Effect of a voluntary food fortification policy on folate, related B vitamin status, and homocysteine in healthy adults[1–3]

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ABSTRACT

Background: Mandatory folic acid fortification of food is effective in reducing neural tube defects and may even reduce stroke-related mortality, but it remains controversial because of concerns about potential adverse effects. Thus, it is virtually nonexistent in Europe, albeit many countries allow food fortification on a voluntary basis.

Objective: The objective of the study was to examine the effect of a voluntary but liberal food fortification policy on dietary intake and biomarker status of folate and other homocysteine-related B vitamins in a healthy population.

Design: The study was a cross-sectional study. From a convenience sample of 662 adults in Northern Ireland, those who provided a fasting blood sample and dietary intake data were examined ($n = 441$, aged 18–92 y). Intakes of both natural food folate and folic acid from fortified foods were estimated; we used the latter to categorize participants by fortified food intake.

Results: Fortified foods were associated with significantly higher dietary intakes and biomarker status of folate, vitamin B-12, vitamin B-6, and riboflavin than were unfortified foods. There was no difference in natural food folate intake (range: 179–197 μg/d) between the fortified food categories. Red blood cell folate concentrations were 387 nmol/L higher and plasma total homocysteine concentrations were 2 μmol/L lower in the group with the highest fortified food intake (median intake: 208 μg/d folic acid) than in the nonconsumers of fortified foods (0 μg/d folic acid).

Conclusions: These results show that voluntary food fortification is associated with a substantial increase in dietary intake and biomarker status of folate and metabolically related B vitamins with potential beneficial effects on health. However, those who do not consume fortified foods regularly may have insufficient B vitamin status to achieve the known and potential health benefits. *Am J Clin Nutr* 2007;86:1405–13.

KEY WORDS Food fortification, folate, folic acid, plasma homocysteine, B vitamins, intakes, biomarkers, dietary folate equivalents, adults

INTRODUCTION

Mandatory folic acid fortification of cereal grains was implemented in 1998 by the US government with the aim of reducing the incidence of neural tube defects (NTDs) in pregnancy (1). This strategy was deemed necessary because alternative policies for NTD protection, which recommended increasing folate intake periconceptionally either from natural food sources or from synthetic folic acid (as supplements or in foods fortified on a voluntary basis), were ineffective (2). Mandatory fortification has resulted not only in improved intake (3) and biomarker status (4, 5) of folate in the general population but, more importantly, in a substantial reduction in pregnancies affected by NTDs (6–8). Some governments in Europe and elsewhere are now considering similar policies (9–11). Although the primary goal of folic acid fortification is to reduce the occurrence of NTDs in women of reproductive age, the potential benefit to the general population in reducing the risk of chronic disease via homocysteine lowering is also highly relevant. Plasma total homocysteine (tHcy) is very responsive to intervention with the B vitamins required for its metabolism: folate (12) and, to a lesser extent, vitamin B-12 (13), vitamin B-6 (14), and riboflavin (15). A recent population-based study suggests that the temporal decline in stroke-related mortality in the United States and Canada coincided with the introduction of folic acid fortification (16). More importantly, a meta-analysis of clinical trials just published shows that supplementation with folic acid reduced the risk of stroke by 18% overall, by 29% in trials with a treatment duration of >36 mo, and by 25% in those trials in subjects with no history of stroke (17), which strongly suggests that either folic acid or tHcy lowering plays a role in the primary prevention of stroke.

Population-based exposure to folic acid through fortification, however, is controversial because of concerns about potential adverse effects on health. The most widely documented concern is the possibility that high-dose folic acid may mask the diagnosis and thereby delay the treatment of vitamin B-12 deficiency in older adults (18). In addition, despite considerable evidence to support a protective role of folate against cancer at a number of sites (19), there is some concern that high-dose folic acid may promote the development of colorectal cancer if precancerous

lesions or neoplasms are already established in the mucosa (20). Thus, mandatory fortification with folic acid remains virtually nonexistent in Europe. Furthermore, although many European countries allow the addition of nutrients to foods on a voluntary basis (ie, at the manufacturer's discretion), others prohibit fortification of any kind. Thus, national fortification policy varies considerably throughout the European Union (EU). The European Commission, however, is aiming in the near future to regulate in its member states the minimum and maximum amounts of vitamins and minerals allowed to be added to foodstuffs (21).

Previous studies investigated the effect of mandatory folic acid fortification on dietary intake (3) or biomarker status (4, 5) of folate, but few have examined the effect of a voluntary fortification policy, particularly in relation to B vitamin biomarkers. The objective of the present investigation, therefore, was to examine the effects of consuming foods fortified with folic acid and related B vitamins on both dietary intake and biomarker status in a healthy adult population living in a country that allows voluntary food fortification on a liberal basis and that has one of the highest rates of NTDs worldwide [11.4 per 10 000 births (22)].

