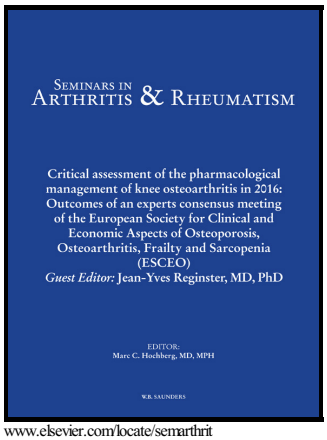

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Serological evolution in women with positive antiphospholipid antibodiesEvolution of antiphospholipid antibodies

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PII: S0049-0172(17)30155-5
DOI: http://dx.doi.org/10.1016/j.semarthrit.2017.05.001
Reference: YSARH51191

To appear in: *Seminars in Arthritis and Rheumatism*

Cite this article as: Leyre Riancho-Zarrabeitia, Germán Daroca, Pedro Muñoz, Marcos López-Hoyos, Ana Haya and Víctor M. Martínez-Taboada, Serological evolution in women with positive antiphospholipid antibodiesEvolution of antiphospholipid antibodies, *Seminars in Arthritis and Rheumatism,* http://dx.doi.org/10.1016/j.semarthrit.2017.05.001

SEROLOGICAL EVOLUTION IN WOMEN WITH POSITIVE ANTIPHOSPHOLIPID ANTIBODIES

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Running title: Evolution of antiphospholipid antibodies

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There was no specific financial support for this study and the authors declare no conflict of interest.

ABSTRACT

Objectives: To explore the clinical and serological course of fertile women with positive aPL, and the factors and therapeutic implications associated with aPL negativization.

Methods: Retrospective study including 105 women with a positive aPL serology between 1995 and 2013 attending the obstetric autoimmune pathology clinic of a tertiary-facility. Patients were classified into 3 groups: patients with primary antiphospholipid syndrome (pAPS, 49), patients with a positive serology for aPL, not meeting clinical criteria (42) and patients with systemic lupus erythematosus and a positive aPL serology (14). They were also classified, according to the serological aPL evolution: persistently negative aPL, transiently positive serology and persistently positive serology according to established criteria.

Results: After a mean follow-up of 114.4 ± 37.2 months, 59% patients had persistently negative antibodies, while 25.7% patients presented persistently positive aPL serology. Multivariate analysis confirmed that smoking (OR 4, 95%CI 1.45-11.08, p=0.008) was an independent risk factor for positive persistence. Persistent positivity, as well as a higher antibody load was associated with higher risk for further pregnancy morbidity. In 29 patients, with persistently negative serology who were asymptomatic, treatment with low-dose aspirin was discontinued. No clinical events related to APS were reported after treatment withdrawal, during the 40.95 months of follow-up.

Conclusions: A significant proportion of fertile women with aPL antibodies became negative during follow-up. Tobacco use and the number of positive antibodies are associated with persistently positive serology. Patients with persistently positive aPL serology suffer more obstetric complications. Treatment withdrawal might be safe in selected patients.

Key Words: antiphospholipid antibodies, serological evolution, antiphospholipid syndrome

Antiphospholipid antibodies (aPL) are a group of autoantibodies that bind to negatively charged phospholipids, phospholipid-binding proteins or both [1]. The most commonly detected antibodies are lupus anticoagulant (LA), anticardiolipin antibodies (aCL), and anti B2 glycoprotein I antibodies (aB2GPI), being the role of other non-criteria aPL recently suggested [2]. Antiphospholipid syndrome (APS) is an autoimmune disease characterized by the presence of aPL and at least a clinical event defined as thrombosis or pregnancy morbidity [3]. It is called primary or secondary APS according to the absence or presence of an underlying connective tissue disorder. The prevalence of aPL in the general population is around 5% [4], being transiently positive in cases of infections and in any inflammatory state [4;5]. In lupus patients aPL are present in up to 41% even in the absence of APS [6].

