Haematologica 2000; 85:632-637
original paper

Hemostasis & Thrombosis

Prevalence and clinical significance of antiprothrombin antibodies in patients with systemic lupus erythematosus or with primary antiphospholipid syndrome

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ABSTRACT

***Background and Objectives*. Antibodies to prothrombin (aII) have been identified in patients with antiphospholipid antibodies, but their clinical significance is not well known. The aim of our study was to investigate their prevalence and association with clinical manifestations of the antiphospholipid syndrome (APS) in patients with primary APS or with systemic lupus erythematosus (SLE).**

***Design and Methods.* A series of 177 patients with autoimmune diseases was studied: 70 with primary APS and 107 with systemic lupus erythematosus. A control group of 87 healthy volunteers were included in the study. aII were investigated in sera by an ELISA, using human prothrombin as antigen fixed in irradiated polystyrene plates.**

***Results.* aII prevalence in patients with autoimmune disease was 47% (57% and 40% in patients with primary APS or with SLE, respectively) significantly higher than in controls (5%) ($p<0.0001$). In the whole series, thrombotic events were more prevalent in patients with aII (45% vs 28%; $p=0.02$). Moreover, aII was found to be an independent risk factor for arterial thrombosis (OR=2.4; $p=0.04$). Similarly, in patients with SLE, aII were associated with both arterial and venous thrombosis (35% vs 14%; $p=0.01$), although only IgG-aII (OR=3.7; $p=0.01$) had an independent value as risk factor for thrombosis. However, a relationship between aII and thrombosis was not found in primary APS. aII were associated with thrombocytopenia only in patients with primary APS (OR=6.7; $p=0.007$).**

***Interpretation and Conclusions.* aII seem to be a serological marker of thrombosis in autoimmune diseases, mainly in SLE patients and/or in the arterial territory.**

Key words: antiprothrombin antibodies, antiphospholipid syndrome, systemic lupus erythematosus, thrombosis, miscarriage, thrombocytopenia

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The association between clinical features of recurrent thrombosis, miscarriage and thrombocytopenia, and antiphospholipid antibodies (aPL) is named the antiphospholipid syndrome (APS).[1] There is now general agreement that antiphospholipid antibodies in autoimmune diseases are not directed towards phospholipids alone, but to complexes of phospholipids and proteins that are bound to these phospholipids and act as cofactors.[2] In this way, anticardiolipin antibodies (aCL) need the β_2-glycoprotein I to bind to cardiolipin[2] and lupus anticoagulants (LA) need β_2-glycoprotein I and/or prothrombin to recognize phospholipids.[3,4] Recently, antibodies to β_2-glycoprotein I and to prothrombin have also been identified in patients with aPL, using these molecules as antigens fixed in the absence of phospholipids in irradiated polystyrene plates.[5,6] Moreover, anti-β_2-glycoprotein I antibodies ($a\beta_2GPI$) seem to be more specific for thrombosis in patients with APS and systemic lupus erythematosus (SLE) than aCL (5). $a\beta_2GPI$, and antiprothrombin antibodies (aII) are frequently found in sera of patients with LA[6] but, so far, available data about the clinical associations and usefulness of aII are controversial.[7-11] It was reported that some non-neutralizing aII associated with LA could cause hypoprothrombinemia and abnormal bleeding by increased clearance of prothrombin-antiprothrombin antibody complexes from the circulation.[7] However, the majority of aII detected in sera of patients with antiphospholipid antibodies are low-affinity antibodies and do not cause hypoprothrombinemia or bleeding.[8] In addition, an association between aII and thrombosis has been suggested.[9-11] The aim of the present work was to study the clinical significance of aII in a large series of consecutive and unselected patients with primary APS or SLE, and to determine whether these autoantibodies could help us to identify a subset of patients with an increased risk of clinical manifestations.

Design and Methods

Patients

We studied 177 patients with autoimmune diseases [153 females and 24 males, mean age 38 years

(range 15-79)]: 70 with primary APS and 107 with SLE. The diagnosis of APS was made according to previously described criteria.[12] The diagnosis of SLE was based on the revised criteria reported by the American College of Rheumatology.[13] The control group was formed of 87 healthy volunteers (73 females and 14 males) without autoimmune disease, bleeding disorders, thrombosis or history of pregnancy losses.

Methods

Blood samples were drawn into trisodium citrate and in non-anticoagulated tubes (Becton Dickinson, Rutherford, NJ, USA). Platelet-free plasma was obtained by double centrifugation [first at 2,000 g (10 min, 22ºC) and then at 5,000 g (10 min, 4ºC)], frozen and stored at –70ºC.

