Low Plasma Levels of Vitamin B_6 Are Independently Associated With a Heightened Risk of Deep-Vein Thrombosis

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Background—Elevated plasma levels of total homocysteine (tHcy) before and after an oral methionine load (PML) are associated with an elevated risk of deep-vein thrombosis (DVT). We investigated whether plasma levels of B vitamins that are involved in Hcy metabolism are associated with an elevated risk of DVT.

Methods and Results—We compared 397 cases with previous DVT with 585 matched healthy controls. The plasma levels of folate, vitamin B_{12}, vitamin B_6, and fasting and PML tHcy were measured. The ORs for DVT associated with high (>95th percentile) fasting levels and PML increases of tHcy were 2.1 (95% CI, 1.2 to 3.4) and 2.4 (95% CI, 1.5 to 3.9) after adjustment for established risk factors for DVT. Fasting plasma levels and PML increases in tHcy correlated negatively with vitamin levels. The crude OR for folate levels in the lowest quartile compared with the highest was 1.5 (95% CI, 1.1 to 2.1), and that for B_6 levels in the lowest and second quartiles compared with the highest was 1.5 (95% CI, 1.0 to 2.1). However, after adjustment for established risk factors and fasting and PML tHcy, the ORs for B_6 levels in the lowest and second quartiles only remained statistically significant (lowest quartile: OR, 1.8; 95% CI, 1.2 to 2.8; second quartile, OR, 1.9; 95% CI, 1.3 to 2.9).

Conclusions—High fasting and PML tHcy and low vitamin B_6 plasma levels are associated with an elevated risk for DVT independently of established risk factors for DVT. The association of low vitamin B_6 levels with the risk for DVT is independent of fasting and PML tHcy levels. **(*Circulation*. 2001;104:2442-2446.)**

Key Words: thrombosis ■ risk factors ■ veins ■ homocysteine ■ vitamin B_6

Case-control studies have shown that elevated plasma levels of homocysteine (Hcy) both before and after an oral methionine load (PML) are associated with a heightened risk for venous thromboembolism.[1] Two meta-analyses of the results of these studies showed that the OR for venous thromboembolism associated with hyperhomocysteinemia is ≈2.5.[2,3] This number is similar to those obtained in a previous meta-analysis of studies of the association of hyperhomocysteinemia with the risk of coronary artery disease and cerebrovascular disease.[4] Although the analogy with the clinical history of patients with homocystinuria resulting from deficiencies of an enzyme involved in Hcy metabolism who suffer from severe episodes of venous and/or arterial thrombosis at a young age[5] strengthens the hypothesis that hyperhomocysteinemia is a risk factor for venous thromboembolism, some questions are still left without a clear answer.

First, prospective studies gave conflicting results. Hyperhomocysteinemia was associated with increased risk of future venous thromboembolic events in patients with factor V Leiden[6] or previous thrombotic episodes[7] but not in patients undergoing total hip replacement surgery[8] or those with systemic lupus erythematosus.[9] Second, the presence of a thermolabile variant of the enzyme methylenetetrahydrofolate reductase resulting from a C-to-T substitution at nucleotide 677 of the encoding gene,[10] which is often associated with mild hyperhomocysteinemia, does not seem to increase the risk of venous thromboembolsim,[1] although it might increase this risk in patients with factor V Leiden.[11,12] Finally, the administration of high doses of vitamin B_6 to patients with homocystinuria caused by deficient activity of cystathionine-β-synthase is associated with considerable reduction in their thrombotic risk, despite the fact that their plasma Hcy levels remain moderately increased.[13]

Although several explanations may be proposed to reconcile the above findings with the hypothesis that moderate hyperhomocysteinemia is a risk factor for venous thromboembolism,[1,14] we should consider the hypothesis that high Hcy levels are either a consequence of venous thrombosis or just a marker of other diseases and/or deficiencies of B vitamins, which can by themselves be responsible for biochemical abnormalities that increase the risk of venous thrombosis independently of circulating Hcy levels.

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TABLE 1. Characteristics of Patients With DVT

n (M/F)	397 (194/203)
Median age (range), y	42 (10–80)
Median age at DVT (range), y	39 (6–74)
Median time elapsed since DVT episode (range), mo	11 (0.5–46)
Idiopathic DVT,* n (%)	163/397 (41)
Recurrences after enrollment, n (%)	61/397 (15)

*DVT episodes were considered idiopathic if they did not occur in association with recent (≤3 mo) surgery, trauma/immobilization, pregnancy/puerperium, or oral contraceptive intake.