According to previous studies [7;8] the titers of aPL frequently decrease and eventually become negative during the follow-up period. The negativization has been related with a lower risk of thrombotic events, allowing for the possible withdrawal of anticoagulant therapy. However, the impact of the negativization on pregnancy morbidities is unknown. This fact may have implications regarding the proper management of these patients.

The aim of this study was to explore the clinical and serological course of fertile women with positive aPL, as well as the factors and the potential therapeutic implications associated with aPL negativization.

MATERIAL AND METHODS

Study population

We conducted a retrospective study which included women attending the obstetric autoimmune pathology clinic of a tertiary-facility serving a population of about 550,000 in Northern Spain. In this clinic, both a gynaecologist and a rheumatologist, attend pregnant women with either a previous diagnosis of an inflammatory condition or with history of pregnancy morbidities. These patients are tested for antiphospholipid antibodies as part of the routine diagnostic work-up. The women included were those with a confirmed positive aPL serology according to Sidney Criteria between October 1995 and December 2013. Laboratory criteria were those of Sydney Criteria: a) LA present in plasma, on two or more occasions at least 12 weeks apart, detected according to the guidelines of the International Society on Thrombosis and Haemostasis, b) aCL of IgG and/or IgM isotype in serum or plasma, present in medium or high titer on two or more occasions at least 12 weeks apart, measured by enzyme-linked immunosorbent assay (ELISA) or c) aB2GPI of IgG and/or IgM isotype in serum or plasma, present on two or more occasions at least 12 weeks apart, measured by ELISA.

Laboratory tests

IgG and IgM aCL and aB2GPI levels were measured by means of ELISA (Aesku diagnostics, Wensdelsheim, Germany) and expressed in GPL or MPL units or U/ml, respectively. Titers were considered to be positive when they were above the 99th percentile, thus corresponding to 20 GPL, MPL or U/ml. We considered positive titer ranges of (20-30) as medium or high (>30) titers, being low titers considered as negative. Lupus anticoagulant was determined according to the standard subcommittee guidelines on Lupus Anticoagulant/antiphospholipid antibody (scientific and standardization

Committee of the International Society of Thrombosis and Haemostasis) [9]. IgA aCL and IgA aB2GPI were not measured, as they are not routinely performed in clinical practice and they are not included in the APS classification criteria [3].

Clinical manifestations

Thrombotic events, both venous and arterial were analyzed. Pregnancy morbidities included in the classification criteria were evaluated. We also analyzed other events not included in the Sydney criteria that could be related to aPL such as less than three early fetal losses, HELLP syndrome, preeclampsia or eclampsia, abruptio placenta, premature birth (<37 weeks), pregnancy hypertension, pregnancy diabetes, early neonatal death (<1 week), intrauterine growth restriction and oligohydramnios.

Study groups

Patients were classified according to 3 different groups: patients with primary APS according to Sydney criteria (pAPS); patients with a positive serology for APL, not meeting clinical criteria (aPL serology) and patients with Systemic Lupus Erythematosus (SLE) according to ACR 1997[10] and a positive aPL serology (SLE+aPL).

We considered time 0 (T0), the date when patient met the serological criteria for APS, meaning the second positive aPL result being at least 12 weeks apart from the first one, according to Sydney criteria. We excluded the determinations that were separate less than 12 weeks during the first 12 months of follow-up and less than 24 weeks during further follow-up. We considered time for negativization the time from the first negative determination (with no further positive aPL serology).

Objectives

The main objective of the study was to describe aPL fluctuations and the rate of negativization, and to determine if there were differences among the three study groups. Patients were classified, according to the serological aPL evolution into three different groups: a) patients with persistently negative aPL (patients with at least the last 2 determinations, drawn more than 6 months apart were negative), b) patients with transiently positive serology (patients in which less than two-thirds of the aPL determinations were positive) and c) patients with persistently positive serology (patients in whom at least two-thirds of the aPL determinations were positive)

The secondary objectives were a) to identify the clinical and serological risk factors for persistently positive aPL serology, as well as its implication in morbidity and mortality during the follow-up period; b) to evaluate the impact of treatment withdrawal in those with persistently negative aPL and a low-risk profile (asymptomatic patients, with no cardiovascular risk factors and unwilling to have further pregnancies).

