



ORIGINAL ARTICLE

The folic acid metabolite L-5-methyltetrahydrofolate effectively reduces total serum homocysteine level in orthotopic liver transplant recipients: a double-blind placebo-controlled study

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Objective: Hyperhomocysteinemia is a described risk factor of cardiovascular diseases. The aim of this study was the treatment of hyperhomocysteinemia in liver transplant recipients with L-5-methyltetrahydrofolate (L-5-MTHF; 1 mg) vs folic acid (1 mg) vs placebo in a double-blind placebo-controlled study and to compare the relative responsiveness of these patients to L-5-MTHF and folic acid.

Subjects/Methods: Patients were recruited from Hepatology-Transplantation-Unit at Johann Wolfgang Goethe-University, Frankfurt. Sixty patients were included in this study and 12 patients dropped out for different reasons. The patients were treated over 8 weeks with supplemental L-5-MTHF or folic acid or placebo. Serum homocysteine (HCY) was analyzed with high-performance liquid chromatography (HPLC) beside routine lab tests.

Results: We observed only a significant decrease of total serum HCY in the L-5-MTHF group during the study period (at week 0: $15 \pm 7.7 \mu\text{M}$; after 8 weeks treatment: $9.41 \pm 2.6 \mu\text{M}$, $P < 0.001$). There was no significant decrease of total serum HCY neither in the folic acid group nor in the placebo group.

Conclusion: The effects of L-5-MTHF are significantly more potent than folic acid itself. Therefore, lowering serum HCY in liver transplant recipients is effective with L-5-MTHF.

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Introduction

Hyperhomocysteinemia is frequently associated with folate deficiency. Homocysteine (HCY) is derived from cellular methionine. Intracellular HCY is normally secreted extracellularly at rapid rates. Elevated concentrations of plasma HCY are related to cardiovascular diseases and to cerebro-

vascular diseases like vascular dementia and mental cognitive dysfunction (Miller *et al.*, 1994; Fassbender *et al.*, 1999; Schachinger *et al.*, 1999; Duthie *et al.*, 2002). High HCY levels have recently been implicated as a risk factor for cancer and might be considered as a new tumor marker (Wu and Wu, 2002) and a pro-proliferative metabolite *in vitro* (Akoglu *et al.*, 2004). Liver transplant recipients have increased HCY levels, probably due to the immunosuppressive therapy, in particular to the use of calcineurin inhibitors cyclosporine A (CyA) and tacrolimus (Tacrolimus). In addition, they have an increased risk for cardiovascular disease because of a high incidence of obesity, arterial hypertension, diabetes mellitus and hyperlipidemia (Johnston *et al.*, 2002; Akoglu *et al.*, 2006). The atherogenic mechanism of HCY as an independent cardiovascular risk factor is not yet well defined, but it might be considered that HCY induces oxidative stress, and thereby, causes injury to vascular wall and other tissues (Stanger *et al.*, 2001).

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Contributors: The study was designed and supervised by BA, DF and WFC. MS produced the study medication in his reputable pharmacy. HB, AJ and EK coordinated the patients and the lab work. BA analyzed the data, interpreted the current findings and wrote the paper.

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HCY metabolism is closely associated with folate metabolism. The folate metabolic pathway has been well elucidated. At a specific point of its metabolic cascade, folic acid is essential for the synthesis of S-adenosylmethionine (SAM), and thereby, is involved in DNA methylation. Furthermore, folate is metabolized (by dihydrofolate reductase) to dihydrofolate and tetrahydrofolate and reduced to L-5-methyltetrahydrofolate (L-5-MTHF) via 5,10-methylenetetrahydrofolate through 5,10-methylenetetrahydrofolate reductase (MTHFR) (Mason, 2003). L-5-MTHF serves as a methyl donor in the remethylation of HCY to methionine, which in turn, is converted into SAM. SAM methylates specific cytosine residues in DNA and therefore regulates gene transcription. Folate (as L-5-MTHF) and vitamin B₁₂ are cofactors of methylation and require two enzymes, methionine synthase and methyltetrahydrofolate reductase (Cravo et al., 1994). Renal function seems to play a key role in HCY clearance. Impaired renal function leads to elevated HCY levels (Friedman et al., 2002).

