Antiprothrombin antibodies: a comparative analysis of homemade and commercial methods. A collaborative study by the Forum Interdisciplinare per la Ricerca nelle Malattie Autoimmuni (FIRMA)

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Abstract

Objective

Prothrombin (PT) is a target for antibodies with lupus anticoagulant (LA) activity, suggesting the possible application of antiprothrombin antibody (aPT) assays in patients with antiphospholipid syndrome (APS). Different methods – both homemade and commercial – for the detection of aPT are available, but they seem to produce conflicting results. The purpose of this study was to compare the performance of different assays on a set of well-characterized serum samples.

Patients and methods

Sera were gathered from 4 FIRMA institutions, and distributed to 15 participating centres. Forty-five samples were from patients positive for LA and/or anticardiolipin antibodies (aCL) with or without APS, and 15 were from rheumatoid arthritis (RA) patients negative for antiphospholipid antibodies. The samples were evaluated for IgG and IgM antibodies using a homemade direct aPT assay (method 1), a homemade phosphatidylserine-dependent aPT assay (aPS/PT, method 2), and two different commercial kits (methods 3 and 4). In addition, a commercial kit for the detection of IgG-A-M aPT (method 5) was used.

Results

Inter-laboratory results for the 5 methods were not always comparable when different methods were used. Good inter-assay concordance was found for IgG antibodies evaluated using methods 1, 3, and 4 (Cohen $k > 0.4$), while the IgM results were discordant between assays. In patients with thrombosis and pregnancy losses, method 5 performed better than the others.

Conclusion

While aPT and aPS/PT assays could be of interest from a clinical perspective, their routine performance cannot yet be recommended because of problems connected with the reproducibility and interpretation of the results.

Key words

Antiphospholipid antibodies, lupus anticoagulant, antiprothrombin antibodies, anticardiolipin antibodies, antiphospholipid syndrome.

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Introduction

Prothrombin was identified as an antigen for autoantibodies with LA activity in the early 1990s (1, 2). This finding led to the development of a variety of solid phase assays designed to detect aPT and thus broaden the battery of diagnostic tools for APS.

Anti-PT antibodies were initially detected by ELISAs that used γ-irradiated plates coated with human PT (3). The presence of relatively high molar concentrations of Ca^{2+} in the coating and washing buffers was suggested to favour a conformational shape naturally displayed by PT (4). Using assays based on this methodology (4), a number of retrospective clinical studies have investigated the clinical significance of aPT but with contradictory results (5, 6). The level of aPT was found to be persistently high in patients with systemic lupus erythematosus (SLE), and some studies found a clinical correlation with thrombosis (5, 7) and pregnancy loss (8, 9). However, other studies reported an association with thrombosis only if LA was also positive (10), or failed to confirm these findings at all (4, 11, 12). Comparable results were found in series of patients with primary APS as well (11-14). An extensive metanalysis on the association between aPT and the risk of thrombosis did not show any statistically significant correlation (15). On the contrary, a study using an *ex vivo* animal model demonstrated that the immunization of mice with thrombin, beta-2 glycoprotein I (β_2GPI) or both resulted in prothrombotic activity (16).

The need to immobilize PT on γ-irradiated plates raised the hypothesis that, like β_2GPI, aPT may be directed against neoepitopes that are exposed when the molecule binds to anionic structures or that a high antigen density may be required because of the low affinity of the autoantibodies (17, 18). Accordingly, a new assay that employed PT coupled to phosphatidylserine (PS)-coated plates was designed (19). This aPS/PT assay was found to detect antibodies directed against the PS/PT complex. Two large studies reported a higher prevalence of aPS/PT in APS and SLE patients and a closer association between thrombotic events and positive LA by the diluted Russel Viper Venom Test (dRVVT) than that detected by the aPT assay (19, 20). However, the possibility that the assay could detect antibodies against phospholipids independently of the reactivity against PT remains an open question.

Interestingly, a recent report underlined that 48% of patients with APS-related clinical features who tested negative using standard antiphospholipid (aPL) assays were found to be positive using aPT and/or aPS/PT (19).

