ABSTRACT

Title of Document: FOLATE INTAKE AND BIOMARKERS AND RISK OF CHRONIC DISEASE

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Background: Folate status of the U.S. population significantly improved after folic acid fortification of enriched cereal-grain products in 1998. Recent evidence suggests that the increased folate levels may have impacts on the risk of chronic disease. The kidneys are highly involved in folate metabolism. Reduced renal function may affect folate metabolism and play a role in the associations between folate and chronic disease.

Objectives: The purpose of this study was to review key events regulating folate homeostasis along folate metabolic pathway. In addition, we examined the associations between folate intake and biomarker levels and the incidence of cancer, stroke and cardiovascular disease (CVD) and between folate biomarker levels and renal function among older adults in post-fortification years.

Design: The Key Events Dose-Response Framework was used to review key steps of folate metabolism. Data of adult participants of the National Health and Nutrition Examination Survey 1999–2002 were used as the baseline data. Incidence of cancer,

stroke and CVD were obtained from the linked Medicare and mortality files. The associations between folate intake and biomarker levels and incidence of cancer, stroke and CVD, and the associations between estimated glomerular filtration rate (eGFR) and folate biomarkers, serum unmetabolized folic acid (UMFA) and plasma homocysteine levels were assessed using Cox proportional hazards regression models and multivariable regression models, respectively.

Results: The saturation of dihydrofolate reductase in the liver is the determining point regulating the release of UMFA in circulation. Lower red blood cell (RBC) folate levels and intake of dietary folate equivalents were associated with a higher cancer incidence. Lower RBC folate and serum folate levels were associated with a higher stroke incidence. No significant associations between folate and CVD were observed. In addition, reduced renal function was associated with higher RBC folate and plasma homocysteine levels among men and women, and higher prevalence of UMFA in blood among women.

Conclusion: High intake of folate may disturb folate metabolism by overwhelming folate regulation mechanisms. Folate may play a protective role against cancer and stroke even at high levels in post-fortification years. Reduced renal function may be implicated in the increased blood folate concentrations.

FOLATE INTAKE AND BIOMARKERS AND RISK OF CHRONIC DISEASE

By

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Chapter 1: Introduction

Folate is a water-soluble B-complex vitamin, which can be found naturally in foods such as green leafy vegetables and citrus fruits. Folic acid is the synthetic and most oxidized form of this vitamin, which is the form added to fortified foods and to dietary supplements. Folic acid must be catalyzed by the enzyme dihydrofolate reductase (DHFR) to the reduced form before participating in intracellular reactions. High intake of folic acid could saturate the enzyme activity and bypass the reduction mechanism, which consequently lead to the release of unmetabolized folic acid (UMFA) in circulation. In the dissertation, folate was used as a collective term for naturally occurring folate and fortified/supplemented folic acid. However, due to their different chemical structures, they may have different impacts on health, thus will be discussed separately, when necessary.

Since the human body does not have the ability to synthesize folate, a lack of dietary folate can lead to folate deficiency. This deficiency may result in many adverse health outcomes. The most notable one is neural tube defects (NTDs) in developing fetus. To reduce the incidence of NTDs, the U.S. instituted the mandate for folic acid fortification of enriched cereal-grain products to the level of 140 µg/100g in 1998. This fortification program is estimated to supply on average an additional 100–200 µg folic acid per person per day based on the average American dietary pattern. The mandatory folic acid fortification program has successfully reduced the incidence of NTDs in newborns by almost 30% (2, 3). Fortification of enriched cereal-grain products with folic acid also significantly improved the nutritional status of folate of the U.S. population. The prevalence of folate deficiency

has dropped to around 5% (4) and mean serum and red blood cell folate (RBC) levels are almost doubled in the post-fortification compared with those in the pre-fortification years (**Figure 1. 1**).

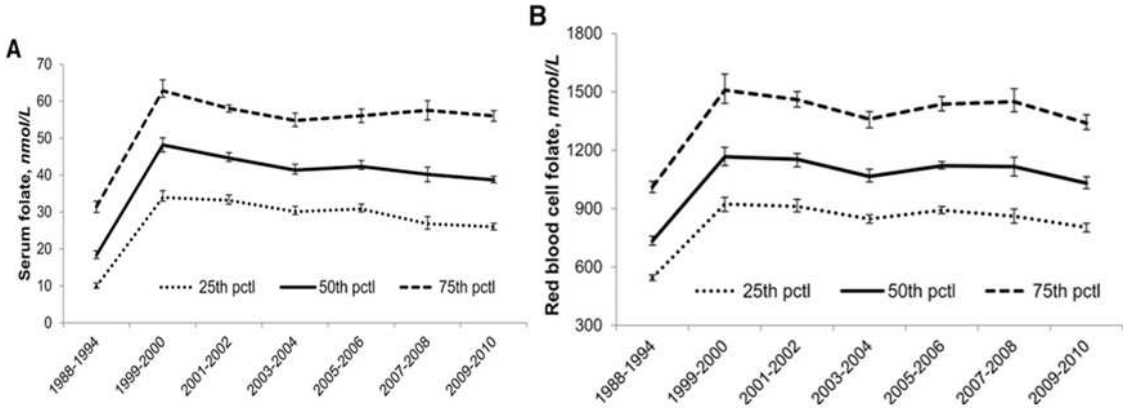


Figure 1. 1 Selected percentiles for pre- and post-fortification serum (A) and red blood cell (B) folate concentrations by survey period (NHANES 1988–2010) (1)

The population targeted by folic acid fortification of cereal-grain products is women of child bearing age. Older adults, however, have relatively higher levels of folate intake and biomarkers than other population groups (5). Safety concerns regarding high intake of folate among older adults has been raised. High levels of folate biomarkers may disturb folate metabolism and result in unintended health consequences. It is yet unclear what these relationships may be because there are still some gaps in our knowledge of folate metabolism. Therefore, studies are needed to address these safety concerns and knowledge gaps.

Altered folate intake either excess or deficient may fail regulation mechanisms and interrupt folate homeostasis, which could ultimately lead to poor health conditions such as cancer and cardiovascular disease. Additionally, high intake of

folic acid may bypass the reduction mechanism and lead to the release of UMFA in blood. UMFA in the blood is reported to be prevalent among older adults as an outcome of high consumption of folic acid in post-fortification years (6). There is a speculation that UMFA could interrupt normal folate metabolism (7-9) and lead to negative health outcomes such as cognitive impairment, decreased immune function and increased cancer risk (10-13)**.** However, metabolism of UMFA and the potential effects on chronic disease have not been fully elucidated.

In addition, high levels of folate intake and biomarkers may increase cancer risk, because high folate levels could promote the progression of pre-existing cancer by providing substrates for DNA replication in rapid cell division (14). In the U.S., approximately 11.3% of population exceeds the Tolerable Upper Intake Level (UL) of 1mg/day as an outcome of food fortification and supplement use (15). An ecological study in the U.S. have shown that an increase in cancer incidence coincided with population-wide increase in intake of folate (16). The safety concerns surrounding the high folate intake emphasize the needs of continued studies and carefully monitoring the effect of folic acid fortification on risk of cancer incidence.

Additionally, folic acid fortification is hypothesized to contribute to the prevention of stroke. It was reported in an ecological study that a decline in stroke mortality paralleled the population-wide increase in serum folate and decrease in blood homocysteine concentrations in post-fortification years in the U.S. (17). However, it is still unclear how much of the decline in stroke mortality is due to the decrease in incidence of stroke. Many factors, such as hospitalization rate of stroke patients and large public health programs promoting stroke prevention, may also

impact stroke mortality (17, 18). Further study particularly longitudinal study is needed to elucidate the effects of folate on risk of stroke incidence in post-fortification years.

Finally, the kidneys are highly involved in maintaining folate homeostasis. Renal insufficiency may affect folate metabolism and this in turn may impact the risk of chronic disease. Currently, approximately 13% of the U.S. population are affected by chronic kidney disease (19). It is of great public health significance to examine the implication of renal insufficiency on folate biomarkers.

In this dissertation, we first examined what we know of the key events and control points in the metabolic pathway of folate and then examined the effects of folate intake and biomarkers on the incidence of cancer, stroke and cardiovascular disease (CVD). Finally, we looked at the potential association between renal insufficiency and the levels of folate biomarkers. The specific aims of the study are:

1) To review the key events in folate metabolic pathway that have major influences on folate metabolism and determining points regulating the release of UMFA using the Key Events Dose-Response Framework.
2) To examine the associations between folate intake and biomarker levels and UMFA and the incidence of cancer by analyzing data of NHANES 1999–2002 and its linked Medicare and mortality files.
3) To examine the associations between folate intake and biomarker levels and the incidence of stroke and CVD by analyzing data of NHANES 1999–2002 and the linked Medicare and mortality files.

4) To examine the associations between renal function and folate biomarkers, UMFA and plasma homocysteine levels by analyzing data of NHANES 1999–2002.

Chapter 2: Literature Review

Folate metabolism

Folate (used as a collective term for naturally occurring folate and fortified/supplemented folic acid) metabolism and homeostasis are regulated by a number of enzymes and transport systems along its metabolic pathway. The hydrolysis and absorption of folate in the small intestine, the uptake and metabolism of folate in the liver and the reabsorption of folate in the kidneys are key events in regulating folate homeostasis. Reduction of folic acid catalyzed by the enzyme dihydrofolate reductase (DHFR) in the liver is the determining point in folate metabolism leading to the release of UMFA in circulation. These regulation systems response to varied folate intake and maintain normal physiological folate levels. Altered folate either access or deficient folate intake may fail these regulation mechanisms and result in disturbance in folate homeostasis. High intake of folic acid can saturate the DHFR activity and lead to the release of UMFA into circulation. Detailed descriptions of key events and determining points of folate metabolic pathway are discussed in the first paper of Chapter 4 (page 23).

Unmetabolized folic acid and disease

UMFA in the blood is reported to be prevalent in approximately 38% of older adults (aged ≥ 60 years) as an outcome of high folic acid intake in post-fortification years, according to the data of NHANES 2001–2002 (6). It has been suggested that UMFA might disrupt folate metabolism by inhibiting folate-dependent enzymes, and consequently result in adverse health outcomes. The accumulation of dihydrofolate,

the first step in folic acid metabolism, may inhibit purine and thymidine synthase and consequently interfere with DNA synthesis, which may trigger cancer process (7, 8). In addition, high folic acid levels may inhibit the methyl donor S-Adenosylmethionine **(**SAM**)** synthesis by inhibiting methylenetetrahydrofolate reductase (MTHFR) and decreasing methylation reactions (9), which may lead to elevated homocysteine levels and hypomethylation of DNA.

Though it is biologically plausible, the potential effect of UMFA on health is yet to be determined. Few epidemiological studies were conducted to examine the associations between UMFA and health outcomes. A study by Selhub et al. in 2010 showed that the presence of UMFA was positively associated with cognitive impairment in older adults (aged ≥ 60 years) (12). UMFA was also reported to be associated with decreased immune function among postmenopausal women (11)**.** Given the fact that UMFA is prevalent in older adults and is hypothesized to be a risk factor for unintended health consequences, it is crucial to examine the effects of UMFA on risk of chronic disease.

Folate and chronic disease

Folate and cancer

Folate functions as a coenzyme in single-carbon, methyl group transfers in the metabolism of nucleic acids. Folate is crucial for normal DNA synthesis, repair and methylation. Folate is believed to have dual effects (promotion and inhibitory effects) on cancer development. A deficiency in folate may lead to an increase in cellular uracil to thymidine ratio, thus, misincorporating uracil into DNA, destabilizing DNA

molecule and potentially leading to an increased risk of malignancy (20). Additionally, DNA methylation altered by folate deficiency was shown to up-regulate proto-oncogene expression and induce cancer (21). High folate levels, however, may promote the progression of pre-existing cancer by providing substrates for DNA replication in rapid cell division (14).

It is hypothesized that high folate intake in the post-fortification years may increase cancer risk. Currently, about 11.3% of the U.S. population exceeds the Tolerable Upper Intake Level (UL) of 1mg/day as an outcome of food fortification and supplement use (15). In some countries with mandatory folic acid fortification, high intake of folate was hypothesized to contribute to an increase in incidence of colon cancer (16, 22). Manson, et al. in 2007 found that fortification of enriched cereal-grain products with folic acid coincided with an increase in the incidence rates of colorectal cancer in the U.S. and Canada (16). The authors found there were about four to six additional colorectal cancer cases per 100,000 individuals in post-fortification years, and this increase in incidence could not be explained by the increased use of endoscopic examination. Similarly, an ecological study in Chile in 2009 indicated that the incidence of colorectal cancer was significantly higher in post-fortification years compared to pre-fortification years (22). In addition, a large, randomized, double blind placebo-controlled clinical trial by Cole et al. reported that, long term and high dosage (1mg/day) of folic acid supplement significantly increased the risk of recurrence of advanced adenoma in the patients with a history of adenomas (13). Additionally, it was also reported that higher intake of folate either from

supplemental folic acid (≥ 400 µg/d) or from dietary folate intake (≥ 312 µg/d) was be associated with an increased risk of postmenopausal breast cancer (23, 24).

Folate and stroke and cardiovascular disease

Folate is considered a preventive factor of atherosclerotic disease via its roles in lowering homocysteine levels. Homocysteine is a sulfur-containing amino acid which is broken down through either the methylation or the trans-sulfuration pathway, where folate serves as a methyl donor and vitamin B6 and vitamin B12 function as coenzymes (25). Therefore, deficient folate, vitamin B6 and vitamin B12 could lead to elevated homocysteine levels, which in turn may result in an increased risk of atherosclerotic disease including stroke. It has been reported that a 25% higher than normal circulating homocysteine concentration was associated with a 19% higher risk of stroke (26).

High levels of folate intake and biomarker in post-fortification is considered to contribute to the prevention of stroke. It was reported in an ecological study that a decline in stroke mortality paralleled the population-wide increase in serum folate and decrease in blood homocysteine concentrations in post-fortification years in the U.S. (17). However, it is unclear how much of the decline in stroke mortality is due to reduced incidence of stroke. Many factors, such as hospitalization rate of stroke and large public health programs promoting stroke prevention, may also impact stroke mortality (17, 18). Further studies are required to elucidate the effects of increase in folate intake and biomarker levels on risk of stroke incidence. In addition, the effect of folate on risk of stroke independent of homocysteine remains to be examined.

Findings of previous epidemiological studies suggest that folate itself could be an important protective factor for stroke (27, 28). It was reported that low serum folate levels were independently associated with a significantly higher risk of stroke among Canadians aged ≥ 65 years (28). Additionally, lower plasma folate levels were found to be independently associated with a significant higher risk of hemorrhagic stroke in the prospective study of the Northern Sweden Health and Disease Study Cohort (27). However, data are not available in post-fortification years in the U.S.

Renal function and folate levels

The kidneys are highly involved in maintaining folate homeostasis. A significant amount of folate are filtered daily and the proximal renal tubular reabsorption of folate prevents extensive urinary excretion of folate (29). Folate receptors and other transporters such as cubilin and megalin located on renal tubule are found to be responsible for folate reabsorption (30). Expression of folate transporters was down-regulated and the uptake and utilization of folate by peripheral tissue and organs were decreased in rat models of chronic kidney disease (31). Absorption of folate by the small intestine was found to decrease in rat models of chronic kidney disease in another study (32). These changes in folate utilization and absorption may result in disturbance in homeostasis of folate.

Renal insufficiency may affect folate metabolism and folate biomarker levels in blood and this in turn may have an impact on risk of chronic disease incidence. Few studies examined the folate biomarker levels among individuals with chronic kidney disease receiving dialysis. Increased blood folate levels were reported in

patients with chronic kidney disease receiving dialysis (33). In other studies, however, decreased serum folate concentrations were observed in dialyzed patients (34, 35). Chronic kidney disease is an increasing health concern that affects about 13% of the U.S. population (19). It is of public health importance to assess the impact of high intake levels of folate on biomarkers of folate among individuals with declining renal functions considering potential impact of altered folate levels on risk of chronic disease.

Chapter 3: Methods

Data of adult participants in the National Health and Nutrition Examination Survey (NHANES) 1999–2002 were used to examine associations between folate intake and biomarkers and incidence of cancer, stroke and CVD, and between folate biomarker levels and renal function. Incidence of these chronic conditions was obtained from NHANES 1999–2002 linked Medicare and mortality files. To preserve the data integrity and privacy of survey participants, proposal of intended studies must be submitted for approval to the National Center for Health Statistics (NCHS). Additionally, NHANES 1999–2002 files and linked Medicare and mortality files are merged at the Research Data Center of NCHS (NCHS-RDC) to ensure that no identifying information is released. All analyses were also conducted at the RDC using SAS for windows (version 9.2, SAS Institute Inc, Cary, NC). All output of analyses was reviewed before their release to investigators. Prior to the initiation of the study, the studies that make up this dissertation obtained approval by the Institutional Review Board of the University of Maryland, College Park.

The National Health and Nutrition Examination Survey (NHANES)

Study design

NHANES is a survey which monitors the nation's health and nutritional status. It is administered by the NCHS of the Centers for Disease Control and Prevention (CDC). Since 1999, the NHANES is continuously in the field and releases data collected in two-year cycles. NHANES uses a complex, multistage, probability

sampling design to select a nationally representative sample of the civilian, non-institutionalized population of the United States. The NHANES sampling procedure consists of 4 stages. At the first stage, primary sampling units (PSUs) are selected. These PSUs are mostly single counties or groups of contiguous counties with probability proportional to a measure size (PPS). At the second stage, segments of the PSUs (generally city blocks or their equivalent) are sampled with PPS. At the third stage, households within each segment are randomly sampled, and individuals in selected households are sampled randomly within designed age-sex-race/ethnicity screening domains at the final stage (36). A sample of about 5,000 persons located in 15 counties across the country is examined each year.

