Vitamin B_{12} Decreases, But Does Not Normalize, Homocysteine and Methylmalonic Acid in End-Stage Renal Disease: A Link With Glycine Metabolism and Possible Explanation of Hyperhomocysteinemia in End-Stage Renal Disease

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The genetic and environmental factors influencing catabolism of homocysteine in end-stage renal disease (ESRD) patients remain poorly understood. This study investigated how genetic and nutritional influences affect the response to high-dose vitamin B_{12} and folate treatment in ESRD patients with hyperhomocysteinemia. We studied 81 hemodialysis patients with hyperhomocysteinemia (> 16 μmol/L) on varied doses of a multivitamin containing 1 mg of folic acid per day. After screening blood work, all patients were switched to daily multivitamin therapy including 1 mg of folic acid for 4 weeks. Vitamin B_{12}, 1 mg/d, was added for an additional 4 weeks. Patients were then randomized to receive folic acid or placebo. The influence of the 3 methylenetetrahydrofolate reductase (MTHFR) 677 C→T genotypes on the efficacy of vitamin therapy was assessed. In addition, we investigated how the metabolic complications of ESRD, including the relationship between methylmalonic acid (MMA) and circulating glycine, may contribute to hyperhomocysteinemia. There was no significant difference in total homocysteine (tHcy) levels between the MTHFR 677 C→T genotypes during the screening phase of the trial. Treatment with a daily multivitamin containing 1 mg folate significantly lowered tHcy levels in all patients by 19.2%. Further supplementation with 1 mg vitamin B_{12} resulted in greater tHcy reduction among subjects with the MTHFR 677 T/T genotype ($P < .01$, T/T *v* C/C or C/T) while lowering MMA equally in all MTHFR genotypes. There was a significant positive correlation between plasma glycine levels and MMA ($P < .05$). High-dose vitamin therapy significantly lowers, but does not normalize, MMA and tHcy levels. The MTHFR genotype, while influencing homocysteine levels, was not responsible for the majority of the elevation in plasma tHcy.

MODERATE ELEVATIONS of total plasma homocysteine (tHcy) are an independent risk factor for cardiovascular disease in patients with and without end-stage renal disease (ESRD).[1,2] The prevalence of hyperhomocysteinemia (85% to 100%) and death from atherosclerotic vascular disease (25% to 60%) in ESRD is significantly greater compared with patients with normal renal function.[3] Pharmacologic doses of vitamin supplementation have failed to normalize circulating tHcy levels in ESRD patients.[4-6] Specifically, treatment with folic acid or its active form, methyltetrahydrofolate (with or without cobalamin) has resulted in only modest reductions in tHcy levels in ESRD.[5-9]

Homocysteine is generated by the demethylation of the essential amino acid, methionine, through the intermediates S-adenosyl-methionine and S-adenosylhomocysteine. Once formed, homocysteine can either be remethylated to methionine via the remethylation pathway or irreversibly converted to cysteine though the B_6-dependent transsulfuration pathway. Hyperhomocysteinemia in ESRD is primarily a result of a defective remethylation and not transsulfuration.[10] Both vitamin B_{12} and 5-methyltetrahydrofolate are essential cofactors in the remethylation of homocysteine to methionine, which is catalyzed by methionine synthase (MS). The methyl group required for this reaction is donated by 5-methyltetrahydrofolate, generated by methylenetetrahydrofolate reductase (MTHFR). MS uses the cofactor cobalamin (vitamin B_{12}) to transfer a methyl group from 5-methyltetrahydrofolate to homocysteine forming methionine and tetrahydrofolate. Severe and mild genetic mutations can independently or in combination with nutritional factors contribute to hyperhomocysteinemia. A common substitution of cytosine for thymine at nucleotide 677 in the MTHFR gene has been reported.[11] This substitution results in increased heat lability and decreased MTHFR enzyme activity, and in patients with low-plasma folate levels, leads to an increase in tHcy.[12]

