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Effects of vitamin B12, folate, and vitamin B6 supplements in elderly people with normal serum vitamin concentrations

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Summary

In a prospective, multicentre, double-blind controlled study, the effect of an intramuscular vitamin supplement containing 1 mg vitamin B12, 1·1 mg folate, and 5 mg vitamin B6 on serum concentrations of methylmalonic acid (MMA), homocysteine (HCYS), 2-methylcitric acid (2-MCA), and cystathionine (CYSTA) was compared with that of placebo in 175 elderly subjects living at home and 110 in hospital. Vitamin supplement and placebo were administered eight times over a 3-week period. Vitamin supplement but not placebo significantly reduced all four metabolite concentrations at the end of the study in both study groups. The maximum effects of treatment were usually seen within 5–12 days. Initially elevated metabolite concentrations returned to normal in a higher proportion of the vitamin than of the placebo group: 92% vs 20% for HYCS; 82% vs 20% for MMA; 62% vs 25% for 2-MCA; and 42% vs 25% for CYSTA.

The response rate to vitamin supplements supports the notion that metabolic evidence of vitamin deficiency is common in the elderly, even in the presence of normal serum vitamin levels. Metabolite assays permit identification of elderly subjects who may benefit from vitamin supplements.

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Introduction

Methylmalonic acid (MMA), homocysteine (HCYS), cystathionine (CYSTA), and 2-methylcitric acid (2-MCA) are metabolites in enzymatic reactions dependent on vitamin B12, folate, and vitamin B6.[1–4] Vitamin B12 (as 5'-deoxyadenosylcobalamin) is an essential cofactor in the enzymatic conversion of methylmalonyl-CoA to succinyl-CoA.[5] The conversion of methylmalonyl-CoA to MMA results in an elevated serum MMA concentration in vitamin B12 deficiency. Folate (as methyl-tetrahydrofolate) is required for methylation of HCYS to methionine catalysed by methionine synthase with vitamin B12 (methylcobalamin) as a cofactor. Vitamin B6 (as cofactor pyridoxal-5'-phosphate) is required for trans-sulphuration of HCYS through cystathionine synthesis, a HYCS catabolic pathway.[6] 2-MCA concentrations are raised in the serum of most patients with vitamin B12 deficiency. Concentrations of CYSTA are elevated in most patients with deficiencies of vitamin B12 and folate, and may be increased in B6 deficiency.[3,4]

In an epidemiological study,[7] we demonstrated that the prevalence of elevated concentrations of these metabolites in an elderly population was higher than the prevalence of low serum vitamin B12, folate, and vitamin B6 values. We postulated that tissue deficiency of these vitamins (shown by elevated metabolite concentrations) may be more common than that estimated by measuring serum vitamin levels. Intracellular vitamin deficiency might be subclinical, but even in patients with normal serum vitamin values, clinical and haematological signs of vitamin deficiency may be found with elevated concentrations of metabolites.[8,9] An elevated HCYS concentration is an independent risk factor for atherosclerotic diseases.[10–15] The prevalence of elevated metabolite concentrations and potential risk factors for related disease, such as neuropsychiatric and thromboembolic disorders, is also high in the elderly.

Diagnosis	Living at home		In hospital	
	Vitamin group (n=88)	Placebo group (n=87)	Vitamin group (n=55)	Placebo group (n=55)
Diabetes mellitus	20 (23)	22 (25)	21 (38)	21 (38)
Ischaemic heart disease	42 (48)	43 (49)	32 (58)	25 (45)
Chronic heart failure	16 (18)	23 (26)	16 (29)	14 (25)
Chronic obstructive lung disease	13 (15)	12 (14)	5 (9)	4 (7)
Hypertension	54 (61)	52 (59)	19 (35)	21 (38)
Neuropsychiatric disorders	17 (19)	13 (15)	18 (33)	17 (31)

Number (%) of subjects; some had more than one diagnosis.

