Effects of Vitamin Therapy on Plasma Total Homocysteine, Endothelial Injury Markers, and Fibrinolysis in Stroke Patients

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Hyperhomocysteinemia linked to B-vitamin deficiency is prevalent and associated with increased risk for stroke. While in vitro studies suggest homocysteine directly injures vascular endothelial thrombomodulin (TM), inhibits von Willebrand factor (vWF) synthesis, and blocks tissue plasminogen activator (t-PA) receptor binding, these mechanisms and their reversibility by vitamin therapy are not established in humans. We investigated the effects of high-dose B-vitamin therapy on endogenous fibrinolysis and endothelial injury markers by randomizing 50 nonvitamin users with prior ischemic stroke to 3 months of treatment with multivitamins either containing folate (5 mg), B6 (100 mg), and B12 (1 mg), or lacking these components. Fasting before noon and post-methionine load plasma total homocysteine (tHcy), t-PA antigen levels, t-PA and plasminogen activator inhibitor (PAI) activities, total vWF antigen, and TM levels were measured before and after vitamin therapy. The primary analysis between treatment groups across time revealed no significant changes ($P > .1$) for any hematologic variables. However, within-groups analysis showed reductions of 23% in plasma TM ($P < .005$) and 27% in fasting tHcy levels ($P < .0001$) and a paradoxical 30% rise in vWF antigen levels ($P < .05$) after high-dose B-vitamin, treatment with no changes in controls. Pooled data revealed a significant and reproducible 20% to 28% decline in plasma t-PA activity after methionine load ($n = 49, P < .02$). Our findings demonstrate methionine load lowers plasma t-PA activity by a plasminogen activator inhibitor (PAI-1) independent mechanism that is not attenuated by 3 months of high-dose B-vitamin treatment. While not improving endogenous fibrinolysis profiles, these results provide initial evidence that B-vitamin treatment may selectively alter markers of vascular endothelial injury after stroke. Key Words: Homocysteine—Stroke—Vitamin therapy—Fibrinolysis—Methionine load—Thrombomodulin.
Increasing epidemiologic data support a relationship between elevated plasma total homocysteine (tHcy) levels and increased risk for vascular disease. In contrast to the rare hereditary disorders producing homocystinuria, attributable in part to B-vitamin deficiency, are prevalent in the stroke population and increase with advancing age. Approximately 30% to 40% of ischemic stroke patients are reported to have hyperhomocysteinemia, which may promote atherosclerosis and a prothrombotic state. Targeted B-vitamin therapies are known to reduce plasma tHcy and subsequent thrombotic events in selected hereditary forms of homocystinuria. However, no controlled studies have investigated whether vitamin therapy to lower tHcy alters markers of endothelial injury or improves endogenous fibrinolysis in the stroke population.

This double-blind, randomized, clinical trial investigates the effects of 3 months of multivitamin therapy with high-dose folate, B₆, and B₁₂ on plasma tHcy, fibrinolysis, and markers of endothelial injury in older individuals with history of prior stroke. In vitro, homocysteine directly injures vascular endothelium, impairs the thrombomodulin (TM)-dependent protein C mechanism, interrupts von Willebrand factor (vWF) synthesis, and inhibits tissue plasminogen activator (t-PA) binding to its specific endothelial receptor. Therefore this pilot study specifically examined the effects of B-vitamin therapy on plasma t-PA activity, TM, and vWF antigen levels, markers of endothelial injury with putative molecular susceptibility to homocysteine-mediated injury. Since elevated tHcy levels while fasting and after protein load may represent distinct, albeit overlapping metabolic conditions preferentially linked to folate/vitamin B₁₂ and pyridoxine deficiency, respectively, we measured fasting and postmethionine load tHcy levels in the stroke patients to better determine efficacy of the vitamin intervention. Because epidemiologic studies suggest that risk for vascular disease is not confined to an upper threshold but increases in a dose-response fashion across normal to higher plasma tHcy levels, we chose to study stroke patients irrespective of their initial plasma tHcy levels.

