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 β_2 glycoprotein I antibodies (anti β_2GPI) 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 β_2GPI 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 β_2GPI 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 β_2GPI (anti β_2GPI) antibodies, 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 β_2GPI 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.

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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 β_2GPI 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/Phospholipid- dependent antibodies. They were recently published [19].

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

Recommendations About
Patients´ selection
Blood collection
Pre analytical Variables
Selection of screening tests
Mixing tests
Confirmatory tests
Expression of results
Transmission of results

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 thromboembolism

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 -70ºC. When frozen plasma has to be assayed it must be thawed totally immersed in a water bath at 37ºC 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 β_2GPI 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 -70ºC 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 determine 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/IIa 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.

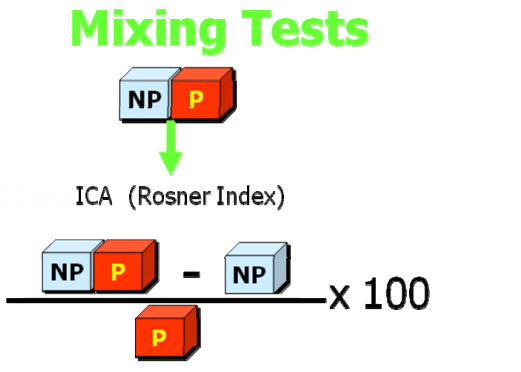

Fig. (1). Index of circulating anticoagulant (ICA).

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 calculate. 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-

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[1]. 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 β_2GPI-dependent and not β_2GPI-dependent LA, because anti β_2GPI 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 phosphatydil serine/choline vesicles, when the LA was dependent on β_2GPI [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 β_2GPI 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 β_2GPI 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[2] 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 β_2GPI in the presence of anti β_2GPI 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 β_2GPI 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%).

[1]Martinuzzo ME, Cerrato G, Iglesias Varela ML, Adamczuk YP, Forastiero RR. The importance of appropriate criteria for Lupus Anticoagulant diagnosis. XXII ISTH Congress, Boston 2009. J Thromb Haemost 2009; 7 (Supplement 2): P-275.

[2]van Os GMA, Meijers JCM, Urbanus RT, DErksen RHWM, de Groot PG. A rapid assay to distinguish between beta2-glycoprotein I depedent and prothrombin dependent lupus anticoagulant. XXII ISTH Congress, Boston 2009. J Thromb Haemost 2009; 7 (Supplement 2): OC-030.

Future Directions of LA Testing

One important point is the evaluation of LA with a quantitative 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 β_2GPI and anti-PT monoclonal antibodies had been used in a External Quality Control Assessment [13], and referential materials by spiking pooled NP with monoclonal antibodies against β_2GPI [54, 55] or enriching NP with purified IgG from patients with LA activity [16, 56] were proposed. But, an international 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 acceptable experimental errors, particularly when a fluorogenic thrombin substrate is used [57, 58]. Very recently Devreese K *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 β_2GPI 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 diagnosis, particularly dRVVT.

Another new coagulation test, not for the detection of LA but to recognize patients with antibodies that reduce the anticoagulant 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 β_2GPI fo IgG or IgM isotype at medium or high titer [1]. It is well established and is a requirement for the diagnosis of APS, that aCL have to be detected in an assay dependent on β_2GPI. This is in fact, a statement not always true, because all aCL assays utilize β_2GPI but almost none include ELISA wells without β_2GPI, 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 rheumatoid factors could produce false positive high titre antibodies 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 reference 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 recommendations to perform the aCL test. The IgA was not included 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 Haemostasis and Thrombosis in Latin America [70]. The Australasian Working party [71] has established that an acceptable performance for aCL testing is a coefficient of variation inter-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 specificity for APS increased [72, 73]. On the other hand, quantification 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 aPhL 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 β_2GPI [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 indeterminate titres of IgM aCL. Interestingly, with both commercial kits evaluated cut off for positive results (> 99th percentile) 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 shown 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 β_2GPI Testing

Considering anti β_2GPI, 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 β_2GPI 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 β_2GPI 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 β_2GPI 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 β_2GPI preparation used to coat the plates. An interesting recent work was performed by Cavazzana *et al.* [101]. They compared 4 different β_2GPI 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 phosphatydil 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 β_2GPI [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 β_2GPI [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, prethrombin 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 β_2GPI in APS patients was extensively investigated by some groups. There is evidence that anti β_2GPI directed against D I are associated with APS [110, 111]. Moreover, the presence of IgG anti D I in patients with β_2GPI 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 β_2GPI. More than 80% of patients fulfilled clinical criteria for APS.