Table 1: **Diagnoses in elderly subjects**

We assessed whether vitamin supplements would lower serum concentrations of HCYS, CYSTA, MMA, and 2-MCA in an elderly population by a double-blind placebo-controlled study with a parenteral vitamin combination.

Subjects and methods

Study population

During an 8-month period, 300 participants aged 65–96 years were enrolled at 13 centres. None had participated in the previous epidemiological study.[7] 180 were living at home and 120 in nursing homes or acute geriatric hospitals. Subjects living at home were recruited by telephone from a general practitioner's register. They were able to carry out all normal daily activities independently or with minimal help and were not acutely ill. A history of concomitant diseases was assessed from the medical records supplied by their general practitioner. Patients living in a nursing home or admitted to hospital were randomly chosen to participate in the study. We excluded patients who had taken vitamins during the previous 3 months or who had a life-threatening disease, history of a coagulation disorder or treatment with anticoagulants, hypersensitivity to lignocaine, or severe renal insufficiency (creatinine clearance of less than 30 mL/min[16]).

The participants received intramuscular injections of 1 mg hydroxocobalamin, 1·1 mg folate, and 5 mg pyridoxine hydrochloride in 4 mL water (vitamin group), or the placebo preparation containing 0·4 mg azorubin, 36 mg sodium chloride, and 4 mL water (placebo group). The two preparations were similar in appearance and were supplied by Medice, Chem Pharmazeutische Fabrik (Iserlohn, Germany). Before injection, both the test drug and the placebo were mixed with 24 mg lignocaine hydrochloride in 1 mL water. Each participant received an intramuscular injection in the morning of days 1, 3, 5, 8, 10, 12, 16, and 20. Fasting blood samples were taken before the first injection on day 1 and before injections on days 5, 12, and 21. The study design was double-blind. All patients included in the analyses if they received at least the first 5 intramuscular injections, with follow-up analysis after 3 weeks.

The protocol was approved by the Freiburger Ethik Kommission in Germany and the ethics committees of the participating academic centres. Informed consent was obtained from all subjects before they joined the study. All patients underwent a complete history and physical examination.

Laboratory investigations

Venous blood was obtained in the morning after an overnight fast. Laboratory tests were done in the same laboratories as described previously.[7] Serum HCYS, CYSTA, MMA, and 2-MCA were assayed by capillary gas chromatography and mass spectrometry. Serum vitamin B12 and folate were measured by a quantitative radioassay method with a purified intrinsic factor and a purified folate-binding protein. Vitamin B6 was measured in deproteinised serum by a radioenzymatic method with tyrosine apodecarboxylase and ^{14}C-tyrosine. The normal reference ranges for the serum vitamins and metabolites were calculated as within 2 SD of the mean in 99 healthy volunteers aged 20–25 years[7]: vitamin B12 103–406 pmol/L; folate 5·4–16·3 nmol/L; vitamin B6 28·7–162 nmol/L; HCYS 5·0–13·9 μmol/L; CYSTA 72–245 nmol/L; MMA 62–247 nmol/L; 2-MCA 62–192 nmol/L.

Analysis

Baseline values and the response to vitamin supplements were examined separately in the subjects at home and in hospital. Data were log transformed before analysis. Means were compared by Student's t test for the evaluation of the baseline characteristics and ANOVA with repeated measures correcting for the baseline value for the changes in the metabolites after therapy. A multiple linear regression model was used to assess the influence of vitamin supplements versus placebo on the metabolite concentrations between days 1 and 21. The χ^2 test was used to compare frequency distributions between groups. A p value of 0·05 was considered statistically significant.

Results

Study groups

Of the 300 participants, 180 were living at home and 120 were in hospital or nursing home. They were randomly assigned to vitamin supplement or placebo so that there were equal numbers in the two treatment arms. 15 subjects were excluded from analysis because of incomplete follow-up or missing results: ten subjects in hospital (5 in each group) and five living at home (1 vitamin and 4 placebo). 285 participants completed the trial (table 1). Vitamin and metabolite concentrations before treatment did not differ between the vitamin and placebo groups (table 2), except that among subjects in hospital CYSTA concentrations were higher in the placebo group (p=0·05). The prevalence of elevated serum metabolite and subnormal serum vitamin concentrations was generally well balanced between the groups (table 3), although there were more subjects with elevated HCYS levels in the placebo group than in the vitamin group among those living at home (p=0·006).