Statistical Analysis

Statistical analysis was performed using IBM SPSS Statistics for Windows version 20 (Armonk, NY: IBM Corp.). Mean values and standard deviations were calculated for the continuous variables. Inter-group differences were analyzed using the Student t-test, ANOVA or the chi square tests. A 2-tailed $p< 0.05$ was considered statistically significant. Logistic regression was used to assess factors associated with the persistence of positive antibodies. Kaplan-Meier plots were used to describe the time-course of antibody positivity.

RESULTS

General characteristics of the study population

We reviewed the clinical charts of 309 patients attending the obstetric autoimmune pathology clinic, and finally included 105 female patients meeting inclusion criteria that had a confirmed positive aPL serology between October 1995 and December 2013. Median age at confirmed serological aPL positivity was 31.8 years (interquartile range 28.9-36.2). Of the 105 patients included, 49 patients had pAPS (82% obstetric pAPS, 14% thrombotic pAPS, and 4% mixed pAPS), 42 patients had a positive serology with no clinical criteria of APS (aPL serology), and 14 had SLE with positive aPL (SLE+aPL).

The main characteristics of the study population are shown in supplementary Table 1. When comparing the baseline characteristics of the three groups studied, we found no differences regarding age and traditional cardiovascular risk factors. The most frequent CV risk factor was tobacco use (including current and former smoker) that was present in 32 (30.8%) patients. Regarding previous treatments, considering those received before T0, the date when patient met the serological criteria for APS, the most common therapy used was antiplatelet therapy (48, 46.2%), followed by heparin (17, 16.3%), antimalarial drugs (16, 15.4%) and corticosteroids (10, 9.6%). As expected, treatment with heparin was more common in the primary APS group ($p=0.051$) and treatment with hydroxychloroquine, steroids, and other immunosuppressants was more frequent in lupus patients ($p<0.001$) (Supplementary Table 1). In line with the immunological profile, all patients were tested for aCL and aB2GPI. LA was also determined in 90 patients out of the 105 patients included. 83% of patients had positive aCL, 71% had aB2GPI and 41% had positivity for LA, not finding any differences between the three groups. Overall, 35%, 42%, and 23% had positivity for one two and three antibodies,

respectively. Moreover, previous thrombotic events and obstetric morbidity were more common in the pAPS group. However, pregnancy morbidities not included in the classification criteria were more commonly seen in patients with positive serology not meeting clinical criteria for APS (p=0.023) (Supplementary Table 2).

In supplementary table 3 and table 3 we describe the incidence of thrombotic events and pregnancy morbidity during follow-up. After a mean follow-up of 114.4 ± 37.2 months (range 12-231), 2 patients (1.9%) suffered thrombotic events and 98 women had 174 new pregnancies. Among them, 21 patients experienced obstetric complications classified as Sydney criteria (16 patients had one or more early pregnancy losses, 5 patients had late pregnancy loss and 3 patients had a live birth with prematurity due to preeclampsia, eclampsia or placental insufficiency). A total of 30 patients experienced obstetric complications not classified as Sydney criteria being prematurity not related with the previous causes (13), oligohydramnios (8), gestational diabetes (6) and intrauterine growth restriction (5) the most frequently seen. There were no differences among the 3 groups in the development of further clinical events, although late fetal losses were more frequent in pAPS (p=0.050) (Supplementary Table 3).

A significant proportion of fertile women with aPL antibodies became negative during follow-up

Patients were classified into 3 groups according to the positive or negative serology during follow-up, as previously described in methods. The mean number of aPL determinations was 6.12 ± 3.61, meaning that aPL serum levels were rechecked 6 times in every patient.