Table 2. Recommendation for Better Standardization of the aPL (including anti β_2GPI) Measurement

Issue	Recommendation
Reproducibility	to perform duplicates because there is a high coefficient of variation
Calibration	to use monoclonal antibodies HCAL (IgG) and EY2C9 (IgM) as standards
Population to set the upper limit of reference range	by checking 50 (preferably 100) healthy volunteers, with a proportion of women more than 50%
Local calculation of cut off	calculate the 99th percentile
Manufacturers	cut-off value must be informed by manufacturers at 95th, 97.5th and 99th percentile
External Quality assessment	by Using also monoclonal antibodies HCAL (IgG) and EY2C9 (IgM) or other mono or polyclonal antibodies

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 β_2GPI . 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 preeclampsia 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 β_2GPI 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 β_2GPI, 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 β_2GPI 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 β_2GPI. 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 or aCL, and anti β_2GPI. 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 β_2GPI 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 β_2GPI, 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 β_2GPI 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

[1] Miyakis S, Lockshin MD, Atsumi T, *et al.* International consensus statement on an update of the classification criteria for definite antiphospholipid syndrome (APS). J Thromb Haemost 2006; 4: 295-306.

[2] Mc Neil HP, Simpson RJ, Chesterman CN, Krilis S. Antiphospholipid antibodies are directed a complex of antigen that includes a lipis binding inhibitor of coagulation: β2glycoprotein I (apolipoprotein H). Proc Natl Acad Sci USA 1990; 87: 4120-24.

[3] Galli M, Comfurius P, Maasen C, Hamker HC, Debates MH, van Breda-Vriesman. Anticardiolipin (ACA) antibodies directed not to cardiolipin but to a plasma protein cofactor. Lancet 1990; 336: 1544-7.

[4] Matsuura E, Igarashi Y, Fujimoto M, Ichikawa K, Koike T. Anticardiolipin cofactors and differential diagnosis of autoimmune disease. Lancet 1990; 336: 177-8.

[5] Bevers EM, Galli M, Barbui T, Comfurius P, Zwaal RF. Lupus anticoagulant IgG's (LA) are not directed to phospholipids only, but to a complex of lipid-bound human prothrombin. Thromb Haemost 1991; 66: 629-32.

[6] Forastiero RR, Martinuzzo ME. Antigen specificity and clinical relevance of antiphospholipid syndrome-related autoantibodies. Current Rheumatol Rev 2005; 1: 177-87.

[7] de Groot PG, Derksen RHWM, De Laat B. Tweenty-two years failure to set up undisputed assays to detect patients with antiphospholipid syndrome. Sem Thromb Hemost 2008; 34: 347-355.

[8] Simmelink MJ, Horbach DA, Derksen RH, *et al.* Complexes between anti-prothrombin antibodies and prothrombin cause lupus anticoagulant activity by competing with the binding of clotting factors for catalytic phospholipid surfaces. Br J Haematol 2001; 113: 621-9.

[9] de Laat B, Derksen RHWM, Urbanus RT, Roest M, de Groot PG. β2- glycoprotein I-dependent lupus anticoagulant hightly correlates with thrombosis in the antiphospholipid syndrome. Blood 2004; 104: 3598-602.

[10] Brandt JT, Triplett DA, Alving B, Scharrer I. Criteria for the diagnosis of Lupus Anticoagulants: an update. On behalf of the of the Subcommittee on Lupus Anticoagulant/Antiphospholipid Antibodies of the Scientific and Standardisation Committee of the ISTH. Thromb Haemost 1995; 74: 1185-90.

[11] Brandt JT, Barna LK, Triplett DA. Laboratory identification of lupus anticoagulants: results of the Second International Workshop for identification of Lupus Anticoagulants. On behalf of the Subcommittee on Lupus Anticoagulant/Antiphospholipid Antibodies of the ISTH. Thromb Haemost 1995; 74: 1597-603.

