Laboratory Criteria of Antiphospholipid Syndrome Need to be Updated or Strictly Followed?

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Abstract: The antiphospholipid syndrome is a very relevant disease that implicates different clinical features and laboratory criteria. Regarding laboratory diagnosis, it has been established in the last consensus that tests included are lupus anticoagulant (LA), anti cardiolipin (aCL) and anti β2 glycoprotein I antibodies (anti β2GPI) of IgG or IgM isotype at medium to high titres. There is not one unique or a gold standard test for APS, so laboratory diagnosis is one of the more important problems. Considering LA testing, new guidelines have been recently published. They include not only the selection of coagulation tests, but also important recommendations about preanalytical variables, mixing and confirmatory studies interpretation, and cut off calculations. Taking into account solid phase assays, the major problem is the low reproducibility and the high inter assays variation. This problem has been recognized early with aCL. Despite many efforts such as the introduction of polyclonal and monoclonal standards, laboratory workshops, etc, the situation is still complicated. This problem was supposed to be solved when anti β2-GPI were introduced, but many collaborative studies and quality control surveys assessment have also demonstrated a lot of problems in standardization. The need of reference materials for LA and solid phase assays has been recognized many years ago but there is no clear advance in this line. Another important aspect is the fact that none of these tests are specific for APS, so they can be present in many clinical settings. Recent advances have been done by developing assays to detect anti domain I β2-GPI antibodies that could be more specific. It is hoped that in the near future, by worldwide collaborative actions, problems on laboratory diagnosis of APS will be overcome.

Keywords: Antiphospholipid antibodies, lupus anticoagulant, anticardiolipin antibodies, antiphospholipid syndrome.

INTRODUCTION

The Antiphospholipid Syndrome (APS) is an autoimmune disease that clearly compromise clinical manifestations and laboratory test for diagnosis. In the last APS consensus clinical and laboratory criteria have been revised and established. Among clinical manifestations venous (VTE) or arterial thrombosis (AT), as well as clear criteria for pregnancy morbidity were introduced. Regarding laboratory criteria lupus anticoagulant (LA), anti cardiolipin (aCL) and anti β2 glycoprotein I antibodies (anti β2GPI) of IgG or IgM isotypes at medium or high titers for both solid phase assays have been included. For APS diagnosis one recent clinical feature (no more than 5 years) and one laboratory criteria repeated at least twice twelve weeks apart are required [1]. Since the recognition of this syndrome a great piece of work have been done for 25 years, however there is still a lot of debate particularly in the laboratory aspects of APS diagnosis.

Initially, these antibodies were thought to be directed against anionic phospholipids (PL), but, since 1990, it is recognized that they are directed against proteins with high affinity for PL. Three different groups described the β2GPI as the main real antigen for anti PL antibodies (aPL) [2-4]. The other protein that has been recognized as a protein antigen involved in APS is human prothrombin (PT) [5]. Many other proteins with high affinity for PL have been found to be recognized by serum of many patients with APS: Annexin A5, Protein S, TFPI, etc, but these assays have not become clinical useful [6].

There are many not solved problems in APS diagnosis [7], many of them because of the clinical criteria that are quite frequent in the population and in most cases not associated with aPL. Additionally aPL can be detected in many clinical settings as epiphenomenon with no clinical relevance. However, the most important problems seem to be dependent on laboratory tests:

1. High variability in the tests performance, mainly inter but also intra laboratory
2. No clear and strict guidelines for performing the assays
3. The absence of reference material for each class of tests to standardize methodologies
4. The low concordance on the calculations of cut off points for each tests
5. The need to demonstrate the persistence of the antibodies detected during time
6. The need of more specific tests for clinical features of APS, clinically and prospectively validated, to identify a pathogenic antibody marker

In the following review we will discuss current knowledge, problems, consensus and future directions of laboratory diagnosis of APS.
LUPUS ANTICOAGULANT TESTING