After a mean follow-up of 114.4 ± 37.2 months (range 12-231), 62 (59%) patients had persistently negative antibodies, 16 (15.2%) patients had transiently positive antibodies,

and 27 (25.7%) patients presented persistently positive aPL serology (**Table 1).** aPL turned negative in 57.1 % of patients with pAPS, in 57.1 % of patients with positive serology not meeting clinical criteria of APS and in 71.4 % of lupus patients with positive aPL serology (p=0.805). As shown in **Table 1** mean age at diagnosis of seropositivity was different among the three serological evolution groups (p=0.019), at expense of differences between persistently negative and transiently positive serological group (Bonferroni posthoc analysis p=0.015). Moreover, smoking was significantly more frequent in patients with persistently positive serology (p=0.008). There were not any differences regarding other CV risk factors or previous treatment. We did not find any differences in previous thrombotic or obstetric events according to the serological evolution, as shown in **Table 2**.

	Total number of patients (n = 105)	**Serological evolution**			*p*
		Persistentl y negative (n = 62)	**Transientl y positive (n=16)**	**Persistentl y positive (n=27)**	
Age at the time of seropositivy mean ± SD	32.17 ± 5.34	33.02 ± 4.53	28.83 ± 3.83	32.19 ± 6.98	0.019
Age (years) mean ± SD	41.55±5.75	42.39±5.07	38.90 ±3.14	41.21 ±7.75	0.09
Family history of thrombotic events	9 (8.6)	6 (9.7)	0 (0)	3 (11.1)	0.403
Traditional CVRF, n(%)					
Tobacco use	32 (30.8)	16 (25.8)	2 (12.5)	14 (53.8)	**0.00**

					8
Hypertension	2 (1.9)	2 (3.2)	0 (0)	0 (0)	0.493
Dyslipidemia	5 (4.8)	4 (6.5)	1 (6.2)	0 (0)	0.403
Diabetes mellitas	1 (1)	1 (1.6)	0 (0)	0 (0)	0.705
Previous treatment					
Heparin	17 (16.3)	8 (13.1)	5 (31.2)	4 (14.8)	0.211
Oral anticoagulants	4 (3.8)	1 (1.6)	2 (12.5)	1 (3.7)	0.132
Antiplatelet therapy	48 (46.2)	29 (47.5)	6 (37.5)	13 (48.1)	0.751
Corticosteroids	10 (9.6)	7 (11.5)	2 (12.5)	1 (3.7)	0.477
Antimalarial drugs	16 (15.4)	8 (13.1)	1 (6.2)	7 (25.9)	0.168
Immunosuppressant drugs	7 (6.7)	5 (8.2)	1 (6.2)	1 (3.7)	0.738

Table 1. Traditional cardiovascular risk factors and previous treatments according to the serological evolution (SD: standard deviation; CVRF: cardiovascular risk factor)

	Total number of	**Serological evolution**			*p*
		Persistently negative	**Transiently positive**	**Persistently positive**	

	patients (n = 105)	**(n = 62)**	**(n=16)**	**(n=27)**	
Previous thrombotic events, n (%)	9 (8.6)	4 (6.5)	3 (18.8)	2 (7.4)	0.284
Previous obstetric events, n (%)	71 (67.6)	44 (71)	9 (56.2)	18 (66.7)	0.529
Obstetric events defining Sidney criteria	53 (58.9%)	33 (61.1%)	6 (42.9%)	14 (63.6%)	0.717
Non-criteria obstetric events	18 (17.1)	13 (21)	1 (6.2)	4 (14.8)	0.354

Table 2. Previous thrombotic and obstetric events according to the serological evolution

Tobacco use and the number of positive antibodies are associated with persistently positive serology

As previously shown we found a clear increased prevalence of smoking in the group with persistently positive serology (p=0.008) **Table 1**. There were no differences when analyzing the other traditional CV risk factors, the presence of thrombotic events or pregnancy morbidity and previous treatments. There was a significant difference when analyzing the number of positive antibodies (aCL, aB2GPI and LA), with a linear association between the number of positive antibodies and the persistence of positive serology (p=0.008) **(Figure 1**).

