Antiphospholipid antibodies and hyperhomocysteinaemia in patients with vascular occlusive disease

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Summary

Hyperhomocysteinemia (HHcy), lupus anticoagulant (LA) and anticardiolipin antibodies (ACA) are independent risk factors for thrombosis. Even though risks are cumulative, the clinical impact of the association is unknown. Preliminary data suggested that HHcy might be associated with transient LA and ACA, disappearing after lowering HHcy. We prospectively evaluated the association of HHcy and LA/ACA, the effect of lowering HHcy with folic acid in LA behavior, and the correlation of the initial dRVVT with LA behavior after folic acid in 210 patients with thrombosis and adverse pregnancy outcomes. Prevalence of HHcy among patients with LA/ACA was 40%. Thirty-one patients exhibited only HHcy (15%; Group 1), 106 (50%; Group 2) had only LA/ACA, while 73 (35%; Group 3) had both. After therapy, 63% and 64% of LA/ACA remained positive in Group 3 and 2, respectively. We observed a trend towards a more positive dRVVT in persistent LA after lowering HHcy. No differences in clinical presentation or in outcomes after two years of follow-up were observed among the groups. Even though the association of HHcy and LA/ACA is common in patients with thrombosis, it might have no prognostic implications if Hcy levels are lowered. Currently, no laboratory findings correlate with LA behavior, which is independent of homocysteine levels and vitamin treatment.

Keywords

Clinical / epidemiological studies, homocysteine, antiphospholipid antibodies, deep vein thrombosis, cerebrovascular disease

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Introduction

Antiphospholipid syndrome (APS) is the most prevalent acquired thrombophilic disorder, leading to venous and arterial thrombosis and adverse pregnancy outcomes (1). Diagnostic criteria require a clinical event and positive, persistent (i.e. at least two determinations, six weeks apart) tests for antiphospholipid antibodies, such as lupus anticoagulant (LA) or anticardiolipin antibodies (ACA) (1). Even though they are usually persistent, many patients with thrombosis present transient antiphospholipid antibodies. Clinical significance of transient antibodies is unknown; infection as well as inflammation has shown to play a role in the genesis of such antibodies (2). Theories explaining antiphospholipid antibodies associated thrombosis include, among others, activation of endothelial cells and oxidant-mediated injury of the vascular endothelium that lead to endothelial perturbation (3, 4).

Hyperhomocysteinemia (HHcy) is an independent risk factor for thrombosis as well. HHcy leads to endothelial dysfunction by direct endothelial and vessel wall damage, oxidative stress generation, and stimulation of a pro-coagulant and pro-inflammatory state (5, 6). Therapy with folic acid would restore endothelial function, probably by reducing oxidative stress, enhancing nitric oxide synthesis and by its anti-inflammatory actions (7).

Galli et al. (8) demonstrated that LA is a stronger risk factor for thrombosis than ACA, irrespective of the site and type of thrombosis, and of the presence of systemic lupus erythematosus. The risk for thrombosis is 5–16 times higher in LA patients than in controls. Similarly, mild HHcy is associated with a 10% higher risk of cardiovascular events, 20% higher risk of stroke, and 27% higher risk of deep venous thrombosis (9).

The association of HHcy and LA/ACA has often been reported in patients with thrombosis, with or without autoimmune

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disorders (10– 14). Even though risks factors for thrombosis are cumulative, the clinical significance of this association is currently unknown. In a cross-sectional study by Kassis et al. (11), the association led to increased risk of both venous and arterial thrombosis in 33 patients. However, as the association has not been evaluated in a large series of cases, it remains unknown whether it implies a higher risk of thrombosis or recurrence.

Moreover, it has not been established whether the relationship between LA/ACA and HHcy is causal or a concomitant finding. In patients with sepsis, McIntyre (15) has shown that LA/ACA appear and disappear subsequent to oxidation-reduction reactions. Preliminary data suggested that in patients with thrombosis HHcy might be associated with transient LA/ACA, probably due to endothelial damage by HHcy, which disappears after lowering Hcy levels (16). Consequently, those patients would not have definite APS but concomitant transient antibodies; prognosis and therapeutic interventions would differ in both cases.

