Assessment of Folate Status in Manitoban Women: Critical Evaluation of Dietary Assessment Methods

by

Aysheh M. Shuaibi

A Thesis submitted to the Faculty of Graduate Studies of

The University of Manitoba

in partial fulfilment of the requirements of the degree of

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Department of Food and Nutritional Sciences

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THE UNIVERSITY OF MANITOBA

FACULTY OF GRADUATE STUDIES

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ABSTRACT

Folate is required for DNA synthesis and for single carbon transfer reactions. Deficiency results in megalobastic anaemia. Low folate status in women is associated with increased risk of neural tube defects (NTDs). Supplements of folic acid (FA) decrease the risk of NTDs. Low folate status may be related to an increased risk of coronary heart disease (CHD). Elevated plasma levels of total homocysteine (tHcy), which increase when dietary folate intake is low and are lowered by FA supplements, are associated with an increased risk of CHD. Low dietary intakes may be implicated in the causation of cancer, depression and osteoporosis in women. With the adoption of fortification of foods with FA, there is a need to develop methods to assess habitual folate intake reliably. The overall objective of this study is to develop and validate a rapid assessment method to assess folate.A rapid dietary assessment method, the Food Choice Map (FCM), which is sensitive to this fortification, is now established, is easy to administer, inexpensive, and a reliable form of dietary analysis. The relative validity, which reflects the assessed agreement between the FCM and the three day food record (3DFR), was expressed as a comparison of the group means, a correlation with corresponding biomarkers, and rankings of study participants according to their intakes and their biomarkers. In general, the FCM produces higher estimates of mean intakes than the 3DFR. There was no significant difference between the two methods in the correlations with serum values. FCM obtained folate intakes ($r = 0.43$, $P<0.01$) exhibited a similar and moderate association with serum folate as did 3DFR obtained folate intakes ($r = 0.39$, $P<0.01$). Similarly, vitamin B-12 intakes obtained with both techniques showed a modest association with serum vitamin B-12 (FCM: $r = 0.40$, $p<0.01$, 3DFR: $r = 0.44$, P

<0.01). The FCM and 3DFR performed similarly and at least 74% of folate or vitamin B-12 intakes were either correctly or closely classified when intakes from supplements were included in the analysis. The Validity Coefficient (VD) assessed the correlation between the estimated dietary intake and the subject's true habitual intake, shows that the FCM may be a more reliable tool to estimate folate and intake in women of reproductive age. At mandated levels of FA fortification, many women of child bearing age are unlikely to meet their special folate requirement from dietary sources alone. Intakes of FA from fortified foods are within the level originally predicted for the fortification. Seventy nine and 91% of women in this study met the Recommended Dietary Allowances (RDA) and Estimated Average Requirement (EAR) for folate, respectively. Only 17% of the study participants met the special recommendation of women capable of becoming pregnant. It is recommended by IOM to consume 400 μg of FA daily from supplements, fortified foods, or both in addition to consuming food folate from a varied diet. No subject met the recommendation when FA intake did not include supplementation. Only 1% of the participants exceed the upper limit (UL) which resulted from the use of 1000g FA. The folates were derived mainly from bakery products (~28%), vegetables (18%), pasta and rice (~15%), juices (~13%), milk and dairy products (~6%), ready to eat cereal (~5%). The biochemical evidence showed that no women were folate deficient, 14% of all participants had red blood cell folate concentration >906 nmol/L, which is associated with very low NTD risk. Hyperhomocysteinaemia > 12.0 μmol/l occurred in 4.2% of women. Determinants affecting the tHcy status for healthy young women were serum and red blood cell folate, and body mass index. It is likely that women of childbearing age need to consume

additional FA through supplementation in order to reach the RBC folate levels where they have the lowest risk of NTDs.

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LIST OF ABBREVIATIONS

AD	Alzheimer's Disease
CHD	Coronary Heart Disease
CNF	Canadian Nutrient File
CSFII	Continuing Survey of Food Intakes by Individuals II
DFEs	Dietary Folate equivalents
DRI	Dietary Reference intakes
EAR	Estimated Average requirement
FA	Folic acid
FFQ	Food Frequency Questionnaire
FCM	Food Choice Map
FDB	Food Composition Databases
IOM	Institute of Medicine
NF	Natural Folate
NHANES	National Health and Nutrition Examination Survey
NTDs	Neural Tube Defects
RDA	Recommended Dietary Allowances
RBC	Red Blood Cell
RNI	Recommended Nutrient Intake
SFA	Synthetic Folic Acud
tHcy	Total Homocysteine
3DFR	Three Day Food Record
VC	Validity Coefficient

UL	Upper limit

CHAPTER 1

1. GENERAL INTRODUCTION

1.1 Introduction

The water soluble vitamin folate plays critical roles in intermediary metabolic pathways. Folate is required for DNA synthesis and for single carbon transfer reactions, including those involved in the metabolic pathways of methionine, serine and glycine (1). Clinical folate deficiency symptoms include megaloblastic anemia and intestinal villous atrophy (2). With respect to sub-clinical deficiency states, poor folate status in women is associated with an increased risk of neural tube defects (NTD) (3) and an increased risk of early spontaneous abortion (4). Supplements of folic acid (FA) have been shown to decrease the risk of NTDs (5). Beyond the association with reproductive health, poor folate status has been linked to an increased risk for cardiovascular disease (6), through a mechanism(s) that may involve the artherogenic amino acid homocysteine (7) which is derived from methionine. Additionally, folate deficiency has been linked to the development of certain cancers (8, 9) and, more recently, an inverse association between depression and folate status has been demonstrated in clinical studies (10). It was shown recently that elevated total homocysteine (tHcy) and low folate levels were associated with reduced bone mineral density in women (11, 12). Consequently, there is a need to develop methods to rapidly assess habitual folate intake reliably. Traditional measures of food intake are lengthy, costly, and generally not practical for use in counseling individuals or in large population surveys (13, 14).

With the adoption of mandatory fortification of foods with FA, a synthetic form of folate, an increasing amount of dietary folate now comes from folate-fortified foods. Any new dietary tool needs to be sensitive to this. In chapter 4, we describe the development and assessment of the reliability and validity of a rapid dietary assessment

tool, the Food Choice Map (FCM), to determine dietary folate intakes of adult women in an environment where folate fortified foods are available in the marketplace.

Low dietary intake is the most common cause of compromised folate status (15). Before fortification, the mean daily intake for all women was 207 ± 2.9 μg, with 92.5% of women in the Second National Health and Nutrition Examination Survey (NHANES II) consuming less than Recommended Dietary Allowances (RDA) of 400 μg FA (16). FA fortification of enriched white flour, pasta products, and selected grain products has been mandatory in North America since 1998 (17, 18). The current level of fortification, 140 μg of FA per 100 g of product (19), was expected to deliver an additional 80-100 μg of FA to the diets of the target population, women of childbearing age (20). The RDA and Estimated Average Intake (EAR) for folate are expressed as Dietary Folate Equivalents (DFEs) which are adjusted for the apparent greater bioavailability of synthetic FA than that for the same amount of naturally occurring food folate. The RDA for folate for women at least of 18 y age is 400 μg of DFEs. For women of childbearing age, the recommendation is to consume 400 μg/d of synthetic FA from fortified food and dietary supplement in addition to naturally occurring folate from food (21). Evidence has accumulated to show that women of childbearing age are not consuming enough FA (400 μg FA/d) (19, 22, 23), and recommendations have been made to fortify at twice the current level (24).

Seven years have passed since the implementation of the fortification and the establishment of the new DFE. There is little information available to determine whether Canadian women meet the current folate recommendations. The intent of chapter 5 was to describe intakes of folate in women of childbearing age while taking account the bioavailability of synthetic FA in dietary supplement and a fortificant as outlined in the

Institute of Medicine (IOM) report (25). In chapter 6 the intent was to determine the (a) folate intakes of women of childbearing age residing in Winnipeg, Manitoba; (b) the effect of supplementation and FA fortification of bread and grain product on their folate intakes; (c) and investigate the pattern of folate intake and major contributors for folate.

To my knowledge, no study has examined the impact of FA fortification of the food supply on folate, vitamin B_{12} and tHcy status for Canadian women of child bearing age. One purpose of this thesis is to provide preliminary data on folate status in women of childbearing age (18 to 25 years of age, n=95) post FA fortification of the food supply. Folate status is assessed by measurement of serum and red blood cell folate and plasma tHcy concentrations, a functional index of folate status, and vitamin B_{12} status assessed by measurement serum vitamin B_{12}.

From a pubic health viewpoint, it is important to identify modifiable factors that influence plasma tHcy levels in women at childbearing age, because elevated concentrations are associated with serious pregnancy complications, including pregnancy-induced hypertension, pre-eclampsia (26), placental abruption (27), early pregnancy loss and neural-tube defects(12, 28). Furthermore, many studies have shown an elevated concentration of tHcy is an independent risk for coronary ischemic disease, stroke, peripheral vascular disease, and stroke (12, 29-31). Recently, elevated tHcy in women was associated with reduced bone mineral density and increased risk of osteoporosis (11, 12, 32). The primary aim of chapter 7 was to investigate the association between tHcy and its dietary determinants (estimated protein, methionine, folate, vitamin B_6, and vitamin B_{12}), some lifestyle factors (physical activity, alcohol, caffeine), biochemical determinants (serum folate, plasma B_6, serum B_{12} and RBC folate) in a group of women aged 18-25 y.

The overall objective of this study is to develop and validate a rapid assessment tool for dietary folate that could be used in health promotion research and epidemiological studies. The thesis will advance the scientific understanding of the dietary assessment process, validation of new dietary assessment tool, implication of folate fortification in the status for women at childbearing age. The following literature review (chapter 2) will provide an overview of folate physiological/chemistry/role in disease prevention, dietary assessment methods, and approaches used to validate the nutrient data as estimated by different dietary assessment methods, folate intakes for women at childbearing age after fortification, and the implication for folate fortification in folate, vitamin B_{12} and tHcy status.

1.2 References

1. Melse-Boonstra A, de Bree A, Verhoef P, Bjorke-Monsen AL, Verschuren WMM. Dietary monoglutamate and polyglutamate folate are associated with plasma folate concentrations in dutch men and women aged 20-65 years. J Nutr 2002;132:1307-1312.
2. Kim Y-I. Role of folate in colon cancer development and progression. J Nutr 2003;133:3731S-3739.
3. Czeizel AE. Prevention of the first occurrence of neural-tube defects by periconceptional vitamin. N Engl J Med 1992;327:1832-1835.
4. George L, Mills JL, Johansson AL, et al. plasma folate levels and risk of spontaneous abortion. JAMA. 2002;16:1867-1873.
5. Czeizel AE. The primary prevention of birth defects: Multivitamins or folic acid? Int J Med Sci 2004;1:50-61.
6. Fairfield K, Fletcher R. Vitamins for chronic disease prevention in adults. JAMA 2002;287:3116-3126.
7. El-Khairy L, Vollset SE, Refsum H, Ueland PM. Plasma total cysteine, mortality, and cardiovascular disease hospitalizations: the Hordaland homocysteine study. Clin Chem 2003;49:895-900.
8. Kim Y. folate and carcinogenesis: evidence, mechanisms, and implications. J Nutr Biochem 1999;10:66-88.
9. Choi SW, Mason JB. Folate Status: Effects on Pathways of Colorectal Carcinogenesis. J Nutr 2002;132:2413S-2418.

10. Bjelland I, Tell GS, Vollset SE, Refsum H, PM. U. Folate, vitamin B12, homocysteine, and the MTHFR 677C->T polymorphism in anxiety and depression: the Hordaland Homocysteine Study. Arch Gen Psychiatry. 2003;60:618-626.

11. Gjesdal CG, Vollset SE, Ueland PM, et al. Plasma total homocysteine level and bone mineral density: the Hordaland homocysteine study. Arch Intern Med 2006;166:88-94.

12. Refsum H, Nurk E, Smith AD, et al. The Hordaland homocysteine study: a community-based study of homocysteine, its determinants, and associations with disease. J Nutr 2006;136:1731S-1740.

13. Hickling S, Knuiman M, Jamrozik K, Hung J. A rapid dietary assessment tool to determine intake of folate was developed and validated. J Clin Epidemiol 2005;58:802-808.

14. Pufulete M, Emery P, Nelson M, Sanders T. Validation of a short food frequency questionnaire to assess folate intake. Br J Nutr 2002;87:383-390.

15. Sauberlich H, Kretsch M, Skala J, Johnson H, Taylor P. Folate requirement and metabolism in nonpregnant women. Am J Clin Nutr 1987;46:1016-1028.

16. Subar A, Block G, James L. Folate intake and food sources in the US population. Am J Clin Nutr 1989;53:508-516.

17. Food and Drug Administration. Foods standards: amendment of standards of identitiy for enriched grain products to require addition of folic acid. 1996,61:8781-8797.

18. Canada. Gazette. Regulatory impact analysis statement, SOR/98-550,132:3029-3033. 1998.

19. Boushey CJ, Edmonds JW, Welshimer KJ. Estimates of the effects of folic-acid fortification and folic-acid bioavailability for women. Nutrition 2001;17:873-879.

20. Quinlivan EP, Gregory JF, III. Effect of food fortification on folic acid intake in the United States. Am J Clin Nutr 2003;77:221-225.

21. Institute of Medicine. Dietary reference intakes: folate, other B vitamin and choline. Washington, DC: National Academy Press, 1998.

22. Gates GE, Holmes TW. Folate intake and supplement use in women of childbearing age. Family Economics and Nutrition Review 1999;12:14-25.

23. French MR, Barr SI, Levy-Milne R. Folate intakes and awareness of folate to prevent neural tube defects: A survey of women living in Vancouver, Canada. J Am Diet Assoc 2003;103:181-185.

24. Caudill MA, Le T, Moonie SA, Esfahani ST, Cogger EA. Folate status in women of childbearing age residing in southern California after folic acid fortification. J Am Coll Nutr 2001;20:129-134.

25. Institute of Medicine. Dietary reference intakes: for thiamin, riboflavin, niacin, vitamin B6, folate, vitamin B12, pantothenic acid, biotin. Washington, DC: National Academy Press., 2000.

26. El-Khairy L, Vollset SE, Refsum H, Ueland PM. Plasma total cysteine, pregnancy complications, and adverse pregnancy outcomes: the Hordaland Homocysteine Study. Am J Clin Nutr 2003;77:467-472.

27. Holmes VA. Changes in haemostasis during normal pregnancy: does homocysteine play a role in maintaining homeostasis? Proc Nutr Soc 2003;62:479-493.

28. Tamura T, Picciano MF. Folate and human reproduction. Am J Clin Nutr 2006;83:993-1016.

29. Herrmann M, Muller S, Kindermann I, et al. Plasma B vitamins and their relation to the severity of chronic heart failure. Am J Clin Nutr 2007;85:117-123.

30. Homocysteine Studies Collaboration. Homocysteine and risk of ischemic heart disease and stroke: A meta-analysis. JAMA 2002;288:2015-2022.

31. Refsum H, Smith AD, Ueland PM, et al. Facts and recommendations about total homocysteine determinations: An expert opinion. Clin Chem 2004;50:3-32.

32. Boushey CJ, Beresford SA, Omenn GS, AG. M. A quantitative assessment of plasma homocysteine as a risk factor for vascular disease. Probable benefits of increasing folic acid intakes. JAMA 1995;13:1049-57.

CHAPTER 2

2. LITERATURE REVIEW

2.1 Folate

2.1.1 Biochemistry of Folates

Dietary folates consist mainly of folylpolyglutamates, which contain from four to seven residues of glutamic acid (1). The folates may bear a formyl group, a methyl group, or a 1-carbon group. The pteridine ring of folate in foods occurs mainly in the reduced tetrahydro-form. Other forms of pteridine include the dihydro-and non-reduced forms (2). Folypolyglutamates do not readily cross cell membranes and are poorly transported by gut, liver, and other cells (3).

2.1.2 Folate Digestion

Naturally occurring folypolyglutamates must undergo enzymatic deconjugation in the small intestine prior to absorption. This reaction is catalyzed primarily by a pteroypolyglutamate hydrolase associated with the jejunal brush border membrane, with possibly some contribution by hydrolase activity from pancreatic secretions (4). The absorption of monoglutamyl folates occurs via a saturable transport process with an acidic pH optimum (Km =1-3 μmol/L). Additionally, there exists an apparently non-saturable absorption mechanism that functions when folate concentrations in intestinal contents exceed 5-10 μmol/L. The pH optimum for folate transport is ~ 5, whereas the pH optimum of enzymatic deconjugation is ~ 6-7 (4).

Nutrient bioavailability is defined as the proportion of a nutrient ingested that becomes available to the body for metabolic processes or storage. The amount of folate absorbed by individual subjects can vary considerably depending on several factors, including folate status: in subjects with low folate status, folate is transported from

plasma to the cell more rapidly and lower amounts of folate are excreted with the urine (5). The rate of folate absorption might be influenced by pathological conditions (malabsorption) or physiologic conditions such as growth, pregnancy and lactation (6). Other factors with an effect on absorption may include the food matrix, pH value at the jejunal mucosal surface, intestinal transit time or inhibition of pteroylpolyglutamate hydrolase activity by unknown food components (7).

2.1.3 Folate Bioavailability

Folate exists in a number of different chemical forms, which can occur naturally in food (concentrated in dark green leafy vegetables, orange juice, legumes) or as FA, which is the synthetic form of the vitamin used in fortified foods and supplements (8). FA differs chemically from endogenous food folate in that there is only one glutamic acid attached to the p-aminobenzoic acid pteridine ring unit and it is the most oxidized and stable form of the vitamin (9). Naturally occurring folates are pteroylpolyglutamates, which contain one to six additional glutamate molecules joined in peptide linkage as explained above. The polyglutamate side chain of food folate must be cleaved before absorption can occur. This converts food folate to the monoglutamate form, which is taken up by a specific carrier located in the membrane of small intestinal enterocytes (10). When synthetic FA is consumed, the vitamin does not require removal of the polyglutamate side chain prior to absorption as food folate does. This fact may explain the large body of evidence that supports the conclusion that synthetic FA is significantly more bio-available than the polyglutamated forms of food folate (11)

The term Dietary Folate Equivalent (DFE) was derived by the IOM committee and used to convert all forms of dietary folate, including synthetic FA in fortified foods,

to an amount that is equivalent to food folate (12). When synthetic FA (a monoglutamate) is consumed as a supplement without food, it is nearly 100% bioavailable (13). In contrast, when FA is consumed with food, as is always the case with fortified cereal-grain products, its absorption is reduced by a small percentage and the approximate bioavailability is 85% (14). It was estimated that FA in fortified products or taken with food is 85/50 or 1.7 times more bio-available than food folate (15). The DFE for mixed diet would therefore be calculated as [μg food folate + (1.7 * μg synthetic FA].

2.1.4 Folates in Blood Stream

Because folates pass inefficiently through biological membranes, the movement of folates through mammalian cell plasma membrane must occur by a mediated process (10). Reduced-folate carrier (RFC; additionally known as FRC-1, FOLT, RFT-1 or SLC19A1) (MR 60 kDa) is a typical transport protein with 12 membrane-spanning domains. The RFC preferentially transports reduced folates, such as 5-methytetrahydrofolate, the most common form of circulating folate. In adult tissues, RFC is expressed in the brush-border membrane of jejunum, ileum, duodenum and colon and in the basolateral membrane of renal tubular epithelium, hepatocytes, choroid plexus and the retinal pigment epithelium (RPE) of the eye (16).

2.1.5 Physiological Functions of Folate in the Cell

Serum folates contain a single glutamate residue; intracellular folates contain a polyglutamate peptide usually consisting of 5-8 glutamate residues that are polymerized through unusual γ-linked peptide bonds. The polyglutamate moiety increases the affinity

of folate for folate-dependent enzyme, aids in sequestering folate within the cell, and may serve as a swing arm that permits metabolic channeling of the cofactor among folate-dependent enzymes (2, 10).

Folate polyglutamates generally act as coenzymes that donate or accept one-carbon units in a set of reactions referred to as one-carbon metabolism, a process that occurs in both the mitochondria and cytoplasm. One-carbon metabolism in the cytoplasm is necessary for the de novo synthesis of purines and thymidylate, and for the remethylation of homocysteine to methionine (6). Methionine can be adenylated to form S-adenosylmethionine (SAM), which is a cofactor and one-carbon donor for numerous other methylation reactions (6). Increasing evidence suggests that the primary role of mitochondrial one-carbon metabolism is to generate glycine and formate from serine. Mitochondria-derived formate traverses to the cytoplasm where it is a major source of one-carbon units for cytoplasmic one-carbon metabolism (6).

Most cellular folate is compartmentalized, with up to half of the folate residing in the mitochondria and the remainder in the cytoplasm. Minor contributors to intracellular folate stores include other organelles, including the nucleus, although this folate does not make significant contributions to total cellular folate concentrations. Several studies have indicated that most cellular folate is protein bound (6, 10).

2.1.6 Folate Catabolism and Excretion

The major route of folate turnover is by catabolism and cleavage of the C9-N10 bond, producing pterdines and para-aminobenzoylglutamate (pABG), which is N-acetylated before excretion (ApABG) (6). Humans turn over approximately 1% of total body folate per day, and this turnover has been assumed to be due to non-enzymatic

degradation of labile folate cofactors (10). Once the cleavage reaction occurs, folate is no longer available to serve as a metabolic cofactor (10). Because folate catabolism represents an irreversible loss of folate, the urinary excretion of folate catabolites has been proposed to be a minimal indicator of folate requirements (17, 18). Caudill and colleagues (18) designed a study to determine if pregnant women in a controlled metabolic study excreted higher quantities of urinary folate catabolites than non-pregnant controls and if catabolite excretion was influenced by folate intake. They found that second trimester pregnant women do not excrete more folate catabolites than non-pregnant controls and that consumption of 450 vs. 850 μg folate/d results in a significant reduction in the quantity of folate catabolites excreted. Factors limiting the accuracy of the estimation of folate requirements depending on measuring ApABG are, based on catabolite excretion is the variable and potentially large fecal excretion of folates from endogenous pool. Another uncertainty is the fact that the excretion of folate catabolites can not be adequately interpreted without considering the potential absorption and fate of pABG formed through the breakdown of dietary folate. Food processing and storage inevitably leads to some loss of folates, at least some of which occurs through cleavage of the C9-N10 bond. It also some has been reported that folate degradation, probably through C9-N10 bond cleavage, also can occur under the conditions of the gastrointestinal tract before absorption (19).

2.2 Risk Factors for Folates Deficiency

Inadequate folate status has been reported in many population groups (20). Clinically, impaired folate status can be associated with gastrointestinal disorders, smoking, alcohol consumption, and antiepileptic drug use (21). In addition, certain

dietary factors can interfere with folate bioavailability (22). Folate deficiency is common during pregnancy (23).

2.2.1 Effect of Dietary Factors on Folate Status

Food folate is a mixture of many derivatives that differ by such factors as oxidation state, one-carbon substitution on the pteridine ring, and the number of attached glutamate residues, as previously mentioned. These differences are associated with different physiochemical properties which, together with certain food constituents, could influence folate bioavailability. One important factor that can influence folate bioavailability is folate stability: Reduced folates, those without any carbon substitution, (i.e. dihydropteroyglutamates, tetrahydropteroylglutamates), generally are the most unstable, followed by 5-methyltetrahydropteroylglutamates, which exhibit intermediate stability (7). On the basis of *in vitro* observations, it appears that low molecular mass organic acids in foods constitute a significant factor that can affect the efficiency of utilization of dietary polyglutamyl folates (8). A folate bioavailability index was developed to reflect the properties of folates in various foods as they pertain to distribution, stability and degree of pteroylpolyglutamation. It was found that this index of folate bioavailability varied widely among foods. The highest index was for egg yolk (72.2%), followed by cow's liver (55.7), orange juice (21.3%), cabbage (6.0%), lima beans (4.5%) and lettuce (2.9%). Yeast folate had the lowest index (0.3%) (7). This bioavailability index has not been rigorously tested in vivo, in either human or animal studies.

Exposure to air, oxidation, heat, and most importantly, the acidic-peptic milieu of the stomach increases folate losses (2, 7). Food preparation can also be a major

contributor to folate loss as the vitamin can be destroyed upon exposure to heat and losses from food can result due to extraction into the cooking water (2).

2.2.2 Genetic Factors

Although folate intake is a key factor in determining folate status, folate concentration depends on gene-nutrient interactions (24). The C-to-T substitution at nucleotide 677 (677C→T mutation) in the gene encoding 5,10-methylenetetrahydrofolate reductase (MTHFR) produces a partially defective enzyme (25). MTFFR activity regulates 5, 10-methylenetetrahydrofolate availability, which is required for the remethylation of homocysteine (26). TT homozygotes have elevated homocysteine concentrations at low folate intakes and may have a greater requirement for folate than do persons with other geno-types (24). The prevalence of this variant is relatively high in the general population; the percentage of individuals homozygous for the 677C→T mutation ranges between 14% and 18% among whites but is considerably lower in African American (on the order of 0-2%) (27). Beer et al (2003) found that at any folate intake level, TT subjects have lower plasma folate concentrations than do CT and CC subjects (25). Folate was significantly lower in the older individuals (≥ 10 years) who were homozygous for the mutation (T/T) than in those who were homozygous for the wild-type allele (C/C). Homocysteine was higher in the T/T group than in both C/C and C/T subgroups aged > 10 y (28). Many other genes involved in folate metabolism are polymorphic such as methionine synthase (*MTR*), methionine synthase reductase (*MTRR*), cystathionine ß-synthase (*CBS*), and thymidylate synthase (*TS*) (29). Other gene polymorphism occur in encoding folate carriers: A $G_{80}A$ polymorphism has been described in the reduced folate carrier gene (*RFC1*) (30)

2.2.3 Alcohol Consumption

Alcohol is known as a folate-antagonist because it inhibits folate absorption and promotes its excretion and catabolism (31). Chronic alcoholism is known to interfere with the enzyme involved in one-carbon metabolism, for which folate serves as coenzyme (32). Correspondingly, decreased blood folate levels and increased blood homocysteine levels have been observed in alcoholism. Cravo and colleagues found that in chronic alcoholism serum and RBC folate concentrations were significantly lower than in the control subjects (32). Studies in man, monkeys and pigs indicate that chronic alcohol ingestion reduces the intestinal absorption of both dietary polyglutamylated folate and its monoglutamatyl FA derivatives (33).

2.2.4 Pregnancy

Pregnancy is associated with a marked acceleration in one-carbon transfer reactions, including those required for nucleotide synthesis and thus cell division, and this in turn provides an explanation for the substantial increase in folate requirements during pregnancy (34). The increase in cell division is associated with the rapidly growing fetus and placenta in addition to an expansion of the number of maternal red cells and size of the reproductive organs. Folate is actively transferred to the fetus during gestation, as evidenced by the higher concentrations of folate during pregnancy for the synthesis of DNA and other one-carbon transfer reactions. Pregnant women are at a higher risk of developing folate deficiency than non-pregnant women (2). It is therefore important to ensure adequate fetal intake during pregnancy to prevent maternal folate depletion, which would thus allow for adequate fetal supplies for growth and development (6, 34). Bonnette et al (1998) conducted a metabolic study in which folate

status was monitored for 12 weeks in second trimester pregnant women and non-pregnant controls who consumed one of two quantities of folate. A folate intake of 600 μg DFE/d was sufficient to maintain both RBC folate concentrations and serum folate concentrations that did not differ from those of non-pregnant controls (35). The conclusion of this study was consistent with the findings from population studies that 600 μg DFE/d is adequate to maintain normal folate status in pregnant women. Brown and his colleagues found that supplementation of diets of women of childbearing potential with 400 μg of FA per day would effectively raise RBC folate to levels associated with a low risk of folate-responsive NTDs (36). They concluded that the efforts to increase FA supplement use and folate consumption among women of childbearing potential must go beyond fortification of refined cereal and grain products and reach women within all educational and income groups.

2.2.5 Vitamin B_{12} Deficiency

Vitamin B_{12} deficiency can induce a deficiency in folate even if folate intake is adequate. In what has been come to be known as the "methyl trap hypothesis", folate deficiency arises due to the fact that vitamin B_{12} is required for the conversion of folate from a form that has limited use (5-methyl-H_4folate) to a form (H_4folate) that can be readily assimilated and used in a variety of reactions (2). Vitamin B_{12} is the cofactor of methionine synthase, the enzyme required for conversion of 5-methyl-H_4folate to H_4folate. The metabolism of folate and that of vitamin B_{12} are, in part, intimately related. Because of this relationship, the symptoms of deficiencies for both of these vitamins are shared (37).

2.3 The Role of Folate in Health and Diseases

2.3.1 Neural Tube Defects

NTDs are a family of birth defects, involving the brain or spinal cord, which arise from mistakes made early in the development of the embryo. The neural tube is a part of the developing human embryo. It begins to form at 18 days after conception. The neural tube seals off and closes, thus forming a tube, by the 30^{th} day after conception. During the second and third week after conception, when the human embryo is 2-3 mm long, a flat structure called the neural plate forms two parallel ridges. These ridges fold over and move towards each other and form a tube (the neural tube) (2). Any drug, nutrient imbalance, or genetic defect that interferes with this stage of development may prevent normal closure of the tube, resulting in defects in the newborn. The most common NTDs are spina bifida (spinal cord at the lumbar vertebra not covered with bone), anencephaly (no brain), and encephalocele (tissue protruding through a hole in the skull) (2, 38). NTDs result in congenital malformations of the nervous system and may lead to spontaneous abortion, stillbirth, death in early infancy or a lifetime of disability (39).

The lifetime medical and financial costs of a patient with spina bifida are very high, and the effect on the family is incalculable (40). NTDs account for 13.5% of infant death in United States and accrue approximately $489 million in national lifetime costs (41).

The link between maternal diet, particularly a lack of folate, and the occurrence of NTDs was first suggested in 1965 by Hibbard and Smithells (42). Accordingly, several observational and intervention studies conducted over the past 40 year have in

general supported a positive association between peri-conceptional use of FA from dietary supplements and a reduced NTD risk. For example, the Medical Research Council (MRC) trial demonstrated a reduction in the risk of recurrence of NTDs in women who had a previous NTD birth from 5 % to 0.6% following vitamin supplementation, an 86% reduction in risk (43). In 1992, Czeizel and Dundas, in a large double-blind randomized controlled trial demonstrated a significant reduction in risk of first occurrence of NTD in women with no previous history of NTD using peri-conceptional FA supplement (approximately 95% of all NTD births occur to women with no previous history of NTD) (44). Since then, extensive research has been conducted and the efficacy of FA in the prevention of NTDs has been ascertained. According to those studies, the amount of FA needed to prevent NTDs was far beyond those needed to prevent classic folate-deficiency syndromes and the effective vehicle was nearly always supplemental FA (pteroylglutamic acid), not folate occurring naturally in food (pteroylpolyglutamic acid (45). It is now accepted beyond any scientific doubt that FA protects against both first occurrence and recurrence of NTDs such as spina bifida or anencephaly. Adequate peri-conceptional intake of this vitamin by women has been shown to lower the risk of disturbances in the early stage of embryonic development (46).

In a population-based retrospective study designed to study all live births, still births and terminated pregnancies in which open NTDs had occurred in Nova Scotia in Canada from Jan. 1, 1991, to Dec. 31, 2000. It was found that the incidence of open NTDs decreased by more than 50%: the mean annual rate was 2.58 per 1000 births during 1991-1997 and 1.17 per 1000 births during 1998-2000 after fortification (40).

In 1992 the US public health service issued a recommendation that all US reproductive-aged women who are capable of becoming pregnant should consume additional FA (400 μg/day) before conception and during the first 12 weeks of pregnancy by eating more folate-rich food and taking a daily supplement of FA (47). Although the precise biological mechanism of FA's protective effect against NTDs is unknown, various hypotheses have been investigated. For example, marginal maternal folate status leads to insufficient products of folate metabolism (as thymidylate) that are needed for rapidly dividing embryonic cells, such as neutral tissue (45). Alternatively, elevated plasma tHcy, which is inversely related to plasma and RBC folate, may be embryotoxic (48).

2.3.2 Spontaneous Abortions

Spontaneous abortions occur in approximately 40% of pregnancies (49). Recurrent early pregnancy loss is a multifactorial disease, and the causes for some of these abortions are still unknown (49). It was found that pregnant women who have low blood folate levels are more likely to have early miscarriages than are pregnant women who have adequate folate levels. In a case-control study, it was found that women with recurrent early pregnancy losses had significantly lower serum folate concentrations than controls (50). In case report and retrospective analysis of hyperhomocysteinemia prevalence in 100 consecutive women with recurrent miscarriages, they found that 12 of 100 patients were hyperhomocysteinemic. Twenty percent had the C677T methylene tetrahydrofolate reductase (gene mutation) genotype and 15% had low plasma folate concentrations. FA administration normalized the homocysteine concentration and favored a successful pregnancy (Quere et al 1998). George and his colleagues found that

women with low folate levels (≤ 2.19 ng/ml) were at increased risk of spontaneous abortion, whereas women with higher folate levels (3.96- 6.16 ng/ml) showed no increased risk of spontaneous abortion (51). All these results lead to the conclusion that elevated tHcy and reduced serum folate concentrations may serve as risk factors for recurrent spontaneous early pregnancy losses. FA supplementation might be beneficial in women with a history of early pregnancy loss.

2.3.3 Total Plasma Homocysteine and Cardiovascular Disease

Homocysteine is a sulphur-containing amino acid formed from the indispensable amino acid methionine. Disturbances of homocysteine metabolism leading to hyperhomocysteinemia can result from inherited or nutritional disorders (52-54), as well as from various metabolic diseases, such as chronic renal disease and hypothyroidism (55, 56). Lifestyle factors such as smoking, alcohol use, caffeine use, body mass index, blood pressure, and antihypertensive medication can be considered as determinants of tHcy status (57).

High concentrations of plasma tHcy are associated with serious pregnancy complications, including pregnancy-induced hypertension, pre-eclampsia, and placental abruption (58), psychiatric disorders, cognitive impairment in the elderly (22, 57) and adverse pregnancy outcomes such as NTDs (57). Furthermore, an elevated plasma tHcy concentration is regarded as an independent risk factor for stroke, coronary and peripheral artery disease (58, 59). For each 5 μmol/L increment in plasma tHcy (approximately 1 standard deviation of the mean for the normal population), there is a corresponding increase of about 40% in the relative risk of developing coronary heart disease (CHD)(60). This increase is comparable to the risk associated with the same

proportional increment in cholesterol and equates homocysteine and cholesterol as risk factors for ischemic heart disease (60).

Normal plasma tHcy levels in the fasting state are in the range of 10 μM.(61). Mildly elevated levels (15-33 μM) have been found in 5-7% of the population (62). Approximately two-thirds of the cases of elevated tHcy have been estimated to be due to a low or moderate concentration of B_6, B_{12} and/or folate vitamins, of which folate is considered the most important (56, 63)

Rimm and his colleagues (64) have suggested that the intake of folate and vitamin B_6 above the current recommended dietary allowance may be important in the primary prevention of CHD among women. Lobo et al (1999) found that FA reduces plasma tHcy by 25% and is maximally achieved by doses of 0.4-0.5 mg daily (65). Other researchers found that grain fortification with FA was predicted to decrease CHD events by 8% in women and 13% in men, with comparable reductions in CHD mortality (66). In studying associations of dietary intake of folate, vitamin B_6, and vitamin B_{12} with the risk of acute coronary events, it was found that a significant inverse association was apparent between quantitative moderate-to-high folate intakes and the incidence of acute coronary events in men, vitamin B_{12} yielded a weak association with respect to a reduced risk of acute coronary events, and a high dietary intake of vitamin B_6 had no significant association with adverse coronary events (57). Venn et al (2002) (67) found that increasing dietary folate from 263 to 618 μg/d improved folate status and decrease tHcy from 12.0 to11.3 μmol/l during 4 weeks. They also found further decreases during follow-up studies, with a final tHcy concentration of 9.7 μmol/l.

The above-mentioned associations have led to the hypothesis that folate treatment may reduce cardiovascular risk by reducing tHcy. Although the roles that

homocysteine plays in artherogenesis, atherosclerosis, and thrombosis are unknown, several recent studies suggest that hyperhomocysteinemia can disrupt functions of the vascular endothelium that may lead to conversion of its usually anticoagulant surface to one that is procoagulant (52). It has also been hypothesized that homocysteine alters endothelial and smooth muscle cell function by generating reactive oxygen species (68). The suggested relationship of tHcy concentrations with vascular disease risk remains controversial (69, 70). Research data suggest that FA improves endothelial function in coronary artery disease acutely be a mechanism largely independent of homocysteine (69).

2.3.4 Cancer

Evidence from several different types of research, including epidemiological studies, research with animal models and clinical intervention studies, has increasingly supported the concept that diminished folate status may predispose to the development of several common cancers. Conversely, this body of literature also suggests that the habitual ingestion of folate at concentrations that are somewhat above present recommendations may be cancer preventative (71, 72). Such associations with folate status have been described for cancers of the colorectum, lungs, pancreas, esophagus, stomach, cervix, colon, and breast, neuroblastoma, and leukemia (73-76).

Currently, it is believed that folate deficiency affects DNA stability principally through two pathways. The first pathway is through altered DNA methylation. Folate, in the form of 5-methyltetrahydrofolate (5-methyl THF), serves as methyl donor in the remethylation of homocysteine to methionine, which in turn is converted to S-adenosylmethionine (SAM). SAM methylates specific cytosines in DNA, and this

regulates gene transcription (75). As a consequence of folate deficiency, cellular SAM is depleted, which in turn induces DNA hypomethylation and potentially induces proto-oncogene expression leading to cancer (75). The second pathway through which folate may alter DNA stability has received less attention. Folate, as 5, 10 methylene THF, donates a methyl group to uracil converting it to thymine, which is used for DNA synthesis and repair. However, if folate is limiting, uracil misincorporation into DNA may occur. This in itself is mutagenic, but DNA instability may occur via a different mechanism. As the cell attempts to repair itself, it breaks the DNA molecule to excise the uracil. If folate is continually limited, imbalances in deoxynucleotide triphosphates in the precursor pool occur. Uracil is misincorporated and repaired in what is a term "a catastrophic repair cycle," which may lead to double-strand breaks, chromosomal damage and cancer (74, 75).

Kim and his colleagues (77) found that increasing dietary folate up to four times the basal requirement led to a progressive reduction in the evolution of macroscopic neoplasms from microscopic foci, although this benefit appeared to be attenuated at higher intakes. In a study done to test the hypothesis that higher folate intake might reduce risk of breast cancer, particularly among the women with greater alcohol consumption, it was found that the risk of breast cancer associated with alcohol intake was strongest among women with total folate intake of less than 300 μg/d. In the group that consumed a FA supplement, a lower breast cancer risk was observed among women who consumed at least 15 g/d of alcohol (78). In a community-based case-control study, blood samples were analyzed for 183 cases of cervical cancer and 540 controls. The researchers found that low blood folate was moderately and consistently associated with an increased risk of invasive cervical cancer (79). Results of previous epidemiological

studies suggested a link between FA and other childhood cancers (80). French and colleagues (81) found that FA fortification was associated with a 60% reduction in neuroblastoma, an embryonic tumor.

2.3.5 Depression

Major depressive disorder is very common, with a lifetime prevalence of ≈ 17% and an occurrence rate almost twice as high in women as in men (82). Over the past 25 years several surveys have shown a high incidence of folate deficiency in psychiatric populations, especially in those with depression (83, 84). Between 10% and 30% of depressed patients may have low folate concentrations, and these patients appear to respond less well to antidepressants (82, 83).

Folate, vitamin B_{12}, and homocysteine are involved in processes important for central nervous system function. Folate metabolism is linked to biopterin dependent neurotransmitter synthesis and S-adenosylmethionine (SAMe)-dependent methylation of biogenic amines and phospholipids in the central nervous system (85). Homocysteine, or its metabolites such as homocysteic acid, may have a direct excitotoxic effect on the N-methyl-d-asparate glutamate receptors, or may inhibit the methylation processes in the central nervous system (85). SAMe is synthesized as part of a multi step pathway involving the vitamins FA and B_{12}. The end product then donates methyl groups in the reactions involved in the synthesis of the key neurotransmitters serotonin, norepinephrine, and dopamine (85). Deficiencies of these neurotransmitters are thought to be involved in the development of depression and other mood disorders. It has been postulated that exogenous addition of SAMe may result in increased synthesis of these neurotransmitters, which may account for its antidepressant effect (82).

Fava and colleagues (86) examined the relationships between levels of three metabolites (folate, vitamin B_{12}, and homocysteine) and both depressive subtype and response to luoxetine treatment in depressed patients. They found that subjects with low folate levels were more likely to have melancholic depression and were significantly less likely to respond to fluoxetine. They found that the level of folate deficiency has also been related to the severity and duration of depression. It was found that hyper-homocysteinemia, vitamin B_{12}, and to a lesser extent, folate deficiency were all related to depressive disorders (87). Moreover, in this study, researchers found that folate but not vitamin B_{12} deficiency was related to loss of appetite.

2.3.6 Alzheimer's Disease

Elevated tHcy levels are considered a risk factor for cerebrovascular disease and may also play an important role in the pathogenesis of Alzheimer's disease (AD). High values of plasma tHcy and low levels of vitamin B_{12} and folate are frequently present in AD patients (88). Clarke et al (89) in a case control study, found that persons ranked in the highest tertile of plasma tHcy had an odds ratio of 4.5 for AD compared to persons in the lowest tertile. High odds ratios were also observed for those participants ranked in the lowest tertile for plasma folate and vitamin B_{12} levels compared to those in the highest tertile. Mizrahi et al (90) found that AD patients consumed significantly less dietary vitamin B_6 ($P= 0.05$) and folate ($p= 0.001$) after age 60 than controls (91). It is believed that the sulphur amino acid homocysteine is a unique candidate for this role because of it its direct neurotoxicity and its association with cerebrovascular diseases

2.4 Dietary Assessment Methods

Significant attention has been focused on the role of folate in maintaining health. Estimates of dietary intake of folate are currently a topic of considerable interest (92), but no rapid validated comprehensive tools are available to assess dietary intakes that are well suited to every health promotion activities and clinical intervention research.

Dietary assessment methods are very important in nutrition research. Since early in this century, researchers have tried to develop tools that could be used to evaluate the food intake of individuals (93). Underreporting of food consumption is a constant problem in dietary intake studies and all methods are subject to this problem to some degree (94-96). To minimize underreporting of foods, subjects are usually using non-biasing food models for portion size estimates, or cup and spoon measures (97).

Two groups of methods are used to measure the food consumption of individuals. The first group, known as quantitative, prospective daily consumption methods, consist of records designed to measure the quantity of the individual foods consumed over a 1 day period (93) and these include weighed and estimated records. Related to this group is the 24-hour recall interview which is a quantitative method, but retrospective. By increasing the number of days assessed, quantitative estimates of the usual intakes of individuals can be obtained, using the same methods (93). The second group of methods includes the dietary history and the food frequency questionnaire (FFQ). Both obtain retrospective information on the patterns of food use during a longer, less precisely defined time period (93). Such methods can be used to assess the usual intake of foods or specific classes of foods. With modifications, they can also provide data on usual nutrient intakes (93).