LA activity is characterized by the presence of an antibody or a mixture of antibodies directed against anionic PL-binding proteins, mainly β2GPI and PT. They interfere in PL-dependent coagulation tests in vitro due to its capacity to increase the already high affinity of the protein to PL, limiting the availability of PL in clotting assays [8, 9]. En 1995 the Criteria for the diagnosis of LA were published on behalf of the Subcommittee on Lupus Anticoagulant / Antiphospholipid Antibodies of the SSC of the ISTH [10] and the results of the Second International Workshop for Identification of Lupus anticoagulants too [11]. These guidelines include 4 important steps:

1- Prolongation of a PL-dependent coagulation test
2- Evidence of an inhibitor demonstrated by mixing studies
3- Confirmation of the PL-dependent nature of the inhibitor
4- To rule out the presence of a specific factor inhibitor

This criteria remained unchanged for more than 10 years but a lot of problem of standardization coexisted with this guidelines due to different tests, different reagents, calculations of cut off, criteria of positivity of different tests, the number of screening test used, the heterogeneity of the antibodies, etc. [12-18].

Very recently, during the last SCC Subcommittee meeting of ISTH in Boston this year, the draft of the new guidelines for LA diagnosis was presented by Dr. Tripodi (Table 1). These guidelines have been proposed and carefully prepared by a group of experts in the area in the frame Scientific and Standardization Committee on Lupus Anticoagulant / Antiphospholipid Antibodies. They were recently published [19].

Table 1. Topics Included in the New Guidelines for LA Diagnosis

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Patients’ Selection

The experts conclude that LA testing should be performed when patients have a significant probability to have an APS or when an unexplained prolonged APTT is observed. They propose to grade the appropriateness of LA searching as:

a) low: elderly patients with arterial or venous thrombembolism
b) moderate: accidentally found prolonged APTT in asymptomatic patients, recurrent early pregnancy losses or patients with a provoked episode of VTE
c) high: unprovoked VTE and unexplained AT in a young patient (<50 years old), thrombosis at unusual sites, late pregnancy loss, any pregnancy morbidity or thrombosis in patients with autoimmune diseases (Systemic lupus erythematosus, rheumatoid arthritis, immune thrombocytopenia, autoimmune haemolytic anaemia)

They do not recommend to study or search for LA in general population or in other categories of patients because of the high probability to find a false positive result without clinical signification, due to the poor specificity of the tests, based on the Italian study results [18]. Ideally, blood should be taken before starting with an anticoagulant therapy or after a long enough period post discontinuation.

Blood Collection and Pre-Analytical Variables

Nothing is included in the guidelines regarding the blood drawing, vacuum systems of collection tubes. It is assumed that current guidelines should be followed [20]. They recommend that blood must be collected in plastic tubes and double centrifugation must be performed in order to obtain platelet poor plasma. After the first centrifugation for 15 min at 2000g, plasma must be transferred, by using plastic pipettes and taking care to leave most of the platelets in the primary tube, to a second plastic tube and centrifuged at 2500g for 10 min. Plasma must be transferred to a definite aliquot to be studied. Filters to eliminate platelets are not recommended because of the variability between filters, the volume which is possible to filter, the loss of factor VIII with von Willebrand factor retained by the filter, etc. [21]. If the sample has to be frozen, it should be done as quickly as possible to avoid labile factors loss, and maintained at -70°C. When frozen plasma has to be assayed it must be thawed totally immersed in a water bath at 37°C for 5 minutes and gently mixed before testing.

Screening Tests

Selection of tests: Due to the increased in false positive results when more than 2 tests are included in the screening [18], the guidelines establish to use only 2 tests based in different principles, dilute Russell viper venom time (dRVVT) and activated partial thromboplastin time (APTT). The first choice should be dRVVT because it has been demonstrated that it was more related to anti β2GPI and thrombotic complications [22] although this observation was not further confirmed [23]. However it is recognized as the most robust test for APS diagnosis [13, 24]. The second test should be APTT by using silica as activator and low PL concentration. The Kaolin is not considered because problems in many coagulometers and because reagents commercially available with kaolin are not very sensitive, and elagic acid reagents are in general insensitive or less sensitive than silica’s ones.