**Patients and Methods**

**Study Groups**

In this double-blind, placebo-controlled, pilot study, community-dwelling stroke patients not already taking vitamin supplements were randomly assigned to 3 months of daily multivitamins that contained folate (5 mg), B₆ (100 mg), and B₁₂ (1 mg) (B-vitamin group), or lacked these B-vitamin components (controls). Patients were 50 to 80 years of age with ischemic stroke occurring >3 months before the study, avoiding the acute-phase effects of stroke on plasma tHcy levels and other hemato-logic measures. Patients were recruited from residents in the 4 ZIP code areas surrounding the University of Maryland by active recruitment and passive screening of outpatient records based on *International Classification of Diseases, 9th Revision* diagnosis codes 433.0-434.9 and 436.0-437.9. Vitamin pill count was obtained upon study completion to document compliance. This study was approved by the Institutional Review Boards at participating hospitals and all patients provided informed consent.

A standardized questionnaire was used to ascertain medical eligibility, stroke risk factors, and alcohol consumption and to detect any recent infection-inflammatory syndromes that may alter hemostatic variables in stroke patients. Resting supine blood pressure, height, and weight were measured. Exclusion criteria consisted of conditions with potential contraindications to vitamin therapy or which could confound interpretation of hemostatic findings, including diabetes; peripheral neuropathy; known B₁₂ deficiency; seizure disorder; human immunodeficiency virus; cancer or myeloproliferative disorder; rheumatoid arthritis; Crohn’s disease; congestive heart, renal, or hepatic failure; sickle cell disease; recent trauma, infection, or inflammation (<2 weeks); or hospitalization <3 months.

**Methionine Load and Laboratory Methods**

At baseline and following 3 months of vitamin therapy, each patient received a standard oral methionine loading test (0.1 g/kg), given in fruit drink with a methionine-free breakfast consisting of 3 low-protein wafers with margarine, jam, or marmalade, and tea or coffee, black or with low-protein milk. Blood was collected in the fasting condition and 3 hours postmethionine load. Antecubital venipuncture after 12-hour overnight fast was performed between 8:00 am and 10:30 am using 21-gauge butterfly needle without tourniquet, after patients were comfortably seated 20 minutes, avoiding alterations in fibrinolysis due to circadian variability, venostasis, orthostatic changes, and physical exertion. The initial 0.5 mL of blood was discarded, and platelet-poor plasma prepared by centrifugation (10,000 x 20 minutes, 4°C) then archived at −70°C until batch assay. Blood for t-PA activity was collected in 130 mmol/L sodium citrate (9:1 vol) acidified <60 seconds by addition of sodium acetate (pH 4.2, 0.5 mol/L, 2:1 vol) to prevent ongoing in vitro inactivation by type-1 plasminogen activator inhibitor (PAI-1) and measured by amidolytic assay (Chromogenix, Franklin, OH). Plasma TM (Diagnostica Stago, Asnieres-Sur-Seine, France) and t-PA antigen (American Bioproducts, Parsippany, NJ) levels were measured from citrate anticoagulated specimens by enzyme immunoassay. Plasma PAI-1 activity was measured by reverse amidolytic activity assay (Chromogenix) and vWF antigen was measured by enzyme immunoassay (American Bioproducts, Parsippany, NJ).
Bioproducts) in plasma prepared using a modification of Files Solution to minimize in vitro platelet activation.14

Plasma tHcy levels were measured from ethylenediaminetetraacetic acid (EDTA)-prepared plasma specimens (9:1 vol) collected while fasting and 3 hours postmethionine load at baseline and after 3 months of vitamin therapy. Plasma tHcy, including both free and protein-bound fractions, was measured using a method involving reduction with sodium borohydride and precolumn derivatization with monobromobimane, followed by high-performance liquid chromatography and fluorescence detection.19 Baseline and posttreatment serum cobalamin and blood folate levels were measured by radioimmunoassay (Diagnostic Products Corp., Los Angeles, CA) and pyridoxal phosphate levels determined by enzymatic photometry with high-performance liquid chromatography separation.20,21 Patients were classified as vitamin-deficient based on their initial cobalamin (<120 pmol/L), blood folate (<180 nmol/L), and pyridoxal phosphate levels (<20 nmol/L) (Mimelab, Sweden).2