Each sampled individual undergoes a household interview, which collects information on demographic and socioeconomic characteristics, health behaviors, and health status and supplement usage. This interview is followed by a visit to the Metabolic Examination Center (MEC) for laboratory tests and physical examinations, including anthropometric measurements, dietary intake and biochemical measures (37, 38). More details about NHANES and its methods can be found elsewhere (39).

Assessment of diet and supplement

Energy and nutrient intakes were estimated from a 24-hour dietary recall. The 24-hour dietary recall is performed in the MEC by trained dietary interviewers in English or Spanish using computer-assisted dietary interview system. The information collected is total energy intake, nutrients, and non-nutrient food components of foods and beverages consumed during the 24-hour period before the

interview. For NHANES 1999–2000, the interview files were imported into the University of Texas Food Intake Analysis System (FIAS) for coding. FIAS version 3.99 with the U.S. Department of Agriculture (USDA) 1994–1998 Survey Nutrient Database was used to process the NHANES 1999-2000 dietary data (39). In addition, for NHANES 2001–2002, FIAS (version 3.99) was used for coding dietary intakes for 2001. The Survey Net, a computer-assisted food coding and data management system developed by USDA, was used for coding dietary intakes for 2002. The USDA FNDDS (version 1.0), a database of foods, their nutrient values and weights for typical food portions, was used for processing the dietary intakes for NHANES 2001–2002.

Intake of dietary supplements was collected through the NHANES Dietary Supplement Questionnaire at the household interview. Participants were asked a series of questions about vitamin or mineral supplements use during the past 30 days. Detailed information is collected on name of manufacturer or distributor, consumption frequency, duration of use, and dosage (39). The average daily intake of folic acid from all dietary supplements was calculated based on the number of days of the consumption of supplements, the amounts taken per day, and the serving size from the product label.

Assessment of socio-demographic and life-style characteristics

Demographic information, including age, gender, self-identified race/ethnicity and educational level, were collected at the household interview. Race/ethnicity was self-identified as Mexican American, other Hispanic, non-Hispanic white, non-

Hispanic black and other race (including Multi-Racial). In this study, three race/ethnicity categories were used: non-Hispanic white, non-Hispanic black and other (Mexican American, other Hispanic and other races). Educational attainment was categorized as less than high school, high school graduate (received a high school or high school equivalency diploma) and greater than high school.

Data on lifestyle, including physical activity, alcohol consumption and cigarette smoking, were obtained through interviewer-administered questionnaires. Physical activity level was self-reported as sedentary, light, and moderate or higher. Alcohol consumption was assessed as grams per day from the 24-hour dietary recall. Cigarette smoking status was categorized as never, former and current. A nonsmoker was defined as a participant who had never smoked ≥ 100 cigarettes during his/ her life; a former smoker was defined as a participant who had smoked ≥ 100 cigarettes and was not smoking at the time of the interview; and a current smoker was defined as a participant who had smoked ≥ 100 cigarettes and was smoking at the time of interview. Body measurement was conducted in the MEC by trained health technicians. Height was measured using fixed stadiometer with a vertical backboard and a moveable headboard. Height was measured on a Toledo digital scale (40). Body mass index (BMI) was calculated as weight in kilograms divided by height in meters squared (kg/m^2).

Biochemical measurements

Serum folate and red blood cell folate concentrations were measured with the Bio-Rad Laboratories "Quantaphase II Folate/vitamin B12" radioassay from BioRad,

Hercules, CA. UMFA in NHANES 1999–2002 participants aged 60 years and over was measured using a revised affinity/ HPLC method with electrochemical (coulometric) detection. The lower limit of detection for UMFA was 0.18 nmol/L and values below the level of detection were set to zero. Serum creatinine was measured based on the Jaffe reaction. Serum creatinine measurements of participants of NHANES 1999–2000 were recalibrated to standardized creatinine measurements (creatinine measurements obtained at the Cleveland Clinic Research Laboratory, Cleveland, Ohio) using the equation: standard creatinine = 0.147 + 1.013 × un-calibrated serum creatinine (mg/dL) (41). Serum alanine aminotransferase (ALT) was measured using an enzymatic rate method (42). Plasma homocysteine was measured by using a fluorescence polarization immunoassay reagent set from Abbott homocysteine assay, Abbott Park, IL. Complete details and documentation for each of these methods are described elsewhere (43, 44).

The Medicare and mortality data linkage

NCHS has linked data of participants of NHANES 1999–2002 to Medicare enrollment and claim records collected from the Centers for Medicare and Medicaid Services (CMS). Medicare enrollment and claims data are available for NHANES respondents who agreed to provide personal identification data to NCHS, and for whom NCHS was able to match personal data with Medicare administrative records between 2005 and 2007 (45). The Medicare Chronic Condition Summary File is a summary of clinical information extracted from the NCHS-CMS linked data for the years 1999–2007. Dates of first occurrence for 21 chronic conditions are available.

Cancer (breast, colorectal, prostate, lung and endometrial cancers), stroke (stroke/transient ischemic attack) and CVD (acute myocardial infarction, heart failure, ischemic heart disease, and stroke/transient ischemic attack) were identified using NCHS designed algorithms of disease codes from Medicare claim records. These are ICD-9 (International Classification of Disease), CPT-4 (Current Procedure Terminology) and/or HCPCS (Healthcare Common Procedure Coding System) codes. More details about algorithms of codes can be found in Appendix B of the NCHS-CMS Medicare Chronic Condition Summary File Data Dictionary (46).

Data of NHANES 1999–2002 participants are also linked to the National Death Index (NDI) through December 31, 2006. Mortality ascertainment is based upon the probabilistic match between the NHANES and National Death Index (NDI) death certificate records (47). The Linked Mortality Restricted-use File provides follow-up data on mortality from the date of survey participation through December 31, 2006. The date of death, underlying and multiple causes of death in ICD-10 are recorded in the Linked Mortality Restricted-use File. More details about the Mortality File can be found elsewhere (48).

Cancer, stroke and CVD cases ascertainment

We used data of NHANES 1999–2002 participants aged 57 years and above to capture all individuals who would potentially reach the age of Medicare eligibility by 2005 to 2007 (Medicare has age-based entitlement at 65 years of age). Cancer, stroke and CVD cases of this population were identified from the NHANES Linked Medicare Chronic Condition Summary File or Mortality Restricted-use File. For

participants who died before 2005, cancer, stroke and CVD cases were identified from the Linked Mortality Restricted-use File using either underlying or multiple causes of death. Date of death from cancer/stroke/CVD was used as the date of disease first occurrence.

Assessment of renal function

Estimated glomerular filtration rate (eGFR) was used to assess renal function. eGFR, normalized to 1.73 m2 of body surface, was estimated using the Chronic Kidney Disease Epidemiology Collaboration (CKD-EPI) formula (49): eGFR $=141\times\min(SCr/k, 1)^{\alpha} \times \max(SCr/k, 1)^{-1.209} \times 0.993^{Age} \times [1.018 \text{ if women}] \times [1.159 \text{ if Black}]$. 'SCr' represents serum creatinine (mg/dL), 'k' is 0.7 for women and 0.9 for men, and 'α' is -0.329 for women and -0.411 for men (49). Individuals with eGFR less than 60 mL/min/1.73 m^2 and those with eGFR between 60 and 90 mL/min/1.73 m^2 were defined as having reduced renal function and mildly reduced renal function, respectively (50). Individuals were categorized based on their CKD-EPI eGFR values in three categories as < 60, 60–90 and ≥ 90 mL/min/1.73 m^2.

Statistical methods

Studies of associations between folate levels and risks of cancer, stroke and CVD

UMFA measurements were categorized into detectable UMFA (UMFA+) and undetectable UMFA (UMFA-) categories. ANOVA for continuous variables and Chi-squared tests for categorical variables were used to examine possible associations between quartiles of serum folate, RBC folate and intakes of dietary folate, folic acid

and dietary folate equivalents and potential confounders. The second quartile, which includes recommended dietary allowance of folate, was used as the reference group in the regression models.

Cox proportional hazards regression models were used to examine hazard ratios (HR) and 95% confidence intervals (95% CI) for incidence of cancer by quartiles of folate intake and blood biomarkers. Follow-up person-years of each participant were estimated from baseline interview to the endpoint (the earliest occurrence of cancer/stroke/CVD), or death, or the end of the follow-up period (December 31, 2007), whichever came first. To examine the association between folate and cancer, we conducted a multivariable Cox proportional hazards regression model for each of the folate intake and biomarker variables of interest. These confounders included age, gender, race/ethnicity, educational attainment, energy intake, BMI, physical activity, smoking status and alcohol intake. We also tested for possible linear association between of folate intake and biomarkers and the incidence of overall cancer using linear regression models. Serum folate, RBC folate, intakes of dietary folate, folic acid and dietary folate equivalents were logarithmically transformed, because these five folate exposure variables were not normally distributed.

In addition, we used a similar approach to assess the associations between folate levels and the incidence of stroke and CVD. We conducted a multivariable-adjusted Cox proportional hazards regression model for each of the folate intake and biomarker variables of interest adjusted for potential confounders. These confounders included age, gender, race/ethnicity, smoking status, alcohol intake, education,

physical activity, BMI, total energy intake, serum total cholesterol and high-density lipoprotein (HDL) and cholesterol. For the independent association between serum and RBC folate and stroke and CVD, plasma homocysteine was included as a cofounder in the regression models. We also tested possible linear associations between folate intake and biomarkers levels and the incidence of stroke and CVD using linear regression models. As indicated above, folate intake and biomarkers variables were logarithmically transformed. In all studies, we evaluate the proportional hazards assumption that there is no significant interaction between predictors and follow-up time; and no violation to this rule was found.

Additionally, in all these studies, data was treated as simple random sampling data and sample weight was not incorporated in data analyses. Due to ineligibility for the linkage to Medicare and mortality files, not all of data of NHANES 1999–2002 participants were included and analyzed. Participants included in our study were not proportionally selected within NHANES 1999–2002 designated age-sex-race/ethnicity screening subdomains. Therefore, the original survey weight is not applicable for analyses of the data of this subpopulation. Adjustment of sample weight for incomplete linkage and non-matches is necessary for the purpose of generating unbiased national estimate. However, our sample was only a subset of the population of interest and was not representative of age, gender or race/ethnicity. Therefore, data was treated as simple random sampling data as described above. Furthermore, there were no significant associations between folate intake and biomarkers with CVD. Therefore, data of results are not presented in this dissertation.

Study of associations between renal function and folate biomarker levels

Data analyses were performed using SAS for windows (version 9.2, SAS Institute Inc, Cary, NC) and SAS-callable SUDAAN (version 10.1; Research Triangle Institute, Research Triangle Park, NC). Analyses were conducted incorporating the four-year sampling weight to account for complex sampling design (51). Data of participants were classified into three categories according to their eGFR values (< 60, 60–90 and ≥ 90 mL/min/1.73 m^2). UMFA measurements were categorized into detectable UMFA (UMFA+) and undetectable UMFA (UMFA-) categories. Variables that were not normally distributed (with a skewness > 4) were log-transformed before group comparison and regression analyses. All tests were stratified by gender (men and women), because there was a statistically significant interaction between gender and eGFR when testing relations between eGFR and blood folate biomarkers of interest. Statistical significance was set at $p < 0.05$. The category eGFR ≥ 90 mL/min/1.73 m2 was set as the reference group in all analyses.

Dunnett's test and Chi-squared tests were used to compare group means of continuous variables and group percentages of categorical variables, respectively. Serum folate, RBC folate and plasma homocysteine levels were assessed in relation to eGFR using multivariable linear regression models. Regression models for serum folate and RBC folate were adjusted for age, gender, race/ethnicity, smoking status, alcohol intake, BMI, physical activity, educational attainment and intake of dietary folate equivalents. Regression model for plasma homocysteine was additionally adjusted for intake of vitamin B12. Test for trend was performed using linear regression models including eGFR as a continuous variable in the model. In addition,

we examined associations between UMFA and eGFR categories. Odds ratios (OR) for the presence of UMFA in the blood and 95% confidence intervals (95% CI) were estimated based on eGFR categories using multivariable logistic regression models. Logistic regression models were adjusted confounders, including age, gender, race/ethnicity, smoking status, alcohol intake, BMI, physical activity, educational attainment and intake of dietary folate equivalents.

Chapter 4: Results

A) Application of the key events dose-response framework to folate metabolism

Abstract

Folate is a vitamin that plays a role as a cofactor and coenzyme in many essential reactions. These essential reactions include nucleic acid synthesis and numerous methyl- transferase reactions such as DNA methylation and amino acid metabolism. These are all interrelated reactions and any change in folate homeostasis could affect other reactions (**Figure 4. 1**). Mandatory folic acid fortification of enriched cereal-grain products implemented in 1998 in the U.S. has been shown to be somewhat effective in reducing neural tube defects (NTD) in newborns. However, studies show that it is not uncommon for individuals in the U.S. to exceed intake of the Tolerable Upper Intake Level (UL) of 1mg/day, as an outcome of food fortification and supplement use (5, 15). Also, the appearance of unmetabolized folic acid (UMFA) in blood circulation is now quite prevalent in the general population (52). This has led to concern that UMFA may disturb cellular folate uptake and normal intracellular folate metabolism which ultimately may lead to negative health outcomes (10-12, 53).

A potential negative outcome of high folic acid intake is the masking of vitamin B12 deficiency among older adults which may lead to cognitive impairment (12, 54). Another concern is that high folic acid intake may increase the risk of recurrence of colorectal adenoma among individuals with history of adenoma (53). The concern that over-supplementation of folic acid may lead to adverse outcomes

makes it crucial to explore ways to identify the minimum effective intake of folic acid that is required to prevent NTDs while minimizing the risk of negative outcomes due to excessive intake among vulnerable populations.

As a step towards that end, we used the framework known as the Key Events Dose-Response Framework (KEDRF), a component of the International Life Science Institute's (ILSI) Global Threshold Project (55), to review what is known about each of the major key events, dose-response characteristics and homeostatic mechanisms along the folate metabolism pathway (**Figure 4. 2** and **Figure 4. 3).** This analytical approach has been applied in a case study with vitamin A (56) and found to be a useful tool in integrating knowledge and identifying research gaps. Since UMFA is a relatively new phenomenon, we examine in this paper its appearance in serum as our outcome of concern, and review the major steps or key events in the metabolic pathway that lead to it.

In this review, we identify control points which are the mechanisms that help maintain a normal physiological environment. The capacity of such mechanisms to keep homeostatic balance is likely to influence the overall dose-response relationship between total folate intake and UMFA (56). Certain control points may play an especially critical role in a given pathway; and if the outcome of these control points greatly influence the likelihood of the ultimate effect of concern, such as the appearance of serum UMFA; then such control points are identified as determining events (55). In addition, we identify gaps in our knowledge of folate metabolism that would be necessary for the determination of a dose-response relationship for folate.

We use folate as a collective term for naturally-occurring folate and fortified/supplemented folic acid; however, due to their different chemical structure, they may undergo different biochemical reactions in certain metabolic steps and thus will be discussed separately, when necessary.

Functions and adverse effects associated with folate

Folate functions as a coenzyme in single-carbon, methyl group transfers in the metabolism of nucleic acids and amino acids. Folate is crucial for normal DNA synthesis and is needed for pyrimidine and purine biosynthesis (**Figure 4. 1**). Pyrimidine nucleotide biosynthesis requires folate co-enzyme in the conversion of deoxyuridylic acid to thymidylic acid. Folate is believed to have dual effects (promotion and inhibitory effects) on cancer development. One of the hypothesis is that a deficiency in folate may lead to an increase in cellular uracil to thymidine ratio, thus, misincorporating uracil into DNA, destabilizing DNA molecule and potentially leading to an increased risk of malignancy (20). Additionally, cytosine methylation altered by folate deficiency was shown to up-regulate proto-oncogene expression and induce cancer (21). Several prospective studies have showed an inverse association between folate and cancer (57-62).

In contrast, results from limited studies showed a positive association between high folate intakes and cancer. Such an association between folate intake and colorectal cancer was hypothesized from the results of two ecological studies (16, 22). However, results of epidemiological studies have been inconsistent (63). Animal studies have also provided critical information concerning the dual modulatory effects

of folate on the development and progression of colorectal cancer (64) and on the protection against colorectal cancer (65). Whether and how folate may exert these dual effects on colorectal carcinogenesis appears to depend on the timing and dose of the folate intervention (14, 66). The mechanism lies in the essential role of folate in DNA synthesis and the biological methylation reactions

Either deficient or excessive folate could also be related to cognitive dysfunctions in the presence of low vitamin B-12. Folate and vitamin B-12 are essential in the conversion of homocysteine to methionine, the precursor of S-adenosyl methionine (SAM). SAM is a methyl donor to a wide range of reactions involving DNA, protein and lipids. Folate acts as the methyl donor where 5-methyltetrahydrofolate (5-MTHF) is converted to tetrahydrofolate (THF) and the methyl group is donated to homocysteine to form methionine (**Figure 4. 1**). The deficiency of folate and vitamin B12 is associated with reduced cellular SAM and elevated homocysteine levels (67, 68), potential disrupted formation of myelin (which is essential for the proper functioning of the nervous system) (69), as well as hindrance of DNA synthesis and cell division (20, 70). Additionally, vitamin B12 is the only acceptor of the methyl group from 5-MTHF and homocysteine is the only acceptor of methyl-B12. Thus, a deficiency in vitamin B12 can generate a large pool of methyl-THF that is unable to undergo reactions and will mimic folate deficiency and accumulate homocysteine. The only way for the 5-MTHF to be recycled to THF, and thus to participate in DNA biosynthesis and cell division, is through the vitamin B12 - dependent enzyme methionine synthase. In its absence, cellular folate will become progressively trapped as 5-MTHF. However, if folic acid is ingested,

nucleotides will be synthesized and the hematological picture of megaloblastic anemia will be normalized but not the neurological symptoms associated with vitamin B12. This may lead to masking of vitamin B12 deficiency and to the progression of potentially irreversible neurological symptoms (dementia, paresthesia and ataxia) (71).

