Effect of supplementation with B vitamins and antioxidants on levels of asymmetric dimethylarginine (ADMA) and C-reactive protein (CRP): a double-blind, randomised, factorial design, placebo-controlled trial.

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ORIGINAL CONTRIBUTION

Effect of supplementation with B vitamins and antioxidants on levels of asymmetric dimethylarginine (ADMA) and C-reactive protein (CRP): a double-blind, randomised, factorial design, placebo-controlled trial

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Abstract

Purpose Cardiovascular risk factors such as elevated levels of asymmetric dimethylarginine (ADMA)/C-reactive protein (CRP) and homocysteine are potentially related to essential micronutrients such as certain B vitamins and antioxidant vitamins. The aim of the present study was to investigate whether supplementation with moderate doses of B vitamins and/or antioxidants could alter either ADMA and/or CRP concentrations in middle-aged, apparently healthy men with mildly elevated homocysteine levels.
Methods A randomised, double-blind, factorial design, intervention study was carried out on 132 men with mildly elevated homocysteine levels, allocated to four groups (a) B vitamins alone—1 mg folic acid, 7.2 mg pyridoxine, 0.02 mg cyanocobalamin daily, (b) antioxidants alone—150 mg ascorbic acid, 67 mg vitamin E, 9 mg β-carotene daily, (c) B vitamins with antioxidant vitamins, or (d) placebo. A total of 101 men completed the study to 8 weeks.

Results When the percentage of baseline ADMA and CRP was examined at 8 weeks, no statistically significant differences were observed between the four groups ($p = 0.21$ and $p = 0.90$, respectively). Similar non-significant results were observed when analysis was stratified based on baseline CRP levels (<1.0 mg/L, $p = 0.10$; ≥ 1.0 mg/L, $p = 0.64$) and smoking status (all $p \geq 0.05$).
Conclusions Supplementation with moderate doses of B vitamins and/or antioxidants did not alter either ADMA or CRP concentrations in these middle-aged, apparently healthy men with mildly elevated homocysteine levels.

Keywords Asymmetric dimethylarginine · C-reactive protein · Cardiovascular disease · Homocysteine · B vitamins · Antioxidant vitamins

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Introduction

Evidence indicates a central role for the endothelium and inflammation in all phases of atherosclerosis. Therefore, a reduction in inflammatory levels and improvement in endothelial function by traditional and novel treatment strategies, such as micronutrients, may lead to a reduction in cardiovascular events.

Increased ADMA, an endogenous inhibitor of nitric oxide synthase, has been observed in subjects with cardiovascular disease (CVD) [1–3], where it results in reduced nitric oxide bioavailability, leading to endothelial dysfunction and CVD [4, 5]. Therapies that lower ADMA and reduce endothelial dysfunction may therefore reduce CVD risk.

Knowledge of ADMA synthesis and metabolism reveals a potential for antioxidants to lower ADMA. ADMA is synthesised when arginine residues in proteins are

methylated by the action of protein arginine *N*-methyl-transferases (PRMTs) [6]. PRMT 1 is involved in the production of ADMA and is expressed in the heart, vascular smooth muscle cells and endothelial cells [6]. Once the proteins are hydrolysed, free methylated arginines are released into the cytosol. ADMA is excreted in the urine to some extent, but the major metabolic pathway is by degradation by the enzyme dimethylarginine dimethylaminohydrolase (DDAH) [6]. Consequently, whereas impaired renal function may result in elevated levels of ADMA, attenuation of DDAH activity may play the key role in increasing ADMA levels in persons without renal complications [7]. Therefore, the attenuation of DDAH activity by oxidative stress [8], whereby a cysteine molecule held in an activated state in the tertiary structure of the enzyme is inactivated by oxidation, may lead to an accumulation of ADMA. Antioxidants have been shown to decrease ADMA levels in cultured human endothelial cells [9] and in vivo rats exposed to native low-density lipoprotein [10]. Consequently, antioxidant therapy may reduce ADMA levels by attenuating the effects of oxidative stress.

Subjects with hyperhomocysteinaemia have also been shown to have elevated ADMA concentrations [11, 12]. Therefore, as homocysteine (tHcy) is lowered through B vitamin supplementation [13], such treatment may also lead to a reduction in ADMA levels.