Our objective was to evaluate prospectively the association of HHcy and LA/ACA in patients with thrombosis. In addition, we investigated the effect of lowering HHcy in the behavior of LA/ACA, and compared it to the LA/ACA behavior in patients without HHcy receiving no folic acid.

We also hypothesized that the initial platelet neutralization procedures on diluted Russell viper venom time (Δ dRVVT-PNP) would correlate with the LA behavior after folic acid supplementation, and that LA with higher Δ dRVVT-PNP would not turn negative after lowering HHcy levels.

Material and methods

Patients

We prospectively investigated patients with vascular occlusive disease referred to our institution for screening of thrombophilia. Vascular occlusive diseases were venous and/or arterial, unusual site thrombosis and adverse pregnancy outcomes (i.e. recurrent pregnancy losses or vascular complications of pregnancy, "APO"). The arterial thrombosis included acute myocardial infarction/ unstable angina and non-embolic cerebrovascular diseases (stroke or transient ischemic attack). Unusual site thromboses were upper extremity deep vein thrombosis, retinal vein occlusion, mesenteric vein thrombosis and cerebral vein thrombosis.

Patients with HHcy and/or positive LA/ACA were included in the study. In all patients, laboratory measurements were performed at least four weeks apart from the acute thrombotic event.

Exclusion criteria were folic acid or vitamin complex intake, active cancer (arbitrarily defined as cancer requiring chemotherapy or radiotherapy, or patients within five years of ending specific treatment, or patients with newly diagnosed cancer undergoing antineoplastic therapy), and patients with prior thromboembolic events with known congenital thrombophilia.

Additional pro-thrombotic risk factors investigated in the study population were APC resistance, FV Leiden and prothrombin G20210A mutation, protein C and protein S deficiency. Concomitant disease and therapies were recorded as well.

Laboratory analysis

Homocysteine (Hcy) levels were measured in fasting blood by high-performance liquid chromatography. HHcy was defined as Hcy > 15 μM. Erythrocytic and serum folate and serum vitamin B_{12} were measured. ACA Ig M and Ig G were determined by ELISA method (Binding Site, UK). ACA were positive if value was greater than 15 GPL or MPL/l. LA was diagnosed according to the SSC-ISTH criteria (17). We used platelet poor plasma samples for LA tests. A sensitive APTT, the PTTLA, evaluated the intrinsic pathway; the diluted Russell viper venom test assayed the final common coagulation pathway, and 1:1 mixtures with normal plasma were performed as well. As confirmatory tests (i.e. demonstrating that the inhibitor effects are phospholipid-dependent) we repeated the coagulation test after addition of a higher concentration of phospholipids to the system. Prothrombin time and thrombin time were carried out as well in order to exclude other abnormalities.

In order to evaluate fulfillment of the Sapporo criteria for APS (1), in patients with positive LA/ACA a second determination was performed at least six weeks apart from the initial test.

According to the initial laboratory results, the study population was divided into three groups. Patients with HHcy only constituted Group 1. Group 2 included patients with LA/ACA but without HHcy. Group 3 included patients with concomitant HHcy and positive LA/ACA. Patients in groups 1 and 3 received folic acid 5mg/day orally. Hcy was measured after at least four weeks in Group 1. In Group 3, measurement of Hcy levels was performed at least after six weeks of vitamins, at the same time as LA/ACA.

Statistical analysis

The sample size was calculated to achieve a power of 80% and 95% confidence. The outcome parameter for the sample size calculation was the frequency of LA becoming negative in the second determination at least six weeks apart from the first one (Group 2) or after at least four weeks of folic acid (Group 3). We aimed to detect an odds ratio of 2.5. We considered 1.5 controls per case. The number of cases (patients with positive antiphospholipid antibodies and HHcy receiving folic acid) and controls (patients with APA but no HHcy, receiving no therapy) to achieve the desired power and confidence was 60 and 90, respectively.

A simple linear and multiple regression models investigated the predictive value of basal Δ dRVVT-PNP. The outcome or dependent variable was LA positive or negative; the following continuous numeric variables: Δ dRVVT-PNP, Hcy, anticardiolipin IgG and IgM levels, and age were the predictors.