Given the results published thus far in the literature, it would appear important to verify whether aPT and/or anti-PS/PT assays can be transferred from the laboratory to use in the clinical workup of patients with thrombo-embolic disorders. Moreover, we wished to study the reliability and compare the performances of different commercial kits. With these goals in mind, we conducted a multi-centre study to compare the results of different assays in the analysis of a single set of sera, focusing on: a) the inter-laboratory variability of each test, b) inter-method agreement in the classification of samples, and c) the possible association of these antibodies with APS-related clinical features.

Patients and methods

Forty-five serum samples were selected. Thirty were aCL and/or LA positive; among these 12 were from patients suffering from thrombosis, 4 were from patients with pregnancy losses, and 3 were from patients with both thrombosis and pregnancy losses, while the remaining 11 samples were from patients with repeated positive test results, but without the clinical features of APS (21). The other 15 samples were collected from patients with rheumatoid arthritis (RA) who were negative for aCL and/or LA. Three unexplained miscarriages were recorded in the clinical notes of one of these patients.

The samples, coming from four different institutions, were collected by FIRMA's central laboratory in Siena, divided into aliquots and sent to the 15 participating centres. Each centre tested all of the samples for aPT using one commercial preparation and, when

available (in 5 laboratories), with a homemade assay. The homemade aPT ELISA was performed using different procedures.

Method 1 was used as previously described (4), with minor modifications, by four different laboratories. Irradiated microtiter plates (Combiplate, Enhanced Binding, Labsystems, Shrewsbury, MA) were coated with 100 µl/well of 10 µg/ml PT (Diagnostica Stago, Asnieres, France) diluted in TRIS 50 mM, NaCl 150 mM, buffer pH 7.4, with $CaCl_2$ 5 mM (Ca-TBS). After overnight incubation at 4°C, the plates were washed 3 times and blocked with 0.1% Tween 20, 1% BSA (Sigma, St. Louis, MO) Ca-TBS. After one hour and 3 subsequent washes, the serum samples diluted 1:50 in Ca-TBS were distributed (100 µl/well). The samples were incubated for 1 hour at 37°C and, after 3 additional washes, alkaline phosphatase-conjugated goat anti-human IgG and IgM (Sigma) were added. After one hour of incubation and 3 final washes, the P-nitrophenyl-phosphate (Sigma) 1 mg/ml in diethanolamine (Farmitalia Carlo Erba, Milan, Italy) pH 9.8 was added. The reaction was read at 405 nm when the positive reference sample reached the expected optical density (OD) (IgG: 1.5 OD; IgM: 2.1 OD).

Method 2 was used in two different laboratories in accordance with Matsuda *et al*. (22) with minor modifications. Microtiter plates (Greiner 655160, Frickenhausen, Germany) were coated with 30 µl of 50 µg/ml phosphatidylserine (PS) and dried overnight at 4 °C. Wells were blocked with 150 µl Tris buffered saline (TBS) containing 1% fatty acid-free bovine serum albumine (BSA) (A-6003; Sigma) and 5 mM $CaCl_2$ (TBS/BSA/Ca) for one hour, to avoid non-specific binding of proteins. After 3 washes with TBS Tween 20 0.05% 5 mM $CaCl_2$, half of each plate received 50 µl of 10 µg/ml human PT (Diagnostica Stago) in TBS/BSA/Ca, and the other half the same volume of TBS/BSA/Ca. Plates were incubated for 1.5 hours at 37°C. After 1 wash, 50 µl of patient serum diluted 1:100 in TBS/BSA/Ca was added in duplicate and the plates were incubated for 1 hour at room temperature. After 3 washes, 50 µl of alkaline phosphatase-conjugated goat anti-human IgG or IgM were added and the plates were incubated for 1 hour at room temperature. After 3 additional washes, enzymatic activity was measured using p-nitrophenyl-phosphate (Sigma) in carbonate buffer. The OD of the wells containing PS alone was subtracted from that of wells containing the PS/PT complex, and results were expressed as the percentage of a positive control. One of the laboratories using this method measured both isotypes while the other evaluated IgG alone.