SUBJECTS AND METHODS

Subject recruitment

Ethical approval was obtained from the University of Ulster Research Ethical Committee, and participants gave written informed consent. As part of a cross-sectional study funded by the UK Food Standards Agency, a convenience sample of 662 healthy adults aged ≥18 y was recruited between February 2003 and December 2005. Participants were recruited from the staff (academic, technical, and ancillary) and student population at the University of Ulster, Coleraine, Northern Ireland, and from the local community (industries, social and recreational clubs, and also for older adults, housing associations permitting independent living within a secure environment). From this cohort, those who provided data on both B vitamin intake and status were included in the current investigation ($n = 441$, age range: 18–92 y). The exclusion criteria were as follows: pregnancy; use of B vitamin supplements in the previous 6 mo; self-reported history of cardiovascular, gastrointestinal, hepatic, renal, or hematologic disease; use of medications that interfere with B vitamin metabolism (eg, methotrexate, sulfasalazine, or anticonvulsants); having visited countries with a mandatory folic acid fortification policy for a period ≥2 wk in the previous 6 mo; plasma creatinine concentrations >130 μmol/L (generally indicative of impaired renal function; Clinical Laboratory, Causeway Health and Social Services Trust, Coleraine, Northern Ireland); and a score of <25 on Folstein's Mini-Mental State Examination [a cognitive function screening test (23)] in those aged ≥60 y, because such subjects could potentially have a reduced ability to recall food intake accurately.

Dietary assessment and anthropometric data

Dietary intake was assessed with the use of a 4-d food diary (for 4 consecutive days, including Saturday and Sunday, to account for the known variation in day-to-day intake) in combination with a food-frequency questionnaire, an approach that was validated against biomarker data at our center. The food-frequency questionnaire, which was designed specifically to investigate fortified foods, requested participants to state the frequency of consumption for food groups or specific branded

products known to be fortified with B vitamins at the time of the study (eg, ready-to-eat breakfast cereals, breakfast cereal bars, breads, and fat spreads). Participants also provided details on the brand names of the products consumed so that the fortification profile of any new products could be established. By combining the 2 dietary collection methods, we were able to estimate both natural food folate intake from all sources and folic acid intake from fortified foods. Generally, when folic acid is added to a food, a number of other B vitamins are also added. Consumers of fortified foods were defined as those participants who, on average, consumed foods fortified with folic acid and other B vitamins on one or more occasions per week. Nonconsumers were classified as those participants who, on average, consumed fortified foods less than once per week.

Each participant received oral and written instructions on how to complete the food diary and food-frequency questionnaire. Any queries or discrepancies between the 2 dietary records were discussed with the participant and were clarified within 1 wk of collection to enhance the accuracy of information regarding usual intake of fortified food. Food portion sizes were estimated by the participant by using household measures and were later quantified by using published food portion size data (24). The food-composition database WEIGHED INTAKE SOFTWARE PACKAGE (WISP, version 3; Tinuviel Software, Anglesey, United Kingdom) was used to calculate mean daily energy and B vitamin intakes. This database does not enable natural food folate to be distinguished from folic acid added to foods by manufacturers, because it generates one value for total folate present in a food. Hence, we had to modify the database as follows. First, we had to customize the database to include folic acid as a new nutrient. Second, we had to generate new food codes and to enter the nutritional information for all foods identified that were fortified with folic acid and other B vitamins. Up-to-date nutritional information was obtained directly from the manufacturers when possible; otherwise, the data were obtained from nutritional labeling on the packaging. In the present study, the term folate is used to refer to the natural form found in food, and folic acid describes the synthetic form added to foods during fortification. Total folate refers to the sum of both forms present in foods.

Height and weight for each participant were measured by using portable approved scales (Seca; Brosch Direct Ltd, Peterborough, United Kingdom), and body mass index was calculated. Basal metabolic rate estimations were derived from the formulas of Schofield (25), which are based on sex and weight for the age groups 18–29, 30–59, 60–74, and ≥75 y. The ratio of energy intake (obtained from the 4-d estimated food record) to basal metabolic rate was calculated on an individual basis, and the revised Goldberg equation (26) was used to assess the validity of the dietary data and to identify those participants likely to be underreporters of energy intake.

Blood sampling and laboratory analysis

A 25-mL fasting blood sample (collected ≥12 h after an overnight fast) was obtained from each participant by a qualified phlebotomist within 10 d, either before or after the dietary record. Blood samples were collected at the University of Ulster or at the participant's home or place of work. Blood samples were collected into 4 tubes: one 8-mL EDTA-containing tube for plasma and red blood cell extraction, two 4-mL EDTA-containing tubes for preparation of red blood cell lysate and measurement of packed cell volume, and one 9-mL serum separation tube for

serum extraction. The 8-mL EDTA-containing tube was wrapped in foil and was placed on ice immediately after collection. Sample preparation and fractionation were performed within 0.5–2.5 h of the time of sampling as described in detail elsewhere (14), and fractions were stored at −70 °C for batch analysis at the end of the study and at −20 °C for extraction of DNA.