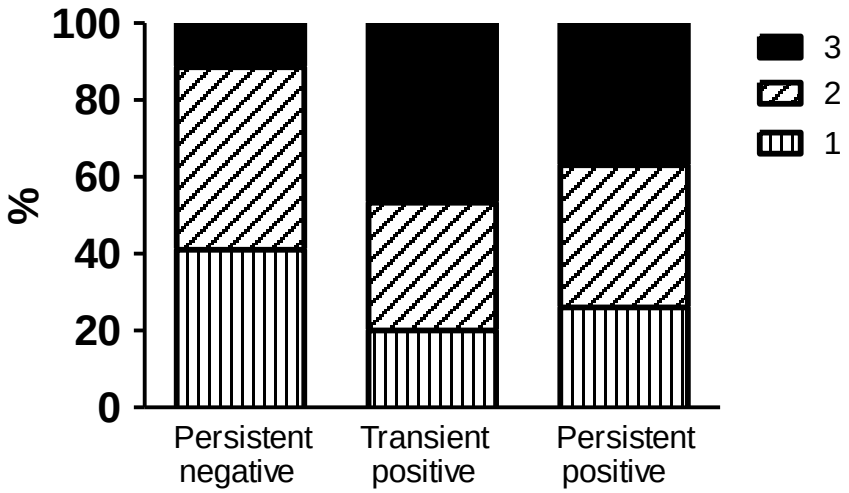

Figure 1. Number of positive antibodies (aCL, aB2GPI and LA) according to the serological evolution.

We also found that the proportion of smokers was higher in those with triple positivity (52.2%) than in those with positivity for one (28.6%) or two antibodies (22.7%) (p=0.041). Besides, smoking was also more commonly seen in those patients with positivity for LA (41.7% vs 20.8%, p=0.033). Additionally, we found an association between the number of positive antibodies and previous treatment with antimalarial drugs (2.8% in women with positivity for one antibody, 18.2% in women with positivity for two antibodies and 30.4% in women with triple positivity; p=0.004). The multivariate analysis using models including the variables with significant association in the univariate analysis confirmed that tobacco use was an independent risk factor for the persistence of positive serology (OR=4; 95%CI 1.45-11.08, p=0.008 **Figure 2**).

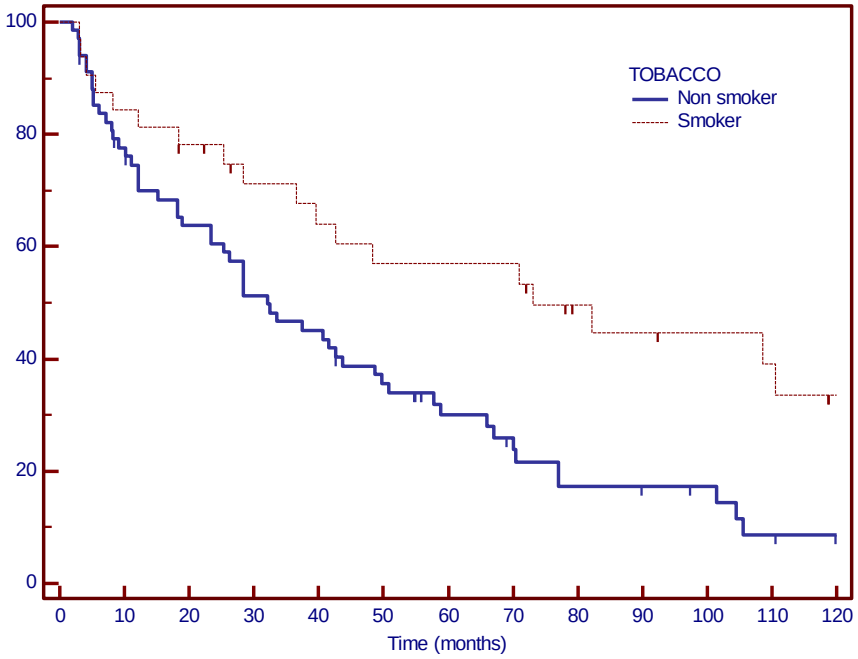

Figure 2. Kaplan Meier Plot of probability of persistently positive serology according to tobacco use.