In addition to being a cofactor for MS, vitamin B_{12} is required for the catabolism of methylmalonic acid (MMA).[13] Methylmalonyl-Co A-mutase (MMM), a mitochondrial protein, uses adenosylcobalamin to convert methylmalonyl-Co A to succinyl-Co A; unlike MS, its activity is independent of folate. Elevations in MMA are therefore a useful marker of vitamin B_{12} tissue sufficiency.[14,15]

In addition to an elevation in MMA levels, mutations in the B_{12}-dependent enzyme MMM result in elevated plasma glycine levels.[16-18] The elevation in glycine is thought to be a result of decreased catabolism by the glycine cleavage system, due to insufficient transport of glycine into the mitochondria or through a direct inhibition of the glycine cleavage system.[19-22]

The aims of this study were 2-fold. First, we examined the influence of the MTHFR 677C→T mutation on baseline tHcy levels and determined whether the MTHFR 677 C→T genotype was predictive of the response to folic acid and vitamin B_{12} therapy. Second, we investigated the metabolic complica-

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Table 1. MMA and tHcy Levels after Screening, Phase 1 (Post-Diavite), Phase 2 (Postvitamin B_{12}), and Phase 3

	MMA (pmol/L [SD])			Homocysteine (μmol/L [SD])		
	C/C (n = 42)	C/T (n = 30)	T/T (n = 9)	C/C (n = 42)	C/T (n = 30)	T/T (n = 9)
Screening	—	—	—	28.3 (9.3)	25.4 (6.6)	32.5 (17.1)
Phase 1 (baseline)	785 (319)	747 (261)	889 (222)	21.2 (4.88)*	22.4 (4.7)*	27.6 (10.7)*,†
Phase 2 (post B_{12})	623 (246)‡	604 (226)‡	671 (113)‡	18.4 (4.52)‡	18.9 (4.5)‡	18.5 (5.3)‡
Phase 3 (folate or placebo)§	616 (245)	588 (184)	654 (114)	17.2 (3.9)	17.8 (3.9)	20.2 (6.6)

NOTE. Postfolate/placebo stratified by the MTHFR 677C→T genotype.

*$P < .05$ using a paired *t* test comparing tHcy levels in phase 2 and phase 1 within the MTHFR genotypes.

†Comparison of the T/T group with the C/C and C/T genotype after phase I using ANOVA and Scheffé post hoc comparison ($P = .01$ T/T *v* C/T; $P = .06$ T/T *v* C/C).

‡$P < .05$ using a paired *t* test comparing tHcy levels in phase 2 *v* phase 1.

§Because there was no significant difference in tHcy lowering between the 2 high-dose folic acid groups and the placebo group, these groups were combined for the purposes of this table.

tions of ESRD, including the relationship between MMA and circulating glycine, which may result in hyperhomocysteinemia.

MATERIALS AND METHODS

Subjects

This study is a substudy of a randomized clinical trial previously conducted by our group.[4] Briefly, 81 hemodialysis patients with hyperhomocysteinemia (> 16 μmol/L) were enrolled. Exclusion criteria included a plasma vitamin B_{12} level less than 133 pmol/L and a red blood cell (RBC) folate concentration less than 450 nmol/L. Subjects were also excluded if they were currently receiving or had received treatment with vitamin B_{12} injections within the last 3 months, more than 1 mg folic acid per day in the past 6 months, or if they were currently using antifolate or antiepileptic medications (ie, phenytoin). At baseline, 92.6% of the enrolled patients were taking supplemental Diavite (R & D Laboratories, Marina del Ray, CA), which contains folic acid 1 mg, vitamin B_{12} 6 μg, vitamin B_6 3 mg, and small amounts of other vitamins. Of those who were taking Diavite, 56% used it daily, and 44% used it 3 times per week postdialysis.