Because no study has so far considered the relationship between the plasma levels of total Hcy (tHcy) and vitamin status and their independent association with venous thrombosis, we investigated the relationship between the plasma levels of tHcy before and after an oral methionine load with vitamin status in patients with deep-vein thrombosis (DVT) and in healthy control subjects. The independent association of folate, vitamin B_{12}, vitamin B_6, and fasting and PML Hcy with the risk of venous thrombosis was also investigated.

Methods

Subjects

Three hundred ninety-seven patients with a first episode of DVT of the lower extremity that occurred between August 1, 1995, and January 1, 1999, were referred to our center for diagnosis and/or treatment of the acute episode, monitoring of oral anticoagulant treatment, or screening for thrombophilic states (Table 1). All diagnoses of DVT had been confirmed by phlebography or compression ultrasonography. Five hundred eighty-five control subjects (243 male subjects, 342 female subjects; median age, 44 years; range, 13 to 77 years) were chosen from the same geographical area and with the same cultural background as the study population. They were partners or friends of the index patients, patients in whom a suspected DVT had been ruled out by objective diagnostic techniques, or laboratory personnel. Previous episodes of venous thromboembolism and arterial occlusive disease were excluded for all control subjects by a structured questionnaire validated for retrospective diagnosis of thrombosis.[15] No subject had abnormal liver or renal function or overt autoimmune or neoplastic diseases. The study was approved by the review committee of the University of Milano. All subjects gave informed consent to the study.

Blood Samples

After an overnight fast, venous blood samples were drawn at about 8 AM into vacuum tubes containing 3.8% trisodium citrate (for coagulation measurements), EDTA (for measurement of tHcy and vitamin B_6 and for extractions of leukocyte DNA), or no anticoagulant (for measurements of anticardiolipin antibodies, folates, and vitamin B_{12}). L-Methionine (3.8 g/m^2 body surface area) was then administered orally in ≈200 mL fruit juice. Four hours later, a second blood sample was drawn for plasma tHcy assay.

Polymorphisms Associated With Heightened Risk of DVT

DNA analyses for G1691A substitution in coagulation factor V gene, responsible for factor V:Q^{506} (factor V Leiden), and for G20210A substitution in coagulation factor II gene were carried out as described.[12]

Measurement of Plasma Levels of tHcy

tHcy levels (free and protein bound) were measured by high-performance liquid chromatography and fluorometric detection as described.[16] The between-run coefficients of variation for this assay were 4.6% for fasting tHcy and 2.7% for PML tHcy.

Pyridoxal-5′-phosphate (PLP), the coenzyme form of vitamin B_6, was measured by the tyrosine decarboxylase method described by Shin-Buehring et al[17]; the between-assay coefficient of variation was 11%. Serum folates and vitamin B_{12} were measured by radioimmunoassay (Becton and Dickinson); the between-assay coefficients of variation were 9.2% for folate and 8.5% for vitamin B_{12}.

Resistance to activated protein C, antithrombin, protein C, and total and free protein S was measured as described.[12] The antiphospholipid syndrome was diagnosed when lupus anticoagulant and/or anticardiolipin antibodies were positive on the basis of standardized criteria.[18,19]

TABLE 2. Median Values of Relevant Parameters in DVT Patients and Healthy Control Subjects

	DVT Patients (n=397)	Control Subjects (n=585)	*P*
Fasting tHcy, μmol/L	9.5 (4.5–223)	8.9 (4.8–51)	0.004
ΔPML tHcy, μmol/L	14.1 (4.9–64)	13.0 (3.6–46)	0.001
Folate, nmol/L	5.5 (1–25)	6.0 (2–24)	0.045
Vitamin B_{12}, pmol/L	403 (45–2400)	390 (89–2400)	0.640
PLP, nmol/L	29.9 (1.1–324)	33.2 (3.7–363)	0.006

Values in parentheses are ranges. The PML test was not performed in 22 patients and 16 control subjects. Data were analyzed with the Mann-Whitney *U* test.

