information you have provided and to help in assessment of the validity of the results obtained. Your name will not be associated with this information. Thank you again for participating in the lactation study.

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1. Did you complete your diet records accurately and honestly?
   _X_ Yes _____ No If no, please provide a brief explanation. _____

   _____

   _____

   _____

2. Did you consume the supplements provided daily throughout the study period? _X_ Yes _____ No If no, approximately how many times per week on average did you take the supplement? _____ times per week

3. Please feel free to share any comments that you have regarding your participation in this study. _____

   _____

   _____

   _____

   _____

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1. Did you complete your diet records accurately and honestly?
   ✓ Yes _____ No If no, please provide a brief explanation. _____

   N/A

2. Did you consume the supplements provided daily throughout the study period? _____ Yes ✓ No If no, approximately how many times per week on average did you take the supplement? 5 times per week

3. Please feel free to share any comments that you have regarding your participation in this study.

   - Sample collection was done well and meticulously.
   - Researcher: very friendly, likeable and understanding.
   - Had to keep reminding myself to take ~~the~~ supplements.
   - Recording dietary intake required dedication & discipline.

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1. Did you complete your diet records accurately and honestly?
   ✓ Yes _____ No If no, please provide a brief explanation. _____

2. Did you consume the supplements provided daily throughout the study period? ✓ Yes _____ No If no, approximately how many times per week on average did you take the supplement? _____ times per week

3. Please feel free to share any comments that you have regarding your participation in this study. I'm real interested in the outcome so please give me a call. Thanks

# VITA

Karen Bernadine Ruggio was born on May 14, 1964 in Syracuse, New York. She attended undergraduate school at the State University of New York at Potsdam where she earned a Bachelors degree in economics with a minor in business. After completing her undergraduate studies, she worked for several years as an accountant in Scottsdale, Arizona. After much thought and soul searching, she decided to return to college for a graduate degree in nutrition. At Virginia Polytechnic Institute and State University she discovered her passion for science and research. In July of 1996, Karen married Ashwin Earl Amanna. Upon completion of her masters degree in human nutrition in December of 1996, she plans to continue her education and to pursue a career in clinical dietetics and research.