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ANTIPHOSPHOLIPID SYNDROME (S ZUILY, SECTION EDITOR)

How to Interpret Antiphospholipid Laboratory Tests

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Abstract

Purpose of the Review This review focuses on the laboratory tests necessary for the diagnosis of antiphospholipid syndrome (APS). For the interpretation of the results of the tests for antiphospholipid antibodies (aPL), understanding of all pitfalls and interferences is necessary.

Recent Findings Progress has been made on the standardization of aPL tests and current guidelines for detection of lupus anticoagulant (LAC), anticardiolipin antibodies (aCL), and antibeta2-glycoprotein I antibodies (aβ2GPI) are useful tools. LAC measurement remains a complex procedure with many pitfalls and interference by anticoagulant therapy. Solid phase assays for aCL and aβ2GPI still show inter-assay differences. Measuring LAC, aCL, and aβ2GPI allows making antibody profiles that help in identifying patients at risk. Other aPL, such as antibodies against domain I of beta2-glycoprotein I (aDI) and antiphosphatidylserine-prothrombin (aPS/PT) antibodies, may be useful in risk stratification of APS patients, but are not included in the current diagnostic criteria as no added value in the diagnosis of APS has been illustrated so far.

Summary The laboratory diagnosis of APS remains challenging. LAC, aCL, aβ2GPI IgG, and IgM should be performed to increase diagnostic efficacy, with an integrated interpretation of all results and an interpretative comment. A close interaction between clinical pathologists and clinicians is mandatory.

Keywords Antiphospholipid syndrome · Anticardiolipin antibodies · Anti-β2 glycoprotein I antibodies · Lupus anticoagulant · Interpretation · Interference

Introduction

Antiphospholipid syndrome (APS) is an autoimmune disease characterized by two major components: the presence of autoantibodies, the so-called antiphospholipid antibodies (aPL), and the occurrence of clinical symptoms defined as thrombosis and pregnancy complications [1]. A patient is classified as an APS patient if at least one clinical criterion and one laboratory criterion is present [1]. In the current classification criteria, lupus anticoagulant (LAC), anticardiolipin (aCL), and antibeta2-glycoprotein I antibodies (aβ2GPI) IgG or IgM are included as laboratory criteria, if persistently present [1, 2]. The incidence of thrombosis and pregnancy complications is high and often determined by underlying factors not related to aPL. Therefore, the diagnosis of APS relies predominantly on the laboratory results; this means the detection of aPL. An accurate laboratory diagnosis is mandatory, since over-diagnosis as well as under-diagnosis has consequences for adequate therapy and for the estimation of the thrombotic risk and recurrence of thrombosis [3, 4]. Moreover, laboratory parameters are very important since the type and level of aPL determine the risk in APS patients [1, 5–11]. Therefore, we need assays with good diagnostic power for identifying aPL.

To detect aPl, we need two test types. First, assays that detect the aPL as inhibitors of coagulation (LAC). Competition of antibody-antigen complexes with the clotting factors for the phospholipids (PL) necessary in clotting assays results in prolonged clotting times by aPL. Second, immunoassays (solid phase assays) that detect aCL and aβ2GPI. Depending on the coating of the solid phase with cardiolipin and beta2-glycoprotein I (β2GPI), or direct coating with β2GPI, aCL or aβ2GPI, respectively, will be detected [2, 12]. The combination of these three tests (LAC, aCL, aβ2GPI) is necessary since aPL are a heterogeneous group

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of antibodies with overlapping characteristics, although not identical. The assays differing in the antigen toward the antibodies are directed as follows: with tests for LAC all aPL are detected, including those binding through β2GPI and those binding by other cofactor proteins such as prothrombin. With the immunoassays, one group of antibodies is detected, those binding toward cardiolipin (in a β2GPI-dependent way) or antibodies directed to the β2GPI protein [2, 12].

The combination of the three tests and the lack of standardization of the assays make the laboratory diagnosis of APS challenging [13–16]. On top of this, LAC measurement remains a complex procedure with a three-step method, including screening, mixing, and confirmatory tests in two coagulation test systems [17]. External quality assessment studies on LAC testing have shown considerable inter-laboratory variability with high rates of false negative and false positive results [18, 19]. Solid phase assays for aCL and aβ2GPI show inter-assay differences [20–22]. Discrepancies in results are contributed to analytical factors (e.g., sample preparation, choice of assays, test procedure, calibration of aCL and aβ2GPI assays), interpretation (e.g., choice of cutoff value, expression of results, clinical value of the antibody potency), and interferences in test procedures (e.g., anticoagulant therapy and acute phase reactants in LAC testing). These pre-, post-, and analytical factors may result in a different interpretation of aPL results and consequently in diagnosis of APS. But, progress has been made on the standardization, harmonization in test procedures, and interpretation following published guidelines on LAC testing and solid phase assays for aCL and aβ2GPI [17, 23–25].