Statistical Analysis

Values of continuous variables were expressed as medians and ranges. Differences between groups were assessed by the Mann-Whitney *U* test. The correlation between tHcy and vitamin levels was assessed by Spearman's correlation test. Hyperhomocysteinemia was diagnosed when either the fasting tHcy plasma levels or their PML increases above the fasting levels were higher than their respective 95th percentiles of distribution among control subjects. The relative risk of DVT associated with hyperhomocysteinemia was expressed as the OR and its 95% CI. The relative risk of DVT associated with low levels of folate, vitamin B_{12}, and vitamin B_6 was assessed by dividing vitamin levels into quartiles and calculating the ORs for different quartiles, with the highest quartile considered the reference. First, we calculated crude ORs by simple cross-tabulation; then, we adjusted for potential confounders by a logistic regression analysis. A value of $P<0.05$ was chosen as the cutoff level for statistical significance.

Results

The median values of plasma levels of fasting tHcy and its PML increases above fasting levels were significantly higher in DVT patients than in control subjects (Table 2). The median values of serum levels of folate and PLP were significantly lower in DVT patients than in control subjects, whereas no statistically significant difference in vitamin B_{12} levels was found between the 2 groups (Table 2).

The fasting plasma levels of tHcy and their PML increases were negatively correlated with serum folate ($\rho=-0.48$ and -0.28, respectively; $P<0.001$) and, although to a lesser extent, with vitamin B_{12} ($\rho=-0.26$, $P<0.001$, and -0.08, $P=0.03$) and PLP ($\rho=-0.13$ and -0.14, $P<0.001$).

The prevalence of hyperhomocysteinemia both before and after an oral methionine load was higher in DVT patients than in control subjects (Table 3). Eight patients and 10 control subjects had both high fasting tHcy levels and high PML tHcy increments. The crude ORs for DVT associated with

TABLE 3. Prevalence of High Fasting Levels of tHcy and PML Increments of tHcy Above Fasting Levels Among Patients With DVT and Control Subjects

	Subjects With High Values,* n (%)		OR (95% CI)		
	DVT Patients	Control Subjects	Crude	Adjusted†	Adjusted‡
Fasting tHcy	38/397 (10)	29/585 (5)	2.1 (1.2–3.4)	1.8 (1.0–3.0)	1.6 (0.9–3.1)
ΔPML tHcy	43/375 (11)	29/569 (5)	2.4 (1.5–3.9)	2.6 (1.5–4.4)	2.1 (1.2–3.7)

The PML test was not performed in 22 patients and 16 controls.

*Higher than the 95th percentile of distribution of values in healthy subjects.

†Adjusted for age, sex, and other risk factors for DVT (factor V Leiden; G20210A factor II; deficiency of antithrombin, protein C, or protein S; presence of the antiphospholipid syndrome).

‡Adjusted for the above plus serum levels of folate and vitamin B_{12} and plasma levels of PLP.

high fasting tHcy and its PML increases were 2.1 (95% CI, 1.2 to 3.4) and 2.4 (95% CI, 1.5 to 3.9), respectively. After adjustment for conventional risk factors for DVT (factor V Leiden; G20210 factor II; activated protein C resistance; deficiency of antithrombin, protein C, or protein S; presence of the antiphospholipid syndrome), the ORs for DVT associated with hyperhomocysteinemia decreased slightly. However, when data were also adjusted for folate, vitamin B_{12}, and PLP levels, the association of PML tHcy increments and the risk of DVT only remained statistically significant (Table 3), whereas the OR for fasting tHcy decreased to 1.6 (95% CI, 0.9 to 3.1).

Table 4 shows the ORs of patients and control subjects after stratification of the levels of folate, vitamin B_{12}, and PLP. The OR for folate levels in the lowest quartile of distribution (<4.4 nmol/L) was 1.5 (95% CI, 1.1 to 2.1) compared with the highest quartile (≥8.0 nmol/L). The relative risk for the lowest quartile of folate decreased to 1.1 (95% CI, 0.7 to 1.5) after adjustment for the presence of established risk factors for DVT and to 0.7 (95% CI, 0.5 to 1.2) after adjustment for risk factors for DVT and fasting and PML hyperhomocysteinemia. No increased risk of DVT was associated with folate in the second and third quartiles.