Even when it has been shown that prolonged kaolin clotting time (KCT) could be associated to anti-PT [18, 22], the guidelines do not recommend the use of KCT because of
lack of reproducibility [25]. In the same way, it is discouraged to include dilute prothrombin time (dPT) in the evaluation of LA because there is a great variability in the reagents so it is difficult to standardized. Tests based on other snake venoms, like Textarin/Ecarin time and Taipan clotting time, are not included because they are not widely used, standardized commercial reagents are not available and more clinical studies are needed.

The presence of LA is considered when one of the screening tests is prolonged above the local cut off, which should be calculated by processing plasmas from 40 healthy donors and calculating the 99th percentile.

Mixing Studies

Preparation of Pooled normal plasma (NP): For the mixing tests NP should be prepared with platelet poor plasma from healthy donors after double centrifugation to obtain a platelet count < 1x 10^7/l and close to 100% of clotting factor activity. NP should be stored frozen at -70°C until use. Commercially available NP could be used if they fulfil these characteristics or has been validated for LA testing.

Mixtures 1:1 of patient plasma in NP are recommended and they have to be processed without preincubation, within 30 minutes. For interpretation of mixing studies it is suggested to use the normalized ratio mixture/NP or the Index of circulating anticoagulant (ICA) (Fig. 1) like that reported by Rosner for KCT [26]. In any case the cut off should be determined in each laboratory by performing mixing studies of plasmas from 40 healthy donors with NP by calculating the 99th percentile.

Thrombin time must always be included in the basic coagulation screening of the patient plasma before the LA detection. Mixing studies should not be performed if the thrombin time is significantly prolonged, because the presence of unfractioned heparin does not correct in mixing tests, particularly when the heparin concentration exceeds the capacity of the heparin neutralizer (polybrene or heparinase) present in commercial reagents. Regarding low molecular weight heparin (LMWH) in the plasma, the guidelines conclude that it could be possible to detect a LA and perform mixing studies when they are present, however it would depend on the anti Xa/Ila activity and heparin concentration. The presence of new anticoagulants, direct Xa and thrombin inhibitors were not evaluated but it is reasonable to think that they could interfere with LA tests.

Confirmatory Tests

The PL dependence of the prolongation should be demonstrated by increasing the concentration of PL in the tests. Guidelines recommend using bilayer or hexagonal phase II PL to increase lipid concentration [27]. It is indicated that freeze/thawed platelets (platelet neutralization procedure) should not be used because the high batch to batch variability.

To interpret the confirmatory tests, the LA ratio (Screen/confirm) or the % of Correction described as (Screen/confirm)/screen x 100 are the most robust criteria [28-30]. These criteria are also recommended for integrated systems at low and high PL concentration. These systems in theory do not need mixing studies, but it is important to do them and even, most of them are described to be performed on patient + NP mixtures [27].

Again, the cut off value should be calculated locally by each laboratory. To do so, at least 40 plasmas from healthy donors should be processed at low (screen test) and at high PL concentration and the % of Correction should be calculated. In a subsequent step, the 99th percentile has to be set as the cut off value. Each result above this cut off must be considered as a positive confirmatory test.

Transmission of Results

LA should be reported with quantitative results of different tests and indexes calculated for mixing and confirmatory studies. In addition, an interpretative conclusion indicating if the results are compatible or not with the presence of LA is highly recommended. It is important because many clinicians do not clearly understand the complexity of procedures, interpretation and limitations of the laboratory tests for LA diagnosis. It is discouraged to inform borderline or dubious LA results, instead of that a recommendation to repeat the test in one week is recommended by the experts. Of course, when LA is diagnosed it must be recommended to repeat testing at least 12 weeks apart as it is established in the APS consensus [1].

Comments on Different Aspects of the Guidelines

It has been recognized for many years that not only the reagent but also the instrument of clot detection plays a role in the sensitivity and specificity of assays for LA [31, 32]. It is clear recommended in the new guidelines the need to calculate locally the cut off points for screening, mixing and confirmatory tests, and not to use cut off values established elsewhere even when they are described for the same reagent and coagulometer.

