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General Article

GENERAL ARTICLE

The dihydrofolate reductase 19-bp deletion modifies the beneficial effect of B-vitamin therapy in mild cognitive impairment: pooled study of two randomized placebo-controlled trials

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Abstract

Background: Higher serum homocysteine is associated with cognitive decline in older people. But homocysteine-lowering trials including folic acid (FA) show inconsistent results on cognitive decline. The reduction of FA to dihydrofolate by dihydrofolate reductase (DHFR) is slow in humans.
Objective: We examined the effects of the *DHFR* 19-bp *deletion/insertion* (*del/ins*) polymorphism on FA-containing treatment on cognitive decline and brain atrophy in older people with mild cognitive impairment (MCI).
Methods: This study used pooled data from two randomized B-vitamin trials on 545 MCI subjects who received either FA-containing B vitamins or placebo for 24 months. Subjects were typed for the *DHFR* genotype. Primary outcome was the Clinical Dementia Rating scale-global score (CDR-global). Secondary outcomes were CDR-sum of boxes score (CDR-SOB), memory and executive *Z*-scores and whole brain atrophy rate by serial MRI.
Results: The proportions of subjects with *del/del*, *del/ins* and *ins/ins* genotype were 29.5, 44.3 and 26.1%, respectively. *DHFR* genotypes modified the effects of B vitamins on CDR-global, CDR-SOB and executive function *Z*-score ($P_{interaction} = 0.017$, 0.014 and 0.052, respectively), with significant benefits being observed only in those with *ins/ins* genotype (Beta = −1.367, −0.614 and 0.315, $P = 0.004$, 0.014 and 0.012, respectively). The interaction was not significant for memory *Z*-score and whole brain atrophy rate. Notably, the supplements only slowed brain atrophy in members of the '*ins/ins*' group who were not using aspirin. *Conclusions:* Our data indicate that the beneficial effects of B vitamins including FA on cognitive function are only apparent in those with *ins/ins* genotype, i.e. relatively better preserved DHFR activity.

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Introduction

Elevated total homocysteine (tHcy) in the circulation is a strong modifiable risk factor for Alzheimer's disease and vascular dementia, and prospectively, tHcy is associated with cognitive decline, dementia, white matter damage and brain atrophy (1). Folate and vitamin B_{12} are both required for homocysteine remethylation and are main determinants of tHcy status (2). Hence much effort has been made to examine the effectiveness of folic acid (FA) with or without vitamins B_{12} and B_6 on cognitive function in older people. The FACIT trial reported that 3-year FA supplementation decreased plasma tHcy concentration by 26% and significantly improved domains of cognitive function including memory, information processing speed and sensorimotor speed (3). In three randomized trials with 400 μg/d FA supplementation, the enrolled older people with mild cognitive impairment (MCI) showed improvement in cognitive performance after 6–24 months (4–6). In the Homocysteine and B vitamins in cognitive impairment (VITACOG) trial, brain atrophy and cognitive decline in the older people with MCI was slowed significantly after 24-month supplementation with FA plus vitamins B_{12} and B_6, and the treatment response was more pronounced in those with higher baseline tHcy (7,8). However, some other trials of FA-containing B-vitamin intervention failed to show significant cognitive benefits despite having lowered tHcy concentration (9–12).

Such inconsistencies may be explained by differences in study designs, e.g. doses of FA, subject selection, status of other related nutrients like omega-3 fatty acids (13). But gene/nutrient interaction may also affect the outcome of trials of FA. Distinct from natural folates, FA is a synthetic oxidized form of folate with no direct biological function in humans. Dihydrofolate reductase (DHFR), primarily in the liver, is the only enzyme that catalyzes the reduction of FA to dihydrofolate and then to bioactive tetrahydrofolate which (together with its one-carbon derivatives) performs the physiological functions of folate. The first step of reduction in humans is extremely slow and rate-limiting (14). Besides, the 19-bp deletion polymorphism in *DHFR* gene is associated with altered DHFR enzymatic activity and compromised folate status and metabolism: Kalmbach *et al.* (15) found that subjects with *del/del* genotype had increased unmetabolized FA in plasma and decreased folate in red blood cells. Unmetabolized FA may also impair folate transport into the brain (14,16). Philip *et al.* (17) reported that the 19-bp deletion polymorphism in *DHFR* modifies the association between folate and memory in a cross-sectional study.

We therefore hypothesized that *DHFR* 19-bp deletion polymorphism has a significant interaction effect on the effects of FA-containing supplements in cognitive function and brain atrophy in older people with MCI. In order to test this hypothesis, we performed a pooled analysis of two similarly designed randomized trials of FA-containing supplements in older people with MCI.

Results

This pooled analysis included 545 older subjects with MCI (266 from the VITACOG trial in the United Kingdom (UK) and 279 from the trial in Hong Kong (HK) Special Administrative Region). The participant flow is shown in Fig. 1. In total 461 (84.6%) subjects completed the 24-month follow-up. Of these, 262 subjects had volunteered for serial brain MRI scans at both baseline and follow-up (168 from the UK trial and 94 from the HK trial).

