

## ORIGINAL COMMUNICATION

# A comparison of the effect of advice to eat either '5-a-day' fruit and vegetables or folic acid-fortified foods on plasma folate and homocysteine

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**Objective:** To assess and compare the effects of natural folate (100 µg) with those of folic acid from fortified sources (100 µg/day) on plasma folate and homocysteine.

**Design:** Randomized controlled trial (parallel groups).

**Setting:** Men and women living in South Wales, UK.

**Subjects:** A total of 135 healthy individuals recruited from the local workforce and blood donor sessions. All subjects possessed the 'wild-type' CC genotype for C677T polymorphism in methylenetetrahydrofolate reductase (MTHFR).

**Interventions:** Subjects underwent one of the following dietary interventions for 4 months: (1) fortified diet—usual diet plus 100 µg/day folic acid from fortified foods; (2) natural folate diet—usual diet plus 100 µg/day folate from natural sources; (3) control—usual diet.

**Results:** The fortified group increased reported intake of folic acid from fortified foods compared to other groups ( $P < 0.001$ ) achieving an extra 98 µg/day (95% CI 88–108). The natural folate group increased reported intake of natural source folates compared with the other two groups ( $P < 0.001$ ), but achieved a mean increase of only 50 µg/day (95% CI 34–66). Plasma folate increased ( $P < 0.01$ ) by a similar amount in both intervention groups compared to controls (fortified group 2.97, 95% CI 0.8–5.1; natural group 2.76, 95% CI 0.6–4.9. Plasma homocysteine, vitamins B<sub>6</sub> and B<sub>12</sub> were not significantly changed.

**Conclusions:** Subjects achieved increases in folate intake using fortified foods more easily than by folate-rich foods, however both sources increased plasma folate by a similar amount. These levels of intake were insufficient to reduce homocysteine concentrations in MTHFR CC homozygotes, but may be more effective in other genotypes.

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### Introduction

Sub-optimal folate status is associated with increased risk of neural tube defects (NTD; Medical Research Council, 1991), cardiovascular disease (CVD; Boushey *et al*, 1995) and cancer (Kim, 1999). Although a causal link has been proven only for NTD (Medical Research Council, 1991), possible health benefits of folate at all stages of the life cycle have stimulated much research into optimal folate nutrition. Plasma homocysteine is inversely related to blood measures of folate and is therefore a responsive marker of folate status (Selhub *et al*, 1993). Epidemiological studies show an association between elevated homocysteine and both NTD and CVD (Stone *et al*, 1999).

Dietary folates occur as natural folates in green leafy vegetables, citrus fruits, liver and dairy products and as added synthetic folic acid in fortified food products. The major function of folates *in vivo* is the transfer of single carbon units, particularly for DNA synthesis and homocysteine remethylation. Homocysteine is regulated by two pathways: transsulfuration catalysed by cystathionine- $\beta$ -synthase with co-factor pyridoxal phosphate (vitamin B<sub>6</sub>) and remethylation catalysed by methionine synthase with co-factor vitamin B<sub>12</sub> and substrate 5-methyltetrahydrofolate (Finkelstein, 1998). Folate is the strongest nutritional predictor of homocysteine concentrations (Boushey *et al*, 1995).

Methylenetetrahydrofolate reductase (MTHFR) converts 5,10-methylenetetrahydrofolate to 5-methyltetrahydrofolate which is required for remethylation of homocysteine. A common mutation in MTHFR (C677T) is present in the homozygous form in approximately 12% of the Caucasian population (Clark *et al*, 1998) and is associated with raised homocysteine concentrations especially when folate intake and plasma folate are low (Harmon *et al*, 1996). Homozygotes for the 'wild-type' C allele (CC genotype) comprise approximately half the general population.

In the UK and EU the reference nutrient intake for folate is 200  $\mu$ g/day for adults (Department of Health, 1991; Commission of the European Community, 1993). It is recommended that women intending pregnancy consume an additional 400  $\mu$ g/day to protect against NTD. Limited success of strategies to increase folate intakes during pregnancy and the possible relationship between folate and chronic disease have stimulated interest in population interventions to increase folate status.

