

# Response of Homocysteine, Cystathionine, and Methylmalonic Acid to Vitamin Treatment in Dialysis Patients

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**Background:** Hyperhomocysteinemia is observed in >80% of hemodialysis patients and is considered a risk factor for cardiovascular disease. Vitamin treatment lowers total homocysteine (tHcy) concentrations in plasma and may therefore reduce the associated risk. Current treatment strategies have not achieved normalization of tHcy in the majority of dialysis patients.

**Methods:** We administered folic acid (5 mg) plus vitamin B<sub>6</sub> (50 mg) and B<sub>12</sub> (0.7 mg) intravenously to 38 hyperhomocysteinemic patients (tHcy >18 μmol/L) after each dialysis treatment. The treatment phase lasted 1 month, and serum concentrations of tHcy, methylmalonic acid (MMA), and cystathionine were measured at weeks 0, 2, 4, 6, 8, and 24.

**Results:** The median serum tHcy concentration decreased significantly, from 26.1 μmol/L at baseline to 13.2 μmol/L at week 4. The median change in tHcy after 4 weeks was 13.4 μmol/L (–51%) compared with baseline. Serum MMA and cystathionine concentrations were reduced by 28% and 26%, respectively, but neither was normalized at 4 weeks. Backward-elimination stepwise regression analysis revealed that higher concentrations of tHcy, MMA, and cystathionine and lower folate at baseline predict changes of tHcy after treatment. Twenty weeks after vitamin withdrawal, tHcy concentrations returned to values comparable to baseline (median, 24.8 μmol/L).

**Conclusions:** The combination of folic acid, vitamin B<sub>12</sub>, and vitamin B<sub>6</sub> used in this study normalized serum concentrations of tHcy in almost all of our hyperhomocysteinemic dialysis patients. This regimen may be used

to investigate the effects of homocysteine normalization on cardiovascular outcomes in hemodialysis patients.

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Increased concentrations of total homocysteine (tHcy)<sup>4</sup> in plasma (>18.0 μmol/L) are very common in patients with end stage renal disease (ESRD) (1–3). Increased tHcy is a risk factor for atherosclerotic vessel diseases in hemodialysis patients (4–6). An accelerated rate of cardiovascular morbidity and mortality has been described in ESRD patients (7), which has been linked to increased concentrations of tHcy (6, 8, 9). Homocysteine-lowering treatment could be an attractive and inexpensive therapeutic strategy for reducing the burden of vascular diseases in uremic patients (10).

Homocysteine is irreversibly metabolized to either methionine or cystathionine. Excess tHcy in plasma is also a proxy measure of inadequate folate, vitamin B<sub>12</sub>, or vitamin B<sub>6</sub> status (3). Additionally, the tHcy concentration is strongly related to indicators of renal function (3). Folic acid is a potent tHcy-lowering agent that has been shown to induce a 16–40% decrease in tHcy in dialysis patients (11–13). Data regarding an additional effect of vitamin B<sub>12</sub> or B<sub>6</sub> administered to dialysis patients are not consistent (13–18). The addition of vitamin B<sub>12</sub> (subcutaneous or intravenous) induced a sizeable decrease in tHcy in folate-replete patients (12, 13). In contrast, tHcy remained unchanged when vitamin B<sub>12</sub> was administered orally or as injections given at wide intervals (14, 15).

Although tHcy concentrations were lowered in most studies, they were not normalized in the majority of ESRD patients (16–19). Considering the dose–response relationship, much higher doses of the vitamin are required in ESRD patients than in individuals with normal renal function to significantly lower tHcy. We investigated the response of tHcy, cystathionine, and methylmalonic acid

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<sup>4</sup> Nonstandard abbreviations: tHcy, total homocysteine; ESRD, end stage renal disease; and MMA, methylmalonic acid.

(MMA) to a 1-month treatment with therapeutic doses of the B vitamin in dialysis patients who initially had hyperhomocysteinemia. In addition, we studied serum concentrations of these metabolites over a 20-week withdrawal phase after stopping the therapy.

