

# Prevalence of antiphospholipid antibodies in systemic sclerosis and association with primitive pulmonary arterial hypertension and endothelial injury

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## Abstract

### Objective

To investigate the prevalence and clinical significance of antiphospholipid antibodies in patients with systemic sclerosis (SSc).

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### Methods

Autoantibodies against cardiolipin (aCL) and  $\beta$ 2-glycoprotein I ( $\beta$ 2-GPI) were detected by enzyme-linked immunosorbent assays (ELISAs) in successively hospitalised SSc patients admitted during a 24-month period. These patients were compared to patients with systemic lupus erythematosus (SLE), rheumatoid arthritis (RA).

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### Results

108 SSc patients were included: 61 had limited cutaneous SSc, 47 had the diffuse sub-type, 16 had primitive pulmonary arterial hypertension (PAH) and 34 had digital ulcerations. The control groups consisted of 37 RA and 38 SLE patients. The prevalence of aCL positivity was lower in SSc patients vs SLE patients (14 vs 47%;  $p < 0.001$ ), lower in RA patients vs SLE patients (19 vs 47%;  $p < 0.001$ ), and not different in SSc vs RA patients (14 vs 19%; NS). The mean aCL titer was also lower in SSc vs SLE patients ( $8 \pm 10$  vs  $15 \pm 20$ ;  $p < 0.001$ ). In SSc patients, positivity for aCL was associated with PAH ( $p = 0.009$ ) and the aCL titer correlated with that of the von Willebrand antigen factor ( $r = 0.23$ ;  $p = 0.045$ ).

The prevalence of anti  $\beta$ 2-GPI positive patients (IgG and/or IgM) was 5% in the SSc group, 18% in the SLE group and 5% in the RA group (SLE vs SSc and SLE vs RA;  $p = 0.005$ ).

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### Conclusion

We found that the prevalence of antiphospholipid antibodies in SSc patients was low. However, aCL antibodies were associated with PAH and endothelial injury.

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### Key words

Systemic sclerosis, antiphospholipid syndrome, anticardiolipin antibodies, von Willebrand factor, pulmonary arterial hypertension, endothelial injury.

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## Introduction

Vascular involvement with generalised microangiopathy, endothelial injury and systemic fibrosis are hallmarks of systemic sclerosis (SSc). Signs of the disease including endothelium cell apoptosis and endothelium activation with cell adhesion molecule expression, inflammatory recruitment and the onset of a procoagulant state, provide strong evidence that immune abnormalities are involved in the emergence of early vascular lesions in SSc (1). Moreover, anti-endothelial cell antibodies are suspected to be associated with endothelial cell apoptosis in SSc (2) and anti-annexin V antibodies are reportedly associated with digital ischemia (3).

The production of antibodies against cardiolipin (aCL) and  $\beta$ 2-glycoprotein I ( $\beta$ 2-GPI) is associated with various adverse events including arterial and venous thromboembolism and recurrent miscarriage. Production of these antibodies is common in many systemic diseases such as systemic lupus erythematosus (SLE) and primary antiphospholipid antibody syndrome (4). Previous reports concerning the production of anti-phospholipid antibodies in SSc cases are scarce and conflicting; the prevalence varying widely from 0 to 41% (5-15). The role played by these antibodies in SSc remains unclear.

The aim of this study was to determine the prevalence of antiphospholipid antibodies in a series of SSc patients and to investigate whether their production was associated with organ involvement in this disease.

## Patients and method

### Study population

We included successively hospitalised SSc patients admitted in the Rheumatology A Department of Cochin Hospital (Paris, France) for a systematic follow-up of the disease during the period from January 2001 to December 2003. They were classified according to the criteria of LeRoy *et al.* (16) as having either limited (lcSSc) or diffuse (dcSSc) cutaneous subset of the disease. Information was collected for each patient concerning the following: skin disease, duration of Raynaud's pheno-

menon, history of digital ulcers and their use of prostacyclins. Patients were questioned about clinical manifestations of antiphospholipid syndrome, in particular as to whether they had suffered any thromboembolic events (phlebitis or pulmonary embolism) or miscarriages.