The problems associated with the assessment of habitual food intake are well documented (98). Weighed food records require high subject motivation, training and

lengthy data handling, while diet histories and 24-h recalls are prone to interview bias, need skilled interviewers and can be costly for large studies (98). Owing to their ease of administration and low burden on the subject, the assessment of dietary intake in epidemiological studies is often assessed by means of an FFQ (99). The main limitation of the FFQ is the prescribed set of foods, which needs to be long enough to allow respondents to represent their diets and short enough to avoid a loss of subject motivation and decreased reliability (98). The FFQs need to be validated for each population.

There are few rapid tools currently available to assess dietary intake of folate. Yen and associates (100) described the performance characteristics and validated a folate-specific focused approach to estimating folate intake. They used a focused dietary recall summary to assess folate intake for 28 women aged 21 to 47 years via telephone and/or email. After seven days of recalls were completed, subjects were asked to complete the FFQ. They found that the focused dietary recall approach is a useful method for collecting information on folate (only one nutrient) intake in women.

Hickling and colleagues in 2005 (92) developed folate intake tool (FIT) to determine dietary intake of folate in adults. The tool development involved i) forming a list of key food items related to dietary intake of folate, ii) selecting frequency response categories, iii) considering serving size of food; and iv) calculating nutrient score. They used the tool to estimate the folate for 568 men and women aged 33-83 years. They found that the folate intake tool provides a valid and reliable measurement of dietary intake of folte for both men and women.

Clifford and colleagues (101) used the semi-quantitative Block DFEs screener to estimate folate intake for 68 women who were at least 18 y old. The 1-page folate-

targets food/supplement intake screener was designed to measure usual and customary intake of DFEs. The questions on the screener were identified by examining the food records reported by individual's ≥ 17 y old in NHANES 1999-2000. Nineteen questions representing foods contributing the top 60% of US DFEs intake were included in the Block DFE screener. They found that RBC folate correlated with total folate intake using a folate-targeted food/supplement screener. (101).

2.4.1 Validation of Dietary Assessment Methods

Accurate and objective estimates of dietary intakes are necessary to assess any effects of nutritional status in epidemiological studies (102). The validation of dietary assessment tools is complicated by the lack of an absolute standard against which the tools can be compared (92). Validity describes the degree to which a dietary method measures what it is intended to measure (103). Dietary methods designed to characterize usual intakes of individuals are the most difficult to validate because the 'truth' is never known with absolute certainty (104) . However, relative validity can be assessed on dietary methods covering both short- and long-term time frames. Relative validity can be defined as the comparison of the "test" method with another method, termed the "reference" method, which has a greater degree of demonstrated validity (105).

Traditionally, the validity coefficient (VC) between the new dietary assessment tools data and the unknown 'true' habitual intake is assessed by the correlation between the intakes achieved by the new tools and the mean value obtained from reference methods (92). However, the correlation between the reference method and the new method may be overestimated, if the sources of errors in these two methods are related (92, 102, 106, 107). Flood et al (108) in their discussion of the issues in assessing the

validity of nutrient data obtained from a FFQ stated that the correlation coefficient should not be used alone to assess the validity of nutrient data.

Using biomarkers of nutritional intake is increasingly important in dietary validation studies (102, 106). Nutritional biomarkers are objective measures of exposure to a certain nutrient and they are not influenced by factors related to misreporting. Therefore, Kaaks et al (109) described that at least two additional measurements are necessary to determine the VC of the method under question, for instance, nutritional biomarkers and dietary records. The VC is the correlation between the dietary intake reported by the method under question and the unknown 'true' dietary intake (109, 110). The method of triads is a triangular approach that uses the correlation between each of the three methods to estimate the VC. Moreover, this approach corrects for bias from the reference method (111). Several studies have used the method of triads to validate food frequency questionnaires (98, 107, 112)

Designing validity studies is difficult. Several factors must be taken into account in the design of such a validation study, including selection of the subjects, age and sex, study objective and time frame, sequence and spacing of test and reference method, socioeconomic and health status, and other factors (93, 113).

Published data of validation of micro-nutrient estimates in the literature using the method of triads are scarce. In a recent study that validated folate and vitamin B_{12} intake carried out with 53 women of reproductive age, a FFQ was filled first (test method) and immediately thereafter, a blood sample was taken to determine serum folate and vitamin B_{12}, followed by three dietary 24-h recalls which were completed during a period of three successive weeks and used as the reference method. The VCs were 0.97 for folate

when serum folate used and 0.75 when RBC folate used and 1 for vitamin B_{12} when serum B_{12} was used (102).

Pufulete and colleagues (98) conducted a validation of a short FFQ to assess folate intake, using a 7-d weighed record (7-dWR) as a reference method in a group of 39 men and women. They found for both women and men, VC for the FFQ were higher than for the 7-d WR when serum folate was the biomarker (FFQ: VC= 0.85 for men and 0.69 for women; 7-dWR VC 0.81 for men and 0.44 for women). Lower VCs were reported when RBC folate used.

In addition to the VC, several researchers have used other methods to validate dietary intakes. Green and colleagues validated a 3-d weighed food record (3-d WFR) and a semi-quantitative FFQ for assessing folate and vitamin B_{12} intakes in 105 women aged 16-19 years. They compared the adjusted and absolute mean intake estimates for the nutrients by the 2 different dietary assessment methods and these nutrient intake estimates with serum, RBC folate and serum vitamin B_{12} concentration by calculating the correlation coefficients and the degree of classification (114). Correlation coefficients between folate intakes and serum folate have been used to validate the FFQ in several studies (98, 115).

Kabagambe and colleagues (107) in their work to validate the FFQ as an indicator of long-term dietary intake applied several methodologies ranging from the method of triads, correlation with the corresponding biomarkers, and correlation between the intakes as estimated by 2 dietary assessment methods. A series of seven 24-hour dietary recalls were used as the reference method. The studies were carried out with male (n=78) and female (n=42) respondents. This study showed that biomarkers did not

perform better than the FFQ and that they should be used to complement the FFQ rather than substitute for it.

In a validation of a new approach to assess folate, Yen and colleagues (100) used a folate-specific recall (focused recall) and validated this against an FFQ and serum folate values using the absolute mean folate intakes estimation. The ranking comparison of focused recalls vs the FFQ demonstrated a concordance of 44% without including intake from supplements and a concordance of 67% with supplements. Estimated folate intake based on focused recalls was correlated significantly with plasma homocysteine ($r=0.443$, $P<0.02$) and there was a marginal significant correlation with plasma folate ($r= 0.354$; $P= 0.06$) (100). Hickling and colleagues (92) validated the Folate Intake Tools by calculating the Pearson correlation between the folate score and serum folate. They found the correlations were moderately high for both prototypes (FIT-A, $r= 0.54$; FIT-B, $r=0.49$). No validation work is described for the Folate-Targeted Semi-quantitative Block DFE screener described by Clifford et al (101).

2.4.2 Absolute Intake vs. Adjusted Intake in Validation Studies

Several investigators have recommended adjusting nutrient intakes by the energy intake prior to correlation analysis (116, 117), as such an approach may account for the underreporting of intakes (93). In its simplest form, the energy adjustment involves calculating the nutrient densities by dividing nutrient values for each subject by the energy content of the diet for that subject (93). These nutrient densities are then used instead of the original nutrient intake values. An alternative and sometimes preferable procedure is to use linear regression with total energy intake as the independent variable (x) and intake of the nutrient of interest as the dependent variable (y) (118). The energy-

adjusted nutrient intake of each subject is determined by adding the residual, that is the difference between the observed nutrient values for each subject and the values predicted from the regression equation, to the nutrient intake corresponding to mean energy intake of the study population (99, 118). Adjustment of nutrient intake to the mean energy intake produces an estimate of dietary composition, rather than of absolute intake. It provides an assessment of nutrient intake that is in most cases sufficiently independent of energy intake, it allows to negligible the influence of the remaining co-linearity on subsequent linear analyses and is suggested to be mostly independent of body size and activity level as well (93, 119). Data for both the test method and reference methods should be calculated in this way in validation studies (93). The energy-adjusted folate and/or vitamin B_{12} intake has been used in several validation studies (98, 102, 107, 114, 115).

The energy-adjusted model has not been used in all folate assessment validation studies. In a validation of the folate-specific recall approach described by Yen and associate (100) and the Folate Intake Tools described by Hickling et al (92), adjustments for energy intake in these estimates were not feasible because the tools were not designed to estimate total energy. Similarly, in validating the FFQ, it was not possible to exclude under-reporters or adjust folate values for energy intake because the FFQ listed only foods rich in folate, excluding energy-rich food sources such as fats (98).

2.4.3 Selecting an Appropriate Reference Method and Number of Days to Estimate Folate in Validation Studies

No method for assessing food or nutrient intakes is devoid of random or systematic errors (93). Errors in the reference dietary method should be independent of

those in the test method and also of the true intake (120). As a result, the selected reference dietary method must be different from the test method. For example, both methods should not rely on memory.

The number of days required in the design depends on the variability of the nutrient content across foods, the relative contributions of foods containing the nutrient to the diet and the day-to-day variations of these contributions. Sempose et al (121) collected food records on 2 randomly selected days per month over a 2-year period in women aged 35-65 years. They found in this group of women, 8 days of records were needed to estimate a "true" sample correlation between dietary folate and another variable with 90% accuracy.

Firth et al (122) examined the variability of folate intake in order to estimate the number of days needed to accurately estimate intake in women of childbearing age. They found 6 days of food records were needed to describe folate intake of these women (18-45 years) with 20% attenuation of a correlation coefficient between dietary folate intake and another biological variable. Based on current knowledge of fortified foods and of intra-person day-to-day variability for most nutrients, the use of one day of recall for assessing folate intake of a given person seems unlikely to be appropriate, but fewer than seven or more than seven days may produce comparable or better results.

2.5 FA Fortification

Different methods have been suggested to improve FA intake in women of childbearing age to prevent first occurrences of NTDs. Public-health experts considered three possible strategies to meet this important goal: 1) behavioural-change programs to increase sources of folate in the diet such as fruits and vegetables, 2) health-education

programs to promote the use of FA containing dietary supplements, and 3) a passive program to increase FA in the general food supply (45, 123). There are at least two major difficulties with "preconception" lifestyle-change strategies (e.g., efforts to increase dietary intake of folate or promote the use of supplements). First, approximately 50% of all pregnancies are unplanned. Second, much research has shown that efforts to promote the use of FA supplements or the use of nutrition counseling to increase dietary sources of folate have not been successful in increasing FA intake in the target population during that critical period (124). Therefore, public health experts vigorously pursued the third option of food fortification, with an initial goal of ensuring that most women consume 400 μg synthetic FA daily before and during the first 4 weeks of pregnancy (45).

Different studies were used to support the specific amount needed to increase folate intake. Daley and colleagues in 1997 conducted a study to determine the minimum dose of FA that would be effective to minimize the incidence of NTDs. They used four groups: placebo, 100 μg, 200 μg, and 400 μg of FA supplement a day. They found an increased amount of FA in RBCs in the treatment groups when compared to the placebo group. They determined that 200 μg would be an adequate amount of FA to implement food fortification because it would decrease the incidence of NTDs and it would be safe for the rest of the population. They estimated that the approval of 100 μg of FA as the value for food fortification by the FDA was safe, but may be insufficient to meet the requirements to decrease the incidence of NTD (125).

Cuskelly et al (123) conducted a study, using an exclusion method of folate fortified foods during a 12 week period, to determine if a low amount of FA would make a difference in folate status in young women. They determined that women who were

consumers of FA (consumed fortified foods at least once/week) had significantly higher folate intake ($p=0.002$) and RBC folate concentration ($p=0.023$) than non-consumers at baseline. After the 12 week exclusion period, women in the consumers group had a decreased folate intake (27%) and RBC folate concentrations (12%) as compared to the non-consumers (4% folate intake; 1% RBC folate concentrations). This study showed that exclusion of FA fortified foods decreased folate intake by 78 ±56 μg/d, a value close to the estimated amount of 70-130 μg FA/d for adults 19 y old that were to be ingested through FA fortified foods (20, 126, 127).

Wald et al (128) analyzed data from different studies to determine the dose-response of FA and the incidence of NTDs. If FA intake increased by 200 μg/d, the risk of NTDs would decrease by 23%, and consumption of 400 μg/d would decrease the risk by 36%.

A study was conducted by Werler et al (129) to evaluate different strategies to enhance folate intake in women of childbearing age proposed by the US Department of Health and Human Science in 1996. They studied a population of women of childbearing age with previous history of a baby with congenital malformation. They found the average amount of folate provided by foods was 250 μg/d and the average amount of FA provided by fortified foods was approximately 130 μg/d.

A Canadian national program was mandated in November 1998 to increase FA fortification of all flour and some corn and rice products (130). The amount of FA added to different products ranges from 95-309 μg/100 g of product. This range of fortification was selected on the basis of a target level 140 μg FA/100 g of the cereal-grain product. The projected average increase of FA intake in the general population attributable to

fortification of enriched cereal–grain products was estimated to be ~ 100 μg/d (127, 131).

Consumption of ready-to-eat cereals has been related to increased FA intake(123, 132). For examples, Cuskelly and colleagues (132) found that the exclusion of FA fortified cereals from diets of young women reduced FA intake about 80 μg/d, and as a consequences, reduced RBC folate concentrations. Gates and Conteras (132) found that higher estimated folate intake in young women was associated with increased consumption of cereals ($r=0.59$; $p<0.001$), fortified foods ($r=0.74$; $p<0.001$) and naturally folate rich food ($r=0.69$; $p<0.001$).

2.5.1 Folate Recommendation

In the U.S and Canada, recommendations for dietary intakes for folate were reported by the IOM in 1998 (12)

. The recommendation referred to as Dietary Reference intakes (DRIs) included the following series of reference values: Estimated Average Requirement (EAR), Recommended Dietary Allowances (RDA), Adequate Intake (AI), and Tolerable Upper Level (UL). The EAR is the amount of nutrient needed to meet the requirement of 50% of the population. When it is possible to estimate the EAR, the RDA is estimated from EAR by correcting for population variance (a coefficient of variation of 10% was assumed for folate) (15) and is defined as the average daily dietary intake sufficient to meet the nutrient needs of approximately 98% of the population. When data are insufficient to determine an EAR, then an AI is estimated. The UL is the highest level of daily nutrient intake not likely to be associated with negative effects in the majority of the population (133).

The type of research data on which the DRI for folate was based included that from both controlled metabolic and epidemiological studies. Sauberlich and colleagues (134) conducted a controlled depletion-repletion metabolic study in 10 adult non-pregnant women (28 days of depletion followed by 64 days of graded repletion phases, each lasting for 21 days). Plasma and RBC folate concentrations continued to fall in response to depletion with 100 μg of food folate (equal to 100 μg DFEs) for 21 days. This continued depletion led to the conclusion that 100 μg of dietary folate is below the average requirement. O'keefe and colleagues (135) in their study designed to estimate the requirement for dietary folate in non-pregnant women concluded that intake of 200 μg/d of folate was not sufficient to maintain folate status of these women. Some of the studies designed to estimate folate requirements provided folate as a combination of food and synthetic FA (61, 135-137), while others used food folate only (134, 138). By using DFEs to estimate folate requirements, an adjustment was made for the differences in absorption. Thus, information about physiological requirements can be compared using a common basis, and this is an essential aspect of the accurate estimation of folate requirements (139).

Epidemiological data support an EAR of approximately 302 μg DFEs/d. A primary example is the study by Selhub and colleagues (140). In this study the prevalence of a tHcy value greater than 12 μmol/L was significantly greater among individuals in the lowest four deciles of folate intake (less than 280 μg/d) as determined from a food frequency questionnaire. Reported intakes in this study were obtained prior to folate fortification and do not include supplements, but they include synthetic FA from ready-to-eat or cooked cereals (which frequently contained added folate) and thus would be higher if expressed in DFEs (133). Results from another study were also

considered in the establishment of an EAR (141). Ancillary information is provided by studies using stable isotope methods to estimate *in vivo* folate pool size and the rate of daily utilization. Through the use of an estimated total body pool folate of 20 mg, as extrapolated from liver folate measurement (142), and the assumption of a 1% daily turnover rate of folate (143), the daily quantity of folate utilized was calculated to be approximately 200 μg. When 200 μg is corrected for the 50% bioavailability of food folate, this represents 400 μg/d of DFE (133)

2.5.2 Recommendation for NTD Risk Reduction

A relationship between folate and NTDs, first suggested a little >50 y ago (144), has become recognized as a result of a large number of clinical investigations (145). A reduced risk of NTD has been observed for women who took a folate supplement of 360 to 800 μg/d in addition to a dietary folate intake of 200-300 μg/d (133). Folate intake is positively associated with erythrocyte folate concentration(125, 146, 147), and NTD risk is inversely associated with both folate intake (44, 123) and erythrocyte folate concentration (148). The recommendation made for women capable of becoming pregnant is for an intake that exceeds the DRI for folate. In particular, it is recommended that women capable of becoming pregnant should consume 400 μg of FA daily from supplements, fortified foods, or both in addition to consuming food folate from a varied diet (39, 133, 149).

2.5.3 Consideration for Fortification

The one negative health outcome associated with high folate intake is the possible masking of B_{12} deficiency, a disease that causes damage to the nervous system,

particularly in elderly population (150). When left untreated, B_{12} deficiency can cause mild to severe neurological problems such as numbness, balance problems, weakness, and paralysis (151). Excessive folate intakes may alleviate the anemia that is associated with B_{12} deficiency, but the neurological symptoms themselves respond only to increased intakes of B_{12}. Known as "masking", this phenomenon could therefore delay the diagnosis and treatment of the deficiency and allow the irreversible neurological manifestations of the disease to progress (12). Also, others have noted that synthetic FA is directly transported into blood and speculate that although the life-long exposure of adult and fetal cells to these synthetic forms have not been investigated, health risks may exist (152). Recently, the use of 5-methyltetrahydrofolate (5MTHF) has showed the same effect as FA in increasing blood folate indexes and lowering plasma tHcy (153, 154). Use of supplemental 5-MTHF may have some advantages over FA. Specifically, 5-MTHF is unlikely to mask vitamin B_{12} deficiency, it will not produce unmetabolized FA in the circulation, and it may be more bioavailable compared to the same dose of FA.

2.6 Folate Intakes for Women of Childbearing Age after Folate Fortification

Folate intake has been evaluated in different populations, and using different methods (155). Gates and Holmes in 1999 analyzed data from the 1994-1995 Continuing Survey of Food Intakes by Individuals (CSFII) to determine the folate intake in 2,086 women of childbearing age estimated by two non-consecutive 24-hour recalls. Folate intake averaged 215 ± 3 μg/d and 50% of the subjects consumed less than 180 μg/d of folate (155).

In 1998, Firth and associates (122) studied folate intake in women of childbearing age. They collected fourteen 1-day randomly selected food records during a 60-day period in the U.S. They found that the mean intake of folate was 288 ± 195 μg/d in a population of 21 women. Only 2 women met the RDA (9%). After simulation of the effect of FA fortification, they found that folate intake increased to 550 ± 279 μg without supplements and 609 ± 327 μg with supplements.

Jacques et al (156) analyzed data from the 5th (1991-1994) and the 6th (1995-1998) examination of the Framingham Offspring Study cohort to assess the effect of FA fortification. Participants examined after FA fortification was implemented formed the study group (n=350; September 1997 to March 1998). Participants examined before FA fortification formed the control group (n=756; January 1995 to September 1996). Baseline data corresponded to the 5th examination and follow up data corresponded to the 6th examination. A FFQ was used to assess the usual folate intake, FA supplement use, and FA from foods. Subjects were separated in two groups based on B vitamin supplement use. Before fortification, study group subjects who used B vitamin supplements had a folate intake of 650 μg/d, and the control group had a folate intake of 651 μg/d. Study group subjects who did not use B vitamin supplements had a folate intake of 266 μg/d, and the control group had a folate intake of 275 μg/d. After the implementation of fortification they found a mean folate intake of 686 μg/d in the study group that used B vitamins, and the control group had a mean folate intake of 675 μg/d. The study group that did not take B vitamins had a mean folate intake of 271 μg/d, and the control group had a mean folate intake of 291 μg/d. They concluded that FA fortification improved folate intake in that population (156).

Yen and coworkers (100) reported that the estimated folate intakes, inclusive of supplements, was 411.4± 192.8 μg/ based on focused recalls and 458 ± 221.5 μg/d based on the FFQ. Exclusive of supplements, estimated folate intake from diet was 327 ± 30.2 μg/d based on the focused recall and 373.5± 155.6 μg/d based on the FFQ for 28 US women aged 18-50 y.

Choumenkovitch et al (131), also using data from the 5th and the 6th examination of the Framingham Offspring Study estimated the effect of FA fortification on 1480 individuals. They found that people exposed to FA fortification without FA supplement consumption had an increased intake of 190 μg/d of FA ($p<0.001$) (131).

Cuskelly and colleagues (123) examined the effect of FA fortification in young women who consumed or did not consume fortified foods. They found at baseline that women who consumed fortified foods had a higher folate intake (265 ± 72 μg/d) than women who did not consume fortified foods (197 ± 72 μg/d, $p=0.002$). After exclusion of fortified foods from the diet for 12 weeks, folate intake decreased in people who consumed fortified foods before the study, but did not change in people who did not consume fortified foods before the study.

Several studies have shown a higher consumption of folate than expected (127, 131, 157). For example, Caudil et al(157) found that women who did not consume FA supplements 12 months prior to the study were consuming 100-200 μg of folate per day more than nationwide estimates before fortification. Quinlivan and Gregory (127) also found that estimated folate intake increased 215-240 μg/d after FA fortification. This is double the expected increase.

Estimating the folate intakes as μg DFEs in post-fortification is limited in the literature because the food composition for the conducted study databases did not

distinguished between naturally occurring food folate and synthetic FA (100, 107, 158). Few studies have been published where folate intake is expressed μg DFEs (101, 131, 159, 160). An 81-item semi-quantitative FFQ was verbally administrated to 148 women aged 18-45 years in Vancouver area of British Columbia. Updated US values were used to estimate the FA content. The mean daily folate intake from food, fortified foods, and supplementation was 609 ± 327 μg DFE (159).

Boushey et al (160) collected dietary data for 289 women 18-89 years old using a quantitative food-frequency questionnaire. They stated that despite the limitations of estimating folate intakes, estimated mean dietary intake of folate increased considerably from 320 to 608 μg DFEs.

Clifford et al (101) estimated the folate in thirty nine women in the US (who were at least 18y old) using a 1-page folate-targeted food/supplement intake screener. The average total folate was estimated to be 745 ± 70 μg DFEs/d.

Choumenkovitch et al (131) used data on food and nutrient intake from 549 individuals who participated in the 6th examination of the Framingham Offspring Cohort Study (exposed to fortification). Published data on total folate in enriched cereal-grain products were used to correct folate content in these foods to reflect fortification. They found the mean daily folate intake was 665 μg DFEs/d for the supplement non users, while the total intake for supplement users was 1031 μg DFEs/d (131).

2.6.1 Folate Intakes from Supplements

Werler et al (129) evaluated FA supplement use after 1992 in 1,136 women of childbearing age. They found that the prevalence of daily FA supplementation during the preconception period was 26% overall, changing from 25% in 1993 to 30% in 1994 and

34% in 1995 (129). When data from the 1994-1995 Continuing Survey of Food Intakes by Individuals were analyzed to determine folate supplement use by women of childbearing age (11-50 years old), they found about 27% of the women consumed daily supplements containing folate, and 15% occasionally consumed this supplement (155). Data from the Food Habits of Canadian Survey 1997-1998 provided evidence that, of 1,530 Canadian adults aged 19-65 years from 5 regions across Canada, the rate of supplement usage was 16% (161)

With respect to FA supplement use after fortification, Firth at al (162) found that of 21 women included in their study, one used a multivitamin supplement containing 200 μg FA (5%) and 6 used supplements containing 400 μg FA (28%). In Canada, French et al in 2003 (159) found that approximately 28% of participants used a supplement containing FA on a daily basis, and another 16% used a supplement at least once weekly. Results indicate that taking FA supplements is a common behavior in this population of young women which has to be taken into account when folate intake is estimated.

2.7 Assessment of Folate Status

Dietary assessment is one component of a nutritional status assessment, provided that accurate dietary intake data are collected, the correct DRI is selected for the assessment, and the results are interpreted appropriately. Ideally, intake data are combined with clinical, biochemical and anthropometric information to provide a valid assessment of an individual's nutritional status (163, 164).

There are different methods to assess folate status in the body. Both serum and red blood cell (RBC) folate can be measured to assess folate status. RBC folate provides

a measure of folate status that is generally insensitive to recent folate consumption (45). The use of serum folate tends to provide a measure of recent folate consumption (165). A serum folate concentration of < 7 nmol/L is indicative of a negative folate balance (133, 166). As a cutoff for adequate RBC folate status, a value of ≥ 304 nmol/L has been used (133). Blood homocysteine values vary depending on folate consumption (167). A normal range of homocysteine for young women is 3.7-10.4 μmol/L (168). The cutoff value for tHcy cited most often is greater than 16 μmol/L, but 14 μmol/L (169) and 12 μmol/L (170) have also been used. Ubbink et al used a prediction model to define a reference range as 4.9-11.7 μmol/L (171). Many studies agree that a tHcy concentration of ≤ 10μmol/L is optimal (172-174). A tHcy concentration of ≤ 12 μmol/L is used as cut-off point for young women (166); given that the risk for cardiovascular disease starts to increase with tHcy concentrations higher than 10.5-11.7 μmol/L (170). Analytical methods to measure plasma tHcy concentrations are varied. High-pressure liquid chromatography with fluorometric detection is the most widely used method (175). Other methods include gas-liquid chromatography, capillary electrophoresis, and immunoassays (175, 176)

2.7.1 Folate Status in Women of Childbearing Age after Folate Fortification

To examine the effect of FA fortification on folate intake and folate status, Jacques and coworkers (156) evaluated plasma folate concentrations in subjects before fortification and after fortification in the Framingham Offspring Study. The control group consisted of 756 subjects examined before fortification (January 1995 to September 1996), and the study group consisted of 350 subjects examined after

fortification (September 1997 to March 1998). They found a considerable increase in plasma folate concentration (4.6 μg/L before fortification to 10.0 μg/L after fortification; $p<0.001$) and a considerable decrease in the prevalence of high homocysteine concentrations after fortification by 50% (18.7% before fortification to 9.8% after fortification; $p<0.001$). This research showed that FA fortification of grain products enhanced the folate status in subjects who participated in the study.

The Centers for Disease Control and Prevention (CDC) reported similar findings with respect to FA fortification and improved folate status among non-pregnant women aged 14-44 years (177). When researchers compare folate levels among women who took part in the NHANES 1999 to women who took part in NHANES III (1988-1994), significant increases in both serum and RBC folate concentrations were found. Mean serum folate levels increase from 6.3 ng/ml to 16.2 ng/ml and mean RBC folate concentrations increased from 181 to 315 ng/ml. Similar findings were observed from NHANES 1988-1994 to NHANES 1999-2000 (178). In the most recent report for the CDC, they found during 2001-2002 and 2003-2004, median serum folate concentration among women aged 15-44 years were 11.4 ng/ml and 10.6 ng/ml, respectively. Thus a statistically significant 16% decline was observed from 1999-2000 (12.6 ng/ml) through 2003-2004 based on comparison of geometric means ($P<0.001$). Similarly, RBC folate concentrations increased 8%, from 225 ng/ml during 1999-2000 to 235 ng/ml during 2003-2004 ($p=0.028$) (179). Changes in laboratory techniques or sampling biases between surveys periods are unlikely to account for the decline in folate levels described in the this report (179). More likely explanations for the observed decline include 1) changes over time in the proportion of women taking supplements containing FA, 2) decreased consumption of foods rich in natural folates or foods fortified with FA, 3)

variations in the amount of FA added to enriched grain products, since fortification was mandated (4), and increases in risk factors associated with lower folate concentrations such as obesity. However, evidence to support these explanations is mixed (179).

Lawrence and colleagues (180) have reported an improvement in folate status since fortification took place. Using data obtained from blood samples submitted to an endocrinology lab in California, they examined serum folate concentrations for 98,351 subjects, 53% of whom were women. The researchers found that from 1994 through 1998, median folate levels increased from 12.6 to 18.7 ng/ml. Because median folate began to increase steadily in 1997, the authors speculated that improvement in folate status likely due to fortification of cereals and grains.

After the introduction of FA fortification in Canada, Ray and colleagues (181) performed a retrospective cross-sectional study using a community database of all Ontario samples analyzed by a laboratory that was a major provider of diagnostic laboratory services in Canada. A total of 711 samples were evaluated; the mean age of all participants was 58.4 years. The median serum folate was39.9 nmol/L, and the median for RBC folate 995 nmol/L, these concentrations were higher than expected and even higher than concentration found in the US.

A cross-sectional study was conducted in Southern California to assess folate status of healthy young women after FA fortification of grain products. Eighty five socio-economically advantaged women and 50 disadvantaged women were used in the study. Results showed that women from both groups benefited from FA fortification, since blood values of folate exceeded the normal values (serum folate >13.6 nmol/L; RBC folate >362 nmol/L) (157).

Quinlivan and Gregory (127) assessed the effect of FA fortification in the US population based on recent studies. They found a greater increase in serum folate (5.4 μg/L (156), 6.0 μg/L (180), 7.9 μg/L (177) than the increase estimated by IOM (1.9-3.5 μg/L (12).

Paralleling the improved serum and RBC folate concentrations found among women of childbearing age following fortification of grain and cereal products has been a decline in the prevalence of spina bifida and anencephaly. The prevalence of NTDs decreased 19% based on a comparison of birth certificate reports of spina bifida and anencephaly before and after mandatory fortification (149). Williams and coworkers (182) used population-based surveillance programs to identify spina bifida and anencephaly cases occurring from 1995 to 1999, and they found that the prevalence of spina bifida and anencephaly had decreased 31% and 16%, respectively, since 1995 (182). In Canada, particularly in Nova Scotia after the fortification was implemented the incidence of open NTDs decreased by more than 50%: the mean annual rate was 2.58 per 1000 births during 1991-1997 and 1.17 per 1000 births during 1998-2000 (40). Studying the incidence of NTDs in Ontario 1986-1999, it was found the total NTD incidence rate increased from 11.7 per 10,000 pregnancies in 1986 to 16.2 per 10,000 in 1995, and it subsequently decreased to 8.6 per 10,000 by 1999 (39). Most recently De Wals et al assessed changes in the prevalence of NTDs in Canada before and after food fortification. The study population included live births, stillbirths, and terminations of pregnancies because of fetal anomalies among women residing in seven Canadian provinces from 1993-2002. On the basis of published results of testing of RBC folate, the study period was divided into pre-fortification, partial fortification, and full-fortification periods. A total of 2446 subjects with NTDs were recorded among 1.9

million births. The prevalence of NTDs decreased from 1.58 per 1000 births before fortification to 0.86 per 1000 during the full fortification period, a 46% reduction (95% Confidence interval, 40 to 51). The decrease was greatest in areas in which the baseline rate was high (183).

Although folate status has improved and NTD rates have declined since the mandatory fortification of grains, folate intakes among women of childbearing age are still below the RDA (20, 129, 159, 160, 184). Boushey and colleagues in their study to estimate the benefits of FA fortification program, found that although the estimated mean dietary intake of folate increased considerably with stimulation of fortification (320 to 608 μg DFEs), more than half of the subjects (61%) did not meet the special RDA for women of childbearing age, where it is recommended to take 400 μg FA/per day (160).

French and associates (159) estimated folate intake in women of childbearing age. One hundred forty-eight women (aged 18 to 45 years) in the Vancouver area of British Columbia, Canada were studied. The Contribution of folate from food, fortified grain products, and supplements was assessed by validated semi-quantitative FFQ. Although 86% of women met the EAR (320 DFE/day) for folate, only 26% met the recommendation (400 μg Synthetic folic acid (SFA)/day) for women capable of becoming pregnant.

When Lewis and colleagues (20) adjusted the food composition data of the 1994-1996 Continuing Survey of Food Intakes buy Individuals (CSFII; 7) and the 1988-1994 NHANES III to reflect fortification of the food supply, they found that the majority of childbearing age women (68-87%) had SFA intakes below the recommendation.

Werler et al showed although FA fortification was implemented, women of childbearing age were not meeting the recommended amount of 400 μg/d of FA to prevent NTDs (129). Less than 10% of women of childbearing age reached the recommended erythrocyte folate concentration of greater than 906 nmol/L that has been shown to be associated with a significant reduction in NTD risk (184).

2.8 Effect of Fortification on tHcy and Vitamin B_{12} Status

The tHcy concentrations post-fortification appeared lower than pre-fortification (169, 185). A direct comparison of plasma tHcy concentrations between NHANES 1999-2000 and NHANES III is not possible because of different in the methods and matrices of the 2 surveys (186). The prevalence of elevated tHcy after FA fortification was 10% comparing with a prevalence of ~ 20% before fortification in the Framingham population (187). What seems even more important, is that nearly 80% of the US population had achieved a plasma tHcy concentration <9 μmol/L, which is considered a desirable concentration (186)

Vitamin B_{12} concentrations did not differ significantly in the young women between the pre-fortification and post-fortification periods. The reference ranges (5^{th}-95^{th} percentiles) for serum B_{12} for females in the US population after the introduction of FA fortification were 179-738 pmol/L (186) and before the introduction of FA were 186-639 pmol/L (168). The range (5^{th}- 90^{th} percentile) for females in Canada after FA fortification was 135-697pmol/L (181), and three percent of the participants were below the optimum level of this vitamin (181). This result is in accord with results of an earlier investigator (186), where they found 3% had marginally low serum B_{12} concentrations.

2.9 References

1. Melse-Boonstra A, de Bree A, Verhoef P, Bjorke-Monsen AL, Verschuren WMM. Dietary monoglutamate and polyglutamate folate are associated with plasma folate concentrations in dutch men and women aged 20-65 years. J Nutr 2002;132:1307-1312.
2. Brody T. Nutrition Biochemistry. Second Edition ed. USA: Academic Press, 1999.
3. Maddox D, Manlapat A, Roon P, Prasad P, Ganapathy V, Smith S. Reduced-folate carrier (RFC) is expressed in placenta and yolk sac, as well as in cells of the developing forebrain, hindbrain, neural tube, craniofacial region, eye, limb buds and heart. BMC Dev Biol. 2003; Epub 2003 Jul 29.
4. Gregory JF. Case Study: Folate Bioavailability. J Nutr 2001;131:1376S-1382.
5. Prinz-Langenohl R, Bronstrup A, Thorand B, Hages M, Pietrzik K. Availability of Food Folate in Humans. J Nutr 1999;129:913-916.
6. Bailey LB, Gregory JF, III. Folate metabolism and requirements. J Nutr 1999;129:779-782.
7. Seyoum E, Selhub J. Properties of food folates determined by stability and susceptibility to intestinal pteroylpolyglutamate hydrolase action. J Nutr 1998;128:1956-1960.
8. Wei M, Gregory J. Organic acids in selected foods inhibit intestinal brush border pteroylpolyglutamate hydrolase in vitro: potential mechanism affecting the bioavailability of dietary polyglutamyl folate. J Agric Food Chem 1998;46:211-219.

9. Krishnaswamy K, Madhavan Nair K. Importance of folate in human nutrition. Br J Nutr 2001;85:S115-124.

10. Suh JR, Herbig AK, Stover PJ. New perspectives on folate catabolism. Annu Rev Nutr 2001;20:255-282.

11. Bailey L. Folate and vitamin B12 recommended intakes and status in the United States. Nutr Rev 2004;62:S14-20.

12. Institute of Medicine. Dietary reference intakes: folate, other B vitamin and choline. Washington, DC: National Academy Press, 1998.

13. Gregory EF. Bioavailability of folate. Eur J Clin Nutr 1997;51:S54-S59.

14. Pfeiffer C, Rogers L, Bailey L, Gregory J. Absorption of folate from fortified cereal-grain products and of supplemental folate consumed with or without food determined by using a dual-label stable-isotope protocol. Am J Clin Nutr 1997;66:1388-1397.

15. Bailey LB. Folate and vitamin B12 recommended intakes and status in the United States. Nutr Rev. 2004;62:S14-20.

16. Chancy C, Kekuda R, Huang W, et al. Expression and differential polarization of the reduced-folate transporter-1 and the folate receptor alpha in mammalian retinal pigment epithelium. J Biol Chem 2000;275:20676-20684.

17. McPartlin J, Halligan A, Scott J, Darling M, Weir D. Accelerated folate breakdown in pregnancy. Lancet 1993;341:148-149.

18. Caudill MA, Gregory III JF, Hutson AD, Bailey LB. Folate catabolism in pregnant and nonpregnant women with controlled folate intakes. J Nutr 1998;128:204-208.

19. Caudill MA, Bailey LB, Gregory JF, III. Consumption of the folate breakdown product para-aminobenzoylglutamate contributes minimally to urinary folate catabolite excretion in humans: investigation using [13C5]para-aminobenzoylglutamate. J Nutr 2002;132:2613-2616.

20. Lewis CJ, Crane NT, Wilson DB, Yetley EA. Estimated folate intakes: data updated to reflect food fortification, increased bioavailability, and dietary supplement use. Am J Clin Nutr 1999;70:198-207.

21. Institute of Medicine. Dietary reference intakes: for thiamin, riboflavin, niacin, vitamin B6, folate, vitamin B12, pantothenic acid, biotin. Washington, DC: National Academy Press., 2000.

22. Refsum H, Smith AD, Ueland PM, et al. Facts and recommendations about total homocysteine determinations: An expert opinion. Clin Chem 2004;50:3-32.

23. Hendricks K, Herbold N. Diet, activity, and other health-related behaviors in college-age women. Nutr Rev 1998;56:65-67.

24. Ashfield-Watt PA, Pullin CH, Whiting JM, et al. Methylenetetrahydrofolate reductase 677C->T genotype modulates homocysteine responses to a folate-rich diet or a low-dose folic acid supplement: a randomized controlled trial. Am J Clin Nutr 2002;76:180-186.

25. de Bree A, Verschuren WM, Bjorke-Monsen A-L, et al. Effect of the methylenetetrahydrofolate reductase 677C->T mutation on the relations among folate intake and plasma folate and homocysteine concentrations in a general population sample. Am J Clin Nutr 2003;77:687-693.

26. Meleady R, Ueland PM, Blom H, et al. Thermolabile methylenetetrahydrofolate reductase, homocysteine, and cardiovascular disease risk: the European Concerted Action Project. Am J Clin Nutr 2003;77:63-70.

27. Amouzou EK, Chabi NW, Adjalla CE, et al. High prevalence of hyperhomocysteinemia related to folate deficiency and the 677C->T mutation of the gene encoding methylenetetrahydrofolate reductase in coastal West Africa. Am J Clin Nutr 2004;79:619-624.

28. Delvin EE, Rozen R, Merouani A, Genest J, Jr, Lambert M. Influence of methylenetetrahydrofolate reductase genotype, age, vitamin B-12, and folate status on plasma homocysteine in children. Am J Clin Nutr 2000;72:1469-1473.

29. Sharp L, Little J. Polymorphisms in genes involved in folate metabolism and colorectal neoplasia: a huge review. Am J Epidemiol 2004;159:423-443.

30. Laverdiere C, Chiasson S, Costea I, Moghrabi A, Krajinovic M. Polymorphism G80A in the reduced folate carrier gene and its relationship to methotrexate plasma levels and outcome of childhood acute lymphoblastic leukemia. Blood 2002;100:3832-3834.

31. Kato I, Dnistrian A, Schwartz M, et al. Epidemiologic correlates of serum folate and homocysteine levels among users and non-users of vitamin supplement. Int J Vitam Nutr Res 1999;69:322-329.

32. Cravo M, Gloria L, Selhub J, et al. Hyperhomocysteinemia in chronic alcoholism: correlation with folate, vitamin B-12, and vitamin B-6 status. Am J Clin Nutr 1996;63:220-224.

33. Halsted CH, Villanueva JA, Devlin AM, Chandler CJ. Metabolic interactions of alcohol and folate. J Nutr 2002;132:2367S-2372.

34. Bailey L. New standard for dietary folate intake in pregnant women. Am J Clin Nutr 2000;71:1304S-1307.

35. Bonnette RE, Caudill MA, Boddie AM, Hutson AD, Kauwell GP, Bailey LB. Plasma homocyst(e)ine concentrations in pregnant and nonpregnant women with controlled folate intake. Obstet Gynecol 1998;92:167-70.

36. Brown J, Jacobs DJ, Hartman T, et al. Predictors of red cell folate level in women attempting pregnancy. JAMA 1997;277:548-52.

37. Rader J. Folic acid fortification, folate status and plasma homocysteine. J Nutr 2002;132:2466S-2470S.

38. Hernandez-Diaz S, Werler MM, Walker AM, Mitchell AA. Neural tube defects in relation to use of folic acid antagonists during pregnancy. Am J Epidemiol 2001;153:961-968.

39. Gucciardi E, Pietrusiak M-A, Reynolds DL, Rouleau J. Incidence of neural tube defects in Ontario, 1986-1999. CMAJ 2002;167:237-240.

40. Persad VL, Van den Hof MC, Dube JM, Zimmer P. Incidence of open neural tube defects in Nova Scotia after folic acid fortification. CMAJ 2002;167:241-245.

41. Hilton J. Folic acid intake of young women. J Obstet Gynecol Neonatal Nurs 2002;31:172-177.

42. Sayers GM, Hughes N, Scallan E, Johnson Z. A survey of knowledge and use of folic acid among women of child-bearing age in Dublin. J Public Health 1997;19:328-332.

43. NoAuthors. Prevention of neural tube defects: results of the Medical Research Council Vitamin Study. MRC Vitamin Study Research Group. Lancet 1991;338:131-137.

44. Czeizel AE. Prevention of the first occurrence of neural-tube defects by periconceptional vitamin. N Engl J Med 1992;327:1832-1835.

45. Neuhouser ML, Beresford SAA. Folic acid: are current fortification levels adequate? Nutr 2001;17:868-872.

46. Wartanowicz M, Ziemlansk iS, Bulhak-Jachymczyk B, Konopka L. Assessment of nutritional folate status and selected vitamin status of women of childbearing age. Eur J Clin Nutr 2001;55:743-7.

47. Cornel M, Erickson J. Comparison of national policies on periconceptional use of folic acid to prevent spina bifida and anencephaly (SBA). Teratology 1997;55:137-137.

48. Stone D, McCarron P, Smith G. Similarities in the epidemiology of neural tube defects and coronary heart disease: is homocysteine the missing link? J Epidemiol Community Health 1999;53:789-793.

49. Sutterlin M, Bussen S, Ruppert D, Steck T. Serum levels of folate and cobalamin in women with recurrent spontaneous abortion. Hum Reprod 1997;12:2292-2296.