### Materials and Methods

#### PATIENTS

Patients were recruited from the Department of Medicine, Division of Nephrology, of the University Hospital of Saarland, Germany. Inclusion criteria were renal patients who had been undergoing hemodialysis for at least 3 months and had a baseline tHcy concentration  $>18 \mu\text{mol/L}$ . Exclusion criteria included short life expectancy, recent stroke, thrombosis or myocardial infarction ( $< 3$  months), and the use of any vitamin treatment in the last 4 weeks before the intervention. No patient had received antifolate or antiepileptic medications. All patients underwent hemodialysis three times a week for a duration of 4 h each. The dialysis membrane was kept constant during the study. The dialysis dose was controlled by an equilibrated double-pool  $Kt/V$  value, which was considered as an indicator for the dialysis dose during the intervention. Patient characteristics and the underlying kidney diseases are shown in Table 1. The study was approved by the Medical Ethical Committee of the University of Saarland, and all patients gave written informed consent to the study.

#### STUDY DESIGN

The study protocol is depicted in Fig. 1 of the Data Supplement that accompanies the online version of this article at <http://www.clinchem.org/content/vol51/issue1/>. In a preliminary phase, tHcy concentrations were measured in 75 patients, 60 of whom had tHcy  $>18 \mu\text{mol/L}$ . Thirty-eight patients met the inclusion criteria and agreed to participate in the study. One month after tHcy screening, patients received 5 mg of folic acid plus 0.7 mg of B<sub>12</sub> and 50 mg of B<sub>6</sub> intravenously at the end of each dialysis session (i.e., three times a week). The first phase of the study (treatment phase) was 1 month in duration and was followed by a 5-month withdrawal period, during which study medications were withheld. After treatment was stopped, the concentrations of tHcy and the other metabolites were followed once every 2 weeks for 1 month, and a final measurement was made after 5 months of withdrawal. None of the patients complained of side effects that were judged to be related to the vitamin intervention. Two patients were hospitalized 2 and 6 weeks after starting the intervention, and both were lost from follow-up. Two patients died, and one underwent renal transplantation in the withdrawal phase. Blood samples were missing on one occasion from five additional patients during the withdrawal phase who were temporarily out of the area for private reasons. All

**Table 1. Patient characteristics and serum markers at start of B-vitamin therapy.**

Characteristics	Value <sup>a</sup>
Patients, n	38
Age, years	64 (45/81)
Female, n (%)	18 (47)
Diabetes, n (%)	16 (42)
Hypertension, n (%)	21 (55)
Coronary vascular disease, n (%)	19 (50)
Duration of dialysis, months	30 (4/82)
$Kt/V$ , single pool	1.13 (0.85/1.46)
Hemoglobin, g/L	115 (98/137)
Hematocrit, %	34.6 (28.5/40.4)
Underlying kidney disease, n	
Diabetic nephropathy	13
Hypertension/renovascular disease	6
Polycystic kidney disease	4
Chronic glomerulonephritis	4
Other types of glomerulonephritis	2
Systemic lupus erythematosus	2
Reflux nephropathy	1
Others	6

<sup>a</sup> Data are the median (10th/90th percentiles) unless otherwise specified.

measurements were available on all occasions in 28 patients.

#### BLOOD COLLECTION AND LABORATORY PROCEDURES

Nonfasting predialysis blood samples were obtained at each time point for the measurement of the metabolites. Blood samples were immediately chilled on ice and centrifuged for 10 min at 2000g and 4 °C within 1 h of collection. Serum samples were stored at  $-70 \text{ }^\circ\text{C}$  until further analysis. Serum concentrations of tHcy, cystathionine, and MMA were measured by gas chromatography–mass spectrometry as described elsewhere (20, 21). The concentrations of vitamin B<sub>12</sub> and folate were determined in serum by a chemiluminescence immunoassay (ADVIA Centaur System; Bayer). Serum concentrations of vitamin B<sub>6</sub> (pyridoxal-5-phosphate) were determined by HPLC with fluorescence detection using reagents from Immundiagnostik.

