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REGULAR ARTICLE

Pilot study of homocysteine and cysteine in patients with thrombosis in different vascular sites. Epidemiology and response to folate

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Abstract

Hyperhomocysteinemia is a risk factor for arterial and venous thrombosis. However, lowering homocysteine (Hcy) with vitamins not only failed to improve outcomes but also may lead to recurrent events. Our objectives were to evaluate Hcy and cysteine (Cys) levels in patients with thrombosis in different vascular sites, and their response to folate.

One hundred and sixty four consecutive patients with thrombosis (42.1% arterial (AT), 36% venous (VT), 4.9% both venous and arterial thrombosis (AVT) and 17% unusual site (UST)) were included. Hcy and Cys were highest in patients with AVT and UST ($p=0.0006$). Ninety-three patients were treated, 70% were followed-up. Hcy levels normalized after therapy in all patients. Cys levels tended to vary after therapy according to the site of thrombosis. We observed a significant correlation between folate and Hcy (r: 0.48; $p=0.005$) among homozygous for MTHFR. A significant inverse relation was observed between Hcy and folate among homozygous and heterozygous (r: 0.462, $p=0.007$ and r: 0.267; $p=0.04$, respectively). No correlation was observed between folate and Cys.

In conclusion, our observations suggest that Hcy and Cys might be implicated in thrombosis in different vascular sites, and respond differently to folate.
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Introduction

Mild hyperhomocysteinemia (HHcy) has been identified as a risk factor for thrombosis both in the arterial

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and venous sites [1,2]. However, lowering homocysteine (Hcy) levels has failed to improve outcomes in patients with cardiovascular arterial disease [3]. Moreover, a recent trial [4] observed a trend towards more myocardial infarctions though fewer strokes among patients receiving folic acid, vitamin B12 and vitamin B6 than among those receiving placebo. In addition, among patients with venous thrombosis, mild HHcy, low folates, and vitamin B12 levels were independent risk factors for thrombosis [5] but multivitamin supplementation failed to reduce recurrent venous thromboembolism (VTE) [6].

These observations suggest that the vascular site and the complex behaviour of the interactive redox-thiol system and its intermediates might play a role in the genesis of thrombosis. In addition, folic acid and vitamins B12 and B6 administration attenuated thrombin generation both in peripheral blood and at sites of hemostatic plug formation [7], suggesting that adverse clinical outcomes might be due to other components of the metabolic pathway, which are either unresponsive to or increase its levels due to vitamin supplementation.

Cysteine (Cys), an intermediate in the trans-sulfuration pathway, is less reactive than Hcy but may also provoke endothelial toxicity by the generation of free radicals and peroxides [1]. Clinical data suggests that high Cys levels would also be a risk factor for thrombosis, independent of Hcy levels [1,8,9].

Moreover, as pro-thrombotic potential is vascular bed-specific [10], the endothelium of different vascular sites would be critical in the local regulation of coagulation. Since both low molecular weight thiols, Hcy and Cys, are intermediates in the same complex metabolic pathway, their evaluation in patients with vascular occlusive disease (VOD) might help enlighten the mechanism of thrombosis in different vascular sites, and add to clinical management.

We hypothesized that Hcy and Cys levels respond differently to vitamin therapy and independently from each other, and that the response should be bed-specific. Such behaviour would explain the different outcomes in different vascular sites after supplementation.

We prospectively investigated Hcy and Cys levels in patients with thrombosis in different vascular sites, and the effect of folate therapy in Hcy and Cys levels.

Patients, materials and methods

We designed a pilot epidemiological analytical study of Hcy and Cys levels among patients with thrombosis in different vascular sites, and their response to standard therapy, i.e.: folate supplementation.

Between September 2002 and November 2003, we included consecutive patients referred to the Thrombosis and Hemostasis Department, Institute of Hematological Research, National Academy of Medicine, Buenos Aires, Argentina for screening of thrombophilia due to VOD.

Patients were eligible if they have had arterial (i.e.: myocardial infarction, unstable angina, non-embolic cerebro-vascular disease and stroke), venous (i.e.: deep venous thrombosis and pulmonary embolism), unusual site (i.e.: upper extremity, mesenteric and cerebral venous thrombosis), or thrombosis in both arterial and venous sites. Patients were included regardless of chronic oral anticoagulant therapy, and at least 90 days from the acute event.

Exclusion criteria were vitamin intake, refusal to participate, and patients younger than 18 years.