Detection of anti-prothrombin antibodies

Microtiter plates Maxisorp (Nunc, Roskilde, Denmark) were coated with 80 µL/well of 10 µg/mL human prothrombin (Diagnostica Stago, Asnières, France) in phosphate buffered saline, pH 7.4 (PBS) overnight at 4ºC. After washing with PBS containing 0.1% Tween 20 (Merk, Munchen, Germany), wells were blocked (1 h, 22ºC) with 150 µL of 1% bovine serum albumin (BSA) (Sigma, St. Louis, MO, USA) in PBS-Tween. After washing, wells were then incubated (1 h, 22ºC) with 100 µL of samples diluted (1:100) in PBS-Tween-1% BSA. After new washing, plates were incubated (1 h, 22ºC) with 100 µL of horseradish peroxidase-conjugated anti-human IgG (1:2,500) and IgM (1:1,500) (Dako, Glostrup, Denmark) in PBS-Tween-1% BSA. Finally, for color developing, 100 µL of 0.04% (0.4 mg/mL) ortho-phenylenediamine dihydrochloride (Sigma) diluted in phosphate-citrate buffer (pH=5.0) containing 25 µL /100 mL H_2O_2 (Sigma) were added. After incubation (10 min, 22ºC), the reaction was stopped with 25 µL of 2N H_2SO_4, and optical density was measured at 492 nm (OD_{492}). Intra and interassay coefficients of variation were 4.56 and 13.3 for IgG, and 4.85 and 9.4 for IgM. In each assay, one positive serum for aII and 8 negative sera were used as controls. OD_{492} values higher than 5 standard deviations (SD) above the mean of negative controls were considered positives: low positive between 5 and 7 SD, moderate positive between 7 and 9 SD, and high positive above 9 SD.

Detection of anti-cardiolipin antibodies

The aCL were measured by a commercially available ELISA (Cheshire Diagnostics, Chester, UK).

Detection of lupus anticoagulant

LA was assessed by coagulation assays using activated partial thromboplastin time, diluted Russell's viper venom time and tissue thromboplastin inhibition. Tests were also performed in mixtures with normal plasmas or phospholipids following the criteria of the *Subcommittee for the Standardization of Lupus Anticoagulants of the International Society of Thrombosis and Hemostasis*.[14]

Detection of anti-β_2-glycoprotein I antibodies

The aβ_2GPI were measured using an ELISA.[5] Briefly, microtiter plates (Maxisorp, Nunc, Roskide, Denmark) were coated (16 h, 4°C) with 10 µg/mL purified human β_2-glycoprotein I (Stago, Asnières, France) in carbonate buffer (pH=9.6). After washing, coated plates were blocked (1 h, 22°C) with 5% BSA (Sigma) in PBS-Tween, and then incubated (1 h, 22°C) with samples diluted in PBS-Tween plus 1% BSA. After new washing, plates were incubated (1 h, 22°C) with horseradish peroxidase-conjugated anti-human IgG and anti-human IgM (Dako) diluted in PBS-Tween-BSA. For color developing, ortho-phenylenediamine dihydrochloride (Sigma) diluted in phosphate citrate buffer (pH=5.0) plus H_2O_2 was added, incubated (10 min, 22°C) and the OD_{492} read. In each assay, 10 negative sera were used as controls and OD_{492} values higher than 5 SD above the mean of them were considered positive: low positive between 5 and 7 SD, moderate positive between 7 and 9 SD, and high positive above 9 SD.

Plasminogen activity

Plasminogen activity was determined using a specific chromogenic assay (Chromogenix, Mölndal, Sweden). Plasminogen present in the plasmas was converted to an active plasminogen-streptokinase complex by an excess of streptokinase. Then, plasminogen-streptokinase complex catalyzed the splitting of *p*-nitroaniline (pNA) from the substrate H-D-Val-Leu-Lys-pNA*2HCl (S-2251) and the pNA release was measured at 405 nm.