Even though detailed recommendations have been issued by these guidelines [17, 24], LAC detection is complicated by a number of unresolved issues; the detection of LAC during anticoagulation is one of the most important and challenging ones. Guidance on how to test during anticoagulant therapy has been recently published [26••]. Adequate detection of LAC is very important since LAC is regarded as the strongest risk factor for thrombosis among all aPL, and also in pregnancy LAC has been reported as the primary predictor of adverse pregnancy outcome in patients with aPL-associated pregnancies [27–30].

Adequate detection of aCL and aβ2GPI allows diagnosis of triple aPL-positive (LAC and aCL and aβ2GPI positive) patients, who are perceived to be the APS patients at highest risk of thrombosis, as well as for a first thrombotic event as for recurrence [8, 10, 31, 32]. Making antibody profiles supports the idea that the combination of aPL rather than the individual test result defines the risk to develop thrombosis or pregnancy complications. Double-positive (LAC negative, aCL and aβ2GPI positive) patients are at lower risk than triple-positive patients, and single-positive patients are less likely to develop APS-related clinical symptoms [2, 9].

Identification of all three groups of aPL enables risk stratification as well as appropriate management of APS patients. In clinical practice, the indication for testing is the choice of the clinicians based on clinical suspicion for APS. However, they may not be aware of the limitations of the tests used for measurement of aPL. On the other hand, the clinical pathologist in the laboratory may not be aware of the use of anticoagulants. Information on the anticoagulation status and the clinical symptoms of the patient is mandatory for good interpretation of results. To prevent misdiagnosis, the diagnostic workup for APS requires collaboration between the clinician and the laboratory. Good laboratory practice together with good clinical practice will contribute to better diagnosing APS.

Lupus Anticoagulant Methodology

The 2009 International Society on Thrombosis and Haemostasis (ISTH) - Scientific and Standardization Committee for Lupus Anticoagulant/Antiphospholipid Antibodies (SSC LAC/aPL) [17], as well as the British Committee for Standards in Haematology (BCSH) [25] and Clinical Laboratory Standards Institute (CLSI) guidelines [24], have contributed to more uniformity in the performance and interpretation of LAC testing. A recent survey illustrated that at some points practice differs, including testing for LAC in patients on anticoagulation and interpretation of results [33]. Based on the responses of this survey and a collaboration of experts in the field, the ISTH-SSC LAC/aPL is currently working on more guidance in LAC testing and interpretation, that will be published soon.

The methodology of LAC detection [17] is based on a three-step procedure including a screening step, a mixing step, and a confirmation step. In the screening step, the presence of aPL is demonstrated by the use of sensitive reagents containing a low concentration of PL. In the mixing step, the presence of an inhibitor is illustrated by mixing the patient plasma with normal pooled plasma in a 1:1 proportion. In the confirmatory step, the PL-dependent character of the inhibitor is illustrated by increasing the concentration of PL used in the screening test. Bilayer or hexagonal (II) phase PL should be used to increase the concentration of PL. All three steps are performed in two test systems with different test principles, since no single test has sufficient specificity and sensitivity. The test systems are limited to the activated partial thromboplastin time (aPTT) and diluted Russell Viper Venom time (dRVVT), to reach more harmonization between the laboratories and to reduce false positive results. The result of each step is expressed as a normalized ratio, and interpreted according to the local cutoff values. The confirmatory step can be expressed by a normalized LAC ratio (screen/confirm) or by percent correction [(screen – confirm)screen]×100 [17]. Some of the integrated tests are designed to measure a difference in

clotting times on a mixture of plasma [34]. The conclusion of positivity for LAC is made if all three steps in at least one test system (dRVVT or aPTT) are positive; it is beyond the locally established cutoff value. If laboratories provide detailed analytical results on the three steps, a final conclusion should be given as positive or negative for LAC. That helps clinicians who are not familiar with the test procedure. Comments as "borderline" results are discouraged, but sometimes necessary. In these cases, repeat testing should be suggested within a shorter time than the repeat testing after 12 weeks for confirmation (see further).

Before starting with the LAC-specific tests, routine coagulation tests comprising of prothrombin time, aPTT, thrombin time, and fibrinogen help identify the presence of anticoagulant therapy, clotting factor deficiencies or specific coagulation factor inhibitors, and acute phase reactants. Laboratory testing for LAC remains complicated with many pitfalls in the test procedure as well as in the interpretation [13]. Automatic reflex testing or algorithms with automated launching of tests, result calculation, interpretation toward the cutoff value, and providing warnings for interference or comments may assist laboratories in LAC measurement according to the guidelines, and hence improve inter-laboratory comparability of LAC results [35].