There was also a non-significant trend for association of previous treatment with hydroxichloroquine (OR 2.55, 95%CI 0.77-8.47; p =0.125). No other factors reached the level of statistical significance.

Patients with persistently positive aPL serology suffer more obstetric complications

No significant differences between the study groups were found regarding the development of thrombotic events **(Table 3)**. However, obstetric complications included in the Sydney criteria (three or more early fetal losses < 10 weeks, late fetal loss >10 weeks or premature birth < 34 weeks due to pre-eclampsia, eclampsia or placental insufficiency) were, overall, more frequent in the group of patients with persistently positive serology, comparing with those with transiently positive or persistently negative serology (37% vs 12.5% or 14.5% respectively, p=0.036). We did

not find any difference in the frequency of obstetric complications not included in Sydney criteria among the serological groups.

	Total number of patients (n = 105)	**Serological evolution**			*p*
		Persistently negative (n = 62)	**Transiently positive (n=16)**	**Persistently positive (n=27)**	
Pregnancies during follow-up (mean ± SD)	1.66±1.01	1.48±0.90	2.06±1.29	1.81±1.00	0.078
Thrombotic events, n (%)	2 (1.9)	1 (1.6)	1 (6.2)	0 (0)	0.338
Obstetric events included in Sidney criteria, n (%)	21 (20)	9 (14.5)	2 (12.5)	10 (37)	**0.036**
Non-criteria obstetric events, n (%)	30 (28.6)	18 (29)	5 (31.2)	7 (25.9)	0.925

Table 3. Thrombotic and obstetric events during follow-up according to the serological evolution**.**

In line with the patients that were diagnosed with APS during follow-up due to obstetric morbidity, two belonged to the persistently negative serology group and the other one to the persistently positive serology.

Higher number of positive antibodies as well as positivity for lupus anticoagulant or anticardiolipin antibodies are associated with the development of further obstetric events

There was a linear association between the number of positive antibodies (aCL,aB2GPI and LA) and the obstetric events suffered during follow-up (p=0.033). **Figure 3.**

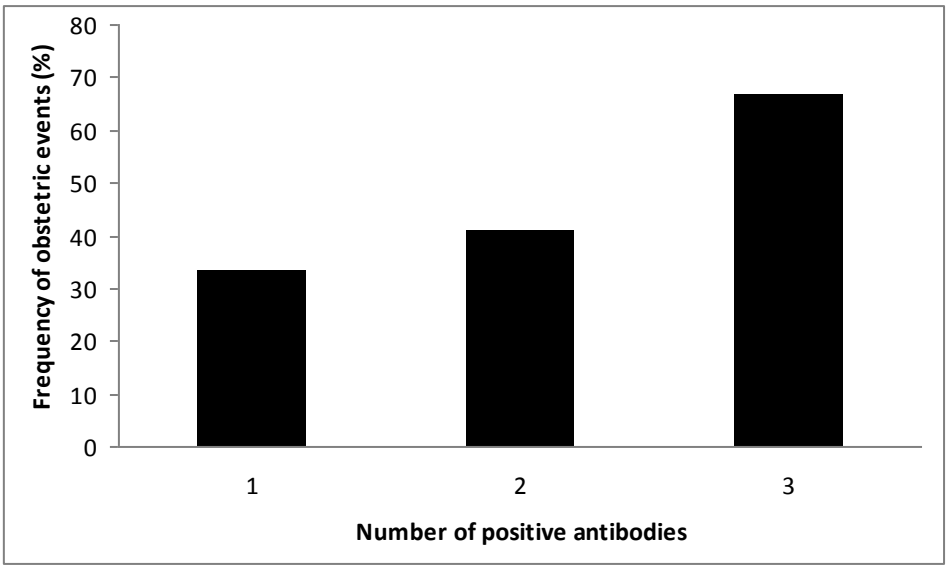

Figure 3. Frequency of obstetric events according to the number of positive antibodies.