Statistical analysis

To study the relationship between autoantibodies and clinical manifestations (thrombosis, fetal miscarriage and thrombocytopenia) the whole series of patients with autoimmune diseases (SLE and primary APS) (n=177) was analyzed. Taken into account that SLE and primary APS are different autoimmune diseases with their own diagnostic criteria, in a second phase we studied patients with SLE and with primary APS separately, and the analysis was repeated considering, first, patients with SLE (n=107), and, second, patients with primary APS (n=70). Results are shown as mean±SD. Chi-square or Fisher's exact tests were used for comparing qualitative variables and ANOVA and the t-test for qualitative ones. A $p<0.05$ was considered statistically significant. Multivariate analysis using a backward stepwise logistic regression was performed and odds ratios (OR) reported. The statistical analyses were performed using the SPSS-PC 6.0 statistical packages for Windows.

Results

Clinical characteristics

Clinical manifestations of patients with APS are shown in Table 1. Primary and secondary APS had a similar clinical spectrum, although thrombocytopenia (platelet count lower than 100×10^9/L) was more frequent in APS associated with SLE (51% vs 30%; $p=0.02$).

Prevalence of antiprothrombin antibodies

aII were positive in 5% (4/87) of healthy volunteers. The prevalence of aII was 47% in all patients with

Table 1. Clinical manifestations of patients with antiphospholipid syndrome.

	Primary APS (70) N(%)	*Secondary APS (43) N(%)*	*Total (113) N(%)*
Miscarriage	31 (60)	24 (60)	55 (60)
Thrombosis	39 (56)	21 (49)	60 (53)
Arterial	22 (31)	11 (26)	33 (29)
Venous	19 (27)	13 (30)	32 (28)
Thrombocytopenia <100x10^9/L	21 (30)	22 (51)*	43 (38)

**p=0.02: Difference between primary and secondary APS. APS: antiphospholipid syndrome.*

Table 2. Prevalence of different antiphospholipid antibodies.

	Autoimmune disease (177) N(%)	*Primary APS (70) N(%)*	*SLE (107) N(%)*
LA	77 (44)	44 (63)	33 (31)
aCL	85 (48)	52 (74)	33 (31)
IgG	71 (40)	44 (63)	27 (25)
IgM	26 (15)	14 (20)	12 (11)
aII	83 (47)	40 (57)	43 (40)
IgG	58 (33)	27 (39)	31 (29)
IgM	41 (23)	22 (31)	19 (18)
a*β_2GPI	60 (34)	32 (46)	28 (26)
IgG	36 (20)	18 (26)	18 (17)
IgM	44 (25)	25 (36)	19 (18)

APS: antiphospholipid syndrome; SLE: systemic lupus erythematosus LA: lupus anticoagulant; aCL: anticardiolipin antibodies; aII: antiprothrombin antibodies; aβ_2GPI: anti-β_2-glycoprotein I antibodies; APS: antiphospholipid syndrome; SLE: systemic lupus erythematosus.

autoimmune disease, and 57% and 40% respectively in patients with primary APS and SLE with and without classical aPL. The IgG class of aII was the most frequent isotype. Table 2 shows the prevalences of the different aPL. A total of 27 patients (15.3%) had aII the autoantibodies (aCL, LA, aβ_2GPI, and aII), and 51 patients (28.8%) had only one antibody (12 aCL, 10 LA, 8 aβ_2GPI, and 21 aII). When we analyzed the correlation between the different aPL in the whole population with autoimmune disease (n=177), we found a close relationship between aII and classic aPL (LA and aCL), preferably between autoantibodies of the same isotype. Specifically, 62% and 64% of patients with LA and aCL, respectively, were aII positive. A good correlation between aII and aβ_2GPI was also observed. Tables 3 and 4 show the relationship among these autoantibodies. A total of 81% (62/77) of patients with LA had aII and/or aβ_2GPI.

Table 3. Relationship between antiprothrombin antibodies and other antiphospholipid antibodies in patients with autoimmune disease (N=177).

	LA (77) N (%)	*aCL (85) N (%)*	*IgG-aCL (71) N (%)*	*IgM-aCL (26) N (%)*	*aβ_2GPI (60) N (%)*	*IgG-aβ_2GPI (36) N (%)*	*IgM-aβ_2GPI (44) N (%)*
aII	48 (62)$^{\#}$	54 (64)$^{\#}$	47 (66)$^{\#}$	17 (65)*	35 (58)*	22 (61)*	28 (64)*
IgG	37 (48)$^{\#}$	37 (44)°	36 (51)$^{\#}$	7 (27)	22 (37)	17 (47)*	16 (36)
IgM	21 (27)	30 (35)$^{\#}$	23 (32)*	11 (42)*	22 (37)°	12 (33)	20 (46)$^{\#}$

*p<0.05; °p<0.01; $^{\#}$p<0.001. LA: lupus anticoagulant; aCL: anticardiolipin antibodies; aβ_2GPI: anti-β_2-glycoprotein I antibodies; aII: antiprothrombin antibodies.