Calculations were performed with SPSS 10 program and Epi Info, 6.04 version from the Center for Disease Control and Prevention (CDC), Epidemiology Program Office, USA.

Patients included in the study were enrolled in a broader prospective investigation of homocysteine metabolism in patients with thrombosis. The Institutional Review Board evaluated the study but, even though there was an "intervention", as the intervention was not experimental but was the standard therapy, it was considered an observational study requiring no formal approval according to Good Clinical Practices. However, informed consent was obtained from all participants. If patients

were underage, the patients gave consent in agreement with their parents.

Results

During the study period (September 2002–November 2004), 335 patients meeting the inclusion criteria were evaluated. Eighty-six patients (26%) had normal results and 39 (12%) were lost for follow-up after an initial positive test. Characteristics of patients lost for follow-up did not differ from those of the patients included in the study.

Two hundred ten patients with positive laboratory findings and complete follow-up were included in the study. Erythrocytic folate, serum folate and serum vitamin B12 levels were within the normal ranges in all patients. Concomitant diseases and medications are listed in Table 1.

An additional thrombophilic disorder was found in 5.2% of study population (11 patients). Seven patients were heterozygous for the FV Leiden mutation (five with LA and VTE, and two with HHcy: one VTE and one retinal vein occlusion), three patients were heterozygous for the prothrombin G20210A mutation (two with HHcy and VTE, and one with recurrent abortions with LA), while one patient with VTE had protein C deficiency and LA.

HHcy was the only finding in 31 patients (15%; Group 1), while 106 (50%) had only LA/ACA (Group 2). Concomitant HHcy and LA/ACA were found in 73 patients (35%) constituting Group 3. Patients' characteristics in each group were comparable (Table 2). The prevalence of HHcy among patients with LA/ACA (Groups 2 and 3) was 40%.

Table 1: Concomitant diseases and medications in the study population.

Diseases/ habits	%	Medications	%
Hypertension	18.5	Oral Anticoagulants	35.7
Smoking	15.2	Aspirin/ anti-platelet agents	20.9
Hypercholesterolemia	10	Angiotensin-converting enzyme inhibitor's	11.4
Thyroid disease	8.1	Hypocholesterolemic drugs	8.5
Immune disease	4.3	Benzodiazepines/ antidepressants	8.5
Obesity	3.8	Thyroid hormone	8.1
Other	3.8	Beta blockers	5.2
Diabetes	2.3	Other	4.7
Prior cancer	1.9	Antiacids	2.8

Table 2: Patients characteristics in the three groups.

	Group 1 (HHcy)	Group 2 (APA)	Group 3 (HHcy + APA)
N	31	106	73
Sex (%)			
female	45	63	64
male	55	37	36
Age (mean ± SD)	48.56 ± 15.07	45.11 ± 14.24	45.97 ± 16.6
Thrombosis site (%)			
venous	22	35	22
arterial	50	34	49
both	3	4	8
unusual site	19	12	12
APO	6	15	8
Oral anticoagulation (%)	30	33	43

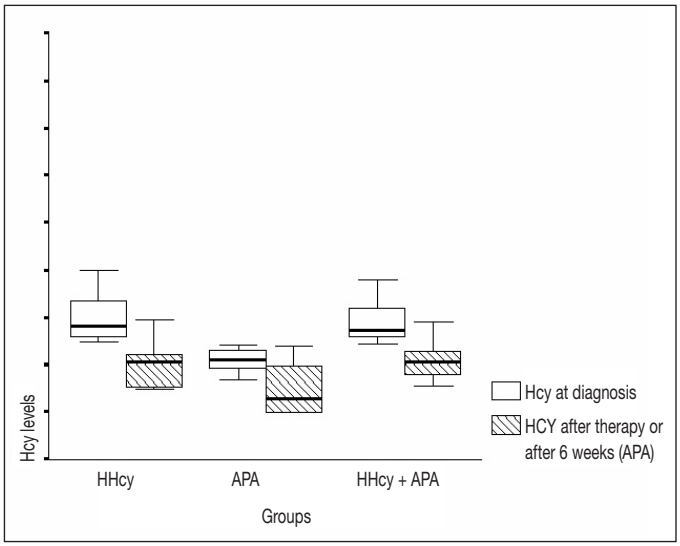

Figure 1: Homocysteine levels at diagnosis, after six weeks or after folic acid supplementation.