#### STATISTICAL ANALYSIS

Data analyses were performed with the software package SPSS (Ver. 11.0). Continuous variables were examined for normality by use of the Kolmogorov–Smirnov test. All continuous variables were skewed and were therefore log-transformed before tests that assume gaussian distribution were used. The effectiveness of the therapy was analyzed by repeated-measures ANOVA. Post hoc comparisons were performed with Bonferroni adjustment for multiple comparisons. The individual relative changes in tHcy caused by the intervention were calculated by dividing individual absolute changes by the corresponding baseline values:

$\Delta$ [tHcy, cystathionine, or MMA]

$$= \frac{(\text{concentration at any given week} - \text{baseline concentration})}{\text{baseline concentration}} \times 100$$

Backward-elimination stepwise regression analysis was performed to identify factors that influenced changes in tHcy and cystathionine concentrations at the end of the intervention (at 4 weeks). The statistical analysis was performed according to the "intention to treat" principle, and  $P < 0.05$  was considered statistically significant.

## Results

### BASELINE CONCENTRATIONS OF METABOLITES AND VITAMINS

Serum concentrations of the metabolites on two independent occasions within 1 month before starting the therapy are shown in Table 2. Serum concentrations of the vitamins were measured only at the start of therapy (Table 2). Pretreatment concentrations of folate ( $<7$  nmol/L), vitamin B<sub>6</sub> ( $<17.4$  nmol/L), and vitamin B<sub>12</sub> ( $<156$  pmol/L) were low in 11%, 19%, and 11% of our patients, respectively. Conversely, all patients had increased serum concentrations of the three metabolites (Table 2). We found strong correlations between serum concentrations of tHcy and MMA at the screening visit and those at the start of therapy (tHcy,  $r = 0.78$ ; MMA,  $r = 0.79$ ;  $P < 0.001$ ). The correlation between cystathionine concentrations at screening and at the start of therapy was also significant ( $r = 0.46$ ;  $P = 0.016$ ).

### CHANGES IN METABOLITE CONCENTRATIONS DURING AND AFTER TREATMENT

Baseline concentrations of tHcy, cystathionine, and MMA were defined as the mean of the two pretreatment measurements for each patient (concentration at screening and that before the first injection). Median tHcy decreased from 26.1  $\mu\text{mol/L}$  at baseline to 13.2  $\mu\text{mol/L}$  at the end of the intervention. A substantial reduction was already evident 2 weeks after the start of therapy (Fig. 1; see also Table 1 in the online Data Supplement). Compared with concentrations at baseline, tHcy was decreased by 50% and 51% at 2 and 4 weeks, respectively. At the end of the intervention, 39% of the patients had tHcy  $<12.0$   $\mu\text{mol/L}$