A large body of evidence also indicates a central role for inflammation in atherosclerotic disease [14, 15]. Multiple epidemiological studies have associated C-reactive protein (CRP), an acute-phase reactant and marker of inflammation, with CVD risk [16]. CRP is synthesised predominately by hepatocytes in the liver in response to interleukin-6 (IL-6), an inflammatory cytokine, which is secreted during acute injury [17]. As for ADMA, therapies that lower CRP as a marker of inflammation may therefore be expected to reduce CVD risk. A number of studies have suggested that antioxidant supplementation may lower CRP [18–23], but this is not always consistent [24–30]. Similarly, increasing levels of tHcy in cell cultures seems to promote IL-6 production by monocytes [31] and endothelial cells [32] and, therefore, B vitamins may also reduce CRP as a result of their tHcy-lowering potential.

Evidence, therefore, indicates that both B vitamins and antioxidants have the potential to reduce ADMA and CRP concentrations as biomarkers of CVD risk. To our knowledge, no intervention studies have yet investigated the effects of B vitamins and antioxidants, both independently and combined, on both ADMA and CRP levels. We have performed a randomised, double-blind, factorial design, intervention study to investigate whether supplementation with B vitamins and/or antioxidants could alter ADMA and/or CRP concentrations in middle-aged, apparently healthy men with mildly elevated tHcy levels. In addition, we tested whether or not the treatment effect depended on baseline CRP levels and smoking status.

Materials and methods

Subjects and study design

This intervention study was originally designed to test the effect of B vitamins and antioxidant vitamins on tHcy concentrations in men with mildly elevated tHcy levels, and the results have previously been published [13]. Therefore, the analyses reported here represent analyses of secondary endpoints.

Briefly, participants were male volunteers aged 30–49 from a single, large, Belfast-based workforce and included all grades of staff from manual to clerical to administrative. The men completed a dietary questionnaire before attending a clinic, at which time a brief medical history was taken and height and weight were determined. Blood pressure was recorded with an automated sphygmomanometer on the right arm, and a fasting venous blood sample was then taken from the left arm. A sample anticoagulated with EDTA was used to provide plasma for tHcy analysis and another sample provided serum for vitamin analysis. The EDTA treated blood samples were processed within 15 min of venepuncture whereas the serum samples were kept in the dark and returned to the laboratory for processing. Those who were diabetic, who had received a general anaesthetic within the past 3 months, or who were taking any form of dietary supplementation were excluded from further study (these factors are known to affect tHcy).

Intervention design

Overall, tHcy was measured in 509 eligible subjects. Men who had tHcy concentrations in the upper third of the distribution were targeted for the intervention study and invited to re-attend by letter ($n = 152$). Of the 152 men chosen for intervention, 132 (87%) were willing to participate. Those who decided to take part had another blood sample taken for baseline measurements. One sample was anticoagulated with EDTA or heparin or left to clot as before. A sample of whole blood was stabilised with EDTA and dithiothreitol, separated and stored in 5% MPA for vitamin C (ascorbic acid) analysis [33]. At this point, the men were randomly allocated in a double-blind fashion in balanced blocks of eight subjects to four groups (a) B vitamins alone—1 mg folic acid, 7.2 mg pyridoxine, 0.02 mg cyanocobalamin daily, (b) antioxidants alone—150 mg ascorbic acid, 67 mg vitamin E, 9 mg β-carotene daily, (c) B vitamins with antioxidant vitamins, or (d) placebo. Doses of micronutrients were selected based on a consideration of the original

primary endpoints. Blinding was carried out in the form of colour coding, and the code was not broken until all analyses had been carried out.

Each subject was given a 4-week supply of low-energy chocolates containing one of the four vitamin combinations outlined above (Sandoz Nutrition Ltd, Berne, Switzerland). Four weeks later, fasting blood samples were taken and subjects were given vitamin supplements for a further 4 weeks. After this period (total of 8 weeks) further fasting blood samples were obtained. Recruitment took place in three phases between January 1995 and March 1995, i.e. in the lowest seasonal supply of vitamin C and therefore possibly co-existing vitamins. The sample size of 25 subjects per group was sufficient to give the study 90% power to detect a statistically significant ($p < 0.05$) 20% reduction in tHcy concentrations for either of the two interventions.