For the screening of LA only 2 tests are recommended, and dPT has been eliminated from this list. It has been demonstrated by us and other groups that when a particular recombinant thromboplastin (Dade Innovin®, Dabe Behring, Marburg, Germany) it was used, the test became really sensitive and specific for LA, even for the APS [33-35]. However, recently a commercial integrated system with a screen and confirm reagents for dPT has been tested, and the sensi-

Fig. (1). Index of circulating anticoagulant (ICA).
tivity was lower compared with that of home-made assay and classical tests like dRVVT and APTT [35]. Some reports showed that extrinsic pathway based assays, (dPT) and activated seven lupus anticoagulant (ASLA) test, could increase the sensitivity of detection of lupus anticoagulants [29, 36, 37]. In our hands ASLA test did not add sensitivity to dPT at least in the obstetric patient setting [36]. In the future, it could be interesting to reconsider the use of dPT performed with a particular recombinant thromboplastin as an additional test, for weak LAs in which the first line assays give not definite results.

On the other hand, tests based in viper venoms like Textarin, Ecarin and Taipan has been also excluded. However Textarin/Ecarin test would be an interesting tool when a patient on oral anticoagulants is evaluated, and Taipan viper venom time when a low levels of factor V is present, due to deficiency or inhibitor against this factor, because this venom is not dependent on factor V to activate prothrombin [38-40]. On the other hand Textarin needs PL and factor V to act.

When considering mixing studies, the guidelines recommend the ICA index for the interpretation of mixing studies. However the percentage of correction calculated as (patient plasma test–1:1 mixture test)/(Patient plasma test-NP test) x 100 has been suggested as one useful tool, particularly in patients with weak LA [41, 42]. Moreover, they can be complementary [42] and cut off of both index corrected by results of ROC curve analysis on a large number of samples evaluated, became more specific for LA not only for screening test [42] but also for mixing criteria of correction as we presented as a poster presentation during the XXII Congress of the ISTH. Another important point is the immediate inhibitory effect of the LAs, compared to the time and temperature dependence of the anti VIII inhibitors.

It is recognized that patients should not be studied during an acute thrombotic episode because they received full anticoagulation with heparin resulting in possible false positive results, and levels of acute phase reactants like fibrinogen and factor VIII are high provoking possible false negative results. The test results interpretation is difficult when patients are receiving vitamin K antagonists and has an INR > 1.5. If INR is > 1.5 and < 3.0 it is important to work with 1:1 mixture with NP. Switching anticoagulation to LMWH is recommended and patients have to receive last LMWH dose more than 12 hours before blood is drawn for LA testing.

Regarding confirmatory tests, commercially available hexagonal (II) phase PL test has high specificity [27], but it is not suitable for non developed countries because the cost is very high. The variability of platelet neutralization procedure among different lots is a problem difficult to solve, but calculating the % correction and performing the normalization of patient coagulation times with NP, this test could play a role in laboratories that cannot access to more expensive reagents.

Coagulation factors should be measured when a specific inhibitor against one factor is suspected. It depends on potency of the inhibitor, but when a LA is present but not when a specific inhibitor is present, the activity of the coagulation factor apparently increases while increasing sample dilution Factor VIII measurement by a chromogenic assay was suggested to be very useful to make distinction between inhibitors against factor VIII and LA, to diagnose LAs in haemophiliac patients or when the presence of both inhibitors is suspected [11, 43].

**The Need for More Specific LA Assays**

LA has been recognized by several authors as the strongest risk factor for thrombotic episodes [44-46]. However, LA can be identified in patients without clinical complications of the APS, for example, in elderly patients [18] or transiently associated to infections or other clinical settings [47, 48]. Therefore, it is of particular importance to find specific tests that recognize LA associated with clinical manifestations of APS for a good clinical management and treatment of those patients.

In the last few years many tests have been developed to distinguish between β2GPI-dependent and not β2GPI-dependent LA, because anti β2GPI are considered more pathogenic (see below). One simple method proposed was based on the shortening of a sensitive APTT in the presence of cardiolipin vesicles but not in the presence of phosphatidylserine/choline vesicles, when the LA was dependent on β2GPI [49]. The presence of this type of LAs highly correlated with thrombosis in APS patients [9], but several methodological problems did not allowed its widely clinical use.

