Clinical significance of phospholipid-cofactor antibodies in patients with systemic lupus erythematosus-associated antiphospholipid syndrome

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Objectives

To establish whether antibodies directed against phospholipid-binding plasma proteins such as β2-glycoprotein I (β2GPI), prothrombin (PT), and Annexin V (AnxV) constitute a risk factor for thrombosis in patients with systemic lupus erythematosus (SLE)-associated antiphospholipid syndrome (SLE/APS).

Patients and methods

A group of SLE patients (with and without APS) and patients with primary APS (PAPS) were included in this study. Fifteen patients with deep vein thrombosis but without antiphospholipid (aPL) antibodies, and another 15 age-matched and sex-matched apparently healthy individuals served as a control group. All patients were investigated for lupus anticoagulants and detection of anticardiolipin (aCL) immunoglobulin G (IgG) and immunoglobulin M (IgM) antibodies. Antibodies against β2GPI (IgG and IgM), PT (IgG and IgM), and AnxV (IgG) were also measured using the respective enzyme-linked immunosorbent assays.

Results

The study included 58 SLE patients (18 SLE/APS patients and 40 patients without APS) as well as 40 patients with PAPS, mean age 43 years (range: 18–74 years). IgG and/or IgM aCL antibodies were detected in all patients with PAPS (100%), whereas the prevalence rates of aPL-cofactor antibodies were as follows: 75% anti-β2GPI, 70% anti-PT, and 25% anti-AnxV antibodies. In SLE patients without APS, aCL antibodies were detected in 17.5%, anti-β2GPI antibodies in 20%, anti-AnxV antibodies in 20%, and anti-PT antibodies in 10% of patients. None of the antibodies measured were detected in deep vein thrombosis cases or healthy controls.

Conclusion

Measurement of antiphospholipid-cofactor antibodies in addition to the more widely used aCL and anti-β2GPI antibodies could be a useful prognostic marker for the risk of thrombosis in SLE/APS patients.

Introduction

Antiphospholipid antibodies (aPL) are associated strongly with thrombosis; although the precise mechanism(s) by which they alter hemostasis to induce a hypercoagulable state remain unclear, numerous theories have been advanced [1]. The most common thrombotic events associated with aPL antibodies are deep vein thrombosis (DVT), pulmonary embolism, and coronary or peripheral artery thrombosis [2].

The discovery that aPL antibodies recognize plasma proteins that bind to phospholipids rather than recognizing phospholipids themselves has been a major advancement in research into the antiphospholipid syndrome (APS). In fact, the term antiphospholipid antibody is a misnomer because these antibodies are directed toward plasma proteins with an affinity for negatively charged phospholipids. It is now established that β2-glycoprotein I (β2GPI) is the most important antigen for aPL antibodies. However, the possible pathologic mechanism is still much debated [3]. The importance of antiprothrombin antibodies (anti-PT) in causing lupus anticoagulant (LA) activity was highlighted in the last two decades. It was observed that LA could inhibit endothelial cell-mediated prothrombinase activity and the immunoglobulin G (IgG) fraction containing LA activity bound to phospholipid–PT complex [4]. Therefore, PT was identified as an antigen for autoantibodies with LA activity as well as β2GPI. Therefore, it could be accepted that antibodies to PT and β2GPI are two major autoantibodies responsible for LA activity, anti-PT antibodies responsible for PT-dependent LA and anti-β2GPI antibodies for β2GPI-dependent LA [5].

Owing to its high affinity for negatively charged phospholipids and its proposed role in hemostasis, it is hypothesized that, besides β2GPI, Annexin V (AnxV) also plays a role in the pathophysiology of APS [6,7].

Several functions have been assigned to AnxV on the basis of its ability to bind with high affinity to negatively charged phospholipids. An in-vitro study showed that AnxV shows anticoagulant properties *in vitro* by forming a two-dimensional crystal on negatively charged spots in the cellular membrane, creating a sort of 'anticoagulant shield'. Antibodies with reactivity for β2GPI can disturb this anticoagulant shield, resulting in increased generation of thrombin [8].