Folic acid is the common form used for supplementation and food fortification. It is the synthetic form of folate and is virtually unknown in nature. Folate absorption is a complex process involving transport across the brush-border membrane, passage through the enterocytes and transport across the basolateral membrane. Active transport systems for folates are located in the intestine. After absorption, folates are transported to the liver and peripheral tissues via the circulatory systems. All absorbed natural food folates are transformed to L-5-MTHF by the intestine following reduction and methylation. Certain amounts of the L-5-MTHF enter the enterohepatic circulation and are reabsorbed by the intestine. However, L-5-MTHF is the common form of folate, which circulates in the blood until it is further metabolized (Chanarin and Perry, 1969). L-5-MTHF, stable in a supplemental form, has now become available (Merck-Eprova, Schaffhausen, Switzerland). L-5-MTHF is the pure crystalline synthetic derivative of the naturally occurring predominant form of folate (Groen and Moser, 1999) and may be more appropriate than folic acid as a fortificant, because it is unlikely to mask vitamin B₁₂ deficiency. Unlike folic acid, L-MTHF has to be converted into tetrahydrofolate (THF) via

the vitamin B₁₂-dependent enzyme methionine synthase before it can participate into other folate-dependent reactions, including those that are essential for normal erythropoiesis (Scott and Weir, 1981; Weir and Scott, 1999). When vitamin B₁₂ is deficient, L-MTHF is not converted into THF and thus is not able to ameliorate megaloblastic anemia. The aim of this study was to investigate the treatment of hyperhomocysteinemia in liver transplant recipients with L-5-MTHF (1 mg) vs 1 mg folic acid vs placebo in a double-blind placebo-controlled study and to compare the relative responsiveness of these patients to L-5-MTHF and folic acid.

Methods

Subjects

Sixty stable liver transplant recipients from our liver transplantation unit were screened for this study. Finally, 48 patients (34 men, age 50 ± 11 years and 14 women, age 52 ± 13 years) were included in the study (Figure 1). Demographic characteristics are listed in Table 1. Local Ethics Committee approved the protocol. All participants gave their written informed consent before study entry.

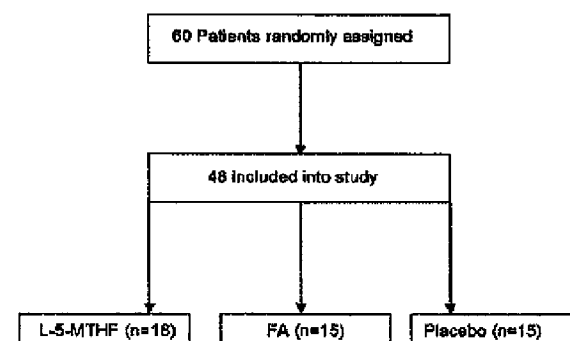


Figure 1 Randomization of 60 patients. After dropouts, 48 patients completed the study.

Table 1 Baseline characteristics of recipients

	Group A, L-5-MTHF	Group B, folic acid	Group C, placebo	Significance
Hemoglobin (g/dl)	12.9 ± 2	14.6 ± 1.9	13 ± 1.5	NS
WBCs (nl ⁻¹)	5.5 ± 1.7	6.13 ± 2.7	6 ± 2.6	NS
Platelets (nl ⁻¹)	223 ± 149	188 ± 90	170 ± 61	NS
MCV (fl)	93.3 ± 9.4	89.1 ± 5.1	89 ± 5	NS
Quick (%)	87 ± 11	89.3 ± 12.4	92.6 ± 7.7	NS
INR	1.11 ± 0.1	1.10 ± 0.1	1.06 ± 0.1	NS
Creatinine (mg/dl)	1.2 ± 0.22	1.11 ± 0.22	1.36 ± 0.2	NS
Urea (mg/dl)	40.9 ± 14	42.3 ± 13.8	47.9 ± 9.9	NS
Vitamin B ₁₂ (pmol)	520 ± 284	405 ± 146	456 ± 254	NS
Vitamin B ₆ (ng/ml)	13.9 ± 2.4	8.37 ± 3	15.4 ± 4	NS
Folate (ng/ml)	8.6 ± 5	8.75 ± 4	12.1 ± 6	NS

Abbreviations: INR, international normalized ratio; MCV, mean corpuscular volume; WBCs, white blood cells.