Another test developed was based in the prolongation of screening coagulation tests for LA dRVVT or dPT [50] in plasmas from patients with anti β2GPI when a low calcium concentration was used. The authors reported these observations in a selected group of patients. Other group (by using APTT) and us (by using dPT) found that the prolongations of clotting times at low calcium concentration is associated to the presence of anti β2GPI measured by ELISA, but not in patients with anti-PT associated LA. Despite these promising results there is a considerable overlapping between groups of patients, particularly when a mixture of antibodies is present [51, 52].

In the last ISTH Congress van Os and coworkers presented as an oral communication a very interesting test based on the shortening of clotting times in APTT, dRVVT and dPT by the addition of recombinant domain I (D1) of β2GPI in the presence of anti β2GPI dependent, but not of anti-PT dependent LAs. This was demonstrated by spiking NP with different monoclonal antibodies, but also in a group of patients with LA and anti D1 antibodies detected by ELISA.

Recently it has been demonstrated in a cohort of more than 200 patients with LA [24] that only the presence of a dRVVT ratio P/NP > 1.60 and the presence of anti β2GPI IgG were associated with clinical criteria of APS, in a multivariate binary logistic procedure, with an OR of 2.39 and 3.4, respectively. They showed that the specificity of dRVVT ratio > 1.6 was 78%, but the sensitivity was lower (53%).

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Future Directions of LA Testing

One important point is the evaluation of LA with a quan-
titative approach. Unfortunately, reference material for such a lupus anticoagulant titration is not available, although it was proposed many years ago by using the lupus ratio [53].

Interestingly a NP spiked with anti β2GPI and anti-PT monoclonal antibodies had been used in a External Quality Control Assessment [13], and referential materials by spik-
ing pooled NP with monoclonal antibodies against β2GPI [54, 55] or enriching NP with purified IgG from patients with LA activity [16, 56] were proposed. But, an interna-
tional standard has not been prepared. Moreover, when this material will be prepared, an international and well designed multicenter study should be performed.

In the last few years the use of global tests for overall evaluation of coagulation has been increasing with accept-
able experimental errors, particularly when a fluorogenic thrombin substrate is used [57, 58]. Very recently Devreese et al. [59] have demonstrated that calibrated automated thrombography could be used as an important tool for detection of LA. They proposed a normalized ratio between two parameters of the thrombogram peak height/ lag time as an accuracy and sensitive index for LA. They found that this ratio detect 59/60 plasma LA positive. They demonstrated that plasmas from patients with transient LA and no features of APS presented a reduced index but this was not modified after the addition of a monoclonal antibody against β2GPI with LA activity, as was seen with plasma from persistence of LA and thrombotic complications. They also proposed to perform the thrombography on the mixture 1:1 of plasma from patients on oral anticoagulants with NP, and found that all 12 plasma evaluated in this condition were detected by the index. It is interested to think that this kind of tests would be useful as a single test to identified LA patients with APS. However they concluded that the response was variable between patients and intra patient over time, so they claimed to use this test with the current assays available for LA diagnos-
sis, particularly dRVVT.

Another new coagulation test, not for the detection of LA but to recognize patients with antibodies that reduce the anti-
coagulant activity of the annexin A5, has been developed and correlated with thrombosis and clinical features of APS [60, 61]. More studies are needed to evaluate the clinical usefulness of this test.