Study Protocol

The study was divided into 3 phases of 1 month each. To ensure standardization of conventional vitamin therapy, during phase 1, all enrolled subjects were supplemented daily with Diavite for 4 weeks. In phase 2, all subjects were treated with Diavite plus oral vitamin B_{12} (Nature Made, Pharmavite, Mississauga, Canada) at a dose of 1 mg/d for 4 weeks. In phase 3, subjects continued to receive Diavite and vitamin B_{12} daily, and in addition, they were randomized to receive either placebo, folic acid 5 mg, or folic acid 20 mg/d (Apotex, Toronto, Canada) for a period of 4 weeks. Plasma vitamin B_{12} and MMA, serum tHcy, and RBC folate were measured before dialysis at the termination of the 3 study phases in all subjects. The plasma glycine concentration was determined at the end of the third phase only. The Conjoint Health and Research Ethics Board at The University of Calgary approved the study protocol.

Biochemical Measurements

tHcy, vitamin B_{12}, and folate levels were measured as previously described.[4] Plasma was precipitated using 5-sulfosalicylic acid, and the treated plasma samples were assayed for glycine using an automated amino acid analyzer. MMA was recovered from plasma using solvent extraction using methyl-^{2}H-malonic acid MMA as the internal standard and quantified using a previously described gas-chromatography-mass spectrometry stable isotope dilution method.[23] DNA was isolated from peripheral lymphocytes for the MTHFR 677C→T genotyping and amplified using the primers and procedures of Frosst et al.[11]

Statistical Analysis

Data are presented as mean ± SD. One-way analysis of variance with Scheffe post hoc analysis was used to compare the mean difference in tHcy, vitamin B_{12}, and MMA at 4, 8, and 12 weeks of study. The above variables at 8 and 12 weeks were similarly compared for the placebo group and the 5- and 20-mg folate treatment groups, respectively. *P* values less than .05 were considered to be statistically significant. Correlation coefficients between MMA and plasma glycine were determined using Pearson's coefficient and simple linear regression analysis.

RESULTS

A detailed description of patient characteristics has been previously published.[4] Briefly, 81 (94.2%) and 78 (90.7%) subjects completed phases 2 and 3 of the study, respectively. The mean age and length of time on hemodialysis of the subjects were 63.5 (95% confidence interval [CI], 60.1 to 66.8) and 3.6 years (95% CI, 2.8 to 4.5), respectively. The mean RBC folate and serum B_{12} concentrations on entry to the study were 1,676 (95% CI, 1,436 to 1,916) and 437 (95% CI, 326 to 548), respectively. The etiology of renal failure was diabetes mellitus (26.8%), glomerulonephritis (28.1%), hypertension (13.4%), interstitial nephritis (including reflux nephropathy) (8.6%), autosomal-dominant polycystic kidney disease (6.1%), miscellaneous (7.3%), and unknown (9.8%). The distribution of the MTHFR 677C→T was 51% (n = 42), 37% (n = 30), and 11%(n = 9) of the patients for the homozygous wild-type C/C, heterozygous C/T, and homozygous mutant T/T genotypes, respectively.

The mean tHcy for the entire group on entry to the study was 27.7 μmol/L (95% CI, 25.5 to 29.8). When segregated according to MTHFR genotype, the screening tHcy levels were not significantly higher in patients with the T/T genotype ($P = .13$) (Table 1).

Plasma tHcy and methylmalonic acid levels after 4, 8, and 12 weeks of treatment are shown in Table 1. With daily Diavite treatment (phase 1), the mean homocysteine of all subjects decreased by 19.2%, although homocysteine levels remained significantly higher in the MTHFR 677 T/T subjects compared with the C/C individuals ($P < .03$). As previously reported,