Methodology of aCL and aβ2GPI Measurement

Recommendations for optimal laboratory detection of aPL by solid phase assays are given in the ISTH-SSC guideline [23].

A large variety of assays are available to measure aCL and aβ2GPI that differ in solid phase, coating, source of β2GPI, blocking agents, and calibration. Originally, aCL and aβ2GPI have been measured by ELISA [1], but nowadays, automated systems have been introduced into the market using different solid phases (magnetic particles, microbeads, membranes, coated polystyrene cups), and various detection systems (chemiluminescence, flow cytometry, multiplex systems) [23]. Automated systems have the advantage that working conditions among the laboratories are comparable, eliminate manual pipetting, and follow a strict protocol on how to perform the assay that may reduce the inter-laboratory variation for one system [36]. ELISAs have shown large inter-laboratory variation and limited consensus in external quality control programs [21, 22].

Both aCL and aβ2GPI have diagnostic value and show significant association with increased risk of thrombosis [8, 37]. β2GPI-Dependent aCL immunoassay can be as specific as an aβ2GPI assay [6, 38]. It is essential to avoid detection of non-cofactor-related aCL by using β2GPI-dependent aCL assays [12]. Testing for aCL that are β2GPI-dependent is primarily to avoid false positive classification of patients as having APS, and thereby preventing unnecessary treatment [2, 12]. β2GPI-Dependent aCL is intended to avoid infectious-related aCL that are not associated with clinical symptoms associated to APS [2, 12, 23].

aCL detection can confirm the positivity of aβ2GPI. Positivity of aCL without aβ2GPI positivity should be interpreted with caution, since both parameters correlate well and discrepant results may result from the use of assays from different manufacturers [13, 20]. Automated systems that provide the four results (aCL and aβ2GPI IgG and IgM) at once, instead of running multiple ELISAs or a combination of test platforms, can improve diagnostic performance [20].

We know from external quality control programs and studies with human monoclonal antibodies that aCL and aβ2GPI IgG/M assays produce variable result [20, 22, 39]. As long as the lack of international calibration standards persists, the comparison between platforms remains challenging [13]. Recently, we performed a comparison of four frequently used solid phase platforms for aPL in a head-to-head comparison on a large patient population including APS patients and different control populations. In this multicenter study, we illustrated that with commercially available solid phase assays, all performed by one technologist in one and the same laboratory and thus eliminating inter-laboratory and inter-operator variation, there is poor agreement in detection of aCL and aβ2GPI IgG/M between platforms [20]. aCL IgG and aβ2GPI IgM detection resulted in the best agreement, however with still a substantial number of samples in disagreement. Interestingly, this was not reflected in the clinical performance of the platforms by odds ratios for thrombosis and/or pregnancy morbidity considering the results of all four aPL together, being globally concordant among the tested platforms [20]. Therefore, it is recommended to measure aCL and aβ2GPI IgG and IgM within the same platform [20]. Nevertheless, detection of patients positive for aCL or aβ2GPI is assay dependent and this poor agreement between available platforms hampers uniform classification of positive aCL/aβ2GPI antibodies. It is important that all workers in the field are aware of these methodological shortcomings. When clinical suspicion is high for APS, and results of aPL are not in line with what is expected, consideration of retesting with another type of solid phase platform can be useful. A sample assigned positive in one assay does not automatically test positive in the same type of assay from a different manufacturer or in another laboratory. Clinicians are not always aware of the sensitivity and specificity of laboratory tests. Test results should always be related to clinical symptoms and an interaction of the laboratory and clinician is mandatory [13].

Antibody Isotype of aCL and aβ2GPI

Current criteria recommend increased levels of IgG and IgM aCL and aβ2GPI to confirm APS [1, 2]. The role of IgM aPL has been discussed based on a less strong association with

thrombosis compared with IgG [27, 40], while for pregnancy morbidity the role of IgM should be further established [28, 41]. The uncertainty about the role of IgM was further explored by a recent systematic review of the literature performed [42]. The review was not able to give a clear answer on the added value of IgM; more significant IgG correlations with thrombosis were confirmed, but significant associations for IgM were found with corresponding IgG. The question of how many APS patients would be missed upon omission of IgM could not be answered [42]. Therefore, to further explore the role of IgM, we evaluated aCL/aβ2GPI IgM measured with four different platforms in a multicenter study on a large patient population including thrombotic and obstetric APS patients and control populations including patients with autoimmune diseases, diseased patient controls for thrombosis, and pregnancy morbidity [43•]. In line with previous literature, we observed that both IgG and IgM aCL and/or aβ2GPI antibodies were significantly correlated with thrombosis and pregnancy morbidity, and higher odds ratios were obtained for IgG compared with IgM positivity. Interestingly, this was independent of the solid phase platform used. To evaluate the added value of IgM, we explored the isolated positivity of IgM that was rare in thrombotic APS and more frequent in obstetric APS. In a multivariate logistic regression analysis of aPL, IgM positivity was found only independently associated with pregnancy morbidity and not with thrombosis. Addition of IgM to LAC and IgG increased the odds ratio for thrombosis, suggesting that testing for IgM might be useful to improve thrombotic risk stratification [43]. These data support testing for IgG and IgM, especially in women suspected for obstetric APS, because in thrombotic patients suspected for APS, first-line testing for IgM has no added value for diagnosis. In a thrombotic patient population, a stepped approach starting with IgG, and if positive also performing IgM, can be a good option, to estimate the risk profile. Previously, it was demonstrated that the presence of aCL and aβ2GPI of the same isotype reinforces the clinical probability of APS [11, 44]. We confirmed that odds ratios for three out of the four tested platforms are higher for triple positivity with concordance of isotype compared with triple positivity including combinations of aCL and ab2GPI irrespective of isotype [45].