Table 1 shows the baseline clinical characteristics and laboratory data of subjects with *DHFR* 19-bp *deletion/insertion* (*del/ins*) genotypes ('*del/del*', '*del/ins*' and '*ins/ins*') in UK and HK. There were no significant differences in clinical, biochemical and cognitive profile between *DHFR* genotypes in both trial sites.

In Table 2, the estimated B-vitamin effects on serum tHcy, holotranscobalamin (holoTC, active vitamin B_{12}), folate and 5-methyltetrahydrofolate (5-MTHF, the downstream bioactive form of folate) of the three genotypes in UK and HK were compared. In both trials, FA-containing B-vitamin supplementation effectively lowered tHcy concentration in each genotype. The differences in tHcy-lowering and serum folate increase in the three genotypes were not significant, but both trials showed a similar trend that the '*ins/ins*' group had the highest folate increase with FA-containing supplements, followed by the '*del/del*' and '*del/ins*' group (325.9, 238.1 and 224.8% increase in the UK trial, $P = 0.567$; 83.5, 75.5 and 57.6% increase in the HK trial, $P = 0.484$). However, among the subjects in the HK trial, the increase of downstream 5-MTHF was significantly different between *DHFR* genotypes with a consistent trend in folate increase (364.3, 311.8 and 174.6% increase in the '*ins/ins*', '*del/del*' and '*del/ins*' group, $P = 0.012$). Serum 5-MTHF increased significantly more with FA-containing supplements in the '*ins/ins*' group than in the '*del/ins*' group after 12 months [Bonferroni corrected *P*-value ($P_{Bonferroni}$) = 0.039 (0.013 × 3), data not shown].

Table 3 shows the results of interaction analyses of *DHFR* genetic polymorphism on the effect of FA-containing supplementation on cognitive function and whole brain atrophy rate. There were significant interaction effects of *DHFR* genotype ('*del/del*', '*del/ins*' and '*ins/ins*') for changes in Clinical Dementia Rating scale-global score (ΔCDR-global) and CDR-sum of boxes score (ΔCDR-SOB) and for Δexecutive function Z-scores with borderline significance ($P_{interaction} = 0.017$, 0.014 and 0.052, respectively; data not shown). The significance was predominantly from interaction with *DHFR* ('*del/ins*' vs. '*ins/ins*'; Beta = −1.509, −0.699 and 0.418, $P_{Bonferroni} = 0.030$, 0.012 and 0.066 for ΔCDR-global, ΔCDR-SOB and Δexecutive function Z-score, respectively); meanwhile, FA-containing supplements tended to interact with *DHFR* ('*del/del*' vs. '*ins/ins*'). The interaction was not significant for Δmemory Z-scores or whole brain atrophy rate. As we recently reported a significant negative interaction effect between aspirin and FA-containing B vitamins in cognitive functioning (12), we performed interaction analyses by splitting into aspirin users and non-users, and found the interaction effects in CDR-global and CDR-SOB to be more pronounced among aspirin non-users (for '*del/ins*' vs. '*ins/ins*': Beta = −2.327 and −0.848, $P_{Bonferroni} = 0.003$ and 0.015, respectively; for '*del/del*' vs. '*ins/ins*': Beta = −1.822 and −0.723, $P_{Bonferroni} = 0.051$ and 0.069, respectively), whereas the interaction effects disappeared among aspirin users (data not shown).

Table 4 shows the group differences in changes in cognitive function and whole brain atrophy rate in the '*del/del*', '*del/ins*' and '*ins/ins*' groups. Among those with the *ins/ins* genotype, as compared with placebo group subjects, active group subjects had significantly more favorable cognitive changes of ΔCDR-global (Beta = −1.367, $P = 0.004$), ΔCDR-SOB (Beta = −0.614, $P = 0.014$) and Δexecutive function Z-scores (Beta = 0.351, $P = 0.012$). In addition, the supplementation decreased whole brain atrophy rate in the '*ins/ins*' group with borderline significance (Beta = −0.318, $P = 0.052$). In participants not using aspirin, the effects of FA-containing supplementation in the *ins/ins* group became more pronounced in ΔCDR-global and ΔCDR-SOB (Beta = −2.293 and −0.826, $P = 0.001$ and 0.014, respectively), as well as in the reduction of whole brain atrophy rate, which now became significant (Beta = −0.520, $P = 0.004$). There was no significant effect of