50. Nelen WL, Blom HJ, Steegers EA, Den Heijer M, Thomas CM, Eskes TK. Homocysteine and Folate Levels as Risk Factors for Recurrent Early Pregnancy Loss. Obstet Gynecol 2000;95:519-524.

51. George L, Mills JL, Johansson ALV, et al. Plasma folate levels and risk of spontaneous abortion. JAMA 2002;288:1867-1873.

52. Jacobsen DW. Homocysteine and vitamins in cardiovascular disease. Clin Chem 1998;44:1833-1843.

53. Lobo A, Naso A, Arheart K, et al. Reduction of homocysteine levels in coronary artery disease by low-dose folic acid combined with vitamins B6 and B12. Am J Cardiol 1999;83:821-825.

54. Mennen LI, de Courcy GP, Guilland J-C, et al. Homocysteine, cardiovascular disease risk factors, and habitual diet in the French Supplementation with Antioxidant Vitamins and Minerals Study. Am J Clin Nutr 2002;76:1279-1289.

55. Koehler KM, Baumgartner RN, Garry PJ, Allen RH, Stabler SP, Rimm EB. Association of folate intake and serum homocysteine in elderly persons according to vitamin supplementation and alcohol use. Am J Clin Nutr 2001;73:628-637.

56. Voutilainen S, Lakka T, Porkkala-Sarataho E, Rissanen T, Kaplan G, Salonen J. Low serum folate concentrations are associated with an excess incidence of acute coronary events: the Kuopio Ischaemic Heart Disease Risk Factor Study. Eur J Clin Nutr 2000;54:424-428.

57. Vollset SE, Refsum H, Ueland PM. Population determinants of homocysteine. Am J Clin Nutr 2001;73:499-500.

58. El-Khairy L, Vollset SE, Refsum H, Ueland P. Plasma total cysteine, pregnancy complications, and adverse pregnancy outcomes: the Hordaland homocysteine study. Am J Clin Nutr 2003;77:467-472.

59. Robinson K, Arheart K, Refsum H, et al. Low Circulating Folate and Vitamin B6 Concentrations: Risk Factors for Stroke, Peripheral Vascular Disease, and Coronary Artery Disease. Circulation 1998;97:437-443.

60. Carmel R, Green R, Rosenblatt DS, Watkins D. Update on cobalamin, folate, and homocysteine. Hematology 2003;2003:62-81.

61. Jacob RA, Gretz DM, Taylor PC, et al. Moderate folate depletion increases plasma homocysteine and decreases lymphocyte DNA methylation in postmenopausal women. J Nutr 1998;128:1204-1212.

62. Pfanzagl B, Tribl F, Koller E, Moslinger T. Homocysteine strongly enhances metal-catalyzed LDL oxidation in the presence of cystine and cysteine. Atherosclerosis 2003;168:39-48.

63. Voutilainen S, Rissanen T, Virtanen J, Lakka T, Salonen, Jukka T. Low Dietary Folate Intake Is Associated With an Excess Incidence of Acute Coronary Events: The Kuopio Ischemic Heart Disease Risk Factor Study. Circulation 2001;103:2674-2680.

64. Rimm E. Folate and vitamin B6 from diet and supplements in relation to risk of coronary heart disease among women. Bibl Nutr Dieta 2001;55:42-45.

65. Lobo A, Naso A, Arheart K, et al. Reduction of homocysteine levels in coronary artery disease by low-dose folic acid combined with vitamins B6 and B12. The American Journal of Cardiology 1999;83:821-825.

66. Tice JA, Ross E, Coxson PG, et al. Cost-effectiveness of vitamin therapy to lower plasma homocysteine levels for the prevention of coronary heart disease: effect of grain fortification and beyond. JAMA 2001;286:936-943.

67. Venn BJ, Mann JI, Williams SM, et al. Dietary counseling to increase natural folate intake: a randomized, placebo-controlled trial in free-living subjects to assess effects on serum folate and plasma total homocysteine. Am J Clin Nutr 2002;76:758-765.

68. Tungtrongchitr R, Pongpaew P, Tongboonchoo C, et al. Serum homocysteine, B12 and folic acid concentration in Thai overweight and obese subjects. Int J Vitam Nutr Res 2003;73:8-14.

69. Doshi S, McDowell I, Moat S, et al. Folic acid improves endothelial function in coronary artery disease via mechanisms largely independent of homocysteine lowering. Circulation 2002;105:22.26.

70. Scott J. Homocysteine and cardiovascular risk. Am J Clin Nutr 2000;72.

71. Choi SW, Mason JB. Folate and carcinogenesis: An integrated scheme. J Nutr 2000;130:129-132.

72. Choi SW, Mason JB. Folate status: effects on pathways of colorectal carcinogenesis. J Nutr 2002;132:2413S-2418.

73. Weinstein SJ, Ziegler RG, Frongillo EA, Jr., et al. Low serum and red blood cell folate are moderately, but nonsignificantly associated with increased risk of invasive cervical cancer in U.S. women. J Nutr 2001;131:2040-2048.

74. Duthie SJ, Narayanan S, Brand GM, Pirie L, Grant G. Impact of folate deficiency on DNA stability. J Nutr 2002;132:2444S-2449.

75. Giovannucci E. Epidemiologic studies of folate and colorectal neoplasia: a review. J Nutr 2002;132:2350S-2355.

76. Kim Y-I. Role of folate in colon cancer development and progression. J Nutr 2003;133:3731S-3739.

77. Kim Y, Salomon R, Graeme-Cook F, et al. Dietary folate protects against the development of macroscopic colonic neoplasia in a dose responsive manner in rats. Gut. 1996;39:732-740.

78. Zhang S, Hunter DJ, Hankinson SE, et al. A Prospective study of folate intake and the risk of breast cancer. JAMA 1999;281:1632-1637.

79. Weinstein SJ, Ziegler RG, Frongillo EA, Jr., et al. Low Serum and Red Blood Cell Folate Are Moderately, but Nonsignificantly Associated with Increased Risk of Invasive Cervical Cancer in U.S. Women. J. Nutr. 2001;131:2040-2048.

80. Lightfoot T, Roman E. Causes of childhood leukaemia and lymphoma. Toxicol Appl Pharmacol 2004;199:104-117.

81. French A, Kim Y, Liu S, Seshadri SK, G. Folic acid food fortification is associated with a decline in neuroblastoma. Clin Phamacol 2003;74:288-294.

82. Mischoulon D, Fava M. Role of S-adenosyl-L-methionine in the treatment of depression: a review of the evidence. Am J Clin Nutr 2002;76:1158S-1161.

83. Alpert J, Fava M. Nutrition and depression: the role of folate. Nutr Rev 1997;55:145-149.

84. Bottiglieri T, Laundy M, Crellin R, Toone BK, Carney MWP, Reynolds EH. Homocysteine, folate, methylation, and monoamine metabolism in depression. J Neurol Neurosurg Psychiatry 2000;69:228-232.

85. Bjelland I, Tell G, Vollset S, Refsum H, Ueland P. Folate, vitamin B12, homocysteine, and the MTHFR 677C->T polymorphism in anxiety and depression: the Hordaland Homocysteine Study. Arch Gen Psychiatry 2003;60:618-626.

86. Fava M, Borus J, Alpert J, Nierenberg A, Rosenbaum J, Bottiglieri T. Folate, vitamin B12, and homocysteine in major depressive disorder. Am J Psychiatry 1997;154.

87. Tiemeier H, van Tuijl HR, Hofman A, Meijer J, Kiliaan AJ, Breteler MM. Vitamin B12, folate, and homocysteine in depression: the Rotterdam Study. Am J Psychiatry 2002;159:2099-101.

88. Postiglione A, Milan G, Ruocco A, Gallotta G, Guiotto G, Di Minno G. Plasma folate, vitamin B(12), and total homocysteine and homozygosity for the C677T mutation of the 5,10-methylene tetrahydrofolate reductase gene in patients with Alzheimer's dementia. A case-control study. Gerontology 2001;47:324-329.

89. Clarke R, Smith AD, Jobst KA, Refsum H, Sutton L, Ueland PM. Folate, vitamin B12, and serum total homocysteine levels in confirmed alzheimer disease. Arch Neurol 1998;55:1449-1455.

90. Ravaglia G, Forti P, Maioli F, et al. Homocysteine and folate as risk factors for dementia and Alzheimer disease. Am J Clin Nutr 2005;82:636-643.

91. Mizrahi EH, Bowirrat A, Jacobsen DW, et al. Plasma homocysteine, vitamin B12 and folate in Alzheimer's patients and healthy Arabs in Israel. J Neurol Sci 2004;227:109-113.

92. Hickling S, Knuiman M, Jamrozik K, Hung J. A rapid dietary assessment tool to determine intake of folate was developed and validated. J Clin Epidemiol 2005;58:802-808.

93. Gibson R. Principle of Nutrition Assessment. New York: Oxford University Press., 2005.

94. Kaczkowski CH, Jones PJH, Feng J, Bayley HS. Four-day Multimedia diet records underestimate energy needs in middle-Aged and elderly women as determined by doubly-labeled water. J Nutr 2000;130:802-805.

95. Martin L, Su W, Jones P, Lockwood G, Tritchler D, Boyd N. Comparison of energy intakes determined by food records and doubly labeled water in women participating in a dietary-intervention trial. Am J Clin Nutr 1996;63:483-490.

96. Kaczkowski C, Jones P, Feng J, Bayley H. Canadian recommendations underestimate energy needs of women over fifty years as determined by doubly-labelled water. Can J Physiol Pharmacol 2000;78:631-635.

97. Rolls BJ, Morris EL, Roe LS. Portion size of food affects energy intake in normal-weight and overweight men and women. Am J Clin Nutr 2002;76:1207-1213.

98. Pufulete M, Emery P, Nelson M, Sanders T. Validation of a short food frequency questionnaire to assess folate intake. Br J Nutr 2002;87:383-390.

99. Willett W. Nutritional Epidemiology. Second Edition ed. New York: Oxford University Press, 1998.

100. Yen J, Zoumas-Morse C, Pakiz B, Rock C. Folate intake assessment: validation of a new approach. J Am Diet Assoc 2003;103:991-1000.

101. Clifford AJ, Noceti EM, Block-Joy A, Block T, Block G. Erythrocyte folate and its response to folic acid supplementation is assay dependent in women. J Nutr 2005;135:137-143.

102. Verkleij-Hagoort A, de Vries J, Stegers M, Lindemans J, Ursem N, Steegers-Theunissen R. Validation of the assessment of folate and vitamin B(12) intake in women of reproductive age: the method of triads. Eur J Clin Nutr 2006;Dec 13; [Epub ahead of print].

103. Kaaks R, Riboli E. Validation and calibration of dietary intake measurements in the EPIC project: methodological considerations. European Prospective Investigation into Cancer and Nutrition. Int J Epidemiol 1997;26:S15-25.

104. Kaaks R, Ferrari P, Ciampi A, Plummer M, Rioli E. Uses and limitations of statistical accounting for random error correlations, in the validation of dietary questionnaire assessments. Public Health Nutr 2002;5:969-976.

105. Bailey K. Methods of Social Research. London: The Free Press, Collier MAcmillan Puplishers, 1987.

106. Kabagambe EK, Baylin A, Allan DA, Siles X, Spiegelman D, Campos H. Application of the Method of Triads to Evaluate the Performance of Food Frequency Questionnaires and Biomarkers as Indicators of Long-term Dietary Intake. Am. J. Epidemiol. 2001;154:1126-1135.

107. Kabagambe EK, Baylin A, Allan DA, Siles X, Spiegelman D, Campos H. Application of the method of triads to evaluate the performance of food frequency questionnaires and biomarkers as indicators of long-term dietary intake. Am J Epidemiol 2001;154:1126-1135.

108. Flood VM, Smith WT, Webb KL, Mitchell P. Issues in assessing the validity of nutrient data obtained from a food-frequency questionnaire: folate and vitamin B12 examples. Public Health Nutr. 2004;7:751-756.

109. Kaaks R. Biochemical markers as additional measurements in studies of the accuracy of dietary questionnaire measurements: conceptual issues. Am J Clin Nutr 1997;65:1232S-1239.

110. Kaaks R, Ferrari P. Dietary intake assessments in epidemiology: can we know what we are measuring? Ann Epidemiol 2006;16:377-380.

111. Ocke M, Kaaks R. Biochemical markers as additional measurements in dietary validity studies: application of the method of triads with examples from the European prospective investigation into cancer and nutrition. Am J Clin Nutr 1997;65:1240S-1245.

112. Ocke M, Bueno-de-Mesquita H, Pols M, Smit H, van Staveren W, Kromhout D. The Dutch EPIC food frequency questionnaire. II. Relative validity and reproducibility for nutrients. Int J Epidemiol 1997;26:S49-58.

113. Block G. human dietary assessment: method and issues. Preventive Medicine 1989;18:653-660.

114. Green TJ, Allen OB, O'Connor DL. A three-day weighed food record and a semiquantitative food-frequency questionnaire are valid measures for assessing the folate and vitamin B-12 intakes of women aged 16 to 19 years. J Nutr 1998;128:1665-71.

115. Ishihara J, Yamamoto S, H I, Inoue M, Tsugane S. Validity of a self-administered food frequency questionnaire (FFQ) and its generalizability to the estimation of dietary folate intake in Japan. [Electronic version]. Nutr J 2005;Oct 5:4:26.

116. McKeown NM, Day NE, Welch AA, et al. Use of biological markers to validate self-reported dietary intake in a random sample of the European prospective investigation into cancer United Kingdom Norfolk cohort. Am J Clin Nutr 2001;74:188-196.

117. Bingham S, Day N. Using biochemical markers to assess the validity of prospective dietary assessment methods and the effect of energy adjustment. Am J Clin Nutr 1997;65:1130S-1137S.

118. Willett W, Stampfer M, J. Total Energy intake: Implication for Epidemiologic Analysis. Am J Epodemiol 1986;124:17-27.

119. Brunner E, Stallone D, Juneja M, Bingham S, Marmot M. Dietary assessment in Whitehall II: comparison of 7 d diet diary and food-frequency questionnaire and validity against biomarkers. Br J Nutr 2001;86:405-414.

120. Kipnis V, Midthune D, Freedman LS, et al. Empirical evidence of correlated biases in dietary assessment instruments and its implications. Am J Epidemiol 2001;153:394-403.

121. Sempose C, Johnson N, Smith E, Gilligan C. Effects of interindividual and interindividual variation in repeated dietary records. Am J Epidemiol 1985;121:120-130.

122. Firth Y, Murtaugh M, Tangney CC. Estimation of individual intakes of folate in women of childbearing age with and without simulation of folic acid fortification. J Am Diet Assoc 1998;98:985-988.

123. Cuskelly G, McNulty H, Scott JM. Fortification with low amounts of folic acid makes a significant difference in folate status in young women: implications for the prevention of neural tube defects. Am J Clin Nutr 1999;70:234-9.

124. Stevenson R, Dean J, Allen W, Kelly M. Prevention program for reducing risk for neural tube defects-South Carolina, 1992-1994. MMWR 1995;44:141-142.

125. Daly S, Mills JL, Molloy AM, et al. Minimum effective dose of folic acid for food fortification to prevent neural-tube defects. Lancet 1997;350:1666-1669.

126. Edmonds J, Boushey CJ, Anderson SL. The effect of folic acid fortification on folate intake in rural women. J Am Diet Assoc 1998;99:A12.

127. Quinlivan EP, Gregory JF, III. Effect of food fortification on folic acid intake in the United States. Am J Clin Nutr 2003;77:221-225.

128. Wald N, Law M, Morris J, Wald D. Quantifying the effect of folic acid. Lancet 2001;358:2069-2073.

129. Werler M, Louik C, Mitchell A. Achieving a public health recommendation for preventing neural tube defects with folic acid. Am J Public Health 1999;89:1637-1640.

130. Ray JG, Vermeulen MJ, Boss SC, DE. C. Declining rate of folate insufficiency among adults following increased folic acid food fortification in Canada. Can J Public Health. 2002;93:249-53.

131. Choumenkovitch SF, Selhub J, Wilson PWF, Rader JI, Rosenberg IH, Jacques PF. Folic Acid Intake from Fortification in United States Exceeds Predictions. J Nutr 2002;132:2792-2798.

132. Gates G, Contreras M. Factors that influence women's folate intake. Top Clin Nutr 2002;17:40:48.

133. Institute of Medicine Research Council. Dietary reference intakes:for thiamin r, niacin, vitamin B6, folate, vitamin B12, pantothenic acid, biotin. Dietary reference intakes:for thiamin, riboflavin, niacin, vitamin B6, folate, vitamin B12, pantothenic acid, biotin. Washington, DC: National Academy Press., 2000.

134. Sauberlich H, Kretsch M, Skala J, Johnson H, Taylor P. Folate requirement and metabolism in nonpregnant women. Am J Clin Nutr 1987;46:1016-1028.

135. O'Keefe CA, Bailey LB, Thomas EA, et al. Controlled dietary folate affects folate status in nonpregnant women. J Nutr 1995;125:2717-2725.

136. Jacob RA, Wu M-M, Henning SM, Swendseid ME. Homocysteine increases as folate decreases in plasma of healthy men during short-term dietary folate and methyl group restriction. J Nutr 1994;124:1072-1080.

137. Caudill MA, Cruz AC, Gregory III JF, Hutson AD, Bailey aLB. Folate status response to controlled folate intake in pregnant women. J Nutr 1997;127:2363-2370.

138. Milne D, Johnson L, Mahalko J, Sandstead H. Folate status of adult males living in a metabolic unit: possible relationships with iron nutriture. Am J Clin Nutr 1983;37:768-773.

139. Suitor CW, Bailey LB. Dietary Folate Equivalents: interpretation and application. J Am Diet Assoc 2000;100:88-94.

140. Selhub J, Jacques P, Wilson P, Rush D, Rosenberg I. Vitamin status and intake as primary determinants of homocysteinemia in an elderly population. JAMA. 1993;270:2693-2698.

141. Gailani SD, Carey RW, Holland JF, O'Malley JA. Studies of folate deficiency in patients with neoplastic diseases. Cancer Res 1970;30:327-333.

142. Hoppner K, Lampi B. Folate levels in human liver from autopsies in Canada. Am J Clin Nutr 1980;33:862-864.

143. Von der Porten AE, Gregory JF, III, Toth JP, Cerda JJ, Curry SH, Bailey LB. In vivo folate kinetics during chronic supplementation of human subjects with deuterium-labeled folic acid. J Nutr 1992;122:1293-1299.

144. Hibbard B. The role of folic acid in pregnancy; with particular reference to anaemia, abruption and abortion. J Obstet Gynaecol Br Commonw. 1964;71:529-524.

145. Pitkin RM. Folate and neural tube defects. Am J Clin Nutr 2007;85:285S-288.

146. Cuskelly G, McNulty H, Scott J. Effect of increasing dietary folate on red-cell folate: implications for prevention of neural tube defects. Lancet 1996;347:657-6659.

147. Brown J, Jacobs D, Hartman T, et al. Predictors of red cell folate level in women attempting pregnancy. JAMA 1997;277:548-552.

148. Daly L, Kirke P, Molloy A, Weir D, Scot tJ. Folate levels and neural tube defects. implications for prevention. JAMA 1995;274:1698-702.

149. Honein M, Paulozzi L, Mathews T, Erickson J, Wong L. Impact of folic acid fortification of the US food supply on the occurrence of neural tube defects. JAMA 2001;286:2236.

150. Weir DG, Scott JM. Brain function in the elderly: role of vitamin B12 and folate. Br Med Bull 1999;55:669-682.

151. Bentley J, Ferrini R, Hill L. American college of preventive medicine public policy statment: folic acid fortification of grain products in the U.S. to prevent neural tube defects. Am J Prev Med 1999;16:264-267.

152. Kelly P, McPartlin J, Goggins M, Weir D, Scott J. Unmetabolized folic acid in serum: acute studies in subjects consuming fortified food and supplements. Am J Clin Nutr 1997;65:1790-1795.

153. Venn BJ, Green TJ, Moser R, Mckenzie JE, Skeaff CM, Mann J. Increases in blood folate indices are similar in women of Childbearing Age Supplemented with [6S]-5-methyltetrahydrofolate and folic acid. J Nutr 2002;132:3353-3355.

154. Lamers Y, Prinz-Langenohl R, Moser R, Pietrzik K. Supplementation with [6S]-5-methyltetrahydrofolate or folic acid equally reduces plasma total homocysteine concentrations in healthy women. Am J Clin Nutr 2004;79:473-478.

155. Gates GE, Holmes TW. Folate intake and supplement use in women of childbearing age. Family Economics and Nutrition Review 1999;12:14-25.

156. Jacques P, Selhub J, Bostom A, Wilson P, Rosenberg I. The effect of folic acid fortification on plasma folate and total homocysteine concentrations. N Engl J Med 1999;13:1449-54.

157. Caudill MA, Le T, Moonie SA, Esfahani ST, Cogger EA. Folate status in women of childbearing age residing in southern California after folic acid fortification. J Am Coll Nutr 2001;20:129-134.

158. Lutsey PL, Steffen LM, Feldman HA, et al. Serum homocysteine is related to food intake in adolescents: the Child and Adolescent Trial for Cardiovascular Health. Am J Clin Nutr 2006;83:1380-1386.

159. French MR, Barr SI, Levy-Milne R. Folate intakes and awareness of folate to prevent neural tube defects: A survey of women living in Vancouver, Canada. J Am Diet Assoc 2003;103:181-185.

160. Boushey CJ, Edmonds JW, Welshimer KJ. Estimates of the effects of folic-acid fortification and folic-acid bioavailability for women. Nutrition 2001;17:873-879.

161. Troppmann L, Gray-Donald K, T. J. Supplement use: is there any nutritional benefit? J Am Diet Assoc 2002;102:818-25.

162. Firth Y, Murtaugh M, angney C. Estimation of individual intakes of folate in women of childbearing age with and without simulation of folic acid fortification. J Am Diet Assoc. 1999;99:985-988.

163. Murphy SP. Dietary Reference Intakes for the U.S. and Canada: Update on Implications for Nutrient Databases. Journal of Food Composition and Analysis 2002;15:411-417.

164. Gibson R. Principles of Nutrition Assessment. New York: Oxford University Press., 2005.

165. Truswell AS, S. K. Quantitative responses of serum folate to increasing intakes of folic acid in healthy women. Eur J Clin Nutr 1997;51:839-845.

166. Wolters M, Hermann S, Hahn A. B vitamin status and concentrations of homocysteine and methylmalonic acid in elderly German women. Am J Clin Nutr 2003;78:765-772.

167. de Bree A, Verschuren WM, Blom HJ, Kromhout D. Association between B vitamin intake and plasma homocysteine concentration in the general Dutch population aged 20-65 y. Am J Clin Nutr 2001;73:1027-1033.

168. Selhub J, Jacques PF, Rosenberg IH, et al. Serum Total Homocysteine Concentrations in the Third National Health and Nutrition Examination Survey (1991-1994): Population Reference Ranges and Contribution of Vitamin Status to High Serum Concentrations. Ann Intern Med 1999;131:331-339.

169. Selhub J, Jacques P, Rosenberg I, et al. Serum total homocysteine concentrations in the third National Health and Nutrition Examination Survey (1991-1994): population reference ranges and contribution of vitamin status to high serum concentrations. Ann Intern Med 1999;131:331:339.

170. Rasmussen K, Moller J, Lyngbak M, Pedersen A, Dybkjaer L. Age- and gender-specific reference intervals for total homocysteine and methylmalonic acid in plasma before and after vitamin supplementation. Clin Chem 1996;42:630-636.

171. Ubbink J, Becker P, Vermaak W, Delport R. Results of B-vitamin supplementation study used in a prediction model to define a reference range for plasma homocysteine. Clin Chem 1995;41:1033-7.

172. Lucock M. Is folic acid the ultimate functional food component for disease prevention? BMJ 2004;328:211-214.

173. Boushey CJ, Beresford SA, Omenn GS, AG. M. A quantitative assessment of plasma homocysteine as a risk factor for vascular disease. Probable benefits of increasing folic acid intakes. JAMA 1995;13:1049-57.

174. Omenn GS, Beresford SAA, Motulsky AG. Preventing coronary heart disease: B vitamins and homocysteine. Circulation 1998;97:421-424.

175. Ubbink J. Assay methods for the measurement of total homocyst(e)ine in plasma. Semin Thromb Hemost. 2000;26:233-241.

176. Zighetti M, Chantarangkul V, Tripodi A, Mannucci P, Cattaneo M. Determination of total homocysteine in plasma: comparison of the Abbott IMx immunoassay with high performance liquid chromatography. Haematologica. 2002;87:89-94.

177. Centers for Disease Control and Prevention. Folate status in women of childbearing age--United States, 1999. MWR Morb Mortal Wkly Rep 2000;49:962-965.

178. Centers for Disease Control and Prevention. Folate status in women of childbearing age, by race/ethnicity--United States, 1999-2000. MMWR Morb Mortal Wkly Rep 2002;13:808-810.

179. Centers for Disease Control and Prevention. Folate Status in Women of Childbearing Age, by Race/Ethnicity--United States, 1999-2000, 2001-2002, and 2003-2004. J Am Med Assoc 2007;297:943-945.

180. Lawrence JM, Bpetitti D, Watkins M, Umekubo MA. Trends in serum folate after food fortification. Lancet 1999;354:915-916.

181. Ray JG, Cole DEC, Boss SC. An Ontario-wide study of vitamin B12, serum folate, and red cell folate levels in relation to plasma homocysteine: is a preventable public health issue on the rise? Clin Biochem 2000;33:337-343.

182. Williams L, Ma iC, Edmonds L, et al. Prevalence of spina bifida and anencephaly during the transition to mandatory folic acid fortification in the United States. Teratology 2002;66:33-39.

183. De Wals P, Tairou F, Van Allen MI, et al. Reduction in neural-tube defects after folic acid fortification in Canada. N Engl J Med 2007;357:135-142.

184. Dietrich M, Brown CJP, Block G. The Effect of Folate Fortification of Cereal-Grain Products on Blood Folate Status, Dietary Folate Intake, and Dietary Folate Sources among Adult Non-Supplement Users in the United States. J Am Coll Nutr 2005;24:266-274.

185. Jacques PF, Rosenberg IH, Rogers G, et al. Serum total homocysteine concentrations in adolescent and adult Americans: results from the third National Health and Nutrition Examination Survey. Am J Clin Nutr 1999;69:482-489.

186. Pfeiffer CM, Caudill SP, Gunter EW, Osterloh J, Sampson EJ. Biochemical indicators of B vitamin status in the US population after folic acid fortification: results from the National Health and Nutrition Examination Survey 1999-2000. Am J Clin Nutr 2005;82:442-450.

187. Jacques PF, Selhub J, Bostom AG, Wilson PW, IH. R. The effect of folic acid fortification on plasma folate and total homocysteine concentrations. N Engl J Med. 1999;13:1449-54.

CHAPTER 3.

3. RATIONALE

3.1 Rationale Introduction

Research recommendations reported by IOM 2002 give high priority to some topics of research related to folate, including: "1) Estimation of folate requirements in high-risk groups for which data are limited and for which public health problems may result from deficiencies. These studies should identify and use new folate status indicators that are linked to metabolic function and traditional indices of folate status; and 2) Development of more precise and reproducible methods of analysis for the estimation of food folate and reversions of the food folate databases"(1).

3.2 Gaps in the literature

3.2.1 Dietary assessment methods

Traditional measures of food intake are lengthy, costly, and not practical for use in counseling individuals or for large population surveys (2). Accurate and objective estimates of dietary intakes are necessary to assess any effects of nutritional status in epidemiologic studies (3). Rapid, reliable, and validated dietary assessment tools are now needed in health promotion research, health promotion practice and in intervention studies. Folate intake in most epidemiological studies is assessed by using FFQ (4). The main limitation of the FFQ is the prescribed set of foods, which needs to be long enough to allows respondents to represent their diets and short enough to avoid a loss of subject motivation and decreased reliability (5). The FFQs need to be validated for each population. The previously described new tools developed to estimate folate intakes in the literature (2, 5-7) included folate rich sources and excluded foods rich in energy, making the adjustment to energy intake not possible. Adjusting dietary intakes to energy

is recommended before conduction correlation analysis (4, 8). Calculating the correlation coefficient is considered a milestone in validating the existing and the new dietary assessment methods (9, 10). The correlation between the reference method and the test method may be overestimated, if the sources of errors in these two methods are related (11). Therefore, the use of biomarkers of nutritional intake is increasingly important in dietary validation studies (11, 12). Research is needed to develop a new dietary method that comprehensively assesses the dietary intakes for the nutrient under question and energy, and to validate the new dietary tool using different approaches.

3.2.2 Folate in the database

In order to achieve valid assessments of folate status, folate food composition data needs to be expressed in the same chemical form and units as the recommendations for folate intakes. The folate values in most food databases have to be revised because the DRI for folate is expressed in μg of DFE, rather than in μg of folate which was used for the 1989 RDAs for the US and Recommended Nutrient Intakes (RNIs) for Canada (13). One μg of folate that is in food is equal to 1.0 DFE. However, a μg of folate consumed in a dietary supplement has greater activity than a μg of folate naturally occurring in food, because the monoglutamate form used for supplement is more bio-available, and 1 μg of folate in a supplement is equal to 2.0 DFE. Folate added to food as fortification is also more active than folate naturally occurring in food, but slightly less active than folate taken as a supplement, and 1 μg of fortification folate is equal to 1.67 DFE. Research is needed to update and validate the folate values in the Canadian Nutrient file 2001(CNF), version 2001b (14).

3.2.3 Impact for folate fortification in the folate intake of women at childbearing age

Beginning in 1998, the US Food and Drug Administration (FDA) and Health Canada mandated that the food supply be fortified with FA, which is achieved through the fortification of all enriched cereal-grain products (15). The enrichment level of 140 μg per 100 g of cereal-grain product was selected to increase the dietary intake of FA in the diets of women of childbearing age but prevent excessive intake among other groups in the population (16). This fortification level was estimated to provide an additional 100 μg of FA to average diet (17). Research is needed to estimate the level of FA in the diets of women of childbearing age in post-fortification.

3.2.4 The impact of fortification of food supply on folate status

No post-marketing surveillance program was mandated by the health authority after the initiation the FA fortification program, these scientists and policy makers must rely on other methods to evaluate the effectiveness of this population wide intervention. Three approaches were proposed to determine the effectiveness of FA fortification for reducing the risk of NTD in pregnant women (18): 1) Monitor NTD rates throughout Canada (ideally, this would be the best approach because it is a direct measure of the outcome of interest), 2) Estimate the absolute intake of total folate from naturally occurring sources, fortified foods, and dietary supplement in a large, random sample of the target population and determine whether those levels provide a total exposure that is consistence with a "protective dose", and 3) Use nutritional biomarkers of FA levels. These measures are important because they can provide objective measures of dietary

intake, which can be used in modeling associations of nutrient exposure and outcomes such as folate and NTDs.

To date, few studies have examined the impact of FA fortification of the food supply on folate status. Jacques and colleagues reported significant an increase in plasma folate concentration and a decrease in plasma tHcy values in Framingham offspring cohort after FA fortification (19). Others found a substantial increased in serum folate concentrations after fortification in blood specimens submitted to Kaiser Permanent's Southern California Endocrinology Laboratory were also observed by Lawrence and colleagues (20). However, because only a small proportion of the populations studied consisted of women younger than 40 years, the impact of FA fortification on folate status in the target population, women of childbearing age, could not be assessed. More research is needed to confirm whether women of childbearing age have adequate folate levels to prevent 50% of NTDs (18, 21).

One of the National Health Objectives in US for 2010 is to increase the proportion of pregnancies for which RBC folate concentration is optimum by increasing the median RBC folate concentration to 499 nmol/L among women aged 15-44 years (objective 16.16b; 52) (22). Regarding the recommended intakes, women of childbearing age should consume 400 μg/d of fortification folate plus to their recommendation (400 μg DFEs/d) in order to reduce the risk of NTDs in the infant (23, 24). Evaluating the intakes of this age group requires an extensive assessment of the DFEs contents of dietary sources and supplements. Research is needed to estimate the total folate intakes (dietary & supplement) and to compare it with special recommendations for this age group. As there are missing folate values for a number of foods in the Canadian Nutrient File database (Health Canada, 20001), and there is

limited information about the effects of different methods of food processing and preparation on the bioavailability of folate(s), it seemed appropriate that the biomarker method might by-pass both the subjectivity of food intake methods, and the incomplete information about folate content of foods. Research that combines the dietary assessment with the biomarkers is needed to assess folate status for women at childbearing age.

3.2.5 Determinants for tHcy status

Hyperhomocysteinaemia has been associated with serious pregnancy complications, including pregnancy-induced hypertension, pre-eclampsia (25), placental abruption (26), early pregnancy loss and NTDs (27, 28). Furthermore, many studies have shown that an elevated concentration of tHcy is an independent risk factor for coronary ischemic disease, peripheral vascular disease, and stroke (28-31). Recently, elevated tHcy in women was associated with reduced bone mineral density and increased risk of osteoporosis (28, 32, 33). Various dietary and lifestyle factors influence tHcy (34). Associations between dietary or lifestyle factors and tHcy have been investigated mainly middle-aged (35-37) and elderly (38, 39) population, it is almost limited for the healthy women at childbearing age.

3.3 Objectives and General Hypotheses

3.3.1 Objectives to address weaknesses in dietary assessment

Hypothesis #1 The proposed dietary assessment method (FCM), compared to the 3DFR will, show more validity using different approaches.

Based on this hypothesis, the objectives for the study presented in chapter 4 were to:

1. introduce the new dietary assessment method: FCM
2. use 3DFR as a standard method for relative validity testing
3. estimate the folate and vitamin B_{12} intakes using the two different methods
4. estimate the absolute and the energy adjusted intake for folate and vitamin B_{12}
5. examine the association between the corresponding bio-markers and the two dietary assessment methods for the intake of folate and vitamin B_{12}
6. evaluate the VC, which defined as the associating between the intake estimated using FCM with the "true" individual intake
7. quantify the extent to which the two dietary methods produce similar rankings of study participants according to the intake of individual nutrients for folate and vitamin B_{12} with their corresponding biomarkers
8. describe the performance characteristics of the FCM as new dietary assessment method.

3.3.2 Objectives to assess folate intake

Hypothesis #2 Manitoba childbearing women are achieving the special recommendation for FA intake (400 μg FA/d) as stated by Institute of Medicine (1).

Hypothesis 3# FA fortification policy is succeeding in delivering 100 μg FA/day for women of childbearing age.

Based on these hypotheses, the objectives for the study presented in chapter 5 were to:

1. estimate the folate intake for women at childbearing age using an updated database that reflects the FA fortification and the different bioavailability od dietary, supplements and fortified food
2. predict the FA-fortification levels
3. determine the prevalence of inadequate and excessive intake

3.3.3 Objectives to assess folate biochemical status

Hypothesis 4# Manitoba childbearing age women are achieving the RBC folate value that associated with low NTD risk.

Based on this hypothesis, the objectives for the study presented in chapter 6 were to:

1. assess the biochemical folate status
2. compare the folate biochemical indexes with the corresponding biomarker cut-off points

3.3.4 Objectives for addressing homocysteine status

Hypothesis 5# The determinants of tHcy are likely to vary among different age, sex, and nutritional status.

Based on this hypothesis, the objectives for the study presented in chapter 6 were to:

1. determine the reference values of homocysteine levels from a sample of healthy women at childbearing age

2. investigate the association between tHcy and its dietary determinants (estimated protein, methionine, folate, vitamin B_6, and vitamin B_{12}), some lifestyle factors (physical activity, alcohol, caffeine), and biochemical determinants (serum folate, plasma B_6, serum B_{12} and RBC folate) in a group of women of childbearing age (18-25 y).

3.4 References

1. Institute of Medicine. Dietary reference intakes: for thiamin, riboflavin, niacin, vitamin B6, folate, vitamin B12, pantothenic acid, biotin. Washington, DC: National Academy Press., 2000.
2. Hickling S, Knuiman M, Jamrozik K, Hung J. A rapid dietary assessment tool to determine intake of folate was developed and validated. J Clin Epidemiol 2005;58:802-808.
3. Sheila AB. Biomarkers in nutritional epidemiology. Public Health Nutr 2002;5:821-827.
4. McKeown NM, Day NE, Welch AA, et al. Use of biological markers to validate self-reported dietary intake in a random sample of the European prospective investigation into cancer United Kingdom Norfolk cohort. Am J Clin Nutr 2001;74:188-196.
5. Pufulete M, Emery P, Nelson M, Sanders T. Validation of a short food frequency questionnaire to assess folate intake. Br J Nutr 2002;87:383-390.
6. Clifford AJ, Noceti EM, Block-Joy A, Block T, Block G. Erythrocyte folate and its response to folic acid supplementation is assay dependent in women. J Nutr 2005;135:137-143.
7. Yen J, Zoumas-Morse C, Pakiz B, Rock C. Folate intake assessment: validation of a new approach. J Am Diet Assoc 2003;103:991-1000.
8. Bingham S, Day N. Using biochemical markers to assess the validity of prospective dietary assessment methods and the effect of energy adjustment. Am J Clin Nutr 1997;65:1130S-1137S.

9. Kaaks R. Biochemical markers as additional measurements in studies of the accuracy of dietary questionnaire measurements: conceptual issues. Am J Clin Nutr 1997;65:1232S-1239.

10. Kaaks R, Ferrari P. Dietary intake assessments in epidemiology: can we know what we are measuring? Ann Epidemiol 2006;16:377-380.

11. Verkleij-Hagoort A, de Vries J, Stegers M, Lindemans J, Ursem N, Steegers-Theunissen R. Validation of the assessment of folate and vitamin B(12) intake in women of reproductive age: the method of triads. Eur J Clin Nutr 2006;Dec 13; [Epub ahead of print].

12. Kabagambe EK, Baylin A, Allan DA, Siles X, Spiegelman D, Campos H. Application of the method of triads to evaluate the performance of food frequency questionnaires and biomarkers as indicators of long-term dietary intake. Am J Epidemiol 2001;154:1126-1135.

13. Council NR. Recommended Dietary Allowances. 10th ed. Washington, DC: National Academy Press, 1989.

14. Health Canada. Food Program. Canadian nutrient file (CNF) {cited 2005}. Available from:http://www.hc.sc.gc.cs/food-aliment/ns-sc/nr-rn/surveillance/cnf-fcen/e_cnf_download.html., 2001.

15. Ray JG, Vermeulen MJ, Boss SC, Cole DEC. Increased red cell folate concentrations in women of reproductive age after Canadian folic acid food fortification. Epidemiology 2002;13:238-240.

16. Boushey CJ, Edmonds JW, Welshimer KJ. Estimates of the effects of folic-acid fortification and folic-acid bioavailability for women. Nutrition 2001;17:873-879.

17. Institute of Medicine. Dietary reference intakes: folate, other B vitamin and choline. Washington, DC: National Academy Press, 1998.

18. Neuhouser ML, Beresford SAA. Folic acid: are current fortification levels adequate? Nutr 2001;17:868-872.

19. Jacques P, Selhub J, Bostom A, Wilson P, Rosenberg I. The effect of folic acid fortification on plasma folate and total homocysteine concentrations. N Engl J Med 1999;13:1449-54.

20. Lawrence JM, Bpetitti D, Watkins M, Umekubo MA. Trends in serum folate after food fortification. Lancet 1999;354:915-916.

21. Caudill MA, Le T, Moonie SA, Esfahani ST, Cogger EA. Folate status in women of childbearing age residing in southern California after folic acid fortification. J Am Coll Nutr 2001;20:129-134.

22. Pfeiffer CM, Caudill SP, Gunter EW, Osterloh J, Sampson EJ. Biochemical indicators of B vitamin status in the US population after folic acid fortification: results from the National Health and Nutrition Examination Survey 1999-2000. Am J Clin Nutr 2005;82:442-450.

23. Gucciardi E, Pietrusiak M-A, Reynolds D, Rouleau J. Incidence of neural tube defects in Ontario, 1986-1999. Canadian Medical Association Journal 2002;167:237-240.

24. Honein M, Paulozzi L, Mathews T, Erickson J, Wong L. Impact of folic acid fortification of the US food supply on the occurrence of neural tube defects. Journal of American Medical Association 2001;286:2236.

25. El-Khairy L, Vollset SE, Refsum H, Ueland PM. Plasma total cysteine, pregnancy complications, and adverse pregnancy outcomes: the Hordaland Homocysteine Study. Am J Clin Nutr 2003;77:467-472.

26. Holmes VA. Changes in haemostasis during normal pregnancy: does homocysteine play a role in maintaining homeostasis? Proc Nutr Soc 2003;62:479-493.

27. Tamura T, Picciano MF. Folate and human reproduction. Am J Clin Nutr 2006;83:993-1016.

28. Refsum H, Nurk E, Smith AD, et al. The Hordaland homocysteine study: a community-based study of homocysteine, its determinants, and associations with disease. J Nutr 2006;136:1731S-1740.

29. Herrmann M, Muller S, Kindermann I, et al. Plasma B vitamins and their relation to the severity of chronic heart failure. Am J Clin Nutr 2007;85:117-123.

30. Homocysteine Studies Collaboration. Homocysteine and risk of ischemic heart disease and stroke: A meta-analysis. JAMA 2002;288:2015-2022.

31. Refsum H, Smith AD, Ueland PM, et al. Facts and recommendations about total homocysteine determinations: An expert opinion. Clin Chem 2004;50:3-32.

32. Gjesdal CG, Vollset SE, Ueland PM, et al. Plasma total homocysteine level and bone mineral density: the Hordaland homocysteine study. Arch Intern Med 2006;166:88-94.

33. Boushey CJ, Beresford SA, Omenn GS, AG. M. A quantitative assessment of plasma homocysteine as a risk factor for vascular disease. Probable benefits of increasing folic acid intakes. JAMA 1995;13:1049-57.

34. Rasmussen LB, Ovesen L, Bulow I, Knudsen N, Laurberg P, Perrild H. Folate intake, lifestyle factors, and homocysteine concentrations in younger and older women. Am J Clin Nutr 2000;72:1156-1163.

35. de Bree A, Verschuren WM, Blom HJ, Kromhout D. Association between B vitamin intake and plasma homocysteine concentration in the general Dutch population aged 20-65 y. Am J Clin Nutr 2001;73:1027-1033.

36. Mennen LI, de Courcy GP, Guilland J-C, et al. Homocysteine, cardiovascular disease risk factors, and habitual diet in the French Supplementation with Antioxidant Vitamins and Minerals Study. Am J Clin Nutr 2002;76:1279-1289.

37. Nygard O, Refsum H, Ueland P, Vollset S. Major lifestyle determinants of plasma total homocysteine distribution: the Hordaland Homocysteine Study. Am J Clin Nutr 1998;67:263-270.

38. Selhub J, Jacques P, Wilson P, Rush D, Rosenberg I. Vitamin status and intake as primary determinants of homocysteinemia in an elderly population. JAMA 1993;270:2693-8.

39. Koehler KM, Baumgartner RN, Garry PJ, Allen RH, Stabler SP, Rimm EB. Association of folate intake and serum homocysteine in elderly persons according to vitamin supplementation and alcohol use. Am J Clin Nutr 2001;73:628-637.