Hcy and Cys levels were measured in fasting blood samples by high-performance liquid chromatography at referral and at least 4 four weeks after supplementation therapy. Normal homocysteine values: 10–15 μm/l; normal cysteine values: 250–350 μm/l [11]. MTHFR genotype of the thermolabile variant (C677T) was investigated by PCR [12].

Count blood cells, renal and hepatic function test, cholesterol and glucose levels were measured in all patients. Erythrocytic folate, serum folate and serum vitamin B12 were measured (IMX, Abbott) before supplementation therapy was initiated.

Supplementation therapy regimens were as follows: patients with Hcy levels between 10 and 15 μm/l (Group A) received folic

Table 1 Patients' characteristics and laboratory parameters according to the site of thrombosis

	Venous	Arterial	Arterial–venous	Unusual
Age (years)	47.6±14.9	45.32±15.6	51.25±12.4	50.28±16.9
Hcy (μm/mL)	13.12± 4.31	14.57±9.34	21.95±14.89	15.13±6.9
Cys (μm/mL)	345.42±64.0	342.74±65.1	374.85±76.3	384.3±106.7
MTHFR (*N*, %)				
Not done	16 (28.1)	14 (20.6)	1 (12.5)	4 (13.8)
Normal	18 (31.6)	22 (32.4)	1 (12.5)	8 (27.6)
Heterozygous C677T	17 (29.8)	22 (32.4)	4 (50)	8 (27.6)
Homozygous C677T	6 (10.5%)	10 (14.7)	2 (25)	9 (31)
Hemoglobin (g/dl)	12.9±0.2	12.8±0.3	11.6±0.5	12.0±0.6
Cholesterol (mg/dl)	225.6±8.0	223.5±8.2	215.0±8.5	234.0±10.8
Glucose (mg/dl)	91.6±2.0	89.2±3.1	93.1±4.4	90.2±2.7
Creatinine (mg/dl)	0.8±0.1	0.7±0.3	3.0±1.1	2.25±0.6
TGP (IU/L)	30.0±2.6	29.5±2.5	31.0±8.0	29.4±3.7
TGO (IU/L)	28.1±1.6	27.4±1.4	27.6±2.4	27.6±1.6
Alkaline phosphatase (IU/L)	201.3±11.7	178.7±9.6	219.1±16.4	187.3±12.1

Table 2 Prevalence of HHCy and HCys according to the site of thrombosis

Site of thrombosis	Hcy levels (mean ± SD)	Cys levels (mean ± SD)	Patients with HHcy (%)	Hcy levels among patients with HHcy (mean ± SD)	Patients with HCys (%)	Cys levels among patients with HCys (mean ± SD)
Venous	12.9 ± 4.3	345.2 ± 63.3	26.6	18.5 ± 3.8	44	403.4 ± 42.5
Arterial	14.6 ± 9.3	341.8 ± 65.9	35.2	23.3 ± 11.4	46.9	397.7 ± 41.7
Venous and arterial	21.9 ± 14.8	374.8 ± 76.3	50	32.5 ± 14.2	42.8	442.3 ± 72.0
Unusual site	15.13 ± 6.9	384.3 ± 106.7	42.8	20.2 ± 6.2	39.2	468.1 ± 92.8

acid 1 mg/d while patients with HHcy (i.e.: Hcy levels >15 μm/l; Group B) received 5 mg/d for 4 weeks at least. When patients persisted with hyperhomocysteinemia, MTHFR genotype was taken into consideration to correct therapy. Patients with normal genotype changed to vitamin B complexes, while if a MTHFR variant was present the dose of folic acid was increased to 10 mg/d.

All participants completed a written questionnaire containing data regarding concomitant diseases (arterial hypertension, thyroid disease, renal failure, hypercholesterolemia, diabetes, etc.), medications, habits (alcohol intake, smoking, and exercise) and diet. The Institutional Review Board approved the study protocol. All subjects gave informed written consent.

Statistical analysis

SPSS 10.0 and Epi Info 6 software were used for all statistical analyses. Descriptive statistics and comparisons were performed among the groups. Hcy and Cys values were logarithmically transformed before the calculation of correlations.

Results

Two hundred and twenty-one patients were referred for evaluation during the study period. One-hundred and eighty three meeting the inclusion criteria were included; 19 (10.3%) were lost for follow-up after diagnosis.