Cross-sectional studies have shown dietary folate intake to be positively associated with plasma folate and inversely with plasma homocysteine (Tucker *et al*, 1996). However, most successful dietary intervention studies have investigated synthetic folic acid either as a supplement or a food fortificant (Brouwer *et al*, 1999a; Dierkes, 1995). Natural folate intervention studies have reported limited success due to lack of compliance with dietary advice, destruction of natural food folates during processing and poor bioavailability (Sauberlich *et al*, 1987; Cuskelly *et al*, 1996). Importantly, Cuskelly *et al* demonstrated that the additional 400  $\mu$ g/day folate intake required for pregnancy could not be achieved using natural source folates alone. Large increases in natural folate intake have been reported, but were achieved under conditions which could not be generally applied to free-living individuals (Brouwer *et al*, 1999b). Low-dose natural folate strategies would be easier to achieve and may produce important increases in folate status in the general population.

Folate intake may be enhanced through other widely publicised health messages such as the 5-a-day fruit and vegetable recommendation of the World Health Organization (WHO, 1990). In the UK, the average daily intake of fruits and vegetables falls short of this target (Department of Health, 2000a). Our pilot data from 85 healthy volunteers

indicated mean intakes of 2.3 portions/day of fruit and vegetables in this region of South Wales. An increase to five portions per day would raise natural folate intake by approximately 100  $\mu$ g/day and may be more easily achieved at the population level than the higher folate doses previously investigated. Alternatively, folate intake may be increased using foods fortified with folic acid. We have previously demonstrated that a diet enhanced with fortified foods (~200  $\mu$ g/day extra folate) significantly increases plasma folate and reduces plasma homocysteine (Ashfield-Watt *et al*, 2001). Low-dose natural and fortified folate approaches have not been directly compared. This study sought to investigate the relative efficacy of 100  $\mu$ g/day folate from natural sources (fruit and vegetables) compared with 100  $\mu$ g/day folic acid from fortified foods in terms of changes in folate status.

## Methodology

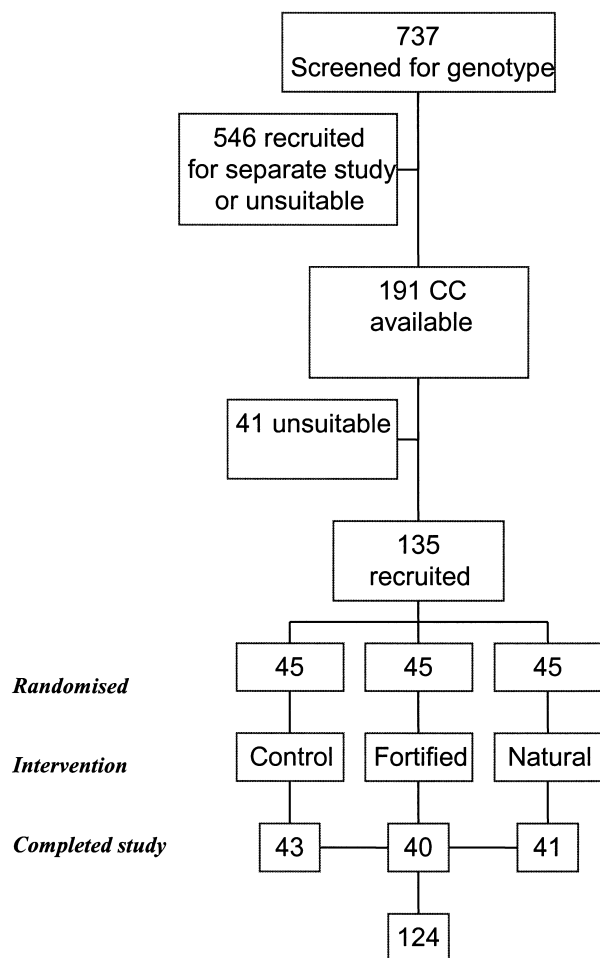
Subjects were healthy men and women who were of the 'wild-type' CC genotype for the MTHFR C677T polymorphism. These were individuals who had previously been screened for, but not recruited to a study of the effect of C677T variant MTHFR genotype on response to folic acid interventions (reported elsewhere, Pullin *et al*, 2001; Ashfield-Watt *et al*, 2002). Buccal cell samples were collected at the screening sessions for determination of MTHFR genotype. The study was approved by Bro Taf Research Ethics Committee. All subjects gave informed, written consent.