The crude and adjusted ORs for vitamin B_{12} in the lowest (<301 pmol/L) and intermediate quartiles of distribution compared with the highest quartile (≥504 pmol/L) were never >1 (Table 4). In contrast, the ORs for PLP in the lowest (<21.7 nmol/L) and second (21.7 to 33.2 nmol/L) quartiles compared with the highest (≥46.5 nmol/L) quartile were 1.5 (95% CI, 1.0 to 2.1) and remained significantly >1 also after adjustment for established risk factors for thrombosis (OR, 1.7; 95% CI, 1.1 to 2.4; and OR, 1.7; 95% CI, 1.1 to 2.5, respectively) or for established risk factors for thrombosis, hyperhomocysteinemia and the plasma levels of folate and

TABLE 4. DVT Risk for Quartiles of Serum Levels of Folate and Vitamin B_{12} and Plasma Levels of PLP

Vitamin Levels	Patients (n=397), n	Control Subjects (n=585), n	OR (95% CI)		
			Crude	Adjusted*	Adjusted†
Folate, nmol/L					
<4.4	132	140	1.5 (1.1–2.1)	1.1 (0.7–1.5)	0.7 (0.5–1.2)
4.4–6.0	63	116	0.9 (0.6–1.3)	0.7 (0.5–1.1)	0.7 (0.4–0.9)
6.1–7.9	92	159	0.9 (0.6–1.3)	0.8 (0.6–1.2)	0.8 (0.5–1.2)
≥8.0	97	154	1.0 (Ref)	1.0 (Ref)	1.0 (Ref)
Vitamin B_{12}, pmol/L					
<301	105	142	0.9 (0.7–1.3)	0.9 (0.6–1.3)	0.8 (0.5–1.2)
301–390	81	145	0.7 (0.5–1.1)	0.7 (0.5–1.1)	0.7 (0.5–1.1)
391–503	89	144	0.8 (0.5–1.2)	0.8 (0.5–1.2)	0.8 (0.5–1.2)
≥504	112	142	1.0 (Ref)	1.0 (Ref)	1.0 (Ref)
PLP, nmol/L					
<21.7	120	146	1.5 (1.0–2.1)	1.7 (1.1–2.4)	1.8 (1.2–2.8)
21.7–33.2	122	146	1.5 (1.0–2.1)	1.7 (1.1–2.5)	1.9 (1.3–2.9)
33.3–46.4	72	147	0.9 (0.6–1.3)	0.9 (0.6–1.4)	1.0 (0.6–1.6)
≥46.5	83	146	1.0 (Ref)	1.0 (Ref)	1.0 (Ref)

Ref indicates reference. Folate levels were available for 384 patients and 569 control subjects; vitamin B_{12} levels, for 387 patients and 573 control subjects; and PLP levels, for all patients and control subjects.

*Adjusted for age, sex, and other risk factors for DVT (factor V Leiden; G20210A factor II; deficiency of antithrombin, protein C, or protein S; presence of the antiphospholipid syndrome).

†Adjusted for the above plus levels of other vitamins, fasting tHcy, and ΔPML tHcy.

vitamin B_{12} (OR, 1.8; 95% CI, 1.2 to 2.8; and OR, 1.9; 95% CI, 1.3 to 2.9).

Discussion

This case-control study of 397 patients with a first episode of DVT confirms previous reports that showed a statistically significant association between hyperhomocysteinemia and an increased risk of DVT[1–3]; in addition, it demonstrates for the first time that low levels of PLP are associated with an increased risk of DVT also independently of the high plasma Hcy levels.

The independent association of hyperhomocysteinemia with venous thromboembolism has been questioned in previous studies.[6,20] Here, we demonstrated that this association is independent of the presence of common and well-established risk factors, including the recently described mutation G20210A of the gene encoding for coagulation factor II.[21] In our study, the OR for DVT associated with hyperhomocysteinemia was ≈2, regardless of whether fasting tHcy levels or its PML increases above fasting levels were considered. These findings match those published in 2 previous meta-analyses of 9 case-control studies, indicating that the enrollment criteria of our cases and controls were similar to those of previous studies.[2,3] Although the magnitude of the relative risk of DVT associated with fasting and PML tHcy was similar, it is important to note that in many instances, the 2 measurements did not identify the same subjects at risk. In fact, fasting tHcy is very sensitive, albeit not exclusively,[22] to abnormalities of the cobalamin- and folate-dependent remethylation pathway, whereas PML tHcy is more sensitive to the PLP-dependent transsulfuration pathway.[23] Moreover, our finding that the association of PML hyperhomocysteinemia with a heightened risk of DVT remained statistically significant after adjustment for the circulating levels of folate, vitamin B_{12}, and PLP suggests that PML tHcy may also be under the control of other less-known variables. These data add to the evidence in the literature that the 2 parameters, far from being interchangeable, should be measured in conjunction for a more accurate evaluation of the risk of thrombosis.[1,24]