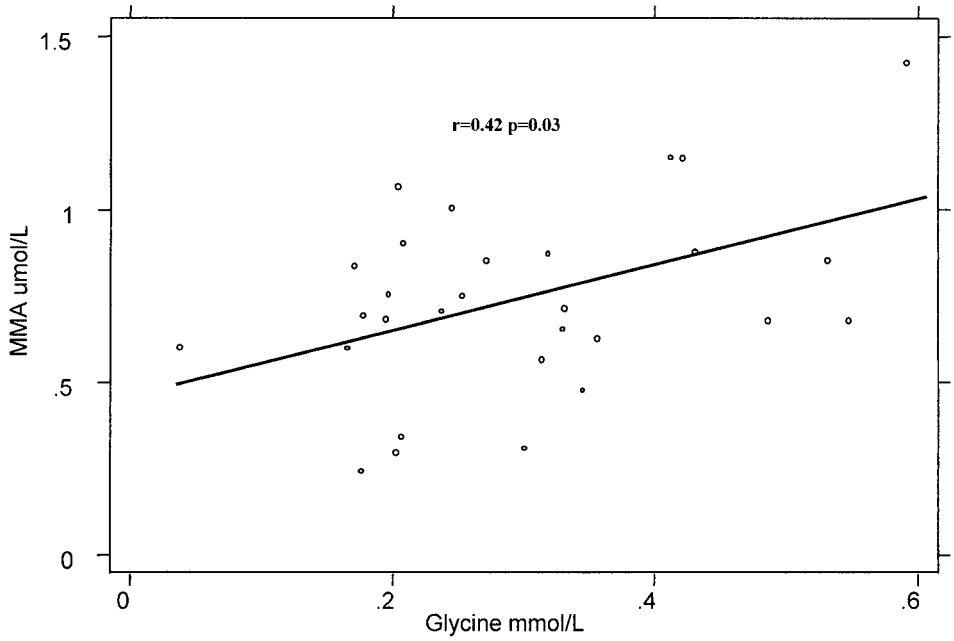

Fig 1. Relationship between glycine and MMA levels in ESRD patients (phase 3). Line represents regression analysis.

RBC folate and vitamin B_{12} levels were well above the normal range during all phases of the trial.[24]

The MMA levels, stratified by the MTFHR genotype, are depicted in Table 1. After phase 1 (Diavite), MMA concentrations in the ESRD patients were 5-fold to 8-fold higher than the 90% percentile of our laboratories normal range (0.84 to 0.115 μmol). There was no statistically significant difference in the MMA concentrations among the MTHFR genotypes during any of the phases. Only additional supplementation with 1 mg B_{12} (phase 2) lowered MMA levels ($P < .05$; Table 1). However the MMA level was not normalized in any subject during the treatment phases. Before treatment with vitamin B_{12}, there was a highly significant negative relationship between B_{12} and MMA ($r = -.04$, $P = .02$). However B_{12} therapy abolished the correlation because the increase in serum B_{12} was not accompanied by a corresponding change in MMA levels. Fully half of the subjects had plasma glycine levels greater than our laboratories upper normal limit of 0.310 μmol/L. As illustrated in Fig 1, a significant positive correlation between plasma MMA and glycine ($r = .42$, $P < .03$) was observed after phase 3.

DISCUSSION

We have previously shown that high-dose vitamin B_{12} and folate fail to normalize tHcy levels in ESRD patients.[24] Data from the present study shows that this treatment also does not normalize MMA levels in ESRD. In agreement with previous studies,[25] we found that the MTHFR T-allele aggravates hyperhomocysteinemia, but its effects on tHcy levels appear to be relatively minor compared with the metabolic complications associated with ESRD. In our study, a folate intake greater than 1 mg/d was not beneficial in lowering tHcy levels in any of the C→T 677 MTHFR genotypes. High-dose vitamin B_{12} was moderately effective in lowering tHcy; its effect was most pronounced in those with the homozygous MTHFR T/T genotype (Table 1). We also present evidence that elevated MMA levels are common in ESRD subjects. Unlike the study of Moelby et al[26] and in concurrence with others,[27] we observed a significant reduction in MMA levels after B_{12} therapy. This modest lowering effect is remarkable given that vitamin B_{12} levels in our population[4] are well above serum B_{12} levels associated with an increase in MMA in subjects with normal renal function.[28] Previous studies have demonstrated a relationship between creatinine and MMA and, therefore, the importance of renal clearance of organic acids, such as MMA.[29,30] Nevertheless, given that we and others[5] have shown that MMA levels can be lowered by vitamin B_{12} supplementation, impaired excretion of MMA in kidney failure cannot solely account for its elevation. Impaired metabolism of MMA, possibly due to tissue B_{12} deficiency, may also play an important role. When one considers the normal B_{12} levels observed in our study, it would be reasonable to conclude that B_{12} levels cannot be relied on as a reliable indicator of tissue availability of B_{12} in patients with ESRD. Further, MMA reference intervals as reported for patients without kidney failure are not relevant in uremic populations.