Subjects were eligible to participate in the study if they fulfilled the following criteria: (1) non-smoking; (2) no history of cardiovascular disease or epilepsy; (3) aged between 18 and 65; (4) not taking supplements containing vitamins B<sub>6</sub>, B<sub>12</sub> or folic acid; (5) not taking any drugs known to interfere with folate metabolism, eg methotrexate, bile acid sequestrants; and (6) women not pregnant nor planning pregnancy during the timeframe of the study.

Subjects were allocated to treatment groups which were balanced with respect to age and gender using a randomization scheme produced by the study statistician (RGN). A total of 135 subjects were assessed at baseline (dietary assessment and venepuncture) and randomly allocated to one of three dietary interventions. Subjects were monitored regularly throughout the intervention period and were reassessed at clinic after 4 months. The study profile is illustrated in Figure 1.

## Dietary assessment and interventions

Folate intake was assessed using a semi-quantitative food frequency questionnaire. This questionnaire was developed from the questionnaire of Yarnell *et al* (1983) which had been validated in this area of South Wales. This questionnaire was modified to include questions on specific folate rich foods such as spinach, Brussels sprouts and other green vegetables, on folate-rich fruit and on vegetable extract spreads, eg Marmite. In total 32 foods were relevant to estimating total folate intakes. Subjects were asked to



**Figure 1** (a) Folic acid diary. (b) Guidelines given to subjects. This example applies to the natural folate diet.

report how often they ate each of these foods (no. of days per week, fortnightly, monthly/rarely). Frequency of intake of folate-rich plus frequently consumed folate poor foods (eg dairy products, meats, unfortified breads) was multiplied by standard average portion sizes (Crawley, 1993) and then by the folate content derived from UK nutrient composition tables to provide folate intake data (Holland *et al*, 1991).

Data on fortified foods were obtained directly from manufacturers and supermarkets and updated regularly. In the UK an overage (additional amount of fortificant added during manufacture to maintain vitamin activity at the level stated on the packet during processing and storage) is added to baked products such as breads. No overage is added to cereal products. We have found in previous studies that fortified cereals are more widely available and therefore the more popular means of increasing folic acid intake. This means that our assessment of folic acid from fortified sources

is unlikely to underestimate the amount of folic acid consumed by subjects in this trial.

Subjects underwent one of three possible dietary interventions for a 4 month period: (1) control diet—subjects were advised to eat their normal diet throughout the study period and asked to pay particular attention to maintaining their usual intake of folic acid-fortified products; (2) fortified diet—subjects were advised to eat an extra 100 µg/day folic acid from fortified food products including fortified cereals and breads and not to exceed this amount; (3) natural folate diet—subjects were advised to eat an extra 100 µg/day folate from natural sources, particularly folate-rich fruit and vegetables and to maintain their usual intake of fortified products.

Due to the amount of variation in folic acid fortification levels both within and between brands, care was taken to assess accurately the baseline folate intake from fortified sources of all subjects. For this reason, a detailed history of cereal intake over the previous month was taken in order to calculate the average baseline folate intake from this source. Subsequently, subjects randomized to control or natural intervention were required to maintain baseline intakes of folic acid-fortified foods throughout the study. Subjects in the fortified group were advised to increase folic acid intake by 100 µg/day (10 points) and not to exceed this amount. All subjects were provided with a comprehensive list of the folate scores derived from information from cereal and bread manufacturers. This enabled subjects to vary their intake of cereal and bread types whilst following the dietary advice given at baseline. The scores were derived by calculating the amount of folate/folic acid per average portion (µg/day), rounding to the nearest 10 µg and then dividing by 10. Each active intervention therefore required an increase of 10 points (100 µg) per day.