Statistical Methods

Data are expressed as mean ± SD. Demographic and stroke risk factors in the 2 groups were compared using chi-square analysis. Unpaired t tests were used to compare the baseline levels of hematologic variables between groups and a nonparametric test (Kruskal-Wallis) was used to examine the ordinal relationships between tHcy, vitamin levels, and selected hemostatic factors. The primary analysis was performed using unpaired t tests to determine the significance of the mean changes in fasting and postmethionine load tHcy, plasma endothelial markers, and endogenous fibrinolysis variables between treatment groups across the 3-month treatment period. However, since this study was designed as a pilot with limited sample size, we also conducted a secondary within-groups analysis utilizing 2 tailed t tests to evaluate the significance of change in these hematologic variables separately in the B-vitamin treatment and control groups, respectively. A similar analysis was performed in the B-vitamin treatment group by median split of initial fasting tHcy levels, to provide insight as to whether the response to vitamin treatment was related to the baseline tHcy level. Paired 2-tailed t tests were further applied to analyze the significance of change in t-PA and PAI activity levels within subjects, which accompanied the methionine load.

Results

Patient Characteristics

Fifty-eight stroke patients, ages ranging from 51 to 80 years, were randomized, and 50 patients completed the study. Dropouts were due to loss to follow-up (n = 4), difficulty swallowing pills (n = 1), fear of constitutional symptoms (n = 1), and 2 were excluded due to recurrent stroke. The mean latency since the most recent stroke was 31 ± 39 months (range 3-202 months) for the B-vitamin treatment group and 25 ± 22 months (range 4-79 months) for the controls. In the B-vitamin treatment group, there were 21 of 27 (78%) incident stroke cases and 6 of 27 (22%) with a history of more than 1 prior stroke, including 3 patients with 2 prior strokes each. In the control group, there were 19 of 23 (83%) incident stroke cases and 4 of 23 (17%) with more than 1 prior stroke, including 2 patients with 2 prior strokes each. Compliance based on pill counts was 98.2% for controls (n = 23) and 97.6% for the B-vitamin treatment group (n = 26). One patient in the B-vitamin group reported full compliance, but no pill count was available for confirmation.

There were no significant clinical or demographic differences between B-vitamin treatment and control groups, with the exception of higher body weight due to a somewhat greater proportion of men in the B-vitamin treatment group (Table 1). Seven patients randomized to each group had vitamin deficiency at baseline. These included deficiencies in B6 (n = 6) and B12 (n = 1) in the B-vitamin treatment group, and B6 (n = 5), folate (n = 1), and all 3 vitamins (n = 1) in the control group. Plasma from 1 patient in the control group was unavailable for vitamin measures. Kruskal-Wallis test applied to baseline values revealed significant inverse relationships between plasma folate (P < .01), B6 (P = .04), and increasing quartiles of fasting plasma tHcy. There were no significant relationships between baseline levels of fasting