Relationship with thrombotic events.

In patients with autoimmune disease, a significant association between aII and thrombosis was found, as shown in Table 4. This association was seen in the IgG isotype, but not in the IgM. The association with thrombosis was also seen for LA, aCL, IgG-aCL and IgG-aβ_2GPI, although LA was the only variable included in the best model for thrombosis in the multivariate analysis (OR=6.4; p<0.0001). For arterial

Table 4. Relationship between antiprothrombin antibodies and thrombosis in different subsets of patients.

	Thrombosis arterial	*venous*	*total*
Autoimmune disease (n=177)	N=34	N=34	N=63
aII:			
Positive (83)	24 (29%)**	17 (21%)	37 (45%)*
Negative (94)	10 (11%)	17 (18%)	26 (28%)
IgG-aII:			
Positive (58)	17 (29%)*	16 (28%)*	29 (50%)°
Negative (119)	17 (14%)	18 (15%)	34 (29%)
IgM-aII:			
Positive (41)	10 (24%)	5 (12%)	14 (34%)
Negative (136)	24 (18%)	29 (21%)	49 (36%)
Primary APS (n=70)	N=22	N=19	N=39
aII :			
Positive (40)	15 (38%)	9 (23%)	22 (55%)
Negative (30)	7 (23%)	10 (33%)	17 (57%)
IgG-aII:			
Positive (27)	9 (33%)	8 (30%)	15 (56%)
Negative (43)	13 (30%)	11 (26%)	24 (56%)
IgM-aII:			
Positive (22)	9 (41%)	3 (14%)	11 (50%)
Negative (48)	13 (27%)	16 (33%)	28 (58%)
SLE (n=107)	N=12	N=15	N=24
aII			
Positive (43)	9 (21%)*	8 (19%)	15 (35%)*
Negative (64)	8 (26%)°	7 (11%)	9 (14%)
IgG-aII:			
Positive (31)	3 (5%)	8 (26%)*	14 (45%)$^{\#}$
Negative (76)	4 (5%)	7 (9%)	10 (13%)
IgM-aII:			
Positive (19)	1 (5%)	2 (11%)	3 (16%)
Negative (88)	11 (13%)	13 (15%)	21 (24%)

**p<0.05; °p<0.01; $^{\#}$p<0.001. aII: antiprothrombin antibodies; APS: antiphospholipid syndrome; SLE: systemic lupus erythematosus.*

thrombosis, we observed a significantly higher prevalence of events in patients with aII and IgG-aII. This relationship was also found for LA, aCL, IgG-aCL, aβ_2GPI, IgG-aβ_2GPI, and IgM-aβ_2GPI, in the univariate analysis. In the multivariate analysis, aII added a significant independent value (OR=2.4; *p*=0.04) to LA (OR=5.9; *p*=0.0001) in the best model for arterial thrombosis. For venous thrombosis, a higher prevalence was found in patients with positive IgG-aII, LA and IgG-aCL, although only LA was included (OR=3.4; *p*=0.002) in the best model.

In the SLE group, aII were significantly associated with thrombosis, as shown in Table 4. Only IgG-aII isotype but not IgM showed significant association. Moreover, the prevalence of thrombosis in patients with LA, IgG-aCL or with IgG-aβ_2GPI was significantly higher than patients who tested negative. IgG-aII (OR=3.7; *p*=0.01) added an independent predictive value to LA (OR=8.0; *p*=0.0001) in the multivariate analysis. When arterial thromboses were analysed, a strong association with aII and its IgG isotype was seen. Furthermore, patients with LA (30% vs 3%; *p*=0.0001) or with IgG-aβ_2GPI (33% vs 7%; *p*=0.004) developed these thrombotic events more frequently than patients without these autoantibodies. Regarding venous thrombosis, only IgG-aII and LA (30% vs 7%; *p*=0.002) showed a significant association. It was not possible to perform a multivariate analysis in the subsets of patients because the number of events was not large enough to have adequate statistical power.

In the primary APS group, no association was found between aII and total thrombosis (Table 4). Only IgG-aCL was associated with thrombosis (66% vs 39%; *p*=0.02). When we studied arterial and venous thrombosis separately, a significant relationship was found only between aCL (39% vs 11%; *p*=0.02) and their IgG isotype (41% vs 15%; *p*=0.02) and arterial thrombosis.