Both fasting and PML tHcy correlated negatively with the circulating levels of folate, vitamin B_{12}, and PLP; as in previous studies of patients with arterial thrombotic diseases and normal subjects, the strongest correlation was found with folate levels,[1] which we measured in serum, although measurement of red cell folate would probably better describe a person's folate status. The median circulating levels of folate and PLP but not those of vitamin B_{12} were significantly lower in DVT patients than in control subjects, demonstrating for the first time that vitamin deficiencies are common not only in patients with atherosclerosis but also in patients with DVT. When the association between vitamin status and the risk of DVT was investigated, a statistically significant association was found for low levels of folate and PLP but not of vitamin B_{12}. However, when other risk factors of DVT and fasting and PML tHcy were included in the multivariate analysis, no association was found between low folate levels and the risk of thrombosis, whereas the association with low PLP levels remained unmodified or even increased (The risk of DVT was ≈2-fold higher for individuals with PLP in the lowest quartile than for those with PLP in the highest quartile). This indicates that although the increased risk of DVT associated with low folate levels may in great part be explained by the associated higher circulating levels of Hcy, the DVT risk associated with low PLP levels is independent of Hcy status.

Although our study is the first to demonstrate an association of low vitamin B_6 levels with the risk of venous thromboembolism, previous case-control and prospective studies found an association of low vitamin B_6 levels with the risk of atherothrombotic diseases.[25–27] In these studies, the risk was independent of fasting[26,27]and PML[26] hyperhomocysteinemia. The hypothesis that low vitamin B_6 levels may increase the risk of thrombosis is not new. Early studies by Rinehart and Greenberg[28] reported that monkeys maintained on diets deficient in vitamin B_6 developed vascular lesions that were similar to those found in humans with atherosclerosis. Although the hypothetical thrombogenic and atherogenic mechanisms of low vitamin B_6 might certainly involve the associated increase in Hcy, additional mechanisms of vascular damage have been advocated.[27,29] In addition, many as-yet-unknown links between vitamin B_6 and the pathogenic mechanisms of atherosclerosis and thrombosis can be hypothesized, considering the >100 enzymatic reactions involved in the metabolism of amino acids, carbohydrates, neurotransmitters, and lipids in which vitamin B_6 functions as a coenzyme.[30] Among the possible effects of vitamin B_6 in thrombogenic mechanisms, an influence on platelet function has been suggested. Ex vivo studies have shown that oral administration of vitamin B_6 inhibits platelet aggregation, probably by interfering with the platelet receptors for ADP, and prolongs bleeding time.[31] It is presently unknown, however, whether platelet function in patients with low plasma levels of vitamin B_6 is enhanced compared with that of individuals with normal vitamin B_6 levels.

Whether or not the association of low vitamin B_6 levels with venous and/or arterial thrombosis is causal cannot be stated on the basis of present knowledge. The most important criterion for establishing a causal relationship between a potential risk factor and a disease is the demonstration that modification of the risk factor influences the risk. Although there are no demonstrations that the normalization of the plasma levels of vitamin B_6 brings about the normalization of the risk for atherosclerosis and thrombosis, there are some indirect indications in the literature that supplementation of high doses of vitamin B_6 may be beneficial. A retrospective study showed that patients with carpal tunnel syndrome or other degenerative diseases who were treated with vitamin B_6 had a significantly lower incidence of myocardial infarction than similar patients not treated with vitamin B_6.[32] Another observational study of 8008 women showed that the use of vitamin preparations containing vitamin B_6 was associated with a lower incidence of atherothrombotic events.[33] In addition, it is well established that the high risk of both arterial and venous thrombosis among patients with homocystinuria resulting from cystathionine-β-synthase deficiency is considerably reduced by the supplementation of high-dose vitamin B_6.[5,13] Because this protective effect can be observed despite the lack of complete normalization of the plasma Hcy

levels,[13] the possibility should be considered that the protective effect of vitamin B_6 is mediated not only by the induced decrease in Hcy levels but also by other unknown mechanisms.

Several clinical trials on the effects of the administration of folate on the risk for thrombosis are ongoing. Because some of them associate the administration of vitamin B_6 to that of folate, including 1 study on patients with previous venous thromboembolic episodes,[34] they might give an indication of whether vitamin B_6 confers an additional antithrombotic effect.

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