IgA aCL and aβ2GPI are not included in the current classification criteria [1, 2, 23]. The role of IgA remains controversial in APS-associated clinical events [46]. A recent systematic review indicated that several studies failed to demonstrate utility of adding IgA aCL and aβ2GPI testing, either because of low prevalence of these antibodies, their association with other aPL, or the lack of improved diagnostic accuracy when IgA antibodies are routinely assessed [47]. In most cases with major APS manifestations (i.e., thrombosis), IgA aPL are usually found in association with IgG and/or IgM [47]. Isolated IgA aPL are in most of the cases linked to non-criteria clinical manifestations of APS [1, 47]. We further investigated the added value of IgA by the results of our multicenter study, in which we also tested aCL and aβ2GPI IgA with four different platforms [48]. Positivity for IgA was detected in 17–26% of the patients with clinical manifestations of APS (depending on the platform) and in 6–13% of the patients with an autoimmune disease and controls. Both aCL and aβ2GPI IgA were significantly associated with thrombosis and pregnancy morbidity. However, a majority of these patients (77–98%) were also positive for LAC, IgG, and/or IgM. Isolated Ig A positivity was rare in patients with clinical manifestations of APS (0.3–5%) and not associated with thrombosis and/or pregnancy morbidity. Addition of IgA to the current criteria panel did not result in higher odds ratios for thrombosis or pregnancy morbidity [48]. This confirmed the findings of the meta-analysis [47].

Based on the data published until now, there is not enough evidence to recommend testing for IgA aCL and/or IgA β2GPI to increase the diagnostic accuracy of the APS.

Interferences in aPL Measurement

Testing during the acute phase of the event is strongly discouraged because of high levels of FVIII [17] or C-reactive protein; the first may mask the LAC by shortening the aPTT and the latter may give false positive results due to interferences with PL in the reagent [49]. For solid phase assays, some interfering substances have been described, such as rheumatic factor, bilirubin, hemoglobin, or triglycerides [23]. Transient antibodies have been described in infectious diseases or drugs and are not of clinical significance [2••]. In the lab report, these possible interfering factors should be mentioned if relevant for the interpretation.

Besides this interference of acute phase proteins, little is known on the effect of thrombosis or pregnancy on the results of aPL, with uncertainty on the best timing for aPL testing, as illustrated in the ISTH-SSC survey [33]. LA positivity may fluctuate over time with or without the presence of systemic lupus erythematosus [50, 51] and during pregnancy, either decreasing or increasing [50, 52, 53]. Test results obtained during pregnancy or in the thrombotic phase should be better repeated post-delivery or at distance of the thrombotic event.

One of the major drawbacks of LAC tests is, because of their coagulation-based principle, their sensitivity to anticoagulant therapy, such as vitamin K antagonists (VKA), heparins, and direct oral anticoagulants (DOAC). Preferably, tests should be postponed until therapy is stopped but requests during therapy still occur very frequently with potentially false positive or false negative results [13]. However, testing during anticoagulant therapy may become important when deciding on duration or type of treatment, for instance VKA treatment or direct oral anticoagulants (DOAC) [26, 54]. Also, even when LAC is evaluated before commencing anticoagulation, the LAC test shall be repeated after 12 weeks to establish the diagnosis of APS [2••].

Some procedures are applied to make LAC testing reliable during VKA therapy. According to the 2009 ISTH-SSC guidelines [17], LAC testing is reliable when the international normalized ratio (INR) is less than 1.5. Testing after a switch to low molecular weight heparin (LMWH) with temporary VKA discontinuation is recommended with the last dose of LMWH administered more than 12 h before the blood is drawn for LAC testing [17, 26]. If the INR is between 1.5 and < 3.0, LAC testing can be performed on a mixture of patient plasma and normal pooled plasma [17]. However, it should be realized that although dilution of the test plasma into pooled normal plasma is widely used, it is not robust enough to help in making a diagnosis of LAC during VKA treatment, and both false negative or false positive results may occur [26, 55]. Other tests such as Taipan snake venom/Ecarin clotting times are not recommended [17, 26] and need further investigation.