Three commercial companies voluntarily participated in this study, and kindly provided kits for use by the centres. Orgentec Diagnostic (Mainz, Germany) (method 3) provided an IgG and IgM anti-PT kit using 2 different plates. Seven laboratories, including that of the company, employed this kit. Corgenix (Cambridge, UK) provided a kit (method 4) consisting of an IgG and IgM anti-PT assay with 2 different plates that was used by five laboratories. Both the Orgentec and Corgenix kits were furnished with plates directly coated with PT.

The Imtec aPS/PT abs Screening test (Imtec Immunodiagnostika, Berlin, Germany) (method 5) was used in five laboratories. This test is based on the immobilization of PT to a solid phase

Table I. Performance of the assays for IgG, IgM and IgG-M-A aPT in different laboratories whose results were considered concordant by the Cochrane test.

Method	Partecipating centres	No. of centres with comparable results	Cochrane test Q	Cochrane test P
		IgG		
1	4	3	7.6	0.01 < P < 0.025
2	2	2	1	0.25 < P < 0.50
3	7	6	12.97	0.01 < P < 0.025
4	5	5	12.73	0.01 < P < 0.025
		IgM		
1	4	3	7.6	0.01 < P < 0.025
3	7	6	5	0.2 < P < 0.5
4	5	3	6.2	0.025 < P < 0.05
		IgG-A-M		
5	5	3	7.8	0.01 < P < 0.025

*Method 2 was performed in only one laboratory for the IgM isotype and their results could not be used in this analysis.

Table II. Comparison of the qualitative (negative/positive) results obtained by different tests on the serum samples examined. When the assay was performed in more than one laboratory, the serum samples yielding different results in different laboratories are indicated as discordant samples and the percentages of discordance are shown.

IgG	Laboratories (no.)	Concordant positive samples	Concordant negative samples	Discordant samples
Method 1	3	6/45 (13.3%)	34/45 (75.6%)	5/45 (11.1%)
Method 2	2	11/40 (27.5%)	28/40 (70.0%)	1/40 (2.5%)
Method 3	6	5/45 (11.1%)	29/45 (64.4%)	11/45 (24.5%)
Method 4	5	8/45 (17.8%)	32/45 (71.1%)	5/45 (11.1%)
IgM	**Laboratories (no.)**	**Concordant positive samples**	**Concordant negative samples**	**Discordant samples**
Method 1	3	3/45 (6.7%)	37/45 (82.2%)	5/45 (11.1%)
Method 3	6	0/45 (0%)	44/45 (97.38%)	1/45 (2.2%)
Method 4	3	12/45 (26.7%)	23/45 (51.1%)	10/45 (22.2%)
IgG, IgA, IgM	**Laboratories (no.)**	**Concordant positive samples**	**Concordant negative samples**	**Discordant samples**
Method 5	3	16/45 (35.6%)	19/45 (42.2%)	10/45 (22.2%)