Uptake from lumen into enterocyte

Digestion in lumen

Most naturally occurring folates are polyglutamate derivatives. These are hydrolyzed to monoglutamates on the surface of intestinal mucosa by the hydrolase enzyme glutamate carboxypeptidase II (GCP II) prior to absorption into the intestinal cells. Folic acid is a monoglutamate, so hydrolysis is not necessary for its absorption.

GCP II is located on the human jejunum brush border and is sufficient to hydrolyze dietary folate intake (10 cm proximal jejunum in humans contains GCPII to hydrolyze 200 µg folate) (72). There is currently no evidence to show that excessive intake of folate will saturate GCP II. There appears to be adaptive up-regulation of the enzyme in case of folate deficiency (73), but the molecular mechanism and threshold dose that would trigger this up-regulation has not been identified. It is unclear whether GCP II is down-regulated in the presence of excess folate.

Thus, GCP II is not an important control point, although it is adaptively regulated. Reisenauer et al. (74) reported that the rate of hydrolysis of polyglutamyl folate by brush-border GCP II is more than 100-fold faster than the rate of

monoglutamyl folate transportation into intestinal cells, which suggests that the transport of the monoglutamate end-product is the rate-limiting step in the process of dietary folate absorption.

Transportation into intestinal cells

Folate monoglutamates are hydrophilic anionic molecules and at physiologic concentrations of luminal folate (<10umol/L), the uptake mainly occurs via a carrier-mediated process (75). In this process, two proteins; the Reduced Folate Carrier (RFC) and the Proton-Coupled Folate Transporter (PCFT), are responsible for intestinal folate uptake in the proximal jejunum and duodenum (76). This process requires energy and is dependent on acidic pH. However, at high amounts of folic acid, passive diffusion through nonsaturable mechanism does occur (75).

RFC is an integral membrane protein and is ubiquitously expressed in tissues to play a central role in tissue folate homeostasis (77). RFC has a high affinity for reduced folate and a low affinity for folic acid (78). PCFT prefers oxidized folate such as folic acid to reduced folates as substrates, although affinities for all folate analogs for PCFT are high at micromolar range (76, 79). Both RFC and PCFT are inversely responsive to folate concentration, so that uptake is reduced when dietary folate intake is high and increased when intake is low (80, 81). In animal and in vitro studies, both mRNA and RFC protein expression increased in a folate deficient environment (73, 82). Similarly, PCFT mRNA levels increased about 13-fold in the proximal small intestine of mice that were fed a folate-deficient diet, as compared to those fed a folate-replete diet (83).

Conversely, down-regulation of RFC and PCFT occurs under conditions of over-supplementation. It has been reported that levels of 100 μmol folic acid/L in culture media lead to significantly reduced folic acid uptake in Caco-2 cells, as well as a significant decrease in RFC and PCFT mRNA levels (84). Similarly, in a rat model, acute folate over supplementation led to significant decrease in intestinal folate uptake by down-regulating the expressions of RFC and PCFT (85). The regulation of intestinal uptake appears to be mediated by transcriptional regulatory mechanisms. It is unclear what amounts of folate and folic acid in humans will saturate the carrier proteins leading to passive diffusion.

In summary, the carrier-mediated intestinal process is important for maintaining normal physiological folate levels and meeting metabolic requirements. Uptake from the lumen into the enterocyte is a homeostatically regulated event, and is a control point influencing folate homeostasis. Folate deficiency and over supplementation lead to a respective up-regulation and down-regulation of folate transporter molecules, as evidenced by respective increases or decreases in their mRNA and protein levels. However, at high levels of folic acid intake, passive diffusion may occur and folic acid enters the cells. The specific mechanisms that dictate how folate levels affect the mRNA and protein levels of folate transporters are not clear. Also, the threshold for folate and folic acid absorption is unclear.

Intestinal intracellular metabolism and distribution

Folate monoglutamate and folic acid are transported into intestinal cells where intracellular metabolism and distribution occurs. Once these folate analogs are in

enterocytes, intracellular metabolism such as reduction, methylation and polyglutamate synthesis takes place. Some of the folic acid will undergo an additional reduction process to dihydrofolate (DHF) and THF catalyzed by dihydrofolate reductase (DHFR). THF will be methylated to 5-MTHF before entering the portal circulation (86, 87). Some of the 5-MTHF will be stored in cells by having glutamate residues added to the molecules to increase their size and hence prevent them from leaving the cells (88).

The capacity of DHFR to reduce folic acid in intestinal cells is low and some of the folic acid in its unmetabolized form will enter the portal circulation. Additionally, DHF is a potent inhibitor of methyl tetrahydrofolate reductase (MTHFR) (9); therefore, high concentrations of folic acid could inhibit the formation of 5-MTHF and lead to a decrease in methionine and SAM (methyl donor) synthesis. In those with poor vitamin B-12 status, methionine synthesis is already compromised, so this mechanism could exacerbate the methyl group deficiency. To our knowledge, no data is available to indicate the amount of folic acid that can saturate DHFR in humans.

After intracellular modifications, monoglutamylfolates, mainly 5-MTHF and unmetabolized folic acid (UMFA) are transported through the enterocyte basolateral membrane into portal circulation. The efflux is transported via a carrier-mediated and active anion exchange mechanism (89). Similar to the brush-border membrane, RFC and PCFT are located at the basolateral membrane responsible for trans-membrane transport (90). However, compared with the brush-border membrane transport system, the transport capacity of RFC is lower at the basolateral membrane in the presence of

intracellular neutral environment (89, 90) and PCFT is believed to be the major carrier at this level (91).

In this key event, monoglutamate and folic acid are biotransformed or leave the cells intact. The DHFR which catalyzes the reduction of folic acid is a control point. DHFR has low capacity and so folic acid may exit the cells for the portal circulation in its unmetabolized form.

Hepatic metabolism and distribution

Enterohepatic circulation of folate

Liver is the major site for folate storage and processing. Folate is taken up from the portal circulation and enters the liver in the form of monoglutamate (THF and primarily 5-MTHF) and UMFA. The transport process is saturable energy-dependent at low folate concentration, while at higher concentration of folate (up to 20 µmol/L), the uptake is not saturable (92). Once taken up by liver, more than 97% of 5-MTHF is rapidly cleared into bile. While 15-20% of THF is retained in liver predominately in the form of polyglutamate for storage (93). The liver contains half of the body's folate. Though a substantial fraction of the folate is shunted to bile, most of the folate in bile is reabsorbed in the intestine via enterohepatic circulation (94).

Folate metabolism in liver

Monoglutamate and folic acid are taken up by the liver primarily by PCFT. Folic acid undergoes conversion to its biologically active form via reduction,

methylation and polyglutamate formation (76). Similarly to what occurs in the enterocyte, DHFR is also the enzyme responsible for reduction of folic acid in the hepatocyte, and its activity is a major control point in the hepatic metabolism of folic acid. Compared to rats, human DHFR activity is quite low (95). It takes about an hour to convert 400 μg of folic acid to the reduced form, while the time to convert 5 mg could take up to 12 hours (96). The limited reduction capacity of DHFR results in the appearance of UMFA in plasma and urine. Bailey and Ayling (2009) reported that a concentration of about 331 μg of folic acid in human liver could saturate available DHFR (96). They suggested that human liver is the fundamental cause for the appearance of UMFA in blood circulation. The authors reported that DHFR activity is low and quite variable in human samples and suggested that the plasma concentration of UMFA and possibly DHF will vary between individuals according to their DHFR activity. Therefore, this control point which may become saturated by excessive intake of folic acid is a determining event as it does not fully control the release of UMFA in blood circulation.

The appearance of UMFA in serum may also be affected by genetic polymorphism. DHFR polymorphism which includes the deletion of a specific 19-bp region of DHFR gene was associated with increased gene expression (97, 98). This could lead to greater one carbon metabolism in favor of DNA synthesis and at the expense of methyl supply. This polymorphism was implicated in increased risk of cancer (97) and decreased risk of neural tube defect (98). In contrast, data from the Framingham Offspring Study showed that individuals with DHFR polymorphism had

higher UMFA (99). Further studies are needed to clarify the role of this polymorphism in humans.

Effort was made to determine the threshold at which UMFA starts to appear in blood circulation. Kelly and colleagues reported that oral folic acid intakes of 266 μg/meal resulted in UMFA in the serum, while no UMFA was observed in serum of individuals taking less than 200μg/meal (100). Similarly, Sweeney et al. detected UMFA in serum of folic acid replete individuals after an oral dose of 200 μg per meal administered twice a day for 7 days (101). The increased appearance of serum folic acid after the second 200 μg per meal dose of folic acid indicate that accumulation may occur over time. In the Framingham Offspring Study serum folic acid was measured before and after mandatory folic acid fortification and the results show that exposure to fortification significantly increased circulating concentrations of folic acid, total plasma folate, and 5MTHF among both supplement and non-supplement users (102).

In this key event, most of the 5-MTHF is circulated back to the intestine via enterohepatic circulation. Folic acid is reduced by DHFR, however, that enzyme has low activity and, intake of 200 μg/meal may lead to the appearance of UMFA in circulation. Thus this event- conversion of folic acid by DHFR to its reduced form- may be responsible for the appearance of UMFA in blood circulation and, is considered a determining event.

Kidney folate reabsorption

Folate is transported in blood primarily as monoglutamate in three forms: free folate, folate bound to folate binding protein and folate bound to albumin, all of which can be filtered by human kidney (29).

Filtered folates are reabsorbed back to circulation from the proximal tubule cells and folates not reabsorbed are finally excreted in urine. Therefore, the homeostatic level of folate in human body is regulated by the reabsorption of filtered folate in the proximal tubule cells. The reabsorption is mediated mainly by three types of receptors; folate receptors (FR), megalin and cubilin, which are all located in the kidney proximal tubule epithelial cells. Of these receptors, FR is the most important one and has high affinity for a number of folate compounds including folic acid (103). The expression of FR is regulated by extracellular folate concentration; however, results of studies are somewhat contradictory depending on experimental model used. In vitro studies show that FR expression on renal epithelial cells was down-regulated with over-supplementation of folic acid at a level of 100 µmol/L culture medium (84), and up-regulated under low-folate conditions (104, 105). In contrast, a low-folate diet led to down-regulation of FRs in studies on mice and rats (106, 107). This down-regulation is suspected to occur due to proteolysis of the membrane anchor for the receptors which become unsaturated as a consequence of the diminished folate concentration in the glomerular filtrate (76). Once folate is taken up by the receptors, the reabsorbed filtered folate crosses the basolateral membrane to blood circulation primarily via RFC which is located on the basolateral membrane of kidney tubules (108).

Renal tubular reabsorption plays a pivotal role in maintaining folate homeostasis by reabsorbing the filtered folate. However, the regulation of tubular folate uptake remains to be established under both excess and deficient folate and folic acid intake.

Tissue uptake, storage and intracellular metabolism of folate

Folate uptake at peripheral tissues occurs via folate receptor FR and membrane carrier RFC (76) and these are overexpressed in the presence of folate deficiency (107, 109-111). Of concern, is that UMFA could compete with 5MTHF for carrier protein and binding protein with special concern for folate metabolism in the brain (112). This mechanism, however, has yet to be demonstrated.

Once monoglutamates enter mammalian cells they are rapidly modified for storage by the addition of several glutamate residues to form long side chains in order to trap the folates within the cells. Polyglutamate synthetase plays a regulatory role in maintaining a relatively constant tissue folate concentration by increasing the polyglutamate chain length in periods of folate deficiency (113). It is suggested that modest increases in cellular concentrations of folate will activate the folate-dependent reactions while large increases may inhibit those reactions and their related enzymes (112, 113).

Little is known about intracellular effects of folic acid. Some reports indicate that folic acid might disrupt folate metabolism by disturbing the balance of folate and several folate-dependent enzymes, where elevated folic acid could act as inhibitor. The accumulation of DHF, the first step in folic acid metabolism, may inhibit purine

and thymidine synthase and consequently interfere with DNA synthesis (7, 8). Additionally, high folic acid also can inhibit SAM (methyl donor) synthesis by inhibiting MTHFR (9) and decreasing methylation reactions.

One of the theories put forward is that excess folic acid among individuals with low vitamin B12 may allow cell division in bone marrow to proceed, as it is independent of methionine synthesis, but these growing cells could place increased demand for methyl group further depleting available methyl groups and worsening the impact on non-proliferating cells of the nervous system (112, 114).

Tissue uptake and storage of folate is a key event of folate metabolic pathway. However, knowledge of the regulatory mechanisms within cells is limited and at this time several theories have been put forward to explain potential impact of excess folic acid on health outcome, however, these have yet to be determined.

Discussion

The Key Events Dose-Response Framework developed by ILSI, is an interesting and flexible analytical approach to reviewing nutrient metabolism because it can be used to integrate research findings within key events and channel this information towards an endpoint of interest. In this paper we use the release of UMFA in blood circulation as our endpoint. There is no evidence that folic acid in circulation occurs naturally, so the appearance of folic acid in the blood indicates that excess folic acid intake has occurred due to supplement use and/or fortified food.

Folate is regulated within each of the key events. The intestine, liver and kidneys each play essential roles in regulating body folate homeostasis. At each of

these key events, however, limited information is available on folate threshold and homeostatic regulation. Additionally, little is known about intracellular effects of folic acid. The determining event in folate metabolism leading to the release of UMFA in serum is the reduction of folic acid to DHF and THF by the liver enzyme DHFR. Excessive intake of folic acid can saturate DHFR. Once the saturation threshold is reached in the liver, UMFA is released into circulation. Results of studies indicate that the lowest amount of folic acid intake that resulted in serum UMFA is an oral dose of 200 μg. There is also some indication that folic acid accumulation occurs over time. It is currently not uncommon to detect UMFA in blood especially since folic acid is present in supplement and fortified food. Intakes exceeding the Tolerable Upper Intake Level (UL) of 1mg/day has been reported (5, 15) and may potentially affect vulnerable populations such as children and older adults.

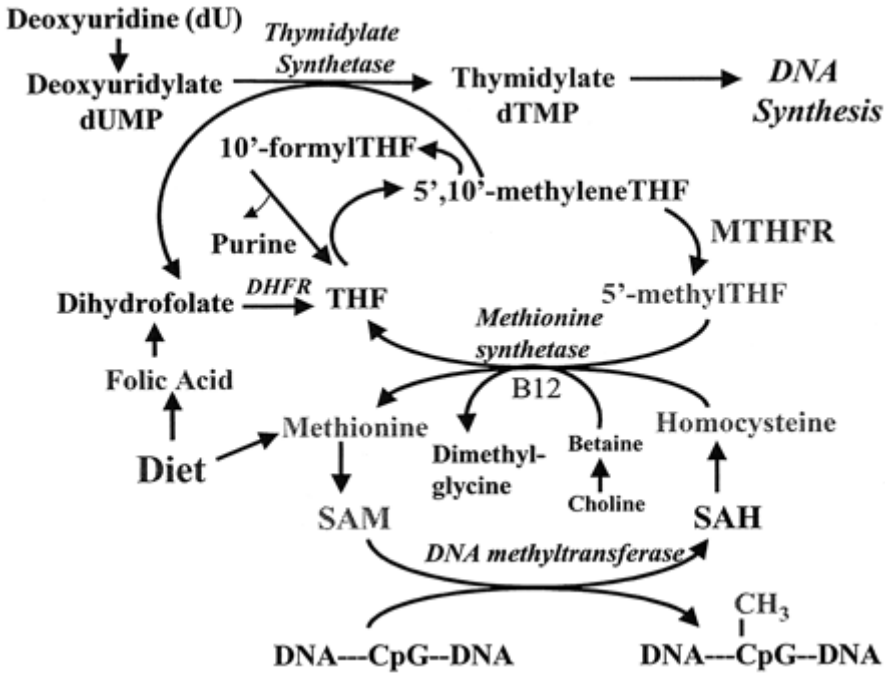
Results of epidemiological studies have shown associations between UMFA and negative health outcomes such as cognitive dysfunction, cancer and stroke. Several theories were put forward to explain the mechanism by which UMFA could lead to negative outcomes. One theory put forward is that high folic acid supply DHF and THF providing substrate to proliferating cells at the expense of methyl reactions. The impact of these changes includes masking of potentially irreversible symptoms of cognitive dysfunction and providing substrate to cancer cells. Alternatively, studies have shown that great increase in total cellular folate may inhibit folate-dependent reactions. Therefore, the question still remains as to whether it is UMFA, increase in total cellular folate or both that exert adverse effect and what are the mechanisms.