Testing during heparin therapy is less prone to interferences. Test reagents (dRVVT, some LAC-specific aPTT reagents) include in their composition heparin neutralizers able to quench unfractionated or low molecular weight heparin up to 1.0 U/mL. LAC tests are less affected by LMWH, but caution is needed in the interpretation of results. A recent study showed that in applying the three-step test procedure for LAC, unfractionated heparin (UFH) did not result in false positive LAC, while enoxaparin caused false positive aPTT-based LAC at supra-therapeutic anti-Xa activity levels that exceeded the heparin neutralizing capabilities of the reagents [56]. The laboratory should always check the anti-Xa activity levels of heparin that are neutralized by inhibitors added in the reagents [56]. Checking anti-Xa activity together with LAC testing can ensure that results are reliable if anti-Xa activity levels are within the therapeutic range [26••].

Since the introduction of DOAC, many studies have illustrated that DOAC interfere with LAC testing [57]. aPTT and dRVVT are prolonged by DOAC, and thus DOAC therapy may result in false positive results, even at low levels of a drug [58]. Strategies to overcome DOAC interference have been investigated, including the use of antidotes or neutralizers [26••]. The pre-treatment of samples by an antidote or neutralizer of DOAC can eliminate the drug effect on LAC tests [59–64]. Antidotes are very expensive, but cheaper charcoal-based compounds appear equally effective in limiting DOAC interference. Some studies indicated that pretreatment with adsorbents may affect clotting times resulting in false positive or negative LAC results, and in some studies, no complete removal of DOAC was observed [56, 62–64]. Pre-treatment of plasma with adsorbents is only advised in DOAC-treated patients, and not as an all-purpose pretreatment procedure for all samples with LAC request [26••]. DOAC levels should also be checked after DOAC adsorption [57]. Sometimes, information of DOAC use is not available; in these conditions, DOAC adsorption can be considered at least for initially positive samples. Clinical and laboratory experience will guide whether the use of these DOAC adsorbents should become standard practice, but this will make the methodology for LAC testing even more complex. This highlights once more the importance of a close collaboration between the clinicians and their laboratory preventing false classification of patients as APS.

Cutoff Values for LAC, aCL, and aβ2GPI

Each result of an aPL above the cutoff values should be considered positive. Imprecision of the method of solid phase assays should be considered, especially for results around the cutoff [23]. Categorizing LAC according to strength does not seem to be appropriate, since there is no established LAC standard available in sufficient quantity. Furthermore, no evidence exists on the relation of "stronger" LAC with clinical symptoms [5]. Nevertheless, LAC ratios slightly above the cutoff value should be interpreted with care, and suggested to be repeated.

In the current guidelines, only medium and high levels of antibodies are included as a diagnostic criterion to improve the specificity of the tests [1, 2]. It is strongly advised to calculate in-house cutoff values by the 99th percentile [17, 23]. To obtain a reliable cutoff value, at least 120 normal donors should be used [23, 65]. However, the high number of normal donors is not feasible for every laboratory. Therefore, an alternative is the transference of the manufacturer's cutoff values after verification using a small number (20 or two times 20) of normal donors [66], as often applied for the solid phase assays [23]. Efforts have been made to establish cutoff values by a multicenter approach for solid phase parameters [36] and for LAC [67–69]. These studies showed that for LAC cutoffs differences were observed between participants, even with the same platform [69], independent of the reagent lot [68] or the set of normal donors used [67]. Unfortunately, we have to conclude that cutoff values for LAC determined elsewhere cannot be transferred from one laboratory to another. However, joint efforts should be continued to calculate universal cutoff values per test/instrument combination, to find a solution for this challenging issue [26••].

Results of aCL and aβ2GPI are reported as numerical values and expressed in units derived from the calibration curve which can differ widely between systems since no international standard is available [13, 23]. Titers do correlate between systems, although the numerical values vary between test platforms [20, 38, 70]. Therefore, a numeric value (> 40 GPL/MPL [1]) cannot be recommended as a general criterion for positivity. Each test result above the local cutoff value should be regarded as positive [2, 23]. External quality programs have shown that solid phase assays measuring the same sample vary in titer but also in classification of the sample as low, medium, or high positive [22]. Medium and high aPL titers are considered to be stronger correlated with clinical outcomes of APS than low titers. Qualitative reporting of results categorizing aCL and aβ2GPI IgG/M titers in low, medium, and high titers could be very useful for the clinician, and

even more if qualitative gradation of results is interchangeable between different systems. So far, no standardized method to define these ranges is available. Reports using gradation of positivity by defining intervals are rare [71], and did not sufficiently investigate whether this can be applied to different solid phase systems.