<table>
<thead>
<tr>
<th>Variable</th>
<th>B-Vitamin Group (n = 26)</th>
<th>Controls (n = 23)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (yr)*</td>
<td>67.7 ± 8</td>
<td>66.7 ± 7.3</td>
</tr>
<tr>
<td>Sex (M:F)</td>
<td>17:10</td>
<td>12:11</td>
</tr>
<tr>
<td>Race (White:Black)</td>
<td>14:13</td>
<td>14.9</td>
</tr>
<tr>
<td>Height (cm)*</td>
<td>168 ± 13</td>
<td>171 ± 11</td>
</tr>
<tr>
<td>Weight (kg)*</td>
<td>80 ± 14</td>
<td>67 ± 7†</td>
</tr>
<tr>
<td>Initial vitamin deficiency</td>
<td></td>
<td></td>
</tr>
<tr>
<td>in folate, B6, and/or B12, % (N)</td>
<td>26 (7/27)</td>
<td>32 (7/22)</td>
</tr>
<tr>
<td>Hypertension history, % (N)</td>
<td>67 (18/27)</td>
<td>78 (18/23)</td>
</tr>
<tr>
<td>Current smoking, % (N)</td>
<td>15 (4/27)</td>
<td>30 (7/23)</td>
</tr>
<tr>
<td>Coronary artery disease, % (N)</td>
<td>25 (7/27)</td>
<td>35 (8/23)</td>
</tr>
<tr>
<td>Aspirin-treated</td>
<td>59 (16/27)</td>
<td>57 (13/23)</td>
</tr>
<tr>
<td>Ticlopidine-treated</td>
<td>4 (1/27)</td>
<td>17 (4/23)</td>
</tr>
<tr>
<td>Warfarin-treated</td>
<td>29 (8/27)</td>
<td>25 (6/23)</td>
</tr>
</tbody>
</table>

*Mean ± SD.
†P < .001.
plasma tHcy and thrombomodulin ($P = .8$), plasma vWF antigen levels ($P = .5$), or any fibrinolysis variables.

**Fasting and Postmethionine Load Homocysteine**

The primary analysis shows that the mean change in fasting plasma tHcy levels with B-vitamin treatment did not achieve statistical significance ($P = .25$) between groups across the 3-month treatment period (Table 2). However, a secondary within-groups analysis revealed a 27% reduction in fasting tHcy ($P < .0001$) and a 20% reduction in the postmethionine load tHcy ($P < .0001$) in the B-vitamin treatment group. There were no significant changes in fasting or postmethionine load tHcy within subjects in the control group (Table 2). The median split value for tHcy in our stroke patients is $\geq 10.5$ μmol/L, similar to that reported in other studies of unselected stroke patients. As anticipated, B-vitamin treatment produced a greater reduction in plasma tHcy levels in the stroke group with higher initial tHcy levels ($n = 12$, $13.4 \pm 1.9$ vs $9.0 \pm 2.2$ μmol/L, pretreatment vs posttreatment, $P = .0003$), but still reduced plasma tHcy in the stroke subgroup with initial tHcy levels below this median split value ($8.9 \pm 1.3$ vs $7.6 \pm 1.4$, $n = 14$, $P < .05$).

**Plasma Markers of Endothelial Injury**

The mean change in plasma TM ($P = .19$) and total vWF antigen levels ($P = .17$) associated with B-vitamin treatment did not reach statistical significance between groups across time. However, a secondary within-groups analysis revealed a 23% decline in the mean circulating TM ($P = .005$) and a 27% elevation in vWF antigen levels ($P < .05$) in the B-vitamin treatment group, but no significant changes in the control group (Table 2). Further analysis excluding all stroke patients meeting the criteria for initial (baseline) vitamin deficiency revealed that B-vitamin treatment still produced an 18% reduction in fasting plasma tHcy ($10.5 \pm 2.8$ vs $8.6 \pm 2$ μmol/L, $n = 20$, $P < .002$), a 30% increase in plasma vWF antigen (81.5 ± 48.4 vs 106.2 ± 55%, $n = 19$, $P < .02$), and a trend of 23% lower plasma TM antigen levels ($14.2 \pm 7.7$ vs $10.9 \pm 5.7$ ng/mL, $n = 19$, $P = .1$).