Effect of supplements on metabolite concentrations

The figure shows the effect of vitamin and placebo injections on serum concentrations of the four metabolites. In the analysis, these values were corrected for the corresponding baseline value (metabolite level on

Characteristic	Living at home		In hospital	
	Vitamin group	Placebo group	Vitamin group	Placebo group
Male/female	36/52	41/46	9/46	19/36
Mean (SD) age in years	72 (6·3)	73·1 (6·7)	79·3 (6·7)	80·4 (6·5)
Geometric mean (95% range) serum concentrations				
Vitamin B12 (pmol/L)	199 (88–450)	189 (76–476)	207 (66–647)	207 (86–495)
Folate (nmol/L)	10·9 (3·4–36)	9·5 (3·4–26)	8·4 (2·5–28)	8·4 (2·5–28)
Vitamin B6 (nmol/L)	65 (19–221)	63 (18–227)	42 (13–142)	42 (10–167)
HCYS (μmol/L)	11·6 (7–19·2)	12·6 (6·1–26)	13·2 (7·1–25)	13·7 (7·4–25·4)
MMA (nmol/L)	222 (65–751)	243 (84–697)	227 (68–762)	254 (57–1129)
CYSTA (nmol/L)	252 (102–625)	256 (92–712)	280 (111–710)	346 (99–213)
2-MCA (nmol/L)	168 (78–362)	167 (70–399)	166 (80–340)	193 (80–471)

Table 2: **Characteristics in the study groups before treatment**

Characteristic	Living at home			In hospital		
	Vitamin group (n=88)	Placebo group (n=87)	Total (n=175)	Vitamin group (n=55)	Placebo group (n=55)	Total (n=110)
Vitamin B12 <103 pmol/L	5 (6)	7 (8)	12 (7)	6 (11)	4 (7)	10 (9)
Folate <5·4 nmol/L	12 (14)	10 (11)	22 (13)	12 (22)	9 (16)	21 (19)
Vitamin B6 <28·7 nmol/L	7 (8)	10 (11)	17 (10)	10 (18)	14 (25)	24 (22)
MMA ≥247 nmol/L	33 (37)	44 (50)	77 (44)	17 (31)	27 (50)	44 (40)
HCYS ≥13·9 μmol/L	17 (19)	33 (38)*	50 (29)	22 (40)	27 (50)	49 (45)
CYSTA ≥245 nmol/L	46 (52)	42 (48)	88 (50)	33 (60)	41 (75)	74 (67)
2-MCA ≥192 nmol/L	31 (35)	29 (33)	60 (34)	22 (40)	26 (47)	48 (44)

Number (%) of subjects. *p=0·006 for comparison with vitamin group.

Table 3: **Abnormal serum concentrations of vitamins and metabolites before treatment**

	Living at home			In hospital			Total (%)		
	Vitamin group	Placebo group	p	Vitamin group	Placebo group	p	Vitamin group	Placebo group	p
MMA	26/33	9/44	<0·0001	15/17	5/27	<0·0001	41/50 (82)	14/71 (20)	<0·0001
HCYS	16/17	7/33	<0·0001	20/22	5/27	<0·0001	36/39 (92)	12/60 (20)	<0·0001
CYSTA	21/46	14/42	0·23	12/33	7/41	0·06	33/79 (42)	21/83 (25)	0·026
2-MCA	21/31	8/29	0·002	13/22	6/26	0·01	33/53 (62)	14/55 (25)	<0·0001

Table 4: **Frequency distribution of patients with elevated pretreatment metabolite values but normal values on day 21**

day 1). The difference in concentrations between the vitamin supplement and placebo groups was greatest at day 12 for all metabolites in subjects living at home and in hospital.

