Original Communication

A micronutrient supplement modulates homocysteine levels regardless of vitamin B biostatus in elderly subjects

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Abstract: Elevated homocysteine (Hcy) levels (≥15 μmol/L) in the elderly are frequently associated with a higher risk of cardiovascular disease and cognitive decline. Several studies have already shown an Hcy-lowering effect of B vitamin supplementation in cohorts deficient in these nutrients. The aim of this randomized, double-blinded 12-week intervention study was to investigate whether Hcy levels in healthy elderly subjects (75.4±4.5 years, n=133) could be lowered with a micronutrient supplement (i.e., 400 μg folic acid, 100 μg cobalamin). Difference in mean initial Hcy levels between intervention (17.6±7.1 μmol/L, n=65) and placebo group (18.9±6.1 μmol/L, n=68) was not significant. The prevalence of cobalamin and folate deficiency in the total study population was low: 27% had serum-cobalamin levels ≤150 pmol/L, 12% holo-transcobalamin (Holo-TC) levels ≤50 pmol/L, 13% low cobalamin status using the aggregated cobalamin marker 4cB12 and 10% red blood cell (RBC) folate ≤570 nmol/L. Nevertheless, the treated subjects still showed improved cobalamin and folate biostatus (serum cobalamin Δt_{12}-t_0: 63±48 pmol/L; Holo-TC Δt_{12}-t_0: 17±19 pmol/L; RBC folate Δt_{12}-t_0: 326±253 nmol/L) and Hcy levels (Δt_{12}-t_0: −3.6±5.7 μmol/L). The effects were statistically significant compared to the placebo group with p=0.005 (serum cobalamin), p=0.021 (Holo-TC), p=0.014 (RBC-folate) and p<0.001 (Hcy). The Hcy-lowering effect was dependent on the initial Hcy levels (p<0.001). Our findings suggest that elevated Hcy levels in elderly subjects can be lowered regardless of the initial cobalamin and folate biostatus.

Keywords: hyperhomocysteinemia, homocysteine, vitamin B12, folate, multivitamin

Background

Homocysteine (Hcy) is an amino acid synthesized as an intermediate product of methionine metabolism. Elevated Hcy levels cause endothelial dysfunction by a) increasing the production of reactive oxygen species [1], b) promoting the oxidation of low-density lipoprotein [2], c) toxic shedding of endothelial cells [3] as well as d) damaging the medial and adventitial layers of the arterial vessel wall [4]. Moreover, Hcy stimulates prothromboplastic activity by increasing platelet aggregation [5], modulates protein function [6], and has both neurotoxic and genotoxic properties [7, 8]. Abnormally high plasma Hcy levels are considered a medical condition called hyperhomocysteinemia [9]. There is no general consensus on the cutoff values for hyperhomocysteinemia [6, 10, 11]. Recent trials have commonly used a cut-off of ≥15 μmol/L [12, 13].

A pathological increase in plasma Hcy levels is considered an independent cardiovascular risk factor [14, 15, 16]. However, studies on B-vitamin intervention for lowering plasma Hcy levels showed no advantages for secondary prevention with regard to cardiovascular disease [17]. Nevertheless, elevated plasma Hcy levels are viewed as componential triggers for a variety of multifactorial diseases, such as atherosclerosis [7], congestive heart failure and illnesses that are typical in the elderly, such as dementia (in particular Alzheimer's disease) [18, 19], age-related macular degeneration and hearing loss [3]. There is also an association with osteoporosis [20, 21, 22]. Altogether, elevated Hcy levels are associated with more than 100 health conditions [23].

Hyperhomocysteinemia is mainly caused by reduced activity of key enzymes involved in Hcy metabolism due to dietary B vitamin deficiencies [24]. In particular, cobalamin (vitamin B12), folate and pyridoxine (vitamin B6) play major roles in Hcy metabolism [25]. Hence, plasma Hcy levels are viewed as an additional functional marker of folate and cobalamin deficiencies [26, 27]. Previous studies have shown that deficiencies in B vitamins and high plasma

Hcy levels are negatively associated and frequent in the elderly [3, 28, 29, 30]. Another cause of disturbed Hcy metabolism is functional gene polymorphisms [24].

Plasma Hcy levels are higher in males compared than in women and increase with age [31]. Therefore, elderly people are at risk of hyperhomocysteinemia [28, 32]. An increase in Hcy levels is possible due to an unbalanced diet or malnutrition, which is frequent in elderly people [33]. On the other hand, an age-related malabsorption of nutrients is a major cause of insufficient B vitamin biostatus and the development of hyperhomocysteinemia [34]. In old age, both autoimmune (type A) and bacterial (*H. pylori*) atrophic gastritis (type B) are frequent (up to 30%) and lead to a reduced formation of intrinsic factor (IF), which normally binds cobalamin and to decreases gastric acid secretion (hypochlorhydria), resulting in a decreased release and absorption of cobalamin [34, 35]. Hcy metabolism is also influenced by the intake of certain drugs [36, 37, 38] and impaired kidney function [39, 40].

Numerous interventional studies observed an Hcy-lowering effect after folate and cobalamin supplementation [41, 42, 43, 44]. However, very few studies have been conducted on seniors aged ≥70 years [34, 45]. To assess the relevance of vitamin supplementation, it is important to measure the prevalence of cobalamin and folate deficiencies [46, 47]. Intervention studies often did not report the initial prevalence of cobalamin- and folate deficiency using valid biomarkers reviewed by Olaso-Gonzalez et al. [48]. It is unclear whether elevated Hcy levels can be lowered in elderly people with a sufficient micronutrient biostatus. In addition, the comparison of results from previous studies is complicated because different biomarkers are used to assess cobalamin and folate biostatus [49]. Frequently, blood levels are measured only in the serum. However, red blood cell folate (RBC folate), holotranscobalamin (Holo-TC) and the combined marker 4cB12 are considered more valid for assessing the long-term biostatus of the two B vitamins [50, 51, 52, 53], since serum cobalamin can fluctuate daily and may inadequately represent cobalamin status in tissues [54, 55, 56]. Consistent with this, a recent study by Campos et al. [52] suggested that Holo-TC should be used as a preferred first-line marker for the detection of subclinical cobalamin deficiency in individuals aged ≥50 years.