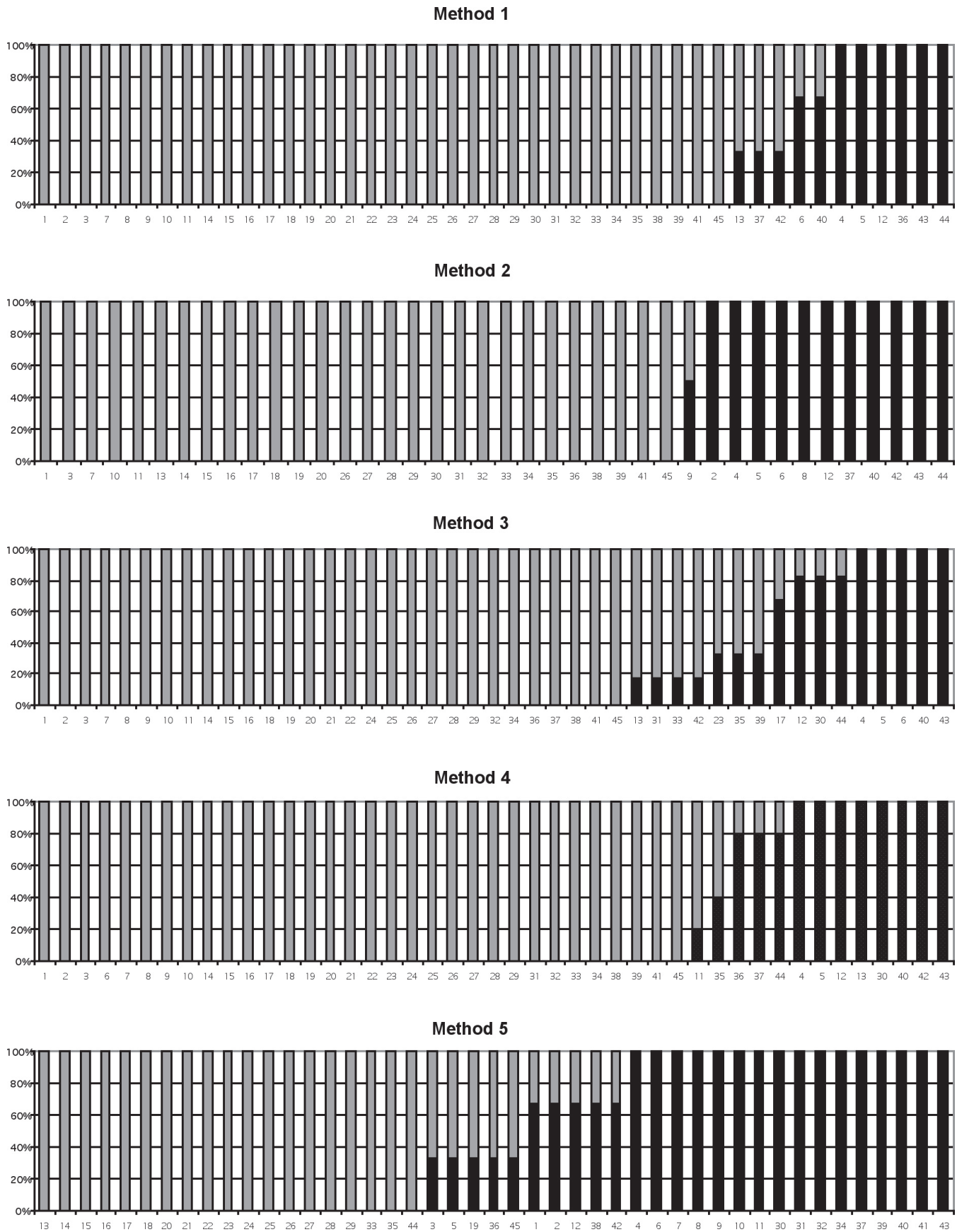

Fig. 1. Inter-laboratory results for IgG (for methods 1, 2, 3, 4) and IgG-A-M (method 5). The bars along the x-axis represent the single samples evaluated using the different methods. The y-axis shows the percentage of concordance for the negative (grey) and positive sera (black).

previously coated with PS. The bound antibodies were detected by a peroxidase-labelled secondary antibody to human IgG-A-M.

Statistical analysis

Cochrane's Q test was used to detect whether there was a significant difference in the results obtained by different centres using the same method. Agreement between methods was evaluated by means of the Cohen's κ statistical test. If κ was > 0.4, the concordance was considered acceptable.

Results

Inter-laboratory concordance for each method

The results for the 45 serum samples obtained using a given method were compared between laboratories to verify the overall performance of the test. If the results of one centre were significantly different from the others by the Cochrane test, this was excluded from the subsequent evaluations. Statistical analysis was conducted only when a method was used by two or more laboratories.

Based on the Cochrane test, the IgG isotype results of one laboratory for methods 1 and 3 were excluded because they were discordant. In addition, the IgM isotype results of one laboratory for methods 1 and 3, and of two laboratories for method 4, were excluded. Two laboratories out of five using method 5 produced discordant results for IgG-A-M and had to be eliminated from the evaluation (Table I).

The results considered to be consistent overall were further analysed, focusing on positive or negative values according to the different protocol recommendations. As shown in Table II, there was still not complete agreement in the sera classification, because varying numbers of samples (from 1 to 11) produced contrasting results in different laboratories. The percentage of discordant results for each assay is presented in Table II.

Inter-method agreement

Cohen's κ test was applied to compare the results obtained using different methods on the same sample. The discordant samples were classified as positive if more than 50% of the laboratories using the test reported a positive result, and negative if fewer than 50% of the laboratories obtained positive results (Fig. 1).