CHAPTER 4

4. VALIDATION OF A FOOD CHOICE MAP AND THREE DAY FOOD RECORD TO ASSESS FOLATE AND VITAMIN B_{12} INTAKE IN COLLEGE-AGED WOMEN [1]

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[1] Submitted

Running Title: Validation of a Food Choice Map

Validation of a food choice map with a three day food record and serum values to assess folate and vitamin B_{12} intake in college-aged women [1 2]* ^.

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KEY words: Validation, Food Choice Map (FCM), Three Day Food Record (3DFR), folate, Dietary folate equivalent (DFE), vitamin B-12 and Validity Coefficient (VC).

4.1 Abstract

Background: Estimate of dietary folate and vitamin B_{12} intakes are currently of considerable interest, but a reliable assessment tool that is faster than the ones currently available would better suit everyday health promotion activities

Objective: To validate a new assessment technique for estimating folate and B_{12} intakes (Food Choice Map - FCM) using serum folate and B_{12} concentrations in a group of 95 females, aged 18-25 years.

Design: The FCM tool was used in a cross-sectional study design to estimate the usual intake of folate and B_{12} intake of each participant. Immediately thereafter, blood samples were taken to determine serum folate and vitamin B_{12} concentrations. Subsequently, three day food records (3DFR) were completed during of three successive weeks and used as a reference method. Using method of triads, validity coefficients were calculated.

Results: There was no significant difference between two methods in the correlations with serum values. FCM obtained folate intakes ($r = 0.43$, $P<0.01$) exhibited a similar and moderate association with serum folate as did 3DFR obtained folate intakes ($r = 0.39$, $P<0.01$). Similarly, vitamin B_{12} intakes obtained from both techniques showed a modest association with serum vitamin B_{12} (FCM: $r = 0.40$, $p<0.01$, 3DFR: $r = 0.44$, $P <0.01$). However, the validity coefficient (VC) was the highest for the FCM for both nutrients - for folate: FCM: VC = 0.97; 3DFR: VC =0.79, and - for vitamin B_{12}: FCM: VC = 0.95; 3DFR: VC= 0.85.

Conclusion: The correlation results suggest that both the FCM and 3DFR are equally valid measures of assessing the folate and vitamin B_{12} intake of young women.

However, the validity coefficients suggest that FCM was found to be a reliable tool to estimate folate and vitamin B_{12} in women of reproductive age. Larger validation studies that include men and other age groups would be needed to further characterize the applicability of the FCM.

4.2 Introduction

The water soluble vitamin folate plays critical roles in intermediary metabolic pathways. Folate is required for deoxyribonucleic acid (DNA) synthesis, for single carbon transfer reactions, including acting as a co-factor in the metabolic pathways of methionine, serine and glycine (1) Clinical symptoms of folate deficiency include megaloblastic anemia and intestinal villous atrophy (2). With respect to sub-clinical deficiency states, poor folate status in women is associated with an increased risk of neural tube defects (NTD) (3) and an increased risk of early spontaneous abortion (4). Supplements of folic acid (FA) have been shown to decrease the risk for neural tube defects (5). Beyond the association with reproductive health, poor folate status has been linked to an increased risk for cardiovascular disease (6), through a mechanism(s) that may involve the atherogenic amino acid homocysteine (7) which is derived from methionine. Additionally, folate deficiency has been linked to the development of certain cancers (8, 9) and, more recently, an inverse association between depression and folate status has been demonstrated in clinical studies (10).

Vitamin B_{12} plays a critical role in key pathways central to energy and amino acid metabolism in animal cells, primarily in its function as a co-factor for the two enzymes methionine synthase and methylmalonyl-CoA mutase (11). Dietary vitamin B_{12} is traditionally provided through the consumption of foods of animal origin, including meat, dairy products and eggs, and is not a normal constituent of plant-based foods (12). As such, vitamin B_{12} intake has the potential to be sub-optimal for individuals restricting foods of animal origin (13). With respect to reproductive health, poor vitamin B_{12} status has been observed in families with NTDs (14), and several

studies have reported decreased vitamin B_{12} levels in the circulation or amniotic fluid of mothers of children with NTDs (15, 16).

With respect to dietary assessment methods, several methods are available, each with their own strengths and limitations. The use of food records requires high participant motivation, training and lengthy data handling, including the maintenance of food lists and portion sizes (17). A major concern is that food records may cause participants to alter their food intake and therefore records may not reflect the participant's usual intake (18). Twenty four hour recall interviews are prone to interviewer bias, need skilled interviewers and need to be repeated at least three times with the same participant, which can be costly for large studies (19).Owing to their ease of a administration and low burden on the subjects, the assessment of dietary intake for folate and vitamin B_{12} in epidemiological studies is often assessed by Food Frequency Questionnaire (FFQ) (20). The chief limitation of the FFQ, however, is that serious errors can occur if the foods governing inter-individual differences in intake of certain nutrients are omitted from the list. Folate is particularly prone to such error, because it is derived from a variety of foods of both animal and plant origin, not all of which can be included in an FFQ (21). Foods that contribute to inter-individual differences may fail to be included in food lists, potentially confounding estimation of folate intake by the FFQ (21). A rapid, reliable and valid dietary assessment method is needed.

The proposed Food Choice Map (FCM) (Sevenhuysen, registered copyright) is a diet assessment interview that is quantified in a process similar to that used for the 24-hour recall, with a structure designed to capture habitual intake. The structure of the FCM interview helps to reduce errors from participant memory lapses and the automated data entry is inexpensive compared to the process of coding food records. The food list

used in the FCM interview is large compared to the number of foods recommended to represent diets of the subjects (22) which avoids the limitations associated with FFQ food. In this paper we compare the estimated nutrient intakes obtained from the FCM and 3 Day food record (3DFR) methods with one another, and with biomarkers of serum folate and vitamin B_{12} in young, college-aged women. The principal aim of this study was to position the FCM as a validated tool for the assessment of habitual intake, and to assess the extent to which the FCM agrees with established 3DFR methodology in the ranking of study participants according to their intakes of folate and vitamin B_{12}. The methods comparison is important for future epidemiological analyses of dietary effects on health.

4.3 Participants and methods

4.3.1 Participants

Ninety-seven women, aged 18-25 years, were recruited from the student population of University of Manitoba, Winnipeg, Canada. The current population was chosen due to the fact that these women can be at risk for poor dietary habits, self-imposed restriction of energy secondary to concerns about weight gain, and the potential also exists for unplanned pregnancies (23). Participants were recruited from October 2004 to March 2005. Exclusion criteria included: current pregnancy, pregnancy lasting more than 20 weeks in the year before the scheduled clinic visit, lactating, and the use of medications known to interfere with folate metabolism (e.g. phenytoin, sulfasalazine, phenobarbital, primidone, and cimetidine). Other exclusion criteria included cancer, gastrointestinal disease, and history of renal insufficiency, diabetes mellitus, and other chronic diseases. Those who were taking oral contraceptives or folate supplements were not excluded. Two participants were excluded from the study for kidney and gastrointestinal disease. Participants were told that the study aimed to measure the adequacy of nutrient intake, but they were not told about the focus on folate and B_{12} intakes or the comparison of assessment methods. The explanations met requirements set out by the Research Ethics Committee of the University of Manitoba which approved the research protocol. Informed written consent was obtained from each participant.

4.3.2 The Food Choice Map

The nature of the FCM differs from most existing methods because respondents are asked to recall their general food behaviors over a week, which yields meal times

and meal composition with alternative and substitute foods, rather than recalling the specific foods eaten at every instance of eating in a set time period. The result is a pattern of eating characterized by the frequencies of meal times, frequencies of foods by meals and throughout the week. These frequencies demonstrate the relative contributions of foods, including alternatives and substitutes, to overall nutrient intakes. They also show the respondent's uses of food and their possible fit with recommended diet structures.

The FCM consists of a food picture board and a food map board, with linkages to a nutrient database, the FCM nutrient database. The food picture board carried 91 magnetic, 1.3 cm^2 food pictures that were used to represent the food choices of participants. The choice of 91 pictures were grouped for easy retrieval during the interview using 8 categories related to familiar food groups (example: dairy, fruit) or cultural use (example: drink) **Figure 4.9.1**. Food categories on the FCM food picture board were designed to facilitate the identification of a variety of foods, including processed foods and menu items containing a variety of food groups (ie: casseroles), and as such do not specifically correspond to food groupings of Canadian (Canada's Food Guide) or US (My Pyramid) dietary guidance materials. The eight groupings included: Drink, Fruit, Vegetable, Extra (i.e. cookies), Mixed (i.e. processed foods, casseroles), Dairy, Staples (i.e. grains, grain products and legumes), and Meat/Fish. The barcode on each food picture, when scanned into a portable laptop computer, displays several food items associated with the picture. The interviewer is able to select the best match for the food item reported from the likely alternatives available in the program database. The 1462 food items listed in the FCM program database represent most of the foods reported by this population in previous studies and included a number of international

and ethnic foods to reflect the multicultural diets of people (24, 25). Both the participant and the interviewer jointly place the pictures that represent the foods consumed by the participant on the food map board. Vertical placement of the food pictures represents time of day of consumption (early, snack 1, mid day, snack 2, end of day, late snack). Horizontally, the placement reflects the number of times the food was eaten **Figure 4.9. 2.** All codes for frequency, time of day, food category, food item, and portion size code are entered electronically using a pen-style bar code scanner which generates real-time results. To start the interview, the participant is asked to consider a food they often eat and pictures are placed on the food map board according to the usual time of day and the usual consumption frequency per week reported by the participant. Follow-up questions build a record of the meal and snacks. Participants are encouraged to participate in creating a visual map of food frequencies by placing food symbols (a generic picture of the food) on a map with grid lines (26). The interview typically requires 20 minutes to place the food pictures on the map and 30 minutes to identify each food in the database and allow the participant to estimate portion size which is also scanned into the program. Participants estimate the amounts consumed from the *Portion Photos of Popular Foods* (27), which contains life-size photos of many food items with several photos of the same food item depicting different portion sizes (small, medium and large portions, or different measures such as cups, spoons and scoops). Where a food is not depicted in the book, participants are referred to the photographs of foods that had similar appearance and density to the food in question. The participants are also prompted to report weights or volumes of frequently consumed packaged items if they know them.

4.3.3 Dietary assessment

After confirming the inclusion/exclusion criteria for study participants, participants completed the FCM interview as described above. The interviewer conducting the FCM was trained by a registered dietitian and interviews took place in a confidential space. Results of a pilot study (data not shown) were used to standardize interview techniques and questions. Participants were given a self-administered 3DFR. Each participant was trained to accurately record the types and quantities of all food and beverages consumed, including brand names and methods of food preparation. The 3DFR were reviewed with each participant to insure the competence of the recorded data. Care was taken to ensure that there was proportional distribution of week and weekend days for the dietary records among participants. Dietary intakes were calculated using the FCM program (Sevenhuysen, registered copyright).

The 3DFR estimates were used for comparison with the FCM estimates because comparison with estimates from an FFQ was not appropriate for four reasons. First, the FCM determined the usual food eaten in a usual week. The assessment relied on the frequencies of foods, and their substitutes and alternatives, as well as the amounts of foods eaten at common meals or snack times. There was no food list in the FCM, as there is in the FFQ, which allowed respondents to represent their diets more comprehensively than is possible in the FFQ. Second, in the FCM, food amounts were established in a way commonly used for 24-hour recalls, rather than the restricted choices for food amounts included in semi-quantified FFQs. Third, in an FFQ designed to estimate folate intakes, it is difficult to exclude under-reporters or adjust folate values for energy intakes, because such FFQs list only foods rich in folate and exclude energy-

rich food sources such as folates (17). The energy adjustment of nutrient intakes prior to correlation analysis is recommended by (20, 28) to minimize the underreporting of intakes. Fourth, in validation studies, errors in the reference dietary method should be independent of those in the test method and also of the "true" intake. The 3DFR was different from the FCM, while the FFQ was a method similar to the FCM because both rely on memory (29).

Information about dietary supplements obtained from the 3DFR and the interview was not used; rather, participants were asked to bring to the clinic visit any nutritional supplements they were currently taking. Information was collected from the supplement container-label by the research staff. Information included the type of the supplement used, the brand name, the dosage, and average use during the previous year and the frequency of use during the week before the survey were obtained during the interview. Average daily intakes of supplemental folate and B_{12} were calculated from the responses and were added to the participant's dietary intakes of these vitamins for both the FCM and 3DFR.

4.3.4 Nutrient assessment

The FCM was designed to estimate nutrient intakes, including folate, B_{12} and energy. As it is recommended to adjust to energy before conducting correlation or regression analyses, the FCM allowed us to adjust nutrient intakes for energy, while the alternative FFQ or other tools developed particularly to assess folate with foods rich in folate and excluding energy rich food sources did not allow this adjustment to be made (30, 31). The FCM was used for vitamin B_{12} estimates for similar reasons. In order to achieve valid assessments of folate status, folate food composition data needs to be

expressed in the same chemical form and units as the recommendations for folate intakes. The folate values in most Food Composition Databases (FCB) have to be revised because the DRI for folate is expressed in μg of Dietary folate equivalent (DFE), rather than in μg of folate which was used for the 1989 Recommended Dietary Allowances (RDAs) for the US and Recommended Nutrient Intakes (RNIs) for Canada (32). The database used with the FCM reflects the fortification and differences in bioavailability between FA and natural folate (NF)). We updated the data base by converting all forms of folate into DFEs.

According to the Institute of Medicine report (33), FA is considered to be more bioavailability than NF. Food-composition values had to be adjusted so that the greater bioavailability of FA than NF. Canadian Nutrient File 2001 (CNF) (34)food composition data were used to provide total folate contents of foods and synthetic folic acid (SFA). NF, which is equivalent to DFE is not available in the CNF. Estimates of NF were calculated by subtracting the SFA from the total folate content values, then the SFA contents of each food were multiplied by 1.7 which is the recommended correction in the IOM report (33) because synthetic folic acid is considered to be more bioavailable than NF. The resulting total folate values were then expressed as DFE, which could be used with the recommended of folate intakes for this age group.

4.3.5 Anthropometric measurements

Anthropometric measurements were taken during the clinic visit (same day of blood sampling). The measurements were taken between 08:00 and 11:00 a.m., after a 12-h fast, with subjects in light clothing and no shoes. Body weights were measured using digital scale (Healthometer) to the nearest 0.1kg. Heights were measured by a

wall-mounted stadiometer (Sera 242) to the nearest millimeter. Body Mass Index was calculated using the standard equation (kilograms per meters squared). Waist circumferences were measured in the standing position, with measurements obtained midway between the lateral lower rib margin and the iliac crest. Measurements were repeated at least 3 times and the averages were used.

4.3.6 Folate and vitamin B_{12} assays

Participants attended an early morning clinic 8:30-10:30 visit after an overnight fast. Blood was collected via venipuncture from participants into evacuated containers with and without anticoagulant. For serum folate and vitamin B_{12} measurements, blood was collected in vacutainers containing no anticoagulant, left to clot for 30 min at room temperature and then placed in ice. All samples on ice were transported within 2 hr to the laboratory and centrifuged at 1800g for 15 min. Serum samples were transferred to plastic vials and stored at -80°C until analyzed. Serum folate and vitamin B_{12} concentrations were measured using a dual isotope kit (Quantaphase Folate/B_{12} Radioassay, bio-Rad Laboratories, Mississauga, Canada). The accuracy of these radioassay methods was checked through serial replication of three levels of control sera (Lyphocheck, Bio-Rad Laboratories, Mississauga, Canada). All control sera fell within the manufacture's stated limit (35).

4.4 Statistical analysis

Statistical analyses were performed by using statistical software Number Cruncher Statistical System (NCSS, 2000 Kaysville, Utah) (36) using a significant probability level of 5%. Biochemical indices and dietary intakes for folate and vitamin B_{12}, as well as biochemical indices of supplement users vs. supplement non-users were normalized using logarithmic transformations. Two calculations of dietary intakes were used: 1) folate and B_{12} intakes of every complete record as reported, 2) folate and B_{12} intakes of every complete record adjusted for energy intake. Adjustment of nutrient intakes to the mean energy intake of the group produces an estimate of dietary composition rather than of absolute intake. The resulting assessment of nutrient intake is independent of body size and activity level, as well as of energy intake itself (18, 37), which allows a comparison of the accuracy of the FCM against that of 3DFR without influence of individual differences in total amounts eaten. Energy-adjusted nutrient intakes were computed as the residual for a participant from the regression model with nutrient intake as the dependent variable and total caloric intake as independent variable. Since the residuals have a mean of zero and include negative values, the expected nutrient intake for the mean caloric intake of the study population was added as a constant (38).

Pearson correlations (r) were generated between each of the nutrient intakes and their respective biochemical indices (39). Partial correlations between nutrient intakes and biochemical measures were determined after adjusting for energy.

The ability of the FCM and the 3DFR to correctly classify participants into quartiles of serum folate or B_{12} levels was determined by assigning participants to

quartiles corresponding to their serum concentrations. Similarly, participants were assigned separately to quartiles of their folate and B_{12} intakes. Participants where considered misclassified if their nutrient intake quartile did not match their respective serum quartile. The proportions of correctly classified and misclassified participants are compared in a 4 x 4 table for each of folate and B_{12} using a chi-square goodness of fit test with 2 degree of freedom to test for the ability of FCM and 3DFR to correctly classify participants for each of the two nutrients.

The validity coefficient (VC) of the FCM was estimated from a triangular comparison of the correlation coefficients between FCM, reference (3DFR) and biochemical measurements with the method of triads. This method defines a hypothetical true intake of a nutrient. The VC is calculated using the correlations between the true intake and a dietary assessment method from the two different dietary methods and the biomarker (40, 41). Several studies have used the method of triads to validate food frequency questionnaires (17, 42, 43)

4.5 Results

Characteristics of the participants are shown in **Table 4.8.1**. Median energy and nutrient intakes expressed on an absolute and energy adjusted basis are presented in **Table 4.8.2**. Based on the intakes of all participants, including supplement users and nonusers, the energy adjusted median intake for folate determined by the FCM was 1.1 times that determined by the 3DFR ($P<0.05$). There was relatively good agreement between median vitamin B_{12} intakes assessed by FCM (4.6 μg/d) vs. the 3DFR (3.6 μg/d)($P>0.05$). The median and range of intakes of supplemental folic acid in this sample was 680 DFE with a range of 73-1700 DFE per day and of vitamin B_{12} was 10 µg with a range of 1.7- 50 ug per day.

Median biochemical levels are presented in **Table 4.8.3**. Using a cut-off value of 7 nmol/L (3 ng/ml) serum folate and 150 pmol/L (200 pg/ml) for serum vitamin B_{12} (44), none of the participants had serum folate concentrations in the deficient range, while one participant had serum B_{12} in the deficient range. Supplement users had significantly higher serum folate and vitamin B_{12} concentrations than supplement non-users.

The correlations between serum folate concentrations and folate intakes are summarized in **Table 4.8.4**. Based on supplement users and non users, serum folate concentrations were significantly correlated with the folate intakes obtained from either the FCM ($r = 0.43$, $p<0.01$) or the 3DFR ($r =0.39$, $p<0.01$). Similarly, serum vitamin B_{12} concentrations were significantly correlated with B_{12} intakes obtained from either method (FCM, $r =0.40$ $p<0.01$; 3DFR, $r =0.44$, $p<0.01$). Both sets of correlations were significant irrespective of whether supplement users were included or excluded from the analysis. Comparing the correlations of serum values with the two sets of intake data

obtained from the FCM and 3DFR methods, it was found that these correlations were not significantly different whether supplement users were included or not.

The ability of the dietary methods to classify individuals into the correct quartile of blood vitamin concentrations is shown in **Table 4.8.5**. The FCM and 3DFR performed similarly and at least 74% of folate or vitamin B_{12} intakes were either correctly or closely classified when intakes from supplements were included in the analysis. Removing supplement users generally decreased the number of correctly classified individuals.

The method of triads was used to calculate the VC of the two dietary assessments of folate and vitamin B_{12} intakes with the corresponding biomarker indices. The VC for folate in the FCM (0.97) was higher than for 3DFR (0.79). Similar observations were made for vitamin B_{12}, with the VC for the FCM (0.95) higher than that observed for the 3DFR (0.85). Generally, the VCs for the two dietary methods were higher than for either of the biomarkers. We did not use the Pearson correlation between the two dietary methods during the validity process. The function of the latter is to measure the strength of the relationship between two variables, but not the agreement between them (45). The correlation between dietary methods was calculated for folate ($r=0.87$, $P< 0.001$) and vitamin B_{12} ($r =0.81$, $p<0.001$) and was used only for the triads calculation.

4.6 Discussion

Folate and vitamin B_{12} intakes estimated using the FCM were in a good agreement with the intakes estimated by the 3DFR. When the VC's of the three measures of folate status, the serum folate, intake assessed by FCM and intake assessed by 3DFR, were determined using the method of triads (42, 43), it was found that that the VC of the FCM was highest of the three measures. The same result was seen for measures of B_{12} status. The VCs obtained for the FCM exceeded the VC for the Food Frequency Questionnaire (0.69) and 7DFR (0.44) when these were used to assess folate intakes in comparison to serum folate in women aged 22-65 y (17). The triad technique assumes that associations between the three measurements are linear and that their measurements of random error are mutually independent (46, 47). This may not be entirely valid because the FCM and the 3DFR may have some sources of error in common. However, the effect of this possible interdependence is seen as negligible because the methodologies of the FCM and 3DFR are different.

There was generally good agreement between energy-adjusted nutrient intakes using either the FCM or 3DFR, whereas the estimate of absolute intake differed markedly. Median daily intakes for folate (505 vs. 459 DFE/d) and vitamin B_{12} (4.2 vs. 3.6 μg/d) estimated from the FCM were significantly higher than those obtained from the 3DFR. There are several explanations. First, the use of 3DFR may have caused study participants to alter and underreport their usual intake. Second, the FCM method is a face to face interview while the 3DFR is a self-administered method, and interaction with the interviewer can help the participant remember foods, snacks or meals, as well as portion sizes. The 3DFR portion size estimation is open-ended for each item

consumed. Third, the probing, memory aids and the computer-assisted identification of foods during the FCM interview may have helped in capturing more details in the FCM record than in the 3DFR record. Fourth, the FCM examines consumption over 7 days compared to the 3 days in the 3DFR.

In this study energy intakes did vary between the two intake assessment methods (FCM = 2085 kcal/d; 3FDR = 1875 kcal/d) suggesting that these explanations can satisfactorily account for the different estimates of folate intakes as determined by the FCM and the 3DFR. The reported energy intake for females aged 18-34 y in a national survey of adult Canadian were 1998 Kcal/d (48), which is closer to the intakes obtained with the FCM.

Results from this study suggest that both FCM and 3DFR are valid measures for assessing the folate intakes of young women aged 18-25 y. Using serum folate concentrations as the sole biochemical criterion, it appears that the FCM was a reliable tool to assess the folate intakes of young women. The correlation coefficient between folate intakes estimated using the FCM and serum folate was 0.43 vs. 0.39 for the 3DFR. Green and his colleagues reported a correlation of 0.48 for Food Frequency Questionnaire (FFQ) and 0.65 for the 3DFR (adjusted for the ratio of inter-participant variation) between folate intake and serum folate in a group of females aged 16-19 y (35, 49). For the FCM data, it was not possible to adjust for day to day variation (inter-participant variation) so unadjusted correlations were used in this study. This may explain the lower correlation values in this study compared with the previously published work (22). In this study, the unadjusted FCM correlation for folate (0.43) is similar to the unadjusted correlation for folate estimated by using the FFQ (0.45) in Kabagambe et al (42). Results from this study also suggest that both the FCM and 3DFR

are valid measures of assessing the vitamin B_{12} intakes of young women aged 18-20 y when both supplement users and nonusers were included in the analysis. The correlation between vitamin B_{12} intakes as determined by FCM ($r=0.40$; $p<0.001$) and a 3DFR ($r=0.44$; $P<0.001$) and serum vitamin B_{12} were similar, and both correlation estimates were higher than that estimated using FFQ for vitamin B_{12} ($r = 0.3$) by Kabagambe et al (42). Our results are consistent with those of Green and colleagues who observed a correlation (adjusted for the ratio of inter-participant variation and energy adjusted) of 0.35 between vitamin B_{12} intakes estimated form a 3DFR and serum vitamin B_{12} concentrations (49).

These findings suggest that both FCM and 3DFR are valid measures of assessing folate and vitamin B_{12} intakes in females aged 18-25 y. On the basis of their ability to correctly assign participants to their respective quartile of serum folate or serum vitamin B_{12}, both methods performed similarly. The low participant burden and ease of administration make the FCM a more attractive method than the 3DFR for the determination of usual folate and vitamin B_{12} intakes in women aged 18-25 y.

The current study has provided evidence that the FCM, a new dietary assessment tool, is a valid instrument for assessing dietary intakes among adult females. Further testing is necessary to determine the impact that specific variables may have on its ability to yield valid dietary information, including testing amongst groups differing in socioeconomic status, gender, and age, and for different nutrient classes.

4.7 References

1. Melse-Boonstra A, de Bree A, Verhoef P, Bjorke-Monsen AL, Verschuren WMM. Dietary monoglutamate and polyglutamate folate are associated with plasma folate concentrations in dutch men and women aged 20-65 years. J Nutr 2002;132:1307-1312.
2. Kim Y-I. Role of folate in colon cancer development and progression. J Nutr 2003;133:3731S-3739.
3. Czeizel AE. Prevention of the first occurrence of neural-tube defects by periconceptional vitamin. N Engl J Med 1992;327:1832-1835.
4. George L, Mills JL, Johansson AL, et al. plasma folate levels and risk of spontaneous abortion. JAMA. 2002;16:1867-1873.
5. Czeizel AE. The primary prevention of birth defects: Multivitamins or folic acid? Int J Med Sci 2004;1:50-61.
6. Fairfield K, Fletcher R. Vitamins for chronic disease prevention in adults. JAMA 2002;287:3116-3126.
7. El-Khairy L, Vollset SE, Refsum H, Ueland PM. Plasma total cysteine, mortality, and cardiovascular disease hospitalizations: the Hordaland homocysteine study. Clin Chem 2003;49:895-900.
8. Kim Y. folate and carcinogenesis: evidence, mechanisms, and implications. J Nutr Biochem 1999;10:66-88.
9. Choi SW, Mason JB. Folate status: effects on pathways of colorectal carcinogenesis. J Nutr 2002;132:2413S-2418.

10. Bjelland I, Tell GS, Vollset SE, Refsum H, PM. U. Folate, vitamin B12, homocysteine, and the MTHFR 677C->T polymorphism in anxiety and depression: the Hordaland Homocysteine Study. Arch Gen Psychiatry. 2003;60:618-626.

11. Cravens DD, Nashelsky J, RC. O. How do we evaluate a marginally low B12 level? The Journal of Family Practice 2007;56:62-63.

12. Bailey L. Folate and vitamin B12 recommended intakes and status in the United States. Nutr Rev. 2004;62:S14-20.

13. Stabler SP, Allen R. Vitamin B12 deficiency as a worldwide problem. Annu Rev Nutr 2004;24:299-326.

14. Wilson A, Platt R, Wu Q, et al. A Common Variant in Methionine Synthase Reductase Combined with Low Cobalamin (Vitamin B12) Increases Risk for Spina Bifida. Mol Genet Metab 1999;67:317-323.

15. Steen MT, Boddie AM, Fisher AJ, et al. Neural-tube defects are associated with low concentrations of cobalamin (vitamin B12) in amniotic fluid. Prenatal Diagnosis 1998;18:545-555.

16. Ronnenberg AG, Goldman MB, Aitken IW, Xu X. Anemia and Deficiencies of Folate and Vitamin B-6 Are Common and Vary with Season in Chinese Women of Childbearing Age. J Nutr 2000;130:2703-2710.

17. Pufulete M, Emery P, Nelson M, Sanders T. Validation of a short food frequency questionnaire to assess folate intake. Br J Nutr 2002;87:383-390.

18. Gibson R. Principle of Nutrition Assessment. New York: Oxford University Press., 2005.

19. Willett W. Nutritional Epidemiology. Second Edition ed. New York: Oxford University Press, 1998.

20. McKeown NM, Day NE, Welch AA, et al. Use of biological markers to validate self-reported dietary intake in a random sample of the European Prospective Investigation into Cancer United Kingdom Norfolk cohort. Am J Clin Nutr 2001;74:188-196.

21. Ishihara J, Yamamoto S, H I, Inoue M, Tsugane S. Validity of a self-administered food frequency questionnaire (FFQ) and its generalizability to the estimation of dietary folate intake in Japan. [Electronic version]. Nutr J 2005;Oct 5:4:26.

22. Dwyer J, Picciano MF, Raiten DJ. Food and Dietary Supplement Databases for What We Eat in America-NHANES. J Nutr 2003;133:624S-634.

23. Bailey L. New standard for dietary folate intake in pregnant women. Am J Clin Nutr 2000;71:1304S-1307.

24. Metka J. The development and validation of a 3-Day food frequency recll. Dep. Foods and Nutrition Disserations. Winnipeg: University of Manitoba, 1992.

25. Kiunga A. Determinants of food choice and other behaviours of participants in a heart health program. Dep. of Foods and Nutrition Dissertations. Winnipeg: University of Manitoba, 1996.

26. Sevenhuysen GP, Gross U. Documenting the reasons people have for choosing their food. Asia Pac J Clin Nutr 2003;12:30-37.

27. Hess M. Portion Photos of Popular Foods. The United States of America: The American Dietetic Association, 1997.

28. Bingham S, Day N. Using biochemical markers to assess the validity of prospective dietary assessment methods and the effect of energy adjustment. Am J Clin Nutr 1997;65:1130S-1137S.

29. Gibson R. Principles of Nutrition Assessment. New York: Oxford University Press., 2005.

30. Yen J, Zoumas-Morse C, Pakiz B, Rock C. Folate intake assessment: validation of a new approach. J Am Diet Assoc 2003;103:991-1000.

31. Hickling S, Knuiman M, Jamrozik K, Hung J. A rapid dietary assessment tool to determine intake of folate was developed and validated. J Clin Epidemiol 2005;58:802-808.

32. National RC. Recommended Dietary Allowances. 10th ed. Washington, DC: National Academy Press, 1989.

33. Institute of Medicine Research Council. Dietary reference intakes:for thiamin r, niacin, vitamin B6, folate, vitamin B12, pantothenic acid, biotin. Dietary reference intakes:for thiamin, riboflavin, niacin, vitamin B6, folate, vitamin B12, pantothenic acid, biotin. Washington, DC: National Academy Press., 2000.

34. Health Canada Food program Canadian Nutrient File (CNF). [cited 200]. Available from:http://www.hc.sc.gc.cs/food-aliment/ns-sc/nr-rn/surveillance/cnf-fcen/e_cnf_download.html., 2001.

35. Green TJ, Allen OB, O'Connor DL. A three-day weighed food record and a semiquantitative food-frequency questionnaire are valid measures for assessing the folate and vitamin B-12 intakes of women aged 16 to 19 years. J Nutr 1998;128:1665-71.

36. Hintze JL. Number Cruncher Statistical System 2000 Statistical System for Windows. 2000 ed. Kaysville, Utah: Number Crunched Statistical Systems., 1999.

37. Brunner E, Stallone D, Juneja M, Bingham S, M. M. Dietary assessment in Whitehall II: comparison of 7 d diet diary and food-frequency questionnaire and validity against biomarkers. Br J Nutr 2001;86:405-414.

38. Willett W, Stampfer M, J. Total Energy intake: Implication for Epidemiologic Analysis. Am J Epodemiol 1986;124:17-27.

39. Snedecor GW, Cochran WG. Statistical Methods. Iowa State: University Press, Ames, IA., 1985.

40. Fraser GE, Butler TL, Shavlik D. Correlations between Estimated and True Dietary Intakes: Using Two Instrumental Variables. Ann Epidemiol 2005;15:509-518.

41. Kaaks R, Ferrari P. Dietary intake assessments in epidemiology: can we know what we are measuring? Ann Epidemiol 2006;16:377-380.

42. Kabagambe EK, Baylin A, Allan DA, Siles X, Spiegelman D, Campos H. Application of the method of triads to evaluate the performance of food frequency questionnaires and biomarkers as indicators of long-term dietary intake. Am J Epidemiol 2001;154:1126-1135.

43. Ocke M, Bueno-de-Mesquita H, Pols M, Smit H, van Staveren W, Kromhout D. The Dutch EPIC food frequency questionnaire. II. Relative validity and reproducibility for nutrients. Int J Epidemiol 1997;26:S49-58.

44. Institute of Medicine. Dietary reference intakes: folate, other B vitamin and choline. Washington, DC: National Academy Press, 1998.

45. Bland M, J, Altman D, G. Statistical Methods For Assessing Agreement Between Two Methods Of Clinical Measurement. Lancet 1986;1:304-311.

46. Kaaks R. Biochemical markers as additional measurements in studies of the accuracy of dietary questionnaire measurements: conceptual issues. Am J Clin Nutr 1997;65:1232S-1239.

47. Ocke M, Kaaks R. Biochemical markers as additional measurements in dietary validity studies: application of the method of triads with examples from the European prospective investigation into cancer and nutrition. Am J Clin Nutr 1997;65:1240S-1245.

48. Gray-Donald K, Jacobs-Starkey L, L. J-D. Food habits of Canadians: reduction in fat intake over a generation. Can J Public Health. 2000 Sep-Oct;91(5):381-5. 2000;91:381-385.

49. Green TJ, Allen OB, O'Connor DL. A Three-Day Weighed Food Record and a Semiquantitative Food-Frequency Questionnaire Are Valid Measures for Assessing the Folate and Vitamin B-12 Intakes of Women Aged 16 to 19 Years. J. Nutr. 1998;128:1665-1671.

4.8 Tables

Table 4.8.1: Characteristics of the study participants (95 women, age 18-25y)

Characteristics	Mean[1] ± SD	Range
Age (y)	20.5 ± 2.0	17-24
Body mass index (kg/m^2)	22.7 ± 4.1	17.5-46.1
Body weight (kg)	61.3 ± 12.4	42.9-124.0
Body height (cm)	163.7 ± 5.8	150.5-176.0
Waist circumferences (cm)	70.3 ± 8.4	57.1-108.6
Current smoker (n), %	(4) 4.2	---
Users of folate-containing supplement (n), %	(25), 26	---
Users of vitamin B_{12}containing supplement (n), %	(25), 26	---

[1]Arithmetic value

Table 4.8.2: Median (1^{st},3^{rd} Quartiles) intakes of energy, folate, and vitamin B_{12}[1] as determined by the FCM and 3DFR in a group of young women 18-25y.

		Absolute intake		Adjusted intake [2]	
Variable	**n**	**FCM**	**3DFR**	**FCM**	**3DFR**
Energy *(kcal/d)* [3]	95	2086 (1733.7, 2591.4) [a]	1876 (1541.3, 2225.9) [b]	----	----
Supplement users+ nonusers [4]					
Folate, *μg DFE/d*		505.4 (419.1, 796.4)	458.9 (373.7, 807.3) [b]	515.7 (405.1, 805.6) [a]	469.0 (388.8, 759.7) [b]
Vitamin B_{12}, *μg/d*		4.2 (2.9, 8.8)	3.6 (2.3, 8.5)	4.6 (3.0 , 8.0) [a]	3.6 (2.3, 8.2) [a]
Supplement nonusers					
Folate, *μg DFE/d*	70	447.9 (379.1, 558.5)	414.8 (343.7, 487.0)	461.7 (383.6 , 558.7) [a]	431.3 (349.3, 479.6) [a]
Vitamin B_{12}, *μg/d*	70	3.5 (2.8, 5.5)	2.8 (2.2, 4.2)	3.5 (2.47, 5.0) [a]	2.8 (2.2, 3.8) [a]
Supplement users					
Folate, *μg DFE/d*	25	963.5 (800.6, 1385.3)	1016.0 (749.2, 1152.5)	1039.2 (827.5, 1311.9)[a]	1031.7 (763.5, 1198.1) [a]
Vitamin B_{12}, *μg/d*	25	14.5 (9.5, 25.4)	15.7 (8.4, 23.9)	14.0 (9.6, 25.4) [a]	15.8 (8.5, 24.2) [a]

Abbreviations: FCM, Food Choice Map; 3DFR, three day food records; n, number of participants.
[1] Nutrient intakes for participants were calculated only if there was a corresponding blood index for that nutrient (e.i., serum vitamin B_{12} for vitamin B_{12} intake and serum folate for folate intake).
[2] Adjusted for energy by residual method reference 25
[3] unlike superscripts denote a significant difference between the mean intake for the FCM and 3DFR ($p< 0.05$), All variables were log-transformed before conducting paired t-test.
[4] Nutrient intake estimates included supplemental sources of vitamins for supplements users.

Table 4.8.3: Median (1^{st}, 3^{rd} quartile) concentrations of serum folate and vitamin B_{12} in a group of young women aged 18-25y.

Biochemical indicators	Supplement nonusers	Supplement users
Serum folate (ng/ml) [2]	13.6 [a 1] (11.2, 16.47)	16.5 [b] (13.7, 18.7)
Serum B_{12} (pg/ml)[3]	541.3 [a] (398.5, 661.6)	710.3 [b] (545.9, 1056.3)

[1] Unlike superscripts denote a significant difference between the mean for supplement nonuser and users ($p< 0.05$), All variables were log-transformed before conducting unpaired t-test.
[2] To convert ng/ml serum folate to nmol/L. multiply by 2.27.
[3] To convert pg/ml to pmol/L, multiply by 0.7375

Table 4.8.4: Correlations between the folate and vitamin B_{12} intakes in a group of women aged 18-25y estimated from FCM and 3DFR vs. serum folate and B_{12}.

	Serum Folate			Serum B_{12}		
Independent variables	r	p-value	n	r	p-value	n
Supplement nonusers						
Folate intake (FCM)	0.26	<0.05	70	---	---	--
Folate intake (3DFR)	0.20	0.11	70	---	---	--
Vitamin B_{12} (FCM)	---	---	--	0.15	0.23	70
Vitamin B_{12} (3DFR)	---	---	--	0.25	<0.05	70
Supplement users + nonusers						
Folate intake (FCM)	0.43	<0.01	95	---	---	--
Folate intake (3DFR)	0.39	<0.01	95	---	---	--
Vitamin B_{12} (FCM)	---	---	--	0.40	<0.01	95
Vitamin B_{12} (3DFR)	---	---	--	0.44	<0.01	95

Abbreviations: FCM, Food Choice Map; 3DFR, three day food records; r, correlation coefficient; n, number of participants

Table 4.8.5: Classification of participants into quartiles of folate and vitamin B_{12} intake based on dietary intake method and serum levels.

Variable		FCM				3DFR			
	n	Correctly Classified (%)	Closely classified%)[1]	Mis-Classified (%)[2]	*P* value[3]	Correctly Classified (%)	Closely Classified (%)	Mis-Classified (%)	*P* value
Including Supplement users									
Serum folate	95	31.5	42.2	26.3	<0.05	34.7	38.9	26.3	<0.01
Serum vitaminB_{12}	95	36.8	36.8	26.3	<0.01	35.8	42.1	22.1	<0.01
Excluding supplement users									
Serum folate	70	27.1	48.7	24.3	<0.01	27.1	41.4	31.4	>0.10
Serum vitamin B_{12}	70	27.2	45.7	27.1	>0.1	32.8	38.6	28.6	>0.10

Abbreviations: FCM, Food Choice Map; 3DFR, three day food records; n, number of participants

[1] Percentage of participants misclassified by one quartile

[2] Percentage of participants misclassified by two or more quartiles.

[3] A P-value <0.05 indicates that there is a significant association between nutrient intake estimates and the appropriate blood nutrient concentration.

4.9 Figures

Figure 4.9.1.A graphic exhibiting the food picture board used in the FCM dietary assessment process.