Hence, in the present study, we aimed to determine and evaluate the effect of multivitamin supplements, including cobalamin and folate, on plasma Hcy levels in healthy elderly subjects aged ≥70 years. This study is part of a larger trial with the overall aim of assessing and improving the status of critical micronutrients in older people and to investigate their impact on a number of health-related biomarkers, including the Hcy level. Folate biostatus was evaluated using RBC folate, while cobalamin biostatus was assessed using serum cobalamin, Holo-TC and 4cB12. In addition, the methylmalonic acid (MMA) concentrations were measured.

Material and methods

Study design and participants

The study was conducted as a single-center, two-armed, double-blinded, and randomized clinical trial at the Institute of Food Science and Human Nutrition, Leibniz University Hannover, Germany according to the guidelines of the Declaration of Helsinki and registered in the German Clinical Register (DRKS00021302).

In total, 133 healthy subjects met all the inclusion criteria (≥70 years, living home dwelling, and independently) and were included in the study (Figure 1). Exclusion criteria were defined as intake of dietary supplements up to three months before the examination, BMI>35 kg/m^2, severe gastrointestinal or cardiovascular diseases, and intake of immunosuppressants or chronic corticosteroids. The participants provided informed consent before enrollment. Subjects were recruited through local press advertisements and announcements in senior network centers and volunteer clubs. Interested subjects were screened for their health status, as well as for the intake of dietary supplements, through a telephone interview (Figure 1).

After controlling for the inclusion and exclusion criteria, the subjects were invited to the institute for examination. Participants completed a questionnaire regarding their medical history, current medical drug intake (frequency and dosage) and health status, and selected questions on general diet and physical activities. Participants described their movement behavior based on the following classification: as "predominantly active" (>2 ½ hours/week movement with middle intensity or >1 ¼ hours/week with high intensity); "predominantly sedentary" (<2 ½ hours/week movement with middle intensity or <1 ¼ hours/week with high intensity) or "regular basis movement" (in approximation to 2 ½ hours/week movement with middle intensity or 1 ¼ hours/week with high intensity).

The examination days included measurement of anthropometric data, including body weight and height (Seca GmbH & Co. KG, Hamburg, Germany), waist and hip circumferences, blood pressure and pulse rate. Consequently, blood pressure and pulse rate were performed after a 5 min rest using volume-plethysmography (Boso ABI-system 100; BOSCH & SOHN, Germany) in the left arm. All the measurements were performed by trained nutritionists. The subjects were asked not to change their diet or physical activity during the intervention. At the final examination, the participants completed a second questionnaire regarding changes in medical drug intake, health status, nutrition,

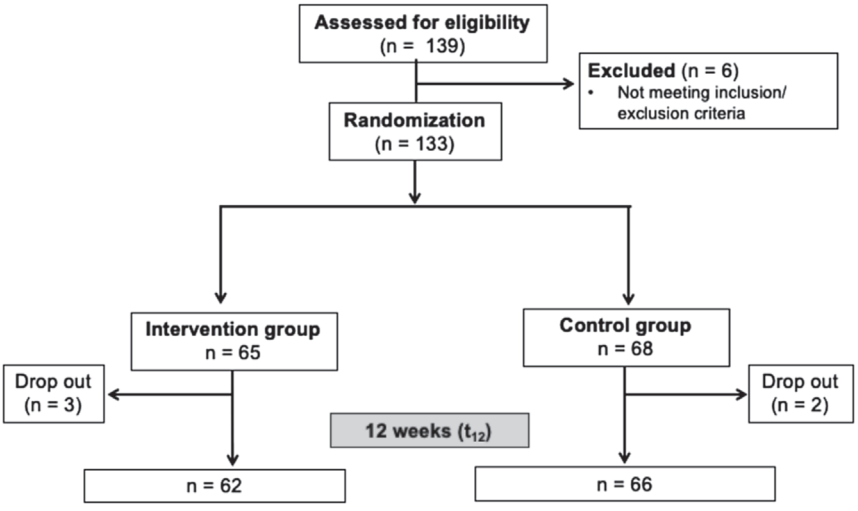

Figure 1. Flow diagram of the study population.

and movement behavior. Subjects reporting major changes were excluded from the analysis.

Supplement

The overall aim of the study was to assess and improve the status of critical nutrients such as numerous vitamins and minerals and to investigate the effects on metabolism, well-being and immune function. As the supply of long-chain omega-3 fatty acids is also unfavorable in elderly people, omega-3 fatty acids have also been supplemented. Accordingly, participants in the intervention group were required to take two different micronutrient supplement capsules per day over the duration of 12 weeks. One capsule contained the following micronutrients: vitamin A:400 µg retinol equivalent (RE), cholecalciferol 50 µg, tocopherol 18 mg (α-TE), vitamin K 30 µg, ascorbic acid 200 mg, thiamine 1.65 mg, riboflavin 2.1 mg, niacin 16 mg niacin equivalent (NE), pyridoxine 2.1 mg, folic acid 400 µg, cobalamin 100 µg, biotin 50 µg, pantothenic acid 6.0 mg, zinc 10 mg, selenium 100 µg, chrome 40 µg, molybdenum 50 µg, iodine 100 µg. The second capsule contained 1.0 g long chain omega-3 fatty acids. Two placebo preparations were administered. The placebo group had to consume one capsule consisting of 500 mg maltodextrin and one capsule with 1.0 g evening primrose oil.