Table III shows a comparison of the IgG results (aPT, methods 1, 3 and 4 and aPS/PT, method 2), in which statistically significant correlations were found (κ > 0.4). In contrast (but as was expected), no correlation was found between the methods searching for the IgG isotype and method 5, which screens for the IgG-A-M isotype.

There was no significant agreement in results between the various assays based on the IgM aPT and aPS/PT methods (Table IV) except that, surprisingly, IgM aPT in method 4 seemed to have a significant concordance with method 5, which investigates IgG-A-M aPS/PT.

Clinical significance of the methods tested

The serum samples used came from a well-defined patient population (Table V) and therefore allowed us to study the relationship between aPT detected by different methods and the principal clinical manifestations of APS (thrombosis and pregnancy losses). However, due to the low number of samples examined and the possibility of selection bias, we cannot draw any definitive conclusions with regard to the specificity and sensitivity of the tests under study. Despite these limitations, based on our data method 5 seems to be the most effective assay for the detection of patients with APS-related clinical features, although it performed negatively in RA patients. One patient with RA and 3 recurrent miscarriages who tested negative using the classical aPL assays was found to be positive for IgM aPT by methods 1 and 4.

Table III. Evaluation of the concordance of IgG aPT (methods 1, 3, 4), IgG aPS/PT (method 2) and IgG-A-M aPT (method 5). Discordant results (κ < 0.4) are indicated in boldface.

Comparison between methods		Cohen's κ
3	4	0.62
3	1	0.78
3	2	0.55
3	5	0.17
4	1	0.67
4	2	0.57
4	5	0.17
1	2	0.61
1	5	0.12
2	5	0.36

Table IV. Evaluation of the concordance of IgM aPT (methods 1, 3, 4), IgM aPS/PT (method 2) and IgG-A-M aPT (method 5). Discordant results (κ < 0.4) are indicated in boldface.

Comparison between methods		Cohen's κ
3	4	0
3	1	0
3	2	0
3	5	0
4	1	0.32
4	2	0.19
4	5	0.41
1	5	0.16
2	5	0.13

Discussion

In the 1990s it became clear that the antibodies responsible for the LA reaction were not directed toward cardiolipin or other phospholipids, but rather to phospholipid binding proteins (1, 23). Beta-2 glycoprotein I and PT were identified as the major targets of the reaction. This observation led to the understanding that some of the coagulation assays used to study LA target anti-β_2GPI LA while others target aPT LA (24). The logical consequence of this was to attempt to translate the different reactions into ELISA methods that directly targeted antibodies to β_2GPI and PT used as antigens in solid phase assays.

The anti-β_2GPI antibody assay was first described in 1992 (25) and, despite several problems with standardization (26), is considered to be a useful tool for the study of patients with thromboembolism or recurrent pregnancy loss. It is now included in the laboratory classification criteria for APS (21).

The situation of the aPT assay is more controversial. The clinical applicability of aPT is still under debate, probably because of the existence of two different methodologies based on two different principles: the binding of PT to empty or to PS-coated microtitre

Table V. Relationship between the positive results obtained using different methods and the clinical features of the patients.

Methods	Pregnancy loss (8 patients)		Thromboses (15 patients)		aPL positive without APS (11 patients)		RA (14 patients)	
	No.	Pos. (%)	No.	Pos. (%)	No.	Pos. (%)	No.	Pos. (%)
3	0	(0%)	4	(26%)	4	(36%)	1	(7%)
4	3	(37%)	8	(53%)	8	(73%)	4	(28%)
1	3	(37%)	4	(26%)	6	(54%)	0	(0%)
2	4	(50%)	5	(33%)	6	(54%)	1	(11%)*
5	6	(75%)	9	(60%)	8	(73%)	0	(0%)

*Method 2 was performed on 9 only RA samples.

plates. Moreover, aPT could represent a heterogeneous group of antibodies directed towards different epitopes and the need to distinguish functional antibodies with LA activity from antibodies without LA activity might be clinically important. Nevertheless, several commercial preparations are now available and the present study was undertaken to investigate whether they produce comparable results. In fact, the discordant results from past studies of aPT or aPS/PT can be at least in part attributed to the absence of standardized methods, as was recently observed (15).