Vascular complications after liver transplantations like chronic heart disease (CHD), stroke, peripheral vascular disease (PVD), ischemic type biliary lesion (ITBL) and sinus vein thrombosis (SVT) were detected from the patients' files. During the study period, there was no acute cardiovascular event detected.

Study design

A 10-week, prospective randomized, double-blind, placebo-controlled, monocentered and investigator-driven study was initiated. Patients were treated over 8 weeks with 1 mg supplemental L-5-MTHF (group A) or 1 mg folic acid (group B) or placebo (group C). The time schedule design was: week 0 pretreatment phase, weeks 1–8 treatment phase and weeks 8–10 posttreatment phase. Supplements were capsuled and divided to portions containing 30 pills per pack (Hirsch Pharmacy, Frankfurt, Germany). Blood samples were taken before treatment (week 0), at weeks 1–8 and 10 weeks after study entry.

Clinical chemistry

Blood samples were obtained after overnight fasting and were immediately analyzed (within 1 h) for total serum HCY according to the manufacturer's instructions (Immunodiagnostik, Bensheim, Germany). In brief, the method consists of the following steps: reduction of the sample with tri-n-butylphosphine, precipitation of proteins, derivatization with ammonium 7-fluorobenzo-2-oxa-1,3-diazole-4-sulphonate and high-performance liquid chromatography (HPLC) separation followed by fluorescence detection (Merck-Hitachi, Darmstadt, Germany; Minniti et al., 1998). Routine parameters for clinical chemistry were assessed at the central laboratory of our center.

Statistical analysis

Results are expressed as mean \pm s.e.m. Differences in continuous variables between two or more groups were analyzed using unpaired *t*-tests and analysis of covariance (ANOVA). Statistical analysis was performed on an Apple Macintosh OsX with InStat 3 Software (GraphPad, San Diego, CA, USA).

Results

A total of 48 patients were randomized in this study: 18 patients were randomly assigned to receive L-5-MTHF (group A), 15 to receive folate therapy (group B) and 15 (group C) to receive placebo (Figure 1). Baseline characteristics were assessed at study entry. After subjects were matched to groups A, B and C, no significant differences were seen between the groups in laboratory values measured at the beginning of the study (Table 1). For mainstay immunosuppression, patients received tacrolimus, CyA, mycophenolate and rapamycin alone or in combination (Table 2). The lowest levels of serum HCY were detected in recipients with tacrolimus mainstay immunosuppression. The HCY levels in patients receiving CyA and rapamycin were significantly elevated compared to the tacrolimus group ($P < 0.01$, $P < 0.05$, *t*-test).

To determine the effects of the study medication, the impact of each treatment chosen was compared among the three groups (Table 3). Overall, there was only a significant decrease of total serum HCY in the L-5-MTHF group (A) during the study period. There was no significant decrease of total serum HCY neither in the folic acid group (B) nor in the placebo group (C). Compared to baseline, the decrease of total serum HCY after week 8 was 37% in the L-5-MTHF group. Less prominent effects were seen in the folic acid group with a decrease of 24% at week 8. Two weeks after wash out (week 10), the previous effects of L-5-MTHF were still ongoing in group A. Total serum HCY levels of patients who received L-5-MTHF before, were still reduced even without any supplementation at week 10 compared to the folic acid group (16 vs 2%; Figure 3).

If we compare the HCY levels between all recipients depending on vascular complications, HCY levels were

Table 2 Mainstay immunosuppression and homocysteine levels

Immunosuppression	N	Homocysteine levels (μ M)
Tacrolimus	36	17 \pm 10
Cyclosporin A	14	22 \pm 4*
Mycophenolate	3	81 \pm 14
Rapamycin	4	41 \pm 10**

* $P < 0.05$. ** $P < 0.01$ vs tacrolimus.