Hence, detection of aPL-cofactor antibodies in addition to the classic anticardiolipin (aCL) antibodies and LA seems to be of considerable clinical importance. However, their role in the pathology of APS and association with thrombosis in systemic lupus erythematosus (SLE) is still controversial [3].

Aim of work

This study aimed to establish whether antibodies directed against phospholipid-binding plasma proteins such as β2GPI, PT, and AnxV constitute a risk factor for thrombosis in patients with SLE and APS.

Patients and methods
Patients' population

A group of SLE patients with and without APS and patients with primary APS (PAPS) were recruited from the outpatient clinics of the Rheumatology department and Vascular surgery unit of Mansoura University hospitals – Mansoura, Egypt. They were enrolled in this study, and informed consent was obtained from each patient. SLE patients were diagnosed according to the 1997 revised American College of Rheumatology criteria for SLE [9], whereas all patients with APS (PAPS and SLE/APS) had a history of IgG and/or IgM aCL antibodies and/or positive LA tests documented on two or more occasions at least 12 weeks apart and fulfilled the Sydney clinical criteria for APS [10]. As controls, we included 15 patients with DVT but without aPL antibodies, and another 15 age-matched and sex-matched apparently healthy controls; none of them had a history of autoimmune disease, thrombosis, or pregnancy loss. The study was approved by medical research ethics committee of Mansoura University. All participants gave written informed consent for participation.

Coagulation assays

All patients were investigated for LA activity, which was measured using a partial thromboplastin time-lupus anticoagulant test and a dilute Russell's viper venom time test (Orgentec Diagnostika GmbH, Mainz, Germany). Patients were classified as positive for LA when they had either a positive partial thromboplastin time-lupus anticoagulant or a positive dilute Russell's viper venom time test.

Serological assays

aCL (IgG/IgM), antibodies against β2GPI (IgG/IgM), and PT antibodies (IgG/IgM) were measured using the respective commercially available enzyme-linked immunosorbent assays (ELISA) (Orgentec Diagnostika GmbH, Mainz, Germany). For the detection of IgG antibodies directed against AnxV, we used a commercial Zymutest anti-Annexin V ELISA kit (Hyphen Bio Med, Andresy, France). All assays were performed according to the manufacturer's instructions.

aCL antibody ELISA

The assays were performed on microtiter plates coated with purified bovine cardiolipin for aCL. IgG/IgM calibrators were used to report aCL antibodies levels as antiphospholipid IgG (GPL) or IgM (MPL) units. Concentrations above 10 GPL phospholipid or 10 MPL phospholipid units were considered positive [11].

Anti-β2GP1, anti-PT, and anti-AnxV antibodies ELISA

Highly purified human antigens were used for anti-β2GP1 [12], anti-PT, and anti-AnxV antibodies assays.

In all assays, diluted serum samples, calibrator, standards, and negative controls are added to their corresponding antigen-coated microtest well. If autoantibodies to the specific protein are present, they bind to the immobilized protein. This is followed by a washing step to remove unbound proteins. After the washing step, a polyclonal rabbit anti-human IgG (or IgM) conjugate, labeled with horse-radish peroxidase, and a 3,3′,5,5′-tetramethylbenzidine enzymatic substrate were used. On adding the substrate, the amount of color developed was directly proportional to the concentration of the present antibodies in the sample. The calibration curve for each antibody was obtained and absorbance was measured photometerically at 450 nm.

Anti-β2GP1, anti-PT, and anti-AnxV antibodies levels were expressed in U/ml. After the study of serum samples from the healthy controls, the cut-off level for anti-β2GP1 was set at 13.5 U/ml for IgG and 11 U/ml for IgM; 12 U/ml IgG and 10 U/ml IgM for anti-PT; and 10 U/ml IgG for anti-AnxV.