Results of studies indicate that the appearance of UMFA in serum varies by individuals. This may be due to genetic makeup and possibly to the presence of DHFR polymorphism. Further studies are needed to clarify the role of this polymorphism in humans and to identify other potential genetic effects on folate metabolism as a consequence of excessive folate intake.

Limited knowledge of the regulation mechanisms and threshold for some key events limit our ability to quantitatively characterize the dose-effect relationship and yet it is critical to do so at this time in light of the petition submitted to FDA by the Centers for Disease Control and Prevention and other organizations to extend food fortification to corn masa flour, a basic ingredient in many foods, such as corn tortillas, that are predominantly, but not exclusively, consumed by the Hispanic population. Compared with other race-ethnicities, Hispanic women have higher rates of infants born with NTDs and lower total folic acid intake (115). This increase in folic acid in the food supply, however, may negatively affect vulnerable populations including Hispanic older adults.

Finally, folate is involved in many reactions that could affect DNA synthesis and amino acid metabolism. Any change such as deficiency or excess could potentially affect a cascade of responses. It would, therefore, be of interest to apply KEDRF to some of the other endpoints in folate metabolism to examine the impact of excess intake on other pathways.

Figures



B12, vitamin B-12; DHFR, dihydrofolate reductase; CH3, methyl group; CpG cytosine-guanine dinucleotide sequence; MTHFR, methylenetetrahydrofolate reductase; SAH, S-adenosylhomocysteine; SAM, S-adenosylmethionine; THF, tetrahydrofolate.

Figure 4. 1 The role of folate cofactors in the methylation cycle (116)

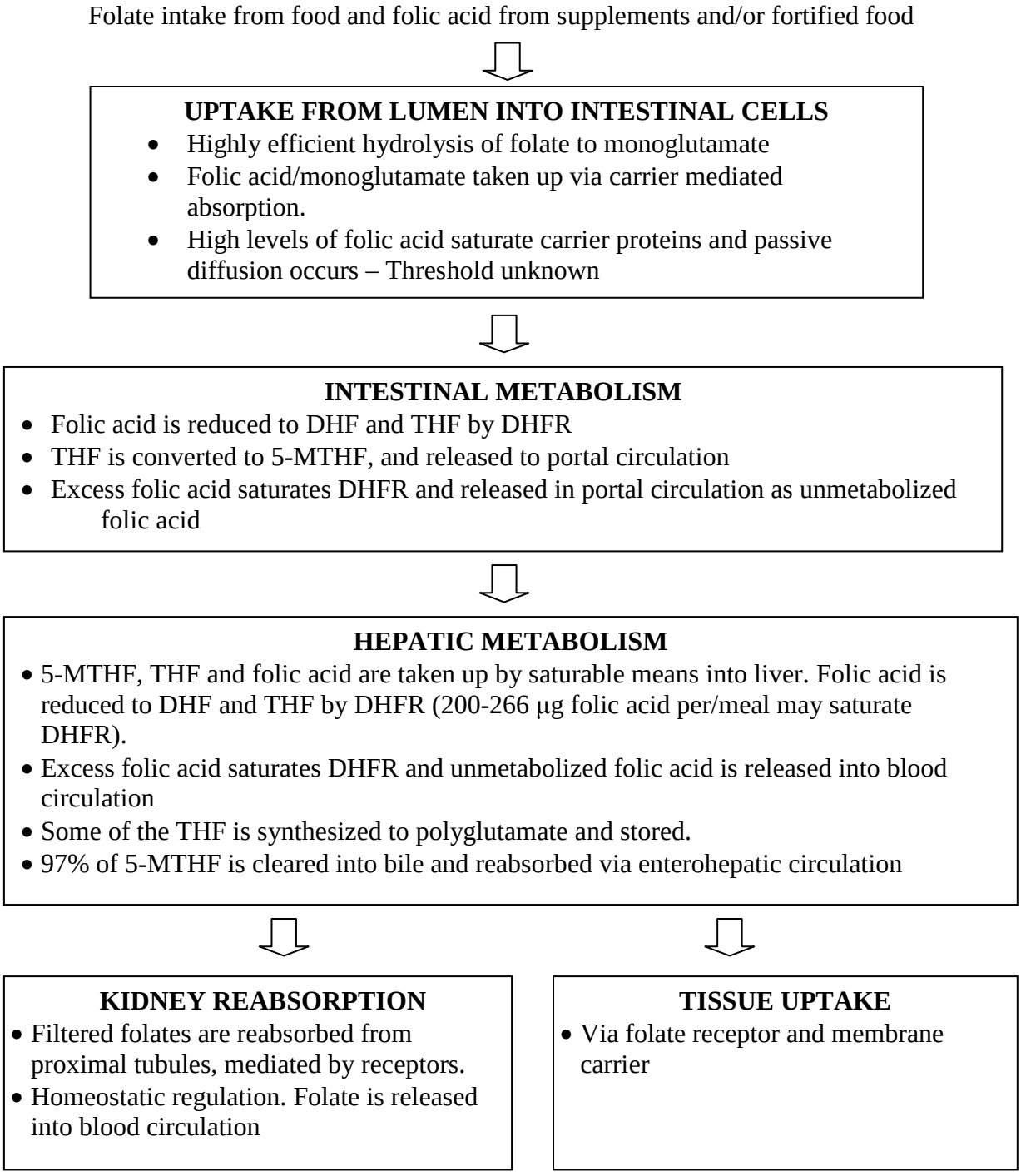


Figure 4. 2 Key Events and control points in the metabolic pathway of folate

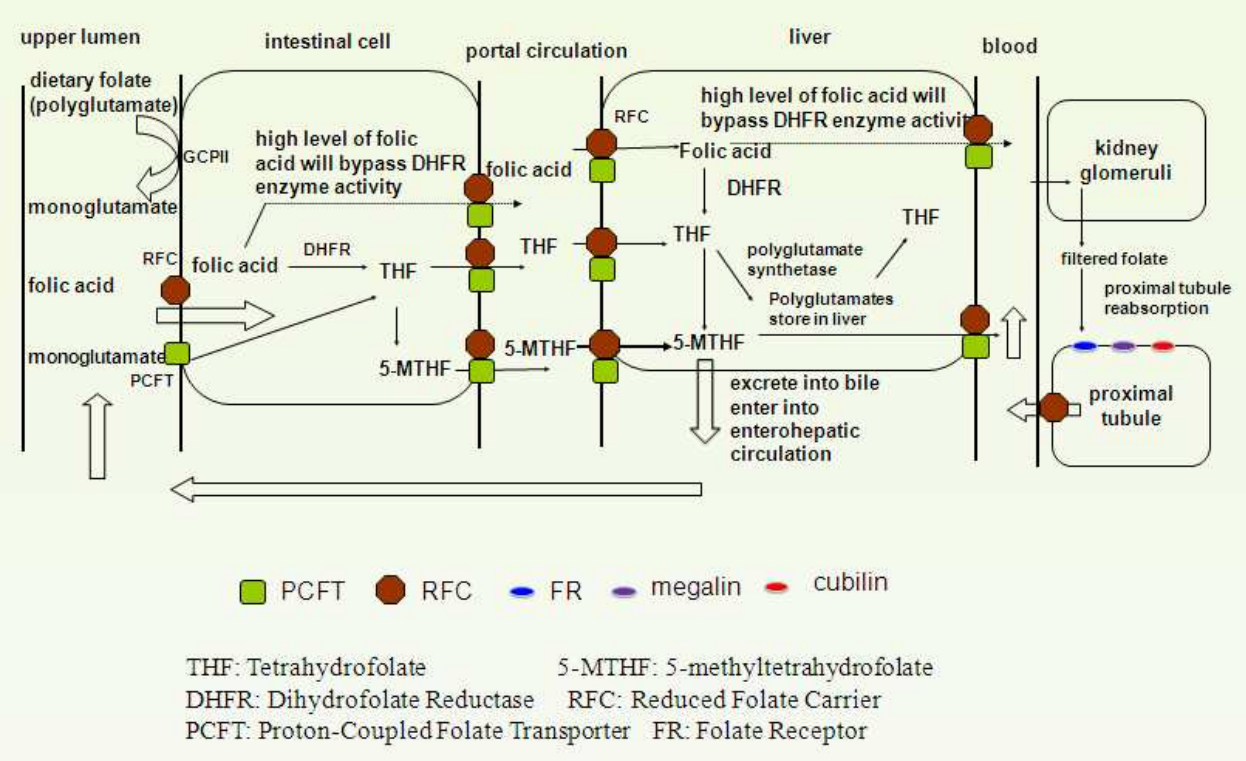


Figure 4. 3 Folate metabolism pathway

B) Associations between folate levels and cancer morbidity among older adults in the U.S.

Abstract

Background: Mandatory folic acid fortification of enriched cereal-grain products in the U.S. has significantly improved the folate status of the population and decreased the incidence of neural tube defects (NTDs). However, safety concerns have been raised that excess consumption of folic acid and high blood folate biomarkers detected in adults in the U.S. may increase the risk of cancer.

Objective: The purpose of this study was to examine associations between folate intake, folate biomarkers levels, and the presence of unmetabolized serum folic acid (UMFA) and the incidence of cancer in a prospective study of older adults in post-fortification years.

Design: Data from participants of the National Health and Nutrition Examination Survey (NHANES) 1999–2002, aged ≥ 57 years were used as the baseline data for this study. Overall incidence of cancer (colorectal, breast, prostate, lung and endometrial cancers) was estimated from the NHANES 1999–2002 linked Medicare and mortality files till December 31, 2007. The associations between folate intake, folate biomarkers and the presence of UMFA and overall cancer incidence were assessed by Cox proportional hazards regression models.

Results: With 8114 person-years of follow-up (median follow-up, 6.3 years), 128 cancer cases were identified. After adjusting for confounders, higher levels of red blood cell (RBC) folate and higher intake of dietary folate equivalents were associated with a lower cancer risk. The hazard ratios (HR) of the highest quartile

versus the reference group (the second quartile) of RBC folate and intake of dietary folate equivalents were 0.54 (95% CI: 0.31–0.93) and 0.54 (95% CI: 0.30–0.95), respectively. No significant associations between the presence of UMFA or intake of food folate and folic acid and cancer incidence were observed.

Conclusion: High folate levels among older population appear to play a protective role against cancer in post-fortification years.

Introduction

Folate is a general term for a group of water-soluble vitamins, which refers to various tetrahydrofolate derivatives naturally found in foods. Folic acid is a synthetic form of folate used in dietary supplements and food fortification. During digestion, folic acid is reduced to biologically active forms which are identical to those from naturally occurring food folate. Folate (used as a collective term for naturally occurring folate and fortified/supplemented folic acid) is essential for numerous bodily functions. Since the human body does not have the ability to synthesize folate, a lack of sufficient dietary folate can lead to folate deficiency. This deficiency can result in many adverse health outcomes. The most notable one is neural tube defects (NTDs) in developing embryos. To reduce the incidence of NTDs, the U.S. government mandated folic acid fortification of enriched cereal-grain products in 1998, which supplies on average an additional 100–200 μg folic acid per person per day based on the average American dietary pattern (117). The folic acid fortification has significantly reduced the prevalence of NTDs (2) and also improved the folate nutritional status of the general population. Mean folate biomarkers such as serum

folate and red blood cell (RBC) folate concentrations increased by approximately 100% and 55%, respectively, in post-fortification compared to pre-fortification years (1).

Though the fortification policy has been successful in reducing the incidence of NTDs and improving folate status of the U.S. population, it also raises safety concerns that high folate intake may have adverse effects such as increasing cancer incidence. High intake of folate could promote the progression of pre-existing cancer by providing substrates for DNA replication in rapid cell division (14). Currently, about 11.3% of the U.S. population exceeds the Tolerable Upper Intake Level (UL) of 1mg/day, as an outcome of food fortification and supplement use (15). In some countries with folic acid fortification programs, high intakes of folate have been hypothesized to contribute to an increase in incidence of colon cancer (16, 22). The results of an ecological study of the U.S. and Canada showed that the increase in incidence of colon cancer coincided with the increase in folate intake after mandatory folic acid fortification in these two countries (16). Similarly, an ecological study in Chile also found that an increased risk of colon cancer coincided with the folic acid fortification program (22). In addition, high dosage of folic acid supplementation (1,000 µg/d) was found to significantly increase the occurrence of multiple and advanced adenomas and total cancers among individuals with history of adenoma in a large clinical trial (14, 53). Another study showed that higher intake of folate from either supplemental folic acid (≥ 400 µg/d) or dietary folate intake (≥ 312 µg/d) was associated with increased risk of postmenopausal breast cancer (23, 24).

Moreover, the fact that unmetabolized folic acid (UMFA) has appeared in blood, as an outcome of high consumption of folic acid, may be of great public health interest. Unlike natural folate, folic acid used in dietary supplements or fortified foods needs to undergo reduction in the liver before participating in intracellular reactions. It is believed that as low as 200 µg of folic acid can saturate the enzyme dihydrofolate reductase (DHFR) which catalyzes folic acid to its reduced form, resulting in detectable unmetabolized folic acid in circulation (118). UMFA is reported to be prevalent in approximately 38% of U.S. adults aged ≥ 60 years, according to data of the National Health and Nutrition Examination Survey (NHANES) 2001–2002 (52). It was speculated that UMFA may be involved in the pathogenesis of cancer by disturbing cellular folate uptake and normal intracellular folate metabolism (10-12, 53). However, the association between UMFA and cancer risk is yet unknown.

We conducted a study to examine the associations between folate intake, folate biomarkers, and the presence of UMFA and overall cancer incidence among adults aged ≥ 57 years who participated in NHANES 1999–2002, using the linked Medicare and mortality data.

Subjects and methods

Baseline data for this study were obtained from NHANES 1999–2002. The NHANES is a nationally representative survey of the health and nutritional status of the non-institutionalized U.S. population, which uses a complex, multistage, probability sampling design. Each NHANES participant undergoes a household interview and a physical examination in a Mobile Examination Center (MEC) (37,

38). The data of NHANES participants were subsequently linked to longitudinal Medicare and mortality data (45, 48). More details about NHANES and its methods can be found elsewhere (39).

Medicare claims and mortality date linkage

Data of NHANES 1999–2002 participants were linked to Medicare enrollment and claim records collected by the Centers for Medicare and Medicaid Services (CMS). The Medicare Chronic Condition File is a summary of clinical information extracted from the NCHS-CMS linked data, which includes the date of first occurrence for 21 chronic conditions, including colorectal, breast, prostate, lung and endometrial cancers from 1999 to 2007. Cancer (breast, colorectal, prostate, lung and endometrial cancers) cases were identified using NCHS designed algorithms of disease codes from Medicare claim records. These are ICD-9 (International Classification of Disease), CPT-4 (Current Procedure Terminology) and/or HCPCS (Healthcare Common Procedure Coding System) codes. More details about algorithms of codes can be found in Appendix B of the NCHS-CMS Medicare Chronic Condition Summary File Data Dictionary (46).

Data of NHANES 1999–2002 participants were also linked to the National Death Index (NDI) through December 31, 2006. The date of death, underlying and multiple causes of death in ICD-10 were recorded in the Linked Mortality Restricted-use File. More details about the linked Medicare file and Mortality file can be found elsewhere (45, 48). The Chronic Condition Summary File information is only available for successfully matched NCHS survey participants who were alive between

2005 and 2007 (45). If participants were not alive by 2005, data on incidence of cancer of these participants were obtained from the NHANES Linked Mortality Restricted-use File.

Cancer cases ascertainment

Cancer cases were identified from the NHANES Linked Medicare or mortality files. For participants whose data were linked to the Medicare Chronic Condition summary file and who were alive between 2005 to 2007, cancer cases and date of first cancer occurrence between 1999 and 2007 were identified from the Summary File (46). For participants who died between 1999 and 2005, their Medicare claims data were not included in the Medicare Chronic Condition Summary File and cancer cases were identified from the Linked Mortality Restricted-use File. Underlying or multiple causes of death from cancer were identified and date of death was used as the date of cancer occurrence.

Study subjects

We used data of NHANES 1999–2002 participants aged 57 years and over (n=3997) to capture all individuals who would potentially reach the age of Medicare eligibility by 2005 to 2007 (Medicare has age-based entitlement at 65 years of age). There were 3192 participants whose data were linked to the Medicare data and 130 participants who died between 1999 and 2005. Data of Medicare beneficiaries who had been enrolled in managed care plans were excluded (n=1034) from the analysis, because claims of these beneficiaries were not collected by CMS. Data were also

excluded if individuals had renal dysfunction (serum creatinine > 131 µmol/L in men and > 115 µmol/L in women, n=84), liver disease (serum alanine aminotransferase > 40 units/L, n=182) or cancer (other than skin cancer, n=213) at baseline examination. We also excluded data of participants who had incomplete folate exposure measurements (dietary folate intake) or blood folate biomarkers (n=275) or incomplete physical exam information (n=109). The final analytic sample was 1425, which included 1384 individuals whose data were linked to the Medicare files and 41 individuals with data linked to Mortality File.

Assessment of diet and supplements

Energy and nutrient intakes including dietary folate and folic acid from fortified foods were estimated from a 24-hour dietary recall, which was administered to each participant by NHANES trained dietary interviewers in the MEC. Data on dietary supplements were collected through the NHANES Dietary Supplement Questionnaire at the household interview. Participants were asked a series of questions about vitamin or mineral supplement use during the past 30 days. Detailed information about frequency of consumption, duration of use, and dosage were collected for each reported dietary supplement. The average daily folic acid intake from dietary supplements and from foods fortified with folic acid were summed to reflect total daily folic acid exposure. In our study, dietary folate equivalents (DFEs) were used as the measurement of total folate intake to account for the difference in the bioavailability of naturally occurring food folate and folic acid. DFEs were

calculated using the equation: DFEs (μg) = food folate (μg) + 1.7 × folic acid from fortified foods or supplements (μg) (119).