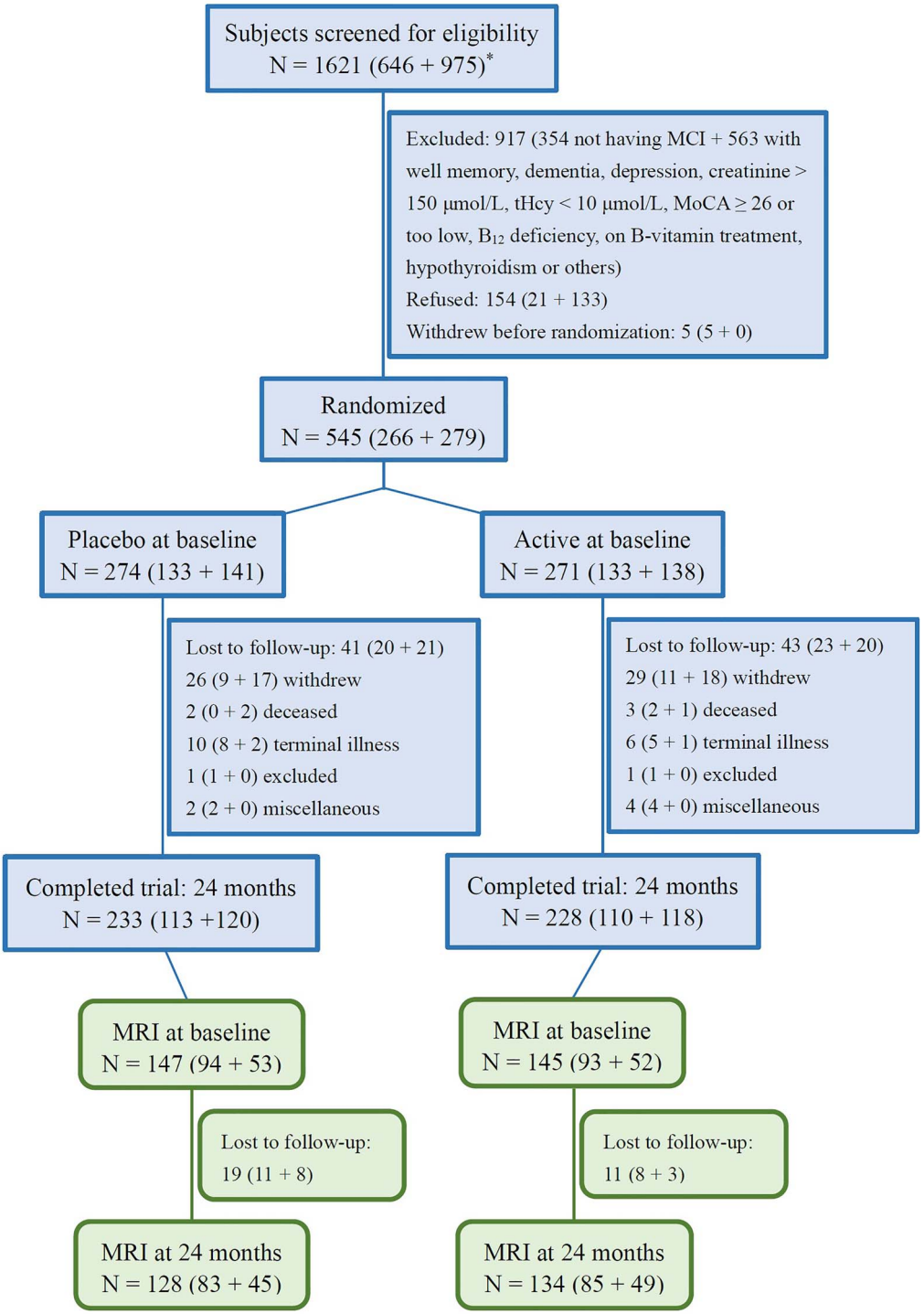

Figure 1. Trial design and participant flow of the UK and HK pooled study. ∗ Data were shown as '*N* = total number (UK + HK)'.

aspirin use in any of the responses to supplementation in the '*del/del*' and '*del/ins*' group.

Discussion

In this pooled analysis, we found that the *DHFR* 19-bp deletion polymorphism had significant interaction effects with FA-containing B-vitamin supplementation in older people with MCI. Thus, FA-containing supplementation improved cognitive function over a 2-year period only among those without *DHFR* 19-bp deletion allele (i.e. *ins/ins* genotype). After excluding the influence of aspirin use, another potential interaction factor with FA-containing B-vitamin use (7,12), the favorable cognitive benefits became more pronounced, and the decrease in whole brain atrophy rate became significant among those with the *ins/ins* genotype.