Blood sampling

Blood samples were collected between 08:00 and 11:00 a.m. at baseline and final examination after overnight fasting (minimum 12 h fasting period). If possible, participants were invited to t12 at the same time of the day as for the t0 examination. Blood samples were obtained by venipuncture of the arm vein using Safety-Multifly® needles (Sarstedt, Nümbrecht, Germany) into serum, EDTA, or S-Monovettes® for tHcy (Sarstedt). All samples were stored at approximately 5 °C and transferred to the laboratory on the same day.

Biochemical analyses

All biomarkers were determined in accredited and certified laboratories (LADR Laborärztliche Arbeitsgemeinschaft, Hannover, Germany; SYNLAB MVZ, Leinfelden, Germany). Cobalamin and Holo-TC levels were determined in serum using the electrochemiluminescence immunoassay method (ECLIA) on cobas® test systems (Roche Diagnostics GmbH, Mannheim, Germany) [57, 58].

Liquid chromatography coupled with mass spectrometry (LC-MS/MS) was performed out [59]. Plasma Hcy was determined by high-performance liquid chromatography (HPLC) with a fluorescence detector [60]. Folate in RBCs was analyzed using ECLIA on Immulite 2000 analyzer series (Diagnostic Products Corporation, Los Angeles, USA) in SYNLAB MVZ (Leinfelden, Germany) [61]. Creatinine was determined enzymatically from the plasma samples (Beckman Coulter AU analyzer).

To determine the cobalamin biostatus, the aggregated marker 4cB12 was calculated based on Holo-TC, serum cobalamin, MMA and Hcy levels, according to the following formula [62]:

$$4cB12 = \log 10\left(\frac{\text{HoloTC} \times \text{B12}}{\text{MMA} \times \text{tHcy}}\right) - \left(\frac{3.79}{1 + (\frac{\text{age}}{230})^{2.6}}\right)$$

Holo-TC and serum cobalamin levels were reported in pmol/L and Hcy and MMA levels were standardized to µmol/L.

Reference levels

The following cut-off points were used for cobalamin deficiency: 150 pmol/L for serum cobalamin and 50 pmol/L for Holo-TC, as suggested in recent studies [12, 39, 63]. MMA levels >270 nmol/L [39] und Hcy levels ≥15 µmol/L [9, 12, 64, 65] were considered elevated. In addition, a cut-off of 15 µmol/L is recommended for Hcy analysis methods with HPLC [21], whereas other cut-offs are applied for other methods. The reference levels for RBC folate are highly dependent on the laboratory-specific assay used [66]. According to the manufacturer's instructions (Diagnostic Products Corporation, Los Angeles, USA), a reference range of 570–1810 nmol/L was specified for the RBC folate analysis method carried out. Consequently, RBC folate levels <570 nmol/L indicate folate deficiency in the current study population.

Statistical analysis

Statistical analyses were performed using SPSS software (IBM SPSS Statistics 28.0; Chicago, IL, USA). Continuous variables are shown as mean±standard deviation (SD), while qualitative variables are presented either as absolute or relative frequencies, or only in relative figures. The Shapiro-Wilk test was used to test for normal distribution [67]. In addition, quantile-quantile plots were created for visual inspection. Intention-to-treat analysis was performed to avoid potential bias owing to the exclusion of subjects. The Student's t-test was used to compare variables in the case of normally distributed data. If logarithmic data transformation failed to obtain a normally distributed dataset, the Mann-Whitney U test was performed to test for differences in non-normally distributed data. The chi-squared test was used to determine the distribution of nominal variables between the intervention and placebo groups. Finally, to assess differences between the two groups after the intervention, one-way analysis of variance (ANOVA) was used. To assess the primary intervention effect, the variable Hcy was explored in an analysis of covariance (one-way ANCOVA), controlling for the covariates of age and sex. Linear regression models were used to detect associations between micronutrient biostatus at baseline and the intervention effect, adjusted for age, sex, BMI and creatinine level. For all analyses, statistical significance was set at p levels <0.05.

Results

The baseline characteristics of the study participants are presented in Table 1. In total, 133 home-dwelling elderly participants between 70 and 100 years of age were included in the study. The study population had a higher proportion of women (72.9%). Participants had a mean age of 75.4 ±4.5 years with an average BMI of 25.7±4.6 kg/m^2. Participants were mostly physically active (regular basis movement, 70.1%; predominantly active, 19.7%; predominantly sedentary, 10.2%; data not shown). Moreover, 90.7% of the participants reported a good to excellent health status and less than 4% were current smokers. Noticeable differences between the intervention and placebo groups were detected only in systolic blood pressure (p=0.044).

The evaluation of the questionnaires showed that the participants did not change their nutritional behavior and lifestyle, including movement behavior, over the intervention period.

Levels of serum cobalamin, Holo-TC, RBC folate, MMA and calculated 4cB12

At the baseline examination, serum cobalamin levels did not differ between the intervention and placebo groups (intervention group: 193±70.8 pmol/L; placebo group: 213±111 pmol/L; p=0.496; Table 2). 26.6% of subjects in the intervention group were deficient according to the cut-off level of 150 pmol/L while in the placebo group, the prevalence was 28.4% (Table 3). For Holo-TC, no difference between groups could be observed at baseline (intervention group: 87.3±32.5 pmol/L; placebo group: 91.1±34.9 pmol/L; p=0.572; Table 2). 12.3% of the participants in the intervention group and 11.8% in the placebo group, Holo-TC levels below the cut-off of 50 pmol/L (Table 3). In accordance, Using the aggregated marker 4cB12 showed that the cobalamin biostatus was predominantly adequate (intervention group: 87.9%, placebo group: 85.3%; Table 3). No significant difference was found between the groups at baseline (p=0.661).