In order to begin to address whether these changes in endothelial markers were related to baseline levels of fasting plasma tHcy, we compared the magnitudes of change in plasma TM and vWF antigen levels in the B-vitamin treated patients with tHcy above versus below the median split of 10.5 μmol/L. B-vitamin treatment was associated with a 25% increase in plasma vWF antigen levels in the stroke group with initial higher plasma tHcy (102.8 ± 50.6 vs 129.1 ± 59%, $n = 12$, $P < .04$), and there was a trend toward increased mean plasma vWF antigen levels in patients with initial tHcy levels below this median split (76.1 ± 45 vs 99 ± 55%, $n = 13$, $P = .08$). There was a 27% reduction in mean plasma TM antigen levels (14.2 ± 6.4 vs 10.4 ± 4.1 ng/mL, $P < .03$) following B-vitamin treatment in the stroke group with initial higher fasting plasma tHcy levels, but no significant change in TM in the stroke group with baseline tHcy levels below this median split value (15 ± 8.1 vs 12 ± 5.9 ng/mL, $P = .28$).

**Endogenous fibrinolysis**

There were no changes in the levels of plasma t-PA, PAI activity, or t-PA antigen in the B-vitamin treatment or control groups (Table 2). Therefore, we performed a pooled analysis including all patients to determine

### Table 2. Primary analysis for change in fasting tHcy and hematological variables between groups

<table>
<thead>
<tr>
<th>Variable</th>
<th>B-Vitamin Group</th>
<th>Controls</th>
<th>$P$ value between groups</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fasting tHcy (μmol/L)</td>
<td>26</td>
<td>22</td>
<td>0.25 (NS)</td>
</tr>
<tr>
<td>Postmethionine tHcy (μmol/L)</td>
<td>22</td>
<td>22</td>
<td>(NS)</td>
</tr>
<tr>
<td>Thrombomodulin (ng/mL)</td>
<td>25</td>
<td>25</td>
<td></td>
</tr>
<tr>
<td>vWF antigen (%)</td>
<td>25</td>
<td>23</td>
<td></td>
</tr>
<tr>
<td>t-PA activity (IU/mL)</td>
<td>25</td>
<td>25</td>
<td></td>
</tr>
<tr>
<td>PAI activity (AU/mL)</td>
<td>25</td>
<td>25</td>
<td></td>
</tr>
<tr>
<td>t-PA antigen (ng/mL)</td>
<td>25</td>
<td>23</td>
<td></td>
</tr>
</tbody>
</table>

NOTE. All data are mean ± SD. The primary analysis for change in fasting tHcy and the hematological variables between-groups across the 3-month treatment period are shown in the table. The secondary statistical analyses (within-groups) for change in values of tHcy and the hematological variables in the B-vitamin treatment and control groups, respectively, are listed below.

Abbreviations: NS, not significant; PAI, plasminogen activator inhibitor; tHcy, total homocysteine; t-PA, tissue plasminogen activator; vWF, von Willebrand factor.

For the within-subject analyses using paired t tests (2-tailed analysis),

* $P < .0001$
† $P = .005$
‡ $P < .05$. 

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**Fasting and Postmethionine Load Homocysteine**

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The mean change in plasma TM ($P = .19$) and total vWF antigen levels ($P = .17$) associated with B-vitamin treatment did not reach statistical significance between groups across time. However, a secondary within-groups analysis revealed a 23% decline in the mean circulating TM ($P = .005$) and a 27% elevation in vWF antigen levels ($P < .05$) in the B-vitamin treatment group, but no significant changes in the control group (Table 2). Further analysis excluding all stroke patients meeting the criteria for initial (baseline) vitamin deficiency revealed that B-vitamin treatment still produced an 18% reduction in fasting plasma tHcy ($10.5 \pm 2.8$ vs $8.6 \pm 2$ μmol/L, $n = 20$, $P < .002$), a 30% increase in plasma vWF antigen (81.5 ± 48.4 vs 106.2 ± 55%, $n = 19$, $P < .02$), and a trend of 23% lower plasma TM antigen levels ($14.2 \pm 7.7$ vs $10.9 \pm 5.7$ ng/mL, $n = 19$, $P = .1$).