Table 3 Response of the treatment groups during intervention period for total serum homocysteine (HCY)

Group	tHCY μ M					
	Week 0	Week 1	Week 2	Week 4	Week 8	Week 10
A, 5-MTHF	15 \pm 7.7	12.6 \pm 3.2	10.9 \pm 3.1*	10.8 \pm 2.4**	9.41 \pm 2.6***	12.5 \pm 4
B, folic acid	15.7 \pm 7.5	14.8 \pm 5	12.7 \pm 4.4	11.7 \pm 4.9	12 \pm 3.1	15.5 \pm 9.3
C, placebo	15.1 \pm 4.1	14.26 \pm 4.9	13.9 \pm 4.2	14.6 \pm 3.2	13.9 \pm 6.9	14.6 \pm 3.9

Mean \pm s.e.m. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, analysis of covariance (ANOVA).

significantly raised in the vascular complications group (23.7 ± 3 vs $16.8 \pm 1 \mu\text{M}$, $P = 0.05$, t -test; Figure 2).

Discussion

Elevated HCY levels are a frequent phenomenon in liver transplant recipients. In addition, elevated HCY concentrations are a well-described risk factor for vascular diseases and are an additional important predictor of mortality in patients with coronary heart disease (Ueland et al., 2000). In transplant recipients, the main causes for late secondary organ loss are often vascular diseases and their complications as well as undesirable effects of chronic rejection (Mahony, 1989). In our patients, HCY levels in recipients with vascular complications were significantly increased.

Folate supplementation has been shown to normalize increased HCY blood levels (Homocysteine Lowering Trialists' Collaboration, 1998). Observations of altered HCY metabolism related to chronic vascular disease (McCully and Wilson, 1975) and some prospective studies have

suggested that there is a relationship between elevated HCY levels and chronic vascular disease (Perry et al., 1995; Petri et al., 1996). Two other studies found a positive correlation between plasma HCY and thickening of the intimal layer of the carotid artery (Malinow et al., 1993) and its narrowing (Selhub et al., 1995). There is accumulating evidence for the preventive effect of folate supplementation. Interestingly, in our cohort, folic acid itself was not potent enough to decrease significantly HCY levels compared to L-5-MTHF.

We used 1 mg L-5-MTHF and folic acid in this study. We could show that a 2.5-fold increased dosage of the daily recommendation of folic acid (400 mg) was not able to decrease significantly HCY levels in liver transplant recipients.

Absorption of folate is mainly located in the jejunum. Food folates occur mostly in the polyglutamated form. Folate polyglutamates are more effective substrates than monoglutamates for most folate-dependent enzymes. Before absorption across the intestinal membrane, folate polyglutamates are hydrolyzed to monoglutamates by a carboxypeptidase. The relative bioavailability of food folates is between 50 and 98% in comparison to the pure monoglutamated form of folic acid. Folate absorption is a complex process involving transport across the brush border membrane, passage through the enterocytes and transport across the basolateral membrane. Active transport systems for folates are located in the intestine. All absorbed natural food folates are transformed to L-methylfolate, following reduction and methylation in the intestine. In the liver, part of the L-methylfolate undergoes the enterohepatic circulation and is reabsorbed by the intestine. L-Methylfolate is the common form of folate, which circulates in the blood until it is taken up by the cells, excreted or partly reabsorbed by the kidney (Saubert et al., 1987; Brouwer et al., 1999).

The protective effects of folates in the prevention of vascular diseases are still unknown. Oxidative stress, for

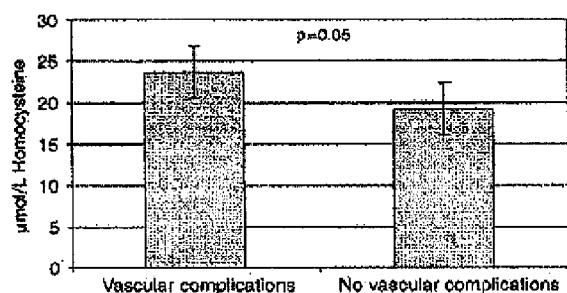


Figure 2 Homocysteine (HCY) levels in patients with or without vascular complications vs no vascular complications.