Plasma tHcy was measured by fluorescence polarization immunoassay (27), and red blood cell folate (RCF), serum folate (28), and serum vitamin B-12 (29) were measured by microbiologic assay at Trinity College Dublin. Plasma vitamin B-6 [pyridoxal-5'-phosphate (PLP)] was measured by reversed-phase HPLC with fluorescence detection (30). Riboflavin status was determined by erythrocyte glutathione reductase activation coefficient (EGRAC), a functional assay that measures the activity of glutathione reductase before and after in vitro reactivation with its prosthetic group flavin adenine dinucleotide (31). Briefly, EGRAC is calculated as the ratio of flavin adenine dinucleotide–stimulated to unstimulated enzyme activity, with values ≥1.3 generally indicating suboptimal riboflavin status. The methylenetetrahydrofolate reductase (MTHFR) 677C→T genotype was identified by polymerase chain reaction amplification followed by *Hin F1* restriction digestion (32). Plasma creatinine was measured by using a standard spectrophotometric assay with the use of a chemistry analyzer (Hitachi; Roche Diagnostics Corporation, Indianapolis, IN). The packed cell volume (required for the calculation of RCF concentration) was obtained by using an automated counter (Coulter Electronics, Hialeah, FL). Measurements of PLP, EGRAC, MTHFR genotyping, creatinine, and packed cell volume were carried out at the University of Ulster, Coleraine. For all assays, samples were analyzed blind, and quality control was provided by the repeated analysis of stored batches of pooled washed red blood cells (for EGRAC), plasma (for tHcy and PLP), serum (for vitamin B-12 and folate), and lysates of RCF covering a wide range of values. For folate status, RCF is considered a more robust marker of tissue stores

TABLE 1
Characteristics of the study participants[1]

	Men (*n* = 147)	Women (*n* = 294)	P^2
General characteristics			
Age (y)	62.0 (46.0, 71.0)[3]	61.0 (38.0, 71.0)	0.443
≥60 y (%)	61	54	0.174
BMI (kg/m²)	27.2 (25.1, 29.7)	25.7 (23.0, 29.3)	0.003
Consumer of folic acid–fortified foods (%)[4]	75	80	0.255
Current smoker (%)	9	12	0.407
MTHFR 677C→T genotype (%)			0.982
CC	43	44	
CT	46	45	
TT	11	11	
Dietary intake			
Energy (MJ/d)	8.34 (7.08, 9.88)	7.00 (5.84, 8.14)	<0.001
Total folate (μg/d)[5]	289 (210, 371)	231 (180, 308)	<0.001
Natural folate (μg/d)	209 (174, 254)	175 (145, 222)	<0.001
Added folic acid (μg/d)	50 (0, 118)	38 (16, 96)	0.023
Vitamin B-12 (μg/d)	4.0 (3.1, 5.0)	2.8 (2.1, 3.9)	<0.001
Vitamin B-6 (mg/d)	2.3 (1.9, 2.8)	1.9 (1.6, 2.3)	<0.001
Riboflavin (mg/d)	1.6 (1.3, 2.0)	1.4 (1.1, 1.8)	<0.001
Nutrient density[6]			
Total folate (μg/MJ)	32 (26, 43)	34 (27, 43)	0.803
Vitamin B-12 (μg/MJ)	0.48 (0.35, 0.60)	0.41 (0.31, 0.55)	0.012
Vitamin B-6 (mg/MJ)	0.28 (0.23, 0.33)	0.27 (0.23, 0.34)	0.878
Riboflavin (mg/MJ)	0.20 (0.15, 0.23)	0.21 (0.17, 0.25)	0.103
B vitamin status			
Red blood cell folate (nmol/L)	909 (689, 1153)	763 (595, 1013)	0.001
Serum folate (nmol/L)	21.7 (14.9, 31.7)	19.3 (13.5, 27.7)	0.118
Serum vitamin B-12 (pmol/L)	267 (193, 325)	254 (186, 336)	0.829
Plasma pyridoxal phosphate (nmol/L)	60.3 (41.3, 74.0)	56.0 (38.0, 71.8)	0.065
EGRAC	1.34 (1.26, 1.43)	1.34 (1.26, 1.46)	0.348
Plasma homocysteine (μmol/L)	10.3 (8.2, 12.5)	10.3 (8.3, 12.6)	0.707

[1] *CC,* wild type; *CT,* heterozygous; and *TT,* homozygous mutant genotype for the 677C→T polymorphism in methylenetetrahydrofolate reductase (MTHFR); EGRAC, erythrocyte glutathione reductase activation coefficient—a functional indicator of riboflavin status.

[2] Differences between the sexes were assessed by using an independent-sample *t* test on log-transformed data, when applicable. Categorical variables were assessed by using chi-square analysis. *P* < 0.05 was considered significant.

[3] Median; interquartile range in parentheses (all such values).

[4] Consumers of folic acid–fortified foods were defined as those who consumed foods fortified with folic acid at least once per week. Generally, these foods were also fortified with several other B vitamins (vitamin B-12, vitamin B-6, and riboflavin).

[5] Total folate intake comprised the intake of folate present naturally in the food and the intake of synthetic folic acid added voluntarily to foods by the manufacturers.

[6] Owing to the significant difference in energy intake between the sexes, mean daily B vitamin intakes were also expressed in terms of nutrient density.

than is serum folate; thus, RCF was used as the main indicator of folate status in the present study.