Several possible explanations for this abnormal association between B_{12} and MMA in ESRD exist, and 2 events in the regulation of B_{12} metabolism need to be discussed. First, the fact that both homocysteine and MMA remain elevated suggests a primary defect in the cellular processing or metabolism of B_{12}. Uremia may interfere with vitamin B_{12} binding to serum transcobalamin II, possibly accounting for the high serum B_{12} levels despite evidence for a cellular deficiency in B_{12}. Alternatively, although not explored in this study, the increased MMA and tHcy levels in ESRD may be a result of defective

B_{12} metabolism as is seen in patients with inborn errors of metabolism, such as cblC and cblD.

Although it is known that inherited defects in MMA alter glycine metabolism, this study demonstrates that MMA levels are positively correlated with serum glycine levels in ESRD pateints (Fig 1). High levels of MMA have previously been demonstrated to interfere with the glycine cleavage system (GCS).[18,31,32] It is thought that the elevated levels of MMA result in an elevation in glycine by interfering with the mitochondrial GCS.[20,22,31] A recent study by Verleysdonk et al[33] has demonstrated that glycine catabolism directly produces serine.[34] De novo serine synthesis produced in the mitochondria may serve as a source of methylgroups for cytosolic serine-glycine hydroxmethyltransferase, which is responsible for regenerating the substrate for MTHFR (5-10-methylenetetrahydrofolate). The importance of serine in the remethylation of folate is highlighted by a study conducted by Wilcken et al,[35] who demonstrated that supplementation of folate in ESRD patients caused a reduction in serine and a corresponding increase in glycine levels. We speculate that an elevation of MMA in ESRD may block the GCS and impair remethylation of tetrahydrofolate in the mitochondria. Although our data is supportive of this hypothesis, further investigation in this area is warranted. Furthermore, we were only able to measure glycine after all treatment phases were completed and, therefore, we may be underestimating the effect of MMA on glycine. Even though we found an association between MMA and serum glycine, it has been demonstrated that circulating glycine levels do not necessarily reflect intracellular and extrcellular compartments.[36,37] Given that cellular glycine levels are significantly elevated[36] in uremic patients, future studies should address the intercompartment variations in glycine and its relationships with cellular MMA levels. In addition, it would be worthwhile to assess the relationship between MMA and glycine in acute renal failure or before vitamin therapy in ESRD to shed light on this issue.

Conclusion

Our study supports previous evidence that subjects receiving hemodialysis not only have hyperhomocysteinemia, but also elevated MMA levels, both only partially corrected with folate and B_{12} therapy. The quantitative effect of the MTHFR 677 C→T genotype on hyperhomocysteinemia was less important than the metabolic complications noted in ESRD. MMA levels were correlated with serum glycine concentrations, and we propose this as a possible mechanism, which may interfere with cellular 1 carbon metabolism and, therefore, folate methylation. Further investigations are needed to clarify this relationship.

The increasing tendency to treat elevated tHcy levels in ESRD patients with high-dose folate may be of concern without adequate additional B_{12} supplementation. We have demonstrated that exogenous supplementation with B_{12} lowers, but does not normalize, MMA and tHcy levels. For hemodialysis patients with hyperhomocysteinemia being treated with folic acid, consideration should be given to supplementation with 1 mg/d B_{12}, regardless of the vitamin B_{12} or MMA levels.

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