Anti-nuclear (ANA) and anti-centromere antibodies were detected by indirect immunofluorescence on Hep-2 cells. Anti-topoisomerase I antibody and anti-ribonucleoprotein antibody (anti-RNP) were determined by counterimmunoelectrophoresis.

Pulmonary involvement was assessed by forced vital capacity (FVC) and by carbon monoxide diffusion capacity to hemoglobin ratio (DLCO/Hb). Pulmonary interstitial fibrosis was defined as bibasilar interstitial fibrosis on computed tomography (CT) scans. Pulmonary arterial hypertension (PAH) was detected by Doppler echocardiography. For the purpose of this study, PAH was defined as a systolic pulmonary artery pressure (sPAP) above 40 mmHg in two separate tests conducted within the period of a few months in patients with no severe pulmonary interstitial fibrosis (FVC > 50%). Left ventricular function was assessed by radionuclide ventriculography.

Control groups were composed of SLE patients who fulfilled the revised ARA criteria (17) and were hospitalised during the 12-month period (year 2003), rheumatoid arthritis (RA) patients fulfilling the ACR criteria (18) who were successively hospitalised during the three month period (October to December 2003) and 20 healthy subjects from the laboratory staff. Clinical manifestations of antiphospholipid syndrome (APS) were investigated in SLE and RA patients. Patients were classified as having a APS if they fulfilled the international criterias defined by one clinical symptom and one antiphospholipid antibody present for at least 6 weeks.

### Detection of anticardiolipin and anti- $\beta$ 2-GPI antibodies

Titers of aCL antibodies (19) and anti  $\beta$ 2-GPI antibodies (20) were measured using previously described homemade solid phase immunoassays (21). The

positive limits of the tests were determined prior to the study using 100 serum samples from healthy subjects. Results were considered positive when the concentrations were higher than 15 U IgG and/or IgM aCL antibodies; and higher than 6 IgG and/or IgM anti  $\beta$ 2-GPI antibodies. This threshold was selected because among 100 sera from different healthy controls tested for these antibodies: 2% or fewer controls were found above 15 aCL units and 6 anti  $\beta$ 2-GPI units. Moreover, patients were definitely considered positive for aCL or anti  $\beta$ 2-GPI antibodies only when a second determination performed at the next hospital visit (3-6 months later) also demonstrated positivity for this investigation.

#### *Measurement of the von Willebrand factor and circulating lupus anti-coagulant*

Coagulation tests included circulating lupus anticoagulant, and von Willebrand factor activity and antigen measurements were conducted to provide markers of endothelial injury (22). The von Willebrand antigen concentration was determined using an ELISA (VIDAS von Willebrand, BioMérieux, Marcy l'Étoile, France). The von Willebrand ristocetin cofactor activity was

measured, using the BC von Willebrand Reagent (Dade Behring, Marburg, Germany), on a PAP4 aggregometer (Biodata). Lupus anticoagulant tests were performed according to the recommendations of the ISTH (23).

#### *Statistical analysis*

Data were analysed with the following nonparametric statistical methods: the Mann Whitney test for comparisons between groups and the Spearman's rank correlation test for assessment of the relationship between quantitative variables. For the comparison of means, the Student's t-test and Anova test were used for variables that followed a normal distribution. These analyses were conducted using the Stat-View statistical analysis software. P values of less than 0.05 were considered significant. All quantitative data are expressed as the mean  $\pm$  SD.

### **Results**

#### *Study groups*

One hundred and eight patients with SSc were included: 93 women and 15 men, with a mean age of  $59 \pm 13$  years [range: 31-92 years]. The mean disease duration (defined by cutaneous involvement) was  $10 \pm 8$  years [range: 0-42] and the mean duration of the Ray-

naud's phenomenon was  $13 \pm 11$  years [range: 0-63]. Clinical and laboratory data for these patients, grouped according to the presence of aCL or PAH, are presented in Table I.