To investigate the effect of vitamin supplements on metabolite concentrations, pretreatment metabolite concentration, age, sex, and group were brought into a multiple linear regression model. Among subjects living at home and in hospital, there was a significant effect of the vitamin treatment over placebo in the concentrations for each of the four metabolites on day 21. Age was significant only for MMA. The numbers and percentages of patients with initially elevated metabolite concentrations that became normal after vitamins or placebo are shown in table 4. For MMA, HCYS, and 2-MCA the concentrations reached the normal range in significantly greater proportions of those receiving vitamins than of the placebo group. More than 80% of the elevated MMA and HCYS concentrations were normal at the end of the study after vitamin supplements; 2-MCA concentrations became normal in more than half and CYSTA in 42%. In vitamin-treated patients whose metabolite concentrations had returned to normal at day 21, a normal concentration was achieved by day 5 (after
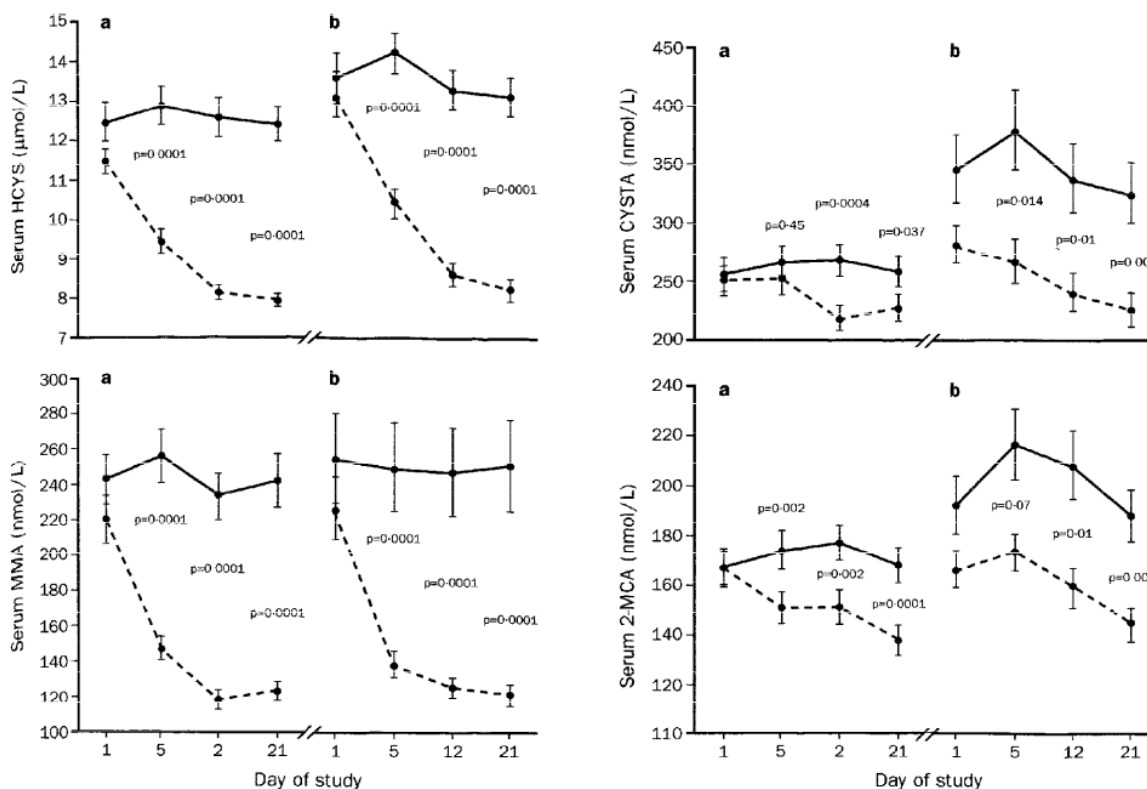

Figure: **Serum metabolite concentrations after vitamin supplementation (- - -) or placebo (——) in elderly subjects (a) at home and (b) in hospital**
Values represent geometric means, error bar antilog of (mean, SEM) logarithmically transformed data

only two vitamin injections) for MMA, HCYS, CYSTA, and 2-MCA, respectively, in 72% (19/26), 88% (14/16), 72% (15/21), and 62% (13/21) of the subjects living at home and 100% (15/15), 65% (13/20), 33% (4/12), and 46% (6/13) of those in hospital.