No significant difference in mean basal Hcy levels was found in patients with HHcy regardless of the presence of LA/ACA (23.18 ± 11.46, 95% CI 18.97–27.53 and 22.90 ± 10.68, 95% CI 19.18–23.91, in Group 1 and Group 3, respectively) (Fig. 1).

Mean Hcy levels after treatment were 10.05 ±4.3 (95% CI 8.43– 11.8) and 10.53 ±4.25 (95% CI 9.44– 11.42), in Group 1 and Group 3, respectively. Therapy was not stopped in any patient with HHcy. In Group 1, mean time between the initiation of folic acid and the first control was 9.3 ± 6.25 weeks while in Group 3 it was 13.68 ± 15.95 weeks (p= NS). All patients with HHcy lowered their Hcy levels after vitamin therapy, regardless of the coexistence of LA/ACA (Fig. 1) or anticoagulant therapy. In Group 2, Hcy levels at diagnosis were significantly higher than after six weeks, even though both were within the normal range (10.68 ± 2.2, 95% CI 10.24–11.12 and 4.84 ± 5.1, 95% CI 3.64–6.05, respectively; $p < 0.0001$) (Fig. 1). Hcy levels after six weeks were independent of LA/ACA behavior (i.e. transient or persistent).

After therapy, 63% of LA/ACA remained positive in Group 3 whereas in Group 2, 64% remained positive after 14.86 ± 13.10 weeks apart from the first determination. Table 3 shows ACA titer before and after six weeks or after vitamin therapy in the three groups. A significant difference was observed in Group 3 only. However, mean values were within normal values because only a few patients showed high ACA titers.

The analysis of LA/ACA behavior according to the site of thrombosis did not show significant differences among the groups.

Mean follow-up (months ± SE) was 23.88 ± 1.48 (95 % CI 20.96–26.81). There were no thrombotic recurrences in either group during follow-up.

In Group 3, there was a non-significant trend towards a higher Δ dRVVT-PNP in patients with persistent LA after lower-

Table 3: ACA titer in the three groups, at diagnosis, after six weeks or after folic acid supplementation (second test).

Group	Mean ACA Ig M titer (range)			Mean ACA Ig G titer (range)		
	At diagnosis	Second test	p	At diagnosis	Second test	p
1	3.9 ± 0.57 (1–14)	1 ± 0.52 (1–7)	NS	4.67 ± 0.61 (1–14)	5.14 ± 1.47 (1–15)	NS
2	15.18 ± 2.65 (6–224)	15.68 ± 5.25 (6–308)	NS	10.10 ± 2.49 (5–180)	9.12 ± 3.28 (7–208)	NS
3	9.0 ± 1.58 (8–107)	5.05 ± 0.88 (4–40)	0.03	10.18 ± 2.11 (10–132)	4.98 ± 0.90 (7–36)	0.03

ing HHcy (mean Δ dRVVT-PNP in persistent LA was 0.27 ± 0.46 vs. 0.19 ± 0.27 in transient LA). However, regression analysis demonstrated no predictive value of basal Δ dRVVT-PNP even after including basal and post therapy Hcy values in the model.

Discussion

Pathogenic mechanisms of LA/ACA in humans are still under investigation. The clinical impact of such antibodies in patients with thrombosis is greatly dependent on the presence of definite APS, which has prognostic and therapeutic implications. However, isolated transient LA/ACA might not be associated with higher risk of thrombosis. Transient antibodies might not be the only cause of thrombosis in patients with vascular occlusive disease, but might contribute to it if a concomitant thrombophilic condition such as HHcy is also present. Transient antibodies might be the result of endothelial damage provoked either by the thrombosis itself or by HHcy; yet, the rate of LA/ACA disappearing was similar in patients with and without HHcy (27 and 26%, respectively).