### Monitoring compliance

To help understand the dietary changes required, all subjects were given a 2 week folate diary at the baseline visit which they completed and returned in a pre-paid envelope. To monitor compliance throughout the study, further food diaries were sent to subjects after 2 months and then 2 weeks before their final visit. Subjects who did not return charts or whose charts indicated poor compliance were contacted by the study nutritionist and encouragement given. The food diary consisted of a chart on which subjects recorded the number of folate points scored from the various food groups over a 2 week period. The folate scores from a wide range of folate-rich foods and frequently eaten moderate folate sources were printed on the reverse of the chart (Figure 2).

All subjects were asked to complete charts during the first 2 weeks of the study to familiarize subjects with the required dietary changes. However, subjects undertaking the natural folate intervention recorded their *usual* folate intake for the first week of the first chart in order to gauge their baseline folate intake. During the second and subsequent weeks they

(a)

Ref: 5-a-day

Name \_\_\_\_\_ Survey No \_\_\_\_\_ Date \_\_\_\_\_

Food item	Sun	Mon	Tue	Wed	Thur	Fri	Sat	Sun	Mon	Tue	Wed	Thur	Fri	Sat
Bread														
Cereal														
Green vegetables														
Carrots														
Beans														
Potatoes														
Other vegetables														
Fruit														
Fruit juice														
Milk, yoghurt, cheese														
Meat														
Bovril/Marmite														
<b>TOTALS</b>														

(b)

There are two main sources of Folic Acid in the diet: some commonly available foods e.g. breads and cereals have been fortified with folic acid, other foods e.g. certain fruits and vegetables contain good amounts of it naturally. Over the next four months we would be grateful if you could enrich your current diet with naturally folate rich foods. Each day you should aim to achieve an extra 100µg folic acid (10 points). This may be achieved by eating 3 extra portions of fruit and vegetables each day. In order to make accurate comparisons, it is important that your folic acid intake from other sources remains relatively constant over the next 4 months. Please do not consume any extra folic acid fortified foods. If you currently eat cereal and you wish to change the brand or type of cereal, please ensure that the new one contains a similar level of folic acid to your old one. If you do not currently eat a cereal and you wish to have one, please have one which contains no added folic acid. Please do not eat folic acid fortified bread unless you currently do so. In order to quantify your folic acid intake, please complete the chart overleaf by referring to the list below for folate scores for individual foods. It may be useful for you to complete the first week of the chart before you change your diet in order to give you an idea of your current intake. The second week of the chart may be completed to help you to modify your diet to achieve the extra dietary folate. A separate sheet of cereal scores will be provided.

**Vegetables**

- Broccoli, 3 spears (135g) = 9 points
- Sprouts, 9 (90g) = 10 points
- Cabbage, small portion (60g) = 2 points
- Carrots, 2 small (60g) = 1 point
- Cauliflower, 9 florets (90g) = 5 points
- Green beans, medium portion (90g) = 5 points
- Peas 2.5 tbsp (70g) = 3 points
- Potatoes, 2 large (120g) = 3 points
- Spinach, 2tbsp (90g) = 8 points
- Sweetcorn, 3tbsp (85g) = 1 point
- Lettuce, 4 small leaves (20g) = 1 point
- Tomato, 1 small = 1 point
- Baked beans, 4tbsp (160g) = 4 points
- Spring greens, medium portion (95g) = 6 points
- Dried, boiled blackeye beans, 2tbsp (60g) = 13 points
- Broad beans, 2tbsp (120g) = 4 points

**Bread**

- 1 slice white = 0.5 points
- 1 slice brown/wholemeal = 1 point

**Fruits**

- Banana, medium (100g) = 2 points
- Grapefruit, ½ (80g) = 2 points
- Orange, medium (160g) = 5 points
- Orange juice, average glass (160g) = 3 points

**Miscellaneous**

- Milk 1/3 pint (200ml) = 1 point
- Yoghurt (150g) = 2 points
- Bovril, 1 cup (9g) = 9 points
- Marmite, scrape on bread (1g) = 1 point
- Cheese (30g) cheddar = 1 point
- Cheese (30g) low fat = 2 points
- Cottage cheese (30g) = 1 point