Control groups were composed of 37 patients with RA (35 women and two men, with a mean age:  $57 \pm 12$  years [range: 35-75]), for whom the mean disease duration was  $11 \pm 9$  years; 38 patients with SLE (36 women and 2 men, with a mean age:  $46 \pm 17$  years [range: 14-76]) for whom the mean disease duration was  $18 \pm 10$  years; and 20 healthy subjects from the laboratory staff (12 women and 8 men with a mean age:  $45 \pm 7$  years).

#### *Measurements of aCL: prevalence and titer*

The prevalence of aCL (IgG and/or IgM) positive patients was significantly higher in SLE patients than in those with SSc [18/38 (47%) vs 15/108 (14%);  $p < 0.001$ ] and also higher in SLE patients than in those with RA [18/38 (47%) vs 7/37 (19%);  $p < 0.001$ ]. Prevalence was not different between SSc and RA patients (14 vs 19%; NS). These results are shown in Table II. None of the control subjects had significant amounts of aCL. The aCL titers were significantly higher (Anova test)

**Table I.** Characteristics of patients with systemic sclerosis (SSc): whole population compared to patients positive for anti-cardiolipin antibodies and patients with primitive pulmonary arterial hypertension.

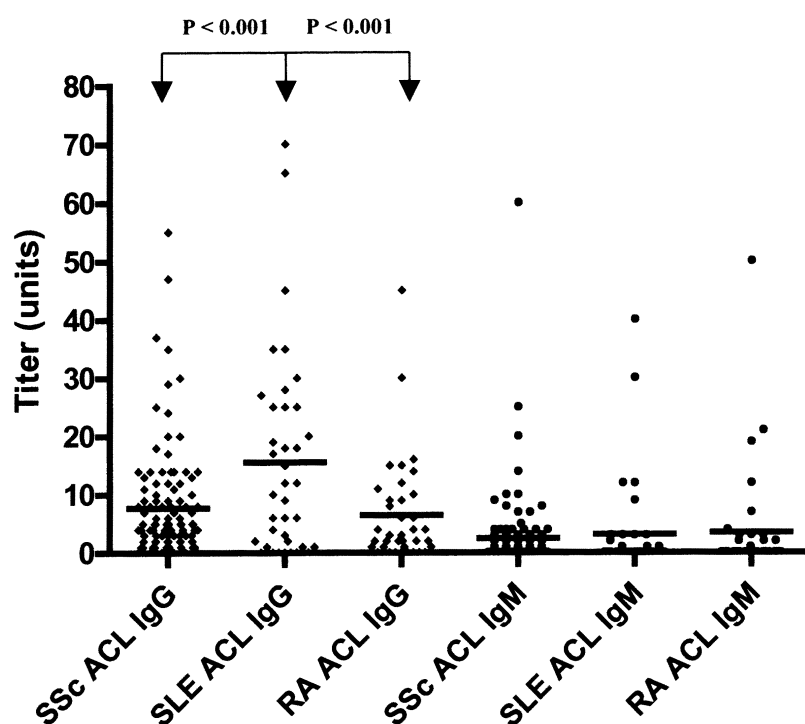
	Whole SSc group (n = 108)	SSc patients with IgG or IgM aCL >15 U (n = 15)	SSc patients with PAH (n = 16)
Cutaneous form of the disease: no. with limited form (%) / with diffuse form (%)	61 (56.5) / 47 (43.5)	8 (53) / 7 (47)	8 (50) / 8 (50)
Age: mean $\pm$ SD (range)	$59 \pm 13$ (31-92)	$61 \pm 17$ (31-86)	$63 \pm 14$ (36-86)
Digital pitting scars: no. of pts. (%)	34 (3.5)	8 (53)	8 (50)
Use of prostacyclin: no. of pts. (%)	21 (19)	3 (20)	9 (56)
sPAP > 40 mm Hg: no. of pts. (%)	16 (15)	7 (47)	16 (100)
Pulmonary fibrosis (CT-scan): no. of pts. (%)	56 (51.8)	12 (80)	15 (93.7)
FCV below 75% of normal values: no. of pts. (%)	28 (26)	7 (47)	6 (37.5)
DLCO/VA < 75%: no. of pts. (%)	43 (40)	10 (67)	11 (69)
Left ventricular ejection fraction % (mean $\pm$ SD)	$67 \pm 10$	$67 \pm 5$	$68.5 \pm 6.5$
C reactive protein (mg/L)	$7 \pm 1$	$9 \pm 2$	$6 \pm 3$
Antinuclear antibodies ( $\geq 1/160$ ): no. (%)	88 (81.5)	14 (93)	14 (88)
Anti-RNP antibodies: no. (%)	9 (8)	2 (13)	4 (25)
Anti-topoisomerase I antibodies/ Anti-centromere antibodies: no. (%)	33 (31) / 16 (15)	6 (40) / 2 (13)	7 (44) / 9 (56)
Von Willebrand activity/ von Willebrand antigen (%)	$170 \pm 68$ / $177 \pm 82$	$194 \pm 57$ / $210 \pm 63$	$240 \pm 95$ / $263 \pm 118$
Mean titer aCL IgG/IgM (IU)	$7.7 \pm 9.6$ / $2.4 \pm 6.7$	$26 \pm 14$ / $2.4 \pm 6$	$13.8 \pm 12.1$ / $2.5 \pm 5.8$