Table 1. Description of the UK and HK populations at baseline by *DHFR* genotype ('*del/del*', '*del/ins*' and '*ins/ins*')

	UK			HK			*P*-value[§]	
	Del/del (*N* = 53)	*Del/ins* (*N* = 117)	*Ins/ins* (*N* = 96)	*Del/del* (*N* = 103)	*Del/ins* (*N* = 117)	*Ins/ins* (*N* = 42)	P_{UK}	P_{HK}
Clinical characteristics								
Age, years	77.1 ± 4.7	76.6 ± 4.8	76.9 ± 5.2	77.7 ± 5.3	76.8 ± 5.4	78.0 ± 5.5	0.846	0.310
Female, *n* (%)	32 (60.4%)	76 (65.0%)	62 (64.6%)	40 (38.8%)	47 (40.2%)	20 (47.6%)	0.835	0.609
Years of education, years	14.9 ± 3.6	14.3 ± 3.4	14.5 ± 3.2	6.1 ± 4.5	7.3 ± 5.2	6.7 ± 4.4	0.546	0.253
BMI, kg/m^2	25.4 ± 3.6	26.6 ± 4.2	25.8 ± 3.9	24.6 ± 3.2	24.8 ± 3.2	24.5 ± 3.5	0.146	0.862
Ever smoking, *n* (%)	20 (37.7%)	60 (52.2%)	45 (46.9%)	31 (30.1%)	38 (32.5%)	10 (23.8%)	0.218	0.576
Aspirin user, *n* (%)	16 (30.2%)	41 (35.0%)	33 (34.4%)	26 (25.2%)	24 (20.5%)	7 (16.7%)	0.817	0.477
HBP, *n* (%)	41 (77.4%)	85 (72.6%)	68 (70.8%)	66 (64.1%)	81 (69.2%)	26 (61.9%)	0.689	0.598
DM, *n* (%)	2 (3.8%)	4 (3.4%)	8 (8.3%)	40 (38.8%)	35 (29.9%)	10 (23.8%)	0.280	0.158
Stroke, *n* (%)	6 (11.3%)	15 (13.3%)	11 (11.5%)	5 (4.9%)	6 (5.1%)	4 (9.5%)	0.901	0.457
Blood biochemistry								
Hb, g/dl	13.8 ± 1.2	13.8 ± 1.3	13.7 ± 1.2	13.5 ± 1.4	13.5 ± 1.4	13.9 ± 1.2	0.936	0.346
MCV, fL	91.7 ± 4.1	92.6 ± 4.6	92.8 ± 4.5	89.9 ± 6.5	88.9 ± 8.8	91.6 ± 6.1	0.332	0.144
TG[#], g/l	1.3 (0.9, 1.7)	1.3 (1.0, 1.7)	1.3 (0.8, 1.7)	1.2 (0.8, 1.6)	1.2 (0.9, 1.5)	1.1 (0.9, 1.7)	0.793	0.991
TC, mmol/l	5.6 ± 0.8	5.5 ± 1.2	5.4 ± 1.1	4.7 ± 1.0	4.6 ± 1.1	4.8 ± 0.9	0.365	0.384
Cr, μmol/l	97.1 ± 19.5	97.0 ± 16.3	96.6 ± 15.3	86.9 ± 20.0	92.6 ± 28.1	86.6 ± 21.5	0.978	0.494
tHcy[#], μmol/l	11.2 (9.6, 13.6)	11.5 (9.7, 13.2)	10.9 (9.5, 13.8)	16.5 (14.7, 19.8)	16.9 (14.2, 20.7)	15.6 (13.5, 19.4)	0.885	0.459
Folate[#], nmol/l	23.8 (14.3, 45.7)	19.0 (12.5, 35.6)	22.1 (13.3, 36.6)	26.2 (22.9, 34.7)	28.4 (21.2, 34.5)	25.4 (21.1, 32.6)	0.397	0.337
HoloTC[#], pmol/l	71.0 (54.5, 95.5)	70.0 (50.0, 89.0)	65.5 (48.3, 91.8)	89.3 (60.9, 124.0)	77.5 (54.4, 105.5)	102.0 (61.5, 128.0)	0.850	0.093
Cognitive function								
CDR-global = 0, *n* (%)	15 (28.3%)	39 (33.3%)	25 (26.0%)	16 (15.5%)	18 (15.4%)	3 (7.1%)	0.495	0.366
CDR-SOB	0.91 ± 0.77	0.86 ± 0.73	0.91 ± 0.81	1.33 ± 0.87	1.23 ± 0.89	1.42 ± 1.18	0.884	0.469
Executive function	−0.04 ± 0.98	0.12 ± 1.01	−0.12 ± 0.99	−0.07 ± 0.91	0.13 ± 1.07	−0.14 ± 0.99	0.219	0.212
Memory	−0.09 ± 1.04	0.05 ± 1.00	−0.01 ± 0.99	−0.07 ± 1.04	0.11 ± 0.94	−0.12 ± 1.08	0.705	0.280
MMSE	28.1 ± 1.7	28.2 ± 1.7	28.2 ± 1.7	25.6 ± 3.0	26.2 ± 3.0	25.4 ± 3.6	0.932	0.251

Data were shown as 'mean ± SD', 'median (Q1, Q3)' or '*n* (%)' as appropriate.
[§]Comparison between the *DHFR* genotypes ('*del/del*', '*del/ins*' and '*ins/ins*') in UK trial (P_{UK}) and HK trial (P_{HK}), respectively.
[#]Use log-transformed data for comparison.
BMI, body mass index; HBP, high blood pressure; DM, diabetes mellitus; Hb, hemoglobin; MCV, mean corpuscular volume; TG, triglycerides; TC, total cholesterol; Cr, creatinine; tHcy, total homocysteine; HoloTC, holotranscobalamin (active vitamin B_{12}); CDR-global, Clinical Dementia Rating scale (CDR)-global score; CDR-SOB, CDR-sum of boxes score; MMSE, Mini-Mental State Examination.