**Meats**

- Beef or lamb (120g) = 2 points
- Chicken (120g) = 1 point
- Liver (120g) = 36 points
- Fish (120g) = 1 point
- Pork (120g) = 1 point

Figure 2 (a) Folic acid diary. (b) Guidelines given to subjects. This example applies to the natural folate diet.

aimed to achieve on average 10 points more per day than during week 1. The changes in fortified foods were easier for subjects to quantify, therefore subjects were not required to maintain baseline intakes during the first week of the study.

**Sample collection**

Following an overnight fast, venous blood was collected into EDTA vacutainers for determination of plasma folate and plasma total homocysteine (Hcy). Samples for measurement

of pyridoxal phosphate and vitamin B<sub>12</sub> were collected into lithium heparin vacutainers. Blood samples were centrifuged within 10 min and the plasma stored at  $-70^{\circ}\text{C}$  until assayed.

Samples were thawed and analysed in batches to ensure that pre- and post-intervention samples for subjects were analysed together to reduce assay variation. Plasma homocysteine was measured by enzymatic immunoassay and plasma folate and vitamin B<sub>12</sub> by competitive protein binding methods using an Abbot IMX instrument (between batch CVs 5.3, 9.3 and 4.0%). Plasma folate results were converted from  $\mu\text{g}/\text{l}$  to  $\text{nmol}/\text{l}$  using a conversion factor of 2.265, based on the formula weight of folic acid.

Plasma pyridoxal phosphate (PLP) was measured as previously described (Bailey *et al*, 1999), with the following modifications: 100  $\mu\text{l}$  of the cyanide derivatives were injected onto the HPLC column (APEX ODS 3  $\mu\text{m}$  (25  $\text{cm}\times 4$  mm). PLP was eluted isocratically at a flow rate of 1 ml/minute using a 2M acetate buffer, containing 1 mM heptane-sulphonic acid, adjusted to pH 3.75 with potassium hydroxide, and detected fluorometrically at an excitation and emission wavelengths of 325 and 418 nm (between batch CV 5.1%).

### Genotyping

Heteroduplex analysis involving DNA extraction from buccal cells, polymerase chain reaction (PCR) and electrophoresis was used for MTHFR genotyping (Clark *et al*, 1998). The

buccal cells were obtained from the inner lining of the cheek using a 'cyto-brush'.

### Statistical methods

The primary statistical test was analysis of covariance (ANCOVA) comparing treatment levels between the three groups using the corresponding pre-treatment value as covariate. Skewed variables which were log-normal (vitamins B<sub>6</sub> and B<sub>12</sub>) were log-transformed before applying parametric tests. Bonferroni correction was applied to multiple comparisons as appropriate. The relation between variables at baseline was assessed using Pearson's correlation co-efficient for normally distributed and log-normal variables. Stepwise regression analysis was used to describe the relationship between homocysteine and B group vitamins. The effect of interventions is reported in the text relative to changes in the control group. Changes reported in the table are the absolute increments calculated in each group as the difference between pre- and post-treatment values.

## Results

### Baseline characteristics

The three dietary groups were balanced with regard to primary variables (see Table 1). Each group comprised 16 males and 29 females. The mean (s.d.) ages for the three