Statistical analysis

Statistical analyses were carried out using the SPSS for windows version 17.0 (SPSS, Chicago, Illinois, USA).

The diagnostic sensitivity and specificity of aCL-cofactor and aPL-cofactor antibodies were calculated. The association between antibody occurrence and a history of thrombosis was assessed using the Fisher exact test. The Kruskal–Wallis analysis of variance test was used to compare antibody concentrations in the different groups. Antibody concentrations are expressed as mean ± SD.

Results

The demographic characteristics of the study population along with the laboratory testing are summarized in (Table 1). The mean levels of aCL antibodies and other aPL-cofactor antibodies were significantly higher in patients with thrombosis (PAPS and SLE/APS) compared with SLE patients without APS, patients with DVT, or healthy controls. The only antibody among SLE patients without APS that showed significantly higher levels compared with DVT patients and controls was aCL (IgG).

The prevalence rates of the various antibodies (IgG and/or IgM) studied are detailed in Table 2. In all SLE patients, positive aCL and anti-β2GP1 antibodies levels were determined in 21 patients (36% each), anti-PT antibodies in 18 patients (31%), and anti-AnxV antibodies in 15 patients (25%).

The association between the various aPL-cofactor antibodies and a history of thrombosis was evaluated in all patients with SLE. aCL (IgG and/or IgM) and IgG anti-PT antibodies were associated with the highest risk of thrombosis ($P < 0.001$) as shown in Table 3.

However, in SLE/APS patients with thrombotic evidence, aCL antibodies were found in 14 patients (77.8%) versus 13 patients (72%) with anti-β2GP1, 13 patients (77.8%) with anti-PT, and seven patients (38.8%) with anti-AnxV antibodies.

Anti-PT antibodies were positive in the four aCL-negative patients with SLE and thrombosis, whereas anti-β2GP1 antibodies were positive in only one aCL-negative patient.

The diagnostic sensitivity and specificity of aCL and different aPL antibodies were calculated among SLE patients. aCL and aPT antibodies showed the highest sensitivity (79%), followed by anti-β2GP1 and anti-AnxV (72.2 and 38.9%, respectively), whereas anti-PT showed the highest specificity (90%) among the aPL-cofactor antibodies assayed (Table 4).

Discussion

Determination of aPL antibodies is commonly used to diagnose APS, to evaluate the risk of thrombosis in patients with SLE, and to test for the causes of thrombotic events in apparently healthy individuals with no risk factors [13]. Although the original concept of aPL antibodies considers that those antibodies were directed against anionic phospholipids, evidence has shown that phospholipid-binding plasma proteins such as β2GPI and PT are the dominant antigenic targets recognized by aPL in patients with the APS. Anti-CL antibodies, anti-β2GPI antibodies, and LA are the laboratory tests considered in the revised criteria for the classification of the APS. However, a number of issues in terms of the definition of 'aPL positive' are in discussion. For example, there would be many in-vitro false positives in LA in daily practice (laboratory false positive). In addition, LA was found in patients with a variety of diseases, such as infectious, malignant, or autoimmune diseases (clinical false positive). Furthermore, there are many patients strongly suspected to have APS by their clinical phenotype, but are negative for any current aPL (laboratorial and/or clinical false negative) [14]. This raises the needs for a more specific and reliable assaying modality in order to identify 'true aPL'.

Table 1 Demographic characteristics and assayed antibody concentrations in the studied groups