The Chinese subjects in the HK trials had significantly lower prevalence of *DHFR ins/ins* genotype as compared with Caucasians in the UK's VITACOG trial (36.1% vs. 15.9%, $P < 0.001$). This is consistent with the report by http://asia.ensembl.org/index.html (rs70991108), that the frequency of *ins* allele and *ins/ins* genotype are 37.1 and 13.8% for East Asian, and 54.9 and 30.1% for non-Finnish Europeans. This implies that the potential beneficial effects of FA may be lower in Chinese than in Caucasians. Indeed the UK trial showed a significant improvement in executive function with FA-containing B vitamins while the HK trials showed no significant effect in any cognitive outcomes (8,12). One should therefore bear in mind this gene/nutrient interaction when interpreting results of FA trials.

The clinical, biochemical and cognitive status at baseline were not significantly different between DHFR genotypes (*ins/ins*, *del/del* or *del/ins*). In particular, the baseline serum concentrations of folate, active vitamin B_{12} and tHcy were comparable between the *DHFR* genotypes. This suggested that subjects with DHFR deletion allele could still obtain adequate folates from dietary sources. Indeed, folates that naturally occur in the diet consist of a complex mixture of reduced tetrahydrofolate [monoglutamates and polyglutamates of pteroic acid and its derivatives; (18)], thus dietary folate could bypass the rate-limiting step of DHFR catalysis of FA to DHF (14).

The extent of tHcy-lowering was not significantly different between the genotypes. But circulating tHcy concentration is predominantly determined by liver and kidney folate homeostasis, therefore not necessarily reflecting folate transport and intracellular folate metabolism (19).

Consistent with the reported impairment of the function of DHFR enzyme with *DHFR* 19-bp deletion polymorphism (15), our data reaffirmed that subjects without deletion allele (*ins/ins* genotype) tended to have greater increase in 5-MTHF upon FA supplementation. 5-MTHF is the predominant active metabolite after FA intake, and is also the primary circulating bioactive folate [accounts for ~98%; (20)]. It is transported across membranes into tissues and across the blood–brain barrier into the brain, and is used at cellular level for homocysteine regulation, cysteine cycle and methionine synthesis (21). Therefore, a greater increase in this active folate may confer more cognitive benefits from FA in those with *ins/ins* genotype.

In addition, the subdued increase in serum 5-MTHF upon supplementation in subjects with deletion allele (especially *del/ins* genotype) implies that more FA might have remained unmetabolized, even though the extent of tHcy-lowering did not differ significantly between genotypes. There have been concerns about the detrimental effects of unmetabolized folic acid [UMFA; (16,19,22)]. In a study of the US National

Table 2. Concentration change (%) of circulating tHcy and B vitamins in the UK and HK trials by *DHFR* genotype ('*del/del*', '*del/ins*' and '*ins/ins*')

Δ (%)	Placebo			Active			*P*-value	
	Del/del ($N^{\dagger}$ = 29, 55)	*Del/ins* ($N^{\dagger}$ = 64, 56)	*Ins/ins* ($N^{\dagger}$ = 40, 20)	*Del/del* ($N^{\dagger}$ = 24, 48)	*Del/ins* ($N^{\dagger}$ = 53, 61)	*Ins/ins* ($N^{\dagger}$ = 56, 22)	P_1	P_2
ΔtHcy [#]								
UK	11.4 (3.4, 18.9)	9.7 (−1.9, 19.4)	6.0 (−4.1, 14.4)	−18.9 (−32.9, −11.3)	−20.0 (−33.8, −8.7)	−24.0 (−32.9, −11.6)	0.301	0.907
HK	0.2 (−18.5, 13.4)	−4.4 (−12.4, 12.5)	−4.2 (−17.8, 26.0)	−37.2 (−47.5, −29.6)	−33.2 (−44.2, −24.4)	−36.1 (−52.5, −23.7)	0.984	0.366
ΔFolate [#]								
UK	0.6 (−24.0, 74.1)	1.8 (−22.2, 30.9)	−3.5 (−35.7, 43.0)	238.1 (46.4, 544.9)	224.8 (124.2, 552.7)	325.9 (127.2, 614.6)	0.747	0.567
HK	−3.8 (−23.8, 12.5)	−6.3 (−18.3, 16.9)	−8.3 (−29.8, 28.9)	75.5 (35.1, 115.9)	57.6 (31.1, 123.1)	83.5 (26.0, 139.6)	0.887	0.484
Δ5-MTHF [#,§]								
UK	/	/	/	/	/	/	/	/
HK	22.5 (−29.1, 49.3)	2.4 (−34.1, 29.9)	−5.6 (−37.7, 83.4)	311.8 (157.4, 433.8)	174.6 (75.5, 348.1)	364.3 (203.2, 562.6)	0.452	0.012*
ΔHoloTC [#]								
UK	12.7 (−1.8, 37.1)	0.0 (−23.1, 25.1)	0.0 (−17.1, 12.1)	108.1 (64.7, 351.9)	227.8 (117.8, 292.7)	214.3 (129.5, 319.2)	0.114	0.253
HK	0.0 (−16.0, 8.1)	0.0 (−13.1, 18.8)	0.0 (−19.3, 19.1)	39.3 (11.6, 102.1)	56.7 (17.5, 114.4)	23.1 (0.0, 77.2)	0.542	0.153