Mean RBC folate baseline levels were also above the threshold for deficiency (<570 nmol/L) and did not differ between the groups (intervention group: 792±212 nmol/L; placebo group: 872±265 nmol/L; p=0.067; Table 2). 9.1% of subjects in the intervention group and 10.3% of the placebo group, RBC folate levels that can be classified as folate deficient (Table 3). With regard to MMA, no statistically significant differences between the two groups were observed at baseline. Mean MMA levels were below the cut-off level of 270 nmol/L (intervention group: 259.9±85.0 nmol/L; placebo group: 286.3±111.2 nmol/L; p=0.469; Table 2). However, 30.8% of subjects in the intervention group and 44.1% in the placebo group had elevated MMA levels (Table 3).

Cobalamin and Holo-TC levels significantly increased in the intervention group after 12 weeks of micronutrient supplementation, whereas no changes were observed in the placebo group (Figure 2, Table 2). Moreover, the prevalence

Table 1. Baseline characterization of the study population

	Total n=133	Intervention group n=65	Placebo group n=68	
	n (%)	n (%)	n (%)	p
Gender				
Female	97 (72.9)	47 (72.3)	50 (73.5)	0.874
Male	36 (27.1)	18 (27.7)	18 (26.5)	
Age groups				
70–74 y	61 (45.9)	34 (52.3)	27 (39.7)	
75–79 y	50 (37.6)	22 (33.8)	28 (41.2)	0.336
≥80 y	22 (16.5)	9 (13.9)	13 (19.1)	
	Mean ± SD	Mean ± SD	Mean ± SD	
Age (y)	75.4 ± 4.5	75.8 ± 5.0	76.1 ± 4.0	0.098[a]
Weight (kg)	70.3 ± 13.9	70.4 ± 13.8	70.3 ± 14.1	0.995[a]
Body mass index (kg/m^2)	25.7 ± 4.6	25.5 ± 4.1	25.8 ± 5.0	0.868[a]
Waist circumference (cm)	92.6 ± 12.3	92.9 ± 11.7	92.4 ± 12.9	0.812[b]
Waist-hip ratio	0.9 ± 0.1	0.9 ± 0.1	0.9 ± 0.1	0.568[a]
Blood pressure (mmHg), n=132				
Systolic	144 ± 16.6	142 ± 15.8	147 ± 17.0	**0.044***[a]
Diastolic	84.6 ± 11.0	84.3 ± 9.8	84.9 ± 12.2	0.748[b]
Pulse rate (per minute), n=131	66.7 ± 9.6	65.8 ± 9.6	67.5 ± 9.6	0.372[b]
Hcy (μmol/l)	18.2 ± 6.7	17.6 ± 7.1	18.9 ± 6.1	0.093[a]
70–74 y	17.3 ± 6.5	16.7 ± 6.8	18.0 ± 6.1	0.360[a]
75–79 y	18.0 ± 5.3	17.5 ± 5.2	18.4 ± 5.6	0.755[a]
≥80 y	21.4 ± 6.7	20.7 ± 11.7	21.9 ± 6.8	0.209[a]

Years (y) of homocysteine (Hcy). Group differences were determined using the chi-square test, unless otherwise stated. [a]Mann-Whitney U test, [b]Student's t-test (for independent samples). *Statistically significant differences between groups.

Table 2. Mean levels of Hcy, serum cobalamin and folate biostatus markers before and after the intervention

	Intervention group		Placebo group			
Time	n	Mean ± SD	n	Mean ± SD	Effect size (95% CI)	p
Hcy (μmol/L)						
t0	65	17.6 ± 7.2	68	18.9 ± 6.1		0.067[a]
t12	62	13.6 ± 3.0	66	18.5 ± 5.2	0.101	**<0.001*[b]**
MMA (nmol/L)						
t0	65	260 ± 85.0	68	286 ± 111		0.469[c]
t12	62	241 ± 64.2	66	288 ± 132	0.067	**0.003*[d]**
Serum cobalamin (pmol/L)						
t0	65	193 ± 70.8	68	213 ± 101		0.496[a]
t12	62	240 ± 85.3	66	189 ± 85.8	0.376	**<0.001*[d]**
Holo-TC (pmol/L)						
t0	65	87.3 ± 32.5	68	91.1 ± 34.9		0.572[a]
t12	62	104 ± 31.3	66	87.3 ± 33.6	0.280	**<0.001*[d]**
4cB12						
t0	65	−0.07 ± 0.4	68	0.07 ± 0.5		0.962[a]
t12	62	0.24 ± 0.4	66	−0.14 ± 0.5	0.055	**<0.001*[d]**
RBC folate (nmol/L)						
t0	53[e]	792 ± 212	64[e]	872 ± 265		0.208[a]
t12	53[e]	1124 ± 223	64[e]	870 ± 252	0.391	**<0.001*[d]**

Homocysteine (Hcy), methylmalonic acid (MMA), holo-transcobalamin (Holo-TC), 4cB12=aggregated marker for cobalamin status calculated based on Holo-TC; serum cobalamin; MMA and Hcy levels, red blood cell folate (RBC folate). P levels reported using [a]Student's t-test (for independent samples), [b]one-way analysis of variance with sex and age as covariates (ANCOVA), [c]Mann-Whitney U Test, [d]one-way ANOVA. *Statistically significant difference between groups. [e]Technically inadequate samples predict a lower number of cases within RBC folate at t0.