Study population included 164 patients with VOD. Sites of thrombosis were arterial (AT, $N=69$, 42.1%), venous (VT, $N=59$, 36%), both arterial and venous (AVT, $N=8$, 4.9%), and unusual site (UST, $N=28$, 17%). Sixty four percent of patients were female (105 female/57 male), median age was 47.3 ± 15.47 years.

Men, regardless of reason for evaluation, had significant higher levels of Hcy (16.3 ± 8.5 μm/l) and Cys (360.8 ± 63.3 μm/l) than women (13.2 ± 7.8 and 336.4 ± 64.9 μm/l, $p=0.034$ and $p=0.039$, respectively). Table 1 shows patients' characteristics and laboratory parameters of study population according to the site of thrombosis. High creatinine levels were observed in patients with AVT and in patients with UST. No differences were observed in the other parameters among the groups. Table 2 shows Hcy and Cys levels, and prevalence of HHcy and hypercysteinemia (HCys) according to the site of thrombosis.

Table 3 Median Hcy levels according to MTHFR genotype before and after therapy

Hcy	MTHFR	Basal	Post therapy	*P*
>10 ≤ 15 μm/l	Heterozygous	12.95 ± 1.5	9.02 ± 2.14	0.0006
	Homozygous	12.81 ± 1.5	10.02 ± 1.9	0.01
>15 μm/l	Normal	18.6 ± 4.2	10.38 ± 4.1	0.0006
	Heterozygous	18.0 ± 7.6	10.6 ± 4.0	0.02
	Homozygous	23.9 ± 12.3	9.5 ± 2.5	0.0001

Factors affecting Hcy and Cys levels

Habits and concomitant diseases

Hcy levels were higher in patients reporting alcohol consumption than in those who did not. Patients drinking "mate", a typical infusion of many countries in Latin America, and those who eat cereals had the lowest Hcy levels. Cys levels were higher in patients that smoke or had hypercholesterolemia.

Site of thrombosis and MTHFR genotype

Patients with AVT and UST had the highest Hcy and Cys levels (Table 2) ($p=0.0006$ and 0.001, respectively).

Homozygous for MTHFR variant had the highest Hcy levels (17.88 ± 10.65 versus 13.09 ± 6.39 and 13.28 ± 4.71 in heterozygous and normal genotypes, respectively, $p=0.01$). Cys levels were higher among homozygous and normal genotypes than in heterozygous, though not statistically significant (349.60 ± 79.45, 347.80 ± 71.22, and 328.45 ± 84.82, $p=0.2$).

More patients with UST than with VT were homozygous for MTHFR variant (31% versus 10.5%, $p=0.01$); 25% of patients with AVT ($p=0.06$) and 14.7% of those with AT (not statistically significant).

Folate and vitamin B12 status

Erythrocytic folate, serum folate and serum vitamin B12 were measured in 157 patients. A significant relation was found between logarithm of folate (relation erythrocytic/serum, log EF/SF) and Hcy levels (r: 0.48; $p=0.005$) in homozygous patients. A significant inverse relation between Hcy logarithm

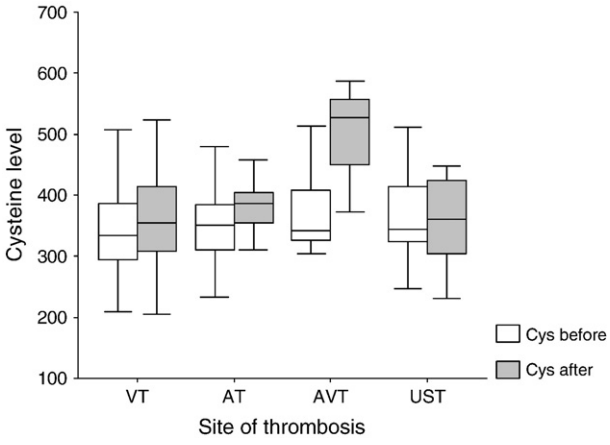

Figure 1 Cys levels before and after vitamin therapy.

and log EF/SF was observed in homozygous and heterozygous patients (r: 0.462, $p=0.007$ and r: 0.267; $p=0.04$, respectively). No relation was found between log EF/SF and Cys.

Response to vitamin supplementation therapy

HHcy was diagnosed and treated in ninety-three patients, 70% were followed-up. No differences in age, thrombosis site, or median Hcy and Cys values were observed between the patients lost for follow-up and those with complete follow-up.