[#]Data were shown as median (Q1, Q3) of percentage of concentration change (Δ, %).
[†]N = number of subjects in the UK and HK trials, respectively.
[§]Δ5-MTHF was concentration change between baseline and month 12 (not month 24) in subjects from the HK trial only.
*$P < 0.05$, P_1: *DHFR* '*del/del*' versus '*del/ins*' versus '*ins/ins*' in the placebo group, P_2: *DHFR* '*del/del*' versus '*del/ins*' versus '*ins/ins*' in the active group.

Table 3. Interaction of B-vitamin supplementation and *DHFR* 19-bp deletion polymorphism ('*del/del*', '*del/ins*' and '*ins/ins*')

Outcomes	Interaction of B vitamins and *DHFR* genotype					
	B vitamins and *DHFR* ('*del/del*' versus '*ins/ins*')		B vitamins and *DHFR* ('*del/ins*' versus '*ins/ins*')		B vitamins and *DHFR* ('*del/del*' versus '*del/ins*')	
	Beta	*P*-value[#]	Beta	*P*-value[#]	Beta	*P*-value[#]
ΔCDR-global	-0.967	0.375	-1.509	0.030*	0.542	0.990
ΔCDR-SOB	-0.562	0.096	-0.699	0.012*	0.138	1.000
ΔExecutive function	0.395	0.138	0.418	0.066	-0.022	1.000
ΔMemory	0.012	1.000	0.120	1.000	-0.108	1.000
Brain atrophy rate	-0.086	1.000	-0.230	0.714	0.144	1.000

[#]Bonferroni corrected *P*-values (original value × 3) with those over 1 shown as 1.000.
*$P < 0.05$.

Table 4. Subgroup analyses of the effects of B-vitamin supplementation with respect to the *DHFR* 19-bp deletion polymorphism ('*del/del*', '*del/ins*' and '*ins/ins*')

	Del/del		*Del/ins*		*Ins/ins*		*P*-value		
	Placebo (*N* = 84)	Active (*N* = 72)	Placebo (*N* = 120)	Active (*N* = 114)	Placebo (*N* = 60)	Active (*N* = 78)	P_1	P_2	P_3
ΔCDR-global	↓ 8 (11.6%) ↔ 52 (75.4%) ↑ 9 (13.0%)	↓ 11 (18.3%) ↔ 46 (76.7%) ↑ 3 (5.0%)	↓ 17 (18.3%) ↔ 68 (73.1%) ↑ 8 (8.6%)	↓ 12 (12.9%) ↔ 73 (78.5%) ↑ 8 (8.6%)	↓ 7 (14.9%) ↔ 32 (68.1%) ↑ 8 (17.0%)	↓ 19 (32.2%) ↔ 35 (59.3%) ↑ 5 (8.5%)	0.290	0.471	0.004*
ΔCDR-SOB	0.21 ± 0.93	0.10 ± 1.04	0.06 ± 0.73	0.19 ± 0.96	0.65 ± 1.73	0.04 ± 0.88	0.690	0.355	0.014*
ΔExecutive function	0.03 ± 0.89	−0.06 ± 0.74	−0.02 ± 0.84	−0.20 ± 0.77	−0.21 ± 0.81	0.01 ± 0.75	0.550	0.649	0.012*
ΔMemory	−0.13 ± 0.99	−0.06 ± 0.96	−0.08 ± 0.88	−0.08 ± 0.83	−0.19 ± 0.80	−0.09 ± 0.90	0.348	0.864	0.433
Brain atrophy rate	1.01 ± 0.68	0.70 ± 0.64	0.85 ± 0.58	0.76 ± 0.61	1.12 ± 0.80	0.77 ± 0.59	0.142	0.351	0.052

Δ, Cognitive change: follow-up minus baseline; ↓, decreased (improvement); ↔, unchanged; ↑, increased (cognitive decline).
*P-value < 0.05, P_1: placebo versus active in *DHFR* '*del/del*', P_2: placebo versus active in *DHFR* '*del/ins*' and P_3: placebo versus active in *DHFR* '*ins/ins*'.