**Table 1** Baseline and follow-up dietary and biochemical parameters

	Dietary group	Baseline	Follow-up	Change <sup>a</sup>	Percentage change <sup>b</sup>
Natural folate ( $\mu\text{g}/\text{day}$ )	Control	197 (67)	186 (65)	-11 (44)	-5.6
	Fortified	195 (50)	203 (67)	7 (53)	4.3
	Natural	200 (58)	251 (60)*	50 (51)	25.2
Fortified folate ( $\mu\text{g}/\text{day}$ )	Control	43 (41)	50 (45)	5 (23)	15.0
	Fortified	47 (37)	144 (49)*	98 (32)	203.7
	Natural	43 (37)	55 (52)	11 (33)	28.8
Total folate ( $\mu\text{g}/\text{day}$ )	Control	241 (88)	236 (89)	-7 (52)	-1.8
	Fortified	242 (63)	347 (91) <sup>†</sup>	104 (68)	43.3
	Natural	243 (74)	306 (85) <sup>‡</sup>	62 (64)	25.9
Plasma folate (nmol/l)	Control	25.9 (7.6)	25.2 (7.8)	-0.9 (4.3)	-2.6
	Fortified	25.7 (7.7)	27.6 (7.7) <sup>§</sup>	2.1 (6.2)	7.1
	Natural	26.1 (7.3)	27.8 (6.5) <sup>§</sup>	1.7 (5.2)	6.8
Plasma hcy ( $\mu\text{mol}/\text{l}$ )	Control	9.0 (2.8)	9.0 (2.7)	-0.1 (1.4)	0.2
	Fortified	9.3 (2.5)	8.8 (2.3)	-0.6 (1.4)	-5.5
	Natural	9.8 (4.3)	8.8 (2.9)	-0.9 (2.7)	-10.1
Plasma PLP (nmol/l)	Control	49.9 (30.7)	53.1 (36.7)	2.4 (20.1)	6.5
	Fortified	46.1 (22.5)	46.9 (21.2)	0.8 (16.9)	1.9
	Natural	41.1 (15.5)	43.5 (18.4)	0.4 (14.0)	6.0
Vitamin B <sub>12</sub> (nmol/l)	Control	347 (144)	348 (167)	3 (73)	0.4
	Fortified	326 (142)	336 (162)	9 (70)	3.2
	Natural	318 (125)	341 (135)	15 (45)	7.4

\*Significantly different from other interventions,  $P < 0.001$ .

<sup>†</sup>Significantly different from natural folate group,  $P < 0.01$ .

<sup>‡</sup>Significantly different from control,  $P < 0.001$ .

<sup>§</sup>Significantly different from control,  $P < 0.05$ .

<sup>a</sup>Change is calculated as absolute change from baseline for each dietary group.

<sup>b</sup>Percentage change from baseline to post intervention, mean values.

Data are presented as mean (s.d.). hcy, homocysteine; PLP, pyridoxal phosphate.

groups were: control 41.2 (11.2), fortified 41.1 (11.6) and natural 41.8 (11.3). The estimated mean (s.d.) total folate intake was 242 µg/day (95% CI 230–255). Eighteen percent of total folate intake was from fortified sources (45 µg/day 95% CI 38–51). Dietary folate intake was moderately correlated with plasma folate at baseline ( $r=0.32$ ,  $P<0.001$ ,  $n=133$ ). Plasma homocysteine was significantly and inversely correlated with plasma folate ( $r=-0.45$ ,  $P<0.001$ ,  $n=128$ ) and log vitamin B<sub>12</sub> ( $r=-0.27$ ,  $P=0.002$ ,  $n=128$ ). There was no significant correlation with log pyridoxal phosphate ( $r=0.04$ ,  $P=0.69$ ,  $n=128$ ).

A regression model was fitted to baseline plasma homocysteine which included plasma folate, log B<sub>12</sub>, log pyridoxal phosphate and pyridoxic acid as predictor variables. Of these, only plasma folate and B<sub>12</sub> significantly predicted homocysteine concentrations. The regression co-efficients for predictor variables were: plasma folate  $-0.449$ ,  $P<0.001$ ; log B<sub>12</sub>  $-4.060$ ,  $P=0.01$ , log pyridoxal phosphate  $0.92$ ,  $P=0.547$ , pyridoxic acid  $0.08$ ,  $P=0.073$ . Plasma folate was the strongest predictor, explaining 19.8% of the variability of homocysteine. Adding vitamin B<sub>12</sub> to the model increased the predictive power to 22.9%.

#### Changes in dietary intake following intervention

Dietary and biochemical data are presented in Table 1. Subjects in the fortified group reported greater increases in folic acid intakes from fortified foods compared to either control (difference 93 µg/day, CI 80–106) or the natural folate group (difference 87 µg/day, CI 73–100),  $P<0.001$  for both. There was a small, non-significant increase (6.7 µg/day) in folate intake from fortified sources in the natural folate group compared to controls.