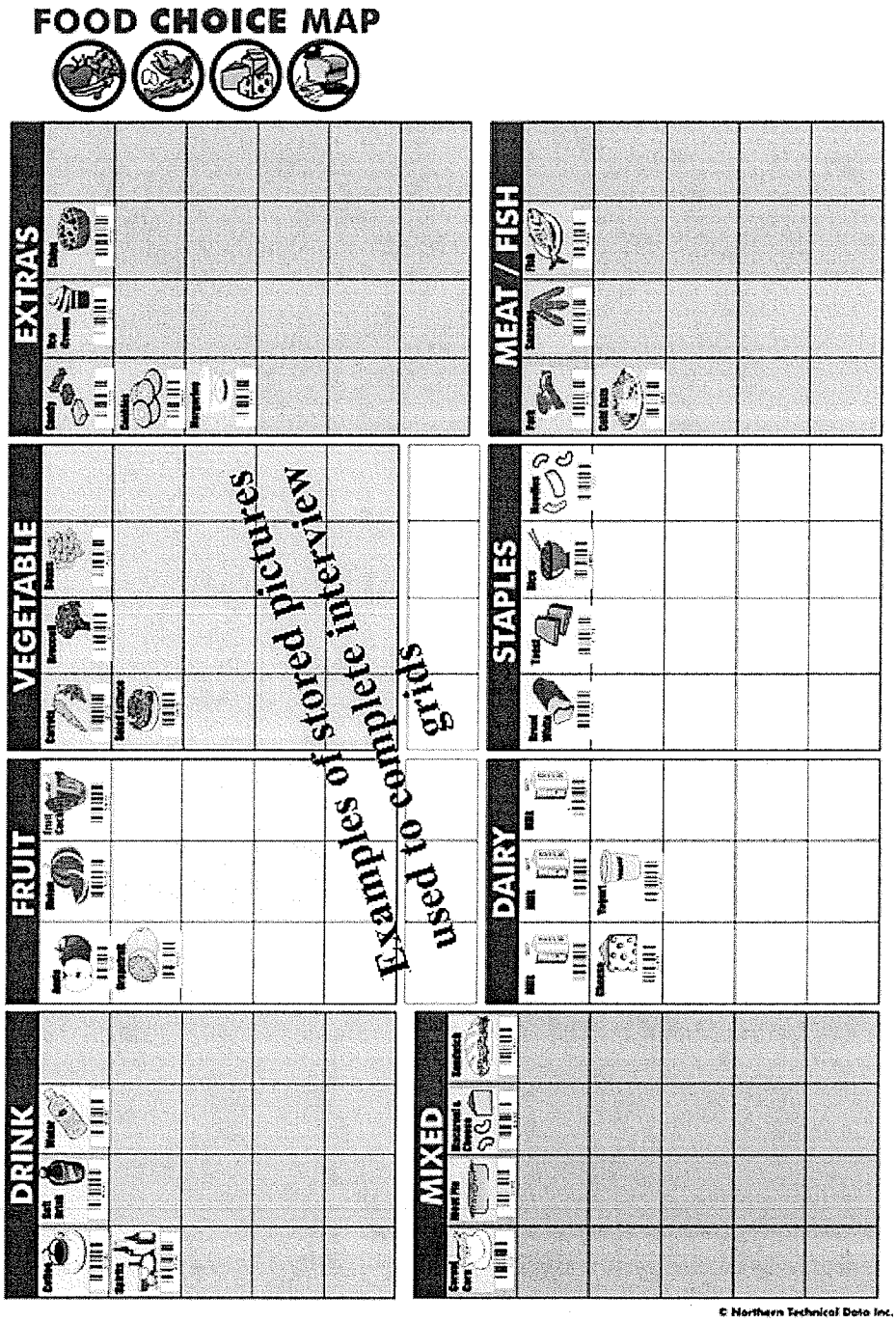
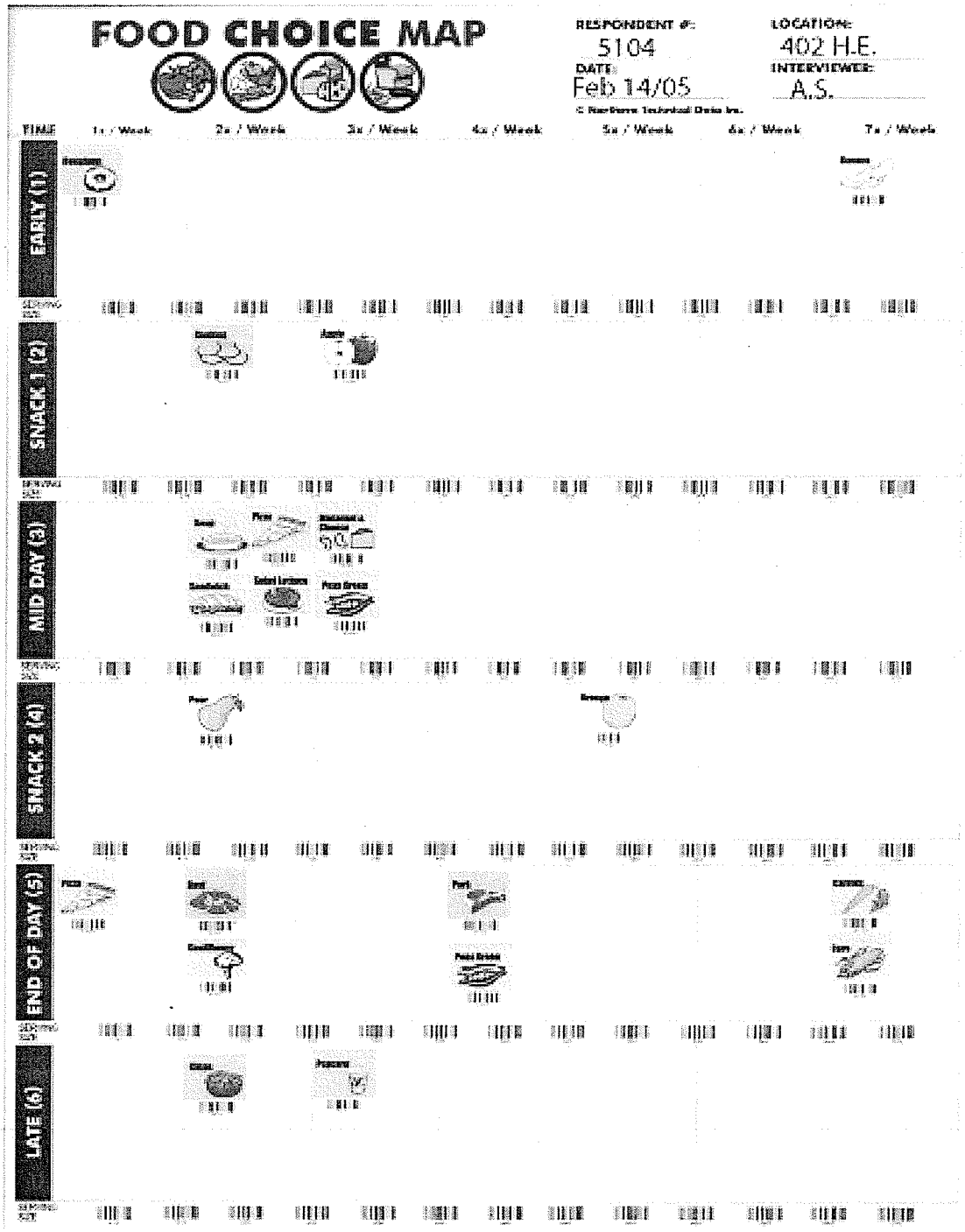


Figure 4.9.2.A graphic exhibiting the food map board used in the FCM dietary assessment process.



CHAPTER 5

5. THE COMPARISON OF FOLATE INTAKE DATA ESTIMATED ON THE BASIS OF TWO DIFFERENT DATABASES: RESULTS AND EXPERIENCES [1]

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[1] Submitted

Running title: Issues in dietary assessment of folate

The comparison of folate intake data estimated on the basis of two different databases: results and experiences [1 2 3] *

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KEY words: folate, Dietary Reference Intake, Upper Level, dietary folate equivalents, and Dietary assessment

5.1 Abstract

In order to achieve valid assessments of folate status, folate food composition data needs to be expressed in the same chemical form and units as the recommendations for folate intakes. Dietary and supplemental folate intakes were assessed twice in a cross-sectional study, using a validated dietary assessment method and two versions of the Canadian Nutrient file (CNF) 2001: the original (folate expressed as μg) and one updated with dietary DFEs (folate expressed as μg DFEs). A fasting blood sample was used to measure serum folate. Statistically significant mean differences were observed in the energy adjusted mean daily folate 471.3 ± 215.8 (μg/d ± SD) from the original CNF and dietary folate 648.0 ± 358.3 (μg DFE/d ± SD) from the updated database, but there was no significant correlation between folate estimates (r= 0.1). When folate intakes included supplemental folic acid, correlation with serum folate was significant with either database. Using Estimated Average Requirement (EAR) as the nutritional cut-off point and the serum folate used as biological cut-off point, intakes estimated as μg DFEs/d predicted folate status better than the μg/d estimation. Assessment of folate nutritional status is incomplete if content values in the food composition database do not account for the bioavailability of the various forms of this vitamin in food and supplements.

5.2 Introduction

Users of nutrient food composition databases (FDBs) frequently compare dietary intakes of individuals or groups with their respective dietary recommendations in order to assess whether the intakes are adequate or excessive. In addition, dietary recommendations are used to plan nutritionally adequate diets. It is important that the FDB variables are expressed in the same form and units as the most current dietary recommendations (1). The Dietary Reference Intakes (DRIs) are nutrient-level recommendations set by the Food and Nutrition board of the Institute of Medicine (2) adopted in the United States and Canada. The DRIs have been updated in a series of reports beginning in 1997 (3-5) . For some nutrients the units of expression and/or the definition of the nutrient, have been changed. In some cases, the form of the nutrient used to determine for the Tolerable Upper Intake Level (UL) is different from the form used to determine for the EAR and DRIs.

The folate values in most FDBs have to be revised because the DRI for folate is expressed in μg of DFE, rather than in μg of folate which was used for the 1989 Recommended Dietary Allowances (RDAs) for the US and Recommended Nutrient Intakes (RNIs) for Canada (6). One μg of folate that is in food is equal to 1.0 DFE. However, a μg of folate consumed in a dietary supplement has greater activity than a μg of folate naturally occurring in food because the monoglutamate form used for supplements is more bio-available, and 1 μg of folate in a supplement is equal to 2.0 DFE. Folate added to food as fortification is also more bioavilable than folate naturally occurring in food, but slightly less active than folate taken as a supplement, and 1 μg of fortification folate is equal to 1.67 DFE.

Hence, separate variables for naturally occurring, fortification forms and supplement forms of folate are needed in FDB. In addition, recommended folate intakes are specific to fortification and supplementation forms. Women of childbearing age should consume 400 μg/d of fortification folate in order to reduce the risk of neural tube defects in the infant (7, 8). Evaluating the intakes of this age group requires an extensive assessment of the folic acid contents of dietary supplements.

5.2.1 Using the DRIs to assess nutrient intakes of individuals

It can be appropriate to compare the intakes of individuals with specific DRIs, even though dietary intake data alone cannot be used to ascertain an individual's nutritional status. Dietary assessment is one component of a nutritional status assessment, provided that accurate dietary intake data are collected, the correct DRI is selected for the assessment, and the results are interpreted appropriately. Ideally, intake data are combined with clinical, biochemical and anthropometric information to provide a valid assessment of an individual's nutritional status (9-11). Serum folate reflects the recent intake while red cell folate indicates tissue content. Serum folate would therefore seem to have the better potential as indicator of dietary intake (12). As there are missing folate values for a number of foods in the Canadian Nutrient File database (13), and there is limited information about the effects of different methods of food processing and preparation on the bioavilability of folate(s), it seemed to us, that the biomarker method might by-pass both the subjectivity of food intake methods, and the incomplete information about folate content.

The objective of this study is to update and validate the folate values in the Canadian Nutrient file 2001(CNF), version 2001b(13), and use both to assess the folate

status for women of childbearing age. This study examines the comparability of folate intake data estimated on the basis of the CNF 2001 database and the updated one, and to clarify the degree of misclassification between dietary intake (dietary sources only) and total intake (dietary plus supplemental sources).

5.3 Materials and Methods

5.3.1 Subjects

The sample comprised 95 healthy female inhabitants of Manitoba aged 18-25 y. Volunteers were recruited from the University of Manitoba. Women were excluded for any history of serious medical conditions or use of medications that would interfere with folate metabolism (dilantin, phenytoin, primidone, metformin, sulfasalazine, triamterene, or methotrexate). Approval of the study was obtained from the Ethics Committee of the University of Manitoba. The subjects gave informed consent before being admitted into the study.

5.3.2 Dietary assessment

The assessment was conducted by an in-person interview for all participants. The Food Choice Map (FCM) was used for evaluation of the intake of foods and nutrients. Participants identified any food as eaten at one of six meal or snack times during the day and as eaten at one of 13 possible frequencies per week (1, 1.5, 2, 2.5, 3, 3.5, 4 , 4.5, 5, 5.5, 6, 6.5, 7). A food could be eaten at more than one mealtime, at different frequencies and portion sizes. The combinations of foods at each meal or snack time showed substitute foods with their respective frequencies, which resulted in a food pattern that was quantifiable (14).

Participants estimated the amounts consumed from the *Portion Photos of Popular Foods* (15), which contained life-size photos of many food items, with several photos of the same food item depicting different portion sizes (small, medium and large portions, or different measures such as cups, spoons and scoops). Where a food was not depicted in the book, participants were referred to the photographs of foods that had similar appearance and density to the food in question. The participants were also prompted to report weights or volumes of frequently consumed packaged items if they knew them.

Trained interviewers used a portable computer to assess intakes using FCM Nutrient Analysis Software (FCM-NAS), which was designed at the University of Manitoba (Sevenhuysen, unpublished copyright). The software standardized the process for identifying foods and beverages reported by participants according to the entries available in the CNF 2001 by quality and preparation techniques and quantity of the amount of each food and beverage. In validity study of the FCM, the FCM was compared with 3-day food records and the biomarkers using method of triads. FCM shows the highest validity coefficient as reported (16).

Subjects were asked if they had taken any form of dietary supplement (nutritive or herbal). Supplement type, name brand, and dose were recorded. Specific nutrient content for all supplements was recorded for all subjects by the research staff. Average daily intake of supplemental folic acid was calculated from the responses and added to the participant's dietary intakes of folate to calculate the total daily intakes obtained with both the CNF 2001 and the updated CNF databases. Adequate information to identify the daily quantity of supplemental folic acid was available from all subjects.

5.3.3 Database development

The database in the FCM-NAS included a subset of 1462 food items selected from the CNF 2001. These food items were selected according to two criteria described by Greenfield and Southgate (17). First, to create a core set of foods and food groups, the frequencies of food items reported by participants using a food frequency questionnaire in the provincially representative Heart Health survey were used to understand the relative frequency ranks of different food groups (18). Second, the food choices of young Manitoba women were used to identify the specific food products that were commonly eaten (19, 20). The number of food items to include in the database was based on the finding by Dwyer and others that about 1000 foods and ingredients account collectively for ~ 80% of the intake for most nutrients, and that for any individual nutritional component, 5-200 foods may account for 80% of the population's intake (21).

The CNF reports 1) total values for dietary folate intake (μg/d) or the sum of naturally occurring folate and folic acid added as a fortificant and 2) folic acid as a fortificant separately. To update the database, dietary folate intakes expressed as μg DFEs/d were added. This unit was developed by the U.S Institute of Medicine to help to account for differences in the bioavailability of naturally occurring food folates and synthetic folic acid (5). Because folic acid ingested with food is estimated to be 85% bioavailable and food folates are thought to be 50% bioavailable, folic acid taken with food is believed to be 85/50 (1.7 times) bioavialble (22, 23). To convert all forms of folate into DFEs, the following calculation was used to update folate values in the database:

$$DFE = FF + (FA \times 1.7)$$

5.3.4 Folate assay

Participants attended an early morning clinic 8:30-10:30 visit after an overnight fast. Blood was collected via venipuncture from participants into evacuated containers with and without anticoagulant. Blood was collected in vacutainers containing no anticoagulant, left to clot for 30 min at room temperature and then placed in ice. All samples on ice were transported within 2 hr to the laboratory and centrifuged at 1800g for 15 min. Serum samples were transferred to plastic vials and stored at -80°C until analyzed. Serum folate was measured using a dual isotope kit (Quantaphase Folate/B_{12} Radioassay, bio-Rad Laboratories, Mississauga, Canada). The accuracy of these radioassay methods was checked through serial replication of three levels of control sera (Lyphocheck, Bio-Rad Laboratories, Mississauga, Canada). All control sera fell within the manufacture's stated limit

5.4 Statistical analysis

Statistical analyses were performed by using statistical software (NCSS, 2000 Kaysville, Utah) (24). A probability level of 5% was chosen as the level of significance. Biochemical indices and dietary intakes for folate of all subjects were normalized using logarithmic transformation. Energy-adjusted nutrient intakes were computed as the residual for subject A from regression model with nutrient intake as the dependent variable and total caloric intake as independent variable. Since the residuals have a mean of zero and include negative values, a constant must added. A logical choice is the expected nutrient intake for the mean caloric intake of the study population (25). To assess for the differences in folate intake as assessed based in the two different databases, the paired- t -test was used.

Pearson correlations (r) were generated between each of the folate intakes estimated using the two databases and their respective serum folate indices. Partial correlations between nutrient intakes and biochemical measures were determined after adjusting for energy.

The ability of the two databases to correctly classify participants into quartiles of blood vitamin levels was determined. For each of the indices, subjects were assigned to a quartile, based on their individual blood vitamin concentration. The nutrient intake values were assigned to quartiles. Finally, the assignments to the quartiles of actual biochemical values were compared with the quartile assignment for the nutrient intake values. Participants where considered misclassified if biochemical values were placed within a different quartile. Under the hypothesis that there is no association between the quartile of actual biochemical value and the quartile based on nutrient intakes, one

would expect 25% of participants to be correctly classified, 37.5 to be closely classified and 37.5 to be misclassified. The actual numbers are compared with expected numbers using a chi-square goodness of fit test with 2 degree of freedom.

5.5 Results

5.5.1 Subject characteristics and dietary intake

The characteristics of the study population as well the dietary intake of energy and macronutrients are shown in **Table 5.9.1.** The mean age of women was 20.5 (18-24). About 26% of women of childbearing age were taking folate supplements. Only one woman reported consuming a separate folic acid supplement; all other supplement users took a multivitamin or B-complex vitamin.

The intakes of dietary and total folate of the 95 women, expressed as μg/d and μg DFEs/d, as well as the difference in the intakes between supplement users and nonusers, are shown in **Table 5. 9.2**. The median intakes were 680.0 μg DFEs/d and 406.4 μg/d. The total intakes expressed as μg DFEs/d were significantly higher than the intake expressed as μg/d. Including folic acid intake from supplements yield a higher significant in the intakes as estimated by the two databases. The correlation between adjusted total CNF folate (μg) and the adjusted total folate expressed as μg DFE/d was (r=0.1; P=0.4).

The correlations between folate intakes (dietary, total) and serum folate concentration are summarized in **Table 5.9.3**. Including supplemental folic acid in the analysis did not lead to significant difference between methods, and folate intakes expressed as μg DFEs/d determined by the updated database showed a similar association with serum folate as the intakes expressed as μg/d ($r = 0.44$, $p<0.01$). Excluding supplemental folic acid from the statistical analysis decreased the association between folate intakes as expressed by both databases and serum folate concentrations (updated database, $r = 0.26$, $p=.01$; CNF 2001, $r = 0.24$, $p=0.05$). The strength of the

correlation as determined by both databases and serum folate concentrations did not differ.

Comparing the calculated folate intake with the corresponding RDA, the dietary intake level that is sufficient to meet the nutrient requirements, overestimates the requirements for around 45% of this age and sex group. Therefore, we also compared the nutrient intake of our sample to the EAR. This is the nutrient intake value that is estimated to meet the requirements of half the healthy persons in a life-stage and sex group (5). The intakes of folate of the 95 women estimated by the two different databases including and excluding the supplemental folic acid, as well as the percentage of women who failed to achieve the RDA and the EAR values, are shown in **Table 5.9.4.** Using the CNF 2001, 45% of the sample did not achieve the RDA value compared to 23% when using the updated database. As many as 21% of women failed to meet average requirement estimated by CNF 2001. Similarly 8% did not achieve the EAR value for the folate expressed as μg DFEs/d.

The ability of the databases to classify individuals into the correct quartile of serum folate concentration is shown in **Table 5.9.5**. Using the updated folate database, 23% of women were misclassified compared to 27% when using the CNF 2001, with intakes from supplements included in the analysis. As shown in **Table 5.9.5**, there was a significant association between the intake and the biomarker classification with supplemental folic acid included in the analysis. Excluding the supplemental folic acid intake dramatically reduced the association, and almost 30% of women were misclassified using either database.

When folate intakes were determined by both databases and supplemental folic acid were included in the analyses, folate intakes correctly or closely classified to their

respective quartile of folate more often than would be expected by chance (more than 73% observed vs. 62.5% expected).

5.6 Discussion

Using a validated dietary assessment method, we compared estimated total and dietary folate intakes obtained with the original CNF 2001 and with the updated database in which DFEs values were added, using the following statistics: (a) median and mean of individual intakes, (b) correlation coefficients with serum folate, (c) quintile rankings with serum folate, and (d) Pearson correlation coefficients for intakes (e) frequency of women deviating from the dietary cutoff points (RDA and EAR).

Mean folate intakes obtained with the two databases were significantly different. Average total folate (± SD) expressed as μg DFEs/d was 648(± 358), and expressed as μg/d was 471(±215.7) μg/d. Comparing the folate intakes (μg DFEs) with those in post-fortification could not be widely performed because the food composition for the conducted study databases did not distinguished between naturally occurring food folate and synthetic folic acid (26-29). The mean daily folate intake from food, fortified foods, and supplementation was 609 ±327 μg DFE for women aged 18-45 years in Vancouver area of British Columbia (30), which was slightly less (39 μg DFE/d) than the mean intake observed in this study. Another study reported that the average total folate was estimated to be 745 ±70 μg DFEs/d for 39 women in the US who are at least 18 years old (31). The explanation for a 97 μg DFE higher intake than recorded in this study may be that the women (n=39) were defined as low-income with a dietary pattern dependent on bread and cereal products, which were the major contributors folate intake. European women were found to have an intake of 318 ± 96.3 μg DFEs/d (32). The probable reason

for this lower intake in Europe is the fact that cereals are not enriched with folate (32). Yen and coworkers reported that the estimated folate intakes, inclusive of supplements, was 411.4± 192.8 μg/d (mean ±SD) for US women aged 18-50 y(27), which agrees with the intake estimated in this study. The reported folate intake for females aged 18-34 y in a national survey of adult Canadian (1997-1998) was 275 μg/d. This mean intake was 196 μg/d lower than observed in this study. Two reasons can explain this difference. The dietary assessment methodology did not record supplement use, and the analysis used a food composition table that was established prior the introduction of fortification.

In the current study, approximately 26% of participants used a supplement containing synthetic folic acid. The median SFA intake provided by supplements for these women (n=25) was 400 μg SFA/day (range = 43-1000 μg SFA/day). The mean ±SD SFA from food was 95.7 ± 63.9 μg/d, and from supplement was 94.9 ± 189.3 μg/d. Only 1% of the participants exceed the UL (data not shown), resulting from the use of 1000 μg folic acid-containing supplements. Higher percentages were reported by Lewis et al (33) and French et al (30) where 5%, and 7% of women, respectively, were found to exceed the UL. The higher supplemental FA intake (median 400, range 10-2400 μg/d) in the later studies may explain the higher percentage of women exceeding the UL. The UL for adults was based on synthetic folic acid, not total folate, because there was no evidence to indicate that excessive intakes of natural folate caused adverse health effect (34).

In this study the correlation coefficients for folate intakes with serum folate and folate intakes including the supplemental folic acid and serum folate were lower than the correlation coefficients between folate intakes and serum folate reported in the literature, which were in the range of 0.43-0.65 (35, 36). The lower correlation values in this study

compared to those previously published (37) can be explained in part by the fact that it was not possible to adjust for day to day variation (inter-subject variation) and unadjusted correlations were used in this study. The finding in this study that total folate intakes (including supplemental folic acid) correlate better with serum folate than did dietary folate intake is expected, because including supplement users increased the range of both vitamin intakes and vitamin concentrations in blood, thereby improving the correlation between the two. Serum folate is influenced by recent changes in folate intake and may not always reflect folate stores (38). In this study, the dietary folate, and supplemental intakes were collected at the time of blood sampling, but the serum folate may be influenced by general characteristics like smoking and using oral contraceptives (10), (39). In this study, some women were smoking or used oral contraceptive, but their biomarker concentration was within the normal ranges (40). The biochemical concentration reflects the individual vitamin status that is determined not only by the metabolism and gene but also by parameters affecting the food intake, such as seasonal variation and pregnancy. These effects on dietary intake are minimized because the study was performed in 95 non-pregnant women between November 2004 and March 2005.

On the basis of the EAR, the prevalence of individuals with inadequate folate intake was 8-22% among individuals as estimated by updated folate database and CNF 2001, respectively. The result for the updated database is similar to the 7% reported by other researchers (34). Using a serum folate concentration cut-off point of < 7 nmol/L (5, 41, 42), no subject was found to have a serum folate value indicative of compromised folate status. Using CNF 2001 folate data resulted in an overestimate of the percentage

of the population below the EAR; the EAR for folate is expressed as micrograms of DFEs.

Folate intakes estimated from the updated database have allowed a more accurate ranking with serum folate comparing to the folate intakes estimated by CNF 2001. The finding in this study that total folate intakes (including supplemental folic acid) classify individuals better with serum folate than did dietary folate intake is not unexpected.

This study had several methodological strengths. First, supplemental (non-dietary) folic acid intake data collection was highly reliable because all subjects brought samples of the vitamin and mineral supplements they were taking and one of the research staff recorded the content and daily dosage from the label on the container. Second, In validity study of the FCM, the FCM was compared with 3-Day Food Records and biomarkers using method of triads and FCM showed the highest validity coefficient (16). Third, based on current knowledge of fortified foods and of intra-person day-to-day variability for most nutrients, the use of 7 days were appropriate to estimate folate intake of women at child bearing age as reported others (27, 36, 43).

Health Canada has issued new CNF 2007 that express as folate intake as μg DFEs. This work has been done to meet the new unit for folate recommendation that account for the different bioavailability for food folate comparing to folic acid (44).

5.7 Conclusions

Nutrient databases must reflect the changes made in DRIs. Adding variables to the databases in response to changes in units, as well as using different forms of the nutrient for the UL, would be an advantageous change for the reliability of nutrient assessments and the clinical interpretation of these assessments. Results indicate that among young women in this population, who are supplement users, taking folic acid supplements is a common behavior, which has to be taken into account when folate intake is evaluated. There is a need for a dietary folic acid supplement database that is analytically tested and verified in order to further increase the reliability of the assessments.

5.8 References

1. Murphy SP. Changes in dietary guidance: implications for food and nutrient databases. J Food Compost Anal 2001;14:269-278.
2. Yates AA, Schlicker S, Suitor C. Dietary reference intakes: The New Basis for Recommendations for Calcium and Related Nutrients, B Vitamins, and Choline. J Am Diet Assoc 1998;98:699-706.
3. Institute of Medicine. Dietary Refernce Intakes for Calcium, Phosphorus, Magnesium, Vitamin D and Fluoride. Washington, DC: National Academy Press., 1997.
4. Institute of Medicine. Dietary Reference Intakes: folate, other B vitamin and choline. Washington, DC: National Academy Press, 1998.
5. Institute of Medicine. Dietary reference intakes: for thiamin, riboflavin, niacin, vitamin B6, folate, vitamin B12, pantothenic acid, biotin. Washington, DC: National Academy Press., 2000.
6. National Research Council. Recommended Dietary Allowances. 10th ed. Washington, DC: National Academy Press, 1989.
7. Gucciardi E, Pietrusiak M-A, Reynolds D, Rouleau J. Incidence of neural tube defects in Ontario, 1986-1999. Can Med Assoc J 2002;167:237-240.
8. Honein M, Paulozzi L, Mathews T, Erickson J, Wong L. Impact of folic acid fortification of the US food supply on the occurrence of neural tube defects. JAMA 2001;286:2236.

9. Murphy SP. Dietary Reference Intakes for the U.S. and Canada: Update on Implications for Nutrient Databases. Journal of Food Composition and Analysis 2002;15:411-417.

10. Gibson R. Principles of Nutrition Assessment. New York: Oxford University Press., 2005.

11. Murphy SP. Dietary reference intakes for the U.S. and Canada: update on implications for nutrient databases. J Food Compost Anal 2002;15:411-417.

12. Truswell A, Kounnavong S. Quantitative responses of serum folate to increasing intakes of folic acid in healthy women. European Journal of Clinical Nutrition. 1997;51:839-845.

13. Health Canada Food program Canadian Nutrient File (CNF). [cited 200]. Available from:http://www.hc.sc.gc.cs/food-aliment/ns-sc/nr-rn/surveillance/cnf-fcen/e_cnf_download.html., 2001.

14. Sevenhuysen GP, Gross U. Documenting the reasons people have for choosing their food. Asia Pac J Clin Nutr 2003;12:30-37.

15. Hess M. Portion Photos of Popular Foods. The United States of America: The American Dietetic Association, 1997.

16. Shuaibi A, Sevenhuysen G, House JD. Validation of a food choice map to assess folate and vitamin B12 intakes in college-aged women. Appl Physiol Nutr Metab 2006;31:356.

17. Greenfield H, Southgate D. Food Composition Data: Production, Management and Use. 2nd ed: FAO, Rome, 2003.

18. Sevenhuysen G, Fieldhouse P, Young T, McNeil C, Gelskey D, McDonald S. The Manitoba Nutrition Survey: fat consumption data. Can J Public Health 1993;84:389-393.

19. Kiunga A. Determinants of food choice and other behaviours of participants in a heart health program. Dep. of Foods and Nutrition Dissertations. Winnipeg: University of Manitoba, 1996.

20. Metka J. The development and validation of a 3-Day food frequency recll. Dep. Foods and Nutrition Disserations. Winnipeg: University of Manitoba, 1992.

21. Dwyer J, Picciano MF, Raiten DJ. Food and dietary supplement databases for what we eat in America-NHANES. J Nutr 2003;133:624S-634.

22. Winkels RM, Brouwer IA, Siebelink E, Katan MB, Verhoef P. Bioavailability of food folates is 80% of that of folic acid. American Journal of Clinical Nutrition 2007;85:465-473.

23. Chun J, Martin JA, Chen L, Lee J, Ye L, Eitenmiller RR. A differential assay of folic acid and total folate in foods containing enriched cereal-grain products to calculate [mu]g dietary folate equivalents ([mu]g DFE). Journal of Food Composition and Analysis 2006;19:182-187.

24. Hintze JL. Number Cruncher Statistical System 2000 Statistical System for Windows. 2000 ed. Kaysville, Utah: Number Crunched Statistical Systems., 1999.

25. Willett W, Stampfer M, J. Total Energy intake: Implication for Epidemiologic Analysis. Am J Epodemiol 1986;124:17-27.

26. Lutsey PL, Steffen LM, Feldman HA, et al. Serum homocysteine is related to food intake in adolescents: the Child and Adolescent Trial for Cardiovascular Health. American Journal of Clinical Nutrition 2006;83:1380-1386.

27. Yen J, Zoumas-Morse C, Pakiz B, Rock C. Folate intake assessment: validation of a new approach. Journal of American Dietetic Association 2003;103:991-1000.

28. Verkleij-Hagoort A, de Vries J, Stegers M, Lindemans J, Ursem N, Steegers-Theunissen R. Validation of the assessment of folate and vitamin B(12) intake in women of reproductive age: the method of triads. European Journal of Clininical Nutrition 2006;online:1-6.

29. Alfthan G, Laurinen M, Valsta L, Pastinen T, Aro A. Folate intake, plasma folate and homocysteine status in a random Finnish population. European Journal of Clinical Nutrition. 2003;57:81:8.

30. French, Melissa R, Barr S, Levy-Milne R. Folate intakes and awareness of folate to prevent neural tube defects: A survey of women living in Vancouver, Canada. J Am Diet Assoc 2003;103:181-185.

31. Clifford AJ, Noceti EM, Block-Joy A, Block T, Block G. Erythrocyte Folate and Its Response to Folic Acid Supplementation Is Assay Dependent in Women. Journal of Nutrition 2005;135:137-143.

32. Wolters M, Hermann S, Hahn A. B vitamin status and concentrations of homocysteine and methylmalonic acid in elderly German women. American Journal of Clinical Nutrition 2003;78:765-772.

33. Lewis CJ, Crane NT, Wilson DB, Yetley EA. Estimated folate intakes: data updated to reflect food fortification, increased bioavailability, and dietary supplement use. Am J Clin Nutr 1999;70:198-207.

34. Choumenkovitch SF, Selhub J, Wilson PWF, Rader JI, Rosenberg IH, Jacques PF. Folic Acid Intake from Fortification in United States Exceeds Predictions. J Nutr 2002;132:2792-2798.

35. Kabagambe EK, Baylin A, Allan DA, Siles X, Spiegelman D, Campos H. Application of the Method of Triads to Evaluate the Performance of Food Frequency Questionnaires and Biomarkers as Indicators of Long-term Dietary Intake. American Journal of Epidemiology 2001;154:1126-1135.

36. Green T, Allen O, O'Connor D. A three-day weighed food record and a semiquantitative food-frequency questionnaire are valid measures for assessing the folate and vitamin B-12 intakes of women aged 16 to 19 years. Journal of Nutrition 1998;128:1665-71.

37. Bailey K. Methods of Social Research. London: The Free Press, Collier MAcmillan Puplishers, 1987.

38. Bailey LB, Gregory JF, III. Folate metabolism and requirements. J Nutr 1999;129:779-782.

39. Willett W. Nutritional Epidemiology. Second Edition ed. New York: Oxford University Press, 1998.

40. Cikot R, Steegers-Theunissen RP, Thomas C, de Boo T, Merkus H, Steegers E. Longitudinal vitamin and homocysteine levels in normal pregnancy. British Journal of Nutrition 2001;85:49-58.

41. Institute of Medicine Research Council. Dietary reference intakes:for thiamin r, niacin, vitamin B6, folate, vitamin B12, pantothenic acid, biotin. Dietary reference intakes:for thiamin, riboflavin, niacin, vitamin B6, folate, vitamin B12, pantothenic acid, biotin. Washington, DC: National Academy Press., 2000.

42. Wolters M, Hermann S, Hahn A. B vitamin status and concentrations of homocysteine and methylmalonic acid in elderly German women. Am J Clin Nutr 2003;78:765-772.

43. Firth Y, Murtaugh M, angney C. Estimation of individual intakes of folate in women of childbearing age with and without simulation of folic acid fortification. J Am Diet Assoc. 1999;99:985-988.

44. Health Canada Food program Canadian Nutrient File (CNF). [cited 2007]. Available from:http://www.hc.sc.gc.cs/food-aliment/ns-sc/nr-rn/surveillance/cnf-fcen/e_cnf_download.html., 2007.

5.9 Tables

Table 5.9.1: Characteristics of study participants

Characteristics	Mean[1] ±SD	Range
Anthropometric (n= 95)		
Age (y)	20.5 ± 1.9	
Body Mass Index (kg/m^2)	22.7 ± 4.1	
Body weight (kg)	61.0 ± 12.4	42.9-124
Body height (cm)	163.7 ± 5.8	150.5-176.0
Waist circumferences (cm)	70.7 ± 8.4	57-108
Dietary intake (n= 95)		
Energy (Kj)	9.1 ± 2.5	3.1-15.6
(Kcal)	(2154 ± 606)	(732-3721)
Protein (g)	91.5 ± 25.5 (16.8% energy)	27.2-187.9
Fat (g)	68.5 ± 30.6 (28.6% energy)	17.2-152.7
Carbohydrate (g)	293.1 ± 93.8 (54.4% energy)	88.5-644.7
Alcohol (g)	2.5 ± 7.8 (0.8% energy)	0-45.734
Other characteristics		
Current smoker (n), %	(4), 4.2%	---
Oral contraceptive users (n), %	(40), 42.1%	---
Oral contraceptive pill nonusers (n), %	(55), 57.9%	---
Users of folate-containing supplement n, %	(25), 26%	---

[1]Arithmetic value

Table 5.9.2: Adjusted folate intake median (1st-9th percentile), and the average expressed as µg DFEs/d and µg/d of the 95 women.

	Dietary intakes			Total intakes[1]		
	Supplement users + nonusers[2,3] (n=95)	Supplement nonusers (n=70)	Supplement users (n=25)	**Supplement users + nonusers (n=95)**	Supplement nonusers (n=70)	Supplement users (n=25)
Folate (*µgDFEs/d*)	**462.5**	463.7	446.7	**515.6**	463.7	1039.1
	(324.6-676.6)	(320.7-683.2)	(327.9-645.1)	**(331.1-1170.2)**	(320.7-683.2)	(661.4-1550.9)
	486.6 a †	485.60	489.4	**648.0 a‡**	485.6	1102.7
Folate (*µg/d*)	**358.2**	353.6	375.5	**406.4**	353.6	689.7
	(239.2-531.9)	(237.4-532.8)	(241.5-514.5)	**(258.6-810.2)**	(237.4-532.8)	(489.8-994.0)
	376.4 b †	379.6	367.1	**471.3 b‡**	379.6	727.9

[1] Nutrient intake estimates included supplemental sources of vitamins for supplements users.

[2] unlike superscripts (a.b) denote a significant difference between the mean intake within the column (µgDFEs/d vs. µg/d) ($p < 0.05$), All variables were log-transformed before conducting paired t-test.

[3] unlike symbols (†‡,) denote a significant difference between the mean intake within the row (dietary vs. total) ($p < 0.05$), All variables were log-transformed before conducting paired t-test.

Table 5.9.3: Correlations between serum folate and the folate intakes of a group of women aged1 18-25 y expressed as µg DFEs/d and µg/d.

	Dietary intakes			**Total intakes**		
	Supplement users + nonusers (n=95)	Supplement nonusers (n=70)	Supplement users (n=25)	**Supplement users + nonusers (n=95)**	Supplement nonusers (n=70)	Supplement users (n=25)
Folate (*µgDFEs/d*)	**0.30 p=0.0035**	0.26 p=0.01	0.37 p=0.070442	**0.43 p=0.00003**	0.26 p=0.01	0.65 0.000487
Folate (*µg/d*)	**0.20 p=0.04568**	0.24 p=0.050249	0.20 p=0.348852	**0.40 p=0.000008**	0.24 p=0.050249	0.65 p=0.000454

Table 5.9.4: Daily folate intakes expressed as µg DFEs/d and µg/d, and frequency of intakes below the RDA and EAR values in the study population[1]

Nutrient	Daily Dietary intake [2]	Women with vitamin intake below RDA	Women with vitamin intake below EAR	Daily Total intake [2]	Women with vitamin intake below RDA	Women with vitamin intake below EAR
		n (%)	n (%)		n (%)	n (%)
Folate *(µgDFE/d)*	486.6 ± 14.9	27 (28.4)	9 (9.5)	648.0± 358.3	22 (23.2)	8 (8.4)
Folate *(µg/d)*	376.4 ± 12.2	62 (65.3)	29 (30.5)	471.3 ± 215.8	45 (47.3%)	22 (23)

[1] RDA and EAR from reference (41)
[2] mean ± SD

Table 5.9.5: Percentage of participants correctly, closely or misclassified into quartiles of folate intakes as expressed as µg DFEs/d and µg/d compared with classification by serum folate indices.

Variable	Dietary intake				Total folate intakes			
	Correctly classified	Closely classified[1]	Mis-classified[2]	*P* value[3]	Correctly classified	Closely classified[1]	Mis-classified[2]	*P* value[3]
µg DFE/d	26.3	43.2	30.5	p >0.1	33.7	43.2	23.1	p<0.005
µg folate/d	29.5	41.0	29.5	p >0.1	33.7	39.0	27.3	p<0.05

[1] Percentage of participants misclassified by one quartile
[2] Percentage of participants misclassified by two or more quartiles.
[3] A P-value <0.05 indicates that there is a significant association between nutrient intake estimates and the serum folate concentration.

CHAPTER 6

6. FOLATE STATUS IN CANADIAN WOMEN OF CHILDBEARING AGE AFTER FOLIC ACID FORTIFICATION [1]

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[1] Submitted.

Running Title: Folate and selected B vitamin status

Folate status in Canadian women of childbearing age after folic acid fortification[1,2,^]

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KEY WORDS: young women, folate, folic acid, vitamin B_6, vitamin B_{12}, homocysteine.

6.1 Abstract

Objective: To investigate folate, vitamin B_6 and B_{12}, and total homocysteine status in healthy women of childbearing age, to investigate their pattern of folate intakes and to estimate the contribution of folic acid to their diets in the post- fortification era.

Design:

In a cross-sectional study design, fasting blood samples were obtained from 95 women of child bearing age. The samples were analyzed for serum folate, vitamin B_{12}, RBC folate, plasma vitamin B_6 (as pyridoxal-5-phosphate) and total homocysteine. Dietary and supplemental intakes for nutrient were assessed.

Results: The biochemical evidence showed that no women were folate deficient. Only 14% reach the recommended RBC folate concentration associated with a significant reduction in NTD risk. Five percent were deficient in vitamin B_6 and 3% had low vitamin B_{12}. Hyperhomocysteinaemia > 12.0 μmol/l occurred in 4.2% of women. Dietary intake mean ± SD of food folic acid were 96 ± 63.9 μg/d, supplemental folic acid was 94. ± 189.3 μg/d, natural folate 314.4± 134.3 μg/d, and the total intake was 645.7±368.4 μg DFEs/d. Only 17% of the participants met the special recommendation of women capable of becoming pregnant. The DFEs were derived mainly from bakery products (28%), vegetables (18%), pasta and rice (15%), juices (~13%), milk and dairy products (6%), and ready to eat cereal (~5%).

Conclusion: These data suggest that women of childbearing age are achieving improvement in folate status in the post fortification era, but it is not enough to reach the recommended RBC folate concentration associated with a significant reduction in NTD risk. Intakes of folic acid from fortified foods are within the level originally predicted for

the fortification. Even with food fortification, women of childbearing age should be advised to take FA containing supplement on a daily basis

6.2 Introduction

In recent years attention has been focused on the role of folates and vitamin B in clinical and public health (1, 2). Suboptimal maternal folate status is linked to neural tube defects (NTD) in newborns, such as spina bifida or anencephaly (3), is a possible risk factor for Down syndrome (4), and is a risk of spontaneous abortion (5). Impaired vitamin B_{12} metabolism has been observed in families with NTD (6). Several studies have reported decrease vitamin B_{12} levels in the circulation or amniotic fluid of mothers of children with NTDs (7, 8). Prenatal vitamin B_6 status may influence birth weight as well (8). Folates are also important factors in homocysteine metabolism, which is an independent risk factor for coronary, cerebral and peripheral atherosclerosis (9). It was shown recently that elevated total homocysteine (tHcy) and low folate levels were associated with reduced bone mineral density in women (10, 11), suggesting that tHcy may be a risk factor for osteoporosis.

Fortification of the food supply with folic acid was considered to be the most effective approach to help women of childbearing age increase their preconception folic acid intake (12). The November 1998 Canadian national program mandated increased folic acid fortification of all flour and some corn and rice products (13), with ranges from 95-309 μg/100 g for different products, and to ensure a target level of 140 μg folic acid/100 g of the cereal-grain product. The projected average increase of folic acid intake in the general population attributable to fortified cereal–grain products was estimated to be ~ 100 μg/d (14). The greater bioavailability of folic acid resulted in the introduction of the concept of "dietary folate equivalent" (DFE) into the dietary reference intakes (DRI) as a way to express a mixed intake of folic acid and natural

folate (15). Thus, it is necessary to distinguish between these two forms of folate to assess the adequacy of folate intake in an individual or population group. It is important to be able to quantify total folic acid intake because the tolerable upper intake levels (UL) and the special recommendation for women at childbearing age are expressed for folic acid alone (16).

The recognition of vitamin B status in women of reproductive age is important not only because nutritional status affects the women's health and productivity but also because deficiencies are associated with adverse pregnancy outcomes. The purposes of this study were to determine the folate, selected vitamin B and tHcy status, understand the existing pattern of folate intake, and estimate the effect of folic acid fortification on the amount of folate consumed by women of childbearing age.

6.3 Materials and methods

6.3.1 Subjects

A total of 95 healthy women aged 18-25 y were recruited from the University of Manitoba. Participants were recruited from October 2004 to March 2005. Exclusion criteria included: current pregnancy, pregnancy lasting more than 20 weeks in the year before the scheduled clinic visit, lactating, and the use of medications known to interfere with folate metabolism. Those who were taking oral contraceptives or folate supplements were not excluded. Approval of the study was obtained from the Ethics Committee of the University of Manitoba. The subjects gave informed consent before admitted into the study.

6.3.2 Assessment of food consumption and nutrient intakes

Food consumption was assessed by FCM by trained interviewers with a portable computer. The "FCM" nutrient analysis program was designed for collecting data on quality, quantity and preparation of foods and beverages in a standardized way (17, 18). In validity study of the FCM, the FCM was compared with 3-Day Food Records and biomarkers using method of triads and FCM showed the highest validity coefficient (18)

In this study the intake of folates from natural folate, foods and folic acid supplements were calculated separately. Dietary intake was calculated from the CNF 2001b (19) for all nutrients except for folate. Participants were asked to bring to the clinic visit any nutritional supplements they were currently taking. Information collected from the supplement containers and a self-administered questionnaire completed by each participant included the type of the supplement used, the brand name, the dosage,

average use during the previous year and the frequency of use during the week before the survey. Average daily intakes of supplemental folate vitamin B_6 and B_{12} were calculated from the responses and were added to the participant's dietary intakes of these vitamins.