Health and Nutrition Examination Survey (1999–2002) where there was a mandatory FA fortification policy, Morris *et al.* (22,23) found that the presence of detectable serum UMFA (in ~33% of the participants) was related to poorer cognitive test performance, whereas serum 5-MTHF was associated with better performance. There is evidence that UMFA could interfere with the endothelial cell uptake and cerebral transport (blood-to-brain) of circulating 5-MTHF *in vivo* (16,19). Although UMFA may only appear in the circulation for a short period [1–2 h; (24)], there is evidence that circulating UMFA may persist longer with sustained FA supplementation (25).

It is unexpected subjects with *del/ins* genotype had even less serum 5-MTHF increase with FA supplement than those with *del/del* genotype. However, such trend between *DHFR* genotypes was consistent with that of folate increase in both trials, as well as the more marked interaction effects of the *del/ins* group (vs. *ins/ins* group) than the *del/del* group in global cognitive functioning. In the Framingham Offspring Study in the USA, among those with high estimated FA intake (>500 μg/day) from fortified foods, only subjects with *del/del* genotype had increased prevalence of high fasting plasma unmetabolized FA, whereas there were no significant differences in red blood cell folate among the three genotypes (15). So far how the 19-bp deletion in intron-1 of the *DHFR* gene alters the regulation of DHFR activity especially upon FA supplementation is not well understood. More mechanistic studies are required to shed light on how this genetic polymorphism influences the cognitive benefits of FA, and indeed the safety of FA-fortified foods.

Another unexpected finding was that placebo group subjects with *ins/ins* genotype appeared to have more cognitive decline than those with *del* allele(s). In contrast, a cross-sectional study reported worse cognitive function in older people with high serum folate and *DHFR 19-bp del/del* as compared with other two genotypes (17). It is biologically not plausible for normal DHFR function to be a cause of cognitive decline. A more likely explanation for this unexpected finding is that the *ins/ins* had more underlying neurodegeneration at baseline.

In the HK trial, a negative interaction between aspirin and FA-containing B vitamins in cognitive functioning was found, and the VITACOG trial also found a borderline significant negative interaction effect of aspirin on the effects of FA-containing B vitamins in brain atrophy (7,12). We have therefore examined the potential confounding influence of aspirin use on our results. First, the proportions of aspirin users were similar between the *DHFR* genotype groups in both trials. Second, after excluding aspirin users, the interaction between FA-containing B vitamins and *DHFR* genotype in cognitive functioning remained significant and became even more pronounced. Notably, the slowing of brain atrophy by the B-vitamin supplements was significant only in participants not using aspirin and who had the *ins/ins* genotype. This suggests that DHFR may be involved in the interaction between aspirin and FA.

The main limitation of this study is that it was a pooled analysis of two clinical trials in different populations and with differences in clinical characteristics. The FA dose used in the HK trial was also lower than that in the VITACOG trial. We have tried to overcome this limitation by comparing between the genotypes in the two trials separately, and adjusting for research site in regression models. The *Z*-scores of memory and executive function were determined in each trial site separately. The analysis for serum tHcy was performed in the same laboratory.

In conclusion, our findings indicate the *DHFR* 19-bp deletion polymorphism has significant interaction effects on the cognitive response to FA-containing supplements in older people with MCI, in that those with *ins/ins* genotype were significantly more likely to improve in cognitive function than those with *del* allele(s). Given that a sizable population carrying the *del/del* or *del/ins* genotype may not gain significant benefit from FA-containing B-vitamin supplements, future trials of folate supplementation for cognitive improvement should consider using alternative forms of folate (e.g. folinic acid or 5-MTHF) which do not require reduction by DHFR.

Materials and Methods

This study used pooled data of two randomized controlled trials (RCTs), including the VITACOG trial (ISRCTN94410159) in the UK and another trial in HK Special Administrative Region [Chi CTR-TRC-13003302; (7,8,12)]. The similarities in the design of the VITACOG (UK) and the HK trials justified pooling of the data: 1) the MCI diagnosis for participants in both trials was made according to Petersen's criteria (26,27); 2) subjects in the UK and HK trials had comparable age at baseline (76.8 ± 4.9 and 77.4 ± 5.3, respectively) and both were randomized in 1:1 to the placebo or active treatment group; 3) both RCTs used FA-containing B vitamins that lower tHcy concentration with the aim of slowing cognitive decline and brain atrophy in older MCI people; 4) the intervention period was 24 months in both trials and 5) both trials examined the effect on cognitive functioning using the CDR scale and shared common cognitive test data for executive function, and both had serial volumetric brain MRI scans to estimate whole brain atrophy rate. Both trials were conducted according to the principles of the Declaration of Helsinki and approved by local ethics committees. The active treatment group in the VITACOG trial received daily 800 μg FA, 500 μg cyanocobalamin and 20 mg vitamin B_6; in the HK trial the treatment was 400 μg FA and 500 μg methylcobalamin.