ANTIPHOSPHOLIPID ANTIBODIES DETECTED BY SOLID-PHASE ASSAYS

Anticardiolipin Antibodies Testing

The current classification criteria for APS include not only aCL but also anti β2GPI fo IgG or IgM isotype at me-
dium or high titer [1]. It is well established and is a require-
ment for the diagnosis of APS, that aCL have to be detected in an assay dependent on β2GPI. This is in fact, a statement not always true, because all aCL assays utilize β2GPI but almost none include ELISA wells without β2GPI, to detect the non specific ones (true aCL). So, in the current assays, aCL developed in APS but also those in infections or other situations not related with APS are detected. Moreover, a very high interassay and interlaboratory variation exists and is particularly important for low and medium titers, and more evident for the IgM isotype. Considering this isotype it is important to take into account that cryoglobulins and rheuma-
toid factors could produce false positive high titre anti-
odies of IgM isotype. This is particularly important when assays do not include wells without CL to discount the non specific binding, as it was established in the home made refer-
ence technique [62]. Regarding home-made assay, it has been recently critically revised and detailed by Pierangeli et al. [63]. In this publication many source of difficulties, errors and variation has been described, as well as a lot of recommenda-
tions to perform the aCL test. The IgA was not in-
cluded as laboratory criteria and could play a role, if any, in the black Afroamerican population [64] or associated to other clinical manifestations not included in the APS criteria in patients with collagen disease [65].

Problems about the high interlaboratory variability was addressed by the European group [66, 67], the Australian group [68, 69], and also by the Cooperative Group of Hae-
mostasis and Thrombosis in Latin America [70]. The Austra-
lasian Working party [71] has established that an acceptable performance for aCL testing is a coefficient of variation in-
ter-runs < 20%, preferentially when testing samples of low and medium titres, and the use of external non kit controls or in house controls are useful to calculate the CV % run to run, lot to lot variation. In case of small laboratories where the access to such controls is not easy at least kit controls should be performed in duplicates. The aCL are present in patients with infections and other clinical manifestations, so they are sensitive but not specific. ELISAs with a mixture of anionic phospholipids as antigen have been developed and the speci-
ficity for APS increased [72, 73]. On the other hand, quanti-
fication of aCL is a problem because most in house ELISA calculated SD of the OD obtained in the control population. The introduction of standards as the aPL ones (GPL, MPL, and APL units) helped to solved partially the problem. But it was recommended to use calibrators prepared by humanized monoclonal antibodies directed to β2GPI [67, 74]. Regarding the cut off value, it is accepted that a titre higher than 99th percentile of the normal population should be considered for APS diagnosis [1, 63, 67]. Recently Budd et al. [75] have evaluated the IgM aCL in a large group of healthy controls and also a group of elderly people and recalculated the cut off for 2 commercial kits and the home made ELISA considering negative < 95th, indeterminate between 95th-99th, and positive > 99th percentile, and shown that nearly 5% of elderly people (similar to younger ones) have presented inde-
terminate titres of IgM aCL. Interestingly, with both com-
mercial kits evaluated cut off for positive results (> 99th per-
centile) were 27 and 38 MPL Units, very close to the titre mentioned in the revising criteria for APS [1]. It is clear that IgG isotype is the most clinically relevant [23], but IgM has not been yet excluded from the APS criteria. However, as mentioned by Galli et al., some prospective studies showed that aCL are not risk factors for venous thrombosis [23]. There are evidence that only high titres of aCL are associated with increased risk of venous thromboembolism [76-78] and arterial thrombosis [46, 76]. The association of aCL with stroke has been refuted recently by the APASS study [79], but definitive conclusions have not been reached because of methodological problems that have been critically rebuttal [80, 81]. Moreover, the same group showed that the presence of aCL in patients with patent foramen oral or cardiac valve
thickening was not associated with subsequent vascular events [82].

A metanalysis shown that aCL of IgG isotype were risk factors for obstetric morbidity [83], and this topic has been also recently reviewed by Tincani et al. [84]. In this patient setting, like in patients with history of thrombosis, low titres of aCL were not considering predictors for subsequent obstetric complication or thrombotic complication [85].