Effects of vitamin supplements in patients with normal serum vitamin concentrations

Of the three vitamins studied, only deficiency of vitamin B12 would be expected to cause an elevated MMA. More than 80% of the subjects with initially high MMA had normal serum vitamin B12 concentrations at the beginning of the study (at home, 29/33 in the vitamin supplement group and 39/44 in the placebo group; in hospital, 13/17 in the vitamin supplement group and 24/27 in the placebo group). The MMA fell to normal in 33 (79%) of the 42 vitamin-treated subjects (home 22/29; hospital 11/13) compared with 12 (19%) of 63 placebo recipients (home 7/39; hospital 5/24) (p<0·0001).

Deficiency of any of the vitamins included in the supplement might have led to raised HCYS concentration. Of the subjects with increased HCYS, 19 had normal pretreatment serum values of all three vitamins. HCYS concentrations became normal after vitamin supplements in 16 (home 8/9; hospital 8/10) compared with only 7 of 35 placebo recipients (home 4/20; hospital 3/15) (p<0·0001).

Discussion

Our data show that intramuscular supplements with vitamin B12, folate, and vitamin B6 reduced raised serum levels of HCYS and MMA in the majority of elderly subjects who had no overt chronic renal failure, irrespective of the serum vitamin concentrations. Serum 2-MCA also fell to within the normal range in most subjects, and CYSTA concentrations fell to normal in nearly half. Similar responses were noted in the subjects living at home and in hospital.

The proportion of subjects with initially elevated metabolites whose values fell to normal by the end of the study was significantly higher in the vitamin group than the placebo group for all four metabolites. Other studies have established the value of measuring serum MMA and HCYS in the diagnosis of clinically significant vitamin B12 and folate deficiency and in follow-up after relevant vitamin substitution.[1,2,8,9,17-23] Furthermore, many studies have shown that elevated homocysteine is an independent risk factor for cerebral, coronary, and peripheral vascular disease.[10-15] In addition to vitamin B12 and folate, vitamin B6 plays an important role in the degradation of HCYS since it is a cofactor for cystathionine-β-synthase, the enzyme that converts HCYS to cystathionine. Serum concentrations of HCYS and CYSTA are elevated in rats deficient in vitamin B6,[24] and serum CYSTA concentrations are increased in the majority of patients with vitamin B12 and folate deficiency,[3] as are 2-MCA values in those with vitamin B12 deficiency.[4]

Our schedule and dosage of 1 mg vitamin B12 and 1·1 mg folate administered in eight injections over 3 weeks is sufficient to treat deficiencies of these vitamins. However, for vitamin B6, the 5 mg dose used is lower than that of previous studies of oral B6 treatment,[12,15] although the recommended dietary allowance is 2·0 mg for men and 1·6 mg for women over age 51.[26] It is possible that a higher dose of parenteral vitamin B6 would have had a greater effect on the HCYS and CYSTA levels in

our subjects. However, serum B6 levels rose to above the normal range (ie, >162 nmol/L) by day 21 in 11 subjects who had had raised pretreatment values; nonetheless, serum CYSTA concentrations remained elevated in 5 of the 11 patients.