The primary outcome was the CDR scale for global cognitive functioning (CDR-global), which is determined by an algorithm of six domain scores (memory, orientation, judgment and problem solving, community affairs, home and hobbies and personal care), and ranged 0–3 with 0.5 indicating an intermediate state between normal cognitive function and dementia (labeled as 'Questionable dementia'), 1 or more indicating clinical dementia (12). CDR-SOB is calculated by summing each of the domain scores and ranged 0–18 (12). Category Fluency Test was used to assess executive function (and semantic memory) in both trials. Episodic memory was assessed by Hopkins Verbal Learning Test–delayed recall (HVLT-DR) in the VITACOG trial and International Shopping List Test (ISLT) in the HK trial (8,12). The raw test scores were standardized into *Z*-scores based on the baseline mean and standard deviation (SD) of the respective trial population, with higher scores indicating better performance (12,28).

In a subgroup of 262 subjects with serial volumetric brain MRI scans (baseline and 24 months), whole brain atrophy rate (%/year) was estimated using SIENA package by calculating percentage brain volume change (PBVC) per year (29). Change in cognitive function was taken as the delta *Z*-score (Δ, post minus pre). ΔCDR-global, categorized into decreased (ΔCDR-global < 0), unchanged ($= 0$) and increased (> 0), was the primary outcome. ΔCDR-SOB, Δexecutive function, Δmemory and annual whole brain atrophy rate were the secondary outcomes.

DNA was extracted using the Wizard DNA Purification Kit (Promega, Southampton, UK) from 266 participants of the

VITACOG trial and extracted with the standard phenol/chloroform method from 279 participants of the HK trial, respectively (7,8,12). Genotype of the *DHFR* 19-bp deletion/insertion polymorphism was determined using the allele-specific PCR as described before with a 7300 RT-PCR system from Applied Biosystems (15,17,30). The following PCR primers were used: forward primer (5′-TCGCTGTGTCCCAGAACATG-3′) and reverse primer (5′-AGCGCAGACCGCAAGTCTG-3′) (15,17). Two TaqMan TAMRA probes from Applied Biosystems were used to target the 19-bp deletion.

Blood was collected without fasting in the VITACOG trial and after an overnight fast in the HK trial. The archived serum in the HK trial was sent to the same laboratory as in the UK trial (7,8) for serum creatinine and sulphur containing amino acids, including homocysteine, which were measured by liquid chromatography–mass spectrometry (LC–MS) as described before (31). Serum folate, holoTC (active vitamin B_{12}) and lipid profile were analyzed at Department of Pharmacology at University of Oxford in the UK and at Department of Chemical Pathology, Prince of Wales Hospital in HK separately (7,8,12).

Serum concentrations of 5-MTHF in the HK trial were determined at Department of Nutrition, University of Oslo by LC–MS/MS using a sample volume of 50 μL and a deuterium labelled isotope as an internal standard (Levomefolic Acid-13C,d3). Ascorbic acid is used to stabilize FA. Cold isopropanol was used for protein precipitation. The extracts were evaporated at 30°C and reconstituted in water. LC–MS/MS was performed using a Shimadzu LC-20ADXR LC system coupled to a Sciex QTRAP5500 mass spectrometer with Turbo V ion source and TurboIonspray probe. Separation of the analytes was achieved using a Accucore Phenyl Hexyl column (100 × 2.1 mm, 2.6 μm) with an aqueous solution of formic acid [0.4%] and methanol + formic acid [0.4%] gradient mobile phase. Positive mode multiple reaction monitoring was used for detection. Linear calibration curves of the peak area ratios of analyte and internal standard in water were used for quantification.

Statistical analysis

Data were presented as 'mean ± SD', 'median [quartile 1 (Q1), Q3]' or 'frequency (%)' as appropriate. Distributions of tHcy, folate, vitamin B_{12} and triglyceride concentration were all skewed and were log-transformed to improve normality before further analysis. For baseline comparison, independent Student's *t*-test was used for continuous variables, and Chi-square test (χ^2, or Fisher exact test) was used for categorical variables. Paired *t*-test was used for comparison between baseline and year two follow-up. The interaction analysis of FA-containing B-vitamin supplementation and *DHFR* genotype was performed using general linear regression model or ordinal regression model with adjustment for age, sex, years of education, body mass index (BMI), smoking status, history of diabetes and stroke at baseline and study site (from UK/HK trial). All statistical analyses were two-sided and *P*-value < 0.05 was considered to be significant (7,8,12,28). Bonferroni correction was considered as appropriate for multiple comparisons. All analyses were performed with SPSS 26.0 for Windows (IBM Corp., Armonk, NY, USA).

Supplementary Material

Supplementary Material is available at *HMG* online.

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Conflict of Interest Statement. YW, NEB, TK, ADS, HR. None declared.

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