	SLE patients ($n=40$)	SLE with APS ($n=18$)	APS ($n=40$)	DVT patients ($n=15$)	Control ($n=15$)	ANOVA test
Age (mean ± SD) (years)	38.3 ± 11.8	42.8 ± 12.7	45.2 ± 11.6	42.6 ± 10.2	44.2 ± 10.5	$P > 0.05$
Sex (χ^2)						
Female	38	17	37	13	13	$P > 0.05$
Male	2	1	3	2	2	
aCL (GPL/ml)	19.8 ± 8.7	22.6 ± 9	70.3 ± 32	6.2 ± 2.3	5.6 ± 2.5	$P < 0.001*$
aCL (MPL/ml)	6.5 ± 3.1	16.3 ± 7.4	29.4 ± 13.5	3.2 ± 1.5	3.4 ± 1.9	$P < 0.01*$
Anti-β2GP1 IgG (U/ml)	8.8 ± 6.2	15.6 ± 7.1	56.8 ± 27.4	4.5 ± 2.1	4.3 ± 2.1	$P < 0.001*$
Anti-β2GP1 IgM (U/ml)	5.5 ± 2.34	10.46 ± 4.8	15.1 ± 6.2	3.3 ± 1.7	2.8 ± 1.2	$P < 0.05*$
Anti-PT IgG (U/ml)	10.2 ± 4.7	18.4 ± 8.5	27.9 ± 12.5	7.5 ± 2.2	6.5 ± 3.1	$P = 0.002*$
Anti-PT IgM (U/ml)	2.4 ± 1.8	4.1 ± 2.2	4.7 ± 2.8	1.4 ± 0.9	1.3 ± 0.7	$P > 0.05$
Anti-AnxV IgG (U/ml)	9.25 ± 3.6	14.6 ± 5.6	16.1 ± 8	5.6 ± 1.64	3.6 ± 1.5	$P = 0.02*$

aCL, anticardiolipin; ANOVA, analysis of variance; AnxV, Annexin V; APS, antiphospholipid syndrome; β2GPI, β2-glycoprotein I; DVT, deep vein thrombosis; IgG, immunoglobulin G; IgM, immunoglobulin M; PT, prothrombin; SLE, systemic lupus erythematosus.
*$P < 0.05$ is significant.

Table 2 Prevalence of aCL-cofactor and aPL-cofactor antibodies in patients with SLE and APS

	SLE patients (n=58) [n (%)]	APS (n=40) [n (%)]
aCL IgG/IgM	21 (36.21)	40 (100)
Anti-β2GP1 IgG/IgM	21 (36.21)	30 (75)
Anti-PT IgG/IgM	18 (31.03)	28 (70)
Anti-AnxV IgG	15 (25.86)	10 (25)

aCL, anticardiolipin; aPL, antiphospholipid; AnxV, Annexin V; APS, antiphospholipid syndrome; β2GPI, β2-glycoprotein I; IgG, immunoglobulin G; IgM, immunoglobulin M; PT, prothrombin; SLE, systemic lupus erythematosus.

Table 3 Prevalence and statistical significance (P) of aCL and various aPL-cofactor antibodies in relation to the presence or absence of thrombosis among 40 SLE patients without thrombosis versus 18 SLE/APS

	Thrombosis		P	OR	CI
	Yes	No			
aCL IgG/IgM					
+	14	7	<0.001*	16.5	4.2–65.5
−	4	33			
Anti-β2GP1 IgG/IgM					
+	13	8	P=0.002*	10.4	2.9–37.8
−	5	32			
Anti-PT IgG/IgM					
+	14	4	<0.001*	31.5	6.9–143.6
−	4	36			
Anti-AnxV IgG					
+	7	8	0.08	2.45	0.75–8.7
−	11	32			

aCL, anticardiolipin; aPL, antiphospholipid; AnxV, Annexin V; APS, antiphospholipid syndrome; β2GPI, β2-glycoprotein I; CI, 95% confidence interval; IgG, immunoglobulin G; IgM, immunoglobulin M; OR, odds ratio; PT, prothrombin; SLE, systemic lupus erythematosus.
*P<0.05 is significant.

Table 4 Sensitivity and specificity of the assayed antibodies

	Sensitivity (%)	Specificity (%)
aCL IgG/IgM	77.78	82.5
Anti-β2GP1 IgG/IgM	72.22	80
Anti-PT IgG/IgM	77.78	90
Anti-AnxV IgG	38.89	80

aCL, anticardiolipin; AnxV, Annexin V; β2GPI, β2-glycoprotein I; IgG, immunoglobulin G; IgM, immunoglobulin M; PT, prothrombin.