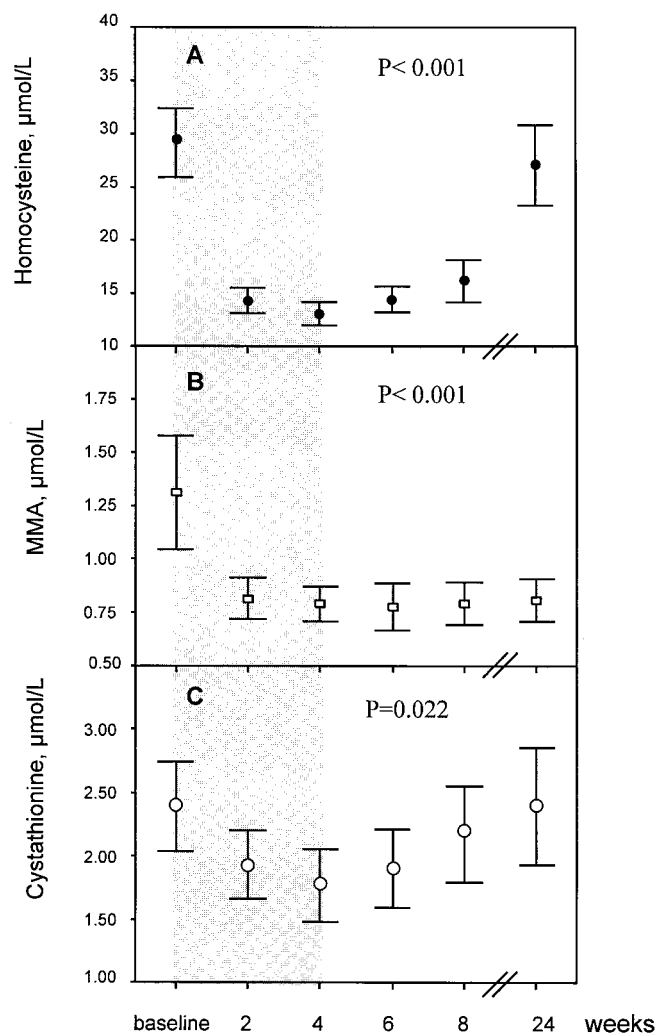


Fig. 1. Mean (error bars, 2 SE) concentrations of tHcy (A), MMA (B), and cystathionine (C) during the treatment and in the withdrawal phase. The shaded area indicates the treatment phase.

and 72% had concentrations  $<15.0$   $\mu\text{mol/L}$ . The extent of decrease in homocysteine was proportional to the baseline concentrations [Spearman correlation coefficient ( $r$ ), 0.92;  $P < 0.001$ ; Fig. 2 in the online Data Supplement]. At the end of the intervention,  $\Delta$ tHcy was higher in patients who had baseline tHcy  $>27.6$   $\mu\text{mol/L}$  compared with

**Table 2. Pretreatment concentrations of the metabolites and vitamins.<sup>a</sup>**

	Screening visit	Before first injection	Difference <sup>b</sup>
tHcy, $\mu\text{mol/L}$	26.7 (19.2/44.4)	25.7 (19.5/49.2)	-0.40 (-5.6/8.4)
Cystathionine, $\mu\text{mol/L}$	1.96 (0.90/3.55)	2.13 (1.25/4.79)	0.19 (-1.84/2.05)
MMA, $\mu\text{mol/L}$	1.00 (0.67/1.84)	1.15 (0.71/2.72)	0.19 (-0.08/0.83)
Folate, nmol/L		21.6 (6.5/100.9)	
Vitamin B <sub>12</sub> , pmol/L		253 (149/517)	
Vitamin B <sub>6</sub> , nmol/L		38.9 (12.1/160.4)	

<sup>a</sup> Data are median (10th/90th percentiles).

<sup>b</sup> Concentration at start of therapy - concentration at screening. The screening and the first injection of vitamin supplements were 1 month apart.

patients whose tHcy was  $<24.0 \mu\text{mol/L}$  (median  $\Delta\text{tHcy} = 64\%$  vs  $34\%$ ;  $P < 0.001$ ).

Although  $\Delta\text{MMA}$  was significant, it was less than  $\Delta\text{tHcy}$  (Fig. 1; see also Table 1 in the online Data Supplement). Thereafter, MMA concentrations decreased further after therapy was stopped and were maintained during the whole duration of the follow-up (5 months; Fig. 1; see also Table 1 in the online Data Supplement). Compared with the baseline concentrations, median  $\Delta\text{MMA}$  was 25% at 2 weeks, 28% at 4 weeks, and 34% at 6 weeks. Normalization of MMA (serum concentration  $<0.27 \mu\text{mol/L}$ ) was not achieved in any patient. The change of MMA at the end of the intervention correlated strongly and significantly with baseline concentrations ( $r = 0.88$ ;  $P < 0.001$ ; Fig. 2 in the online Data Supplement). On the other hand, the response of cystathionine to treatment was smaller than that of tHcy and MMA (Table 1 in the online Data Supplement). The decrease was significant compared with baseline only at the end of the intervention phase (at 4 weeks; median,  $2.18$  vs  $1.65 \mu\text{mol/L}$ ;  $\Delta\text{cystathionine} = 26\%$ ). Unlike tHcy and MMA, cystathionine increased markedly after treatment was stopped (Fig. 1).