Biochemical measurements

RBC folate and serum folate concentrations were measured with the Bio-Rad Laboratories "Quantaphase II Folate/vitamin B12" radioassay from BioRad, Hercules, CA. UMFA concentrations were determined in NHANES 1999–2002 only for participants aged 60 years and over by using a revised affinity/ HPLC method with electrochemical (coulometric) detection. The lower limit of detection for UMFA was 0.18 nmol/L and values below the level of detection were set to zero. Serum creatinine was measured based on the Jaffe reaction. Serum alanine aminotransferase (ALT) was measured using an enzymatic rate method. Complete details and documentation for each of these methods are described elsewhere (43, 44).

Socio-demographic and lifestyle variables

Individuals were classified by non-Hispanic white, non-Hispanic black and others (Mexican American, other Hispanic and other race/ethnicity). Educational attainment was categorized as less than high school, high school graduate (received a high school or high school equivalency diploma) and greater than high school. Physical activity level was self-reported as sedentary, light, and moderate or higher. Alcohol consumption was assessed as grams per day from the 24-hour dietary recall. Cigarette smoking status was categorized as never, former and current. A nonsmoker was defined as a participant who had never smoked ≥ 100 cigarettes during his/her

lifetime; a former smoker was defined as a participant who had smoked ≥ 100 cigarettes and was not smoking at the time of the interview; and a current smoker was defined as a participant who had smoked ≥ 100 cigarettes and was smoking at the time of interview. Body mass index (BMI) was calculated as weight in kilograms divided by height in meters squared (kg/m^2).

Statistical analysis

Baseline data from NHANES 1999–2002, incidence of cancer obtained from the Medicare Chronic Condition Summary File and mortality data obtained from the Linked Mortality Restricted-use File were merged into a single file at the National Center for Health Statistics-Research Data Center (NCHS-RDC) computer lab. Analyses were performed using SAS (version 9.2; SAS Institute Inc, Cary, NC) and $p < 0.05$ was considered statistically significant.

UMFA measurements were categorized into detectable UMFA (UMFA+) and undetectable UMFA (UMFA-) categories. ANOVA for continuous variables and Chi-squared tests for categorical variables were used to examine associations between quartiles of folate biomarkers (serum folate and RBC folate), dietary folate intake (food folate, folic acid and dietary folate equivalents) and characteristics of survey participants.

Cox proportional hazards regression models were used to examine hazard ratios (HR) and 95% confidence intervals (95% CI) for incidence of cancer by quartiles of folate intake and blood biomarkers. The second quartile, which includes recommended dietary allowance of folate, was used as the reference group in the

regression models. The follow-up period of participants in survival analyses was estimated from the time of baseline data collection to the endpoint (the earliest occurrence of any cancer: lung, prostate, breast, colorectal or endometrial cancers). People who were alive or died of other causes were censored at the end of the follow-up period (December 31, 2007) or at the date of death.

For each of the folate intake and biomarker variables, we conducted a multivariable Cox proportional hazards regression model adjusted for confounders. These confounders included age, gender, race/ethnicity, educational attainment, energy intake, BMI, physical activity, smoking status and alcohol intake. We also tested for possible linear association between variables of folate intake and biomarkers and the incidence of overall cancer using linear regression models. Serum folate, RBC folate, intakes of dietary folate, folic acid and dietary folate equivalents were logarithmically transformed, because these five folate exposure variables were not normally distributed. The proportional hazards assumption that there is no significant interaction between predictors and follow-up time was assessed, and no violation to this rule was found.

Results

With 8114 person-years of follow-up (median follow-up, 6.3 years), 128 cancer cases were identified; four of these cases were identified from the mortality files using underlying or multiple causes of death. The characteristics of the cohort by quartiles of RBC folate and dietary folate equivalents are summarized in **Table 4. 1.** RBC folate concentrations tended to be higher among non-Hispanic white women

with higher educational attainment, nonsmokers, and users of vitamin supplements. Participants who had higher intake of dietary folate equivalents tended to be non-Hispanic white man, with higher educational attainment, more physically active, former smokers, and users of vitamin supplements.

Results from linear regression analyses showed that there were significant inverse association between both RBC and serum folate and risk of cancer incidence ($p < 0.01$) (**Table 4. 2**). Individuals whose RBC folate levels were in the fourth quartile (≥ 422.0 ng/ml) had a significantly lower cancer incidence, compared to the reference group (237.8–318.0 ng/ml): the adjusted HR was 0.54 (95% CI: 0.31–0.93). Although there was a linear association between serum folate levels and risk of cancer incidence, there were no associations by serum folate categories. The association between the highest serum folate category and risk of cancer incidence approached significance (HR: 0.59, 95% CI: 0.33–1.05). In addition, individuals with intake of dietary folate equivalents in the fourth quartile (≥ 836.4 µg/d) had significantly lower cancer incidence: the adjusted HR for those in the highest quartile relative to those in the reference group (291.6–<467.0 µg/d) was 0.54 (95% CI: 0.30–0.95), but no significant linear association was observed (**Table 4. 2)**. There were no associations observed between intake of dietary folate, total folic acid or the presence of UMFA and risk of cancer incidence (data not shown).

Discussion

This study showed inverse linear associations between both RBC folate and serum folate concentrations and overall cancer risk. The highest quartile of RBC

folate was inversely associated with overall cancer incidence. There was also a significant association between the highest quartile of dietary folate equivalents and lower risk of incident cancer.

Folate is an essential vitamin in the maintenance of normal DNA function. It is a cofactor in DNA synthesis, repair and methylation. Folate deficiency is considered a risk factor for cancer. Inadequate folate may increase cancer risk by disturbing the synthesis of thymidylate and purines (20, 21), which may cause uracil misincorporation into DNA. The uracil misincorporation may destabilize DNA and increase risk of malignancy. A deficiency in folate also affects methylation of DNA, which in turn influences gene expression and triggers carcinogenesis (21). The mechanisms through which folate influences DNA function suggest dual effects of folate on cancer, whereby low folate concentrations could trigger cancer initiation, while excessive folate intake could promote more rapid cancer progression following onset by providing DNA synthesis substrates (112).

Several epidemiological studies conducted in pre-fortification years in the U.S. suggest that low folate intake (< 200 µg/day) are associated with an increased risk of cancer incidence (58, 59, 62). In our study, we did not observe a higher cancer risk in the lowest quartile (< 292 µg/day) compared to the reference group which included the recommended daily intake value (400 µg/day). However, due to folic acid fortification, only about 10% of individuals had total folate intake less than 200 µg per day in our study. The small number of individuals with lower intake of folate may not provide sufficient statistical power to detect significant association.

We found that individuals in the highest quartile of total folate intake tended to have a lower cancer risk. This finding does not support the hypothesis that fortification of enriched cereal-grain products with folic acid may increase the incidence of cancer. Safety concerns have been raised that high folate intake may promote carcinogenesis. Several studies have reported positive associations between high folate intake levels and cancer risk. In two ecological studies, the increase in intake of folate due to folic acid fortification was found to parallel an increase in the incidence of colorectal cancer in the U.S., Canada (16) and Chile (22). In clinical trials, administration of folic acid supplementation (1000 mg/d) in patients with a history of adenoma was found to accelerate the growth of adenomas and increase the risk of cancer (53, 120). However, a recent meta-analysis which pooled data of around 50,000 participants in randomized clinical trials found that folic acid supplementation did not significantly increase or decrease the incidence of site-specific cancer during the first 5 years of treatment. The doses used in these trials were higher than the average amounts consumed due to folic acid fortification of enriched cereal-grain products (117).

In addition, results of our study suggest linear inverse associations between both RBC and serum folate and risk of cancer incidence. A study conducted among NHANES participants in pre-fortification years (NHANES III 1988–1994) (121) also found an inverse association between serum folate of 3.0–4.3 ng/ml and cancer mortality, but not at lower levels. In our study, the lowest quartile of serum folate concentration was higher than the levels observed in their study and an inverse linear association between serum folate and overall cancer morbidity was observed.

Finally, we did not find UMFA to be significantly associated with cancer risk. Folic acid, a synthetic form of folate, needs to be reduced to its biologically active form (tetrahydrofolate) by the enzyme dihydrofolate reductase (DHFR) before taking part in intracellular reactions. High intake of folic acid could saturate the enzyme and result in the buildup of UMFA in circulation (96). In post-fortification years, UMFA is reported to be prevalent in about 38% of U.S. adults aged ≥ 60 years due to high consumption of folic acid from dietary supplements and fortified foods (52). However, little is known about intracellular effects of UMFA. UMFA has been proposed to interrupt normal folate metabolism through several mechanisms, including inhibiting folate-dependent enzymes and interfering with DNA synthesis and methylenetetrahydrofolate reductase (MTHFR) (7-9). One study found that high blood UMFA concentration associated with decreased natural killer cell cytotoxicity among postmenopausal women (11). Our study showed no association between UMFA and the incidence of cancer. The absence of a relationship may be due to insufficient power to detect a significant association, as UMFA was only measured for participants aged 60 years and over in NHANES 1999–2002. Additionally, because not all participants had detected UMFA in the blood, we examined the association between the presence of UMFA rather than the amount of UMFA and risk of cancer incidence. Further study on larger samples of population is needed to examine the possible dose-response association between UMFA and risk of cancer.

A limitation of this study is that cancer cases were identified using algorithms of disease codes from medical claim records. Medicare claims data were collected for billing purposes, and not for epidemiological study. There could be biased or

incomplete hospital coding. Therefore, health insurance claim records may not reflect precise disease occurrence (122, 123). However, the reliability in identifying cancer incidence has been verified by several studies (124, 125). Additionally, the strength of Medicare claims data are not subject to recall bias. Another limitation of our study is that we used overall cancer morbidity as the health outcome because the number of site-specific cancer cases was too small. Folate may have different effects on the etiologies of different cancers.

Additionally, the date of death from cancer was used as the date of cancer occurrence for individuals who died between 1999 and 2005 as morbidity data were not available on participants who died before 2005. However, only four cancer cases were identified through the mortality files and could not have much impact on the results. Finally, due to our exclusion criteria, the study sample is not nationally representative.

In conclusion, our findings suggest that folate may have a protective role against cancer even at post-fortification levels. UMFA detected in serum was not associated with cancer risk.

Tables

Table 4. 1 Characteristics of NHANES 1999–2002 participants by quartiles of red blood cell (RBC) folate and intake of dietary folate equivalents[1]

	RBC folate (ng/ml)				Dietary folate equivalents (µg/d)			
	Q1	**Q2**	**Q3**	**Q4**	**Q1**	**Q2**	**Q3**	**Q4**
Characteristics[2]	< 237.8	237.8 –< 318.0	318.0 –< 422.0	≥ 422.0	< 291.6	291.6 –< 467.0	467.0 –< 836.4	≥ 836.4
Days of follow up	2193 ± 39	2096 ± 42	2179 ± 39	2187 ± 40	2162 ± 45	2193 ± 41	2201 ± 40	2091 ± 33
Age (year)	68.6 ± 0.4	69.2 ± 0.4	70.8 ± 0.5	71.2 ± 0.5	70.2 ± 0.5	69.7 ± 0.4	69.9 ± 0.5	69.6 ± 0.4
Gender, man (%)	59.2	55.1	50.6	44.9	44.3	54.1	57.6	55.6
Race/ethnicity (%)								
Non-Hispanic white	41.4	55.2	69.1	75.4	49.3	56.9	63.5	71.1
Non-Hispanic black	29.6	15.4	8.9	5.3	21.1	16.2	12.0	10.1
Other race	29.0	29.4	22.1	19.3	29.6	26.9	24.5	18.9
Education level (%)								
< High school	54.1	48.7	33.2	32.5	55.1	45.6	36.5	31.1
High school	18.9	20.5	27.2	23.4	19.1	23.8	22.5	25.7
> High school	26.9	30.8	39.5	44.2	25.8	30.6	41.0	43.2
Smoking (%)								
Current	24.0	14.9	12.3	10.5	21.2	18.1	10.3	11.8
Former	34.6	40.6	36.4	36.8	33.9	35.4	40.7	41.7
Never	41.4	44.5	51.3	52.6	44.9	46.5	49.0	46.5
Physical activity (%)								
Sedentary	29.0	31.1	25.8	29.5	35.5	30.3	27.9	24.9
Light	58.3	55.7	59.3	55.6	54.5	56.4	57.3	57.1
Moderate/high	12.7	13.2	14.9	14.9	9.7	13.1	15.0	18.1
Alcohol intake (gm/d)	6.5 ± 1.2	6.5 ± 1.1	4.0 ± 0.7	4.5 ± 0.9	4.2 ± 0.6	5.9 ± 1.1	6.1 ± 1.1	5.2 ± 0.9
BMI (kg/m^2)	27.6 ± 0.3	28.4 ± 0.3	28.1 ± 0.3	28.0 ± 0.3	28.3 ± 0.3	28.1 ± 0.3	28.0 ± 0.3	27.7 ± 0.3
Total energy intake (kcal/d)	1730 ± 45	1797 ± 42	1807 ± 40	1760 ± 41	1186 ± 24	1725 ± 31	2044 ± 40	2111 ± 47
Folic acid supplement, users (%)	7.1	19.3	13.8	62.6	13.8	17.7	27.4	74.6

[1] Frequencies do not add up to 100% because of rounding and missing values.

[2] Mean ± SE for continuous variables, and percentages for categorical variables presented by quartiles of RBC folate and intake of dietary folate equivalents.

Table 4. 2 Hazard ratios (HR) of overall cancer and 95% confidence intervals (95% CI) by quartiles of red blood cell (RBC) folate, serum folate and intake of dietary folate equivalents

	Quartiles of folate levels				p for trend[2]
	Q1	**Q2**	**Q3**	**Q4**	
Red blood cell folate (ng/ml)	< 237.8	237.8 –< 318.0	318.0 –< 422.0	≥ 421.0	
Number of all-cancer cases	38	39	27	20	
HR adjusted for covariables (95% CI) [1]	0.98 (0.61–1.57)	1.0	0.68 (0.41–1.14)	0.54 (0.31–0.93)	< 0.01
Serum folate (ng/ml)	< 10.7	10.7 –< 15.6	15.6 –< 22.9	≥ 22.9	
Number of all-cancer cases	42	32	30	19	
HR adjusted for covariables (95% CI)[1]	1.44 (0.90–2.31)	1.0	0.88 (0.53–1.47)	0.59 (0.33–1.05)	< 0.01
Dietary folate equivalents (µg/d)	< 291.6	291.6–< 467.0	467.0 –< 836.4	≥ 836.4	
Number of all-cancer cases	31	38	38	18	
HR adjusted for covariables (95% CI) [1]	0.85 (0.51–1.41)	1.0	0.95 (0.60–1.52)	0.54 (0.30–0.95)	0.15

[1] Adjusted for age, gender, race/ethnicity, smoking status, alcohol intake, energy intake, educational attainment, physical activity and BMI.

[2] Test for trend were performed using linear regression models by including log-transformed RBC folate, serum folate or dietary folate equivalents as a continuous variable.

C) Associations between folate levels and stroke morbidity among older adults in the U.S.

Abstract

Background: Mandatory folic acid fortification of enriched cereal-grain products in the U.S. has significantly improved the folate status of the general population. Folate is hypothesized to have a protective effect against stroke by lowering homocysteine levels. Few population-based studies have examined the associations between folate intake, folate biomarkers and homocysteine and the risk of stroke incidence following fortification of enriched cereal-grain products with folic acid.

Objective: The purpose of this study was to examine longitudinal associations between folate intake, folate biomarker levels and homocysteine levels and the incidence of stroke among older adults in post-fortification years.

Methods: Data of participants of the National Health and Nutrition Examination Survey (NHANES) 1999–2002 aged ≥ 57 years were used as the baseline data for this study. Incidence of stroke was obtained from the NHANES 1999–2002 linked Medicare and mortality files up to December 31, 2007. The associations of folate intake and biomarkers levels with the incidence of stroke were assessed by Cox proportional hazards regression models.

Results: With 8129 person-years of follow-up (median follow up, 6.3 years), a total of 123 stroke cases were documented. Results showed that individuals with lower red blood cell (RBC) folate or serum folate tended to have a higher risk of stroke incidence. After adjusting for potential confounders including plasma homocysteine, the hazard ratios (HR) for individuals in the lowest quartile versus those in the second quartile of RBC folate and serum folate were 2.45 (95% CI: 1.34–4.49) and 2.38

(95% CI: 1.33–4.24), respectively. Individuals with higher plasma homocysteine levels tended to have a higher risk of stroke. The adjusted HR for individuals in the highest quartile versus the second quartile of plasma homocysteine was 2.00 (95% CI: 1.14–3.53). No significant associations between intake of dietary folate equivalents, food folate or total folic acid intake and risk of stroke incidence were observed.

Conclusion: This study suggests a protective role of folate, independent of homocysteine, against stroke. Maintenance of normal blood folate levels may be important for prevention of stroke in the U.S. population.