In order to begin to address whether these changes in endothelial markers were related to baseline levels of fasting plasma tHcy, we compared the magnitudes of change in plasma TM and vWF antigen levels in the B-vitamin treated patients with tHcy above versus below the median split of 10.5 μmol/L. B-vitamin treatment was associated with a 25% increase in plasma vWF antigen levels in the stroke group with initial higher plasma tHcy ($102.8 \pm 50.6$ vs $129.1 \pm 59%$, $n = 12$, $P < .04$), and there was a trend toward increased mean plasma vWF antigen levels in patients with initial tHcy levels below this median split (76.1 ± 45 vs 99 ± 55%, $n = 13$, $P = .08$). There was a 27% reduction in mean plasma TM antigen levels (14.2 ± 6.4 vs 10.4 ± 4.1 ng/mL, $P < .03$) following B-vitamin treatment in the stroke group with initial higher fasting plasma tHcy levels, but no significant change in TM in the stroke group with baseline tHcy levels below this median split value (15 ± 8.1 vs 12 ± 5.9 ng/mL, $P = .28$).

**Endogenous fibrinolysis**

There were no changes in the levels of plasma t-PA, PAI activity, or t-PA antigen in the B-vitamin treatment or control groups (Table 2). Therefore, we performed a pooled analysis including all patients to determine
whether methionine load may affect endogenous fibrinolysis profiles in older stroke patients. Pooled analysis of baseline (prerandomization) data revealed a 20% decline in mean plasma t-PA activity levels following methionine load (n = 49, 1.82 ± 1.10 vs 1.46 ± 0.63 IU/mL, premethionine v postmethionine load, P = .013). In those patients that were available for repeated fibrinolysis measures at study completion, we observed a similar 28% decline in mean plasma t-PA activity with methionine load (n = 46, 1.72 ± 0.74 v 1.33 ± 0.51 IU/mL, pmethionine v post-methionine load, P < .001). As anticipated based on circadian variability, there was a modest decline in plasma PAI activity (n = 49, 18.6 ± 4.1 v 16.6 ± 4.8 au/mL, P < .001) across the 3-hour methionine load conducted at baseline, and again, a similar decline following methionine load performed at the time of study completion (n = 46, 19.4 ± 4.9 v 18.1 ± 1, P < .001). Three months B-vitamin treatment did not alter the fibrinolytic response to methionine load in these stroke patients (P = .4).

Discussion

Although in vitro studies suggest that homocysteine may alter vascular endothelial cell function, impairing the TM-dependent protein C antithrombotic mechanism, inhibiting vWF synthesis, and blocking t-PA binding to its specific receptor site, these vascular mechanisms and the effects of vitamin therapy to lower tHcy are not established in humans. In a randomized pilot study in stroke patients, we compared the effects of 3 months of multivitamin treatment with high-dose B-vitamin therapy that included 5 mg of folate, 100 mg of B6, and 1 mg of B12 to daily multivitamins lacking these 3 B-vitamin components and found no statistically significant differences in plasma tHcy, TM, vWF antigen, or fibrinolysis markers between groups across time. However, a secondary within-groups analysis revealed reductions in plasma tHcy and circulating TM antigen levels, a marker of endothelial injury, in the B-vitamin treatment group only. Further, we observed a significantly reduced plasma t-PA activity level following 3-hour methionine load, but report that B-vitamin treatment does not alter either the decline in t-PA activity following methionine loading or fasting before noon fibrinolysis profiles. These findings suggest that methionine load may lower endogenous plasma t-PA activity in older stroke patients and provide initial evidence that B-vitamin treatment may selectively alter markers of vascular endothelial injury after stroke.