Statistical analysis

All statistical analysis was performed by using SPSS software (version 11; SPSS UK Ltd, Chersey, United Kingdom). Consumers of fortified foods were divided into tertiles on the basis of their folic acid intake to form low, medium, and high categories. For numerical variables, the differences between categories were examined by using independent sample t tests or analysis of covariance (controlling for sex) with Scheffe's post hoc test. For categorical variables, differences were examined by using chi-square tests. To compare the proportion of women of reproductive age achieving an optimal RCF status between the different fortified food categories, we used a chi-square test for trend. Correlations between variables were performed by using Pearson's correlation coefficients. For normalization purposes, variables were log transformed as appropriate before statistical analysis, and $P < 0.05$ was considered significant. A total of 25 participants had missing data for ≥ 1 marker; all other participants provided complete data on all markers of interest.

RESULTS

The characteristics of the study participants are described in **Table 1**. Participants were predominantly female (67%) and >60 y of age (57%), and >75% consumed foods fortified with

folic acid alone or in combination with other B vitamins more often than once a week. Body mass index was the only general characteristic to differ significantly between the sexes, with higher values in men. Energy and nutrient intakes of all 4 B vitamins were significantly higher in men than in women; hence, nutrient intakes were adjusted for energy. After adjustment, no differences in intakes of the B vitamins were noted except intakes of vitamin B-12, which remained significantly higher in men. The biomarker status of B vitamins was generally higher in men than in women, although the sex difference was only significant for RCF. Plasma tHcy concentrations did not differ between men and women.

Of the fortified foods identified at the time of the study, ready-to-eat breakfast cereals were the most commonly consumed food, with 63% of participants consuming these products at least once per week (data not shown). We calculated that a typical serving provided, on average, 88 μg folic acid/d, 0.3 μg vitamin B-12/d, 0.6 mg vitamin B-6/d, and 0.5 mg riboflavin/d; these estimations were based on nutritional data supplied by the manufacturers of the 5 most commonly consumed ready-to-eat cereals. The second most commonly consumed product fortified with B vitamins was fat spread, with 25% of consumers regularly eating these products. Daily intakes ranged from 5 to 50 g, in turn providing a folic acid intake of 50–500 μg/d [25–250% of the UK reference nutrient intake value (33)]. Other fortified products were generally consumed in amounts that were insufficient to make any notable contribution to overall folic acid intakes.
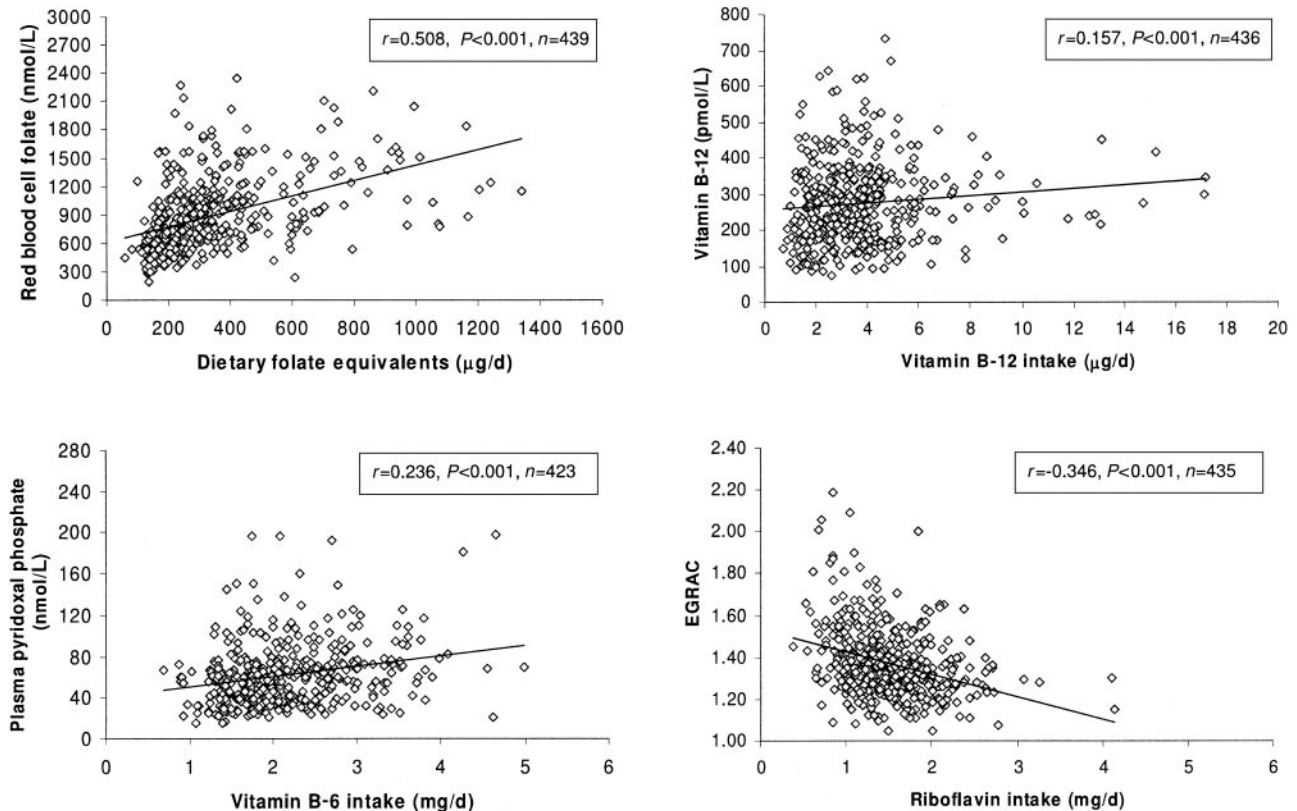