SSc: systemic sclerosis; PAH: pulmonary arterial hypertension; sPAP: systolic pulmonary arterial pressure; aCL: anticardiolipin antibodies; n patients: number of patients.

**Table II.** Prevalence (%) of patients with positive anticardiolipin (aCL) and anti  $\beta$ 2-GPI antibodies.

	SSc n = 108	SLE n = 38	RA n = 37
IgG aCL: no. (%)	12 (11)	17 (45)*	5 (13)
IgM aCL: no. (%)	3 (3)	2 (5)	(5)
IgG anti $\beta$ 2-GPI: no. (%)	5 (5)	5 (13)	1 (3)
IgM anti $\beta$ 2-GPI: no. (%)	2 (2)	2 (5)	1 (3)

\*p < 0.001 for SLE vs. SSc and SLE vs. RA patients.

**Fig. 1.** The titers of IgG and IgM anticardiolipin antibodies in patients with systemic sclerosis (SSc), systemic lupus erythematosus (SLE) and rheumatoid arthritis (RA). Data points represent the titers in individual patients and the bar represents the median titer of the group.**Table III.** Titers of anticardiolipin antibodies in positive patients in the different groups (NS).

	SSc (n = 15/108)	SLE (n = 18/38)	RA (n = 7/37)
IgG titer of aCL (u)	26 ± 14	29 ± 23	18 ± 15
IgM titer of aCL (u)	2.4 ± 6	3.2 ± 8	3.5 ± 9

for SLE patients than for those with SSc ( $15.4 \pm 20$  vs  $7.7 \pm 9$ ;  $p = 0.01$ ), and also higher for SLE patients than for those with RA ( $15.4 \pm 20$  vs  $6.4 \pm 9$ ;  $p = 0.02$ ). The individual aCL titers in the different groups of patients are shown in Figure 1. Considering only positive patients for aCL antibodies there was no difference in the titers of IgG or IgM antibodies between the 3 groups of patients (Table III). All patients with positive results at the first determina-

tion were also aCL positive at the second one.

#### Measurement of anti $\beta$ 2-GPI and circulating lupus prevalence

The prevalence of IgG and/or IgM anti  $\beta$ 2-GPI antibodies was 5/108 (5%) in SSc patients, 7/38 (18%) in SLE patients, 2/37 (5%) RA patients and 0/20 in healthy subjects (SLE vs SSc and SLE vs RA;  $p = 0.005$ ) (Table II). Prevalence was not different between SSc

and RA patients (5 vs 5%; NS). None of the patients in the SSc and RA groups had circulating lupus anticoagulants, whereas 4/38 (11%) were positive in the SLE group. All patients with positive results at the first determination were also anti  $\beta$ 2-GPI positive at the second one.