6.3.3 Correction for the bioavailability of folic acid from food

According to the IOM report(15), folic acid (FA) is considered to be more bioavailability than natural folate (NF). Food-composition values had to be adjusted so that the greater bioavailability of FA than NF was reflected in the total folate value for the food, which was then expressed as μg DFEs. Canadian Nutrient File 2001 (CNF) (19) food composition data were used to provide total folate contents of foods and synthetic folic acid (SFA). Natural folate (NF), which is equivalent to dietary folate equivalent (DFE) is not available in the CNF 2001. Estimates of NF were calculated by subtracting the SFA from the total folate content values, where the SFA contents of each food were multiplied by 1.7 which is the recommended correction in the IOM report (15) because synthetic folic acid is considered to be more bioavailable than natural folate. The resulting total folate values were then expressed as DFE, which could be used with the recommended of folate intakes for this age group.

6.3.4 Food sources and contributors for the folate

To determine how to group food items we identified the major contributors of folate from other studies (20-22). For example, skim milk and whole milk contain about the same amount of folate (1.9 DFE/cup) so we reported all fluid milk as one group. If the amount in the subgroup different, we used each subgroup separately. For example,

bread (5 μgDFE/100 g) and rice (0.5 μg DFE/100 g) were analyzed as separate subgroups. We selected 24 food groups and subgroups in this attempt. The total amount of folate in each of the selected groups or subgroup was estimated for each participant then divided by the total folate intake from all foods. Foods were ranked by percentage contribution to dietary folate intake.

6.3.5 Collection of blood

Fasting peripheral blood samples were collected by venipuncture by trained personal in the period October 2004 to March 2005. A hundred μl of well-suspended blood was diluted to 1 ml in freshly made 0.4% ascorbic acid solution to measure RBC folate. The plasma obtained from the EDTA specimens was used to measure total homocysteine and vitamin B_6. For serum folate, and vitamin B_{12} measurements, blood was collected in vacutainers containing no anticoagulant, left to clot for 30 min at room temperature and placed in ice. Then it was transported within 2 hr to laboratory where centrifuged at 1800g for 15 min. Serum and plasma were transferred to plastic vials and stored at $^{-}80°C$ until measured.

6.3.6 Chemical analysis

Plasma total homocysteine was measured according to the reverse phase-HPLC method of Araki and Sako (23), with modifications as suggested by Gilfix et al (24). Plasma pyridoxal 5′-phosphate (PLP) concentrations were measured using a commercial radio enzymatic assay (Vitamin B-6 PLP 3H-REA, ALPCO). RBC folate, serum folate and vitamin B_{12} were measured by competitive protein binding (Bio-Rad Laboratories,

Mississauga, Canada). The maximum reporting limit for serum folate was 45 nmol/L, while that for RBC folate was 1450 nmol/L.

6.3.7 Cut-off values

A serum folate concentration of < 7 nmol/L indicates a negative folate balance (15). As a cutoff for adequate RBC folate status, a value of ≥ 304 nmol/L was chosen (15). The U.S. Food and Nutrition Board (IOM, 2000) chose a cutoff of < 20 nmol/L for plasma PLP as indicative of inadequacy (15) and 120-180 pmol/L (170 to 250 pg/ml) for serum vitamin B_{12}(15). We defined a tHcy concentration of ≤ 12 μmol/L as normal because other researchers have proposed age and gender-specific reference intervals (15). It is given that the risk for cardiovascular disease starts to increase with tHcy concentrations at 10.5-11.7 μmol/L (25).

6.4 Statistical analysis

Biomarker, nutrient and food intake data were analyzed by using statistical software (NCSS, 2004 Kaysville, Utah) (26). Descriptive statistics for serum and red blood cell folate, serum B_{12}, plasma B_6 and tHcy included their arithmetic mean, 10^{th}, 50^{th} and 90^{th} centile values. Normal distributions of data were checked by using the Kolmogorov-Sminrnov test. Log transformations were used to normalize skewed data. To assess for the differences in vitamin intake between supplement users and nonusers the independent-sample t test was used. A probability level 5% was chosen as the level of significance

6.5 Results

6.5.1 Subject characteristics and dietary intake

The characteristics of the study population as well the dietary intake of energy and macronutrients are shown in **Table 6.8.1**. The mean age of women was 20.5 (18-24). About 26% of women of childbearing age were taking folate vitamin B_{12} and B_6 supplements. Only one woman reported consuming a separate folic acid supplement, and another one reported consuming a separate vitamin B_{12}; all other supplement users took a multivitamin or B-complex vitamin.

The intakes of folate as NF, dietary FA, supplemental FA and DFEs, vitamin B_6 and B_{12} of the 95 women, as well as the difference in the intakes between supplement users and nonusers, are shown in **(Table 6.8.2)**. Women who took daily supplements containing FA consumed 14 μg less natural folate than did women who not currently consumed supplement ($P>0.05$). The intake of natural folate expressed as μg was 1.6 times more than the of folic acid (food and supplement) intake expressed as μg, and almost the same when expressed as DFEs. The total intakes for folate, vitamin B_6 and B_{12} were significantly higher for supplement users compared to supplement nonusers.

6.5.2 B vitamin status

The mean ± S.D median and the range (10^{th} and 90^{th} percentile) for serum, RBC folate, plasma vitamin B_6, serum vitamin B_{12}, and tHcy are listed in **Table 6.8.3**. Although mean vitamin B_6 and B_{12} concentrations were within the normal range, deficiencies of these vitamins were observed in 5% and 3% of women respectively. Hyperhomocysteinaemia, defined as > 12.0 μmol/l, occurred in 4.2% women.

6.5.3 Folate sources

Sources of folates in the diets of Canadian women of childbearing age shown in **Figure 6.9.1**. The major sources of folate were bread and bakery products, vegetables, juices, pasta and rice, ready to eat cereal. Some of the other contributors of folate (e.g., milk, non-alcoholic beverages) are not rich sources of this vitamin, but these foods usually were consumed by most women. Other foods that are naturally rich sources of folate (e.g., dark green vegetables, lettuce) did not contribute as much folate to the diet, because few women consumed these foods.

6.6 Discussion

6.6.1 Folate Status in relation to fortification

We investigated folate and vitamin B status following folic acid fortification of the food supply in 95 healthy women of childbearing age residing in Manitoba. No woman was found to have a serum folate value indicative of compromised folate status. Mean serum folate concentration for all supplement users and nonusers was 14.6 ng/ml (33.1 nmol/L). These results are similar to those of two Canadian studies. House et al reported the median (5^{th} and 95^{th} percentile) for serum folate was 11.1 ng/ml (3.5 - 19.4) for women of the same age (27). Ontario study (28) reported the mean (10^{th} and 90^{th} percentiles) serum folate for 308 females aged 57 y to be 16.3 ng/ml (10.5-19.8). Jacques et al (29) reported that the prevalence of plasma folate values < 3 μg/L (< 6.8 nmol/L) improved to a greater extent among nonusers of supplements compared users of dietary supplements after fortification was implemented. Although the proportion of people with a suboptimal folate status in other studies has varied between 0-79% (30-34), this variability is due in part to analytical methods, populations and criteria and population characteristics (34).

The mean red cell folate concentration was 325.7 ng/ml (706.7 nmol/L) and was almost 2.3 times higher than the level deemed acceptable 140 ng/ml (304 nmol/L). Fourteen percent of all participants had red blood cell folate concentration >906 nmol/L, which is associated with very low NTD risk (35). Similar results for the median RBC folate in Canadian women of the same age group was reported by House et al (27) where the median of RBC folate was 650 nmol/L. Ray et al (28) reported median value for RBC folate of 995 nmol/L.

6.6.2 Other vitamin status in relation to fortification

Previous studies of vitamin B_6 in young women are sparse. The mean plasma vitamin B_6 obtained in this study was 74.5nmol/L, was 2.5 times higher than the mean reported by other investigators for the same group (36).

The median serum vitamin B_{12} in this study was higher compared to values reported for Canadian women (27) and older women aged 57 y (28). Three percent of the older women were below the optimum level of vitamin B_{12}, which is similar to results of an earlier investigator (37) who found 3% had marginally low serum B_{12} concentrations. The higher serum vitamin B_{12} in this study could be explained by the 25% of women taking supplements, while neither of the other two studies recorded the use of vitamin B_{12} supplement and age-related decline in vitamin B_{12} absorption (38). Another study found similar mean serum vitamin B_{12} as this study in women aged 25- 64 y among both supplement users and non users (39). Pfeiffer et al (37) found that serum vitamin B_{12} concentrations did not differ significantly in the young women between the pre-fortification and post-fortification periods with the 5^{th} - 95^{th} percentiles at 186-639 pmol/L (31) and at 179-738 pmol/L (37), respectively. The 5^{th} and 90^{th} percentiles for females in Canada are 135-697.3 pmol/L (28).

The lower median tHcy concentration reported in this study compare to the median for Canadian women in Ray et al study (8.4 μmol/L) and the median for young women (7.6 μmol/L) reported by Rasmussen (32), is likely due to the younger group with almost sufficient vitamin B intakes and good vitamin B biomarkers. Intakes and blood concentrations of folate, vitamin B_6 and B_{12} were found to be negatively associated with tHcy concentrations (40-43). tHcy concentrations after fortification

appeared lower than before. The prevalence of elevated tHcy after FA fortification was 10% comparing with a prevalence of 20% before fortification in the Framingham population (29). These results may not be generalized to all women of childbearing age, as this was a non-random, convenience sample form one geographic location. In addition, this study lacked a pre-FA fortified control group and comparisons of serum folate and red blood cell folate results obtained from different laboratories and methods may not be directly comparable

6.6.3 Folate, vitamin B_6 and B_{12} intakes

Mean daily folate intake from food, fortified foods, and supplements was 645.7 ±368.4 μg DFE/d. The reported intake for similar age and gender group living in Vancouver was 812 ± 710 μg DFEs/d (44), and the daily intake reported for American women aged 18-39 y was 745 ± 70 μg DFEs/d (45). The apparently lower average intake in our study may relate to the use of different dietary assessment methods.

The approximately 26% (n=25) of participants who took a supplement containing folic acid had a median intake from supplements of 400 μg SFA/day (range = 43-1000 μg SFA/day). The contribution of SFA to total folate intake, when calculated with using of the bioavailability correction factor for SFA was 51%. The dietary folate intakes of supplement users from food alone were similar to those of nonusers. This finding agreed with the results of Bree and collegues (46), where the mean dietary folate intakes were similar among supplement users and nonusers.

With the use of folic acid-containing supplements, and the 1998 folic acid fortification program, 79% and 91% of women in this study met the DRA and EAR for folate, respectively. Lewis et al (47) found that 85% of, women aged 20-49 y in

NHANES III met or surpassed the EAR 75% of childbearing age women did so in the Continuing Survey of Food Intakes by Individuals (CSFII;7). In the current studt only 17% of study participants met the special recommendation for women capable of becoming pregnant and no one met this recommendation when folic acid intake did not include supplementation. Lewis et al (47) estimated that 16% of women of childbearing age from NHANES III data and 31% from the CSFII study were consuming this special recommendation for childbearing age women.

One concern with the fortification program is that it will increase the population with potentially un-safe folic acid intakes. In the current study, one of the participants exceeds the UL, resulting from the use of 1000 μg folic acid-containing supplements. These findings are slightly lower than reported by Lewis et al (47) and French et al (44) where they found only 5% and 7%, respectively, of women exceeded the UL.

The increase in folic acid consumption due to fortification reported in this study was 96 μg/d. This is consistent with the prediction by the FDA that the fortification policy will deliver 70-130 μg folic acid/d for adults 19 y old (48) and similar to results of other recent studies (47, 49). These results are encouraging because they suggest that the fortification program is reaching women of reproductive age.

A daily median (range) dietary intake of vitamin B_6 for all subjects was 1.9 mg/d (0.62- 4.2 mg/d), which is above the EAR of 1.1 mg/d for this group (50). The intakes of vitamin B_6 were below the RDA in 6% of all participants, and below the EAR in 3% of the total participants. A daily median (range) dietary intake of vitamin B_{12} for all subjects was 3.6 μg/d (0.56 -15.3 μg/d), which is above the EAR of 2.0 mg/d for this group (50). The intakes of vitamin B_{12} were below the RDA in 13.1 % of participants, and below the EAR in 11.5% of participants.

6.6.4 Folate sources

The foods which provided the most folate for women in this study were similar to the major sources of folate reported in earlier national studies (22). Vegetables contribute 17.7% of the total folate consumed by these women. The top folate contributors were bread and bakery products, vegetables, juices, pasta and rice, and ready to eat cereal. The food groups that contributed more than 6% of total folate intakes identified in NHANES 1999-2000 data were bread, rolls and crackers, vegetables, breakfast cereal, mixed dishes, fruit and juice, rice and other grain products, pasta, beans and peanuts (22). In a recent study, it was found that women consumed the greatest proportion of total dietary folate from grains (41%). And tht fruit and vegetables (excluding orange juice) contributed the next largest fraction of dietary folate (21%) (51).

Canada began its mandatory fortification of all flour and some corn and rice products with folic acid in November 1998 which had a significant effect on the risk for NTD. Based on the national birth certificate data, Honein et al (52) reported that the prevalence of NTD decreased by 19% in the US when comparing pre- and post-FA fortification data (52). In Ontario, Canada, the NTD incidence decreased by 47% from 1995-1999 (53). Most recently, De Wals et al assessed changes in the prevalence of NTDs in Canada before and after food fortification in seven Canadian provinces from 1993-2002. On the basis of published results of testing of RBC folate, the study period was divided into prefortification, partial fortification, and full-fortification periods. A total of 2446 subjects with NTDs were recorded among 1.9 million births. The prevalence of NTDs decreased from 1.58 per 1000 births before fortification to 0.86 per

1000 during the full fortification period, a 46% reduction (95% confidence interval, 40 to 51). The decrease was greatest in areas in which the baseline rate was high (54)

6.7 Conclusion

These preliminary data suggest that folic acid fortification of the food supply is resulting in significant improvements in folate status in women, but it was not enough to reach the recommended RBC folate concentration associated with a significant reduction in NTD risk for women of productive age. Only 17% of the participants met the special recommendation of women capable of becoming pregnant. Almost 100 μg of folic acid is being delivered to the target population, that meet the projected average increase of folic acid intake in the general population attributable to fortification of enriched cereal–grain products (14). It seems necessary to distinguish between food folate forms in advising an increase in folate intake from the diet. The partitioning of SFA intakes from total folate intakes allows the extent to which the new recommended DFE intake to be estimated. Nutritional monitoring for folate status remains important, not only for women of childbearing age but also for children and persons of all ages.

6.8 References

1. Nourhashemi F, Gillette-Guyonnet S, Andrieu S, et al. Alzheimer disease: protective factors1. Am J Clin Nutr 2000;71:643s-649.
2. Hartman TJ, Woodson K, Stolzenberg-Solomon R, et al. Association of the B-Vitamins pyridoxal 5'-Phosphate (B6), B12, and folate with lung cancer risk in older men. Am J Epidemiol 2001;153:688-693.
3. Czeizel AE, I. D. Prevention of the first occurrence of neural-tube defects by periconceptional vitamin supplementation. N Engl J Med. 1992;24:1832-1835.
4. Eskes TKAB. Abnormal folate metabolism in mothers with Down syndrome offspring: Review of the literature. European Journal of Obstetrics & Gynecology and Reproductive Biology 2006;124:130-133.
5. George L, Mills JL, Johansson AL, et al. Plasma folate levels and risk of spontaneous abortion. JAMA. 2002;288:186-1873.
6. Wilson A, Platt R, Wu Q, et al. A common variant in methionine synthase reductase combined with low cobalamin (Vitamin B12) increases risk for spina bifida. Mol Genet Metab 1999;67:317-323.
7. Steen MT, Boddie AM, Fisher AJ, et al. Neural-tube defects are associated with low concentrations of cobalamin (vitamin B12) in amniotic fluid. Prenatal Diagnosis 1998;18:545-555.
8. Ronnenberg AG, Goldman MB, Aitken IW, Xu X. Anemia and deficiencies of folate and vitamin B-6 are common and vary with season in Chinese women of childbearing age. J Nutr 2000;130:2703-2710.

9. Homocysteine Lowering Trialists' Collaboration. Dose-dependent effects of folic acid on blood concentrations of homocysteine: a meta-analysis of the randomized trials. Am J Clin Nutr 2005;82:806-812.

10. Gjesdal CG, Vollset SE, Ueland PM, et al. Plasma total homocysteine level and bone mineral density: the Hordaland homocysteine study. Arch Intern Med 2006;166:88-94.

11. Refsum H, Nurk E, Smith AD, et al. The Hordaland homocysteine study: a community-based study of homocysteine, its determinants, and associations with disease. J Nutr 2006;136:1731S-1740.

12. Cuskelly GJ, McNulty H, Scott JM. Fortification with low amounts of folic acid makes a significant difference in folate status in young women: implications for the prevention of neural tube defects. Am J Clin Nutr 1999;70:234-239.

13. Ray JG, Vermeulen MJ, Boss SC, DE. C. Declining rate of folate insufficiency among adults following increased folic acid food fortification in Canada. Can J Public Health. 2002;93:249-53.

14. Choumenkovitch SF, Selhub J, Wilson PWF, Rader JI, Rosenberg IH, Jacques PF. Folic Acid Intake from Fortification in United States Exceeds Predictions. J. Nutr. 2002;132:2792-2798.

15. Institute of Medicine. Dietary reference intakes: for thiamin, riboflavin, niacin, vitamin B6, folate, vitamin B12, pantothenic acid, biotin. Washington, DC: National Academy Press., 2000.

16. Institute of Medicine. Dietary reference intakes: folate, other B vitamin and choline. Washington, DC: National Academy Press, 1998.

17. Sevenhuysen GP, Gross U. Documenting the reasons people have for choosing their food. Asia Pac J Clin Nutr 2003;12:30-37.

18. Shuaibi A, Sevenhuysen G, House JD. Validation of a food choice map to assess folate and vitamin B12 intakes in college-aged women. Appl Physiol Nutr Metab 2006;31:356.

19. Health Canada Food program Canadian Nutrient File (CNF). [cited 2001]. Available from:http://www.hc.sc.gc.cs/food-aliment/ns-sc/nr-rn/surveillance/cnf-fcen/e_cnf_download.html., 2001.

20. Gates GE, Holmes TW. Folate Intake and Supplement Use in Women of Childbearing Age. Family Economics and Nutrition Review 1999;12:14-25.

21. Melse-Boonstra A, West CE, Katan MB, Kok FJ, Verhoef P. Bioavailability of heptaglutamyl relative to monoglutamyl folic acid in healthy adults. Am J Clin Nutr 2004;79:424-429.

22. Dietrich M, Brown CJP, Block G. The Effect of Folate Fortification of Cereal-Grain Products on Blood Folate Status, Dietary Folate Intake, and Dietary Folate Sources among Adult Non-Supplement Users in the United States. J Am Coll Nutr 2005;24:266-274.

23. Araki A, Sako Y. Determination of free and total homocysteine in human plasma by high performance liquid chromatography with fluorescence detection. J Chromatogr 1987;422:422-452.

24. Gilfix BM, Blank DW, Rosenblatt DS. Novel reductant for determination of total plasma homocysteine. Clin Chem 1997;43:687-688.

25. Rasmussen K, Moller J, Lyngbak M, Pedersen A, Dybkjaer L. Age- and gender-specific reference intervals for total homocysteine and methylmalonic acid in plasma before and after vitamin supplementation. Clin Chem 1996;42:630-636.

26. Hintze JL. Number Cruncher Statistical System 2000 Statistical System for Windows. 2000 ed. Kaysville, Utah: Number Crunched Statistical Systems., 1999.

27. House JD, March SB, Ratnam S, Ives E, Brosnan JT, Friel JK. Folate and vitamin B12 status of women in Newfoundland at their first prenatal visit. CMAJ 2000;162:1557-1559.

28. Ray JG, Cole DEC, Boss SC. An Ontario-wide study of vitamin B12, serum folate, and red cell folate levels in relation to plasma homocysteine: is a preventable public health issue on the rise? Clin Biochem 2000;33:337-343.

29. Jacques PF, Selhub J, Bostom AG, Wilson PW, IH. R. The effect of folic acid fortification on plasma folate and total homocysteine concentrations. N Engl J Med. 1999;13:1449-54.

30. Brussaard JH, Lowik MR, van den Berg H, Brants HA, W. B. Dietary and other determinants of vitamin B6 parameters. Eur J Clin Nutr. 1997;51:S39-45.

31. Selhub J, Jacques PF, Rosenberg IH, et al. Serum Total Homocysteine Concentrations in the Third National Health and Nutrition Examination Survey (1991-1994): Population Reference Ranges and Contribution of Vitamin Status to High Serum Concentrations. Ann Intern Med 1999;131:331-339.

32. Rasmussen LB, Ovesen L, Bulow I, Knudsen N, Laurberg P, Perrild H. Folate intake, lifestyle factors, and homocysteine concentrations in younger and older women. Am J Clin Nutr 2000;72:1156-1163.

33. Brouwer DAJ, Welten HTME, Reijngoud D-J, van Doormaal JJ, Muskiet FAJ. Plasma folic acid cutoff value, derived from its relationship with homocyst(e)ine. Clin Chem 1998;44:1545-1550.

34. Alfthan G, Laurinen MS, Valsta LM, Pastinen T, A. A. Folate intake, plasma folate and homocysteine status in a random Finnish population. Eur J Clin Nutr 2003;57:81:8.

35. Daly LE, Kirke PN, Molloy A, Weir DG, JM. S. Folate levels and neural tube defects. Implications for prevention. JAMA. 1995;274:1698-702.

36. Mennen LI, de Courcy GP, Guilland J-C, et al. Homocysteine, cardiovascular disease risk factors, and habitual diet in the French Supplementation with Antioxidant Vitamins and Minerals Study. Am J Clin Nutr 2002;76:1279-1289.

37. Pfeiffer CM, Caudill SP, Gunter EW, Osterloh J, Sampson EJ. Am J Clin Nutr 2005;82:442-450.

38. Andres E, Loukili NH, Noel E, et al. Vitamin B12 (cobalamin) deficiency in elderly patients. CMAJ 2004;171:251-259.

39. Wartanowicz M, Ziemlanski S, Bulhak-Jachymczyk B, L. K, Related Articles L. Assessment of nutritional folate status and selected vitamin status of women of childbearing age. Eur J Clin Nutr. 2001;55:743-7.

40. de Craen AJM, Stott DJ, Westendorp RGJ, et al. Homocysteine, B Vitamins, and Cardiovascular Disease. N Engl J Med 2006;355:205-211.

41. Selhub J, Jacques PF, Wilson PW, Rush D, IH. R. Vitamin status and intake as primary determinants of homocysteinemia in an elderly population. JAMA. 1993;270:2693-8.

42. Bjelland I, Tell GS, Vollset SE, Refsum H, PM. U. Folate, vitamin B12, homocysteine, and the MTHFR 677C->T polymorphism in anxiety and depression: the Hordaland Homocysteine Study. Arch Gen Psychiatry. 2003;60:618-626.

43. Brouwer IA, van Dusseldorp M, Thomas CMG, et al. Low-Dose folic acid supplementation decreases plasma homocysteine concentrations: a randomised trial. Indian Heart J 2000;52:S53-58.

44. French MR, Barr SI, Levy-Milne R. Folate intakes and awareness of folate to prevent neural tube defects: A survey of women living in Vancouver, Canada. J Am Diet Assoc 2003;103:181-185.

45. Clifford AJ, Noceti EM, Block-Joy A, Block T, Block G. Erythrocyte folate and its response to folic acid supplementation is assay dependent in women. J Nutr 2005;135:137-143.

46. Troppmann L, Gray-Donald K, T. J. Supplement use: is there any nutritional benefit? J Am Diet Assoc 2002;102:818-25.

47. Lewis CJ, Crane NT, Wilson DB, Yetley EA. Estimated folate intakes: data updated to reflect food fortification, increased bioavailability, and dietary supplement use. Am J Clin Nutr 1999;70:198-207.

48. Quinlivan EP, Gregory JF, III. Effect of food fortification on folic acid intake in the United States. Am J Clin Nutr 2003;77:221-225.

49. Edmonds J, Boushey CJ, Anderson SL. The Effect of Folic Acid Fortification on Folate Intake in Rural Women. J Am Diet Assoc 1998;99:A12.

50. Institute of Medicine Research Council. Dietary reference intakes:for thiamin r, niacin, vitamin B6, folate, vitamin B12, pantothenic acid, biotin. Dietary

reference intakes:for thiamin, riboflavin, niacin, vitamin B6, folate, vitamin B12, pantothenic acid, biotin. Washington, DC: National Academy Press., 2000.

51. Sherwood KL, Houghton LA, Tarasuk V, O'Connor DL. One-Third of Pregnant and Lactating Women May Not Be Meeting Their Folate Requirements from Diet Alone Based on Mandated Levels of Folic Acid Fortification. J Nutr 2006;136:2820-2826.

52. Honein MA, Paulozzi LJ, Mathews TJ, Erickson JD, LY. W. Impact of folic acid fortification of the US food supply on the occurrence of neural tube defects. JAMA 2001;286:2236.

53. Gucciardi E, Pietrusiak M-A, Reynolds DL, Rouleau J. Incidence of neural tube defects in Ontario, 1986-1999. CMAJ 2002;167:237-240.

54. De Wals P, Tairou F, Van Allen MI, et al. Reduction in Neural-Tube Defects after Folic Acid Fortification in Canada. N Engl J Med 2007;357:135-142.

6.9 Tables

Table 6.9.1: Characteristics of study participants

Characteristics	Mean[1] ±SD	Range
Anthropometric (n= 95)		
Age (y)	20.5 ± 1.9	17-24
BMI (kg/m^2)	22.7 ± 4.1	17.5-46.1
Body weight (kg)	61.0 ± 12.4	42.9-124
Body height (cm)	163.7 ± 5.8	150.5-176.0
Waist circumferences (cm)	70.7 ± 8.4	57-108
Dietary intake (n= 95)		
Energy (Kcal)	2154 ± 606	732.-3721.
Protein (g)	91.5 ± 25.5 (16.8% of energy)	27.2-187.9
Fat (g)	68.5 ± 30.6 (28.6% of energy)	17.2-152.7
Carbohydrate (g)	293.1±93.8 (54.4% of energy)	88.5- 644.7
Alcohol (g)	2.5 ± 7.8 (0.8% of energy)	0-45.734
Meal preparation (n= 95)		
By subjects	38	
By others	57	
Lifestyle factor		
Current smoker (n), %	(4) 4.2%	---
Oral contraceptive users (n), %	(40) 42.1%	---
Oral contraceptive pill nonusers (n), %	(55) 57.9%	---
Users of folate-containing supplement n, %	(25), 26 %	---
Users of vitamin B_{12}containing supplement (n),%	(25), 26%	---
Users of vitamin B_6containing supplement (n),%	(24), 25%	---
Current use of alcohol n, %	46, 48.4%	---

[1]Arithmetic value

Table 6.9.2: Estimated daily folate, vitamin B_{12} and B_6 intakes for 95 women of childbearing age.

	Supplement users and nonusers			Supplement nonusers [1,2]			Supplement users		
Daily intakes	Mean ±SD	Median	Range	Mean ±SD	Median	Range	Mean ±SD	Median	Range
Natural folate (µg/d)	314.4± 134.3	294.5	84.1 -875.5	317.9 ±135.6	290.6	124.4-875.5	304.3± 132.8	314.4	84.2-662.4
Folic acid from food (µg/d)	95.7± 63.9	83.1	4.1- 311.6	94.9 ±57.8	86.34	4.1-311.6	97.9 ± 79.8	65.4	16.4- 302.2
Folic acid from supplement (µg)	94.9 ± 189.3	0	0-1000	0	0	0	360.8 ± 201.0	400.0	43-1000
Total DFE	645.7 ±368.4	505.3	194.8-2277.1	486.4 ±170.1[a]	448.0	194.8-1215.2	1092.0± 408.5 [b]	963.5	458.5-2277.1
Vitamin B_6 (mg/d)	4.0 ± 7.9	2.3	0.619 - 54.1	2.1 ± 0.6 [a]	2.0	0.6 -3.8	9.7 ±14.5 [b]	4.5	2.4-54.1
Vitamin B_6 µg/g protein	44.19 ± 86.5	24.4	13.3- 695.4	23.17 ± 6.6 [a]	21.4	13.3 - 53.3	106.7 ± 158.2 [b]	51.6	30.1-695.4
Vitamin B_{12} (µg/d)	8.4 ± 11.2	4.2	0.6 -54.0	4.0 ± 2.4 [a]	3.5	0.6-15.3	20.8 ± 16.2 [b]	14.5	5.5-54.1

[1] Means compared using independent t test between supplement nonusers and users. Values with different subscripts in row differ significantly.
[2] Supplement nonusers for B_6 71, supplement nonusers for B_{12} 70, and supplement nonusers for Folic acid 70

Table 6.9.3: Mean serum concentration of vitamins and total homocysteine in women aged 18-25 y (n=95)

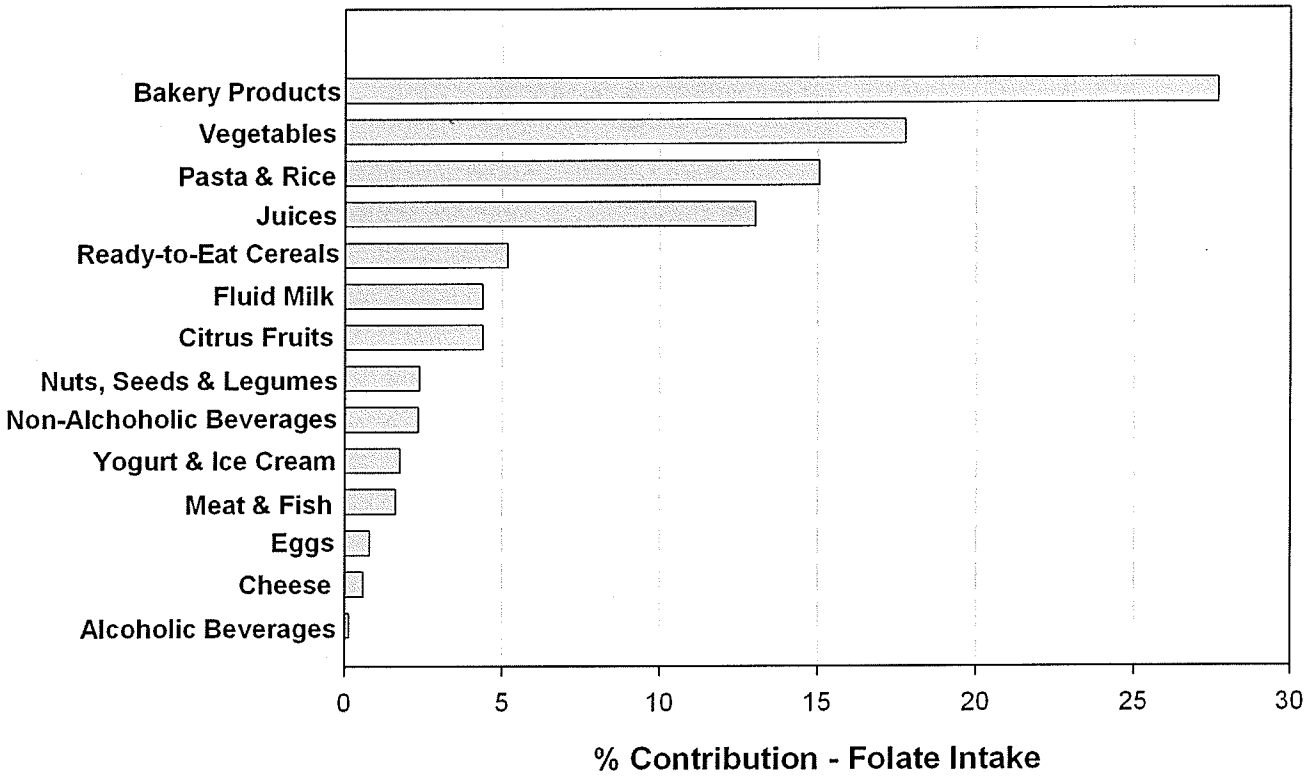
	Means ± SD	Median	Range (10^{th} and 90^{th} percentile)
Folate (serum)			
ng/ml	14.6 ± 3.5	14.1	10.0 - 19.3
(nmol/l)	33.0 ± 7.9	32.0	22.6 - 43.8
Folate (RBC)			
ng/ml	325.7 ± 143.1	299.6	188.3- 483.2
(nmol/L)	706.7 ± 310.6	650.2023	408.6- 1048.5
Vitamin B6[1]			
(nmol/L)	74.5 ± 50.0	63.2	32.6 -122.8
Vitamin B_{12}			
pg/ml	669.8 ± 452.3	563.2	312.8- 1085.0
(pmol/L)	494.0 ± 333.6	415.3	230.7-800.2
Total homocysteine (μmol/L)	5.3 ± 1.81	5.0	3.5- 7.5

[1] As pyridoxal-5-phosphate.

To convert nmol/L folate to ng/ml, multiply nmol/L by 0.441. To convert ng/ml folate to nmol/L by 2.27

6.10.1 Figures

Figure 6.9.1: Sources of folate in the diet for Canadian women of childbearing age



CHAPTER 7

7. DETERMINANTS OF TOTAL HOMOCYSTEINE IN YOUNG WOMEN.[1]

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[1] Prepared in manuscript format.

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Determinants of total homocysteine in young women [1, 2, 3]

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Key Words: Homocysteine, tHcy, folate, folic acid, dietary intake, vitamin supplement, alcohol, serum and red blood cell folate, vitamin B_{12}, body mass index.

7.1 Abstract

Background: An elevated total homocysteine (tHcy) concentration is considered to be an independent risk factor for cardiovascular diseases. Among women, raised tHcy level is associated with decreased bone mineral density, pregnancy complications and adverse pregnancy outcomes.

Objective: To evaluate the determinants of tHcy in healthy young women.

Design: tHcy concentrations were measured in 95 young women aged 17-25 y. All participants completed questionnaires about factors including lifestyle. Dietary and supplemental intakes for nutrients were assessed. Fasting blood samples were analyzed for serum folate, vitamin B_{12}, red blood cell folate, plasma vitamin B_6 (as pyridoxal-5-phosphate) and total homocysteine.

Results: Median tHcy was 6.9 μmol/L (range 3.6- 14.0). Overall hyperhomocysteinaemia, defined as 12.0 μmol/L, occurred in 4.2% women. Serum folate ($p= 0.0010$) and RBC folate ($p= 0.0124$) were inversely associated with log tHcy in multiple linear regression analyses. Body weight was positively associated with log tHcy ($p=0.0039$). Supplement users had a slightly lower mean tHcy than did nonusers (NS), but had significantly higher serum folate and serum vitamin B_{12} concentrations ($p= 0.005$, $p= 0.03$ for serum folate and Vitamin B_{12}, respectively).

Conclusions: The significant predictors of tHcy concentration were serum and RBC folate and body weight for healthy young women.

Key Words: Homocysteine, tHcy, folate, folic acid, dietary intake, vitamin supplement, alcohol, serum and red blood cell folate, vitamin B_{12}, body mass index.

7.2 Introduction

Hyperhomocysteinaemia has been associated with serious pregnancy complications, including pregnancy-induced hypertension, pre-eclampsia(1), placental abruption (2), early pregnancy loss and neural-tube defects(3, 4). Furthermore, many studies have shown an elevated concentration of tHcy is an independent risk for coronary ischemic disease, stroke, peripheral vascular disease, and stroke (4-7). Based on a meta-analysis including 16 prospective studies, there is a 23% increase in heart disease risk (Combined OR = 1.23) for every 5 μmol/L increase in tHcy (8). Several possible mechanisms that may underlie the positive association between tHcy concentration and risk for heart disease include oxidation of LDL cholesterol, toxic effects on endothelial cells, impaired platelet activity, and increase cell proliferation (9, 10). Recently, elevated tHcy in women was associated with reduced bone mineral density and increased risk of osteoporosis (4, 11, 12).

Various dietary and lifestyle factors influence tHcy (13). A high folate intake from diet or supplemental folic acid was associated with a decreased tHcy concentration in intervention (14-16) and cross-sectional studies (17). Intakes and blood concentrations of vitamin B_6 and vitamin B_{12} were found to be negatively associated with tHcy concentrations (5, 18). These associations are consistent with homocysteine metabolism. Homocysteine is either remethylated to methionine predominantly by methionine synthase (5-methyltetrahydofolate-homocysteine S-methyl-transferase) in a reaction that requires methyltetrahydofolate (as a methyl donor) and vitamin B_{12} (as an enzyme cofactor) or is transsulfurated to cysteine in reaction that requires pyridoxal-P, the

coenzyme from of vitamin B_6 (19).Protein intake was also found to be inversely associated with tHcy concentration (20).

Beside diet, other lifestyle factors such as smoking (9, 21), physical activity (4), and consumption of alcohol (9, 21, 22), coffee, and tea (21, 22) may have an effect on tHcy levels in the general population. Associations between dietary or lifestyle factors and tHcy have been investigated mainly in elderly or middle-aged persons. The determinants of tHcy, however, will likely to vary among cultures and countries (23). The primary aim of this study was to investigate the association between tHcy and its dietary determinants (estimated protein, methionine, folate, vitamin B_6, and vitamin B12), some lifestyle factors (physical activity, alcohol, caffeine), biochemical determinants (serum folate, plasma B_6, serum B_{12} and RBC folate) in a group of childbearing age women 18-25 y.

7.3 Material and methods

7.3.1 Subjects

A total of 95 healthy women aged 18-25 y were recruited from the University of Manitoba. Participants were recruited from October 2004 to March 2005. Exclusion criteria included: current pregnancy, pregnancy lasting more than 20 weeks in the year before the scheduled clinic visit, lactating, and the use of medications known to interfere with folate metabolism. Those who were taking oral contraceptives or folate supplements were not excluded. Approval of the study was obtained from the Ethics Committee of the University of Manitoba. The subjects gave informed consent before being admitted into the study.

7.3.2 Assessment of food consumption and nutrient intakes

Food consumption was assessed by Food Choice Map (FCM) by trained interviewers with a portable computer. The "FCM" nutrient analysis program was designed at the Department of nutrition for collecting data on quality, quantity and preparation of foods and beverages in a standardized way (24, 25). In a validity study of the FCM, the FCM was compared with 3-Day Food Records and biomarkers using method of triads and the FCM showed the highest validity coefficient (25).

In this study the intake of folates from natural folate, foods and folic acid supplements were calculated separately. Dietary intake was calculated from the CNF 2001b for all nutrients except for folate. Participants were asked to bring to the clinic visit any nutritional supplements they were currently taking. Information collected from the supplement containers and a self-administered questionnaire completed by each

participant included the type of the supplement used, the brand name, the dosage, average use during the previous year and the frequency of use during the week before the survey. Average daily intakes of supplemental folate vitamin B_6 and B_{12} were calculated from the responses and were added to the participant's dietary intakes of these vitamins.

7.3.3 Physical activity

Physical activity was reported by self-administered questionnaires, and included four levels of weekly activity: 0= mainly sedentary, 1= light (2-4 light physical activity weekly), 2= moderate (>4hrs light or 2-4 strenuous physical activity weekly) and 3= vigorous (>4 hrs strenuous physical activity weekly).

7.3.4 Correction for the bioavailability of folic acid from food

According to the IOM report (26), folic acid (FA) is considered to be more bioavailability than natural folate (NF). Food-composition values had to be adjusted so that the greater bioavailability of FA than NF was reflected in the total folate value for the food, which was then expressed as μg DFEs. Canadian Nutrient File 2001 (CNF) (27) food composition data were used to provide total folate contents of foods and synthetic folic acid (SFA). Natural folate (NF), which is equivalent to dietary folate equivalent (DFE) is not available in the CNF. Estimates of NF were calculated by subtracting the SFA from the total folate content values, where the SFA contents of each food were multiplied by 1.7 which is the recommended correction in the IOM report (26) because synthetic folic acid is considered to be more bioavailable than natural

folate. The resulting total folate values were then expressed as DFE, which could be used with the recommended of folate intakes for this age group.

7.3.5 Collection of blood

Fasting peripheral blood samples were collected by venipuncture by trained personal in the period October 2004 to March 2005. A hundred µl of well-suspended blood was diluted to 1 ml in freshly made 0.4% ascorbic acid solution to measure RBC folate. The plasma obtained from the EDTA specimens was used to measure total homocysteine and vitamin B_6. For serum folate, and vitamin B_{12} measurements, blood was collected in vacutainers containing no anticoagulant, left to clot for 30 min at room temperature then placed in ice. The samples were transported within 2 hr to laboratory where centrifuged at 1800g for 15 min. Serum and plasma were transferred to plastic vials and stored at ˉ80°C until measured.

7.3.6 Chemical analysis

Plasma total homocysteine was measured according to the reverse phase-HPLC method of Araki & Sako (28), with modifications as suggested by Gilfix *et al.*(29). Plasma pyridoxal 5′-phosphate (PLP) concentrations were measured using a commercial radioenzymatic assay (Vitamin B-6 PLP 3H-REA, ALPCO). RBC folate, serum folate and vitamin B_{12} were measured by competitive protein binding (Bio-Rad Laboratories, Mississauga, Canada). The maximum reporting limit for serum folate was 45 nmol/L, while that for RBC folate was 1450 nmol/L.

7.4 Statistical analysis

Results are expressed as average ± SD. Two-sample t test and Pearson correlations (or nonparametric alternatives as noted) were used for descriptive statistics. Pearson correlations and plots of the variables were used to examine variable nonlinearity. The relation between tHcy concentration and the independent variables was determined by using linear regression in which log-transformed tHcy was the outcome variable. A model was constructed with all variables that significantly correlated with log tHcy (body weight, waist circumferences, serum folate, RBC folate, serum vitamin B_{12}, food intake for vitamin B_{12}), putative determinant (age, height, plasma B_6, systolic and diastolic blood pressure, energy, alcohol, caffeine, protein, methionine, food intakes for vitamin folate and vitamin B_6, and the total intake for folate, vitamin B_6 & B_{12}), and other variables potentially associated with tHcy (BMI) to determine which variables were more effective in predicting log tHcy concentration. The generalized linear models were used to estimate geometric means of tHcy adjusted for confounders with all covariates set to their respective sample mean. P values <0.05 (two=sided) were considered statistically significant. All statistical tests were performed with NCSS statistical software (NCSS, 2004 Kaysville, Utah) (30). Because protein, methionine, folate, vitamin B_6 and B_{12} were highly correlated with energy, they were adjusted for energy by using the residual method described by Willett and Stampfer (31)

7.5 Results

7.5.1 Participant characteristics

Characteristics of the study population are shown in **Table 7.8.1.** The mean age of the participants was 20.5 y. About twenty-six percent used supplements containing one of the 3 B vitamins (folate, vitamin B_6 and B_{12}). Only ~ 4% smoked cigarettes, and they were supplement nonusers. Supplement users had a slightly lower mean tHcy than did nonusers (NS), but had significantly higher serum folate and serum vitamin B_{12} concentrations. Supplement user had a significantly lower BMI comparing to the nonusers.