This study was approved by the Research Ethics Committee of the Faculty of Medicine, Dentistry and Biomedical Sciences, Queen's University Belfast who followed the guidelines of the Royal College of Physicians of London. Informed consent was obtained from all subjects for the study.

Laboratory methods

Total tHcy, both free and protein-bound, was assayed by HPLC [34]. Serum total cholesterol was estimated using an enzymatic CHOD-PAP kit, while serum triglycerides were measured using the Peridochrom GPO-PAP kit (both Boehringer Mannheim, Germany (now Roche Applied Science)). All cholesterol assays were carried out on the Cobas Fara auto-analyser. Plasma vitamin B_{12} (cobalamin) and folate concentrations were measured by competitive protein binding utilised by the SimulTRAC-SNB radioassay kit (ICN Pharmaceuticals, California, USA). Concentrations of serum vitamin E (α-tocopherol) and β-carotene were measured by HPLC [35, 36]. Vitamin C was measured on a Cobas FARA centrifugal analyser with a fluorescent attachment [33]. The active form of vitamin B_6, pyridoxal-5-phosphate, was quantified in serum using a HPLC method [37]. Plasma CRP was determined using an ultra sensitive assay (Biokit, Barcelona, Spain) on the ILab 600 Chemistry System (Instrumentation Laboratory, Cheshire, UK). ADMA along with symmetrical dimethylarginine, arginine and homoarginine were assayed by HPLC [38]. All assays were externally quality assured, and in-house quality control samples were also included in every run.

Statistical analysis

Continuous variables were checked for normality and were logarithmically transformed where necessary. Data are presented as mean (95% CI) or geometric mean (95% CI) where indicated. Chi-square statistics were carried out on categorical variables, such as comparing smoking status in the four intervention groups. The four intervention groups were compared on baseline characteristics such as tHcy and vitamin concentrations using one-way analysis of variance (ANOVA) followed by a Student-Neuman-Keuls multiple-range test. The effect of intervention on the change in each endpoint of interest was compared between the four intervention groups using ANOVA, and smokers were compared to non-smokers with regard to baseline CRP and ADMA concentrations using the independent samples t test. $p < 0.05$ was used as the threshold for significance. Pearson correlation coefficients were calculated to estimate colinearity between baseline vitamin status with ADMA and CRP.

Individuals with raised CRP levels (>10 mg/L) were excluded from statistical analyses. This cut point was selected based on guidelines issued by the Centers for Disease Control and the American Heart Association for the use CRP in clinical and public health practice [15]; CRP concentrations of >10 mg/L represented acute infection or inflammatory disease, possibility from a non-cardiovascular origin. All analyses were performed with the use of SPSS 14.0 for Windows (SPSS Inc, Chicago, USA).

Results

tHcy was measured in 509 eligible subjects, of which 152 were targeted for the intervention study based on the inclusion criteria (tHcy concentrations in the upper third of the distribution). Of the 152 men chosen for intervention, 132 (87%) were willing to participate. A total of 101 men completed the study to 8 weeks.

Baseline ADMA was inversely associated with baseline folate ($r = -0.282$, $p = 0.005$), vitamin C ($r = -0.212$, $p = 0.038$) and α-carotene ($r = -0.217$, $p = 0.034$) concentrations, whilst CRP was inversely associated with β-carotene concentrations only ($r = -0.216$, $p = 0.031$).

There was no significant difference between the four groups at baseline (indicating randomisation was successful) with the exception of systolic blood pressure which was significantly lower in the group receiving B vitamins ($p = 0.03$) compared with the other three groups (Table 1).

Baseline vitamin status and values after 8 weeks of supplementation in the four groups are shown in Table 2. Supplementation for 8 weeks with B vitamins increased plasma folate, pyridoxal-5-phosphate and vitamin B_{12} status significantly, which resulted in an expected, concomitant, lowering in plasma tHcy [13]. As expected, the antioxidant supplementation significantly improved the initially poor plasma vitamin C status and also significantly