[12] Roussi J, Roisin JP, Goguel A. Lupus anticoagulants. First French interlaboratory Etalonorm survey. Am J Clin Pathol 1996; 105: 788-93.

[13] Arnout J, Maijer P, Vermylen J. Lupus anticoagulant testing in Europe: an analysis of results from the first European Concerted Action on Thrombophilia (ECAT) survey using plasma spiked with monoclonal antibodies against human beta2-glycopreotein I. Thromb Haemost 1999; 81: 929-934.

[14] Jacobsen EM, Barna-Cler L, Taylor JM, Triplett DA, Wisløff F. The evaluation of clotting times in the laboratory detection of lupus anticoagulants. Thromb Res 2001; 104:275-82.

[15] Jennings I, Greaves M, Mackie IJ, Kitchen S, Woods TA, Preston FE. Lupus anticoagulant testing improvements in performance in a UK NEQAS proficiency testing exercise after dissemination of national guidelines on laboratory methods. Br J Haematol 2002; 119: 364-9.

[16] Tripodi A, Biasiolo A, Chantarangkul V, Pengo V. Lupus anticoagulant (LA) testing: performance of clinical laboratories assessed by a national Survey using lyophilized affinity-purified immunoglobulin with LA activity. Clin Chem 2003; 49: 1608-14.

[17] Wong RC, Adelstein S, Gillis D, Favaloro EJ. Development of Consensus guidelines for anticardiolipin and lupus anticoagulant testing. Semin Thromb Hemost 2005; 31: 39-48.

[18] Pengo V, Biasiolo A, Gresele P, *et al.* On behalf of participating centers of Italian Federation of Thrombosis Centers (FCSA). Survey on lupus anticoagulant diagnosis by central evaluation of positive plasma samples. J Thromb Haemost 2007; 5: 925-30.

[19] Pengo V, Tripodi A, Reber G, *et al.* On behalf of the Scientific and Standardization Committee on Lupus Anticoagulant/Phospholipid-dependent antibodies. Update of the Guidelines for Lupus Anticoagulant detection. J Thromb Haemost 2009; 7: 1737–40.

[20] Clinical and Laboratory Standards Institute (CLSI). Collection, Transport and Processing of Blood Specimens for Testing Plasma-Based Coagulation Assasys and Molecular Hemostasis Assays: Approved Guideline- 5th ed. CLSI Document H21-A5 (ISBN 1-56238-657-3), Clinical and Laboratory Standards Institute, 940 West Valley Road, Suite 1400, Wayne Pensylvania 19087-1898 USA 2008.

[21] Favaloro EJ. Preanalytical variables in coagulation testing. Blood Coagul Fibrinol 2007; 18: 86-9.

[22] Galli M, Finazzi G Norbis F, Marziali S, Marchioli R, Barbui T. The risk of thrombosis in patients with lupus anticoagulants is predicted by their specific coagulation profile. Thromb Haemost 1999; 81: 695-700.

[23] Galli M, Borrelli G, Jacobsen EM, *et al.* Clinical significance of different antiphospholipid antibodies in the WAPS (warfarin in the antiphospholipid syndrome) study. Blood 2007; 110-1178-83.

[24] Lambert Marc, Ferrard- Sasson G, Dubucquoi S, *et al.* Diluted Russell viper-venom time improves identification of antiphospholipid syndrome in a lupus anticoagulant-positive patient population. Thromb Haemost 2009; 101: 577-81.

[25] Urbanus RT, Derksen RH, de Groot PG. Current insight in the diagnostic and pathophysiology of the antiphospholipid syndrome. Blood Rev 2008; 2: 93-105.

[26] Rosner E, Pauzner R, Lusky A, Modan M, Many A. Detection and quantitative evaluation of lupus circulating anticoagulant activity. Thromb Haemost 1987; 57: 144-7.

[27] Triplett DA, Barna LK, Unger GA. A hexagonal (II) phase phospholipids neutralization assay for lupus anticoagulant identification. Thromb Haemost 1993; 70: 787-93.