The first aspect that we examined was the concordance of the results obtained by different laboratories using the same assay. As far as the IgG isotype was concerned, one of the four laboratories using method 1 and one of the seven laboratories using method 3 produced discordant results. For the IgM isotype, aberrant results were produced by one laboratory using methods 1 and 3, and two laboratories using method 4. Finally, five laboratories used method 5 (aPS/PT, IgG-A-M), but only three produced comparable results. These findings reflect the generally better agreement of ELISAs for IgG antibodies, but also underline the problems associated with both the homemade and commercial aPT assays kits in terms of inter-laboratory agreement. Interestingly, method 2 (an aPS/PT home made assay) showed good overall concordance, but this finding is of limited significance because the method was used only by two laboratories for the IgG isotype.

Statistical analysis allowed us to discard significantly aberrant results and to select those centres that produced comparable results, which could then be used to evaluate the intra-assay concordance of the positive and negative values for every serum sample.

The percentage of discordant results from the IgG aPT assays (Table II) was related to the number of laboratories performing the assays. In fact, the highest proportion (24% discordant samples) was found for method 3, which was used by six laboratories. This pattern was not observed for the IgM isotype results, where the highest number of discordant samples was found for method 4, which was performed only in three laboratories. It is worthwhile to mention that method 3, with the lowest proportion of discordant results among laboratories (1 sample, 2.2%), was negative for IgM in 44 out of the 45 examined samples. Moreover, method 5, despite the previous exclusion of two of the five laboratories using this kit, produced an uncertain classification for 22% of the samples, thus demonstrating the presence of some unresolved methodological problems. Finally, the highest proportion of discordant samples was observed for method 3 (IgG), method 4 (IgM) and method 5 (IgG-A-M), which were all commercial kits. This could reflect the settings of the assays, which are still under debate by scientists and difficult to sort out for companies, or the lack of experience of the users, who may have been employing these assays for the first time, despite our effort to select those centres that provided the most homogeneous results.

The evaluation of the inter-assay agreement, which was limited to the attribution of a positive or negative value to each sample, was satisfactory for the IgG isotype. The results showed a good overall concordance for methods 1, 2, 3 and 4 and a significant but expected difference in results for method 5 (IgG-A-M). Interestingly, method 2 (aPS/PT) was not found to be significantly different from methods 1, 3 or 4 (aPT). However, as expected, the Cohen's test comparing method 2 (aPS/PT) with methods 1, 3 and 4 (aPT) showed lower (although still significant) values than those obtained by comparing methods 1, 3 and 4 (all aPT) among themselves. Remarkably, no inter-assay agreement was found for the IgM isotype using both aPT and aPS/PT. Even the borderline concordance of IgM aPT (method 4) with IgG-A-M aPS/PT is rather difficult to interpret. We are forced to conclude that, at least for the IgM isotype, these assays cannot be considered reliable as yet.

Although our objective was to perform a methodological evaluation of the homemade and commercial assays available, we were also able to compare the performance of the different tests in serum samples from: (i) patients with APS-related clinical features, (ii) patients with an unrelated disease, and (iii) patients without APS who tested positive using classical aPL assays. Method 5 showed the closest correlation with thromboses and pregnancy losses and was not positive in patients with RA. Interestingly, we observed that one RA patient with 3 previous miscarriages and no successful pregnancies, who was negative for LA, aCL and anti-β_2GPI, was found to be IgM aPT positive by methods 1 and 4. This finding suggests that the aPT ELISA might be used in patients with the clinical features of APS whom standard assays classify as "seronegative", as already suggested by a recent paper (9).

In conclusion, despite evidence showing that many APS patients are positive for aPT and aPS/PT antibodies, the diagnostic value of the assays for these antibodies is still debated. In this study, none of the methods was entirely reliable and all of them still need to be validated and standardized. There-

fore, for now these tests must be applied with caution, and their use should probably be limited to clinical studies. Progress in the standardization of these tests will hopefully be made through a close collaboration between research institutions and companies involved in the field.

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