Table 3. Prevalence of micronutrient deficiencies and elevated biomarker levels in intervention and placebo groups at baseline and after 12 weeks

	Time	Intervention group %	Placebo group %	p
Hcy				
Elevated (≥15 µmol/L)	t0	60.0	66.2	0.460
	t12	35.5	72.7	**<0.001***
MMA				
Elevated (>270 nmol/L)	t0	30.8	44.1	0.112
	t12	23.0	39.4	**0.046***
Serum cobalamin				
Deficient (≤150 pmol/L)	t0	26.6	28.4	0.818
	t12	12.9	33.8	**0.005***
Holo-TC				
Deficient (<50 pmol/L)	t0	12.1	11.8	0.923
	t12	1.6	10.6	**0.021***
RBC folate				
Deficient (<570 nmol/L)	t0	9.1	10.3	0.948
	t12	0	9.2	**0.014***
4cB12				
Elevated (>1.5)	t0	0	0	/
	t12	0	0	/
Adequate (−0.5 to 1.5)	t0	87.9	85.3	0.661
	t12	98.4	87.9	**0.022***
Low (−1.5 to −0.51)	t0	12.1	14.7	0.661
	t12	1.6	10.6	**0.022***
Possible deficiency (−1.51 to −2.5)	t0	0	0	/
	t12	0	1.5	/
Probable deficiency (<−2.5)	t0	0	0	/
	t12	0	0	/

Homocysteine (Hcy), methylmalonic acid (MMA), holo-transcobalamin (Holo-TC), red blood cell folate (RBC folate) and 4cB12=aggregated marker for cobalamin status calculated based on Holo-TC, serum cobalamin, MMA and Hcy levels. Group differences were assessed using the chi-squared test. *Statistically significant differences within groups.

of low Holo-TC levels decreased in the intervention group from 12.1% to 1.6%. Within the placebo group, we observed a decrease from 14.7% to 10.6% (Table 3).

Similarly, RBC folate levels were significantly increased in the intervention group (p<0.001; Figure 2, Table 2). After 12 weeks of intervention, no subject had folate deficiency based on RBC folate levels (Figure 2), while the mean RBC folate levels even dropped slightly in the placebo group (Table 2).

MMA levels were reduced by 7.4% on average in the intervention group (p=0.003), whereas no changes in the placebo group were observed (Table 2). The change in MMA levels was significantly associated with baseline MMA (p<0.001), Holo-TC (p=0.010) and RBC folate (p=0.018) levels, but not with cobalamin levels (data not shown).

Plasma Hcy levels

At baseline, the mean plasma Hcy level in the study population was 18.2 µmol/L with wide ranges from 8.5 to 51.1 µmol/L (Table 2). The differences in plasma Hcy levels between the intervention and placebo groups were not significant (Figure 3a, Table 2). 63.2% of the total population was hyperhomocysteinemic with plasma Hcy levels ≥15 µmol/L, while no differences between the intervention group (60.0%) and placebo group (66.2%) could be observed (Table 3). Plasma Hcy levels were on average 3.1 µmol/L higher in men than in women (p=0.031) and differed significantly between the age group 70–74 y (17.3 ±6.5 µmol/L) and ≥80 y (21.4±6.7 µmol/L; p=0.025; Table 1). Using adjusted linear regression (adjusted model for sex, BMI and creatinine), plasma Hcy levels were found to be significantly associated with age (p=0.021, beta=0.201; data not shown), serum cobalamin (p=0.036; beta=−0.180) and Holo-TC (p=0.017; beta=−0.210), but not with RBC folate (p=0.411; beta=−0.074; Table E1 in Electronic Supplementary Material 1).

Table 4 displays the micronutrient biostatus at baseline in relation to the normal and elevated plasma Hcy levels. Overall, the prevalence of micronutrient deficiency and increased MMA levels was almost the same in the Hcy-subgroups, except for serum cobalamin. The prevalence of serum cobalamin deficiency was significantly higher in