[28] Jacobsen EM, Barna-Cler L, Taylor JM, Triplett DA, Wisloff F. The Lupus Ratio test: an interlaboratory study on the detection of Lupus anticoagulants by an APTT-based integrated and semiquantitative test. Fifth International Survey of Lupus Anticoagulants ISLA-5. Thromb Haemost 2000; 83: 704-8.

[29] Moore GW, Smith MP, Patel Y, Savidge GF. The activated seven lupus anticoagulant (ASLA) assay: a new test for lupus anticoagulants (LAs). Evidence that some LAs are detectable only in extrinsic pathway-based assays. Blood Coagul Fibrinol 2002; 13: 261-269.

[30] Greaves M, Cohen H, Machin SJ, Mackie I. Guidelines on the investigation and management of the antiphospholipid syndrome. Br J Haematol 2000; 109: 704-15.

[31] Lawrie AS, Mackie IJ, Purdy G, Machin SJ. The sensitivity and specificity of commercial reagents for the detection of lupus anticoagulant show marked differences in performance between photo-optical and mechanical coagulometers. Thromb Haemost 1999; 81:758-62.

[32] Gardiner C, MacKie IJ, Malia RG, *et al.* The importance of locally derived reference ranges and standardized calculation of dilute Russell's viper venom time results in screening for lupus anticoagulant. Br J Haematol 2000; 111:1230-5.

[33] Arnout J, Vanrusselt M, Huybreschts E, Vermylen J. Optimization of the dilute prothrombin time for detection of the lupus anticoagulant by use of a recombinant tissue thromboplastin. Br J Haematol 1994; 87:94-9.

[34] Forastiero RR, Cerrato GS, Carreras LO. Evaluation of recently described tests for detection of the lupus anticoagulant. Thromb Haemost 1994; 72: 728-33.

[35] Devreese KMJ. Evaluation of a new commercial dilute protrombin time in the diagnosis of Lupus Anticoagulants. Thromb Res 2008; 123: 404-11.

[36] Martinuzzo ME, Adamczuk YP, Iglesias Varela Ml, Pombo G, Forastiero RR. Poor sensitivity of the Activated Seven Lupus Anticoagulant (ASLA) test for the detection of Lupus Anticoagulant in women with obstetric morbidity. Thromb Haemost 2005, 93: 1007-9.

[37] Moore GW, Rangarajan S, Savidge GF. The activated seven lupus anticoagulant assay detects clinically significant antibodies. Clin Appl Thromb Hemost 2008; 14: 332-37.

[38] Rooney AM, McNally T, Mackie IJ, Machin SJ. The Taipan snake venom time: a new test for lupus anticoagulant. J Clin Pathol 1994; 47: 497-501.

[39] Parmar K, Lefkou E, Doughty H, Connor P, Hunt BJ. The utility of the Taipan snake venom assay in assessing lupus anticoagulant status in individuals receiving or not receiving an oral vitamin K antagonist. Blood Coagul Fibrinol 2009; 20: 271-5.

[40] Moore GW. Combining Taipan snake venom time/Ecarin time screening with the mixing studies of conventional assays increases detection rates of lupus anticoagulants in orally anticoagulated patients. Thromb J 2007; 5: 12.

[41] Cahng S, Tillema V, ScherrD. A "percent correction" formula for evaluation of mixing studies. Am J Clin Pathol 2002; 117: 62-73.

[42] Devreese KMJ. Interpretation of normal plasma mixing studies in the laboratory diagnosis of lupus anticoagulants. Thromb Res 2007; 119: 369-76.

[43] Blanco AN, Peirano AA, Grosso SH, Gennari LC, Bianco PR, Lazzari MA. A Chromogenic substrate method for detecting and titrating anti factor VIII antibodies in the presence of lupus anticoagulant. Haematologica 2002; 87: 271-8.

[44] Horbach DA,van Oort E, Donders RC, *et al.* Lupus anticoagulant is the strongest risk factor for both venous and arterial thrombosis in patients with systemic lupus erythematosus. Comparison between different assays for detection of antiphospholipid antibodies. Thromb Haemost 1996; 76: 916-24.

[45] Wahl DG, Guillemin F, de Maistre E, *et al.* Methanalysis of the risk of venous thrombosis in individuals with antiphospholipid antobodies without underlying autoimmune disease or previous thrombosis. Lupus 1998; 7: 15-22.