Introduction

Folate is considered a preventive factor against stroke because it is a major determinant of homocysteine levels (126, 127). Folate serves as a methyl donor for homocysteine remethylation to methionine. Deficient folate could lead to elevated homocysteine levels, which have been associated with an increased risk of atherosclerotic disease including stroke. A 25% higher than normal circulating homocysteine concentration has been associated with a 19% higher risk of stroke (26). Since folate can lower homocysteine levels and that homocysteine is positively associated with risk of atherosclerotic disease, it has been suggested that folic acid fortification of enriched cereal-grain products may contribute to prevention of stroke by lowering blood homocysteine concentrations. It was reported in an ecological study that a decline in stroke mortality paralleled the population-wide increase in serum folate and decrease in blood homocysteine concentrations in post-fortification

years in the U.S. (17). However, it is unclear how much of the decline in deaths from stroke is due to reduced incidence of stroke. Many factors, such as the hospitalization rate of stroke patients, may also impact stroke mortality (17, 18). Studies, particularly prospective studies, are needed to elucidate the effects of the increase in folate biomarker levels on the risk of stroke incidence.

In addition, folate itself could be an important factor in stroke prevention independent of blood homocysteine levels. Folate is considered an important factor in maintaining normal vasculature epithelial function. It functions as an antioxidant in reducing the oxidative stress on the vasculature (128), and could restore levels of nitric oxide, an important protective molecule in the vasculature (129). The protective role of folate independent of homocysteine on stroke has been supported in several epidemiological studies (27, 28). Low serum folate levels were independently associated with a significantly higher risk of stroke among Canadians aged ≥ 65 years (28). Additionally, lower plasma folate levels were found to be independently associated with a significantly higher risk of hemorrhagic stroke in the prospective study of the Northern Sweden Health and Disease Study Cohort (27). However, data are not available in post-fortification years in the U.S.

This study was conducted to examine longitudinal associations between folate intake and biomarker levels and homocysteine levels and the risk of stroke incidence among older adults in post-fortification years, using data of NHANES 1999–2002 and the linked Medicare and mortality files.

Subjects and methods

Study population

Baseline data were obtained from NHANES 1999–2002. NHANES is a nationally representative survey of the health and nutritional status of the non-institutionalized U.S. population, and uses a complex, multistage, probability sampling design. Each NHANES participant undergoes a household interview and a physical examination in a Mobile Examination Center (MEC). Date of participants was subsequently linked to longitudinal Medicare claims and mortality data. More details about NHANES and its methods can be found elsewhere (37, 38, 45).

Medicare and mortality data linkage

Data of NHANES 1999–2002 participants were linked to Medicare enrollment and claim records collected by the Centers for Medicare and Medicaid Services (CMS). The Medicare Chronic Condition File is a summary of clinical information extracted from the NCHS-CMS linked data, which includes the date of first occurrence for 21 chronic conditions, including stroke from 1999 to 2007. Stroke (stroke/transient ischemic attack) cases were identified using NCHS designed algorithms of disease codes from Medicare claim records. These are ICD-9 (International Classification of Disease), CPT-4 (Current Procedure Terminology) and/or HCPCS (Healthcare Common Procedure Coding System) codes. More details about algorithms of codes can be found in Appendix B of the NCHS-CMS Medicare Chronic Condition Summary File Data Dictionary (46).

Data of NHANES 1999–2002 participants were also linked to the National Death Index (NDI) through December 31, 2006. The date of death, underlying and multiple causes of death in ICD-10 were recorded in the Linked Mortality Restricted-use File. More details about the linked Medicare file and Mortality file can be found elsewhere (45, 48). The Chronic Condition Summary File information is only available for successfully matched NCHS survey participants who were alive between 2005 and 2007 (45). If participants were not alive by 2005, data on incidence of stroke of these participants were obtained from the NHANES Linked Mortality Restricted-use File.

Ascertainment of stroke cases

Stroke cases were identified from the NHANES Linked Medicare Chronic Condition Summary File or Mortality Restricted-use File. For participants whose data were linked to the Medicare Chronic Condition Summary file and who were alive between 2005 and 2007, stroke cases and date of first stroke occurrence were identified from the Chronic Condition Summary File (46). However, if participants died before 2005, their Medicare claims data were not included in the Medicare Chronic Condition Summary File. For participants who died between 1999 and 2005, stroke cases were identified from the Linked Mortality Restricted-use File. Underlying or multiple causes of death from stroke were identified and date of death was used as the date of cancer occurrence.

Study subjects

We used data of participants in NHANES 1999–2002 aged 57 years and over (n=3997) to capture all individuals who would potentially reach the age of Medicare eligibility by 2005 to 2007 (Medicare has age-based entitlement at 65 years of age). There were 3192 participants whose data were linked to the Medicare Chronic Condition Summary and 130 participants who died between 1999 and 2005. Data of Medicare beneficiaries who had been enrolled in managed care plans were excluded (n=1034) from the analysis, because claims of these beneficiaries were not collected by CMS. Data were also excluded if individuals had liver disease (serum alanine aminotransferase > 40 units/L, n=182), renal dysfunction (serum creatinine > 131 µmol/L in men and > 115 µmol/L in women, n=84) or cardiovascular disease (n=468) at the baseline examination. We also excluded data of participants who had incomplete folate exposure measurements (dietary folate intake) or folate biomarkers (serum or RBC folate) (n=199) or incomplete physical exam information (n=74). The final analyses were conducted with data on 1281 persons.

Baseline Socio-demographic and Lifestyle Variables

Individuals were classified as non-Hispanic white, non-Hispanic black and others (Mexican American, other Hispanic and other race/ethnicity). Educational attainment was categorized as less than high school, high school graduate (received a high school or high school equivalency diploma) and greater than high school. Physical activity level was self-reported as sedentary, light, and moderate or higher. Alcohol consumption was assessed as grams per day from the 24-hour dietary recall. Cigarette smoking status was categorized into never, former and current. A

nonsmoker was defined as a participant who had never smoked ≥ 100 cigarettes during his/her lifetime; a former smoker was defined as a participant who had smoked ≥ 100 cigarettes and was not smoking at the time of the interview; and a current smoker was defined as a participant who had smoked ≥ 100 cigarettes and was smoking at the time of interview. Body mass index (BMI) was calculated as weight in kilograms divided by height in meters squared (kg/m^2).

Assessment of diet and supplements

Energy and nutrient intakes, including dietary folate and folic acid from fortified foods, were estimated from a 24-hour dietary recall, which was administered to each participant by NHANES trained dietary interviewers in the MEC. Data on dietary supplements were collected through the NHANES Dietary Supplement Questionnaire at the household interview. Participants were asked a series of questions about vitamin or mineral supplement use during the past 30 days. Detailed information about frequency of consumption frequency, duration of use, and dosage were collected for each reported dietary supplement. In our study, the average daily folic acid from supplemental sources and from fortified foods was summed to reflect total daily folic acid exposure. Dietary folate equivalents (DFEs) were used as the measurement of total folate intake to account for the difference in the bioavailability of naturally occurring food folate and folic acid. DFEs were calculated using the equation: DFEs (μg) = food folate (μg) + 1.7 × folic acid from fortified foods or supplements (μg) (119).

Biochemical measurements

RBC and serum folate concentrations were measured with the Bio-Rad Laboratories "Quantaphase II Folate/vitamin B12" radioassay from BioRad, Hercules, CA. Plasma homocysteine was measured by using a fluorescence polarization immunoassay reagent set from Abbott Homocysteine assay, Abbott Park, IL. Serum creatinine was measured based on the Jaffe reaction. Serum alanine aminotransferase (ALT) was measured using an enzymatic rate method. Complete details and documentation for each of these methods are described elsewhere (43, 44).

Statistical Analyses

Baseline data from NHANES 1999–2002 files, incidence of stroke obtained from the Linked Medicare Chronic Condition Summary File and mortality data obtained from Mortality Restricted-use File were merged into a single analytical file at the National Center for Health Statistics-Research Data Center (NCHS-RDC) computer lab. Data analyses were performed using SAS (version 9.2; SAS Institute Inc, Cary, NC) and $p < 0.05$ was considered statistically significant.

ANOVA for continuous variables and Chi-squared tests for categorical variables were used to examine associations between baseline quartiles of folate biomarkers (serum folate and RBC folate), dietary folate intake (food folate, folic acid and dietary folate equivalents), plasma homocysteine levels and potential confounders. Cox proportional hazards regression models were used to determine hazard ratios (HR) and 95% confidence intervals (95% CI) for stroke incidence by quartiles of blood biomarkers, intake of dietary folate and plasma homocysteine

levels. The second quartile, which includes recommended dietary allowance of folate, was used as the reference group in the regression models. Follow-up person-years of participants were estimated from the time of baseline data collection to the endpoint (the earliest occurrence of stroke). Participants who died of other causes during the follow-up or were alive till the end of follow up were censored at the date of death or at the end of the follow-up period (December 31, 2007).

For each of the folate intake and biomarkers variables of interest, we constructed a multivariable model adjusted for confounders, which included age, gender, race/ethnicity, smoking status, alcohol intake, education, physical activity, BMI, total energy intake, serum total cholesterol and high-density lipoprotein (HDL). Tests for trend were performed using linear regression models including folate intake and biomarkers variables as continuous variables. In these linear regression models, serum folate, RBC folate, food folate, and folic acid and dietary folate equivalents were logarithmically transformed, because these five folate exposure variables were not normally distributed. In addition, we constructed multivariable models further adjusted for plasma homocysteine to test the associations of folate levels with stroke independent of plasma homocysteine. The proportional hazards assumption that between predictor does not interact with the follow-up time was tested and no violation to the rule was found.

Results

With 8129 person-years of follow-up (median follow up, 6.3 years), a total of 123 stroke cases were documented; two of these cases were identified from mortality

files using cause of death. Baseline characteristics of participants by quartiles of serum folate and RBC folate concentrations are summarized in Table **4. 3**. Individuals with higher RBC and serum folate concentrations tended to be non-Hispanic white women, with higher educational attainment, never smokers, users of vitamin supplements, and those with higher educational attainment. Individuals with RBC and serum folate in the lowest quartile tended to have higher plasma homocysteine as compared to those in the second quartile.

As shown in **Table 4. 4**, the incidence of stroke was significantly higher among participants with RBC concentrations in the lowest quartile (< 239.08 ng/ml): the adjusted HR for those in this quartile relative to those in the second quartile (239.08–317.83 ng/ml) was 2.54 (95% CI: 1.40–4.62). After including plasma homocysteine in the regression model, the adjusted HR was slightly attenuated and remained statistically significant, the adjusted HR was 2.45 (95% CI: 1.34–4.49). HR estimates for those in third and highest quartile of RBC folate concentration did not statistically significantly differ from 1.0. In addition, individuals whose serum folate levels were in the lowest quartile (< 10.9 ng/ml) had a significantly higher risk of stroke, as compared to those of the second quartile (10.9–15.6 ng/ml). The adjusted HR for those in lowest quartile relative to those in the second quartile was 2.43 (95% CI, 1.37–4.33). After including plasma homocysteine in the regression model, the adjusted HR was slightly attenuated but still significant: HR= 2.38 (95% CI: 1.34–4.25) and the linear association also remained significant (p= 0.03). HR estimates for those in third and highest quartile of serum folate concentration did not statistically significantly differ from 1.0. Finally, individuals with plasma homocysteine levels in

the highest quartile had a higher risk of stroke as compared to those in the second quartile (**Table 4. 4**): the adjusted HR for those in the highest quartile (≥ 11.36 µg/ml) relative to those in the second quartile (7.56–9.31 µg/ml) was 2.00 (95% CI: 1.14–3.53).

Discussion

This study showed that lower serum and RBC folate and higher plasma homocysteine folate levels are associated with a significantly higher risk of stroke incidence.

Our findings did not show a significantly lower incidence of stroke among individuals with circulating folate (serum and RBC folate) levels in the highest quartile. The preventive effects of folate against stroke are considered to be via its roles in lowering homocysteine levels. However, among individuals who are not folate-depleted, higher folate levels do not appear to be associated with a further reduction in homocysteine levels and in turn not associated with a further reduction in the incidence of stroke (130). Our results support such findings and suggest that high blood folate concentrations above a certain level may not have additional beneficial effects against stroke in post-fortification years.

Furthermore, we observed that incidence of stroke was significantly higher among those with serum or RBC folate in the lowest quartile compared to those with circulating folate in the second quartile. Our findings support those of previous studies that lower folate biomarker may be a risk factor of stroke.

Low serum folate levels (< 3.8 ng/ml) were found to be independently associated with

a significantly higher risk of stroke among Canadians aged ≥ 65 years (28). In the U.S., a study conducted in the pre-fortification years also reported that low serum folate levels (≤ 4.1 ng/ml) were associated with an increased risk of ischemic stroke among participants of the first National Health and Nutrition Examination Survey Epidemiologic Follow-up Study (1975–1987) (131). In our study, the cutoff value of the lowest quartile of serum folate of our participants is 10.9 ng/ml, which is much higher than the lowest level of those two studies. These levels of serum folate, however, were still associated with a significant higher risk for stroke.

In addition, our results also showed that an elevated blood homocysteine concentration is a predictor of stroke. Findings of some previous epidemiological studies (132-135), but not all, (136-139) suggested that elevated homocysteine levels are associated with an increased risk of atherosclerotic disease. Homocysteine can only be broken down through either the trans-sulfuration or the remethylation pathway. Folate serves as a methyl donor for homocysteine remethylation to methionine. (25). Therefore, folate is considered to play a protective role against stroke by reducing homocysteine levels. Folate itself, however, may be an independently risk factor of stroke.

Our results showed a protective role of folate against stroke independent of blood homocysteine levels. After further adjusting for plasma homocysteine concentrations in multivariable regression models, the risk estimates for those with RBC or serum folate in the lowest quartiles relative to those with RBC or serum folate in the second quartiles were slightly attenuated and remained statistically significant. These findings suggest a protective role of folate, possibly in addition to

its effects on blood homocysteine concentrations, against stroke. Mechanisms involved in the association between folate and stroke were examined in previous studies, but not yet fully established. It was reported that plasma folate, as an antioxidant, plays an important role in reducing the oxidative stress on the vasculature (128). In addition, folate was found to be able to restore levels of nitric oxide, an important protective molecule in the vasculature (129).

Finally, we did not find significant associations between intake of dietary folate and the risk of stroke incidence. Previous studies have shown mixed results. In the Nurses' Health Study, no association was found between folate intake and all strokes or stroke subtypes among women (140). However, in the Health Professionals Follow-up Study, a significant inverse association between food folate and ischemic stroke was observed among men (141). Similar inverse associations between food folate and ischemic stroke were also observed in the prospective NHANES I study and the Northern Sweden Health and Disease Cohort Study (27, 142). In a prospective nested case-control study, food folate intake was inversely related to hemorrhagic stroke but not ischemic stroke. It should be noted that none of these studies were conducted in post-fortification years, when dietary folate intake of the U.S. population significantly increased. Intake of dietary folate was almost doubled in post-fortification compared to pre-fortification years (143). We cannot exclude the possibility that the lack of associations between intake of dietary folate and incidence of stroke is due to insufficient statistical power to detect significance and, therefore, additional studies are needed.

A limitation of this study is that stroke cases were identified using algorithms from medical claims records. Medicare claims data were collected for billing purposes, and not for epidemiological study. There could be biased or incomplete hospital coding. Therefore, health insurance claim records may not reflect precise disease occurrence (122, 123). However, the strength of these data is that Medicare claims data are not subject to recall bias. Another limitation is that the date of death from stroke was used as the date of first stroke occurrence for individuals who died before 2005. The Medicare Chronic Condition Summary File does not include data on stroke incidence of Medicare beneficiaries who died before 2005. Only two stroke cases, however, were identified from either underlying cause of death or multiple causes of death and would not have much impact on the estimation of follow-up person-years.

In addition, due to lack of data on genetic polymorphisms, we did not adjust for genetic factors which may play important roles in the association between folate and stroke (such as MTHFR polymorphisms C677T and A1298C) (144). Additionally, our study only assessed the associations of folate with all-stroke risk but not with stroke subtypes (ischemic or hemorrhagic stroke). Folate, however, may unequally impact the risk of them (27, 141). Finally, the study sample is not nationally representative. Data included in this study were of participants in NHANES 1999–2002 that linked to Medicare files and not enrolled in managed care plans.

In conclusion, adequate blood folate levels appear to have protective effects against stroke among older adults and higher folate biomarkers levels may not have

additional beneficial effects on stroke. Maintenance of normal blood folate levels may be important for prevention of stroke in the U.S. population.