Table 1 Baseline characteristics of intervention subjects

	Placebo	B vitamins	Antioxidants	B + antioxidants
n	33	32	33	34
Age (years)	38.9 (36.9, 40.9)	39.3 (37.0, 41.6)	40.1 (37.8, 42.4)	40.6 (38.2, 42.9)
BMI (kg/m^2)	25.6 (24.6, 26.6)	26.1 (25.0, 27.1)	26.5 (25.5, 27.1)	26.7 (25.4, 28.1)
Systolic blood pressure (mm Hg)*	129 (125, 134)	122 (118, 127)	130 (125, 136)	132 (127, 136)
Diastolic blood pressure (mm Hg)	81 (78, 84)	78 (75, 82)	82 (80, 85)	83 (79, 87)
Cholesterol (mmol/L)	5.6 (5.1, 6.0)	5.6 (5.2, 5.9)	6.0 (5.6, 6.4)	6.1 (5.7, 6.5)
Triglycerides (mmol/L)	1.44 (1.22, 1.69)	1.34 (1.14, 1.58)	1.65 (1.40, 1.93)	1.56 (1.32, 1.85)
Smoking (% smokers)[a]	27.3	18.8	15.2	29.4

Mean (95%CI), except triglycerides given as geometric mean (95%CI)

* $p = 0.03$ for difference between the four intervention groups. One-way ANOVA except [a]Chi-square

Table 2 Baseline and post-supplementation plasma total homocysteine and vitamin concentrations

	Placebo	B vitamins	Antioxidants	B + antioxidants
n				
Week 0	33	32	33	34
Week 8	26	22	25	28
Homocysteine (μmol/L)				
Week 0	9.22 (8.46, 10.04)	9.12 (8.22, 10.11)	8.78 (7.77, 9.91)	10.31 (8.85, 12.00)
Week 8	8.69 (7.91, 9.55)	6.67* (6.20, 7.17)	8.70 (7.64, 9.91)	7.49* (6.33, 8.87)
Folate (nmol/L)				
Week 0	10.04 (8.81, 11.43)	9.53 (8.40, 10.81)	11.86 (10.07, 13.96)	9.50 (8.24, 10.96)
Week 8	10.51 (8.96, 12.31)	29.67* (23.67, 37.21)	14.14 (11.76, 16.99)	34.25* (27.79, 42.18)
Vitamin B$_{12}$ (pmol/L)				
Week 0	264 (228, 305)	243 (208, 285)	270 (236, 307)	267 (231, 308)
Week 8	253 (216, 298)	382* (326, 449)	267 (227, 314)	368* (313, 433)
Pyridoxal-5-phosphate (nmol/L)				
Week 0	34.4 (26.9, 44.0)	33.7 (26.1, 43.4)	33.4 (24.7, 45.1)	46.1 (33.3, 63.9)
Week 8	45.6 (35.4, 58.7)	72.4* (58.2, 90.1)	34.4 (26.2, 45.3)	117.3* (89.2, 154.2)
Vitamin C (μmol/L)				
Week 0	20.7 (15.2, 28.2)	18.2 (12.3, 27.0)	19.2 (14.1, 26.1)	22.9 (16.9, 30.9)
Week 8	23.3 (16.2, 33.5)	16.1 (9.7, 26.8)	52.1** (45.4, 60.1)	49.8** (42.8, 58.1)
Vitamin E (μmol/L)				
Week 0	26.3 (23.5, 29.3)	26.2 (23.6, 29.2)	29.2 (25.9, 33.0)	31.0 (27.1, 35.5)
Week 8	29.0 (25.4, 33.0)	29.2 (25.8, 33.0)	44.7** (38.7, 51.8)	45.7** (40.2, 52.0)
Lipid standardised vitamin E (μmol/L)				
Week 0	26.4 (23.5, 29.7)	26.7 (23.9, 29.9)	27.2 (24.7, 29.9)	28.6 (25.8, 31.8)
Week 8	29.3 (26.3, 32.6)	30.6 (27.3, 34.3)	39.5** (34.7, 45.0)	42.9* (38.7, 47.6)
β-carotene (μmol/L)				
Week 0	0.180 (0.130, 0.233)	0.175 (0.120, 0.233)	0.170 (0.121, 0.221)	0.210 (0.136, 0.290)
Week 8	0.198 (0.148, 0.250)	0.238 (0.131, 0.355)	1.339** (0.930, 1.836)	1.343** (0.940, 1.829)

Geometric mean (95%CI)

* $p < 0.01$ significantly different from placebo and antioxidants groups. One-way ANOVA

** $p < 0.01$ significantly different from placebo and B vitamins groups. One-way ANOVA

increased vitamin E and β-carotene status [39]. Nevertheless, despite expected improvements in nutrient status, supplementation with B vitamins and/or antioxidants had no significant effect on the ADMA or CRP concentrations ($p = 0.21$ and $p = 0.90$, respectively) (Table 3).