[46] Galli M, Luciani D, Bertolini G, *et al.* Lupus anticoagulants are stronger risk factors for thrombosis that anticardiolipin antibodies in the antiphospholipid syndrome a systematic review of the literature. Blood 2003; 101: 1827-32.

[47] Hunt BJ. Pediatric antiphospholipid antibodies and antiphospholipid syndrome. Semin Thromb Hemost 2008; 34: 274-81.

[48] Devreese KMJ. Is there evidence for persistent or transient positive lupus anticoagulants according to the prolongation of clotting tests? Thromb Res 2008; 112: 576-9.

[49] Simmelnik MJ, Derkesen RHWM, Arnout J, de Groot PG. A simple method to discriminate between beta2- glycoprotein I and prothrombin dependent lupus anticoagulants. J Thromb Haemost 2003; 1: 740-7.

[50] Pengo V, Biasiolo A, Pegoraro C, Iliceto S. A two-step coagulation test to identify anti- β2- Glycoprotein I lupus anticoagulants. J Thromb Haemost 2004; 2: 702-7.

[51] Devreese KMJ. A functional coagulation test to identify anti β2-glycoprotein I dependent lupus anticoagulants. Thromb Res 2007; 119: 753-9.

[52] Martinuzzo ME, Cerrato GS, Adamczuk YP, Iglesias Varela ML, Forastiero RR. La prolongación del tiempo de protrombina diluido (dPT) a baja concentración de iones calcio no identifica a todos los anticoagulantes lúpicos (AL) asociados a anticuerpos anti β2 glicoproteína I. Hematología 2006; 10: 88-92.

[53] Schjetlein R, Wisloff F. Choice of standard plasma for diagnosis and quantification of lupus anticoagulants. Thromb Res 1993; 72: 287-94.

[54] Arnout J, Vanrusselt M, Wittevrongel C, Vermylen J. Monoclonal antibodies against beta-2 glycoprotein I: use as a referential material for lupus anticoagulant tests. Thromb Haemost 1998; 79: 955-8.

[55] Le Querrec A, Arnout J, Arnoux D, *et al.* Quantification of lupus anticoagulants in clinical samples using anti-beta 2GPI and anti-prothrombin monoclonal antibodies. Thromb Haemost 2001; 86: 584-9.

[56] Tripodi A. Laboratory testing for lupus anticoagulants: a review of issues affecting results. Clin Chem 2007; 53: 1629-35.

[57] Hemker HC, Al Dieri RA, de Smedt E, *et al.* Thrombin generation, a function test of the haemostatic-thrombotic system. Thromb Haemost 2006; 96: 553-61.

[58] Chantaragankul V, Clerici M, Bressi C, *et al.* Thrombin generation assessed as endogenous thrombin potential in patients with hyper and hypo-coagulability. Haematologica 2003; 88: 547-54.

[59] Devreese K, Peerlinck K, Arnout J, Hoylaerts MF. Laboratory detection of the antiphospholipid syndrome *via* calibrated automated thrombography. Thromb Haemost 2009; 101: 185-96.

[60] Rand JH, Wu XX, Quinn AS, Taaties DJ. Resistance to Annexin A5 anticoagulant activity: a thrombogenic mechanism for antiphospholipid syndrome. Lupus 2008; 17: 922-30.

[61] de Laat B, Wu XX, van Lummel M, Derksen RH, de Groot PG, Rand JH. Correlation between antiphospholipid antibodies that recognized domain I of β2-glycoprotein I and a reduction in the anticoagulant activity of Annexin A5. Blood 2007; 109: 1490-4.

[62] Harris EN. The Second International Anticardiolipin Standardization Workshop/The Kingston Antiphospholipid Antibody (KAPS) Study Group. Am J Clin Pathol 1990; 94: 476-84.

[63] Pierangeli SS, Harris EN. A protocol for determination of anticardiopipin antibodies by ELISA. Nat Protoc 2008; 3: 840-8.

[64] Diri E, Cucurull E, Gharavi A, *et al.* Antiphospholipid (Hughes') syndrome in African-Americans: IgA aCL and anti bet2 glycoprotein I is the most frequent isotype. Lupus 1999; 8: 263-8.