We[7] and others[27] have found that the majority of the healthy elderly subjects with elevated MMA levels have normal vitamin B12 levels. Moreover, in 79% of patients with a normal serum vitamin B12 concentration and an elevated MMA level in the present study, the MMA fell to normal within 3 weeks of vitamin supplements, in contrast to only 19% of the patients treated with placebo. The results for patients with elevated HCYS and normal levels of all three vitamins were equally striking. Hence, our findings add further support to the view that many elderly people have metabolic evidence of early vitamin B12, folate, or vitamin B6 deficiency despite normal serum vitamin concentrations. Serum metabolite concentrations were also found to be a more sensitive indicator of early vitamin B12 deficiency than vitamin levels in patients with pernicious anaemia who were withdrawn from monthly maintenance therapy.[8] In addition, about 5% of patients with unequivocal haematological or neurological syndromes caused by cobalamin deficiency have normal serum vitamin B12 values at the time of presentation although serum MMA and HCYS are increased.[8]

Elevated MMA and HCYS concentrations fell substantially within 5 days after only two injections, with a maximum effect seen by day 12. The clinical use of this response during substitution might be a rapid and objective test to confirm the presence of vitamin deficiency. Although decreases in concentrations of MMA and 2-MCA were presumably caused by vitamin B12, it is not possible from our study design to indicate which particular vitamin brought about the reductions in HCYS and CYSTA concentrations.

Other factors may alter serum concentrations of the four metabolites. Although patients with frank renal failure were excluded from our study, mild to moderate renal dysfunction may have contributed to elevations in metabolites in some subjects,[14,27] especially for CYSTA and 2-MCA,[3,4] in which the responses to vitamin supplements were less uniform. Increases in HCYS concentrations may also be seen after treatment with various drugs,[13,17] and any of the metabolites may be affected by inborn errors of metabolism.[2-6,13,17]

The response to vitamin supplements in patients with elevated MMA and HCYS and normal serum vitamin B12, folate, and vitamin B6 values, provides further evidence that vitamin deficiency in the presence of normal serum levels is more common than previously thought. To what extent reduction of these metabolite concentrations to normal values is associated with clinical improvement remains unknown.

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Hormone replacement therapy and serum angiotensin-converting-enzyme activity in postmenopausal women

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The mechanisms by which hormone replacement therapy (HRT) reduces the risk of coronary heart disease (CHD) are incompletely understood, but may include direct arterial effects. We examined the effect of oestrogen/progestagen HRT on serum angiotensin-converting-enzyme (ACE) activity in postmenopausal women. After 6 months, ACE activity was reduced by 20% (p<0·001) on average in 28 treated women but remained unchanged in 16 controls. Serum ACE activity is modifiable by gonadal steroids and changes in serum ACE may represent a novel mechanism by which HRT reduces CHD risk in women.

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Oestrogen replacement therapy is associated with reduced risk of coronary heart disease (CHD) in postmenopausal women.[1] Whilst beneficial changes in lipid and carbohydrate metabolism, body-fat distribution, and haemostasis may bring about this effect, there is also evidence for direct arterial effects of oestrogen.[2] Elevated activity of serum angiotensin-converting enzyme (ACE) and deletion polymorphism in the ACE gene may both be independently associated with increased risk of CHD in men.[3,4] Serum ACE levels may vary four-fold between healthy individuals, with up to 50% of inter-individual variation attributable to the polymorphism.[5,6] In longitudinal studies, ACE concentration appears constant within groups composed predominantly of men.[7] Whether serum ACE is similarly constant in women or may be influenced by menopause has not been studied. We hypothesised that circulating levels of gonadal steroids may determine ACE activity in women and that serum ACE activity may thus be a modifiable CHD risk-factor. Accordingly, we measured serum ACE activity in postmenopausal women before and during hormone replacement therapy (HRT) and in a control group of untreated women.

The study population comprised the initial recruitment of eligible patients into a prospective HRT study. The population consisted of 28 postmenopausal women assigned to be treated with continuous combined HRT (2 mg oestradiol valerate and 0·7 mg norethisterone orally daily) and 16 postmenopausal women who did not wish to receive HRT, who were recruited concomitantly to serve as controls. All women were postmenopausal (>12 months' amenorrhoea), confirmed by elevated serum follicle-stimulating hormone, and none had received any oestrogen preparations within 12 months or implants within 3 years. The women were apparently healthy