Report of the Results

Solid phase aCL and aβ2GPI assays should be interpreted together with coagulation LAC tests to assess the clinical significance [17, 23]. Although in the current classification criteria, it is sufficient to have one out of the three aPL positive, combining the aPL may benefit risk assessment [1, 2]. The concept of antibody profiles was already recommended in the Sydney criteria by the categorization of patients according to whether they had positive findings for one or multiple tests, and revised by Pengo et al. taking into account the type and the number of positive tests [1, 11]. Triple positivity is defined as LAC, aCL, and aβ2GPI positive, double positivity indicates aCL and aβ2GPI positivity, and single positives have only one aPL positive [11]. Combined positivity for LAC, aCL, and aβ2GPI antibodies (i.e., triple positivity) has been shown to be associated with a high risk of both a first thrombotic event and recurrence [8, 31, 32]. Also, in asymptomatic carriers, the number of events was much higher in triple positives, compared with double and single positives [10, 32]. Double-positive (LAC-negative) patients are at lower risk than triple-positive patients, and single-positive patients are less likely to develop APS-related clinical symptoms [2, 9]. Except for myocardial infarction, ischemic stroke, and pregnancy morbidity, isolated LAC is not a risk factor and often observed in the absence of clinical symptoms, in elderly patients, or on a first occasion not confirmed after 12 weeks [9, 30, 72–74].

In making antibody profiles, we should be aware of inter-assay and inter-laboratory variabilities of the assays. We illustrated differences between solid phase platforms result in differences in numbers of triple-positive samples [20, 45]. Nonetheless, in this cohort, a strong correlation between triple positivity and thrombosis was confirmed, as triple positivity was significantly correlated with thrombosis independent of the platform used with odds ratios higher for triple-positive patients compared with LAC, irrespective of the isotype of aCL and aβ2GPI [45]. However, the association with thrombosis of triple positivity with IgM depended on the platform [45].

Coagulation assays for LAC and solid phase assays tend to be performed in different laboratory departments: the hemostasis laboratory and the immunology laboratory. It is strongly recommended to perform all three assays at once on the same sample. This often requires an interdisciplinary cooperation between laboratory departments [13]. Besides, all assays can be performed on citrated plasma. Although most of the laboratories use serum to perform solid phase assays, both serum and citrated plasma can be used as dilution by citrate is negligible, on the condition that assay specifications (including cutoff values) are validated for the corresponding sample type [23].

In the final report, besides the individual results of LAC, aCL IgG/IgM, and aβ2GPI IgG/IgM, the antibody profile should be discussed. LAC is reported with a final conclusion as positive/negative, based on the three steps of screening, mixing, and confirmatory tests, and detailed results of these steps should be reported with their corresponding cutoff values. aCL and aβ2GPI are reported with their titer and indication of the cutoff value.

Positive results of LAC, aCL, or aβ2GPI need to be confirmed on a second occasion, after 12 weeks to confirm persistent positivity. Transient antibodies have been described in infectious diseases or drugs and are not of clinical significance; therefore, re-testing was originally meant to avoid over-diagnosis of APS patients who were not persistently positive [1, 2]. Although triple-positive patients usually have a persistent antibody profile on follow-up testing after 12 weeks [75, 76], re-testing for confirmation after 3 months is still recommended [2••]. Reproducing the same test results as for the initial positive test after 3 months renders the test result more reliable, which is important in the context of poor standardization and interferences with effect on the test result [2, 13].

A report with an explanation of the results should be given: interpretive comments can be given, for instance when the laboratory feels this is a "doubtful" result; for instance, doubtful results are those with clotting time ratios just above the cutoff values and negative aCL and abeta2GPI. Any comments on possible interferences, retesting, and repeat testing after 12 weeks goes into the interpretive report. The results should always be related to the clinical context, and interpreted related to the anticoagulation status of the patient. A close interaction between the laboratory and the clinician is mandatory [13].

Other aPL

Publications are available on various non-criteria aPL (e.g., antiphosphatidic acid, antiphosphatidyl-choline, antiphosphatidyl-ethanolamine, antiphosphatidyl-glycerol, antiphosphatidyl-inositol, antiphosphatidyl-serine, anti-prothrombin, anti-prothrombin/phosphatidyl serine, anti-annexin A5, anti-protein S, anti-annexin A2, …), but only anti-prothrombin/phosphatidyl serine (aPS/PT) is a group of aPL that merits more attention based on recent literature describing their association with thrombosis and pregnancy morbidity [77, 78]. aPS/PT antibodies are strongly associated with LAC and frequently present in APS patients [72, 79]. Patients and asymptomatic carriers with triple positivity show persistently positivity for aPS/PT, indicating their potential role in risk stratification [80, 81]. aPS/PT of isotype IgM is strongly correlated with isolated LAC in APS patients, as well as in asymptomatic carriers, suggesting that aPS/PT IgM may be less pathogenic [79, 81].