[65] Tajima C, Suzuki E, Mizushima Y, Ichikawa Y. Clinical significance of immunoglobulin A antiphospholipid antibodies possible association with skin manifestations and small vessel vasculitis. J Rheumatol 2001; 28: 2637-43.

[66] Reber G, Arvieux J, Comby E, *et al.* Multicenter evaluation of nine commercial kits for the quantification of anticardiolipin antibodies. Thromb Haemost 1995; 73: 444-52.

[67] Tincani A, Allegri F, Sanmarco M, *et al.* Anticardiolipin antibody assay: a methodological analysis for a better consensus in routine determination. A cooperative project of the European Antiphospholipid forum. Thromb Haemost 2001; 86: 575-83.

[68] Wong RCW, Wilson RJ, Pollock W, Steele RH, Gillis D. Anticardiolipin antibody testing and reporting practice among laboratories participating in a large external quality assurance program. Pathology 2004; 36: 174-81.

[69] Wong R, Favaloro E, Pollock W, *et al.* A multicentre evaluation of the intra-assay and inter-assay variation of commercial and in-house anti-cardiolipin antibody assay. Pathology 2004; 36: 182-92.

[70] Forastiero RR, Blanco AN, Otero AM. Estudio interlaboratorio de los ensayos para anticuerpos antifosfolípidos. Hematología 2002, 6: 27-35.

[71] Wong RC Australasian aCL Working Party. Consensus guidelines for anticardiolipin antibody testing. Thromb Res 2004; 114: 559-71.

[72] Harris EN, Pierangeli SS. A more specific ELISA assay for detection of antiphospholipid. Clin Immmunol News 1995; 15: 26-8.

[73] Merkel EN, Chang Y, Pierangeli SS, Harris EN, Polisson RP. Comparison between standard anticardiolipin antibody test and a new phospholpid test in patients with a variety of connective tissue diseases. J Rheumatol 1999; 26: 591-6.

[74] Ichikawa K, Tsutsumi A, Atsumi T, *et al.* A chimeric antibody with human gamma 1 constant region as a putative standard for assays that detect IgG β2 glycoprotein I antibodies. Arthritis Rheum 1999; 42: 2461-70.

[75] Budd R, Harley E, Quarshie A, Henderson V, Harris EN, Pierangeli SS. A re-appraisal of anticardiolipin IgM test. J Thromb Haemost 2006; 4: 2210-4.

[76] Ginsburg KS, Liang MH, Newcomer L, *et al.* Anticardiolipin antibodies and the risk of schemic stroke and venous thrombosis. Ann Intern Med 1992; 117: 997-1002.

[77] Neville C, Rauch J, Kassis J, *et al.* Thromboembolic risk in patients with high titer anticardiolipin and multiple antiphospholipid antibodies. Thromb Haemost 2003; 90: 108-115.

[78] Finazzi G, Brancaccio V, Moia M, *et al.* Natural history and risk factors for thrombosis in 360 patients with antiphospholipid antibodies: a four-year prospective study from the Italian registry. Am J Med 1996; 100: 530-6.

[79] Levine SR, Brey RL, Tilley BC, *et al.* APASS investigators. Anticardiolipin antibodies and the risk of subsequent thrombo-oclussive event in patients with ischaemic stroke. JAMA 2004; 291: 576-84.

[80] Wahl D, Regnault V, de Moerloose P, Lecompte T. Antiphospholipid antibodies and recurrent vascular events. JAMA 2004; 291: 2701-2.

[81] Ruis-Irastorza G, Khamashta MA, Hughes GR. Antiphospholipid antibodies and risk of recurrent vascular events. JAMA 2004; 291: 2701-2.

[82] Rajamani K, Chaturvedi S, Jin Z, *et al.* Patent foramen ovale, cardiac valve thickening and antiphospholipid antibodies as risk factors for subsequent vascular events: the PICSS-APASS study. Stroke 2009; 40: 2337-42.

[83] Opatrny L, David M, Kahn SR, Shrier I, Rey E. Association between antiphospholipid antibodies and recurrent fetal loss in women without autoimmune disease: a metaanalysis. J Rheumatol 2006; 33: 2214-21.