This analysis also tested whether the treatment effect depended on baseline CRP levels (Table 4). Among those with baseline CRP < 1.0 mg/L, no statistically significant difference was observed between the four groups ($p = 0.10$) when the percentage of baseline CRP was examined at 8 weeks. Among those with increased CVD

risk, as represented by CRP ≥ 1.0 mg/L, when the percentage of baseline CRP was examined at 8 weeks, again no statistically significant difference was observed between the four groups ($p = 0.64$).

Because of the baseline associations described above, analyses were carried out to determine whether B vitamins and/or antioxidants resulted in a change in ADMA or CRP concentrations depending on whether subjects initially had low concentrations of baseline folate (<7 nmol/L); [40], vitamin C (≤22.7 μmol/L); [39], or α-carotene (<0.1 μmol/L); [41] for ADMA; and β-carotene

Table 3 Percentage of baseline plasma ADMA and CRP (excludes subjects with CRP > 10 mg/L) concentrations after 8 weeks of intervention

Intervention group	n	Baseline ADMA (μmol/L)	Post-intervention ADMA (μmol/L)	Percentage of baseline ADMA at 8 weeks*
Placebo	26	0.89 (0.75, 1.05)	0.87 (0.74, 1.01)	98 (94, 103)
B vitamins	22	0.85 (0.74, 0.98)	0.87 (0.77, 1.02)	102 (95, 107)
Antioxidants	22	0.79 (0.57, 1.08)	0.73 (0.57, 0.95)	92 (74, 106)
B + antioxidants	25	0.86 (0.70, 1.02)	0.88 (0.65, 1.09)	99 (90, 110)
Intervention group	n	Baseline CRP (mg/L)	Post-intervention CRP (mg/L)	Percentage of baseline CRP at 8 weeks**
Placebo	26	1.11 (0.77, 1.40)	1.22 (0.86, 1.59)	110 (92, 145)
B vitamins	20	1.15 (0.87, 1.36)	1.19 (0.83, 1.76)	103 (82, 117)
Antioxidants	23	1.37 (0.94, 1.51)	1.49 (1.02, 1.95)	109 (92, 137)
B + antioxidants	27	1.31 (0.83, 1.89)	1.52 (0.90, 2.43)	116 (92, 137)

Geometric mean (interquartile range)

* $p = 0.21$ for differences in percentage of baseline ADMA between the four groups after 8 weeks. One-way ANOVA

** $p = 0.90$ for differences in percentage of baseline CRP between the four groups after 8 weeks. One-way ANOVA

Table 4 Percentage of baseline plasma CRP concentrations after 8 weeks of intervention (stratified by baseline CRP and excludes subjects with CRP > 10 mg/L)

Intervention group	n	Baseline CRP for subjects with CRP < 1.0 mg/L	Post-intervention CRP for subjects with CRP < 1.0 mg/L at baseline	Percentage of baseline CRP at 8 weeks*
Placebo	13	0.78 (0.69, 0.92)	0.92 (0.78, 0.99)	119 (100, 136)
B vitamins	9	0.84 (0.75, 0.96)	0.85 (0.69, 1.03)	102 (84, 115)
Antioxidants	10	0.89 (0.82, 0.97)	1.32 (0.86, 1.92)	148 (101, 207)
B + Antioxidants	13	0.82 (0.72, 0.91)	0.94 (0.66, 1.21)	114 (92, 129)
Intervention group	n	Baseline CRP for subjects with CRP ≥ 1.0 mg/L	Post-intervention CRP for subjects with CRP ≥ 1.0 mg/L at baseline	Percentage of baseline CRP at 8 weeks**
Placebo	13	1.58 (1.19, 2.09)	1.62 (1.00, 2.09)	103 (72, 163)
B vitamins	12	1.75 (1.06, 2.75)	1.84 (0.93, 2.62)	105 (82, 150)
Antioxidants	13	1.90 (1.16, 2.29)	1.64 (1.12, 2.11)	86 (88, 122)
B + Antioxidants	14	2.03 (1.36, 3.05)	2.39 (1.42, 4.62)	118 (75, 171)