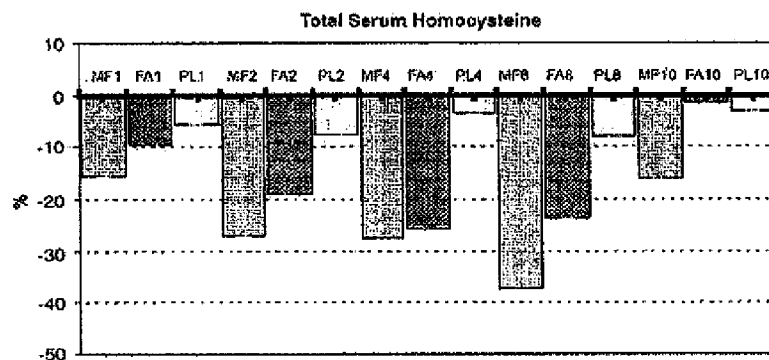


Figure 3 Percentage decrease of total serum homocysteine (HCY) during treatment (MF(1-10), L-5-methyltetrahydrofolate (L-5-MTHF) during weeks 1-10, FA(1-10), folic acid during weeks 1-10 and PL(1-10), placebo during weeks 1-10).

example, caused by elevated HCY levels in the vascular membrane seems to be the most likely explanation for this still controversially discussed issue. Beside the HCY lowering effect of folates, which induces reduced antioxidative effects in tissues, the folate metabolite L-5-MTHF is described to have antioxidative potential itself, including the improvement of tetrahydrobiopterine (BH4) availability, a direct superoxide-scavenging capacity and its ability to reduce superoxide generation by the 'uncoupled' endothelial nitric oxide synthase (eNOS), switching the enzyme to its 'coupled' state and thus restoring nitric oxide (NO) synthesis and NADPH oxidation, in a BH4-dependent manner. Both enantiomers of 5-MTHF (L[6S] and the D[6R]) are shown to have these specific effects (Stroes et al., 2000; Doshi et al., 2001).

Sudan et al. (1999) described causes of late death in liver transplant recipients, 5 or more years after transplantation. The mortality due to cardiovascular events was 19% (Sudan et al., 1999). Other publications quoted the mortality from cardiovascular disease between 1.5 and 3% of the total number of patients alive at 1 year. In heart transplant recipients, the main causes for late secondary organ loss are vascular diseases and their complications (Abbasoglu et al., 1997; Pruthi et al., 2001; Rabkin et al., 2001; Eisen et al., 2003). It is true that classical cardiovascular risk factors like hypertension, obesity, diabetes mellitus and so on were also responsible for these incidences.

Ischemic-type biliary lesions (ITBLs) lead to considerable morbidity after orthotopic liver transplantation. Its exact pathogenesis is still unknown. Poor condition of the arterial system in elderly patients may impair regular perfusion of the bile duct capillaries. A large part of ITBLs might be classified as a kind of ischemic biliary lesions (IBLs). In this sense, IBLs can occur as either macroangiopathic (arterial occlusion, stenosis, thrombosis) or microangiopathic biliary lesions (inadequate perfusion of small vessels) (Moench et al., 2003). Cases of diffuse bile duct injury with adequate perfusion during organ procurement should continue to be classified as ITBLs. However, in our patients with I(T)BL the HCY levels are elevated ($21 \pm 3 \mu\text{M}$, data not shown), and the incidence is comparable to other studies (18%) (Moench et al., 2003). HCY might therefore be considered as an additional risk factor for macroangiopathic or microangiopathic biliary lesions in patients after liver transplantation.

In conclusion, we emphasize the important role of impaired folate metabolism and elevated HCY levels, respectively in liver transplant recipients. Probably owing to our sample size, the used dosage and a drop out rate of 20%, we could not show any significant effects of folic acid itself. Lowering total serum HCY by folic acid itself was not efficient compared to its reduced metabolite L-5-MTHF. Therefore an efficient supplementation with folates in liver transplant recipients seems to be more effective with L-5-MTHF. Further long-term studies are needed to figure out if patients with elevated HCY levels have different rates of survival compared to recipients with lower levels. Further-

more, it is unknown and controversially discussed, if treatment with substances like folic acid are able to change the morbidity and mortality, in general, and after liver transplantation by lowering total serum HCY, in particular.

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