[84] Tincani A, Bazzani Ch, Zingarelli S, Lojacomo A. Lupus and the antiphospholipid síndrome in pregnancy and obstetrics: clinical characteristics, diagnosis, pathogenesis and treatment. Semin Thromb Hemost 2008; 34: 267-73.

[85] Silver RM, Porter TF, van Leeuween I, Jeng G, Scott JR, Branch DW. Anticardiolipin antibodies: clinical consequences of "low titers". Obstet Gynecol 1996; 87: 494-500.

[86] Martinuzzo ME, Forastiero RR, Carreras LO. Anti β_2 Glycoprotein I antibodies: Detection and association with thrombosis. Br J Haematol 1995; 89: 397-402.

[87] Galli M, Luciani D, Bertolini G, Barbui T. Anti β2 glycoprotein I, antiprothrombin antibodies and the risk of thrombosis in the antiphospholipid syndrome. Blood 2003; 102: 2717-23.

[88] Forastiero R, Martinuzzo M, Pombo G, *et al.* A prospective study of antibodies to β_2 glycoprotein I and prothrombin and risk of thrombosis. J Thromb Haemost 2005; 3: 1231-8.

[89] Sanmarco M, Roll P, Gayet S, *et al.* Combined search for anti β2 glycoprotein I and anticardiolipin antibodies in antiphospholipid syndrome: contribution to diagnosis. J Lab Clin Med 2004; 144:141-7.

[90] Nash MJ, Camilleri RS, Kunka S, *et al.* The anticardiolipin assay is required for sensitive screening for antiphospholipid antibodies. J Thromb Haemost 2004; 2:1077-81.

[91] Forastiero RR, Martinuzzo ME, Admaczuk Y, Carreras LO. Occurrence of anti-prothrombin and anti-β_2 glycoprotein I antibodies in patients with history of thrombosis. J Lab Clin Med 1999; 134: 610-5.

[92] Sanmarco M, Soler C, Chistides C, *et al.* Prevalence and clinical significance of IgG isoptype anti-beta 2-glycoprotein I antibodies in antiphospholipid syndrome: a comparative study with anticardiolipin antibodies. J Lab Clin Med 1997; 129: 499-506.

[93] Carreras LO, Forastiero RR, Martinuzzo ME. Which are the best biological markers of the antiphospholipid syndrome? J Autoimmun 2000; 15:163-72.

[94] de Larrañaga G, Forastiero RR, Martinuzzo ME, *et al.* High prevalence of antiphospholipid antibodies in leprosy: evaluation of antigen reactivity. Lupus 2000; 9: 594-600.

[95] Reber G, Schousboe I, Tincani A, *et al.* Inter-laboratory variability of anti-beta2-glycoproten I measurement. A collaborative study in the frame of the European Forum on Antiphospholipid Antibodies Standardization Group. Thromb Haemost 2002; 88: 66-73.

[96] Roubey RAS, Eisenberg RA, Harper MF, Winfield JB. "Anticardiolipin" autoantibodies recognize β_2-Glycoprotein I in the absence of phospholipid. Importance of antigen density and bivalent binding. J Immunol 1995; 154: 954-60.

[97] Reddel SW, Wang YX, Krilis SA. Anti β2 glycoprotein I autoantibodies requiere an antigen density threshold, consistent with divalent binding. Lupus 2003; 12:37-45.

[98] de Laat B, Derksen WM, de Groot P. High avidity anti β2 glycoprotein antibodies highly correlate with thrombosis in contrast to low aviditiy anti β2 glycoprotein antibodies. J Throm Haemost 2006; 4: 1619-21.

[99] Tincani A, Allegri F, Balestrieri G, *et al.* Minimal requirements for antiphospholipid antibodies ELISAs proposed by the European Forum on antiphospholipid antibodies. Thromb Res 2004; 114: 553-8.

[100] Reber G, Tincani A, Sanmarco M, de Moerloose P, Boffa MC. Proposals for the measurement of anti-beta 2-glycoprotein antibodies. Standardization group of the European Forum on Antiphospholipid Antibodies. J Thromb Haemost 2004; 2: 1860-2.

[101] Cavazzana A, Pengo V, Tonello M, *et al.* Anti-β2-glycoprotein I ELISA assay: the influence of different antigen preparations. Thromb Haemost 2009; 101: 789-91.