In this study, we selected three groups of patients: one group with PAPS, one with SLE, stratified by the presence or absence of thrombotic events. In all patients and control groups, aCL, in addition to anti-cofactor antibodies (anti-β2GP1, anti-PT, and anti-Anx V), were measured in order to evaluate their diagnostic sensitivity and specificity and to investigate the practical usefulness of their inclusion in the diagnostic and prognostic profile of patients with SLE.

The prevalence of anti-β2GPI antibodies was lower than that of aCL antibodies in the group of patients with PAPS (75 vs. 100%), but they were equal in the group with SLE (36.2% each). In addition, anti-β2GPI antibodies were positive in only one of the aCL antibodies-negative patients with SLE and thrombosis. Similar findings were obtained by Theodoridou et al. [15] and by Hsieh et al. [16].

In all SLE patients, we found that anti-PT antibodies of the IgG class were nearly prevalent as aCL and anti-β2GPI antibodies. IgG anti-PT antibodies were also positive in 77.8% of the SLE patients who had suffered a thrombotic event, similar to aCL antibodies, whereas anti-β2GPI antibodies were positive in 72% and anti-AnxV antibodies in only 50% of patients. Moreover, anti-PT antibodies of the IgG class were also found to be more specific (90%) than aCL and anti-β2GPI antibodies for the presence of thrombosis. This means that in patients with SLE and SLE/APS, IgG anti-PT antibodies have the greatest specificity over all the various autoantibodies directed against the phospholipids or their cofactors, confirming the results of the study by Puurunen et al. [17], who reported the presence of anti-PT antibodies in 34% of patients with SLE and found a positive correlation with DVT in this population. Numerous other researchers have found an association between anti-PT antibodies and thrombosis. Bertolaccini et al. [18] found that thrombotic events were more prevalent in patients with anti-PT antibodies and that these antibodies represented an independent risk factor for thrombosis in patients with SLE or PAPS [19]. Nojima et al. [20] reported the presence of anti-PT antibodies in SLE patients for whom, together with LA, they constituted the only risk factor for venous thromboembolism.

However, Horbach et al. [21] who studied 175 patients with SLE, found that both IgG and IgM anti-PT antibodies were more frequent in patients with a history of thrombosis, but the correlation was not significant when examined by a multivariate analysis. Pengo et al. [22] and Swadzba et al. [23] also found no correlation between the presence of anti-PT antibodies and thrombosis in patients with APS or with SLE.

In this study, we found that although aCL antibodies and anti-β2GPI antibodies were significant risk factors for thrombosis in SLE, the highest risk was associated with anti-PT antibodies. The best results in terms of diagnostic accuracy were obtained by combining aCL antibodies and anti-PT antibodies; actually, the presence of one or the other antibodies brings the sensitivity for thrombosis to 100%.

Finally, the results of this study indicate that anti-PT antibodies are very sensitive markers associated with thrombosis in SLE patients. Specifically, anti-PT antibodies could detect 22% of patients with SLE and thrombosis who were negative for aCL antibodies, and the combined measurement of aCL and anti-PT

antibodies could provide a 100% sensitivity for thrombosis. Therefore, their measurement can be usefully associated with the more widely used aCL antibodies in the prognostic evaluation of SLE patients. The main limitation of this study may be the small number of SLE/APS patients included, especially the aCL antibodies-negative patients.

Conclusion

Anti-PT antibodies are specific and sensitive markers associated with thrombosis in SLE patients. Measurement of aPL-cofactor antibodies, particularly anti-PT antibodies in addition to the more widely used aCL antibodies, could be a useful prognostic marker for a higher risk of thrombosis in SLE/APS patients.

Prospective and longitudinal studies with larger populations are needed to clarify which antibody is not only associated with, but also predictive of, the development of thrombotic complications.

Acknowledgements

Conflicts of interest
There are no conflicts of interest.

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