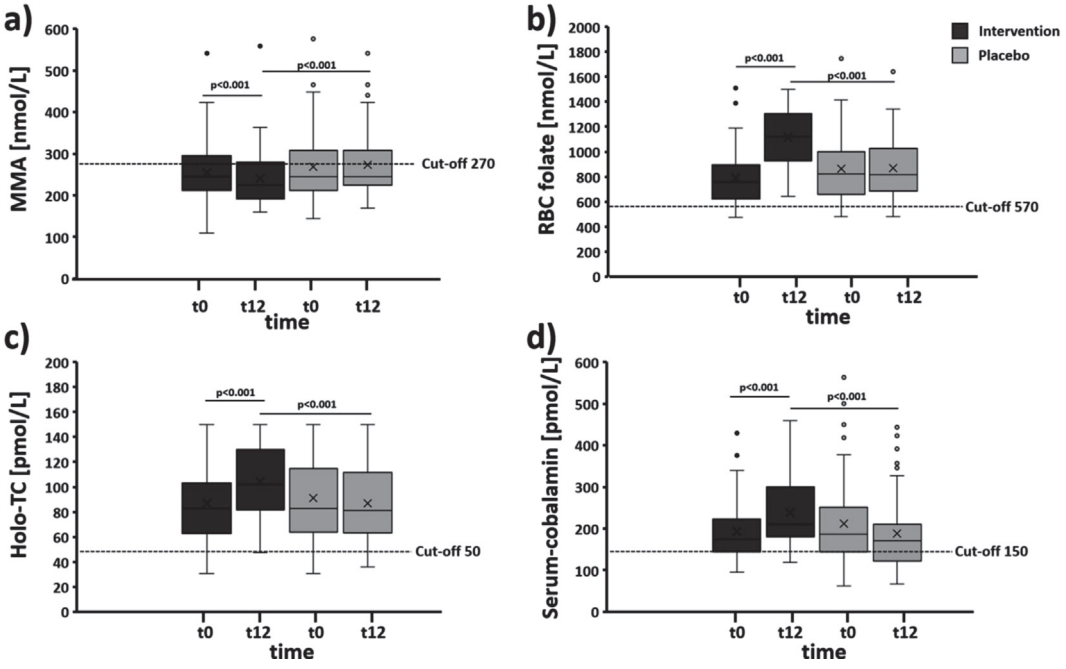

Figure 2. Levels of a) methylmalonic acid (MMA), b) red blood cell folate (RBC folate), c) holo-transcobalamin (Holo-TC), and d) serum cobalamin in the intervention and placebo group initial (t0) and after 12 weeks (t12) of intervention. a) Statistically significant difference between mean t0 and mean t12 MMA levels within the intervention group with p<0.001 and significant difference between mean t12 MMA levels of intervention group and mean t12 levels of placebo group with p=0.007, b) statistically significant difference between mean t0 and mean t12 RBC folate within the intervention group with p<0.001 and significant difference between mean t12 RBC folate of intervention group and mean t12 RBC folate of placebo group with p=0.001, c) statistically significant difference between mean t0 and mean t12 Holo-TC within the intervention group with p<0.001 and significant difference between mean t12 Holo-TC of intervention group and mean t12 Holo-TC of placebo group with p=0.001, d) statistically significant difference between mean t0 and mean t12 serum cobalamin within the intervention group with p<0.001 and significant difference between mean t12 serum cobalamin of intervention group and mean t12 serum cobalamin of placebo group with p=0.001. x: within the boxplot denotes the mean of the dataset.

subjects with elevated plasma Hcy levels (p=0.042). In addition, no significant difference between normal and elevated plasma Hcy levels was observed with respect to medical drug intake (p=0.668).

Discussion

In this study, we showed that an intervention with a micronutrient supplement, including cobalamin and folate, over 12 weeks significantly decreased elevated plasma Hcy levels in an elderly population that was mostly well supplied with cobalamin and folate even before intervention. Overall, there is a lack of studies on Hcy reduction with cobalamin and folate supplementation in participants aged ≥70 years. The mean plasma Hcy reduction was −3.6±5.7 μmol/L corresponding to a relative reduction of 23%.

The Hcy-reducing effect of micronutrient supplementation depends on many factors, which complicate the interpretation of intervention studies. Differences in the duration of the intervention time (weeks to months), the composition and dosage of the supplements and the characteristics of the population (e.g., age, baseline Hcy, biostatus of cobalamin, folate and pyridoxine; healthy vs. renal dysfunction) make it difficult to compare the study results and are the cause for varying effect sizes in plasma Hcy reduction. Moreover, the intake of micronutrients via the background diet, including fortified foods, varies significantly between countries. Certain foods in countries such as the US, Canada and South Africa are generally fortified with folate [68]. This makes it difficult to compare study results between different countries and underlines the necessity of measuring vitamin B biostatus with valid markers. All these factors have led to heterogeneous results in previous studies [34, 39]. The decrease in plasma Hcy in response to B vitamin supplementation, therefore, highly varied between 9.8% and 48.6% [48].

The observed mean plasma Hcy reduction of 23% was in line with results of previous studies. The decrease in Hcy with our micronutrient supplement is comparable to our previous work with younger subjects, where the Hcy-lowering effect was also dependent on initial plasma Hcy values [69]. We determined a significant and biologically relevant Hcy reduction in the study cohort with an average relatively good cobalamin and folate biostatus. This did not agree with