Relationship with miscarriage

In the autoimmune disease group constituted by 153 women, miscarriages occurred more frequently in women with positive aII than in women who tested negative, although this difference did not reach statistical significance (43% vs 35%; *p*=0.2). Only aCL appeared to be significantly associated with miscarriages (49% vs 31%; *p*=0.02).

In the group of 101 women with SLE, no association with aII (28% vs 28%; *p*=0.9) was observed. Miscarriages occurred in 42% of women with positive LA compared to 21% with negative LA (*p*=0.03). Moreover, they were significantly more frequent in women with positive aCL than with negative aCL (43% vs 21%; *p*=0.02). The multivariate analysis included aCL in the best predictive model (OR=2.9; *p*=0.02).

In the group of 52 women with primary APS, no significant association between aPL and miscarriage was found. Sixty-three percent of women with positive aII had miscarriages compared to 55% of those who tested negative (*p*=0.5).

Relationship with thrombocytopenia

In the autoimmune disease group, thrombocytopenia (<100×10^9/L) was not significantly associated with aII. Only LA (42% vs 16%; *p*=0.0001) and IgG-aβ_2GPI (42% vs 23%; *p*=0.03) emerged as being associated with thrombocytopenia.

Thrombocytopenia was detected in 25% of patients with SLE, but it was not significantly associated with aII. Only LA was statistically associated with thrombocytopenia (42% vs 18%; *p*=0.007).

In the primary APS group, thrombocytopenia was significantly associated with aII (45% vs 10%; *p*=0.0009) and their two isotypes, IgG (44% vs 21%; *p*=0.03) and IgM (50% vs 21%; *p*=0.01). Also, it was associated with LA (41% vs 12%; *p*=0.006), aCL (37% vs 11%; *p*=0.03) and IgG-aβ_2GPI (56% vs 21%; *p*=0.007). The multivariate analysis showed aPT (OR=6.7; p=0.007) and IgG-aβ_2GPI (OR=4.0; *p*=0.02) as the only two independent predictor factors of thrombocytopenia.

Plasminogen activity

All samples had a normal plaminogen activity with a mean of 109±17%. Differences between groups were not observed, and no relationship with thrombotic events or with aII was found.

Discussion

The clinical relevance of aII has not yet been established and available data are controversial. Some of them, but not all, show LA activity,[15,16] suggesting that these autoantibodies are heterogeneous. Our results, showing that aII are detected in 62% of samples with LA, and, taken together, aII and aβ_2GPI are found in 81% of samples with LA, suggest that the ELISA methods used in the present work cannot identify all the samples with LA activity, and, consistently, could not replace the detection of LA in the study of aPL.

In patients without autoimmune disease, Palosuo *et al.*[9] found a close relationship between aII levels and deep venous thrombosis and pulmonary embolism in middle-aged men. Vaarala *et al*.[10] also found that aII imply a risk of myocardial infarction in middle-aged men. Recently, Bertolaccini *et al.*[11] found aII in 28% of patients with SLE, and the presence of aII correlated with a history of thrombosis: 53% of patients with aII had thromboses compared to 32% of those without aII. Contrariwise, Eschwège *et al.*[17] did not find a relationship between aII and a history of venous thrombosis in unselected patients; aII could be demonstrated in only 4% of the 122 plasmas tested, which is a similar prevalence to that found by us in the group of healthy people. Our results showed that aII were associated with arterial thromboses in the patients with autoimmune diseases and with both arterial and venous thromboses in patients with SLE. Moreover, IgG isotype but not IgM was useful as a thrombosis marker, unlike the results obtained by Bertolaccini *et al.*,[11] who found an important correlation between the presence of both isotypes and vascular events. However, in agreement with previous reports,[18] LA seems to have the strongest predictive value for thrombosis. Moreover, Horbach *et al*.[19] also showed that LA correlates best with both arterial and venous thromboses in patients with SLE, and neither aII nor aβ_2GPI gives additional information about a thrombotic risk. Pengo *et al.*[20] reported an aII prevalence of 50% in patients with