Geometric mean (interquartile range)

*$p = 0.10$ for differences in percentage of baseline CRP between the four groups after 8 weeks. One-way ANOVA

**$p = 0.64$ for differences in percentage of baseline CRP between the four groups after 8 weeks. One-way ANOVA

Table 5 Comparison of baseline ADMA and CRP (excludes subjects with CRP > 10 mg/L) concentrations by smoking status

	Smokers	Non smokers	p value
ADMA (μmol/L)	n = 25	n = 75	
	0.96 (0.77, 1.08)	0.81 (0.72, 0.99)	0.01
CRP (mg/L)	n = 23	n = 73	
	1.26 (0.91, 1.48)	1.22 (0.85, 1.54)	0.85

Geometric mean (interquartile range)

Smokers compared to non-smokers using independent samples t test

(<0.4 μmol/L); [41] for CRP (data not shown). However, when the change in either ADMA or CRP was compared by the above nutrients within each intervention group, there were no significant effect of supplementation found

($p = 0.154$ for change in ADMA for those with initially low folate; $p = 0.064$ for change in ADMA for those with initially low vitamin C; $p = 0.233$ for change in ADMA for those with initially low α-carotene; and $p = 0.920$ for change in CRP for those with initially low β-carotene).

Baseline ADMA, but not CRP concentrations differed significantly between smokers and non-smokers ($p = 0.01$ and $p = 0.85$, respectively) (Table 5), with smokers having higher ADMA levels. Response to B vitamin and/or anti-oxidant supplementation was examined according to smoking status (Table 6). When the percentage of baseline ADMA and CRP was examined at 8 weeks, no statistically significant differences were observed between the four groups, for either smokers ($p = 0.26$ and $p = 0.40$, respectively) or non-smokers ($p = 0.48$ and $p = 0.92$, respectively).

Table 6 Percentage of baseline plasma ADMA and CRP (excludes subjects with CRP > 10 mg/L) concentrations after 8 weeks of intervention (stratified by smoking status)

Intervention group	Smokers (n)	Baseline ADMA (μmol/L)	Post-intervention ADMA (μmol/L)	Percentage of baseline ADMA at 8 weeks*
Placebo	7	0.89 (0.70, 1.06)	0.90 (0.73, 1.08)	101 (95, 105)
B vitamins	6	0.94 (0.79, 1.14)	0.98 (0.86, 1.14)	104 (95, 113)
Antioxidants	4	1.04 (0.83, 1.35)	0.84 (0.63, 1.20)	80 (66, 95)
B + antioxidants	8	1.01 (0.76, 1.09)	1.00 (0.83, 1.12)	99 (84, 133)
	Non smokers (n)	Baseline ADMA (μmol/L)	Post-intervention ADMA (μmol/L)	Percentage of baseline ADMA at 8 weeks**
Placebo	19	0.89 (0.77, 1.04)	0.86 (0.75, 0.98)	96 (92, 102)
B vitamins	16	0.82 (0.74, 0.96)	0.83 (0.77, 1.00)	102 (93, 107)
Antioxidants	18	0.74 (0.55, 1.04)	0.70 (0.54, 0.95)	95 (77, 108)
B + antioxidants	17	0.80 (0.65, 1.02)	0.82 (0.61, 1.04)	103 (92,108)
	Smokers (n)	Baseline CRP (mg/L)	Post-intervention CRP (mg/L)	Percentage of baseline CRP at 8 weeks[†]
Placebo	7	1.00 (0.70, 1.48)	1.09 (0.87, 1.39)	109 (91, 136)
B vitamins	6	1.58 (1.10, 2.92)	1.51 (0.96, 2.28)	96 (78, 117)
Antioxidants	3	0.98 (0.91, 1.09)	1.71 (0.86, 3.19)	174 (89, 294)
B + antioxidants	7	1.45 (0.83, 1.62)	1.79 (0.95, 4.32)	123 (81, 137)
	Non smokers (n)	Baseline CRP (mg/L)	Post-intervention CRP (mg/L)	Percentage of baseline CRP at 8 weeks[‡]
Placebo	19	1.15 (0.81, 1.38)	1.27 (0.85, 1.93)	111 (92, 151)
B vitamins	14	1.01 (0.84, 1.32)	1.07 (0.74, 1.50)	108 (81, 124)
Antioxidants	20	1.44 (0.95, 1.81)	1.46 (1.02, 1.94)	102 (93, 128)
B + antioxidants	20	1.27 (0.78, 1.91)	1.44 (0.90, 1.99)	113 (92, 143)