The findings of reduced plasma TM by within-groups analysis in the B-vitamin treatment group raises the possibility of reduced endothelial injury. TM is an endothelial membrane glycoprotein which binds thrombin, terminating its prothrombotic actions, yielding an enzyme-cofactor complex that generates circulating activated protein C (APC) from its zymogen precursor form. Plasma TM is composed of major fragments representing the products of proteolytic injury at the endothelial cell surface. Elevated plasma TM is reported in a variety of disease conditions characterized by endothelial injury, including disseminated coagulation, active systemic lupus erythematosus, diabetic microangiopathy, and atherosclerotic disease. Prospective studies provide evidence that elevated plasma TM levels are an independent predictor for increased risk of serious hemorrhagic events and increased vascular mortality in patients receiving long-term warfarin therapy. In a nonrandomized study of 18 adults <50 years of age with peripheral arterial occlusive disease (PAOD), van den Berg et al. found that 1 year of treatment with folate (5 mg) and B6 (250 mg) lowered plasma TM antigen by 21%, findings interpreted as consistent with reduced vascular endothelial injury. Similarly, we observed a 23% reduction in plasma TM levels in our high-dose B-vitamin treatment stroke group only. Although this finding did not achieve statistical significance between groups across time in this small study, this degree of reduction in TM levels would be scientifically and clinically important. Thus, larger studies are needed to determine whether high-dose B-vitamin therapy has the potential to lower markers of endothelial injury in populations at high risk for cardiovascular disease.

The potential mechanism(s) and biologic significance underlying reduction in plasma TM levels remain uncertain. Lowered plasma TM levels, as suggested by the data in our B-vitamin treatment group and reported by van den Berg et al. in PAOD patients, could reflect a reduction in homocysteine-mediated vascular injury. In monkeys, the combination of diet-induced hyperhomocystinemia and hypercholesterolemia upregulates aortic TM expression, a process not reversed by B-vitamin therapy. Similarly, it seems unlikely that B-vitamin treatment could lower TM expression by reversing generalized atherosclerosis across such a brief 3-month treatment window in these elderly stroke patients. In vitro, homocysteine interrupts asparagine-linked oligosaccharide TM processing and damages susceptible disulfide bonds in epidermal growth factor-like regions, reducing functional TM expression at the endothelial cell surface. Although this reduces antithrombotic function, total TM protein synthesis and mRNA levels rise with prolonged homocysteine exposure. Hence, B-vitamin treatment to lower tHcy may reduce TM turnover while improving integrity of the TM-dependent protein C mechanism. However, these in vitro effects occur at much higher tHcy concentrations than encountered in our patients, and we cannot rule out the possibility that B vitamins may alter plasma TM levels by other mechanism(s) independent of lowering tHcy.

In contrast to van den Berg et al., we observed a paradoxical 27% increase in plasma total vWF antigen
levels in stroke patients in the B-vitamin treatment group only. These results are surprising, since elevated plasma vWF levels are considered a marker of endothelial injury and linked to increased risk for vascular events.\textsuperscript{32,33} In vitro, homocysteine interferes with pro-vWF asparagine-linked oligosaccharide processing and disulfide bond formation at the vWF dimer amino terminus, markedly reducing endothelial vWF synthesis and release.\textsuperscript{10} It is possible that we observed a treatment effect with short-term B-vitamin therapy leading to increased vWF levels by attenuating homocysteine-mediated suppression of normal endothelial vWF synthesis and release. Again, these dose-dependent in vitro effects on vascular endothelial cell vWF synthesis are reported at supraphysiologic homocysteine levels. Moreover, our findings in this pilot study are limited by a small sample size and must be interpreted with caution. The present study also cannot determine the mechanism for altered plasma vWF levels, nor address the possibility that a longer duration of B-vitamin therapy may have potential to lower vWF by reducing homocysteine-mediated endothelial injury, as suggested by van den Berg et al.\textsuperscript{30}

Impaired fibrinolysis has been proposed as a mechanism linking elevated tHcy to increased thrombotic risk.\textsuperscript{11,34,35} However, few studies have investigated the effects of B-vitamin therapy to lower tHcy on endogenous fibrinolysis profiles. In a noncontrolled study of PAOD patients, 12 months of high-dose folate and vitamin B\textsubscript{6} lowered plasma tHcy but did not affect t-PA antigen levels, suggesting that B-vitamin therapy does not alter endogenous fibrinolysis.\textsuperscript{30} The present study extends these observations that B-vitamin treatment does not lower plasma t-PA antigen levels or alter fasting morning plasma t-PA or PAI activity levels in stroke patients.