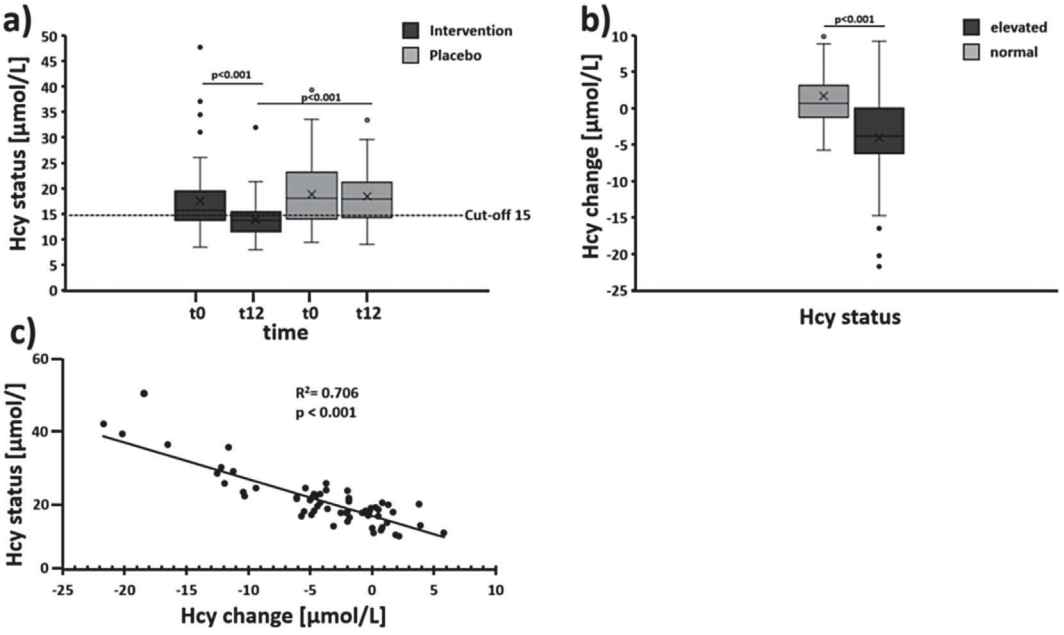

Figure 3. Plasma homocysteine (Hcy) levels in the intervention and placebo group. a) initial (t0) and after 12 weeks of intervention (t12). Statistically significant difference between mean t0 and mean t12 plasma Hcy levels within the intervention group with p<0.001 and significant difference between mean t12 plasma Hcy levels of intervention group and mean t12 plasma Hcy levels of placebo group with p=0.001 b) Change of plasma Hcy levels in subjects with normal (<15 µmol/L) and elevated (≥15 µmol/L) plasma Hcy levels at baseline differ statistically significant with p<0.001. c) Linear regression analysis (adjusted model for gender, BMI, creatinine and age) of plasma Hcy level decrease dependent on Hcy levels at baseline (beta=−0.955; r=0.741; p<0.001). x: within the boxplot denotes the mean of the dataset.

the cardinal principle of nutrition that only subjects with an initial deficient status benefit from supplementation [70]. However, previous studies also showed an Hcy-lowering effect in subjects without deficiencies, but these studies failed to analyze valid cobalamin and folate biomarkers, as in this study [71].

The prevalence of cobalamin and folate deficiency in our otherwise healthy study population is in line with the prevalence of vitamin B deficiency in comparable studies with elderly German subjects [45, 72]. In this study, we used long-term biomarkers, in particular, RBC folate, Holo-TC and the aggregated biomarker 4cB12, for the assessment of vitamin B supply, which, to the best of our knowledge, has not been used in any other trial to date. Therefore, there is no comparative data from other studies on the biostatus of Holo-TC and 4cB12. Moreover, many current Hcy-lowering trials failed to assess cobalamin and folate biostatus with valid long-term biomarkers, linked to the prevalence of deficiencies before and after intervention [73, 74, 75, 76].

The biostatus of cobalamin and folate is inversely correlated with Hcy plasma levels [77, 78]. However, we found that the majority of subjects in the present cohort demonstrated elevated plasma Hcy levels despite Holo-TC, 4cB12 and RBC folate levels being within the normal reference range. Of course, it should not be neglected that a significant amount of 12% (based on Holo-TC), 13% (based on 4cB12), or 10% (based on RBC folate) of the subjects had a cobalamin or folate deficiency and elevated Hcy levels. Only a few comparable studies have arrived at similar conclusions. De Koning et al. [73] have shown that healthy elderly people have elevated levels of plasma Hcy and MMA, whereas serum levels of related vitamins are within the normal range. Smith et al. [79] also reported sufficient cobalamin and folate biostatuses in an elderly study population using serum cobalamin and serum folate as biomarkers. After intervention with B vitamins, they found a difference in mean plasma Hcy levels of 31.7% between intervention and placebo.

One potential explanation for the Hcy-lowering effect, despite sufficient cobalamin and folate biostatus, might be age-related impairment in the activity of enzymes [especially methionine synthase or 5-, 10-methylenetetrahydrofolate reductase (MTHFR)] that are involved in the regeneration of Hcy to methionine [33, 80]. Similarly, additional folate may compensate for the restricted availability of 5-methyltetrahydrofolate in subjects with genetic MTHFR polymorphism [81]. Another explanation for the study effects could be that our micronutrient supplement included not only B vitamins but also antioxidants (e.g., ascorbic acid, tocopherol, zinc and selenium), cholecalciferol and omega-3 fatty acid. Plasma Hcy levels were reported to be inversely related to omega-3 supplementation [82]. To date, the physiological background of this

Table 4. Prevalence of cobalamin and folate deficiency, elevated MMA levels, and medical drug intake in subjects with normal and elevated plasma Hcy levels at baseline

	Normal Hcy (<15 μmol/L) n=49	Elevated Hcy (≥15 μmol/L) n=84	p
Serum cobalamin			
Deficient (≤150 pmol/L)	16.3%	32.5%	**0.042***
Holo-TC			
Deficient (<50 pmol/L)	16.3%	9.5%	0.245
4cB12			
Adequate (−0.5 to 1.5)	72.2%	62.0%	0.405
Low (−1.5 to −0.51)	27.8%	38.0%	
RBC folate			
Deficient (<570 nmol/L)	7.5%	3.0%	0.370
MMA			
Elevated (>270 nmol/L)	28.6%	45.3%	0.057
Medical drug intake			
Yes	79.2%	75.9%	0.668
No	20.8%	24.1%	

Homocysteine (Hcy), holo-transcobalamin (Holo-TC), 4cB12=aggregated marker for cobalamin status calculated based on Holo-TC; serum cobalamin; MMA and Hcy levels, red blood cell folate (RBC folate), and methylmalonic acid (MMA). The Hcy subgroups were formed from the total study population. Group differences were assessed using the chi-squared test. *Statistically significant differences between groups.

condition is not fully understood. Omega-3 fatty acids may induce enzymes involved in Hcy metabolism via modulation of gene expression [82]. Dawson et al. [83] reviewed the significant plasma Hcy-lowering effect associated with omega-3 fatty acids, but not all studies showed a positive change in plasma Hcy levels. In principle, this effect was more pronounced when omega-3 fatty acid supplementation was combined with vitamin B supplementation.