FIGURE 1. Relation between dietary intake and biomarker status of B vitamins. Correlations were carried out on log-transformed data and were calculated by using Pearson's correlation coefficients (r). Correlations for which $P < 0.05$ were considered significant. Total folate intake was expressed as dietary folate equivalents (DFEs) introduced in the United States to account for the higher bioavailability of synthetic folic acid added to food than of natural food folate; DFEs were calculated as μg natural folate plus 1.7 × μg added folic acid (34). EGRAC, erythrocyte glutathione reductase activation coefficient, a functional indicator of riboflavin status. A higher EGRAC ratio indicates a lower riboflavin status (values ≥ 1.3 generally indicate suboptimal status).

The relation between dietary intakes of each of the 4 B vitamins and the corresponding blood concentrations was examined (**Figure 1**). When folate data were reexamined separately for folic acid and natural folate intakes, the relation between intake and status was stronger for added folic acid intake (ie, from fortified food) than for natural food folate intake (**Figure 2**). As anticipated, plasma tHcy was found to be inversely related to the biomarker status of folate (RCF: $r = -0.390$, $P < 0.001$; serum folate: $r = -0.433$, $P < 0.001$), vitamin B-12 ($r = -0.406$, $P < 0.001$), and vitamin B-6 ($r = -0.244$, $P < 0.001$) (data not shown).

Data for the participants classified as nonconsumers of fortified foods (0 μg folic acid/d) were compared with data for the consumers of fortified foods, who were further subdivided into tertiles on the basis of their folic acid intake to form low, medium, and high categories (**Table 2**). Across the 4 groups, the proportion of men to women differed significantly; hence, all statistical analyses were adjusted for sex. No significant differences in age or in any other general characteristic were observed between the 4 groups. There was a graded increase in folic acid intake and consequently in RCF and serum folate status in the consumers of fortified foods, with participants in each tertile of folic acid intake having significantly higher status than did the participants of the previous category. There was no significant difference between the groups with respect to the intake of natural folate. Significant increases in dietary intakes of vitamin B-12, vitamin B-6, and riboflavin were also found with increased fortified food intake. Although this was generally reflected in higher biomarker status in consumers of fortified foods than in nonconsumers, the differences between the 3 fortified food categories were not always statistically significant. Plasma tHcy concentrations were found to be 2 μmol/L lower in consumers with higher intakes of fortified foods (middle and top tertile) than in nonconsumers. Although up to 27% of the study population had energy intake data indicative of potential underreporting, reanalysis of the data in Table 2 after the removal of possible underreporters did not change any of the outcomes (data not shown).

RCF status was examined separately in women of reproductive age (comprising 25% of the total study population) according to their intake of fortified foods (ie, nonconsumers and low, medium, and high consumers). The mean (95% CI) for RCF in each of these fortification categories was 592 (516, 667), 784 (703, 864), 815 (709, 922), and 1024 (877, 1172 nmol/L), respectively. The percentage of women with an RCF value >907 nmol/L [400 μg/L; the maternal RCF concentration that is associated with the lowest risk of having an NTD-affected pregnancy (35)] showed a significant increasing trend with increasing fortified food intake (**Figure 3**). None of the women had an RCF concentration below the cutoff for biochemical deficiency of 340 nmol/L (ie, 150 μg/L; data not shown).

DISCUSSION

The current study shows that a voluntary but liberal food fortification policy is associated with a significant increase in dietary intake and biomarker status of folate and the metabolically related B vitamins (vitamin B-12, vitamin B-6, and riboflavin) in a convenience sample of adults who were nonusers of B vitamin supplements. The higher B vitamin status in consumers of fortified foods was in turn associated with lower plasma tHcy concentrations. These findings are important because they are relevant to 2 areas of current debate: mandatory folic acid fortification of food (a worldwide concern) and the emerging legislation on the voluntary addition of nutrients to food (an EU concern).