[102] Galli M, Barbui T. Antiprothrombin antibodies: detection and clinical significance. Blood 1999; 93: 2149-57.

[103] Arvieux J, Darnige L, Reber G, Bensa JC, Colomb MG. Development of an ELISA for autoantibodies to prothrombin showing their prevalence in patients with lupus anticoagulants. Thromb Haemost 1995; 74: 1120-5.

[104] Forastiero RR, Martinuzzo ME, Carreras LO. Binding properties of antibodies to prothrombin and β_2-glycoprotein I assayed by ELISA and Dot blot. Clin Exp Immunol 1999; 118: 480-6.

[105] Atsumi T, Ieko M, Bertolaccini ML, *et al.* Association between autoantibodies against phosphatydilserine/prothrombin complex with manifestations of the antiphospholipid syndrome and the presence of lupus anticoagulant. Artritis Rheum 2000; 43: 1982-93.

[106] Donohoe S, Mackie TJ, Isenberg S, Machin SI. Antiprothtombin antibodies assay conditions and clinical association with the antiphospholipid syndrome. Br J Haematol 2001; 113: 544-9.

[107] Nojima J, Iwatani Y, Suehisa E, Kuratsume H, Kanakura Y. The presence of antiphosphatidylserine/prothrombin antibodies as risk factor for both arterial and venous thrombosis in patients with systemic lupus erythematosus. Haematologica 2006; 91: 699-702.

[108] Bizzaro N, Ghirardello A, Zampieri S, *et al.* Antiprothrombin antibodies predict thrombosis in patients with systemic lupus erythematosus: a 15-year longitudinal study. J Thromb Haemost 2007; 5: 1158-64.

[109] Oku K, Atsumi T, Amengual O, Koike T. Antiprothrombin antibody testing: Detection and Clinical Utility. Semin Thromb Hemost 2008; 34: 335-9.

[110] Iverson GM, Victoria EJ, Marquis DM, *et al.* Anti-beta2 glycoprotein I (beta2GPI) autoantibodies recognize an epitope on the first domain of beta2GPI. Proc Natl Acad Sci USA 1998; 98: 15542-6.

[111] de Laat B, Derksen RH, Urbanus RT, de Groot PG. IgG antibodies that recognize epitope Gly40-Arg 43 in domain I of beta 2-glycoprotein I cause LAC, and their presence correlates strongly with thrombosis. Blood 2005; 105: 1540-5.

[112] de Laat B, Pengo V, Pabinger I, *et al.* The association between circulating antibodies against domain I of beta2-glycoprotein I and thrombosis: an international multicenter study. J Thromb Haemost 2009; 7: 1767-73.

[113] Pengo V, Biasiolo A, Gresele P, *et al.* A comparison of lupus anticoagulant positive patients with clinical picture of antiphospholipid syndrome and those without, Arterioscler Thromb Vasc Biol 2007; 27: 309-10.

[114] Galli M, Reber G, de Moerloose P, de Groot PG. Invitation to a debate on the serological criteria that define the antiphospholipid syndrome. J Thromb Haemost 2008; 6: 399-401.

[115] Wahl D, Thiebaugeorges O, Regnault T, Dalloul A, Lecompte T. Pursuing the debate on the serologic criteria that define the antiphospholipid syndrome. J Thromb Haemost 2008; 6: 1433-5.

[116] Pengo V. A contribution to the debate on the laboratory criteria that define the antiphospholipid syndrome. J Thromb Haemost 2008; 4: 1048-9.

[117] Boffa MC, Boinot C, De Carolis S, *et al.* Laboratory criteria for obstetrical antiphospholipid syndrome. Data from a multicentre prospective European women cohort. Thromb Haemost 2009; 102: 25-28.

[118] Moffat K, Kraemer LM, Plumhoff E, *et al.* Are laboratories following published recommendations for lupus anticoagulant testing? An international evaluation of practice. Thromb Haemost 101; 178-84.

[119] Wong RCW, Favaloro EJ. A consensus approach to formulation of guidelines for laboratory testing and reporting antiphospholipid assays. Semin Thromb Hemost 2008; 34: 361-72.

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