Moreover, supplementation with antioxidants could have a beneficial effect on the study outcomes. Breilmann et al. [84] examined the effect of antioxidant supplementation on ascorbic acid, tocopherol and β-carotene on plasma Hcy levels in an aging population. The authors reported that ascorbic acid levels were a relevant predictor of plasma Hcy levels; however, intervention with antioxidants was not associated with Hcy levels. An inverse relationship between antioxidants and elevated Hcy levels has also been reported by Floegel et al. [85]. Racek et al. [86] reported that antioxidant supplementation did not affect Hcy concentrations, but improved antioxidative defense and inhibited peroxidation. Further studies are necessary to clarify the effects of antioxidants on Hcy levels. However, the mechanism of action is unclear.

Several micronutrients are described to improve renal function [87, 88, 89]. Considering that older age is correlated with reduced kidney function, the decrease in Hcy might be partly explained by the regeneration of kidney function through micronutrient supplementation. Considering that the kidney plays an important role in Holo-TC metabolism, improved kidney function leads to reduced biological loss of Holo-TC via the urine [90].

Although Holo-TC and RBC folate are considered reliable biomarkers to reflect the biostatus of micronutrients in tissues [91, 92], there is debate that Holo-TC and RBC folate levels might not reflect the intracellular bioavailability of cobalamin and the metabolically active form of folate (5,10-methyltetrahydrofolate) [93]. Concerning cobalamin biostatus, the aggregated biomarker 4cB12, however, can be regarded as the most comprehensive marker of B12 deficiency, as this combined index has been described as more reliable than any single biomarker [51].

Besides the study supplement, other dietary or lifestyle-associated factors may also have potentially influenced Hcy metabolism during the intervention period. The participants were instructed not to change their diet, physical activity, or other lifestyle variables during the intervention. Nevertheless, it cannot be ruled out that mere participation in a nutrition study may have led to increased health awareness causing a plasma Hcy-lowering effect (e.g., lower coffee, protein, and alcohol consumption, methionine overload, less/no smoking and increased physical activity) [80, 94]. However, it is unlikely that these confounding variables occurred exclusively in the supplement group since the plasma Hcy levels remained almost constant in the placebo group. Moreover, according to self-disclosure using questionnaires, the study participants were compliant and no changes in these variables were found.

As described above, reduced kidney function is a major reason for elevated plasma Hcy levels in the elderly, since regeneration of Hcy mainly takes place in the kidney. Reduced renal function may be identified by elevated serum creatinine concentration [95]. However, the mean serum creatinine concentration in our study population was 0.88±0.24 mg/dL and thus, well below the cut-off level for an adverse renal function of >1.4 mg/dL. Therefore, the influence of kidney damage as a confounder was limited.

Nevertheless, we adjusted all relevant statistical models for creatinine level.

Drugs such as metformin or proton pump inhibitors may cause reduced micronutrient availability and may directly interact with Hcy metabolism [37, 96]. We could not identify an association between any medical drug intake and the measured plasma Hcy levels. Medical drugs such as metformin or proton pump inhibitors were only taken by a few subjects (7% of the total population), which is why a systemic influence on the plasma Hcy level can be ruled out. Ham et al. [97] observed that the clinical influence of medication on plasma Hcy levels was smaller than generally assumed.

Strengths and limitations

The strength of the study is the clear and fairly well-characterized cohort, especially considering the applied state-of-the-art biomarkers to evaluate cobalamin and folate biostatus. This study has several potential limitations. We did not analyze genetic mutations in the Hcy-metabolizing enzymes. In detail, isoforms of MTHFR cause reduced folate metabolism and therefore require a higher dose of folate to normalize elevated Hcy levels [98, 99]. However, in our study, the prevalence of low RBC folate levels (<570 nmol/L) was low, so we cannot expect a high influence of this genetic mutation in our population since the prevalence of the heterozygous genotype is approximately 10% anyway [100].

The biostatuses of pyridoxine and riboflavin were not evaluated. Since both vitamins are also involved in Hcy metabolism and are associated with plasma Hcy levels [101, 102], the influence of the pre-interventional supply biostatus of these two vitamins on the plasma Hcy level (e.g., different pyridoxine and riboflavin baseline levels between the verum and placebo groups) cannot be excluded. However, the existing literature indicates that Hcy lowering with pyridoxine is only effective under exceptional circumstances (e.g., methionine overload and severe pyridoxine deficiency) but with a small response [103]. Riboflavin biostatus appears to be a potent modulator in cases of genetic polymorphisms of MTHFR [101, 104]. Thus, there is evidence that the correlation between these vitamins and Hcy levels is considerably low [105, 106]. Consequently, pyridoxine and riboflavin play minor roles in the development of hyperhomocysteinemia in the elderly population. Furthermore, it should be noted that the supplement included both vitamins.

Conclusion

Micronutrient intervention, including several B vitamins, significantly lowered elevated plasma Hcy levels in an elderly population without B vitamin deficiency. Contrary to expectations based on the literature, micronutrient biostatus did not appear to be a determinant of Hcy-lowering effects in this elderly population. At least the studied population of elderly subjects with elevated plasma Hcy levels could benefit from multinutrient supplementation regardless of their nutritional status. The clinical relevance of these findings needs to be investigated in further studies, which should focus on subjects without vitamin B deficiencies. Further investigations are needed to determine the causes of elevated plasma Hcy levels despite the absence of cobalamin and folate deficiencies.

Electronic Supplementary Material

The electronic supplementary material (ESM) is available with the online version of the article at https://doi.org/10.1024/0300-9831/a000777

ESM 1. Linear regression analysis of Hcy levels and levels of serum cobalamin, Holo-TC, and RBC folate at baseline (Table E1).

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Informed Consent Statement
Informed consent was obtained from all subjects involved in the study.

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