Notably, we observed a reduction in plasma t-PA activity levels following oral methionine load. Normally, fibrinolysis is circadian-dependent with the lowest plasma t-PA activity levels in the morning due to greater inhibition by PAI-1 and highest t-PA activity levels by evening hours as PAI-1 activity declines.\textsuperscript{36} We observed the decline in t-PA activity levels despite the measured circadian-related reduction in plasma PAI activity across the 3-hour time period, suggesting that methionine load may lower plasma t-PA activity by a PAI concentration-independent mechanism. In vitro, homocysteine inhibits t-PA binding to its specific vascular endothelial membrane receptor, annexin II, with half-maximal blockage occurring at 11 \textmu{mol}/L, a value similar to the median fasting plasma tHcy in our stroke patients and well below the plasma tHcy levels produced by methionine load.\textsuperscript{11,37} It is possible that homocysteine-mediated blockage of t-PA, as reported by Hajjar et al.,\textsuperscript{39} could lead to subsequent altered kinetics of t-PA neutralization by inhibitors or hepatic clearance (i.e., reducing endogenous t-PA activity by mechanisms independent of plasma PAI-1 regulation). However, the present study is limited, as we can neither determine the mechanism nor discriminate between increased plasma methionine or tHcy as the agent responsible for any reduction in endogenous t-PA activity with the methionine load. Regardless, our findings indicate the hypofibrinolytic response to methionine load is not attenuated by B-vitamin treatment in stroke patients. Notably, oral methionine load constitutes a supraphysiologic stimulus to increase plasma tHcy.\textsuperscript{17} Further studies are needed to determine whether a hypofibrinolytic response is produced under the physiologic conditions of a high-protein-load meal.

Results from this randomized pilot study must be interpreted with caution. Our primary analysis showed no statistically significant differences in plasma tHcy, markers of endothelial injury, or fibrinolysis between treatment groups across time. Only the secondary analysis within the B-vitamin treatment group revealed declines in TM and tHcy and increased vWF antigen levels, weak evidence supporting a treatment effect. However, a power analysis based on data from our trial indicates there was a high probability of type II error in the statistical assessment of the between-group differences. Furthermore, the biologic effects of B-vitamin treatment on endothelial function may be diminished in this stroke cohort that had essentially normal baseline mean plasma tHcy levels (11.3 \textmu{mol}/L).\textsuperscript{38-40} The latter is supported by our findings that reductions in plasma TM and increased vWF antigen levels achieved significance only within that subgroup of B-vitamin treated stroke patients that had baseline fasting tHcy levels above the median split. Nevertheless, these results are considered preliminary and a larger randomized trial is underway to further investigate these findings.

In summary, this randomized, placebo-controlled trial does not establish that high-dose B-vitamin therapy can significantly alter tHcy or markers of vascular endothelial injury in unselected older stroke patients. However, our findings of reduced plasma TM, within-subjects in the treatment group only, corroborate a prior study that high-dose B-vitamin therapy lowers this circulating marker of vascular endothelial injury.\textsuperscript{30} This study also provides evidence that methionine load may reduce endogenous plasma t-PA activity levels in older stroke patients by a mechanism that is independent from the usual circadian physiological regulation by PAI. Since impaired endogenous fibrinolysis is an independent predictor for increased risk of stroke and myocardial infarction,\textsuperscript{38-40} this mechanism warrants further investigation. Our findings further suggest against any beneficial effect of short-term (i.e., 3 months) high-dose B-vitamin therapy on plasma-endogenous fibrinolysis profiles. Further studies in larger populations are needed to understand and confirm the effects of B-vitamin treatment on endothelial function in high atherothrombotic risk populations and across the spectrum of aging.
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References