Measurement of aPS/PT in patients with LAC may be useful to identify subgroups of patients at different thrombotic risks. Although the association of aPS/PT IgG and IgM with clinical manifestations of APS looks strong, further studies are needed to identify the added value of aPS/PT on top of the criteria aPL in diagnosis of APS [78•]. Therefore, search for aPS/PT in daily practice is not recommended yet.

Anti-domain I β2GPI IgG antibodies (aDI) are a subgroup of aβ2GPI antibodies directed against domain I of β2GPI [82]. Some years ago, two studies demonstrated that aDI correlated well with thrombosis and obstetric complications [83, 84]. These studies were performed with an in-house ELISA not available for routine practice, and more clinical studies and commercially available assays were to wait before aDI could be introduced in

Table 1 Key messages for clinicians on interpretation of antiphospholipid antibody (aPL) testing

1. Laboratory criteria
   - Classification criteria for APS include lupus anticoagulant (LAC), anticardiolipin (aCL), and antibeta2-glycoprotein I antibodies (aβ2GPI) IgG or IgM
     • Positive results of LAC, aCL, or aβ2GPI need to be confirmed on a second occasion, after 12 weeks to confirm persistent positivity
     • Identification of all three groups of aPL enables risk stratification
     • Triple positivity is defined as LAC, aCL, and aβ2GPI positive, double positivity indicates aCL and aβ2GPI positivity, and single positives have only one aPL positive
     • Triple-positive patients are at the highest risk to develop APS-related clinical symptoms
     • Medium and high aPL titers are considered to be stronger correlated with clinical outcomes of APS than low titers. It is strongly advised to calculate in-house cutoff values by the 99th percentile
     • All three parameters (LAC, aCL, aβ2GPI) should be performed at once on the same sample
   - Other aPL are not included in the laboratory criteria
     • aPS/PT antibodies are strongly associated with LAC and frequently present in APS patients. Further studies are needed to identify the added value of aPS/PT on top of the criteria aPL in diagnosis of APS
     • Anti-domain I β2GPI IgG antibodies significantly correlates with clinical symptoms of APS and are associated with triple-positive high-risk population. The lack of added value to the aβ2GPI makes that these antibodies are not recommended to be applied in daily routine
   - To prevent misdiagnosis, the diagnostic workup for APS requires collaboration between the clinician and the laboratory
2. LAC
   - LAC is detected by a combination of phospholipid-dependent coagulation assays in a three-step procedure, including screening, mixing, and confirmation steps
   - LAC is regarded as the strongest risk factor for thrombosis and aPL-related pregnancy complications
   - Information on the anticoagulation status of the patient is mandatory for good interpretation of LAC results
   - Routine coagulation tests (aPTT, PT, thrombin time) help identify the presence of anticoagulant therapy
   - If laboratories provide detailed analytical results on the three steps of LAC testing, a final conclusion should be given as positive or negative for LAC
3. aCL and aβ2GPI
   - aCL and aβ2GPI are measured by solid phase assays
   - Detection of aCL and aβ2GPI IgG/M shows poor agreement between platforms
   - Numerical values vary between test platforms. Therefore, a numeric value (> 40 GPL/MPL) cannot be recommended as a general criterion for positivity. Local cutoff values (> 99th percentile) are recommended
   - It is recommended to measure aCL and aβ2GPI IgG and IgM within the same platform
   - When clinical suspicion is high for APS, and results of aPL are not in line with what is expected, consideration of retesting with another type of solid phase platform can be useful
   - IgM aCL and/or aβ2GPI antibodies are significantly correlated with thrombosis and pregnancy morbidity, but higher odds ratios are obtained for IgG compared with IgM positivity
   - aCL/aβ2GPI IgM positivity was found independently associated with pregnancy morbidity but not as an independent risk factor for thrombosis
   - Presence of aCL and aβ2GPI of the same isotype reinforces the clinical probability of APS
   - There is not enough evidence to recommend testing for IgA aCL and/or IgA β2GPI to increase the diagnostic accuracy of the APS
   - Imprecision of the method of solid phase assays should be considered, especially for results around the cutoff
4. Interferences of aPL testing
   - Results of LAC testing during an acute phase response or during pregnancy should be interpreted with caution, as false positive and negative results can occur. Repeat testing post-delivery or at distance of the thrombotic event is recommended
   - For solid phase assays for aCL and aβ2GPI, some interfering substances have been described, such as rheumatic factor, bilirubin, hemoglobin, or triglycerides
   - LAC testing in patients receiving anticoagulant treatment (direct oral anticoagulants (DOAC), antivitamin K therapy (VKA), heparins) can result in false positives and false negatives. Preferably, tests should be postponed until therapy is stopped
   - Testing during heparin therapy is less prone to interferences. If measurement of anti-FXa activity indicates that the patient's anti-FXa is within the therapeutic interval, LAC testing can be performed if reagents contain heparin neutralizers
   - DOAC result in false positive LAC. The use of DOAC adsorbents in the test procedure is a promising solution
   - LAC testing during VKA is reliable when international normalized ratio is less than 1.5 (after temporary discontinuation of the VKA, with consideration of LMWH bridging). Dilution of patient plasma into pooled normal plasma before testing can result in false negative or false positive LAC results
5. Report of results
   - aCL and aβ2GPI assays should be interpreted together with coagulation LAC tests to assess clinical significance
   - Individual results of LAC, aCL IgG/IgM and aβ2GPI IgG/IgM, and the antibody profile should be discussed
   - Results are interpreted according to the local cutoff values stated in the report. aCL and aβ2GPI are reported with their titer. Each result above the local cutoff should be considered positive
   - LAC is reported with a final conclusion as positive or negative
   - Report with an explanation of the results should be given and interpretive comments should be included (e.g., on possible interferences)
   - Information on the anticoagulation status and the clinical symptoms of the patient is mandatory for good interpretation of results