For all subjects, there were significant positive correlations between body weight, waist circumferences, body mass index (BMI) and tHcy. There were significantly negative correlation between serum folate, RBC folate, and serum vitamin B_{12} concentrations. Association between tHcy and life style factors showed that activity level and caffeine consumption had the strongest linear association with tHcy concentration in a univariate analysis(r= -0.11, NS)

7.5.2 Dietary intake

The distributions of reported intakes of energy and nutrients are shown in **Table 7.8.2** (not energy adjusted). Median of total folate intake was 505 μg DFEs/d and median of folate intake from food (dietary) was 448 μg DFEs/d. Ninety-two percent of participants had a total folate intake < 400 μg DFEs/d and seventy-nine percent had an intake 320 μg DFEs/d (not shown). The most common supplement folate dose was 400 μg FA (680 μg DFEs) which was taken by 12 (46%) of the supplement participants; 4

participants (15%) took > 400 μg folic acid. The common supplemental vitamin B_{12} doses were 6, 10, and 20 μg/d, taken by 6 (19%), 4 (15%), 3 (12%) of supplements participants, respectively; 5 (19%) took >25 μg/d. The common vitamin B_6 does was 2 mg/d, taken by 8 (31%) of supplemented participants; 3 participants (11.5%) took > 20mg/d. After energy adjustment, there was no significant association between any of the dietary intakes and tHcy concentration.

7.5.3 Multivariable linear regression models

As illustrated in **Table 7.8.3** serum and RBC folate had highly significant negative coefficients, while body weight had a significant positive coefficient and the model explained 0.27 of the variance in tHcy.

In **Figure 7.9.1** we identified the potential impact of low folate concentration on high tHcy concentration; we predicted tHcy across a range of serum folate concentration, with the other variables held constant. The range serum folate consists of the 10^{th}, 20^{th}, 30^{th}, 40^{th}, 50^{th}, 60^{th}, 70^{th}, 80^{th}, 90^{th}, 99^{th}, and cutoff value for serum folate (3 ng/mL), which indicates a negative folate balance (26, 32).

In **Figure 7.9.2** we identified the potential impact of low RBC folate concentration on high tHcy concentration; we predicted tHcy across a range of RBC folate concentration, with the other variables held constant. A value of equal or more than 140 ng/mL of RBC folate was chosen as the cutoff point for adequate folate (26).

7.6 Discussion

In this study, we found that 4.2% of women presented plasma homocysteine levels greater than 12 μmol/L. This percentage is lower than in the US healthy adult women (33), in which a lower cutoff points was applied (10.4μmol/L) and in the Finnish population , where elevated tHcy (>14 μmol/l) was found in 11% of subjects (34).

We defined a tHcy concentration of ≤ 12 μmol/L as normal because other researchers have proposed age and gender-specific reference intervals (35). It is used by others for the same age and gender (32), and given that the risk for cardiovascular disease starts to increase with tHcy concentrations at 10.5-11.7 μmol/L (35).

The lower median tHcy concentration reported in this study (6.9μmol/L) compared to the median for Canadian women in Ray et al study (8.4 μmol/L), the median for US young women (7.6 μmol/L) reported by Rasmussen et al (13). The median for German healthy young women (7.7 μmol/L) reported by Dierkes (36), is likely due to include younger group with almost sufficient vitamin B intakes and good vitamin B biomarkers.

Serum folate, red blood cell folate and serum vitamin B_{12} are good predictors of tHcy (9, 13, 17, 18, 22), although often divergent when analyzing by gender. Thus, Mennen et al. reported an inverse relationship between RBC folate and tHcy only in men (23) . However, we found this is significant relationship among women. In case of Mennen et al, serum folate was not measured where as this was our best predictor of tHcy. At lower ranges of serum and RBC folate, tHcy decreases as folate concentration increases, but at higher level of folate concentration, tHcy reaches a low level and remains the same as folate concentration increases (37)

Body weight in this study showed a significant positive coefficient after adjusting for all other variables. Tungtrongchitr et al found that there were statistically significant higher levels of serum tHcy in overweight (n=149, BMI 31) than a control group (n=113, BMI=22)(38). Rasmussen et al found the BMI related significantly to tHcy in the younger group (25-30y) but not in the elderly group (60-65y) (13). Others found a slight relationship between these variables (22, 39), although in some they disappear when adjusted for certain variables (40) and in others there is none (41).

Dietary intake for folate vitamin B_6 and B_{12} have been shown to be positively associated with tHcy (42). However, neither any of them was associated with tHcy concentration in the present study. Almost all participants in this study obtained the recommended amount of folate, vitamin B_6 and B_{12} (43). Thus, the reason that the intake of this vitamin did not influence tHcy could be that the intakes in general were sufficient. Protein and methionine intakes have been shown a non -significant inverse association with tHcy as reported by others (22) and that agrees with the finding in this study.

Subjects who did not use supplements had 8.1% higher tHcy concentration than did those who use supplements, which agrees with 10.9% difference in tHcy between supplement users and nonusers (9, 13).

High levels of alcohol consumption increase tHcy concentration (9, 44). However; in this study, there was no statistically significant association between alcohol consumption and tHcy. This could be due to the small range of alcohol intake in women. The same finding reported in the literature for healthy young women (9, 18). Ganji and Kafai suggested that alcohol consumption $\leq$ 1drink/d may not inversely influence tHcy (9). The fact that we observed no association in women could indicate a dose effect of

ethanol. On the other hand, the type of alcoholic beverage consumed could be important (9, 18).

The potential association between physical activity and tHcy has received little attention. Bree et al found a weak positive relation between activity and tHcy level in women (21). This contrasts with the protective effect observed in the Hordaland Homocysteine study (40). Similar to the findings in this study, others found no association between activity level and tHcy (13, 17). Intervention studies might be able to clearly elucidate the effect of physical activity on tHcy concentration.

Strengths of the current study include methodological rigor, the use of fasting blood samples, and assessment of nutrient intake on the basis of a validated-dietary assessment method. Also the data used in this study were collected after folate enrichment became mandatory in Canada

In conclusion, folate status measures as serum and RBC folate and body weight have been found to be associated with tHcy for healthy young women. Several other variables were not consistently associated with tHcy. These results may no be generalized to all women of childbearing age, as this a non-random, convenience sample from one geographic location. Further aspects beyond the adequacy of vitamin factors (body composition, physical activity) should received intensive investigation.

7.7 References

1. El-Khairy L, Vollset SE, Refsum H, Ueland P. Plasma total cysteine, pregnancy complications, and adverse pregnancy outcomes: the Hordaland homocysteine study. Am J Clin Nutr 2003;77:467-472.
2. Holmes VA. Changes in haemostasis during normal pregnancy: does homocysteine play a role in maintaining homeostasis? Proc Nutr Soc 2003;62:479-493.
3. Tamura T, Picciano MF. Folate and human reproduction. Am J Clin Nutr 2006;83:993-1016.
4. Refsum H, Nurk E, Smith AD, et al. The Hordaland homocysteine study: a community-based study of homocysteine, its determinants, and associations with disease. J Nutr 2006;136:1731S-1740.
5. Herrmann M, Muller S, Kindermann I, et al. Plasma B vitamins and their relation to the severity of chronic heart failure. Am J Clin Nutr 2007;85:117-123.
6. Homocysteine Studies Collaboration. Homocysteine and risk of ischemic heart disease and stroke: A meta-analysis. JAMA 2002;288:2015-2022.
7. Refsum H, Smith AD, Ueland PM, et al. Facts and recommendations about total homocysteine determinations: An expert opinion. Clin Chem 2004;50:3-32.
8. Wald DS, Law M, Morris JK. Homocysteine and cardiovascular disease: evidence on causality from a meta-analysis. BMJ 2002;325:1202-1206.
9. Ganji V, Kafai MR. Demographic, health, lifestyle, and blood vitamin determinants of serum total homocysteine concentrations in the third National

Health and Nutrition Examination Survey, 1988-1994. Am J Clin Nutr 2003;77:826-833.

10. Jacob RA, Gretz DM, Taylor PC, et al. Moderate folate depletion increases plasma homocysteine and decreases lymphocyte DNA methylation in postmenopausal women. J Nutr 1998;128:1204-1212.

11. Gjesdal CG, Vollset SE, Ueland PM, et al. Plasma total homocysteine level and bone mineral density: the Hordaland homocysteine study. Arch Intern Med 2006;166:88-94.

12. Boushey CJ, Beresford SA, Omenn GS, AG. M. A quantitative assessment of plasma homocysteine as a risk factor for vascular disease. Probable benefits of increasing folic acid intakes. JAMA 1995;13:1049-57.

13. Rasmussen LB, Ovesen L, Bulow I, Knudsen N, Laurberg P, Perrild H. Folate intake, lifestyle factors, and homocysteine concentrations in younger and older women. Am J Clin Nutr 2000;72:1156-1163.

14. Brouwer IA, van Dusseldorp M, Thomas CMG, et al. Low-Dose folic acid supplementation decreases plasma homocysteine concentrations: a randomised trial. Indian Heart J 2000;52:S53-58.

15. Venn BJ, Mann JI, Williams SM, et al. Dietary counseling to increase natural folate intake: a randomized, placebo-controlled trial in free-living subjects to assess effects on serum folate and plasma total homocysteine. Am J Clin Nutr 2002;76:758-765.

16. Lamers Y, Prinz-Langenohl R, Bramswig S, Pietrzik K. Red blood cell folate concentrations increase more after supplementation with [6S]-5-

methyltetrahydrofolate than with folic acid in women of childbearing age. Am J Clin Nutr 2006;84:156-161.

17. Henriquez P, Doreste J, Deulofeu R, Fiuza MD, L. S-M. Nutritional determinants of plasma total homocysteine distribution in the Canary Islands. Eur J Clin Nutr 2007;61:111-8.

18. de Bree A, Verschuren WM, Blom HJ, Kromhout D. Association between B vitamin intake and plasma homocysteine concentration in the general Dutch population aged 20-65 y. Am J Clin Nutr 2001;73:1027-1033.

19. Selhub J. Homocysteine Metabolism. Annu Rev Nutr 1999;19:217-246.

20. Stolzenberg-Solomon RZ, Miller ER, III, Maguire MG, Selhub J, Appel LJ. Association of dietary protein intake and coffee consumption with serum homocysteine concentrations in an older population. Am J Clin Nutr 1999;69:467-475.

21. Bree Ad, Verschuren WMM, Blom HJ, Kromhout D. Lifestylefactors and plasma homocysteine concentrations in a general population sample. Am J Epidemiol 2001;154:150-154.

22. Koehler KM, Baumgartner RN, Garry PJ, Allen RH, Stabler SP, Rimm EB. Association of folate intake and serum homocysteine in elderly persons according to vitamin supplementation and alcohol use. Am J Clin Nutr 2001;73:628-637.

23. Mennen LI, de Courcy GP, Guilland J-C, et al. Homocysteine, cardiovascular disease risk factors, and habitual diet in the French Supplementation with Antioxidant Vitamins and Minerals Study. Am J Clin Nutr 2002;76:1279-1289.

24. Sevenhuysen GP, Gross U. Documenting the reasons people have for choosing their food. Asia Pac J Clin Nutr 2003;12:30-37.

25. Shuaibi A, Sevenhuysen G, House JD. Validation of a food choice map to assess folate and vitamin B12 intakes in college-aged women. Appl Physiol Nutr Metab 2006;31:356.

26. Institute of Medicine. Dietary reference intakes: for thiamin, riboflavin, niacin, vitamin B6, folate, vitamin B12, pantothenic acid, biotin. Washington, DC: National Academy Press., 2000.

27. Health Canada Food program Canadian Nutrient File (CNF). [cited 2001]. Available from:http://www.hc.sc.gc.cs/food-aliment/ns-sc/nr-rn/surveillance/cnf-fcen/e_cnf_download.html., 2001.

28. Araki A, Sako Y. Determination of free and total homocysteine in human plasma by high performance liquid chromatography with fluorescence detection. J Chromatogr 1987;422:422-452.

29. Gilfix BM, Blank DW, Rosenblatt DS. Novel reductant for determination of total plasma homocysteine. Clin Chem 1997;43:687-688.

30. Hintze JL. Number Cruncher Statistical System 2000 Statistical System for Windows. 2000 ed. Kaysville, Utah: Number Crunched Statistical Systems., 1999.

31. Willett W, Stampfer M, J. Total Energy intake: Implication for Epidemiologic Analysis. Am J Epodemiol 1986;124:17-27.

32. Wolters M, Hermann S, Hahn A. B vitamin status and concentrations of homocysteine and methylmalonic acid in elderly German women. Am J Clin Nutr 2003;78:765-772.

33. Selhub J, Jacques P, Rosenberg I, et al. Serum total homocysteine concentrations in the third National Health and Nutrition Examination Survey (1991-1994): population reference ranges and contribution of vitamin status to high serum concentrations. Ann Intern Med 1999;131:331:339.

34. Alfthan G, Laurinen MS, Valsta LM, Pastinen T, A. A. Folate intake, plasma folate and homocysteine status in a random Finnish population. Eur J Clin Nutr 2003;57:81:8.

35. Rasmussen K, Moller J, Lyngbak M, Pedersen A, Dybkjaer L. Age- and gender-specific reference intervals for total homocysteine and methylmalonic acid in plasma before and after vitamin supplementation. Clin Chem 1996;42:630-636.

36. Dierkes J, Kroesen M, Pietrzik K. Folic acid and vitamin B6 supplement and plasma homocysteine concentration in healthy women. Internat J Vit Nutr Res 1998;68:98-103.

37. Institute of Medicine. Dietary reference intakes: folate, other B vitamin and choline. Washington, DC: National Academy Press, 1998.

38. Tungtrongchitr R, Pongpaew P, Tongboonchoo C, et al. Serum homocysteine, B12 and folic acid concentration in Thai overweight and obese subjects. Int J Vitam Nutr Res 2003;73:8-14.

39. Jacques PF, Bostom AG, Wilson PW, Rich S, Rosenberg IH, Selhub J. Determinants of plasma total homocysteine concentration in the Framingham Offspring cohort. Am J Clin Nutr 2001;73:613-621.

40. Nygard O, Refsum H, Ueland P, Vollset S. Major lifestyle determinants of plasma total homocysteine distribution: the Hordaland Homocysteine Study. Am J Clin Nutr 1998;67:263-270.

41. Lussier-Cacan S, Xhignesse M, Piolot A, Selhub J, Davignon J, Genest J, Jr. Plasma total homocysteine in healthy subjects: sex-specific relation with biological traits. Am J Clin Nutr 1996;64:587-593.

42. Selhub J, Jacques P, Wilson P, Rush D, Rosenberg I. Vitamin status and intake as primary determinants of homocysteinemia in an elderly population. JAMA 1993;270:2693-8.

43. Shuaibi AM, House JD, Sevenhuysen G. B vitamin status and concentrations of homocysteine in Canadian women of childbearing age. FASEB J 2007;21:A705.

44. Cravo M, Gloria L, Selhub J, et al. Hyperhomocysteinemia in chronic alcoholism: correlation with folate, vitamin B-12, and vitamin B-6 status. Am J Clin Nutr 1996;63:220-224.

7.8 Tables

Table 7.8.1: Characteristics of the participants [1]

Characteristics	Value	Correlation with tHcy* (r)[2]	By supplement use No(n = 69)	Yes(n = 26)
Current cigarette smokers [n, %]	4, 4.2%	---	4, 42.%	0
Supplement users [n %]	25, (26)	---	---	---
Age (y)	20.5 ±1.9	0.09	20.6 ± 1.9	20.3 ± 1.9
Weight (kg)	61.0 ± 2.4	0.3 [3]	62.0 ± 13.4	58.5 ± 9.0
Height (cm)	163.7 ± 5.8	0.0928	163.2 ± 5.5	165.0 ± 6.5
Waist circumferences (cm)	70.7 ± 8.4	0.3019 [4]	71.7 ± 9.1	68.0 ± 5.6
Body mass index (kg/m^2)	22.7± 4.1	0.3206 [5]	23.2 ± 4.5	21.5 ± 2.6 [6]
tHcy (μmol/L)	7.2 ± 1.9	---	7.4 ± 2.1	6.8± 1.3
Serum folate (nmol/ml)	14.6 ± 3.5	-0.41 [3]	13.9 ± 3.5	16.1 ± 2.9 [7]
RBC folate (ng/mL)	325.7 ± 14.30	-0.32 [4]	303.8 ± 97.1	383.8± 212.6
Plasma B_6 [8](nmol/L)	74.5 ± 50.0	0.0579	70.0± 44.5	86.2± 61.7
Serum vitaminB_{12} (pg/ml)	669.8 ± 452.3	-0.2711 [4]	610.3 ± 455.7	827.9 ± 410.6 [9]
Systolic blood pressure (mmHg)	109.0 ± 9.7	0.1036	109.0 ± 9.5	109.1 ± 10.6
Diastolic blood pressure (mmHg)	71.7 ± 7.6	0.0374	71.8 ± 7.6	71.5 ± 7.6
Activity level	1.35 ± 0.9	-0.11	---	---

[1] average ±SD. Supplement use was defined as taking supplement of all 3 of the B vitamin folate, vitamin B_6, and vitaminB_{12}, either separately or as part of multivitamin. tHcy, total homocysteine, RBC, red blood cell.

[2] Pearson correlation coefficient for log (tHcy) with variable [or log (variable), except Spearman correlation coefficient for log (tHcy) with activity level

[3] $P<0.001$

[4] $P<0.01$

[5] $p<0.05$

[6, 7, 9] Significantly different from no (t test of means); [6] $P = 0.0251$, [7] $P = 0.0053$, [9] $P = 0.0301$

[8] As pyridxal-5-phosphate

Table 7.8.2: Percentiles of dietary intakes and correlations between intakes and tHcy for 95 women of childbearing age 17-24 y.[1]

	Percentile					
Daily dietary intake	10th	25th	50th (median)	75th	90th	Correlation with tHcy(r) [2]
Energy (kcal)	1432.2	1728.5	2082.5	2594.5	2953.0	-0.03
Folate						
Total intake (μg DFEs)	329.0	419.1	505.4	796.4	1200.6	-0.13
Food intake (μg DFEs)	243.8	377.3	447.6	570.6	700.6	-0.04
Vitamin B_6						
Total intake (mg/d)	1.4	1.8	2.3	3.2	5.3	-0.17
Food intake (mg/d)	1.3	1.5	1.9	2.4	2.9	-0.10
Vitamin B_{12}						
Total intake (μg/d)	1.6	2.9	4.2	8.9	18.6	-0.20
Food intake (μg/d)	1.0	2.8	3.6	5.2	6.3	-0.21[3]
Protein (g)	62.2	74.0	89.9	104.8	126.8	-0.07
Methionine (g)	0.80	1.3	1.6	1.9	2.5	-0.06
Alcohol (g)	0	0	0	0	6.734	-0.005
Caffeine (g)	0	1.6	30.5	111.2	340.2	-0.11

[1]n=95. Median and other percentiles shown are not energy adjusted, but data were energy adjusted for calculation of correlation coefficient. tHcy, total homocysteine concentration

[2]Pearson correlation coefficient for log(tHcy) with log (energy-adjusted dietary intake). Intakes adjusted for energy by residual method

[3]$P<0.05$

Table 7.8.3: Linear regression model predicting log (tHcy)

Independent variables	Regression coefficient	SE regression coefficient	p
Intercept	0.7003	0.2927	0.0189
Serum folate	-0.0102	0.0030	0.0010
Body weight	0.3840	0.1294	0.0039
RBC folate	-0.1564	0.0612	0.0124

n=95. Adjusted R^2= 0.27

7.9 Figures

Figure 7.9.1: Predicted total homocysteine concentration (tHcy) according to serum folate concentration (n=95). Predicted tHcy is from the model in Table 3 for a woman weighting 61 kg with RBC folate of 325 ng/mL. The dashed line shows the cutoff value for deficient levels of serum folate and the solid line showsthe median serum folate in the model.

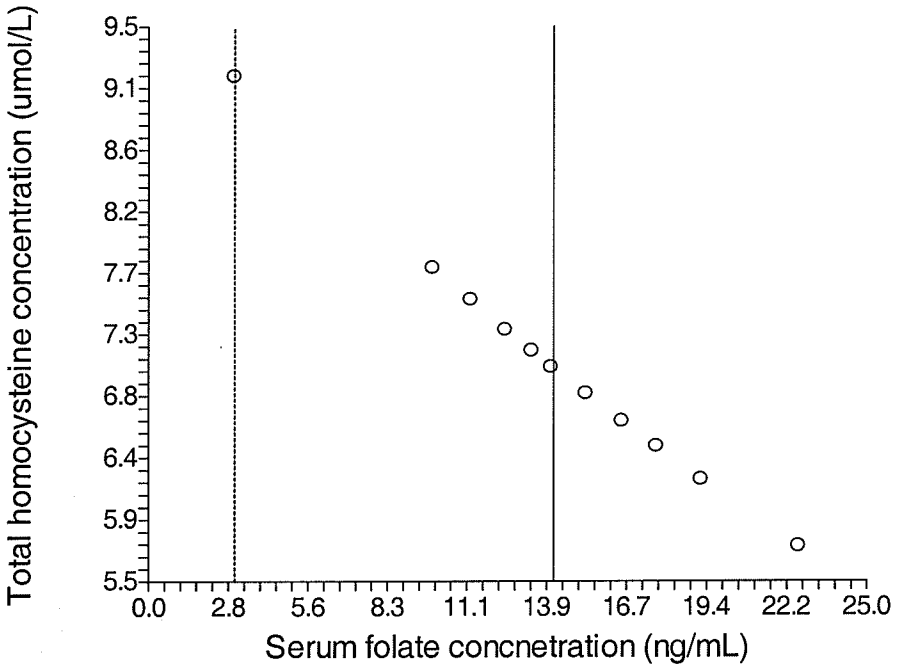
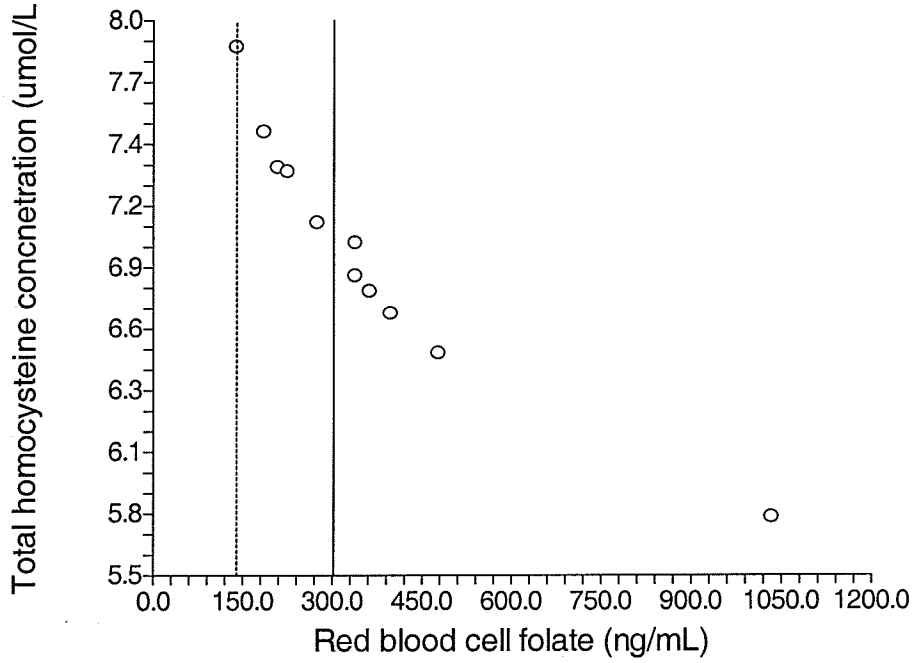


Figure 7.9.2: Predicted total homocysteine concentration (tHcy) according to RBC folate concentration (n=95). Predicted tHcy is from the model in Table 3 for a woman weighting 61 kg with serum folate of 14.5ng/mL. The dashed line shows the cutoff value for deficient levels of RBC folate, and the solid line showed the median RBC folate in the model.



CHAPTER 8

DISCUSSION

8.1 Contribution to present state of knowledge

The studies encompassed within this thesis were designed to address a number of issues and objectives in propelling knowledge related to methods of dietary assessment, the assessment of the validity of nutrient intake data and folate databases, and the impact of FA fortification on folate, vitamin B_{12} and tHcy status in college-aged women in Manitoba.

The first study focused on the development and validation of a novel method for the assessment of folate and vitamin B_{12} intakes in young women. With existing dietary assessment methods, there is the potential for both the underestimation and overestimation of folate intakes, depending on which method is used (1). As a result of the current research, a rapid dietary assessment tool, FCM has now been established as a useful, reliable, and valid form of dietary analysis for folate and B_{12} intake.

The nature of the FCM differs from most existing methods because respondents are asked to recall their general food behaviors over a week, which yields meal times and meal composition with alternative and substitute foods, rather than recalling the specific foods eaten at every instance of eating in a set time period. The result is a pattern of eating characterized by the frequencies of meal times, frequencies of foods by meals and throughout the week. These frequencies demonstrate the relative contributions of foods, including alternatives and substitutes, to overall nutrient intakes. They also show the respondent's uses of food and their possible fit with recommended diet structures. Previous studies of quantifying portion sizes have shown that the use of a series of photographs is associated with smaller errors in portion size perception than when using single average photograph or no graphs at all (2). Therefore, the

quantification of portion sizes in the FCM interview was carried out with the *Portion Photos of Popular Foods* (3), which contains life-size photos of many food items with several photos of the same food item depicting different portion sizes (small, medium and large portions, or different measures such as cups, spoons and scoops).

FCM tool offers several technical improvements for measuring the food consumption of individuals including the use of photographs for both foods and portion size as memory aids, the automation of data entry, and standardization of identification and description of foods during the interview. Such developments aim to reduce respondent burden and hence increase compliance; reduce errors resulting from memory lapses and incorrect estimation of portion sizes; and, in the case of electronic devices, reduce interviewer biases and minimize the tedious process of coding and entering the food record.

Among the important characteristics of any instrument such as the FCM is the need to have accurate assessments of target nutrient intake, a requirement for relatively low levels of subject burden, and the ability to accommodate persons with low levels of reading and mathematical literacy. Young women, an important target group for improving folate status, are generally overburdened with work, child care, school, and social obligations. As a result, low subject burden is of particular importance in this population.

Recently, the aim of most dietary validity studies has been the estimation of the correlation between the assessed nutrient value and the subject's "true" habitual intakes- the VC (4). Results from the current study suggest that both 3DFR and the FCM are valid methods for assessing folate and vitamin B_{12} intakes of young women aged 18-25 y. Using the VC as the sole criterion, it appears that the FCM was a more reliable and

valid tool for the assessment of folate and vitamin B_{12} intakes of young women, compared to the 3DFR.

The current results suggest that an updated folate database could predict folate nutritional status in a healthy population. The updated database achieved a valid assessment of folate intakes, with the content or intake of naturally occurring food folate reported separately from that of FA form in fortified foods and supplements. The latter point allowed the computation of DFEs for the assessment of intake and diet planning, allowed the computation of synthetic FA intake and comparisons with the UL for folic acid, and the special recommendation for women at childbearing age.

At mandated levels of FA fortification of the food supply, many Canadian women of child bearing age are unlikely to meet their special folate requirement from dietary sources alone. It is recommended by IOM that women consume 400 μg of FA daily from supplements, fortified foods, or both in addition to consuming food folate from a varied diet (5). Results of this study and others (6, 7) suggest that the fortification has delivered an additional 100 μg/day of folic acid to the diets of women of childbearing age. Initial projection of the effect of FA fortification on folate intake predicted an average increase ~ 80- 130 μg FA for adults > 19 years old (8, 9).These results are encouraging because they suggest that the fortification program is reaching its primary target, women of reproductive age.

One concern with the fortification program is that it will increase the proportion of the population with potentially un-safe FA intakes. In the current study, one of the participants exceeds the UL, resulting from the use of 1000 μg FA-containing supplements. The UL is established for FA not natural folate for the first time in the IOM report 1998 at the level of 1000μg (5).

Preliminary data on folate, vitamin B_{12} and tHcy status in women of childbearing age (18-25 years age, n= 95) post FA fortification of the food supply were obtained. No subject was found to have a serum folate value indicative of compromised folate status. The mean red cell folate concentration was approximately 2.3 times higher than the level deemed acceptable 140 ng/ml (304 nmol/L) (5). Fourteen percent of all participants had red blood cell folate concentration >906 nmol/L, which is associated with very low NTD risk (10) Three percent of the women were below the optimum level of vitamin B_{12}. Serum vitamin B_{12} concentrations (494.0 pmol/L) in this study were higher than those obtained from an Ontario cross sectional study, where the mean serum vitamin B_{12} for females was 399.1 pmol/L(11, 12). Vitamin B_{12} concentrations have significantly improved in the post-fortification era in Canada (12) .Mean plasma tHcy concentrations (5.3 μmol/L) fell within the lower end of normal range (5, 7, 13), and reflected the sufficient folate and other B vitamins status of the participants in this study.

The changes in folate status examined in this study were assessed by both nutrient intakes and biochemical measures. Blood biomarkers of folate status such as the levels of folate in serum and RBC and tHcy in plasma provided the best evidence of the effectiveness of the FA-fortification program, due to their relatively high sensitivities in relation to their specificities as markers of folate status. In addition, these biomarkers might provide information about the risks of other disease related to folate status such as cardiovascular disease and osteoporosis. Previous results have shown that an elevated concentration of tHcy is an independent risk for coronary ischemic disease, peripheral vascular disease, and stroke (14-17). Recently, elevated tHcy in women was associated with reduced bone mineral density and increased risk of osteoporosis (17-19).

This study is among a limited number of studies in which the association between dietary intakes of the B vitamins involved in homocysteine metabolism and the fasting plasma tHcy concentration was investigated for healthy women aged 18-25 y. Our results indicate that after adjustment for confounders, serum and RBC folate were inversely associated with plasma tHcy concentration. Our finding in this study supports previously reported inverse relationship between serum and RBC folate and tHcy (20-24). Furthermore, we showed that body weight was positively associated with plasma tHcy in women at childbearing age.. This supports the reported findings by others (22, 25), where they found the BMI related significantly to tHcy.

Strengths of the current investigation of the tHcy determinants include methodological rigor, the use of fasting blood samples, and the assessment of nutrient intake on the basis of a validated-dietary assessment method. Additionally, the data used in this study were collected after folate enrichment became mandatory in Canada, which helps in assessing the impact of FA fortification on tHcy status.

Paralleling the improved serum and RBC folate concentrations found among women of childbearing age following fortification of grain and cereal products has been a decline in the prevalence of spina bifida and anencephaly. The incidence rate of NTDs in Ontario, 1986-1999, decreased from 11.7 per 10,000 pregnancies in 1986 to 16.2 per 10,000 in 1995, and it subsequently decreased to 8.6 per 10,000 by 1999 (26). Most recently De Wals et al (27) assessed changes in the prevalence of NTDs in Canada before and after food fortification, The prevalence of NTDs decreased from 1.58 per 1000 births before fortification to 0.86 per 1000 during the full fortification period, a 46% reduction (95% Confidence interval, 40 to 51).

8.2 Summary of major contributions

The main findings of this research are:

1. The FCM, as adapted for folate and vitamin B_{12} intake, is a valid method to estimate these B-vitamins in women of childbearing age.
2. The validity coefficient of the FCM for folate and vitamin B_{12} intake is comparable with the validity coefficient in other studies (28, 29).
3. Reinforcing the importance of addressing the bioavailability of synthetic FA in addition to fortification and supplements in assessing the folate intakes
4. The updated folate database could predict folate nutritional status.
5. Women of childbearing age are achieving improvement in folate status in the post fortification era, but it is not enough to reach the recommended RBC folate concentration associated with a significant reduction in NTD risk.
6. Intakes of FA from fortified foods are within the level originally predicted for the fortification.
7. Among young women in this population, who are supplement users, taking FA containing supplements is a common behavior, which has to be taken into account when folate intake is evaluated.
8. Hyperhomocysteinaemia > 12.0 μmol/l occurred in 4.2% of women.
9. The significant predictors of tHcy concentration were serum and RBC folate, and body weight for healthy young women.

8.3 Conclusions

In conclusion, these findings indicate that the FCM is a valid method for the estimation of the dietary intake of folate and vitamin B_{12} in women of reproductive age. The VC analysis used to assess the validity of the dietary assessment methods in this study provides additional information that cannot be obtained from correlation coefficients only. Nutrient databases must reflect the changes made in DRIs. Adding variables to the databases in response to changes in units, as well as using different forms of the nutrient for the UL, would be an advantageous change for the reliability of nutrient assessments and the clinical interpretation of these assessments. Although FA intakes from fortified foods are within the level originally predicted for the fortification, many Canadian women of childbearing age are not likely to meet their requirements for folate. Considering blood biomarkers to examine the changes in folate status, 14% of all participants had red blood cell folate concentration >906 nmol/L, which is associated with very low NTD risk. Plasma tHcy concentrations fell within the lower end of normal range, and reflected the positive folate, vitamin B_6 and B_{12} balance of the participants in this study. Among several dietary, biochemical, and lifestyle determinants of tHcy status, it has been found that serum and RBC folate were inversely associated with tHcy concentration, and body weight was positively associated with tHcy concentration in healthy adult women.

8.4 Implications and suggested future research

The new dietary assessment method, FCM, introduced in this study proved to be a useful method for collecting data on folate intake in young women. This easily administered method may serve as a practical tool for both researchers and dietetics professionals in public health and clinical practice. By providing accurate data on folate

intake, the FCM method can form the basis for dietary counseling for this essential micronutrient.

The FCM assesses both nutrient intake and food patterns. This will help to meet the increased interest in the identification of dietary patterns as an alternative and complementary approach to single-nutrient analyses in relation to disease outcomes (30-32). Because of the high co-linearity among many of the nutrients in foods, it is often difficult to identify the effect of a single nutrient on health outcomes. Dietary patterns are characterized on the basis of habitual food consumption (30) and therefore present the opportunity to examine both nutrients and the foods that contain the nutrients. Therefore, the use of dietary patterns may represent a more powerful predictor for health outcomes than the analysis of any single nutrient alone. Furthermore, dietary pattern analysis offers an approach for better understanding the complexities of eating behaviors of different population groups and subgroups, and may be used to effectively develop dietary intervention strategies for target groups (33). The FCM will also enable diet counselors to quickly translate foods into nutrient intakes, which is essential in assessing performance and establishing goals.

Further testing is necessary to determine the impact that specific variables may have on the FCM ability to yield valid dietary information, including testing amongst groups differing in socioeconomic status, gender, and age, and for different nutrient classes.

Flexibility is key when designing a nutrient database that can easily adapt to changing dietary recommendation. In particular, disaggregating of both natural nutrient and nutrient added to food can provide a variety ways to calculate or combine variables. For example, if an availability factor for a nutrient such as folate changes in the future, it

will be possible to recalculate the DFEs if there are separate variables for the different forms of folate (naturally occurring in food, fortification added to food, and supplements). However, if the nutrient database contains only a single variable for DFE, it will take a substantial effort to recalculate the new values.

In general, it is useful to disaggregate nutrient values into at least the three major sources (food, fortification, and supplements), even if the bioavailability factors are currently believed to be the same. Not only will this approach provide more flexibility if the bioavailability assumptions change in the future, but nutrient database users could also be provided with informative new variables. For example, a better understanding of the amount of a nutrient that is provided by fortification (or supplementation) could lead to changes in nutrition education programs or to re-evaluation of the level of food fortification for some nutrients.

To allow users of nutrient databases to provide relevant evaluations of dietary data, developers will need to consider the bioavailability of the nutrient forms, and the source of the nutrient (food versus added or supplemental nutrients). There is a need for a dietary FA supplement database that is analytically tested and verified in order to further increase the reliability of the assessments. Questions regarding differences in bioavailability among food folate forms remain largely unresolved.

This study provides information about the folate intakes for women at childbearing age, using a validated dietary assessment method. This information can be used to help nutrition educators and policy markers in addressing inadequate folate intake by women of childbearing age. Increasing folate intake by encouraging women to add foods that are naturally rich in folate is a challenge for nutrition educators. Perhaps, a more successful approach is to encourage women to substitute good sources of folate for low-

folate choices. Encouraging all women of childbearing age to take supplements containing FA is another approach to improving folate status in this high risk group.

Further research should identify the best strategies to use when shaping nutrition intervention to increase women's intake of foods that are naturally rich or fortified with folates or to increase women's use of folic acid supplements. In addition to having information on the foods that are being consumed, we need to understand better the behaviours and environmental factors that shape food intake and supplement use. Only then can interventions and nutrition education programs be designed to effect an increased intake of folates.

Additional efforts will be needed to monitor the safety and effectiveness of the folate content of the folate fortification program. Such efforts include continued determinations of the folate content of foods and studies involving the stability and bioavailability of folate in foods. Studies need to be undertaken to determine the impact of the fortification on indicators of folate status in specific subgroups of the population, including those who might be at risk of excess intakes. Careful monitoring over time will be necessary to ensure that fortification does not have unintended consequences.

Further studies are needed to establish the determinants of tHcy in various ethnic populations and in population at risk of heart disease. Defining the determinants of tHcy concentrations is essential for understanding to what extent the plasma tHcy concentration can be lowered through dietary and lifestyle interventions. This should help in establishing recommendations for a cardio-protective lifestyle.

Despite the role of FA in decreasing the prevalence of NTDs, and potential risk of developing CHD (via homocysteine lowering), the authorities in many other countries are choosing not to introduce similar fortification legislation because of the concerns of

potentially adverse effects of FA discussed earlier in the consideration for fortification (34). Recent findings show that use of 5-methyltetrahydrofolate (5MTHF) has the same effect as FA in increasing blood folate indexes and lowering plasma tHcy (35, 36), and it may have some advantages over FA- Specifically, 5-MTHF is unlikely to mask vitamin B_{12} deficiency, it will not produce unmetabolized FA in the circulation, and it may be more bioavailable compared to the same dose of FA. A study in progress is being conducted to determine the relative absorption of 5MTHF from a fortified egg with an equivalent amount of the vitamin provided as a folic acid after a single ingestion in folate saturated subjects (for more details, please see appendix F).

Overall, this research has introduced a new dietary assessment method, FCM, which comprehensively assessed the dietary intakes for folate and vitamin B_{12} and energy and validated the new tool using different approaches. This research also estimated the level of FA in the diets of women of childbearing age in post-fortification, combined the dietary and biochemical assessment to address the implications of folate fortification. The findings in this research can be used to help nutrition educators and policy markers in addressing inadequate folate intake by women of childbearing age.

8.5 Strengths and limitations

Despite the demonstrated validity of the new FCM tool, the strengths and limitations of the validity study should be considered. The size of the study population is comparable with other validation studies (95 versus, 34 and 53, respectively) (28, 29). However, validation studies have been performed with up to 200 subjects, but without the use of the method of triads (37-39). The quantification of portion sizes in the FCM interview was carried out using a series of life-size photograph, which associated with

smaller errors in portion size perception than using single average photograph or no graphs at all (2). Using the same food and nutrient database to estimate the dietary intake for both the 3DFR and the FCM eliminate the databases added variability. The triad technique assumes that associations between the three measurements are linear and that their measurements of random error are mutually independent (4, 40). This may not be entirely valid because the FCM and the 3DFR may have some sources of error in common. However, the effect of this possible interdependence is seen as negligible because the methodologies of the FCM and 3DFR are different.

These results may not be generalized to all women of childbearing age, as this was a non-random, convenience sample form one geographic location. In addition, this study lacked a pre-FA fortified control group and comparisons of serum folate and red blood cell folate results obtained from different laboratories and methods may not be directly comparable.

8.5 References

1. Boushey CJ, Edmonds JW, Welshimer KJ. Estimates of the effects of folic-acid fortification and folic-acid bioavailability for women. Nutrition 2001;17:873-879.
2. Emma F, John NS M, Michael N, Julie M H, John C M, Ashley J A. Accuracy of estimates of food portion size using food photographs – the importance of using age-appropriate tools. Public Health Nutr 2006;9:509:514.
3. Hess M. Portion Photos of Popular Foods. The United States of America: The American Dietetic Association, 1997.
4. Ocke M, Kaaks R. Biochemical markers as additional measurements in dietary validity studies: application of the method of triads with examples from the European prospective investigation into cancer and nutrition. Am J Clin Nutr 1997;65:1240S-1245.
5. Institute of Medicine. Dietary reference intakes: folate, other B vitamin and choline. Washington, DC: National Academy Press, 1998.
6. Jacques P, Selhub J, Bostom A, Wilson P, Rosenberg I. The effect of folic acid fortification on plasma folate and total homocysteine concentrations. N Engl J Med 1999;13:1449-54.
7. Caudill MA, Le T, Moonie SA, Esfahani ST, Cogger EA. Folate status in women of childbearing age residing in southern California after folic acid fortification. J Am Coll Nutr 2001;20:129-134.

8. Quinlivan EP, Gregory JF, III. Effect of food fortification on folic acid intake in the United States. Am J Clin Nutr 2003;77:221-225.

9. Choumenkovitch SF, Selhub J, Wilson PWF, Rader JI, Rosenberg IH, Jacques PF. Folic Acid Intake from Fortification in United States Exceeds Predictions. J Nutr 2002;132:2792-2798.

10. Daly L, Kirke P, Molloy A, Weir D, Scot tJ. Folate levels and neural tube defects. implications for prevention. JAMA 1995;274:1698-702.

11. Ray JG, Cole DEC, Boss SC. An Ontario-wide study of vitamin B12, serum folate, and red cell folate levels in relation to plasma homocysteine: is a preventable public health issue on the rise? Clin Biochem 2000;33:337-343.

12. House J, March S, Ratnam M, Crowley M, Friel J. Improvements in the status of folate and cobalamin in pregnant Newfoundland women are consistent with observed reductions in the incidence of neural tube defects. Can J Public Health 2006;97:132-135.

13. Ueland P, Refsum H, Stabler S, Malinow M, Andersson A, Allen R. Total homocysteine in plasma or serum: methods and clinical applications. Clin Chem 1993;39:1764-1779.

14. Herrmann M, Muller S, Kindermann I, et al. Plasma B vitamins and their relation to the severity of chronic heart failure. Am J Clin Nutr 2007;85:117-123.

15. Homocysteine Studies Collaboration. Homocysteine and risk of ischemic heart disease and stroke: A meta-analysis. JAMA 2002;288:2015-2022.

16. Refsum H, Smith AD, Ueland PM, et al. Facts and recommendations about total homocysteine determinations: An expert opinion. Clin Chem 2004;50:3-32.

17. Refsum H, Nurk E, Smith AD, et al. The Hordaland homocysteine study: a community-based study of homocysteine, its determinants, and associations with disease. J Nutr 2006;136:1731S-1740.

18. Gjesdal CG, Vollset SE, Ueland PM, et al. Plasma total homocysteine level and bone mineral density: the Hordaland homocysteine study. Arch Intern Med 2006;166:88-94.