Geometric mean (interquartile range)

* $p = 0.26$ for differences in percentage of baseline ADMA between the four groups after 8 weeks. One-way ANOVA

** $p = 0.48$ for differences in percentage of baseline ADMA between the four groups after 8 weeks. One-way ANOVA

[†] $p = 0.40$ for differences in percentage of baseline CRP between the four groups after 8 weeks. One-way ANOVA

[‡] $p = 0.92$ for differences in percentage of baseline CRP between the four groups after 8 weeks. One-way ANOVA

Discussion

This analysis has found that supplementation with moderate doses of B vitamins and/or antioxidants for 8 weeks had no significant effect on ADMA or CRP concentrations in middle-aged, apparently healthy men with mildly elevated tHcy levels. Additionally, no effect of intervention was observed when the analysis was stratified by baseline CRP status or smoking status.

To date, only two studies have been carried out to examine the effect of antioxidants alone on ADMA levels in humans. Saran et al. demonstrated that supplementation with vitamin E (800 IU/d) for 8 weeks may have the potential to lower ADMA concentrations in patients with chronic kidney disease (lowering effect seen in six out of eight patients) [42]; while Nanayakkara et al. [43] found that when separate treatment effects were examined in a multiple intervention design, vitamin E (300 mg/d) significantly decreased ADMA levels by 4% compared with the placebo group. However, the former study was an open-label, small-scale pilot study [42] and the latter a secondary analysis where mean plasma ADMA levels were relatively low at baseline (0.53 μmol/L) [43]. Therefore, these results should be treated with caution and warrant further investigation.

tHcy-induced endothelial dysfunction may result in increased synthesis of ADMA [11]. Folic acid and vitamin B_{12} decrease tHcy concentrations by stimulating remethylation into methionine, and vitamin B_6 stimulates the breakdown of tHcy into cysteine through the transsulfuration pathway. Therefore, a possible lowering effect of tHcy with B vitamins may also lead to a reduction in ADMA concentrations. However, even though it has been shown that B vitamin supplementation leads to a significant decrease in tHcy concentrations [13], no effect has been reported on ADMA levels [44–47], with only one study showing an effect of B vitamin supplementation on lowering ADMA levels in human subjects [48]. This study was a relatively small-scale study ($n = 42$), uncontrolled, with no placebo group and suffers from the design weaknesses associated with this type of intervention. The lack of statistically significant results from human intervention trials is backed up by a randomised, double-blind, placebo-controlled intervention using a combined B vitamin and antioxidant supplement [44], and also the current study. It is therefore highly unlikely that B vitamins can lower ADMA, with Schmitt et al. actually suggesting a mechanism whereby B vitamin supplementation might even promote ADMA generation through increasing metabolic flux through the trans-sulphuration and remethylation pathways [44].

Associations between antioxidant vitamins and CRP concentrations have been reported in some [49, 50], but not in all epidemiological studies [51, 52]. Intervention studies have tried to clarify these ambiguous results; but these have also been inconclusive. Of those looking at the effects of vitamin E, three studies found statistically significant treatment effects [18–20]; and four found no treatment effect [24–27]. Most of these studies were conducted in disease state subjects, especially those which reported an effect, unlike the apparently healthy men with mildly elevated tHcy levels used in the current study; healthy subjects were also used in three of the four studies reporting no effect [24, 25, 27]. Additionally, those which reported an effect often used doses of vitamin E that were higher than those employed in the current study (e.g. 200 mg/d [18], 800 IU/d [19] and 1,200 IU/d [20]). With regard to vitamin C, three studies found statistically significant treatment effects [21–23], and two found no treatment effect [28, 29]. Firm conclusions are difficult to draw from these studies due to the wide variation in doses used and the differing lengths of each intervention. Two studies have examined combined vitamin C and E supplementation [22, 30], but neither of these studies found significant reductions in CRP levels.