a non-research setting. Nowadays, an automated assay based on the chemiluminescent technique (CLIA) has become available to measure aDI, and since then, various studies have been published exploring the clinical value of the assay [85•]. Although some studies have illustrated a stronger correlation with thrombosis (even though estimates varied between published studies) compared with the full-length aβ2GPI assay, others could not verify that there is added value in performing the commercial aDI assay on top of the aβ2GPI [85•]. Three studies looked into detail to the added value of aDI by adding the aDI, or replacing the aβ2GPI in the current aPL panel, and did not find higher risk for thrombosis nor indicate aDI as an independent risk factor [86–88]. Experiments with monoclonal antibodies against a specific epitope G40-R43 in domain I of β2GPI revealed that the original two-step ELISA measured a more specific aDI antibody population directed against G40-R43, compared with the commercially available aDI CLIA assay detecting all antibodies to domain I, and not those specifically against the epitope G40-R43 [85•]. We hypothesized that this explains the added clinical value of the aDI positivity by the in-house two-step aDI ELISA compared with the full-length aβ2GPI, demonstrated by the higher OR for thrombosis and pregnancy morbidity [83, 84]. Interestingly, aDI detected by the commercial assay are frequently present in triple-positive patients and show higher titers in this high-risk patient population, compared with the titers in single or double positives [86, 88, 89]. Even though aDI significantly correlate with clinical symptoms of APS and are associated with triple-positive high-risk population, the lack of added value to the aβ2GPI assay makes that aDI are not recommended for application in daily routine, and not included in the current laboratory criteria [2••]. But, the high correlation between aDI and triple positivity confirms the patients are at higher risk for clinical events in APS.

Conclusions

The laboratory diagnosis of APS relies on the detection of aPL, and requires persistently positivity of LAC, aCL, or aβ2GPI IgG or IgM. Not every test has an equal diagnostic importance; therefore, antibody profiles based on the combination of these three groups of aPL help in identifying the patients at risk for APS-related clinical symptoms. At present, other aPL such as antibodies against domain I of β2GPI and aPS/PT antibodies are not included in the diagnostic criteria as they have no added value in the diagnosis of APS.

All assays have to performed according to the guidelines and only high titers should be considered. We should be aware of inter-assay and inter-laboratory variabilities and the performance characteristics of the assays. Solid phase assays for aCL and aβ2GPI show inter-assay differences. The combination of coagulation assays for LAC makes the procedure complex and prone to interference, for instance with anticoagulant therapy. Methodological issues make the laboratory diagnosis of APS challenging, although progress has been made on the standardization and interpretation as reflected in published guidelines on LAC testing and solid phase assays for aCL and aβ2GPI.

To prevent false classification of patients as APS patients, an interaction between the clinicians and their laboratory is very important. Clinicians should inform the clinical pathologists on the anticoagulation status of the patient. The clinical pathologist should deliver a report with an interpretative comment, with a warning about possible interference, and suggestion for repeat testing to confirm the positivity. The results of LAC should always be related to the results of aCL and aβ2GPI to assess the risk profile in the clinical context. A close collaboration and sharing of clinical information as well as information on test result interpretation between the laboratory and the clinician is mandatory. The key messages for the clinicians are summarized in Table 1.

Compliance with Ethical Standards

Conflict of Interest The author declares that she has no conflict of interest.

Human and Animal Rights and Informed Consent This article does not contain any studies with animal subjects performed by the author. For the studies with human subjects referred to with the author as co-author, compliance with ethical guidelines were addressed in the referred articles.

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- • Of importance
- •• Of major importance

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