19. Boushey CJ, Beresford SA, Omenn GS, AG. M. A quantitative assessment of plasma homocysteine as a risk factor for vascular disease. Probable benefits of increasing folic acid intakes. JAMA 1995;13:1049-57.

20. Ganji V, Kafai MR. Demographic, health, lifestyle, and blood vitamin determinants of serum total homocysteine concentrations in the third National Health and Nutrition Examination Survey, 1988-1994. Am J Clin Nutr 2003;77:826-833.

21. Jacob RA, Gretz DM, Taylor PC, et al. Moderate folate depletion increases plasma homocysteine and decreases lymphocyte DNA methylation in postmenopausal women. J Nutr 1998;128:1204-1212.

22. Rasmussen LB, Ovesen L, Bulow I, Knudsen N, Laurberg P, Perrild H. Folate intake, lifestyle factors, and homocysteine concentrations in younger and older women. Am J Clin Nutr 2000;72:1156-1163.

23. Henriquez P, Doreste J, Deulofeu R, Fiuza M, Serra-Majem L. Nutritional determinants of plasma total homocysteine distribution in the Canary Islands. Eur J Clin Nutr 2007;61:111-8.

24. de Bree A, Verschuren WM, Blom HJ, Kromhout D. Association between B vitamin intake and plasma homocysteine concentration in the general Dutch population aged 20-65 y. Am J Clin Nutr 2001;73:1027-1033.

25. Tungtrongchitr R, Pongpaew P, Tongboonchoo C, et al. Serum homocysteine, B12 and folic acid concentration in Thai overweight and obese subjects. Int J Vitam Nutr Res 2003;73:8-14.

26. Gucciardi E, Pietrusiak M-A, Reynolds DL, Rouleau J. Incidence of neural tube defects in Ontario, 1986-1999. CMAJ 2002;167:237-240.

27. De Wals P, Tairou F, Van Allen MI, et al. Reduction in neural-tube defects after folic acid fortification in Canada. N Engl J Med 2007;357:135-142.

28. Verkleij-Hagoort A, de Vries J, Stegers M, Lindemans J, Ursem N, Steegers-Theunissen R. Validation of the assessment of folate and vitamin B(12) intake in women of reproductive age: the method of triads. Eur J Clin Nutr 2006;Dec 13; [Epub ahead of print].

29. Pufulete M, Emery P, Nelson M, Sanders T. Validation of a short food frequency questionnaire to assess folate intake. Br J Nutr 2002;87:383-390.

30. Hu FB. Dietary pattern analysis: a new direction in nutritional epidemiology.Nutrition and metabolism. Curr Opin Lipidol 2002;13:3-9.

31. Gao X, Yao M, McCrory MA, et al. Dietary pattern is associated with homocysteine and B vitamin status in an urban Chinese population. J Nutr 2003;133:3636-3642.

32. Tucker KL, Chen H, Hannan MT, et al. Bone mineral density and dietary patterns in older adults: the Framingham Osteoporosis Study. Am J Clin Nutr 2002;76:245-252.

33. Millen B, Quatromoni P, Gagnon D, Cupples L, Franz M, D'Agostino R. Dietary patterns of men and women suggest targets for health promotion: the Framingham Nutrition Studies. Am J Health Promot 1996;11:42-52.

34. Pentieva K, McNulty H, Reichert R, et al. The short-term bioavailabilities of [6S]-5-methyltetrahydrofolate and folic acid are equivalent in men. J Nutr 2004;134:580-585.

35. Venn BJ, Green TJ, Moser R, Mckenzie JE, Skeaff CM, Mann J. Increases in blood folate indices are similar in women of Childbearing Age Supplemented with [6S]-5-methyltetrahydrofolate and folic acid. J Nutr 2002;132:3353-3355.

36. Lamers Y, Prinz-Langenohl R, Moser R, Pietrzik K. Supplementation with [6S]-5-methyltetrahydrofolate or folic acid equally reduces plasma total homocysteine concentrations in healthy women. Am J Clin Nutr 2004;79:473-478.

37. Ishihara J, Yamamoto S, H I, Inoue M, Tsugane S. Validity of a self-administered food frequency questionnaire (FFQ) and its generalizability to the estimation of dietary folate intake in Japan. [Electronic version]. Nutr J 2005;Oct 5:4:26.

38. Brunner E, Stallone D, Juneja M, Bingham S, Marmot M. Dietary assessment in Whitehall II: comparison of 7 d diet diary and food-frequency questionnaire and validity against biomarkers. Br J Nutr 2001;86:405-414.

39. Hickling S, Knuiman M, Jamrozik K, Hung J. A rapid dietary assessment tool to determine intake of folate was developed and validated. J Clin Epidemiol 2005;58:802-808.

40. Kaaks R. Biochemical markers as additional measurements in studies of the accuracy of dietary questionnaire measurements: conceptual issues. Am J Clin Nutr 1997;65:1232S-1239.

APPENDICES

Appendix A

UNIVERSITY OF MANITOBA

RESEARCH SERVICES & PROGRAMS
Office of the Vice-President (Research)

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Winnipeg, MB R3T 2N2
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APPROVAL CERTIFICATE

20 July 2004

TO: James House
Gustaaf Sevenhuysen
Principal Investigators

FROM: Wayne Taylor, Chair
Joint-Faculty Research Ethics Board (JFREB)

Re: Protocol #J2004:114
"Effect of Folic Acid-Enriched Egg Consumption on the Folate Status of Manitoba Women of Childbearing Age"

Please be advised that your above-referenced protocol has received human ethics approval by the **Joint-Faculty Research Ethics Board** which is organized and operates according to the Tri Council Policy Statement. This approval is valid for one year only.

Any significant changes of the protocol and/or informed consent form should be reported to the Human Ethics Secretariat in advance of implementation of such changes.

Please note that, if you have received multi-year funding for this research, responsibility lies with you to apply for and obtain Renewal Approval at the expiry of the initial one-year approval; otherwise the account will be locked.

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Appendix B

CRITERIA CHECKLIST

Name: **Code:----------**

Phone: (For researcher purpose)

E-mail:

Questions	Response
Are you a female between 18-25 years old?	❑ Yes ❑ No
Are you a student at the University of Manitoba during the 2004-2005 year?	❑ Yes ❑ No
Are you pregnant? Are you planning a pregnancy in the next year? Were you pregnant or breastfeeding in the past 6 months?	❑ Yes ❑ No ❑ Yes ❑ No ❑ Yes ❑ No
Do you have arthritis, cancer, diabetes, epilepsy or any gastrointestinal condition?	❑ Yes ❑ No
Do you have any kidney problems?	❑ Yes ❑ No
Have you had any major surgery in the past 6 months?	❑ Yes ❑ No
Are you currently on medication? If yes, please list the names of the drugs and the purpose of using.	❑ Yes ❑ No

Appendix C

Manitoba College-Aged Women's Nutrition Study

**** All information contained in this questionnaire will be kept strictly confidential ****

Name (Last, First):	Code:
Street Address:	Postal Code:

Telephone number(s):

Home _____ **Work** _____ **Cell** _____

E-mail address:

Age (years):	Date of Birth (Year/Month/Day):

Marital Status:

❑ **Single** ❑ **Married**

Are you studying at the University of Manitoba during the 2004-2005 year?

❑ **Yes** ❑ **No**

If yes, please indicate faculty and department of study:

Faculty: _____

Department: _____

What year of study are you currently enrolled in?

Year _____ **of a** _____ **year program**

Do you have any of the following medical conditions? (Please check)

❑ **arthritis** ❑ **diabetes** ❑ **hypothyroidism**
❑ **cancer** ❑ **epilepsy** ❑ **liver disease**
❑ **chronic renal disease** ❑ **gastrointestinal disease/disorder**

Do you have any history of high cholesterol? **❑ Yes ❑ No**
Do you have any family history of high cholesterol? **❑ Yes ❑ No**
Do you take any medications or prescription drugs? **❑ Yes ❑ No** **If yes, please provide name and dosage:**
Do you take Aspirin or acetaminophen (Tylenol) on a regular basis? **❑ Yes ❑ No** **If yes, please indicate type and frequency of use:**
Have you ever given birth to a premature infant? **❑ Yes ❑ No**
Have you ever given birth to a low-birth-weight infant? **❑ Yes ❑ No**
Have you ever given birth to an infant with a neural tube defect? **❑ Yes ❑ No**
Have you ever had a miscarriage? ❑ Yes ❑ No
How would you describe your periods? **❑ regular to the same day** **❑ regular within several days** **❑ irregular**
When was the date of your last menstruation?
Do you use oral contraceptives? ❑ Yes ❑ No **If yes, please indicate type and length of use:**

How would describe yourself?

❑ a vegan (eating only vegetable products)
❑ a vegetarian (eating anything except meat, poultry, and/or fish)
❑ an omnivore (eating anything including some meat, poultry, and fish)

Do you follow a particular diet? (eg. Atkin's, South Beach, etc.)
❑ Yes ❑ No

If yes, please indicate type of diet:

Do you have any food allergies or intolerances?
❑ Yes ❑ No

If yes, please indicate which foods and reason:

Do you restrict any particular foods from your diet?
❑ Yes ❑ No

If yes, please indicate which foods and reason:

How is the food you eat normally prepared?

❑ prepared by self
❑ prepared by others and/or self in same residence
❑ prepared by a cafeteria

How often do you eat breakfast?
Please check or indicate amount:

❑ every day ❑ _____ times per week

Do you take any vitamins?
❑ Yes ❑ No

If yes, please indicate type and frequency:

Brand Name: _____

Type: _____ **(multi-vitamin, single vitamin eg. Vit.C)**

Frequency: Number of times _____ per _____ (day / week)

Do you take your vitamins with food?
❑ Yes ❑ No

Do you take any minerals?
❑ Yes ❑ No

If yes, please indicate type and frequency:

Brand Name: _____

Type: _____ (eg. Iron, Calcium, etc.)

Frequency: Number of times _____ per _____ (day / week)

Do you take your minerals with food?
❑ Yes ❑ No

Do you take any herbal supplements?
❑ Yes ❑ No

If yes, please indicate type and frequency:

Brand Name: _____

Type: _____ (eg. echinacea, gingko biloba, etc.)

Frequency: Number of times _____ per _____ (day / week)

Do you take your herbal supplements with food?
❑ Yes ❑ No

Do you take any dietary supplements?
❑ Yes ❑ No

If yes, please indicate type, amount, frequency, and purpose:

Brand Name: _____

Type: _____ (eg. meal replacement shake, energy bar)

Amount: _____

Frequency: Number of times _____ per _____ (day / week)

Purpose for taking dietary supplement:

If you are not currently taking any supplements, have you taken any on a regular basis in the past 6 months?
❑ Yes ❑ No

If yes, please indicate type, amount, and duration:

Type: _____

Amount: _____

Duration: _____ (number of months, years)

What amount and how often do you purchase eggs?

Number of cartons (one carton = 12 eggs): _____

Number of times _____ per _____ (week, month)

Do you eat eggs?
❑ Yes ❑ No

If yes, please indicate how often:

Number of eggs _____ per _____ (day/week/month)

If you eat eggs how do you normally prepare them?

Do you drink beer?
❑ Yes ❑ No

If yes, please indicate the amount and frequency:

Number of bottles/cans _____ per _____ (day/week/month)

Do you drink wine?
❑ Yes ❑ No

If yes, please indicate the amount and frequency:

Number of glasses _____ per _____ (day/week/month)

Do you drink liquor?
❑ Yes ❑ No

If yes, please indicate the amount and frequency:

Number of glasses _____ per _____ (day/week/month)

Ounces of alcohol per glass: _____

Do you drink coffee?
❑ Yes ❑ No

If yes, please indicate type, amount and frequency:

Regular coffee: Number of cups _____ per _____ (day/week/month)

Cappuccino: Number of cups _____ per _____ (day/week/month)

Latte: Number of cups _____ per _____ (day/week/month)

Do you drink tea?
❑ Yes ❑ No

If yes, please indicate type, amount and frequency:

Tea type: _____

Number of cups _____ per _____ (day/week/month)

Do you smoke?
❑ Yes ❑ No

If yes, please indicate amount and frequency:

Number of cigarettes _____ per _____ (day, week, month)

How would you describe your level of physical activity?

❑ mainly sedentary
❑ 2-4 hours light physical activity weekly
❑ >4 hours light or 2-4 hours strenuous physical activity weekly

❑ >4 hours strenuous physical activity weekly
Please indicate type of exercise:
Comments:

Thank you

Appendix D

Manitoba College-Aged Women's Nutrition Study

INSTRUCTIONS FOR 3-DAY FOOD RECORD

1) Write down all the foods you eat and drink for 3 complete days: 2 weekdays and 1 weekend day on the sheets provided. Choose days which represent your *usual* eating patterns (not days when you have a family barbeque or wedding). **The 3 days to NOT have to be consecutive.**

2) Record the place foods and/or supplements are consumed. If you are eating at a restaurant, please indicate which one.

3) Use the *sample food record* as an example and to make sure your food record is as accurate as possible.

4) Look over the *checklist* to make sure you have included the necessary details about your food intake.

5) Include all condiments, snacks and beverages consumed with or between meals.

6) Include amounts for *everything* (eg. sugar, jelly, creamer, salt, butter, oil, etc.)

7) Be specific about everything indicating amount and description. For example, instead of just recording "large bowl of cereal", record the type of cereal, amount, and what type of milk you used (1%, whole, skim). The *checklist* can help with providing detail.

8) If you are taking vitamin/mineral supplements, please write down what you take, and amount of the nutrient.

9) If you have any questions do not hesitate to call the study staff at 474-6423.

10) Be specific about everything indicating amount and description. For example, instead of just recording "large bowl of cereal", record the type of cereal, amount, and what type of milk you used (1%, whole, skim). The *checklist* can help with providing detail.

11) If you have any questions do not hesitate to call the study staff at 474-6423.

DESCRIBE WITH AS MUCH DETAIL AS YOU CAN
CHECKLIST FOR 3-DAY FOOD RECORD

Use this checklist to ensure you have included all necessary details for the 3-Day Food Record.

Type of Food	**Did you specify…**
All	Amount eaten? Use cup, tablespoon, ounce, deck of cards, and slice of bread or other common measures. Use size (dimensions such as length, width, thickness). Record quantity or weight
Cereal	Serving size? Brand name? Instant or ready-to-eat? Remember to include additions such as fruit, sugar, milk, or nuts.
Baked Goods	Homemade or commercial? From scratch or mix? Brand? Frosting or topping? Include size and/or weight.
Beverages/Drinks	Brand? Amount? Sweetened or diet? If juice, fresh, canned, or frozen? Is drink 100% fruit juice or a juice drink? Remember to include drinks with alcohol.
Eggs	How prepared? Any fat/oil added? Size?
Fats and Oils	Brand? Type (canola, olive, butter, shortening, etc.)? Stick, tub, diet, whipped, squeeze, or liquid margarine?
Fruit	Fresh, cooked, canned, frozen, or dried? Peeled? Size (small, medium, large)?
Meat, poultry, fish	How cooked? Added fat? Type of cut? Regular, lean, extra lean? Water or oil packed tuna? With or without fat or skin? Cooked weight or size of amount eaten (3 oz = size of a deck of playing cards)?
Milk, cheese, yogurt	Amount? Skim, whole, or low-fat milk? Percent fat? Liquid or powdered? Type of cheese? Added fruit or flavour to yogurt? Milk substitute?
Mixed dishes	Homemade, frozen, or restaurant-prepared? Brand? Cooking method? Added fat? Did you list individual ingredients such as meat, noodles, cheese, etc. and amounts?
Restaurant Meals	Refer to "Mixed dishes". Name of restaurant? Include nutrient information if available.
Sandwiches	Type and amount of bread, meat, cheese? Amount and type of condiments (ketchup, mayonnaise, mustard, etc.)? Lettuce, tomato, onion, pickles, etc.
Snacks	Brand, size, weight, number eaten? Include a label if possible.
Vegetables	Serving size? Fresh, frozen, canned? Cooked? Sauces or other additions? If lettuce, what kind?

Appendix E

UNIVERSITY
OF MANITOBA

Human Ecology Bldg.
University of Manitoba
Winnipeg, Manitoba
Canada R3T 2N2
Phone: (204) 474-9523
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_house@umanitoba.ca

Participant Information and Consent Form - Survey

Research Project Title: Manitoba College-Aged Women's Nutrition Study

Researcher(s): Dr. James House, Dept. of Human Nutritional Sciences
University of Manitoba

Dr. Gustaaf Sevenhuysen, Dept. of Human Nutritional Sciences
University of Manitoba

Aysheh Shuaibi, PhD student, Dept. of Human Nutritional Sciences
University of Manitoba

Sponsors: Canadian Egg Marketing Agency
Agri-Food Research Development Initiative (ARDI)

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This consent form, a copy of which will be left with you for your records and reference, is only part of the process of informed consent. It should give you the basic idea of what the research is about and what your participation will involve. If you would like more detail about something mentioned here, or information not included here, you should feel free to ask. Please take the time to read this carefully and to understand any accompanying information.

~~~~~~

Purpose

This research study is being conducted to study the intakes and status of a specific vitamin in women of childbearing age. The study is being conducted because the vitamin of interest is related to various health aspects, particularly relating to those of women of childbearing age. Recent data indicate that the intake of this vitamin is

insufficient in a large part of the population. Intakes and status will be assessed using two different diet analysis tools and will be related to nutrient recommendations. Intake will be related to vitamin status within the body. This research may lead to better information of vitamin intake of the population and if necessary development of strategies to improve it's intake.
If you meet certain criteria and are interested, you may be eligible to participate in the second part of this research study, an intervention trial.

Procedures

Subject
We will recruit a random sample of eighty to ninety healthy, non-pregnant women from the student population at the University of Manitoba. Participants will be aged 18-25 and enrolled in the 2004-2005 academic year. Participants will be required to fit certain inclusion criteria, which will be checked using a Criteria Checklist. You will not be able to participate if you are pregnant or if you are taking medications or have any conditions that are known to interfere with the absorption of the specified vitamin.

If you take part in this study, you will have the following tests and procedures:

You will be asked to attend three interviews. These interviews will take place on the University of Manitoba Fort Garry campus. For interview number one you will attend an information session about the research study. You will fill out a short questionnaire to ensure you meet study inclusion criteria. You will complete a general life questionnaire, which will include questions about your lifestyle, use of medications, medical history and diet information. You will be asked to complete a Selected Foods Checklist. We will provide you with a form to choose the consumption of selected foods. You will also be asked to complete a 3-Day Food Record. We will provide you with a form for recording all the food and beverages that you consume during three days (2 weekdays plus 1 weekend day). During this visit we will record your body weight, height, blood pressure, and waist circumference. You will also be provided with a schedule entailing all your responsibilities for the duration of the study.

For interview number two you will participate in a Food Choice Map interview. The Food Choice Map is a modern diet analysis tool used to assess your usual food intake. You will be asked to bring along all supplements and medications that you are taking. Food Checklist for will be provided to complete and bring it next interview.

For interview number three you will be required to give a fasting blood sample. You will be required to fast overnight (no food or beverages except water for 12 hours). You will arrive at University Health Services between 8:30 and 10:00 am the next morning. You will give a fasting blood sample (40 mL or 8 teaspoons). The blood will be taken from the vein on the forearm. The blood sampling will be done by a trained medical technologist or nurse at the clinic. The blood will be stored in a -80°C freezer until analysis.

Participation in the study will be for two or three weeks.

Participation in the study will involve

1. Information session (approximately 20-30 minutes total for Study Information, research Participant Information and Consent Form, Study Procedures), and measurement of body weight, height, and waist circumference (approximately 5 minutes)
2. Recording food consumption for three days with a 3-Day Food Record (approximately 20 minutes)
3. Completing Selected Foods Checklist (approximately 10 minutes)
4. Participating in a Food Choice Map interview (approximately 50 minutes)
5. Blood sample (approximately 5-10 minutes)

Risks and Discomforts

Since we will be taking a blood sample from the vein on the forearm, this procedure will result in a needle prick to the arm and there is a risk that it could result in a small bruise. All the blood sampling will be done by a trained nurse or medical technologist. The amount of the blood sample (40 ml or 8 teaspoons) is similar to when you give blood for medical tests.

Confidentiality

Research records and information that contain your identity will be treated as confidential. Your name, identification code and all information gathered during the study will be kept in a locked cabinet by the research staff. Only the research staff will have access to this information. Research staff will follow those guidelines as set out in the Personal Health Information Act (PHIA) of Manitoba and those in the Freedom of Information and Protection of Privacy Act (FIPPA) of Manitoba to maintain your confidentiality.

Your blood sample will be handled by a laboratory technician who only sees the identification code on each sample. Blood analysis conducted will be directly related to the research being conducted.

Information gathered in this research study may be published or presented in public forums. However, your name and other identifying information will not be used or revealed. Data analysis with the use of computers will be conducted using code numbers to protect your identity. No information revealing any personal information such as your name, address or telephone number will leave the University of Manitoba. The University of Manitoba Joint Faculty Research Ethics Board may review research-related records for quality assurance purposes. Your identity will remain confidential and any personal information gathered will be destroyed. All data collected and analyzed will be destroyed after 3 years.

Feedback

You will be received an individual dietary analysis and blood analysis report. A summary report of study findings will be available on request once the data analysis is completed.

Remuneration
You will receive $50 CDN upon completion of the survey interviews.

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Your signature on this form indicates that you have understood to your satisfaction the information regarding participation in the research project and agree to participate as a subject. In no way does this waive your legal rights nor release the researchers, sponsors, or involved institutions from their legal and professional responsibilities. <u>You are free to withdraw from the study at any time, and /or refrain from answering any questions you prefer to omit, without prejudice or consequence.</u> Your continued participation should be as informed as your initial consent, so you should feel free to ask for clarification or new information throughout your participation.

If you require more information please contact us:
Dr. House at 474-9523
Dr. Sevenhuysen at 474-9704
Study staff at 474-6423

This research has been approved by the Joint-Faculty Research Ethics Board. **If you have any concerns or complaints about this project you may contact any of the above-named persons or the Human Ethics Secretariat at 474-7122, or e-mail margaret_bowman@umanitoba.ca. A copy of this consent form has been given to you to keep for your records and reference.**

____
_

Participant's Signature Date

____
_

Researcher's Signature Date
~~~~~~

Appendix F

Egg 5-Methyltetrahydrofolate Bioavailability Study

1) **Proposal: The Short-Term Bioavailability of 5-Methyltertrahydrofolate and folic acid in young women**
2) **References**
3) **IRB Approval**
4) **Consent form**

1) Proposal: The Short-Term Bioavailability of 5-Methyltertrahydrofolate and folic acid in young women

KEY WORDS: • *[6S]-5-methyltetrahydrofolate [5-MTHF]* • *folic acid [FA]* • *plasma folate* • *short-term folate bioavailability*

Introduction

Folic acid taken before and during early pregnancy reduces a women's risk of neutral tube defects (NTD) affected pregnancy (1). A high rate of unplanned pregnancies led Canada to mandate fortification of grain based foods with folic acid in 1998(2). Mandatory fortification of foods has not occurred in many other countries because of concern about excessive folic acid intakes, a major concerns is that folic acid could mask the hematological signs of vitamin B_{12} deficiency, delaying diagnosis and allowing progression of neurological problems (3). Recently, a reduced form of folate, 5-methyltetrahydrofolate (5-MTDF), has become available that may be safer as a food fortification because it is unlikely to mask a vitamin B_{12} deficiency (4).Folic acid is able to mask the hematological signs of vitamin B_{12} deficiency because it is readily converted to tetrahydrofolate. In this way, Folic acid can by-pass the normal vitamin B_{12} dependent

control step and be metabolized and utilized for DNA biosynthesis even in vitamin B_{12} deficient cells, thereby allowing full remission of the anemia of vitamin B_{12} deficiency, while 5-MTHF requires a vitamin B_{12}-dependent enzyme, methionine synthase, for conversion to tetrahydrofolate (5). Thus, the question of whether of whether FA and 5-MTHF have equivalent bioavailability require further investigation. Eggs are an inexpensive and low calorie source of nutrients such as folate, riboflavin, selenium, choline and vitamin B_{12}. And eggs are also one of the few exogenous sources of vitamin K and D. Furthermore, eggs are a source of high quality protein, and the lipid matrix of the yolk serves to enhance the bioavailability of nutrients such as lutein and zeaxanthin (6). Therefore, there is possibility to extend their current fortification profile to include 5-MTHF. Dr. House's lab in animal Science department has developed egg yolk powder enriched with 5-MTHF(House, 2002 #158). However, the bioavailability of this 5-MTHF carried in the egg matrix has never been scientifically determined.

The **hypotheses** to be tested are:

1) Egg 5-MTHF has more bioavailability comparing to an equivalent dose of folic acid.

The **objectives** to be achieved are:

1) Enroll 22 healthy adult female.

2) Determine the relative absorption of 5MTHF from fortified egg with an equivalent amount of the vitamin provided as a folic acid after a single ingestion in folate saturated subjects.

Subjects and methods

Subjects

Female volunteers (n>22) 18-45 y will be recruited through advertisements among the staff and student population of the University of Manitoba. Exclusion criteria will be implemented to avoid the inclusion of subjects in the study who could give an abnormal response to the ingested folic acid or 5MTHF. All recruited volunteers will be interviewed using a short questionnaire regarding general health, supplement and medication use and blood sample will be taken for screening blood tests. Subjects will be excluded if they had a history of hepatic, gastrointestinal, renal vascular, hematological, or neuro-psychiatric disease, those who are taking folic acid containing supplements or medicine known to interfere with folate metabolism (aspirin, antiacides, aminopterin, pyrimethamine, phenytoin, methotrexate, sulphasalazine, Phenobarbital, primidon), those homozygous for the C677T (so called thermo labile) variant of the methylenetetrahydrofolate reeducates (MTHFR) gene, those with high plasma homocysteine concentration or low status of folate or related B vitamins (Table 1). Participants who meeting the inclusion criteria (n=22) will be included in the study.

Table 1 Subject characteristics

Category	Reference range	Reference
Age (y)	18-45	
Plasma folate (nmol/l)	6.80-45.23	Neuhouser et al; 2001 (8)
Hemoglobin value g/dl	12.1-15.1	
Red cell folate (nmol/l)	340-2266	Choumenkovitch et al; 2001(9)
Plasma B_{12} (pmol/l)	111-738	
Plasma pyridoxal phosphate (nmol/l)	$\geq$ 30	
Plasma total homocysteine (μmol/l)	$\leq$15.0	Wartanowics et al., 2001(10)
MTHFR genotype	CC/CT	
Cholesterol	200 mg/dL	Clinical biochemistry reference range

Clinical biochemistry reference ranges are given for all parameters except for plasma total homocysteine the cutoff for hyperhomocysteinemia defined by Wartanowics et al., 2001(10) has been used.

Study design and treatments.

In a cross-over design, subjects will receive three different treatments in random order, with an interval of at least 1 wk between each test day. The treatments will be as follows:

1) 30g folic acid fortified egg yolk powder (contains ~ 308.4 μg 5-MTHF) (equimolar to 297.6 μg FA), 2) 297.6 μg crystalline folic acid plus placebo powder, 3) No folate placebo powder)

The egg yolk powder for the fortified egg will be supplied from Dr. House lab. The egg yolk powder will be used to prepare smoothie and will be consumed by the participants through their breakfast, early in the morning after an overnight fast.

To minimize inter-individual differences in baseline plasma folate concentration, a presaturation regimen with Folic acid will be administrated before the start of the study (5mg/d Folic acid for 1 wk, followed by 2 folic acid free days before treatment) and during the intervals between treatments (5 mg folic acid for 5 days, followed by 2 folic acid-free days). This a presaturation regimen had been used by other research group (11, 12). To monitor compliance, subjects will receive the folic acid in a 7-d tablet organizer box and will be asked to return the box at each visit; any missed tablets will be recorded.

During each of the test days, subjects will consume an identical specially prepared low-folate diet **(Table 2).** Energy and nutrient contents of all meals and snack will be estimated using the FCM program

The low folate diet will be comprised of breakfast, and lunch, and provided two-thirds of energy requirements for female subjects with a sedentary lifestyle. the weighted mean of energy intake for Canadian women as estimated in the national survey of adult Canadian 1893 kcal/d (13)

The lunch meal will be prepared in advance in one batched and kept frozen at -20°C for the duration of the study. To decrease folate content all the ingredients of the lunch meal will be boiled three times, with the water discarded after each boiling, then to make the meal more palatable the dish will be seasoning and stir-frying before serving.

A duplicate portion of low folate diet (which included breakfast, two snacks and a lunch dish) will be analyzed for folate content. The 5MTHF content of the fortified egg yolk, the nonfortified egg yolk powder will also be determined.

Table 2: Food, energy and nutrient composition of the low folate diet which will be provided to subjects during each test day.

Food	Weight	
breakfast		
Smoothie (ml)	250	
Lunch		
Chicken breast (g)	100	
Carrots (g)	80	
Onion (g)	60	
Mushrooms(g)	40	
Oil (ml)	10	
Pasta (g)	150	
Afternoon snack		
Tea/coffee without sugar	1 small cup	
Energy and nutrient composition	**Estimated**	**measured**
Energy (kcal)	1240	x
Protein (g)	58	x
Fat (g)	59	x
Carbohydrate (g)	150	x
Cholesterol (mg)		x
Total folate (μg) for lunch*		56.5

* The folate content was analyzed and reported from Medallion Labs

Blood sampling and analysis

Subjects will be cannulated and blood sample will be taken by a nurse before the consumption of test treatments and afterwards at regular intervals over a period of 7-8 h (0.5, 1, 1.5, 2, 2.5, 3, 5, and 8 h).The 7-8-h period pattern was chosen based on similar

studies, which found that the serum folate returned to baseline value after 7-8 h (11, 12). Blood samples will be collected into-foil-wrapped Monoject tubes with EDTA. The samples will immediately place on ice, and plasma will be separated by centrifugation (2000×g, 10 min) within 30 min and stored at –80°C until analysis. All samples collected from each subject (8×3 days) will be analyzed in one assay to remove inter-assay measurement variability.

Analytical methods

1. The folate content in egg yolk powder and the food consumed by subject.
2. Serum folate

Sample size and statistical analysis

Sample size was calculated using a statistical program by PASS (2002) and inputting the expected means and standard deviations for the main outcome (serum folate). The smallest sample requires detecting a minimum difference based on the difference in serum folate between 5-MTHF and folic acid (3.7 nmol/l) with an estimated deviation of 6.1 nmol/l using one-sided one sample t-test. these values taken from similar study by Pentieva et al, 2004 (11) is 18, at alpha of 0.05 and power 0.8, the minimum sample size estimate is 18. To allow for 20% dropout 22 participants will be recruited. All results will be expressed as mean ± sd. The differences in plasma folate concentrations between baseline and each of the time points over 8 h within one treatment will be tested by repeated-measures ANOVA followed by Dunnett's post-hoc test. Differences among treatments will be evaluated by repeated-measures ANOVA and Scheffé's post-hoc test. Plasma folate response vs. time will be plotted, and the

maximum plasma folate response (Rmax) after both folate treatments will be determined by inspection of individual values at different time points. The area under the curve (AUC) for plasma folate response will determined by the by area under the curve (AUC) 0 -8 hr using (FIGP 2.98 software; BIOSOFT, Canada). AUC: An intuitive method of finding the area under a curve with fasting levels as the baseline and truncate at zero. The AUC calculation allows comparison of the integrated effects of the test meals over a fixed time period and the differences in AUC among the 3 treatments will be evaluated by 1-way ANOVA followed by Scheffé's post-hoc test. All statistical analysis will performed using the Statistic Analysis Package, Number Crunch Statistical System (NCSS) (2002, USA). The level of significance will set at $P<0.05$.It is expected that 5MTHF will have 5-7% more increase in serum folate than folic acid.

Significance of the study

The implications are that the natural folate derivative (5MTHF) could have all the beneficial effects associated with FA, but without the potential disadvantage of masking the anemia of vitamin B_{12} deficiency. Importantly, 5MTHF is a natural folate derivative, a normal constituent of the body, and safety and tolerance of high doses are not issue of concern.

References

1. Czeizel AE. The primary prevention of birth defects: Multivitamins or folic acid? *Int J Med Sci* 2004;1:50-61.
2. Ray JG, Meier C, Vermeulen MJ, Boss S, Wyatt PR, Cole DE. Association of neural tube defects and folic acid food fortification in Canada. *Lancet* 2002;360:2047-2048.
3. Ray JG, Vermeulen MJ, Langman LJ, Boss SC, Cole DEC. Persistence of vitamin B_{12} insufficiency among elderly women after folic acid food fortification. *Clin Biochem* 2003;36:387-391.
4. Pentieva K, McNulty H, Reichert R, Ward M, Strain JJ, McKillop DJ, McPartlin JM, Connolly E, Molloy A, Kramer K, Scott JM. The Short-Term Bioavailabilities of [6S]-5-Methyltetrahydrofolate and Folic Acid Are Equivalent in Men. *J Nutr* 2004;134:580-585.
5. Weir DG, Scott JM. Brain function in the elderly: role of vitamin B_{12} and folate. *Br Med Bull* 1999;55:669-682.
6. Herron KL, Fernandez ML. Are the Current Dietary Guidelines Regarding Egg Consumption Appropriate? *J Nutr* 2004;134:187-190.
7. House J, Braun K, Ballance D, O'Connor C, Guenter W. The enrichment of eggs with folic acid through supplementation of the laying hen diet. *Poult Sci. 2002 Sep;81(9):1332-7.* 2002;81:1332-7.

8. Choumenkovitch SF, Jacques PF, Nadeau MR, Wilson PWF, Rosenberg IH, Selhub J. Folic Acid Fortification Increases Red Blood Cell Folate Concentrations in the Framingham Study. *J Nutr* 2001;131:3277-3280.

9. Wartanowicz M, Ziemlansk iS, Bulhak-Jachymczyk B, Konopka L. Assessment of nutritional folate status and selected vitamin status of women of childbearing age. *Eur J Clin Nutr* 2001;55:743-7.

10. Wartanowicz M, Ziemlanski S, Bulhak-Jachymczyk B, L. K. Assessment of nutritional folate status and selected vitamin status of women of childbearing age. *Eur J Clin Nutr* 2001;55:743-7.

11. Hannon-Fletcher MP, Armstrong NC, Scott JM, Pentieva K, Bradbury I, Ward M, Strain J, Dunn AA, Molloy AM, Kerr MA, McNulty H. Determining bioavailability of food folates in a controlled intervention study. *Am J Clin Nutr* 2004;80:911-918.

12. Pentieva k, McKillop D, Duffy N, De Deckere E, Jacobs R, Van der Put N, McNulty H. Acute absorption of folic acid from a fortified low-fat spread. *European Journal of Clinical Nutrition* 2003;57:1235-1241.

13. Gray-Donald K, Jacobs-Starkey L, L. J-D. Food habits of Canadians: reduction in fat intake over a generation. *Can J Public Health. 2000 Sep-Oct;91(5):381-5.* 2000;91:381-385.

14. Pentieva K, McKillop D, Duffy N, de Deckere E, Jacobs RvdP, NMJ, McNulty H. Acute absorption of folic acid from a fortified low-fat spread. *Eur J Clin Nutr* 2003;75:1235-1241.

Approval certificate

05 September 2006

Canadian Egg Marketing

Agency

ARDI

TO: **James House**
Gustaaf Sevenhuysen
Principal Investigators

FROM: **Wayne Taylor, Chair**

Joint-Faculty Research Ethics Board (JFREB)

Re: **Protocol #J2006:086**
"The Short-Term Bioavailability of 5-Methyltertrahydrofolate and Folic Acid in Young Women"

Please be advised that your above-referenced protocol has received human ethics approval by the **Joint-Faculty Research Ethics Board**, which is organized and operates according to the Tri-Council Policy Statement. This approval is valid for one year only.

Any significant changes of the protocol and/or informed consent form should be reported to the Human Ethics Secretariat in advance of implementation of such changes.

Please note:

- **if you have funds pending human ethics approval, the auditor requires that you submit a copy of this Approval Certificate to Kathryn Bartmanovich, Research Grants & Contract Services (fax 261-0325), including the Sponsor name, before your account can be opened.**

- **if you have received multi-year funding for this research, responsibility lies with you to apply for and obtain Renewal Approval at the expiry of the initial one-year approval; otherwise the account will be locked.**

UNIVERSITY
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Participant Information and Consent Form - Survey

Research Project Title: Manitoba College-Aged Women's Metabolic Study

Researcher(s): Dr. James House, Dept. of Human Nutritional Sciences
University of Manitoba

Dr. Gustaaf Sevenhuysen, Dept. of Human Nutritional Sciences
University of Manitoba

Aysheh Shuaibi, PhD student, Dept.of Human Nutritional Sciences
University of Manitoba

Sponsors: Canadian Egg Marketing Agency
Agri-Food Research Development Initiative (ARDI)

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This consent form, a copy of which will be left with you for your records and reference, is only part of the process of informed consent. It should give you the basic idea of what the research is about and what your participation will involve. If you would like more detail about something mentioned here, or information not included here, you should feel free to ask. Please take the time to read this carefully and to understand any accompanying information.

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Purpose

The study is being conducted because the vitamin of interest is related to various health aspects, particularly relating to those of women of childbearing age. Recent data indicate that different forms of folate have different bioavailability. This research study is being conducted to determine the bioavailability of different forms of folate in women. It is necessary to be able to quantify folate bioavailability in order to establish recommendations for folate intake.

If you meet certain criteria and are interested, you may be eligible to participate in this study.

Procedures

Who can participate?

- young women between age 25-45 years
- no self-reported incidence of hypertension or any other systemic or mental illness,
- no regular usage of any prescription medications except oral contraceptives
- no chronic intake of medication which interferes with folate metabolism, and/or any other over-the-counter supplements (including mineral vitamin or herbal),
- should have a history of a regular menstrual cycle,
- Have their biomarker values within the following range.

Category	Reference range
Hemoglobin	12.1 to 15.1 gm/dl
Plasma folate (nmol/l)	6.80-45.23
Red cell folate (nmol/l)	340-2266
Plasma B_{12} (pmol/l)	111-738
Plasma pyridoxal phosphate (nmol/l)	≥ 30
Plasma total homocysteine (μmol/l)	≤ 15.0
MTHFR genotype	CC/CT
Cholesterol	200 mg/dL

Clinical biochemistry reference ranges are given for all parameters except for plasma total homocysteine the cutoff for hyperhomocysteinemia defined by Wartanowics et al., 2001 has been used.

Who can not participate?

- Lactating and/or pregnancy.
- Have a history of hepatic, gastrointestinal, vascular, hematological, or neuropsychiatry disease.
- Have a history of renal insufficiency.
- Women taking folic acid containing supplement
- Women taking medicine interfere with folate metabolism (aspirin, antiacides, aminopterin, pyrimethamine, phenytoin, methotrexate, sulphasalazine, Phenobarbital, primidon).

Participants will be excluded during the study if they required using a supplement folic acid or any of the medication that interfere with folate metabolism.

Description of Procedures
I may be involved in this study which will take 24 days if I

a) Pass all the criteria for selection into the study.
b) Agree to participate in this study.

During this time, the following will happen to me:

The following treatments and procedures will occur at University of Manitoba:

1. I will be asked to visit the research staff in the 4th floor Human Ecology for orientation and completion the inclusion-exclusion form.

2. I will be given instructions on how to fast for blood sampling in the screening stage.

3. Upon my biomarker values, I may be asked to take part in this study.

4. I will be asked to complete a health questionnaire regarding smoking, alcohol consumption, and medical status. And I will be asked to have anthropometric measurements.

5. I will be given instruction regarding consuming the supplement pill, and providing with 7-day tablet organizer box.

6. I will be asked to return the box at each time I have it.

7. After a week of providing you with the supplement, I will be asked to come to the human Ecology building for 7-8 hrs.

8. Early in the morning, I will be cannulated by an intravenous catheter in my hand vein for
blood sampling. We chose this procedure to avoid you frequent needle pricking.

9. I will be asked to provide a fasting blood sample (1teaspoon) and afterwards at a regular intervals over a period 8 hr (0.5, 1, 1.5, 2, 2.5, 3, 5, and 8 h). The total collected blood will be less than (8 teaspoons) which is similar to when you give blood for medical tests.

10. In the same day, I will be asked to eat a breakfast (smoothie) around 8:30-9 am, and then I will be provided by 2 snacks and a lunch. All food will be prepared in advance in the sensory laboratory in the 4th Human Ecology building. A nutritionist will prepared all the food.

11. for the next day, I will be given instruction regarding consuming the supplement pill, and providing with 7-day tablet organizer box.

12. After a week of providing me with the supplement, I will be asked to come to the human Ecology building for 7-8 hrs for the second time. Repeating the exact procedure as mentioned above.

Risks and Discomforts

Since we will be taking a blood sample from the vein on the arm, this procedure will result in a needle prick to the arm and there is a risk that it could result in a small bruise. All the blood sampling will be done by a trained nurse or medical technologist. The amount of the blood sample (40 ml or 8 teaspoons) is similar to when you give blood for medical tests. Hemoglobin will be assessed to make sure the participants are not anemic.

Confidentiality

Research records and information that contain your identity will be treated as confidential. Your name, identification code and all information gathered during the study will be kept in a locked cabinet by the research staff. Only the research staff will have access to this information. Research staff will follow those guidelines as set out in the Personal Health Information Act (PHIA) of Manitoba and those in the Freedom of Information and Protection of Privacy Act (FIPPA) of Manitoba to maintain your confidentiality.

Your blood sample will be handled by a laboratory technician who only sees the identification code on each sample. Blood analysis conducted will be directly related to the research being conducted.

Information gathered in this research study may be published or presented in public forums. However, your name and other identifying information will not be used or revealed. Data analysis with the use of computers will be conducted using code numbers to protect your identity. No information revealing any personal information such as your name, address or telephone number will leave the University of Manitoba. The University of Manitoba Joint Faculty Research Ethics Board may review research-related records for quality assurance purposes. Your identity will remain confidential and any personal information gathered will be destroyed. All data collected and analyzed will be destroyed after 3 years.

Feedback

You will be received an individual blood analysis report. A summary report of study findings will be available on request once the data analysis is completed.

Remuneration

You will receive a moderate honorarium upon completion of the study (100$/day).

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Your signature on this form indicates that you have understood to your satisfaction the information regarding participation in the research project and agree to participate as a subject. In no way does this waive your legal rights nor release the researchers, sponsors, or involved institutions from their legal and professional responsibilities. You are free to withdraw from the study at any time, and /or refrain from answering any questions you prefer to omit, without prejudice or consequence. Your continued participation should be as informed as your initial consent, so you should feel free to ask for clarification or new information throughout your participation.

If you require more information please contact us:

Dr. House at 474-9523
Dr. Sevenhuysen at 474-9704
Study staff at 474-6423

This research has been approved by the Joint-Faculty Research Ethics Board. **If you have any concerns or complaints about this project you may contact any of the above-named persons or the Human Ethics Secretariat at 474-7122, or e-mail margaret_bowman@umanitoba.ca. A copy of this consent form has been given to you to keep for your records and reference.**

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Participant's Signature Date

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Researcher's Signature Date