Tables

Table 4. 3 Characteristics of NHANES 1999–2002 participants by quartiles of red blood cell (RBC) folate and serum folate[1]

	RBC folate (ng/ml)				Serum folate (ng/ml)			
	Q1	Q2	Q3	Q4	Q1	Q2	Q3	Q4
Characteristics[2]	< 239.1	239.1 –< 317.8	317.8 –< 413.6	≥ 413.6	< 10.8	10.8 –< 15.5	15.5 –< 22.5	≥ 22.5
Days of follow up	2165 ± 42	2233 ± 39	2163 ± 42	2191 ± 42	2084 ± 44	2261 ± 36	2191 ± 41	2212 ± 43
Age (year)	68.2 ± 0.4	68.8 ± 0.5	70.4 ± 0.5	71.2 ± 0.5	68.4 ± 0.5	68.7 ± 0.5	69.2 ± 0.5	72.3 ± 0.5
Gender, man (%)	60.7	52.3	47.1	38.1	61.8	57.0	43.6	36.8
Race/ethnicity (%)								
Non-Hispanic white	41.9	54.5	69.2	76.6	47.3	55.4	62.3	77.1
Non-Hispanic black	29.2	16.5	9.4	4.5	26.4	16.2	11.8	5.5
Other race/ethnicity	28.9	29.0	21.4	18.9	26.4	28.3	25.9	17.4
Education level (%)								
< High school	54.2	43.0	31.2	30.1	53.4	43.3	32.8	29.7
High school	17.5	21.3	28.3	22.4	17.6	21.3	26.9	24.2
> High school	28.3	35.2	40.6	47.4	29.0	35.4	40.3	46.1
Smoking (%)								
Current	22.7	14.5	10.7	10.3	22.3	16.2	10.2	10.0
Former	34.4	42.9	38.0	32.0	38.5	41.1	39.3	29.4
Never	42.9	42.6	51.3	57.7	39.2	42.7	50.5	60.7
Physical activity (%)								
Sedentary	26.6	27.1	23.7	27.2	32.8	21.7	24.9	24.8
Light	60.7	57.7	61.0	58.0	55.7	61.5	55.7	64.2
Moderate/high	12.7	15.2	15.3	14.7	11.5	16.9	19.3	11.0
Folic acid supplement, users (%)	8.5	16.2	41.0	69.0	8.5	16.2	41.0	69.0
Alcohol intake (gm/d)	6.4 ± 1.2	8.2 ± 1.3	4.5 ± 0.8	5.0 ± 1.0	7.6 ± 1.3	6.8 ± 1.2	5.1 ± 0.9	4.9 ± 1.1
BMI (kg/m^2)	27.6 ± 0.3	28.2 ± 0.3	28.0 ± 0.3	27.7 ± 0.3	28.6 ± 0.4	28.3 ± 0.3	27.7 ± 0.3	26.8 ± 0.3
Total energy intake (kcal/d)	1729 ± 45	1840 ± 48	1803 ± 42	1791 ± 45	1792 ± 46	1823 ± 47	1794 ± 45	1737 ± 42
Total cholesterol (mg/dL)	213.7 ± 2.4	213.9 ±2.3	212.5 ±2.2	209.1 ±2.1	213.0 ±2.4	211.2 ± 2.3	210.5 ± 2.2	215.1 ± 2.1
HDL-cholesterol (mg/dL)	52.5 ± 0.9	54.1 ± 1.0	53.4 ± 0.9	54.7 ± 1.0	51.1 ± 0.9	51.9 ± 0.8	55.1 ± 1.0	56.5 ± 1.0
Plasma homocysteine (μg/ml)	11.4 ± 0.3	9.7 ± 0.2	9.3 ± 0.1	9.4 ± 0.2	11.5 ± 0.2	9.9 ± 0.2	9.2 ± 0.2	9.3 ± 0.2

[1] Frequencies do not add up to 100% because of rounding and missing values. Q, quartile.

[2] Mean ± SE for continuous variables, and percentages for categorical variables by quartiles of RBC and serum folate.

Table 4. 4 Hazard ratios (HR) of stroke and 95% confidence intervals (95% CI) by quartiles of red blood cell (RBC) folate, serum folate and plasma homocysteine

	Quartiles of folate levels				P for trend[3]
	Q1	Q2	Q3	Q4	
Red blood cell folate (ng/ml)	< 239.1	239.1–< 317.8	317.8 –< 413.6	≥ 413.6	
Number of stroke cases	41	18	27	32	
Adjusted HR1[1] (95% CI)	2.54 (1.40–4.62)	1.0	1.46 (0.77–2.76)	1.49 (0.80–2.77)	0.05
Adjusted HR2[2] (95% CI)	2.45 (1.34–4.49)	1.0	1.49 (0.78–2.84)	1.49 (0.77-2.87)	0.05
Serum folate (ng/ml)	< 10.9	10.9 –< 15.6	15.6 –< 22.5	≥ 22.5	
Number of stroke cases	38	21	26	33	
Adjusted HR1[1] (95% CI)	2.43 (1.37–4.33)	1.0	1.45 (0.78–2.68)	1.31 (0.71–2.39)	0.02
Adjusted HR2[2] (95% CI)	2.38 (1.33–4.24)	1.0	1.49 (0.80–2.77)	1.35 (0.74–2.50)	0.03
Plasma homocysteine (µg/ml)	< 7.6	7.6 –< 9.3	9.3 –< 11.4	≥ 11.4	
Number of stroke cases	22	24	28	45	
Adjusted HR[1](95% CI)	1.21 (0.64–2.28)	1.0	1.38 (0.76–2.49)	2.00 (1.14–3.53)	0.18

[1]Adjusted for age, gender, race/ethnicity, smoking status, alcohol intake, educational attainment, physical activity, BMI, total energy intake, serum total cholesterol and high-density lipoprotein (HDL).
[2] Further adjusted for plasma homocysteine concentrations.
[3] Test for trend were performed using linear regression models by including log-transformed RBC folate, serum folate or plasma homocysteine levels as a continuous variable.

D) Associations between folate biomarker levels and renal function among adults in the U.S.

Abstract

Background: Levels of folate biomarker of the U.S. population significantly increased since folic acid fortification of enriched cereal-grain products in 1998. Since kidneys are highly involved in maintaining folate homeostasis, the effect of folic acid fortification may be affected by reduced kidney function. However, it is unclear what the impact of reduced kidney function is on folate status.

Objective: The purpose of this study was to investigate the associations of renal function with levels of serum folate, red blood cell (RBC) folate, and plasma homocysteine and the presence of serum unmetabolized folic acid (UMFA) in U.S. adults.

Design: Data of participants of the National Health and Nutrition Examination Survey (NHANES) 1999–2002 aged ≥ 40 years were used in this study. Associations between levels of folate biomarkers and homocysteine and the presence of UMFA and three estimated glomerular filtration rate (eGFR) categories (< 60, 60–90 and ≥ 90 mL/min/1.73 m^2) were assessed using multivariable regression models, controlled for confounders. The Chronic Kidney Disease Epidemiology Collaboration (CKD-EPI) formula was used to estimate eGFR.

Results: In men and women, RBC folate and plasma homocysteine levels were significantly higher among individuals with eGFR < 60 ml/min/1.73 m^2 compared with those with eGFR ≥ 90 ml/min/1.73 m^2; serum folate levels were not significantly different between eGFR groups. In women but not in men, UMFA was significantly

more likely to appear in the blood of individuals with eGFR < 60 ml/min/1.73 m^2 compared to those with GFR ≥ 90 ml/min/1.73 m^2 (OR: 2.2, 95% CI: 1.2–4.0). **Conclusion:** Reduced renal function may be implicated in the increased blood folate concentrations. Until more information is available, caution should be taken in consumption of folic acid-enriched foods and folic acid supplements among individuals with renal insufficiency.

Introduction

Folate is a general term for a group of water-soluble vitamins, which refers to various tetrahydrofolate derivatives naturally found in foods. The kidneys are highly involved in maintaining folate homeostasis. A significant amount of folate is filtered daily and the proximal renal tubular reabsorption of folate prevents extensive urinary excretion of folate (29). Folate receptors and other transporters such as cubilin and megalin located on renal tubule are believed responsible for folate reabsorption (30). Expression of folate transporters was down-regulated and the uptake and utilization of folate by peripheral tissue and organs were decreased in rat models of chronic kidney disease (31). Absorption of folate by the small intestine was found to decrease in rat models of chronic kidney disease in another study (32). These changes in folate utilization and absorption may result in disturbance in homeostasis of folate.

Folate biomarker levels of the U.S. population have significantly increased since folic acid fortification of enriched cereal-grain products in 1998. Mean serum folate and red blood cell (RBC) folate are estimated to have increased by approximately 100% and 55%, respectively, in post-fortification years compared to

pre-fortification years (1). In addition, about 11.3% of the U.S. population exceeds the Tolerable Upper Intake Level (UL) of 1 mg/day, by consuming fortified foods and dietary supplements (5, 15). It has been reported that unmetabolized folic acid (UMFA) was prevalent in approximately 38% of U.S. adults in 2001–2002 as an outcome of high folic acid consumption (145).

The effect of folic acid fortification, however, may be different in individuals with renal dysfunction, given that reduced renal function may have an impact on folate homeostasis. Studying the association between kidney function and folate levels may be of public health significance because chronic kidney disease (CKD) is an increasing health concern that currently affects about 13% of the U.S. population (19). Additionally, altered folate status such as low folate levels have been considered as a risk factor for adverse health outcomes such as cancer (21, 64). Increased folate levels (13, 14, 16) and the presence of UMFA (10-12, 23) have also been associated with increased risk of diseases, such as cancer and cognitive impairment.

To date, few studies examined the associations between renal function and folate status, and the findings are not conclusive (33-35). In addition, no study has examined the association between renal function and UMFA. Therefore, this study was undertaken to further understand the associations of renal function with folate biomarkers and UMFA using data from NHANES 1999–2002.

Subjects and methods

Study population:

The National Health and Nutrition Examination Survey (NHANES) is a nationally representative, cross-sectional survey of the health and nutritional status of non-institutionalized U.S. population. It uses a complex, multistage, probability sampling design. Each NHANES participant undergoes a household interview and a physical examination performed in a Mobile Examination Center (MEC). More details about NHANES and its methods can be found elsewhere (37-39).

Data of NHANES 1999–2002 participants aged ≥ 40 years were used in this study (n= 6671). Data of individuals undergoing dialysis (n=30) or had liver disease (serum alanine aminotransferase > 40 units/L, n=516) were excluded. Also excluded were data of individuals with incomplete dietary information (n=872), folate biomarkers (n=318) or serum creatinine (n=46) measurements. This study was conducted with the data of 4889 persons. In addition, analysis was also conducted on a subsample of NHANES 1999–2002 participants aged ≥ 60 years who had a UMFA measurement (n=2419).

Assessment of diet and supplements

Energy and nutrient intakes, including dietary folate and folic acid from fortified foods, were estimated from a 24-hour dietary recall, administered in the MEC. Data on dietary supplements were collected through the NHANES Dietary Supplement Questionnaire at the household interview. Participants were asked a series of questions about vitamin or mineral supplement use during the past 30 days. The average daily intake of folic acid from all dietary supplements was calculated based on the reported number of days of supplement consumption, amount taken per

day and the serving size unit from the product label (146). In our study, the average daily folic acid intake from dietary supplements and from foods fortified with folic acid were summed to reflect total daily folic acid exposure. Dietary folate equivalents (DFEs) were used as the measurement of total folate intake to account for difference in the bioavailability of naturally occurring food folate and folic acid. DFEs were calculated using the equation: DFEs (μg)= food folate (μg) + 1.7 × folic acid (μg) (119).

Measures of blood biochemical and kidney function

Serum folate and RBC folate concentrations were measured with the Bio-Rad Laboratories "Quantaphase II Folate/vitamin B12" radioassay from BioRad, Hercules, CA. UMFA concentrations were determined in NHANES 1999–2002 participants aged ≥ 60 years by using a revised affinity/ HPLC method with electrochemical (coulometric) detection. The level of detection for UMFA was 0.18 nmol/L and values below the level of detection were set to zero. Plasma homocysteine was measured by using a fluorescence polarization immunoassay reagent set from Abbott Homocysteine assay, Abbott Park, IL. Serum alanine aminotransferase was measured by using an enzymatic rate method. (42). Serum creatinine was measured based on the Jaffe reaction. Creatinine measurements of NHANES 1999–2000 were recalibrated to standardized creatinine measurement values (obtained at the Cleveland Clinic Research Laboratory, Cleveland, Ohio), using the equation: standard creatinine = 0.147+1.013 × uncalibrated serum creatinine (mg/dL) (147). Estimate glomerular filtration rate (eGFR) was calculated using the Chronic Kidney Disease Epidemiology

Collaboration (CKD-EPI) creatinine formula (148). Complete details and documentation for each of these methods are described elsewhere (43, 44).

Baseline socio-demographic and lifestyle variables

Individuals were classified by race/ethnicity as non-Hispanic white, non-Hispanic black and others (Mexican American, other Hispanic and other race/ethnicity). Educational attainment was categorized as less than high school, high school graduate (received a high school or high school equivalency diploma) and greater than high school. Physical activity level was categorized as sedentary, light, and moderate or higher by using answers to the question "Average levels of physical activity each day" in the physical activity questionnaire. Alcohol consumption was assessed as grams per day from one 24-hour dietary recall. Cigarette smoking status was categorized as never, former and current. A nonsmoker was defined as a participant who had never smoked ≥ 100 cigarettes during his/her lifetime; a former smoker was defined as a participant who had smoked ≥ 100 cigarettes and was not smoking at the time of the interview; and a current smoker was defined as a participant who had smoked ≥ 100 cigarettes and was smoking at the time of inquiry. Body mass index (BMI) was calculated as weight in kilograms divided by height in meters squared (kg/m^2).

Statistical analyses:

Data analyses were performed using SAS for windows (version 9.2, SAS Institute Inc, Cary, NC) and SAS-callable SUDAAN (version 10.1; Research Triangle

Institute, Research Triangle Park, NC). Analyses were conducted incorporating the four-year sampling weight to adjust for complex study design (51). Individuals with eGFR less than 60 mL/min/1.73 m^2 and those with eGFR between 60 and 90 mL/min/1.73 m^2 were defined as having reduced renal function and mildly reduced renal function, respectively (50). Data of participants were classified into three categories according to their eGFR values (< 60, 60–90 and ≥ 90 mL/min/1.73 m^2). UMFA measurements were categorized into detectable UMFA (UMFA+) and undetectable UMFA (UMFA-) categories. Serum folate and plasma homocysteine levels were not normally distributed (with a skewness > 4), and, therefore, these two variables were log-transformed before group comparison and regression analyses. All tests were stratified by gender (men and women), and the eGFR ≥ 90 mL/min/1.73 m^2 category was set as the reference group. Statistical significance was set at $p < 0.05$.

Dunnett's test and Chi-squared tests were used to compare group means of continuous variables and group percentages of categorical variables, respectively. Serum folate, RBC folate and plasma homocysteine levels were assessed in relation to eGFR using multivariable linear regression models. Regression models for serum folate and RBC folate were adjusted for age, gender, race/ethnicity, smoking status, alcohol intake, BMI, physical activity, educational attainment and intake of dietary folate equivalents. Regression model for plasma homocysteine was additionally adjusted for intake of vitamin B12. eGFR was also examined as a continuous variable in association with folate biomarkers and the association was represented as p for trend. In addition, we examined associations between UMFA and eGFR categories. Odds ratios (OR) for the presence of UMFA in the blood and 95% confidence

intervals (95% CI) were estimated based on eGFR categories using multivariable logistic regression models. Logistic regression models were adjusted confounders, including age, gender, race/ethnicity, smoking status, alcohol intake, BMI, physical activity, educational attainment and intake of dietary folate equivalents.

Results

The mean age of the study population was 57.3 years, and 44.8% were men. Prevalence of individuals with eGFR < 60 mL/min/1.73 m^2 was 10.9 %. Individuals with eGFR < 60 mL/min/1.73 m^2 were more likely to be older, non-Hispanic white, nonsmokers (in women), former smokers (in men), less physically active, have lower educational attainment, and users of folic acid supplements (in women) (**Table 4. 5**). There was no significant difference in intake of dietary folate or vitamin B12 by eGFR categories.

In both men and women, RBC folate levels were significantly higher in the eGFR < 60 mL/min/1.73 m^2 group compared to reference group ($p < 0.05$), after adjustment for age, gender, race/ethnicity, smoking status, alcohol intake, BMI, physical activity, educational attainment and intake of dietary folate equivalents (**Table 4. 6).** There were no significant differences in serum folate by categories of eGFR. Plasma homocysteine levels were significantly higher among participants in the eGFR < 60 and 60–90 mL/min/1.73 m^2 category compared to the reference group ($p < 0.001$) after controlling for confounders including intake of vitamin B12 (**Table 4. 6)**. Among women but not men with eGFR < 60 mL/min/1.73 m^2, there was a

significantly higher prevalence of UMFA compared to those in the reference group (OR: 2.2, 95% CI: 1.2–4.0).

Discussion

Results of this study showed that reduced renal function as determined by eGFR was associated with higher RBC folate and plasma homocysteine levels among men and women, and higher prevalence of UMFA among women, as compared to individuals in the highest eGFR category. Additionall1y, even individuals in the intermediate eGFR category (60–90 mL/min/1.73 m^2) had significantly higher plasma homocysteine levels as compared to those in the highest eGFR category.

Results suggest that reduced renal function was associated with a higher RBC folate levels. RBC, which has a lifespan of 120 days, accumulates folate only during erythropoiesis, the process by which the bone marrow produces red blood cells (149). Therefore, red blood cell folate concentrations respond slowly to changes in folate intake and are indicative of long-term folate status. RBC uptake of folate is believed to be through FBP (folate binding protein) (150) and RFC (reduced folate carrier) (151) transport systems. Expression of folate receptors including RFC was found to be down-regulated in peripheral tissues and organs including the small intestine, heart, liver and brain, in rat models of chronic kidney disease. Such down-regulation of folate receptors can result in reduced folate uptake and utilization of folate by peripheral tissues and organs (31). However, it is yet unknown what is the impact of chronic kidney disease on RBC folate uptake systems.

Previous evidence suggests that RBC folate levels may not be affected significantly by renal insufficiency. Results of previous studies showed that folate levels could maintain normal without folate supplementation among individuals with renal insufficiency, even among those undergoing dialysis (153, 154). Additionally, it was reported in 1999 that RBC folate levels of over 80% of dialysis patients were within the normal range (155). Our findings indicate higher RBC folate levels among individuals with renal insufficiency. This association may be due to widely used folic acid supplements in individuals with renal insufficiency (156). However, the association between RBC folate and lower eGFR remained significant even after controlling for confounders including folic acid and dietary folate intakes.