Moreover, patients with LA positivity suffered more obstetric events, both criteria and non-criteria, than those without LA (64.9 vs 35.1; p=0.010). Similarly, 48.8% patients with aCL developed obstetric events during follow-up, whereas only 22.2% of those with negative aCL suffered obstetric events (p=0.066). This tendency was confirmed when the analysis was restricted to non-criteria obstetric events where all patients who suffered those events had positive aCL (p=0.001).

Treatment withdrawal might be safe in selected patients

All patients included in the study received antiplatelet or anticoagulant therapy previously and/or during follow-up. Among them, 69 were still on treatment at the end of the study, in 29 patients treatment was discontinued and in 7 patients information was missed. Regarding the 29 patients in whom antiplatelet therapy was discontinued, 13 belonged to the pAPS group, 13 had positive serology and 3 had SLE+aPL. All them fit the required criteria for antiplatelet therapy discontinuation (aPL were persistently negative, patients were asymptomatic for at least 12 months, had no cardiovascular risk factors, and were not willing for further pregnancies). They were all on low-dose aspirin therapy (100 mg daily). Patients who discontinued low-dose aspirin were

followed during a median follow-up of 40.95 months (range 9-135). Three patients were excluded due to less than 6 months of follow-up after treatment discontinuation. None of them experienced any clinical manifestations related to APS.

DISCUSSION

There is a scarcity of information about the evolution of aPL serology and its associated factors as well as its consequences regarding thrombotic and obstetric events. We studied 105 female patients with a positive aPL serology that were attended in our autoimmune obstetric clinic. During almost ten years of follow-up, only 25% remained persistently positive, and more than 50% became persistently negative. When analyzing possible factors implied, we identified tobacco use as an independent risk factor for positivity persistence. Moreover, those women with persistently positive serology were at higher risk for further obstetric events, as well as those with a higher number of positive antibodies. This finding might have a relevant clinical impact not only on the planning of future pregnancies but also to decide the need for long-term antiplatelet therapy.

Martinez-Berriotxoa et al [6] studied a cohort of 237 lupus patients with positive aPL serology (excluding positivity for LA) showing that only 10% of patients remained persistently positive during follow-up. In contrast, a previous report by Erkan et al [11], showed that aPL remained stable in at least three quarters of subsequent tests, during a follow-up period ranging from 1 to 3.5 years depending on the antibody tested. Recently, Yelnik et al [12] reported that after a median follow-up of 13 years, 27% of patients had a complete aPL disappearance, aCL being the most commonly negativized, and suggesting that triple aPL positivity frequency remained stable over time.

It is well known that the levels of aPL fluctuate over time, and many factors have been proposed to play a role in such oscillation. Infections, such as CMV [4;13] are considered inductors of transient aPL positivity. Pregnancy has also been suggested as an inductor of transient aPL by AlBalushi et al [14]. In contrast, Yelnik et al [15] showed in a multicenter prospective study a modest decrease in all aPL during the course of pregnancy that was not associated with pregnancy outcomes. Overall any inflammatory state has been proposed as a promoter of aPL positivity, as suggested in SLE patients [16;17]. Some treatments have been proposed to decrease the levels of aPL, such as hydroxychloroquine and corticosteroids [18] although other studies did not confirm this association [19].