aCL and thrombosis but no association with thrombosis was found. Forastiero *et al.*[21] observed that aβ_2GPI, but not aII, give an increased risk for venous thrombosis in patients with aPL. Finally, Swadzba *et al.*[22] reported no correlation between aII and thrombosis in patients with SLE. The differences between the above mentioned works can be explained by the different selection criteria of their populations, the number patients'proved, and technical variations in aII ELISA. aII are more frequently detected in ELISA when prothrombin is bound to phosphatidylserine-coated ELISA plates using calcium ions than when prothrombin is bound to γ-irradiated or high-activated PVC plates.[15] Furthermore, we should not forget that many works are retrospective and they analyze the relationship between the presence of an autoantibody, such as aII or aβ_2GPI, in a unique sample of patients with a history of thrombosis. Therefore, prospective studies in large series of patients are needed to determine the risk that subjects carrying aII and other autoantibodies have of developing thrombosis. According to our results, aII could be a serologic marker of thrombosis mainly in patients with SLE.

The role of aII in the pathogenesis of thrombosis is unknown. It was suggested that aII showing LA activity cause prolongation of clotting tests, mainly the kaolin clotting time, by inhibiting both the prothrombinase and tenase complexes.[23] However, this coagulation profile seems to confer a weak risk of thrombosis, in contrast with the dilute Russell's viper venom time, which selectively evaluates prothrombin conversion and is prolonged in the presence of aβ_2GPI.[24] The aII seem to increase the affinity of prothrombin for negatively charged phospholipids competing with the binding of other coagulation factors for them and inhibiting the conversion of prothrombin into thrombin and, thus, probably hampering protein C activation.[23] However, it has been recently suggested that aII are unable to inhibit protein C activity.[25] More recently, Puurunen *et al.*[26] found that aII crossreact with plasminogen in patients developing myocardial infarction. These crossreactive aII can interfer with fibrinolytic function of plasminogen and favor the development of thrombosis. However, our results do not support this hypothesis, because we found a normal plaminogen activity in all samples and no relationship was found with aII.

The relationship between aII and miscarriage is still less clear. We did not find an association between aII and fetal losses either in primary APS or in SLE patients, similarly to results reported by Forastiero *et al.*[21] and Falcon *et al*.[27] in women with aPL and by Bertolaccini *et al*.[11] in women with SLE. However, Ailus *et al.*[28] showed that increased levels of aII were associated with secondary abortions in women who had already delivered at least one live child. These discrepancies are probably due to differences in baseline characteristics of the samples of studied patients. It has been suggested that vascular thrombosis and pregnancy loss are due to the reduction of surface-bound annexin V by aPL,[29] leading to a hypercoagulable state in the placenta and vascular endothelium. Binding of aPL to vascular endothelium can be mediated by phospholipid-binding proteins, such as β_2-glycoprotein I[30] and other cofactors, although whether aII is involved remains uncertain.

Finally, we observed an association between aII and thrombocytopenia in patients with primary APS but not in those with SLE, in agreement with previous reports.[11,22] Although it can not be excluded that aII could bind to platelets and cause thrombocytopenia, it seems that the presence of antibodies directed to specific platelet glycoproteins could be the main mechanism of thrombocytopenia, similar to the situation in idiopathic thrombocytopenic purpura.[31]

In conclusion, aII seem to be a serologic marker of thrombosis in autoimmune diseases, particularly in patients with SLE. Prospective studies are needed to confirm these results.

Potential implications for clinical practice

1. aII are a serologic marker of thrombosis in autoimmune diseases, particularly in patients with SLE. These autoantibodies could, therefore, be useful together with the classical aPL for evaluating the potential risk of thrombosis.
2. aII are not associated with other manifestations of primary or secondary APS, such as miscarriage or thrombocytopenia.

Funding

Supported by Spanish grants FIS 96/0580, FIS 97/0525, FIS 98/0171 and FIS 99/0148. Francisco J. Muñoz-Rodríguez was a research fellow sponsored by a grant from the Hospital Clínic of Barcelona, Spain.

Contributions and Acknowledgments

FJMR, JCR, JF and DT designed the study, interpreted the results and wrote the paper. FJMR, JCR, DT and GE performed the laboratory studies. FJMR, RC, JF, GE and JCR recruited patients and controls and were the clinicians responsible for the clinical management of the patients and clinical data acquisition. FC and JB took care of the pregnant patients and were responsible for the obstetrical program and data. JB, AO and MI are the heads of their respective departments and gave final aproval for the manuscript submission. All the authors critically revised the paper. The order of authors was decided on the basis of the size of their contribution.

Disclosures

Conflict of interest: none.

Redundant publications: no substantial overlapping with previous papers.

Manuscript processing

Manuscript received November 9, 1999; accepted April 4, 2000.

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