Subjects in the natural folate group reported significantly greater natural folate intakes compared with either the fortified group (difference 45 µg/day, 95% CI 24–65) or controls (difference 62 µg/day, 95% CI 42–83),  $P<0.001$ .

Total dietary folate intake (the sum of fortified and natural folates) increased significantly in both intervention groups compared with controls ( $P<0.001$  for both), but the increase in total folate in the fortified group was significantly greater than in the natural folate group (difference 43 µg/day, 95% CI 16–70,  $P<0.01$ ).

Both interventions significantly raised plasma folate concentrations compared with controls ( $P=0.01$ ). The change in plasma folate was similar in both intervention groups (fortified group 2.97 nmol/l, 95% CI 0.8–5.1, natural folate group 2.76 nmol/l, 95% CI 0.6–4.9).

There was no statistically significant effect of intervention on plasma homocysteine ( $P=0.65$ ). Compared with controls, the fortified and natural folate interventions reduced homocysteine by 0.47 and 0.61 µmol/l, respectively. There were no significant differences in effects on plasma B<sub>6</sub> or B<sub>12</sub> concentrations between groups at follow-up.

#### Discussion

Plasma concentrations of homocysteine, folic acid and the other B group vitamins measured were similar to those reported elsewhere for healthy subjects in Britain (Chambers *et al*, 2000) and Europe (Graham *et al*, 1997) with plasma folate being the strongest predictor of homocysteine concentrations. Vitamin B<sub>12</sub>, but not B<sub>6</sub>, was weakly predictive of homocysteine concentrations. This is in keeping with other studies which have indicated a stronger relationship between B<sub>6</sub> and post-methionine load homocysteine concentrations than with fasting homocysteine concentrations (Boushey *et al*, 1995).

Subjects had dietary folate intakes which were similar to reported national average values, 242 vs 238 µg/day (Department of Health, 2000a). Although less than half (47%) of the subjects ate cereals fortified with folic acid at baseline, this intervention group complied well with advice to increase folate intake from fortified sources and reported no difficulties. The target of 100 µg extra folic acid daily was generally achieved with a mean increase in the fortified group of 98 (s.d. 32) µg/day.

Subjects in the natural folate group reported more difficulty in attaining the target level of intake. Only 13% achieved the target 100 µg/day extra natural food folates. Several of those unable to reach the target already had high intakes of fruit and vegetables at baseline. However, some subjects may have achieved greater folate intakes than indicated by the dietary folate estimate as nutrient composition data are not available on the wide range of exotic fruits now available. Also, changes to portion sizes are difficult to detect using the structured format of the food frequency questionnaire. Diet charts indicated greater compliance in some subjects than did food frequency questionnaire estimates. The difficulty experienced by subjects in this study, however, reflects the small response to government policies to increase fruit and vegetable intakes (Cox *et al*, 1996) and limited success of natural dietary interventions intended to achieve larger increases in dietary folate intake (>100 µg) in free-living, 'unsupervised' subjects (Cuskelly *et al*, 1996).

We observed a significant increase in natural folate intake, albeit short of the target, which was associated with a significant increase in plasma folate. This finding supports the promotion of fruit and vegetables as valuable sources of natural folate. The similar change in plasma folate in both intervention groups was unexpected given the widely reported poorer bioavailability of natural source folates compared with folic acid. The bioavailability of folate has been more widely studied than that of many other micronutrients, but remains contentious. Sauberlich *et al* estimated that natural folates have only approximately 50% of the bioavailability of folic acid, although more recently, Brouwer *et al* estimated that the relative bioavailability of natural folates compared to synthetic folic acid is much higher (98%; Brouwer *et al*, 1999b). These authors suggest that their higher estimate may be due to the source of natural folate in their study (citrus fruit and vegetables) compared with the

wider range of foods used in the former study. The almost exclusive use of fruit and vegetables to increase folate intake in the current study may have produced an increase in the more bioavailable folates.