#### BASELINE FACTORS AFFECTING RESPONSE TO TREATMENT

The highest  $\Delta\text{tHcy}$  (64%) at the end of the therapy was in patients whose  $\Delta\text{cystathionine}$  and  $\Delta\text{MMA}$  were also the highest (Fig. 3 in the online Data Supplement). Backward-elimination stepwise-regression analysis revealed that pretreatment tHcy concentration was the strongest predictor of  $\Delta\text{tHcy}$ , followed by the baseline concentrations of MMA, cystathionine, and folate in that order (Table 2 in the online Data Supplement). Neither the concentration of vitamin  $\text{B}_{12}$  nor that of  $\text{B}_6$  at baseline was a significant factor in determining  $\Delta\text{tHcy}$  resulting from the therapy. On the other hand, the strongest predictors of  $\Delta\text{cystathionine}$  were the concentrations of cystathionine and vitamin  $\text{B}_6$  at baseline. The regression analysis included all possible confounding factors, such as, age, sex,  $Kt/V$ , type of dialysis membrane, the presence of hypertension, and history of cardiovascular disease. None of these factors had a significant influence on  $\Delta\text{tHcy}$  resulting from the therapy (data not shown).

#### Discussion

Interest in lowering tHcy in dialysis patients has recently increased with the growing emphasis on disease prevention (22, 23). Conclusive evidence from prospective studies that tHcy-lowering treatment may reduce vascular disease in the dialysis population is still awaited. Very low concentrations of some nutritional markers, such as cholesterol, creatinine, albumin, and probably homocysteine, may be inversely or paradoxically related to poor outcome in dialysis patients. Conditions related to undernutrition in dialysis patients, such as hypoalbuminemia, have suggested that the "reverse epidemiology" may

contribute to differences among results from different trials (24). In recent years, various combinations and doses of the B vitamins have been tested in ESRD patients, but normalization of tHcy was rarely achieved.

The most notable finding in our study is that at the end of the intervention, 72% of the patients had tHcy concentration  $<15 \mu\text{mol/L}$  and only three patients had tHcy concentrations between 15 and  $20 \mu\text{mol/L}$ . Homocysteine concentrations decreased by a median of 50% after only 2 weeks of therapy (Table 2 in the online Data Supplement). Thereafter, prolonged vitamin treatment had no appreciable tHcy-lowering effect. In line with previous reports, tHcy increased after the cessation of vitamin supplementation (11, 19). Because residual renal function is negligible in dialysis patients and the dialysis dose and conditions were constant during the trial, the decrease in tHcy during the study was most likely related to improved metabolism. This suggestion is supported by the finding that pretreatment tHcy, MMA, and cystathionine were significant predictors of the response of tHcy to treatment (Table 2 in the online Data Supplement).