For protection against first occurrence of NTDs, the public health recommendation in place for nearly 15 y worldwide is to take an additional 400 μg/d folic acid periconceptionally. However, evidence indicates that a supplemental folic acid intake of 200 μg/d is as effective as 400 μg/d in improving RCF status to a concentration of $\geq$907 nmol/L [400 μg/L (36)], the maternal RCF concentration associated with the lowest risk of having an NTD-affected pregnancy (35). Participants in the current study in the top tertile of fortified food intake were found to have a median folic acid intake of 208 μg/d. This intake level is comparable with the estimated increase of 190 μg/d folic acid as a result of mandatory fortification in the United States (3), a level nearly twice that originally predicted (1). Furthermore, the biomarker status of folate in these participants was similar to that in
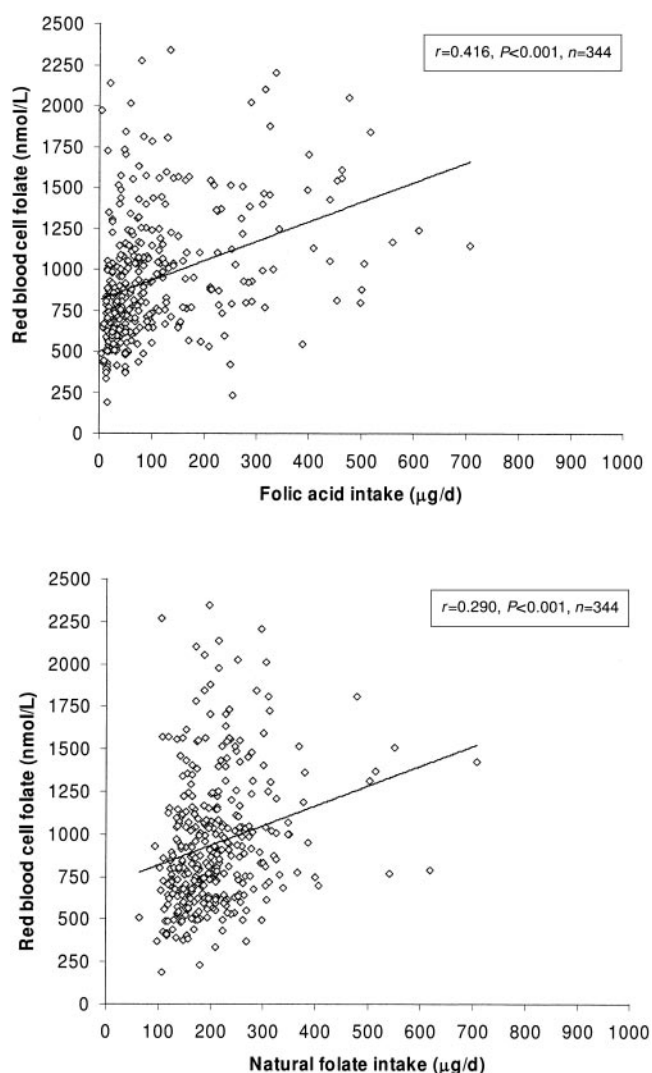

FIGURE 2. Relation between folate intake and red blood cell folate status in consumers of fortified foods according to folic acid intake and natural folate intake. Correlations were carried out on log-transformed data and were calculated by using Pearson's correlation coefficients (r). Correlations for which $P < 0.05$ were considered significant.

TABLE 2
Comparison of B vitamin intake and biomarker status of participants grouped by intake of folic acid–fortified foods[1]

	Nonconsumers (n = 97) (intake = 0 μg/d)	Low consumers (n = 111) (intake = 1–39 μg/d)	Medium consumers (n = 118) (intake = 40–98 μg/d)	High consumers (n = 115) (intake ≥99 μg/d)
General characteristics				
Age (y)	62.0 (49.0, 71.0)[2]	61.0 (40.0, 71.0)	59.0 (34.3, 69.3)	62.0 (39.0, 72.0)
≥60 y (%)	67	56	49	57
Male (%)[3]	38	21	37	37
BMI (kg/m²)	26.8 (24.2, 29.7)	26.0 (23.3, 29.3)	26.0 (23.4, 28.0)	27.2 (24.2, 30.3)
TT genotype (%)	10	15	9	11
Dietary intake				
Energy (MJ/d)	7.12 (5.91, 8.41)	7.11 (5.89, 8.54)	7.74 (6.38, 9.18)	7.77 (6.42, 8.88)
Total folate (μg/d)[4]	186 (142, 223)[a]	206 (173, 246)[a]	259 (212, 310)[b]	422 (333, 549)[c]
Natural folate (μg/d)	186 (142, 223)	179 (151, 215)	196 (150, 248)	197 (157, 238)
Added folic acid (μg/d)	0 (0, 0)[a]	25 (17, 33)[b]	60 (50, 75)[c]	208 (125, 291)[d]
Vitamin B-12 (μg/d)	2.8 (1.7, 3.8)[a]	2.7 (1.9, 3.9)[a]	3.4 (2.5, 4.3)[b]	3.9 (3.1, 4.8)[b]
Vitamin B-6 (mg/d)	1.6 (1.3, 2.0)[a]	1.8 (1.5, 2.1)[a]	2.0 (1.8, 2.5)[b]	2.8 (2.3, 3.3)[c]
Riboflavin (mg/d)	1.1 (0.9, 1.5)[a]	1.4 (1.1, 1.7)[b]	1.7 (1.4, 2.0)[c]	1.6 (1.3, 2.0)[c]
B vitamin status				
Red blood cell folate (nmol/L)	653 (532, 830)[a]	697 (564, 857)[a]	862 (680, 1082)[b]	1040 (798, 1413)[c]
Serum folate (nmol/L)	15.1 (10.0, 21.1)[a]	16.2 (11.6, 22.1)[a]	22.6 (16.7, 30.5)[b]	30.1 (21.5, 45.5)[c]
Serum vitamin B-12 (pmol/L)	239 (183, 297)[a]	234 (168, 316)[a]	287 (208, 365)[b]	276 (206, 338)[ab]
Plasma pyridoxal phosphate (nmol/L)	52.0 (36.6, 65.9)[a]	55.0 (37.9, 67.7)[ab]	60.3 (42.7, 77.3)[b]	65.3 (43.0, 77.5)[b]
EGRAC	1.38 (1.31, 1.54)[a]	1.32 (1.27, 1.44)[b]	1.32 (1.26, 1.43)[b]	1.31 (1.24, 1.43)[b]
Plasma homocysteine (μmol/L)	11.5 (9.4, 13.9)[a]	10.7 (8.9, 13.4)[a]	9.6 (7.8, 11.2)[b]	9.4 (7.7, 12.0)[b]