#### Prevalence of patients who fulfilled the international criteria for antiphospholipid syndrome

The prevalence of patients who fulfilled the international criteria for antiphospholipid syndrome was (3/108) 3% in the SSc group, (7/38) 18% in the SLE group and (1/37) 3% in the RA group. SSc patients had significantly less thromboembolic events than patients with SLE ( $p < 0.001$ ). The APS manifestations in the 3 groups of patients are detailed in table IV.

#### Clinical significance of aCL in SSc patients

There was an association between PAH and IgG and/or IgM aCL antibody positivity: 7/16 (44%) patients with PAH were positive for aCL antibodies, whereas only 8/92 (9%) patients without PAH were positive for aCL antibodies ( $p = 0.009$ ). Patients with PAH had also a higher mean titer of aCL closed to statistical significance:  $13 \pm 12$  U in patients with PAH vs  $7 \pm 8$  U in patients without PAH ( $p = 0.06$ ). Among the 16 patients with increased sPAP measured by echocardiography at the time of this study, 8/16 had severe clinical symptoms and PAH confirmed by right heart catheterism; these 8 patients did not exert higher mean titer of aCL than the other 8 with increased sPAP not evaluated by catheterism ( $14 \pm 11$  vs  $12 \pm 12$  U; NS). There was no correlation between sPAP values measured by echocardiography and aCL antibody titers. Patients with PAH produced larger amounts of von Willebrand factor antigen and had higher von Willebrand factor activities than patients without PAH ( $263 \pm 119$  vs  $171 \pm 76$ ;  $p = 0.002$  and  $240 \pm 95$  vs  $164 \pm 63$ ;  $p = 0.01$  respectively). The IgG aCL titer correlated with that for the von Willebrand antigen factor ( $r = 0.23$ ;  $p = 0.045$ ), and there was also a trend between IgG

**Table IV.** Prevalence of APS manifestations in the different groups.

	SSc (n = 108)	SLE (n = 38)	RA (n = 37)
Phlebitis: no. (%)	5 (5)	8 (21)	1 (3)
Pulmonary embolism: no. (%)	5 (5)	1 (3)	1 (3)
Miscarriages: no. (%)	7 (6)	1 (3)	3 (8)
Others*	0 (0)	6 (16)	0 (0)
Any APS manifestations: no. (%)	13 (12)	14 (37) <sup>‡</sup>	6 (16)
APS events in positive aCL patients: no. (%)	3/15 (20)	7/18 (39) <sup>‡</sup>	1/7
APS prevalence: no. (%)	3/108 (3)	7/38 (18)	1/37 (3)

\* including stroke, Libman Sachs endocarditis.

<sup>‡</sup>  $p < 0.01$  for SLE vs SSc and SLE vs RA patients.

APS: antiphospholipid syndrome; SSc: systemic sclerosis; SLE: systemic lupus erythematosus; RA: rheumatoid arthritis.

aCL titer and von Willebrand factor activity ( $r = 0.21$ ;  $p = 0.07$ ).

Positivity for the aCL antibody was not associated with sex, age, disease duration, duration of Raynaud phenomenon, or cutaneous subtype (dcSSc or lcSSc), digital ulcerations, interstitial lung disease, left ventricular ejection fraction, or the presence of anti-topoisomerase I, anti-centromere or anti-RNP antibodies.

## Discussion

We found that the prevalence of aCL was low and that aCL-related clinical events occurring among this series of SSc patients were low. However, these antibodies seem to be associated with vascular involvement and endothelial injury as reflected by their association with PAH and elevated levels of von Willebrand factor.