The three studies [21–23] that reported an effect of vitamin C on CRP levels were the only studies of either vitamin C or E to exclude subjects with CRP > 10 mg/L, as these subjects are likely to have acute infection or inflammatory disease [15]. It would also seem logical to hypothesise that biomarkers such as CRP would be more likely to be reduced when initially elevated. Therefore, two of three studies above [21, 23] also conducted stratified analysis by baseline CRP above and below 1.0 mg/L. Both studies reported no treatment effect in those subjects with initially low CRP levels, CRP < 1.0 mg/L, but a significant effect in those with initially higher levels. However, we found no effect of treatment even when the analysis was stratified by baseline CRP.

Few epidemiological studies have looked at B vitamins in relation to CRP levels, and, again, results have not been consistent [49, 51, 52]. Intervention studies carried out evaluating the effect of B vitamins on CRP concentrations have been conducted in subjects who were undergoing haemodialysis [53], or stable coronary artery disease patients [54], or those with hyperhomocysteinemia [55]. Two out of three reported no significant treatment effect on CRP levels [54, 55]. However, Chang et al. demonstrated that a 3-month intervention with a combined B vitamin supplement effectively lowered tHcy and CRP concentrations in haemodialysis subjects, although this study was a randomised, open-labelled study which used doses higher than those in this current study (folic acid (5 mg/d) and vitamin B complex) [53].

It was hypothesised that smokers might respond more strongly to the intervention, because smoking is associated

with oxidative damage leading to lipid peroxidation and endothelial dysfunction [56]. Inflammatory changes may increase turnover of micronutrients so that blood concentrations are lower in smokers than non-smokers [57]. Unlike the majority of previous studies such as [58, 59], we did not see significantly higher baseline CRP levels among smokers within this study; although a current meta-analysis should confirm the relationship between smoking status and CRP [60], as results have not always been consistent [61]. However, we did show that smokers had significantly higher baseline ADMA levels than non-smokers within this population, which has been previously reported in some [62–64], although not all [65, 66] studies. When the analysis (percentage of baseline ADMA and CRP at 8 weeks) was stratified by smoking status, we observed no statistically significant differences between the four groups in response to supplementation. However, it should be noted that the number of subjects in this sub-group analysis was very small and these were exploratory analyses as the study was not originally powered for such stratification.

There are limitations to the current study. This study was a small-scale intervention study of apparently healthy men in Northern Ireland with only mildly elevated tHcy levels, involving a short supplementation period of 8 weeks. Additionally, this intervention was originally designed to test the effect of B vitamins and antioxidant vitamins on tHcy concentrations and, therefore, the secondary analyses presented in this paper may lack power and some of the robustness of larger, more specifically designed intervention studies. Comparing the result of this study with others is difficult due to the differences in the following: (1) the specific populations studied (ranging from healthy volunteers through to haemodialysis patients and diabetics); (2) the duration of the intervention period; (3) the combinations of micronutrients used; and (4) the doses of micronutrients used. These factors could all have had an influence on the findings of each individual study. For example, Block et al. [21] used 1,000 mg/d vitamin C and 800 IU/d (536 mg/d) vitamin E whereas we used a combination of 150 mg/d vitamin C and 67 mg/d vitamin E and also included β-carotene (9 mg/d). None of the studies have examined the dose-dependent effects of B vitamins or antioxidants on ADMA or CRP. In terms of the study population, our participants were apparently healthy, although they had mildly elevated tHcy levels (mean, 9.66 μmol/L), and had CRP levels which were already quite low at baseline (mean, 1.23 mg/L); therefore, a lowering effect of supplementation may have been difficult to achieve.

In summary, we have shown that treatment with B vitamins or antioxidants, whether administered independently or combined, for 8 weeks in male subjects with mildly elevated tHcy levels had no effect on ADMA or CRP concentrations. Additionally, no effect of intervention was observed when analysis was stratified by baseline CRP levels or smoking status. Results from further intervention studies are needed to confirm our results, although future studies may be better to focus on individuals with initially elevated ADMA and CRP levels, where effects of intervention are more likely to be detected, as there may be a threshold concentration above which there is the potential for reduction in ADMA and CRP levels with micronutrient supplementation.

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