In addition, our results did not show any significant difference in serum folate concentrations between lower eGFR (eGFR < 90 mL/min/1.73 m^2) versus the normal category. A few studies examined plasma or serum folate levels among chronic kidney disease patients but results were inconclusive. In one study among patients with chronic kidney disease receiving dialysis showed increased plasma folate levels (33), while other studies reported decreased serum folate concentrations among dialyzed patients (34, 35). The mechanism which leads to altered serum/plasma folate levels in individuals with renal insufficiency were examined but not fully established. It was reported that intestinal folate absorption was significantly reduced in a rat model of chronic kidney disease (32). Also, folate uptake and utilization of folate by peripheral organs and tissues was found reduced in a rat model of chronic kidney disease (31). It is speculated that reduced intestinal absorption of folate, diminished

utilization by peripheral organs and tissues may possibly contribute to normal serum folate levels.

Additionally, in our study individuals who had lower renal function (eGFR < 90 mL/min/1.73 m^2) were at increased risk of higher homocysteine levels. This result supports other studies, which showed that declining renal function is associated with elevated plasma homocysteine levels (157-160). A study by Bostom et al. showed that hyperhomocysteinemia was prevalent in 83% of patients with renal insufficiency (25). Nutrient deficiency, impaired renal excretion of homocysteine and disturbance of metabolism of amino acids required for breakdown of homocysteine are hypothesized to contribute to hyperhomocysteinemia among individuals with renal insufficiency (161, 162). However, the mechanisms through which renal insufficiency lead to elevated homocysteine levels have not been fully elucidated (157).

Elevated homocysteine levels are usually found along with vitamin deficiency, especially folate deficiency, because folate serves as a methyl donor in the homocysteine remethylation pathway (25). However, in our study individuals with higher homocysteine concentrations, paradoxically, had higher RBC folate levels. One possible explanation for such findings may be that tissue uptake of folate is reduced in renal insufficiency (31). Intracellular inadequacy of folate may consequently lead to the accumulation of homocysteine. Another explanation may be that higher folate levels do not appear to further reduce homocysteine levels among individuals who are not folate-depleted (130). In our study, we found that individuals in the eGFR < 60 mL/min/1.73 m^2 category had good nutritional status of folate (163). Only 1.2% of participants had deficient folate levels (RBC folate < 140 ng/ml)

possibly as an outcome of folic acid fortification. Therefore, high folate levels may coexist with elevated homocysteine levels in individuals with renal insufficiency.

Finally, we found that UMFA was significantly more likely to appear in the blood of women with lower eGFR. Folic acid intake significantly increased after folic acid fortification of enriched cereal-grain products in 1998 and UMFA is reported to be prevalent among older adults aged ≥ 60 years (145). Folic acid needs to be reduced to its biologically active form before taking part in intracellular reactions. Excess intake of folic acid could bypass the conversion mechanism and lead to the appearance of UMFA in circulation (96). To date, little is known about the specific intracellular impacts of UMFA. It has been speculated, however, that UMFA may be associated with some adverse effects, including cognitive impairment in older population (12) and decreased immune function in postmenopausal women (11). The mechanism by which reduced eGFR might be associated with an increase in UMFA still remains to be studied.

In summary, reduced renal function is associated with increased levels of RBC folate and plasma homocysteine. Reduced renal function is also associated with the presence of UMFA in circulation among women. More studies are needed to understand the consequences of high intake of folic acid among people with reduced renal function and the health implications of high RBC folate. At this time, however, caution must be taken in consumption of folic acid-enriched foods and folic acid supplements among individuals with renal insufficiency.

Tables

Table 4. 5 Baseline characteristics of NHANES 1999–2002 participants aged ≥ 40 years by eGFR categories[1]

	eGFR (mL/min/1.73 m^2)			
	All subjects	**< 60**	**60 –< 90**	**≥ 90**
Men				
n	2332	328	1179	825
Age	56.7 (0.3)	72.8 (0.9) ***[3]	58.9 (0.4) ***	49.8 (0.3)
Race/ethnicity (%)				
Non-Hispanic white	79.7 (76.1-82.8)	86.3 (83.1-89.1) ***	85.6 (82.0-88.7) ***	70.1 (65.0–74.7)
Non-Hispanic black	8.3 (6.6–10.3)	8.0 (5.4–11.6)*	6.0 (4.7–7.6)**	11.5 (8.9–14.7)
Other race/ethnicity	12.0 (9.0–15.9)	5.7 (2.9–11.1) ***	8.4 (5.7–12.2) ***	18.4 (14.2–23.6)
Education level (%)				
< High school	21.3 (18.8–24.2)	35.1 (28.0–42.8)*	18.2 (14.8–22.2)	22.2 (18.7–26.2)
High school	23.1 (20.3–26.2)	23.3 (17.3–30.7)	23.0 (20.5–25.6)	23.2 (18.8–28.3)
> High school	55.6 (51.6–59.5)	41.6 (32.2–51.8)*	58.8 (54.2–63.4)	54.6 (49.5–59.6)
Smoking (%)				
Current	21.9 (19.3–24.7)	12.6 (7.3–21.0) ***	16.6 (13.9–19.9) ***	31.1 (27.2–35.2)
Former	40.1 (37.5–42.8)	53.4 (47.1–59.6) ***	42.5 (38.5–46.6)**	33.7 (29.8–37.9)
Never	38.1 (34.8–41.5)	34.0 (29.2–39.2)	40.9 (36.0–46.0)	35.3 (31.2–39.6)
Physical activity (%)				
Sedentary	24.5 (22.0–27.2)	32.4 (25.8–39.7)**	27.8 (24.4–31.5)**	18.3 (15.5–21.3)
Light	49.4 (46.5–52.3)	51.8 (44.9–58.6)	49.1 (44.4–53.8)	49.3 (44.9–53.7)
Moderate/high	26.1 (23.4–28.9)	15.9 (11.6–21.3)***	23.1 (20.0–26.5)**	32.5 (27.9–37.4)
Alcohol intake (gm/d)	14.1 (1.0)	7.0 (1.7)**	13.6(1.1)	16.4(1.8)
BMI (kg/m^2)	28.2 (0.2)	28.0 (0.3)	28.1 (0.2)	28.2 (0.3)
Folic acid supplement user (%)	33.8 (31.1–36.6)	35.7 (29.5–42.4)	36.1 (32.2–40.1)*	30.3 (26.8–34.0)
Dietary vitamin B12 (µg/d)	6.1 (0.3)	5.2 (0.4)	6.1 (0.3)	6.2 (0.6)
Total dietary folic acid (µg/d)[2]	298.5 (9.2)	315.9 (22.2)	307.7 (11.9)	282.0 (11.9)
Dietary folate equivalents (µg/d)	757.6 (17.4)	744.4 (41.1)	774.2 (22.5)	738.8 (23.1)
Women				
n	2541	373	1117	1051
Age	57.8 (0.4)	74.0 (1.0) ***	60.1 (0.7)***	50.4 (0.4)
Race/ethnicity (%)				
Non-Hispanic white	76.6 (72.2–80.5)	83.0 (76.7–87.8) ***	84.1 (79.9–87.6) ***	66.2 (60.4–71.6)
Non-Hispanic black	9.4 (6.8–12.9)	8.1 (5.6–11.6)**	6.3 (4.2–9.3)*	13.4 (9.8–17.9)
Other race/ethnicity	14.0 (10.1–19.2)	8.9 (4.4–17.2) ***	9.6 (6.1–14.8) ***	20.4 (15.5–26.5)
Education level (%)				

< High school	23.8 (21.9–25.9)	37.7 (31.4–44.5)**	19.6 (16.1–23.5)	24.6 (21.8–27.6)
High school	25.5 (23.4–27.7)	29.7 (25.0–34.8)**	29.0 (26.0–32.2)**	20.3 (16.9–24.3)
> High school	50.7 (48.1–53.3)	32.6 (26.4–39.5)***	51.5 (47.4–55.6)	55.1 (50.6–59.5)
Smoking (%)				
Current	17.4 (15.2–19.8)	7.2 (4.5–11.2) ***	15.5 (12.7–18.7)**	22.5 (19.0–26.4)
Former	26.0 (23.1–29.1)	29.8 (23.6–37.0)	27.2 (23.5–31.2)	23.5 (19.0–28.7)
Never	56.7 (53.7–60.0)	63.0 (56.8–68.8)**	57.4 (54.1–60.6)	54.0 (50.1–57.9)
Physical activity (%)				
Sedentary	29.0 (26.1–32.1)	37.4 (30.9–44.4)	27.2 (23.3–31.5)	28.6 (24.4–33.3)
Light	55.8 (52.8–58.6)	53.3 (46.5–60.0)	55.7 (51.5–60.0)	56.6 (51.7–61.3)
Moderate/high	15.2 (13.0–17.7)	9.3 (6.3–13.6)*	17.1 (14.0–20.8)	14.8 (12.3–17.7)
Alcohol intake (gm/d)	5.2 (0.6)	1.9 (0.8)**	5.4 (0.8)	6.0 (0.9)
BMI (kg/m^2)	28.5 (0.3)	28.7 (0.6)	28.4 (0.3)	28.6 (0.4)
Folic acid supplement user (%)	41.1 (37.9–44.4)	44.5 (39.5–49.7)*	43.1 (38.5–47.9)*	37.8 (34.3–41.4)
Dietary vitamin B12 (µg/d)	4.2 (0.2)	3.8 (0.3)	4.3 (0.3)	4.3 (0.3)
Total dietary folic acid (µg/d)[2]	271.0 (7.4)	277.2 (20.5)	275.5 (10.3)	264.0 (11.4)
Dietary folate equivalents (µg/d)	659.1 (14.0)	645.9 (36.4)	667.2 (17.8)	653.8 (24.0)

[1] All values are means and SEs or percentages and 95% CIs by eGFR categories.

[2] Total dietary folic acid represents the sum of dietary and supplemental folic acid consumption.

[3] Dunnett's test and Chi-squared test were used to test the differences between eGFR categories for continuous variables and categorical variables, respectively. Difference between the eGFR categories and the reference group is statistically significant: *$p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$.

Table 4. 6 Red blood cell (RBC) and serum and folate, plasma homocysteine levels and prevalence of unmetabolized folic acid (UMFA) of NHANES 1999–2002 participants aged ≥ 40 years by eGFR categories[1]

	eGFR (mL/min/1.73 m^2)			
	< 60	**60 –< 90**	**≥ 90**	**p for trend[3]**
Men				
RBC folate (ng/ml)	361.2 (16.4)*	324.6.5 (6.9)	314.9 (5.0)	0.02
Serum folate (ng/ml)[2]	2.6 (0.03)	2.6 (0.02)	2.6 (0.02)	0.19
Plasma homocysteine (μmol/L)[2]	2.5 (0.03)***	2.3 (0.01)***	2.2 (0.02)	< 0.001
UMFA+ (%)[4]	28.8 (22.0–36.8)	25.3 (20.8–30.3)	30.9 (21.3–42.6)	--
Odds ratio[5]	0.7 (0.4–1.5)	0.7 (0.4–1.2)	1.0	--
Women				
RBC folate (ng/ml)	373.3 (12.4)*	339.0 (5.1)	333.1 (5.0)	0.21
Serum folate (ng/ml)[2]	2.7 (0.05)	2.7 (0.02)	2.7 (0.02)	0.34
Plasma homocysteine (μmol/L)[2]	2.4 (0.03)***	2.1 (0.01)***	2.0 (0.01)	< 0.001
UMFA+ (%)[4]	35.7 (30.1–41.7)	31.5 (25.3–38.3)	21.0 (14.6–29.4)	--
Odds ratio[5]	2.2 (1.2–4.0)	1.7 (0.9–3.0)	1.0	--

[1] All values, except odds ratios, are least square means and SEs or percentages and 95% CIs by eGFR categories.
[2]Significance levels were obtained at the log-transformed scales of variables. Group comparisons of folate biomarkers were performed adjusted for age, gender, race/ethnicity, smoking status, alcohol intake, BMI, physical activity, educational attainment and dietary folate equivalents. Group comparisons of plasma homocysteine were also adjusted from dietary vitamin B12. * and ***, difference between eGFR category and the reference group (eGFR ≥ 90 mL/min/1.73 m2) was statistically significant at $p < 0.05$ and $p < 0.001$, respectively.
[3] Test for trend were performed using linear regression models by including eGFR as a continuous variable.
[4] Analyses for UMFA were performed on a subsample (n=2419) of NHANES 1999–2002 participants aged ≥ 60 years who had UMFA measurements
[5] Odds ratios and 95% CIs for the presence of UMFA in blood based on eGFR categories. The eGFR ≥ 90 mL/min/1.73 m2 is the reference group.

Chapter 5: Summary and Implications

A) Summary

Fortification of enriched cereal-grain products with folic acid has significantly improved the nutritional status of folate of the general population in the U.S. However, there are safety concerns regarding high intake of folate particularly among older adults, who consume high levels of cereals and dietary supplements containing folic acid. High levels of folate biomarkers may disturb folate metabolism and results in unintended health consequences. Furthermore, there are still some gaps in our knowledge of folate metabolism. Therefore, studies are needed to address these safety concerns and knowledge gaps. In this dissertation, we first examined what we know of the key events and control points in the metabolic pathway of folate. We then examined the effects of folate intake and biomarkers on the incidence of cancer, stroke and cardiovascular disease. Finally we looked at the potential association between renal insufficiency and the levels of folate biomarkers.

In the study cohort of older adults ($\geq$ 57 years) from NHANES 1999–2002, a total of 128 cancer and 123 stroke cases were identified from the linked Medicare claims data with an average 6.3 years of follow-up.

Folate was found to have a protective effect against cancer. The highest levels of RBC folate and intake of dietary folate equivalents were associated with a significantly lower risk of cancer incidence. No significant associations between UMFA, serum folate and cancer risk were observed. We also observed that lower serum folate and RBC folate levels were associated with a higher risk of stroke and such significant associations were also independent of plasma homocysteine levels.

No significant associations between risk of stroke incidence and intake of dietary folate equivalents, food folate and total folic acid were observed. Finally, we did not find significant associations between folate intake and biomarkers and incidence of CVD.

Additional analyses were conducted to examine the impact of reduced renal function found in relatively large segments of the U.S. population and folate status. The results showed that renal insufficiency was associated with increased RBC folate and plasma homocysteine levels. RBC folate and plasma homocysteine levels were significantly higher in individuals with reduced renal function, compared to those with normal renal function, after adjustment for potential confounders. Among women but not in men, UMFA was significantly more likely to appear in the blood of individuals with reduced renal function compared to those with normal renal function.

The strength of this study is that we obtained data from the NHANES, which has information on health and nutrition status collected on a large sample of the U.S. population and these data are linked to Medicare and mortality follow-up data. Incidence of cancer, stroke and CVD were extracted from these linked files, which are not subject to recall bias. However, the results are not representative of the U.S. population because Medicare data was not available on all older participants because some of them chose managed care health insurance and their claims data are not collected by CMS.

Despite the large NHANES dataset, participants' follow-up was on average about 6 years, so cancer outcomes were on a small sample and we could not examine associations between folate and site-specific cancers. Finally, no causative

relationship between renal function and folate levels could be established because the data used were from the cross-sectional NHANES survey.

B) Implications

In addition to the U.S., 52 countries mandated fortification of enriched cereal-grain products with folic acid and more countries are discussing initiating folic acid fortification (164). Discussion of the benefits and safety concerns regarding possible harmful consequences of this fortification program require careful and continued monitoring of its effects especially in vulnerable populations, such as older population and individuals with renal insufficiency. Additionally, a petition was presented to the Food and Drug Administration to extend folate fortification to corn masa flour to target the Hispanic population which has not benefited as much in the decline in NTDs from folic acid fortification (165). Before additional fortification of food with folic acid, further studies must be conducted.

Results of this study do not show additional negative impact on cancer, stroke and CVD. But these are not definitive results and more studies are required. Additionally, for some subpopulation, such as those with renal insufficiency, it is important to examine the implication of much higher levels of folate biomarkers and until more information becomes available, caution must be taken in consumption of folic acid enriched foods and folic acid supplements among this subpopulation.

Future investigation of the association between UMFA and chronic disease in a larger study sample is needed. The hypothesis has been raised that UMFA is associated with unintended health consequences by disrupting folate metabolism and

the balance of folate and several folate-dependent enzymes (10-12, 53). This disturbance may consequently result in increased risk of certain diseases, such as cancer. Even if it is biologically plausible, the effect of chronic exposure to UMFA is not conclusive in population-based studies. Until the impact of UMFA on health is fully understood, UMFA might be used as a biomarker for monitoring folic acid exposures.

Further studies are also needed to assess the effects of high intake of folate on cancer particularly among high-risk subpopulations in post-fortification years. Folate plays essential roles in DNA synthesis and repair. Adequate folate is considered an important protective factor against cancer. However, the timing of folate supplement administration also could affect how and whether folate promotes cancer progress. High folate intake could promote the development of carcinogenesis after the onset of cancer. The population group targeted by folic acid fortification is women of childbearing age. However, individuals with existing cancer or pre-cancerous conditions also consume foods enriched with folic acid. It is essential to determine if these groups of people are at a higher risk of accelerated cancer progress.

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