**Anti β2GPI Testing**

Considering anti β2GPI, it was early described an association between the presence of these antibodies and thrombosis [86] and it is well known that they correlated very well with clinical manifestations of APS [6, 9, 23, 87]. For this reason anti β2GPI were included in the revised criteria for APS [1]. In a prospective study conducted in our laboratory, 194 consecutive patients with AL and/or aCL were followed for a long period, more than 10 years [88]. We demonstrated that the presence of medium or high titre of IgG anti β2GPI was an independent risk factor for the development of a new thrombotic episode in patients with a previous thrombosis and also for the first episode in asymptomatic ones [88]. These antibodies are present most of times associated with LA or aCL, as was seen by the low frequency of them in patients with history of thrombosis presenting LA and aCL negative results [89-91]. Although they are more specific for APS [92, 93], they become positive in some chronic infections like leprosy [94]. On the other hand, despite using a protein antigen, the interassay and interlaboratory variation is high as was investigated by the European forum [95]. Interestingly, it could be due to calibrators and β2GPI preparations used in each assay. Another important point is the antigen density in the plate, and the need of irradiated plates to achieve this density, because these are low affinity antibodies that required bivalent binding [96, 97]. Other characteristic that seems to be important is the avidity of the antibodies because it has been shown that those with high avidity are more specific for APS detection [98].

Some recommendations were published by the European Forum [99, 100] (Table 2).

Another important point to consider is the β2GPI preparation used to coat the plates. An interesting recent work was performed by Cavazzana et al. [101]. They compared 4 different β2GPI preparations obtained by different methods of purification were which were tested in 3 different centres through the home made ELISA. The degree of purification was different and the cut off value calculated by the 99th percentile of 100 sera from healthy volunteers were different. However, the agreement between results obtained with different preparations when positive controls, negative controls and samples of 107 patients fulfilling clinical criteria for APS were tested was very good, indicating the importance to correctly set the cut off value of the test.

**Anti Prothrombin Testing**

Several anti-PT ELISAs were described using both, human PT or phosphatidyil serine/calcium/PT complexes [102-106], with a lot of different methodological characteristics. We found in a retrospective study that anti-PT were associated to venous thrombosis even in patients testing negative for LA, aCL and anti β2GPI [91]. However, not consistent association with thrombosis has been recognized [87]. In contrast, in our prospective study their presence add some thrombotic risk to LA and anti β2GPI [88] and in recent studies IgG anti-PT, has been associated to venous thrombosis [23, 105]. Moreover, it has been shown that its presence became a risk factor for both venous and arterial thrombosis in SLE patients [107, 108].

It is not clear which are the relevant epitopes recognized by anti-PT. Binding to fragment 1, prothrombin 1 and the whole PT molecule, but not thrombin has demonstrated. A good specificity but low sensitive for APS diagnosis have been described and review recently [109]. The role of anti-PT as criteria for APS is still under debate, it is considered as a complementary test, but at the moment the recommendation is not to include this assay in the routine work up for APS. More prospective studies should be conducted in order to clarify its clinical usefulness.

**Look Forward to Finding More Specific Solid Phase Assays: Anti Domain I (anti D I) Antibody Testing**

The localization of epitope that is recognized by anti β2GPI in APS patients was extensively investigated by some groups. There is evidence that anti β2GPI directed against D I are associated with APS [110, 111]. Moreover, the presence of IgG anti D I in patients with β2GPI dependent LA activity strongly correlated with thrombosis, with an OR 18.9 [111], whereas antibodies directed against other domains of the molecule did not. Very recently, a multicenter study conducted by the same group has validated this association [112]. They studied 442 samples from 9 European laboratories which had been tested positive for anti β2GPI. More than 80% of patients fulfilled clinical criteria for APS.

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<td>Reproducibility</td>
<td>to perform duplicates because there is a high coefficient of variation</td>
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<td>Calibration</td>
<td>to use monoclonal antibodies HCAL (IgG) and EY2C9 (IgM) as standards</td>
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<td>Population to set the upper limit of reference range</td>
<td>by checking 50 (preferably 100) healthy volunteers, with a proportion of women more than 50%</td>
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<td>Local calculation of cut off</td>
<td>calculate the 99th percentile</td>
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<td>Manufacturers</td>
<td>cut-off value must be informed by manufacturers at 95th, 97.5th and 99th percentile</td>
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<tr>
<td>External Quality assessment</td>
<td>by Using also monoclonal antibodies HCAL (IgG) and EY2C9 (IgM) or other mono or polyclonal antibodies</td>
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They found that IgG anti D I were present in 55% of patients, and clearly associated with thrombosis, OR 3.3 (2.1-5.2, 95% confidence interval) by evaluating patients positive for IgG anti β2GPI. Additionally, from the whole population 201 women had been pregnant and when the presence of anti D I were evaluated, a significant association were found for late pregnancy loses and premature birth due to preecclampsia or placental insufficiency, with an OR of 2.1 and 2.0 respectively. In this study a selected population was used because participating laboratories were reference centres in their countries, and also because only patients testing positive to anti β2GPI were included. So, it would be interesting to search which is the performance of the tests as a first line study for APS.