Decreases in MMA concentrations after vitamin  $\text{B}_{12}$  supplementation are a metabolic sign of restored cellular vitamin  $\text{B}_{12}$  status (25). The residual increase in MMA at the end of the therapy (median slightly  $>0.70 \mu\text{mol/L}$ ) is probably related to renal dysfunction (26). The mean baseline serum concentration of vitamin  $\text{B}_{12}$  was  $214 \text{ pmol/L}$  in patients who showed the largest decrease in MMA at 4 weeks ( $n = 15$ ; mean  $\Delta\text{MMA} = -1.04 \mu\text{mol/L}$ ; range,  $-0.34$  to  $-4.01 \mu\text{mol/L}$ ). Pretreatment  $\text{B}_{12}$  was  $<156 \text{ pmol/L}$  in only 3 of these 15 patients. Obviously, the prolonged subcellular vitamin  $\text{B}_{12}$  deficiency was profound enough to cause a metabolic dysfunction, indicated by increased MMA, which was in the range usually associated with neurologic complications (27). Vitamin  $\text{B}_{12}$  is required for efficient utilization of 5-methyltetrahydrofolate in the conversion of tHcy into methionine. Vitamin  $\text{B}_{12}$  deficiency causes retention of 5-methyltetrahydrofolate (28). Accordingly, serum folate concentrations decreased in vitamin  $\text{B}_{12}$ -deficient ESRD patients treated with vitamin  $\text{B}_{12}$  (17).

An important observation in this study is that tHcy increased significantly after treatment was stopped; however, MMA concentrations were maintained during the withdrawal phase. The estimated storage capacity for vitamin  $\text{B}_{12}$ , especially in the liver, is high (29), and depletion of the vitamin takes a long time to become metabolically or clinically expressed (30). Vitamin  $\text{B}_{12}$  deficiency in renal patients has been related to a reduced cellular uptake of the vitamin (31). Considering that we used an appreciably higher dose of  $\text{B}_{12}$  than in previous studies (17, 26), prolonged exposure of the tissues to a supraphysiologic amount of vitamin  $\text{B}_{12}$  may be an additional factor that could have influenced the long-term response of MMA. Conversely, folic acid is a small molecule that may diffuse and be lost through the dialysis filter. In addition, the storage capacity for folate is much

lower than that for B<sub>12</sub>, and accelerated depletion of folate is expected when ingestion or folate therapy is stopped (11). Therefore, in the presence of an adequate vitamin B<sub>12</sub> status at the end of the washout phase, folate became a rate-limiting factor, which may partly explain the increase in tHcy after the vitamin therapy was stopped.

Cystathionine is highly increased in renal patients, and its response to vitamin treatment is minimal compared with the other two metabolites, tHcy and MMA (3, 19, 32). Cystathionine accumulation in renal patients may be related to an accelerated rate of production in addition to a reduction in its rate of removal into cysteine. In this study, we observed a higher  $\Delta$ cystathionine in patients with lower vitamin B<sub>6</sub> at the start of therapy (Table 2 in the online Data Supplement). Therefore, the decrease in cystathionine can be rationalized as an improvement in its irreversible disposal via cystathioninase. Vitamin B<sub>6</sub> deficiency inhibits the transsulfuration pathway and is particularly associated with increased tHcy after a methionine loading test (33).

Compared with previous similar studies, we achieved a much lower posttreatment tHcy concentration by use of a considerably lower dose of folic acid (11, 34). Data from different studies are difficult to compare when different doses, combinations, and route of administrations have been applied. In general, oral administration of the vitamin seems less effective than intravenous administration. Symptoms of malabsorption are very common in uremic patients and are related to chronic gastritis and *Helicobacter pylori* infection (35). In addition, patients' poor compliance with oral treatment is a major problem that may affect the inter- and intraindividual responses (11).

In summary, we observed an effective correction of hyperhomocysteinemia in dialysis patients after intravenous administration of folic acid, vitamin B<sub>12</sub>, and vitamin B<sub>6</sub>. Serum concentrations of folate, vitamin B<sub>12</sub>, and vitamin B<sub>6</sub> within the population-based reference interval may not be sufficient to promote movement of the vitamin into the cells, thereby promoting important intracellular vitamin-dependent reactions in patients with ESRD. We anticipate that vitamin B<sub>6</sub> doses similar to those used in patients with homocysteinuria (250 mg) may be necessary to improve the transsulfuration pathway in dialysis patients. The effect of a longer duration of vitamin supplementation on serum concentrations of cystathionine should be also tested. Further investigations regarding the underlying mechanisms of this generalized resistance to vitamin in renal dialysis patients are needed.

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