[1] Nonconsumers were defined as those participants who consumed foods fortified with folic acid less than once per week. Consumers of folic acid–fortified foods were defined as those who consumed foods fortified with folic acid at least once per week. Generally, these foods were also fortified with several other B vitamins (vitamin B-12, vitamin B-6, and riboflavin). Consumers were stratified into 1 of 3 groups according to tertiles of added folic acid intake. TT, homozygous mutant genotype for 677C→T polymorphism in methylenetetrahydrofolate reductase (MTHFR); EGRAC, erythrocyte glutathione reductase activation coefficient, a functional indicator of riboflavin status. Values in the same row with different superscript letters are significantly different, $P < 0.05$.

[2] Median; interquartile range in parentheses (all such values).

[3] Chi-square analysis showed a significant difference in sex distribution between the groups ($P = 0.014$). Differences were therefore adjusted for sex by using ANCOVA with Scheffes post hoc tests on log-transformed data when applicable.

[4] Total folate intake comprised the intake of folate present naturally in the food and the intake of synthetic folic acid added voluntarily to foods by the manufacturers.

populations exposed to mandatory folic acid fortification (37, 38). Thus, the current findings show the potential of a voluntary fortification policy to increase folate intake and status of adults to a similar degree as achieved in countries with mandatory fortification. Evidence from such countries proves that this strategy has substantially reduced NTDs (6–8). When we examined RCF concentrations specifically in women of reproductive age, we showed that with increasing fortified food intake there was a significant increase in the proportion of women achieving optimal folate status (ie, an RCF value >907 nmol/L; 35). Thus, it is highly likely that voluntary fortification would be effective in reducing NTD risk in women who regularly consume fortified foods. However, women of reproductive age who are nonusers of folic acid supplements and who do not consume fortified foods on a regular basis are unlikely to have sufficient folate status for optimal protection against NTDs. Few women correctly take folic acid supplements as universally recommended (39); most, in fact, rely on dietary intake to meet their folate requirements periconceptionally; however, there is considerable evidence that natural food folates have poorer bioavailability than does folic acid added to foods (40, 41). In the current cohort, folic acid was found to be a much more important determinant of RCF concentration than was natural food folate, a result that supports the US approach of setting folate recommendations on the basis of accounting for the greater bioavailability of folic acid added to food

than of natural folate, ie, expressed as dietary folate equivalents (34). Furthermore, only participants with higher fortified food intakes were able to achieve an average RCF concentration that would be considered optimal. Evidence from several European populations shows that, in the absence of mandatory folic acid fortification, public health strategies to prevent NTDs have been ineffective (22, 42). Our findings, therefore, support the view expressed by many that mandatory fortification with folic acid is the only means of ensuring that all women of reproductive age can benefit in terms of reduced risk of NTDs.

Apart from the primary aim of folic acid fortification to reduce the incidence of NTD-affected pregnancies, the potential benefit to the general population in reducing the risk of cardiovascular disease (CVD) via tHcy lowering is also highly relevant. Although a number of secondary prevention trials published recently failed to show a benefit of tHcy lowering therapy on CVD events generally (43–45), one of these showed (but largely overlooked) a significant reduction in the risk of stroke (44). Furthermore, these trials are now widely recognized as being substantially underpowered for heart disease (46). In fact, new evidence from a meta-analysis of clinical trials confirms that folic acid supplementation reduced the risk of stroke by 18% overall and by 25% in those trials in persons with no history of stroke (17). Thus, the case for folic acid or tHcy lowering in CVD is stronger for stroke than for heart disease and possibly strongest of all for the
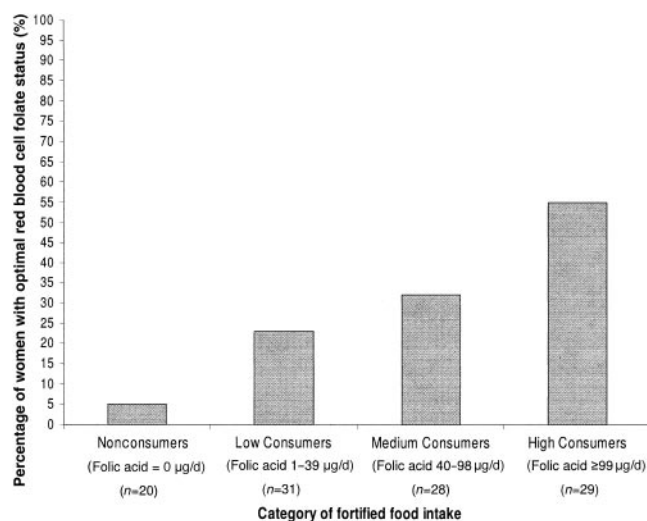
FIGURE 3. Proportion of women of reproductive age with optimal red blood cell folate status according to fortified food intake. The cutoff for optimal red blood cell folate status of women of reproductive age was taken to be ≥907 nmol/L (≥400 μg/L), which is the maternal concentration associated with the lowest risk of a neural tube defect–affected pregnancy (35). Nonconsumers were defined as those participants who consumed foods fortified with folic acid less than once per week. Consumers of folic acid fortified foods were defined as those who consumed foods fortified with folic acid at least once per week. Consumers were stratified into tertiles of added folic acid intake: low consumers (median: 25 μg/d), medium consumers (median: 60 μg/d), and high consumers (median: 208 μg/d). The values under each bar are ranges of intakes for each fortification category. Chi-square analysis for trend showed a significant difference between the 4 groups ($\chi^2 = 14.99$, $P < 0.0001$).