In our study, we evaluated the clinical factors that could influence the serological fluctuations. We found that tobacco use was clearly associated with the persistence of positive serology, that could be partially explained as tobacco is considered to be a second hit in the pathogenesis of APS [20;21] and has been proposed as an aPL inductor in SLE patients [22]. We also described that a higher number of positive antibodies was associated with the persistence of positive serology, as previously suggested by Gianakoppoulos et al and Yelnik et al[23;24]. However, treatment with hydroxychloroquine was not associated with lower probability of persistently positive antibodies as previously reported [25;26]. On the contrary, treatment with antimalarials was an independent risk factor for positive serology persistence. This fact could be explained as treatment with hydroxychloroquine is frequently added in lupus patients and also in those refractory APS patients. The fact that antimalarials are used in more severe patients, usually with persistently positive serologies, could represent a confounding factor that would explain our findings.

During follow-up there were 2 thrombotic events. The rate of obstetric complications included in the classification criteria decreased from 41.9 % to 20 %, thus reflecting the effect of therapy and medical care. These findings are consistent with those reported by Cervera *et al* [27] from the Euro-Phospholipid project that showed an increase in the rate of successful pregnancies from 47.6% to 72.9%. The rate of obstetric complications included in the classification criteria was shown to be more frequent in those women with persistently positive serology than in those with transiently positive or persistently negative serology. To our knowledge, this has not been previously reported in the literature. However, similar findings were reported in lupus patients where the risk of thrombosis was associated with the presence of positive LA or persistently positive aCL [6]. Anyway, patients with persistently negative serology are somehow still at risk for thrombotic events, because traditional cardiovascular risk factors and chronic inflammation also play a crucial role. Moreover, aPL has been, although not consistently, linked to the development of accelerated atherosclerosis.

We also studied the incidence of obstetric complications that, although not included in the classification APS criteria, have been suggested to be associated with the syndrome [28-31] such as HELLP syndrome, pre-eclampsia, and intrauterine growth restriction. We found that 30 % of patients suffered these complications without any significant difference among the clinical or serological groups. This increase in late pregnancy morbidities contrasts with the decline in the number of fetal losses and highlights the need for multidisciplinary units to guarantee a proper management of these patients, especially during the third trimester.

Recommendations for treatment of APS include indefinite anticoagulation for thrombotic APS and low-dose aspirin for obstetric APS. In our study, 29 patients

discontinued antiplatelet treatment. All of them were asymptomatic for at least 12 months, had persistently negative aPL serology, no cardiovascular risk factors and were unwilling to have further pregnancies. They were followed for more than three years, showing no thrombotic events. This supports the idea reported by Criado Garcia et al [32] that described six patients with primary APS who had persistently negative serology and low-risk profile and discontinued anticoagulation showing no recurrence of thrombotic events after 21 months of follow-up. Coloma Bazan *et al* [33] reported similar findings in a series of 11 patients with primary APS and persistently negative serology in which thromboprophylaxis was discontinued.

Our study has some limitations due to the retrospective design of the study. First, some data as concomitant risk factors that could influence the presence of aPL antibodies, such as obesity, infections or the use of contraceptive drugs were not systematically reported in the clinical charts. Also in some patients data regarding the date of antiplatelet therapy discontinuation was missing. Finally, the follow-up was not homogeneous among the group of patients. However, the high number of patients included in the study, the homogeneity of the population studied, as well as the long duration of follow-up support our original results with clear clinical implication.

In summary, our study suggests that among fertile women, aPL remains persistently positive in only one-quarter of patients. In these patients with persistently positive serology, tobacco use was identified as an independent risk factor for this persistence. The persistence of aPL serology is associated with pregnancy morbidity, treatment with antiplatelet therapy being efficient in decreasing early pregnancy complications. However, late pregnancy morbidity is still frequent thus supporting the need for multidisciplinary units for monitoring these patients. In patients with persistently

negative serology and with a low-risk thrombotic profile discontinuation of antiplatelet could be considered a safe choice.

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