Several authors have reported the association of LA/ACA and HHcy in patients with thrombosis (10–12). Avivi et al. (10) found that the combination of both defects led to an increased risk of life-threatening thrombotic initial events in 50% of patients with primary anti-phospholipid syndrome fulfilling Sapporo criteria. Life-threatening events were mostly venous, i.e. pulmonary embolism, abdominal vein thrombosis and cerebral thrombosis. However, the sample size was small (16 patients), mean Hcy values in both groups were not provided, patients received no therapy, and whether the risk persisted during follow-up was not evaluated. In contrast, Kassis et al. (11) observed that the association led to an increased risk of arterial thrombosis. We observed no difference in clinical presentation or in outcomes after long-term follow-up among the three groups in the present study.

Ames et al. (12) observed a non-significant difference in Hcy levels among patients with APS, non-thrombotic antiphospholipid antibodies carriers, and healthy controls. In our series of patients with vascular occlusive disease we observed no difference in Hcy levels, basal or in response to therapy, in either group.

Our study is the first to investigate the effect of lowering HHcy on the LA/ACA behavior. In spite of our initial hypothesis and observations (16), we could not demonstrate a direct effect of HHcy on LA/ACA behavior or an effect of LA/ACA behavior on Hcy levels. Prevalence of persistent and transient antibodies was similar in patients with and without HHcy, before and after therapy, and regardless of the thrombosis site. However, Hcy levels were higher in the first determination of LA/ACA in patients in Group 2 compared to Hcy levels at the second LA/ACA determination. Whether this finding suggests that Hcy levels increase in response to endothelial damage caused by the antibodies requires further investigation.

We observed a trend towards higher ACA titers as well as higher Δ dRVVT-PNP in patients with persistent LA after lowering HHcy. There are several explanations for this last observation. Firstly, even though the sample size was powered to show differences among the groups, it might not be powered enough to show differences in the Δ dRVVT-PNP. Secondly, there were few "potent" (i.e. high Δ dRVVT-PNP) LA. Finally, as not all LA/ACA are equal, tests might be incapable of discriminating them. However, our current findings are in agreement with the retrospective analysis by Galli (18) that found no correlation between dRVVT ratios and the risk of thrombosis. Regardless of the Δ dRVVT-PNP value, a LA persisting after six weeks (regardless of HHcy) is consistent with APS and not with a reactive antibody. Nonetheless, since a trend towards a higher Δ dRVVT-PNP was observed among persistent LA, further investigation is required in order to assess the predictive value of this confirmatory test in this patient population.

In addition, we evaluated the effect of both pro-thrombotic conditions on patient's outcomes. In contrast to previous reports, we found that the association of LA, either transient or persistent, and HHcy had no effect on the severity of symptoms at presentation. Moreover, the risks of each pro-thrombotic condition were not cumulative, and did not increase the risk of thrombosis recurrence during long-term follow-up. The heterogeneity of thrombotic events might explain, at least in part, the lack of any recurrent event during follow-up. In addition, more than one third of patients were already under anticoagulant therapy and 20% were on aspirin. Besides, as hyperhomocysteinemia was normalized in all patients, the risk of recurrence was also decreased. Finally, all patients were instructed to receive prophylaxis for thrombosis during risk situations such as surgery, etc.

The patients included met the characteristics of a population of patients with thrombosis that would be evaluated for antiphospholipid syndrome and HHcy. Nevertheless, referral bias leading to a highly selected population cannot be discarded. If this were the case, our results would have low external validity, except for other specialized services. However, the selection bias would have been towards a high-risk population.

The rational for choosing Δ dRVVT-PNP as the test that would correlate with LA behavior was that this test is a confirmatory test and, if useful, it would be practical from a clinical point of view. However, this election was arbitrary and this study has demonstrated that a greater sample size or, perhaps, combination with other tests would be required in order to evaluate its predictive value.

In conclusion, diagnosis of concomitant HHcy and LA/ACA is common in patients with thrombosis. However, this finding might have no prognostic or therapeutic implications if Hcy levels are lowered. The observation that, in patients with antiphospholipid antibodies but without HHcy, homocysteine levels increase, sustains the hypothesis of a relationship between endothelial damage and the antibodies. Currently, no laboratory finding can help to predict LA behavior, which is independent of Hcy levels and vitamin treatment.

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