primary prevention of stroke. In the current study, consumers of fortified foods had plasma tHcy concentrations that were 2 μmol/L lower than those of nonconsumers of fortified foods. Furthermore, those with a median intake of 60 μg/d folic acid (middle tertile) had tHcy concentrations similar to those with intakes of 200 μg/d (top tertile), which suggests that long-term exposure to additional folic acid, even at very low doses, in combination with metabolically related B vitamins can have a substantial effect on tHcy concentrations. Although this was an observational study and therefore cannot confirm a causal relation, the higher RCF status and lower tHcy concentration observed in consumers of fortified foods were probably the direct result of fortification rather than of a generalized positive dietary pattern (such as higher fruit and vegetable intakes) in consumers of fortified foods. In support of this, we found no difference in natural food folate intakes between the different fortified food categories; only folic acid intakes were found to increase, and fortified foods are the only source of the latter. Therefore, apart from any benefit in the reduction of NTD risk in women of reproductive age, the implementation of folic acid fortification may be of potential benefit to the general population in lowering tHcy for the primary prevention of CVD, in particular stroke.

In the current study, there was considerable variation in folic acid intake in consumers of fortified foods (range: 5–708 μg/d), with no participant consuming >1000 μg/d, an amount considered by experts to be the tolerable upper intake (34). However, none of the participants in the present study took folic acid supplements, and we did not consider the potential additional folic acid intake through "overage" by manufacturers [assumed to be at a level of 20% to 32% depending on the fortified food (11)]

Available evidence is inconsistent as to whether mandatory folic acid fortification has had any adverse effects with respect to masking vitamin B-12 deficiency or neurologic damage, the major concern in relation to overexposure to high-dose folic acid (47, 48). However, any fortification strategy (either voluntary or mandatory) that includes vitamin B-12 along with folic acid could overcome this potential adverse effect for all but those with the most severe form of vitamin B-12 deficiency (ie, pernicious anemia, estimated to be present in <2% of the older population; 49). Furthermore, vitamin B-12 has been shown to be a risk factor, independent of folate status, in NTD occurrence (50, 51) and has become the main determinant of plasma tHcy in post-fortification populations (52, 53) or in subjects with optimized folate status (54). A recent expert report from the United States recommended mandatory fortification with vitamin B-12 to provide an additional intake of 1.0 μg/d (55); this happens to be the increment in vitamin B-12 intake shown in the current study to be associated with the higher fortified food intakes.

Apart from the universal interest in mandatory folic acid fortification because of its established role in preventing NTDs and its potential role in preventing CVD, the general question of harmonizing voluntary fortification policy across EU countries is also important. Current policies vary considerably, with many European countries opposed to fortification in the belief that low or deficient nutrient intakes can be rectified by changes in dietary habits alone (56). However, the current results, which show that consumers, compared with nonconsumers, of fortified foods had higher dietary intakes of not only folate but of vitamin B-12, vitamin B-6, and riboflavin, which in turn were reflected in an enhanced biomarker status of all 4 B vitamins, show that voluntary fortification may have clear benefits for European consumers not taking supplements. Our findings are consistent with those of previous studies, ie, that consumers of fortified breakfast cereals have higher dietary intakes (57–59) and a better biomarker status (59) of micronutrients than do nonconsumers of fortified breakfast cereals, and are important in the current debate concerning the regulation of the amounts of vitamins and minerals to be added to foodstuffs in European countries (21). They suggest that the availability of fortified foods throughout the EU could increase the biomarker status of B vitamins and thus offer potential health benefits to consumers.

In summary, voluntary fortification of food with folic acid can be as effective as mandatory fortification in increasing folate intake and biomarker status in consumers who choose to eat these foods regularly. However, women of reproductive age and other subgroups who do not consume fortified foods regularly are unlikely to have optimal folate status for protection against disease. Implementing a policy of mandatory folic acid fortification can be predicted not only to reduce the prevalence of NTDs, but may also contribute to the primary prevention of CVD. Including vitamin B-12 along with folic acid in any new policy could not only alleviate most of the concern regarding the masking of vitamin B-12 deficiency, but also potentially cause further reductions in both CVD and NTD rates.

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