Median Hcy levels before and after therapy were 17.95 ± 8.85 and 10.65 ± 3.95 μm/l, respectively ($p<0.00001$), median treatment duration was 8.65 ± 6.52 weeks.

Response to therapy was evaluated according to basal Hcy levels (≥10 but <15 μm/l, Group A, and >15 μm/l, Group B) and MTHFR genotype (Table 3).

In patients in Group A with normal genotype, no difference was observed among Hcy and Cys levels pre and post therapy with folic acid 1 mg/d. Median basal Hcy level in heterozygous was 12.95 ± 1.5 μm/l while after therapy it was 9.02 ± 2.14 μm/l ($p=0.0006$). In homozygous for the MTHFR variant, Hcy levels pre and post supplementation were 12.81 ± 1.5 y 10.02 ± 1.9 μm/l, respectively ($p=0.01$).

Patients in Group B showed a statistically significant difference in Hcy levels before and after therapy with folic acid 5 mg/d regardless of MTHFR genotype. In patients with normal genotype median Hcy levels were 18.6 ± 4.2 and 10.38 ± 4.1 μm/l, pre and post therapy, respectively ($p=0.0006$), while in heterozygous it was 18.0 ± 7.6 and 10.6 ± 4.0 μm/l, respectively ($p=0.02$) and in homozygous 23.9 ± 12.3 and 9.5 ± 2.5 μm/l, respectively ($p=0.0001$).

Despite the basal Hcy levels, supplementation regimen and MTHFR genotype, Cys levels did not vary significantly (Fig. 1). However, a trend to increase Cys levels after therapy was observed in AT (346.08 ± 65.75 and 375.35 ± 54.2, pre and post therapy) and AVT (374.85 ± 76.39 and 495.33 ±110.67, pre and post therapy). Conversely, Cys levels decreased in patients with UST (421.53 ± 121.26 and 375.41 ±112.10, respectively) while remained unchanged in patients with VT (358.25 ± 72.34 and 352.69 ± 104.04, before and after supplementation therapy respectively).

There were no thrombotic recurrences in any group of patients during a median of 36 months of follow-up.

Discussion

Our study evaluated both Hcy and Cys in patients with thrombosis in different vascular sites, including unusual site thrombosis. In addition, the therapeutic response to vitamins of both low molecular weight thiols together has not been investigated before.

HHcy has been identified as a cardiovascular and venous thrombosis risk factor [13], an observation that suggests that its thrombotic potential is not only due to atherosclerosis. In an analysis of recent trials, Loscalzo [14] suggested that Hcy should not be considered an isolated event, but rather as a component of the interactive redox-thiol system that includes other low molecular weight thiols. As the impairment of any component of the system would lead to vascular lesions, it has been suggested that Hcy and Cys should be evaluated concomitantly [1,15].

As in previous reports, HHcy was a prevalent finding among patients with vascular occlusive disease, and Cys levels were higher in patients than in normal population [16].

In our population, patients with AVT and UST had the highest levels of both Cys and Hcy. High Hcy levels have been associated to the risk of UST, such as upper extremity, cerebral or retinal vein thrombosis [17–19]. In addition, El-Khairy et al. [9] observed an increased cardiovascular risk in patients with low Cys and high Hcy levels, and in patients with high Cys and Hcy levels.

It has been shown that renal function is a determinant of Hcy levels [20] in general population. However, if the mildly impaired renal function observed among patients with AVT and UST has an effect in Hcy and Cys levels, requires further evaluation.

We observed a correlation between folate levels and Hcy levels but not with Cys levels. No significant changes in Cys levels were observed between patients with low and normal vitamin levels, or after supplementation. However, we observed a trend towards a variation of Cys levels after supplementation that was different according to the site of thrombosis. This observation is preliminary but deserves consideration. Whether endothelium of different regions of the vascular tree, with its different extra-cellular signals and cellular responses plays a role in Cys levels warrants further investigation as well as the clinical implications of these observations.

Lastly, the effect of drinking mate on Hcy has not been reported before and might be one of the determinants of Hcy in people in this latitude.

In conclusion, our pilot study suggests that intermediates of Hcy metabolism might exhibit a different response to therapy in different vascular sites. Further studies with enough power are required to demonstrate if HHcy as well and increased Cys levels are risk factors for thrombosis in specific sites, and if investigating both Hcy and Cys levels is a valid and helpful approach for the management of those patients.

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