**INCREASING SPECIFICITY THROUGH THE APL PROFILES**

It is important to consider LA results as a member of the whole family of aPL, taking into account the concept: triple positive patients (LA + aCL +anti β2GPI, particularly of IgG isotype) are at higher risk for thrombotic events [113]. It is not clear if this concept is true for obstetric morbidity. It is more frequent to find isolated weak LAs in patients without clinical manifestations of APS and in elderly people [18]. In concordance, in our prospective study the incidence of thrombotic events was significantly higher in patients with anti β2GPI and anti-PT in addition to LA [88].

**UNDER DEBATE**

During the last 3 or 4 years the discussion about excluding aCL as a laboratory criterion for APS was present. Recently, Galli et al. [114] published and invitation to discuss serological criteria for APS and suggested the need to revise the LA testing guidelines, to eliminate aCL and the IgM aCL and anti β2GPI. Many opinions have been published regarding this proposal, but one said not to eliminate aCL [115], and the other to keep aCL but only IgG and to eliminate IgM isotype [116]. It has also been demonstrated by Nash et al. [90], who studied a group of patients with persistent LA and aCL, and anti β2GPI. They found that 26% of patients that fulfilled the clinical criteria for APS, had aCL positive alone, although they were mainly of low titre. It is important to point out that most of these opinions are based in studies which evaluated thrombotic manifestations. Interestingly, a recent study on a European cohort of 109 women with obstetric complication 46% presented isolated antibodies, and 31% presented aCL alone [117]. Moreover 3 patients presented only anti β2GPI of IgM isotype. These findings and those from Opatrny [83] suggest that perhaps laboratory criteria for obstetric morbidity are different to that established for thrombotic manifestations of APS and in this setting of patients no changes in laboratory criteria should be done.

**TO FOLLOW THE GUIDELINES, IT IS MANDATORY**

Many evidences have been accumulated about the variability and reliability of APS diagnosis. It was clear that the possibility to have false negative results for LA improves when guidelines are followed. This was early demonstrated by Jennings et al. [15], and also by Moffat et al. [118] by using questionnaires, that many laboratories in North America for clinical practice declared they were following the ISTH 1995 guidelines but they did not followed many aspects of these guidelines. Many laboratories did not perform mixing studies, or factor assays to evaluate factor deficiencies or specific inhibitors. More important was the fact that an important number of laboratories accepted to change their clinical practice after their participation in the survey. On the other hand, many recommendations and consensus have been developed in the last decade [15, 17, 119], and these activities are important tools to change mind of people performing aPL tests.

**CONCLUDING REMARKS**

APS is an autoimmune syndrome characterized by thrombotic and/or obstetric clinical complications. It is rather common and its correct diagnosis is very important because of their clinical and therapeutic consequences. It has been very problematic because of the lack of a gold standard test, the high variability between laboratories not only for solid phase assays to detect aCL and anti β2GPI, but also for coagulation tests for LA. Moreover, these antibodies can be present in other clinical settings, infections, drugs, neoplasm, etc., not related with the APS, so none of these tests is specific. Some important collaborative work and quality control surveys have added recommendations and guidelines about these tests. It is important to strictly follow the guidelines and the recommendations in order to minimize the source of variability and to increase reliability of the tests. Now a day, many efforts are doing to clarify the laboratory aspects of APS: the establishment of clear and detailed guidelines for LA testing, the development of more specific assays which are under investigation, the recommendations regarding technical aspects for aCL and anti β2GPI testing and the suggestion to take the whole laboratory results to diagnose an APS. The future of laboratory diagnosis of APS seems to be promising and further improvement could be achieved through multicenter collaborative prospective studies.

**REFERENCES**