Both interventions increased plasma folate by approximately 3 nmol/l (95% CI approximately 1–5) compared with controls, a change which we consider to be real given the high degree of subject matching at baseline. Two other studies which have investigated the effect of similar levels of folic acid fortified foods on plasma folate and homocysteine have reported conflicting results. Malinow *et al* (1998) reported a 3.8 nmol/l increase following intervention with fortified cereal providing 127 µg/day folic acid which produced a non-significant 0.5 µmol/l reduction in homocysteine concentrations in patients with coronary artery disease. These results are very similar to the effects which we observed in healthy subjects in the current study. However, Jacques *et al* reported a much greater increase in plasma folate (12 nmol/l) and significant reduction in homocysteine (0.7 nmol/l) in a group of subjects assessed following early implementation of mandatory fortification regulations in the USA compared with a group of subjects assessed before mandatory fortification was implemented (Jacques *et al*, 1999). Fortification in the USA is estimated to increase folic acid intakes by approximately 100 µg/day (Institute of Medicine, 1998).

Methodological differences between these studies complicates comparison. The cross-over study of Malinow *et al* used a 5 week intervention period which may have been too short to attain new steady state folate concentrations. Schorah *et al* (1998) reported significant changes in plasma folate after 4 weeks of folic acid supplementation and in homocysteine after 8 weeks. The current study had a much longer intervention period (4 months) and therefore should have been adequate for equilibration of cellular folate stores. The larger effect observed in the study of Jacques *et al* may indicate that the average increase in dietary folate intakes in the USA as a result of mandatory fortification is greater than the estimated 100 µg. This has been suggested by other authors (Rader *et al*, 2000; Lewis *et al*, 1999).

It has been suggested that folate and homocysteine responses to folate interventions depend on baseline concentrations (Graham *et al*, 1997). Ward *et al* (1997) suggest 'a threshold of plasma homocysteine in terms of ability to respond to folic acid'. They observed that there was no response to folate interventions in those with the highest baseline plasma folate and lowest homocysteine concentrations. The results reported here are in keeping with a concentration-dependent effect.

A further important factor which is likely to have influenced folate and homocysteine responses in the current study was the genetic composition of the group. Subjects for this study were CC 'wild-type' homozygotes for the C677T MTHFR polymorphism. It is widely accepted that subjects with the CC genotype have the highest plasma folate and lowest homocysteine concentrations of the three

MTHFR genotypes. TT homozygotes occupy the extreme opposite positions while CT heterozygotes have intermediate concentrations (Harmon *et al*, 1996; Jacques *et al*, 1996). We have recently shown that, in subjects drawn from the same population as the current study, MTHFR genotype modulates the homocysteine lowering response to folate enhancing interventions with TT homozygotes responding most (Ashfield-Watt *et al*, 2002). This study, which comprised equal numbers of subjects with each MTHFR C677T genotype ( $n = 42$  of each), supports previous observations made in cohorts where only a small number of TT homozygotes were studied (Malinow *et al*, 1997; Nelen *et al*, 1998). Fohr *et al* (2002) have recently reported that subjects with the MTHFR TT genotype have a greater response to synthetic folates than other genotypes, but did not include a dietary intervention in their study. The gene frequency of C677T variant MTHFR in South Wales is 0.32, giving genotype frequencies of 12% TT, 40% CT and 48% CC (in keeping with other Caucasian populations; Clark *et al*, 1998). This is in contrast to the current study where the frequency of the CC genotype was 100%. Therefore, a greater reduction in homocysteine can be expected in the general population because of the greater number of TT homozygotes.

The level of fortification of staple foodstuffs recommended in the UK by the Committee on Medical Aspects of food (COMA) for health reasons is 240 µg/100 g flour product which is estimated to produce an increase of 201 µg/day folic acid intakes generally (Department of Health, 2000b). While it is unlikely that this level of extra folate intake could be achieved by increasing natural dietary folate, it is encouraging that achievable increases from natural folate sources can significantly increase folate status. Such measures are therefore complementary to the current situation of voluntary fortification of cereal products and promote health by providing a wide range of other nutrients.

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