Previous studies of aCL antibodies in SSc patients gave varying and conflicting results, with the prevalence of aCL ranging from 0 to 41% (5-15). The disparity between these results could be explained by interlaboratory variability in aCL methodology. Our results for the control groups are in accordance with those from previous studies: aCL positivity ranged from 4 to 49% in RA patients and from 17 to 61% in systemic lupus erythematosus patients, with most groups reporting a prevalence above 30% (7).

In this study, we investigated production of both the aCL and anti  $\beta$ 2-GPI antibodies. We found that only 14% of the SSc patients were aCL positive and this frequency was even lower for the anti  $\beta$ 2-GPI antibody. Our results are very similar to those of Schoenroth et

al. who found that 12% of cases were positive for aCL and 8% for anti  $\beta$ 2-GPI (15). Both sets of data suggest that aPL antibodies have a low frequency in SSc.

However, we found a significant association between aCL production and PAH in SSc patients. This association has been found previously, not only in SSc patients (13, 24) but also in systemic lupus erythematosus patients with APS (25) and in 29% of patients with precapillary PAH irrespective of its cause (26). We retrospectively looked at aCL measurements taken in our PAH patients prior to development of this symptom: these measurements were taken over a mean period of ten years (range 1-15 years), and consisted of two or three aCL tests. The majority of these patients (4/7) were negative for aCL prior to the development of PAH. This suggests that production of aCL is not predictive of PAH but is concomitant with this vascular complication. Patients with severe pulmonary fibrosis and elevated PAP were not taken into account for PAH group which then only reflects vascular associated intrinsic PAH lesion.

Endothelial cell injury in SSc patients is accompanied by an elevation in the level of von Willebrand factor (27). Our results show that there is significant correlation between the IgG aCL titer and the amount of von Willebrand antigen produced. We also found an association between patients having PAH and the amount of von Willebrand factor produced. This suggests that aCL positivity is associated with endothelial injury and PAH in our patients. The fact that the titers of aCL were not

correlated with sPAP and that patients with severe PAH did not exert higher aCL values than patients with mild PAH rather support an association than a causative role of aCL with PAH vascular lesions but more data are required to determine whether production of these antibodies represent a primary or a secondary event with respect to vascular injury of systemic and pulmonary circulation in SSc patients.

We could not find an association between anti  $\beta$ 2-GPI, PAH and von Willebrand factor levels; this seems to be related to the low frequency of these antibodies in SSc patients which did not allow powerful statistical analysis. It could be explained by the higher specificity of anti  $\beta$ 2-GPI for APS whereas aCL could rather reflect aspecific endothelium injury; this hypothesis is emphasised by the results from a recent study suggesting that the presence of aPL with or without clinical findings is linked to increased levels of P-selectins, a marker of endothelial activation (28).

The cause of vascular occlusion in patients with aCL is unknown, but hemostatic disorder and/or endothelial damage are possible mechanisms. Thus, it has been suggested that aCL might inhibit the production of prostacyclin and act as an antibody against endothelium, interacting with different coagulation factors (29,30). By this action aCL may participate for the vascular lesions and hemostatic abnormalities in SSc patients and thus constitute a link between the immunological and vascular disorders associated with this disease. There are data supporting an association between these autoantibodies and other vascular diseases, such as in atherosclerosis (31). However, as in SSc, it remains unknown in patients with coronary artery disease whether aCL positivity constitutes a primary event or is merely a secondary event resulting from endothelial abnormalities (32).

We did not find any association between the production of aCL or anti  $\beta$ 2-GPI and the clinical manifestations associated with APS. Among the aCL positive patients, those suffering from SSc had significantly fewer thrombotic events than patients with systemic lu-

pus erythematosus. These results are consistent with previous studies (5-10) and may be explained by the low prevalence of anti  $\beta$ 2-GPI, which, in comparison to aCL, is associated more strongly with a history of thrombosis.

## Conclusion

We found that the frequency of the antiphospholipid antibody was low and not associated with thrombotic events in patients with SSc. However, production of aCL antibody was associated with primitive pulmonary arterial hypertension and the titer of von Willebrand factor. Thus, production of this antibody might reflect vascular injury.

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