



# Role of antiphospholipid antibodies in the diagnosis of antiphospholipid syndrome

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## ABSTRACT

The diagnosis of antiphospholipid syndrome (APS) relies on the detection of antiphospholipid antibodies (aPL). Currently, lupus anticoagulant (LA), anticardiolipin (aCL), and antibeta2-glycoprotein I antibodies (aβ2GPI) IgG or IgM are included as laboratory criteria, if persistently present. LAC measurement remains a complicated procedure with many pitfalls and interfered by anticoagulant therapy. Solid-phase assays for aCL and aβ2GPI show interassay differences. These methodological issues make the laboratory diagnosis of APS challenging. In the interpretation of aPL results, antibody profiles help in identifying patients at risk. Other aPL, such as antibodies against the domain I of beta2-glycoprotein (aDI) and antiphosphatidylserine-prothrombin (aPS/PT) antibodies have been studied in the last years and may be useful in risk stratification of APS patients. Because of the methodological shortcomings of immunological and clotting assays, these non-criteria aPL may be useful in patients with incomplete antibody profiles to confirm or exclude the increased risk profile. This manuscript will focus on the laboratory aspects, the clinical relevance of assays and interpretation of aPL results in the diagnosis of APS.

## 1. Introduction

The antiphospholipid syndrome (APS) is an auto-immune disease defined by the clinical presentation of thromboembolic complications and/or pregnancy morbidity. The classification criteria for APS include, besides the clinical criteria, the presence of persistently positive antiphospholipid antibodies (aPL). The clinical and laboratory diagnosis of APS is challenging, and has preoccupied haematologists, rheumatologists, obstetricians and pathologists since its first description in 1983 until today. Although the Sydney criteria were meant as classification criteria, the laboratory parameters are used as diagnostic criteria. It took many years to further define these diagnostic laboratory criteria that are still prone to discussion and need for regular updates.

The laboratory diagnosis of APS relies on the detection of aPL. APL are a heterogeneous group of autoantibodies, but in the current classification criteria only lupus anticoagulant (LA), anticardiolipin (aCL) and antibeta2-glycoprotein I antibodies (aβ2GPI) IgG or IgM are defined as laboratory criteria, if persistently present [1,2]. The confirmation of a positive result after twelve weeks was added to the classification criteria

to avoid overdiagnosis, and to exclude patients with transient aPL to be regarded as APS. Clinical criteria of APS, thrombotic events as well as pregnancy morbidity, are common in the general population, and often not caused by aPL presence, causing a great need for reliable aPL assays [3]. Moreover, laboratory criteria are very important since the type and level of aPL determine the risk in APS patients [4–7]. A huge number of publications on which laboratory tests to use have been published during the past years, sometimes with conflicting information. The current classification criteria limit the laboratory parameters to three groups of aPL: LA, aCL and aβ2GPI IgG and IgM. However, optimization of laboratory diagnosis and risk stratification opens the field towards so-called “non-criteria aPL”. Antiphosphatidylserine-prothrombin (aPS/PT) antibodies gain importance during the last year based on clinical studies illustrating their role in thrombotic events as well as pregnancy complications [8]. Other aPL such as antibodies against the domain I of beta2-glycoprotein (aDI) are pathogenic and confirm the risk for clinical APS related manifestations [9–12].

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## 2. Patient selection for testing for antiphospholipid antibodies

Testing for aPL in the context of APS, should focus on patients with a high probability of having APS. Indiscriminate testing is strongly discouraged to avoid incidental findings [13,14]. Patient selection is summarized in Table 1.

## 3. Lupus anticoagulant

LA measurement detects all aPL binding to phospholipid of cell membranes, independent of the phospholipid binding protein that acts as ligand for aPL. Consequently, LA are a heterogeneous group of aPL. Amongst all antibody aPL cofactors,  $\beta_2$ glycoprotein I and prothrombin have the most significant association with pathogenicity [15]. LA represents an intriguing paradox [16,17]. This class of aPL causes a phospholipid-dependent prolongation of the clotting time but is associated with an increased risk of thrombosis and pregnancy morbidity.

The methodology described in the 1990's has essentially not changed and uses on a multistep procedure [14,18–20]. As laboratory test, LA is challenging. The analysis of LA is complex, with many pitfalls in the preanalytical conditions, applied procedure, and interpretation [21,22]. Guidelines for LA detection has been proven to benefit harmonization of methods [19,23,24]. A comparative study on analyzing samples showed that good agreement in LA performance between laboratories can be achieved by using the same test protocols and test systems [25]. A survey on methodology of LA initiated by the International Society on Thrombosis and Haemostasis Scientific and Standardization Subcommittee on LA/aPL (ISTH-SSC) showed that there was good agreement on sample preparation, choice of assays, repeat testing, and the use of in interpretative comments. However, on other issues, such as testing in patients on anticoagulation, cutoff values, and calculation and interpretation of results, there was more variability between labs [26]. A recent update on how to test for LA with guidance on aspects of methodology, choice of assays, cutoff values, calculation and interpretation of results, and timing of testing in relation to thrombosis and pregnancy,

**Table 1**

Patient selection for antiphospholipid antibody testing in patients likely to have APS [13,14,19,84].

1. LA, aCL and  $\text{a}\beta_2\text{GPI}$  should be tested in:
  - younger patients (<50 years) with unprovoked venous thromboembolism (VTE)
  - VTE at unusual sites
  - younger patients (<50 years) with ischaemic stroke, transient ischaemic attack or other evidence of brain ischaemia
  - arterial thrombosis in other sites in younger patients (<50 years)
  - microvascular thrombosis
  - recurrent VTE unexplained by subtherapeutic anticoagulation, patient non-adherence or malignancy
  - pregnancy morbidity: fetal loss after 10 weeks, recurrent early (first trimester) miscarriages, prematurity (<34 weeks' gestation) associated with severe (pre) eclampsia, HELLP syndrome, placental insufficiency (fetal growth restriction), stillbirth
  - systemic lupus erythematosus: testing for LA, aCL and  $\text{a}\beta_2\text{GPI}$  is part of the diagnostic criteria
2. LA, aCL and  $\text{a}\beta_2\text{GPI}$  testing could be considered in the following situations:
  - Younger patients (<50 years) with non-criteria clinical manifestations [84], i.e. those not included in the Sydney criteria – e.g. immune thrombocytopenia, particularly with presence of arthralgias or arthritis, hair loss, sun sensitivity, mouth ulcers, rash, thromboembolism with positive serological markers for autoimmune disease
  - livedo reticularis, particularly with presence of symptoms/laboratory markers of other systemic autoimmune diseases or mild thrombocytopenia
  - cognitive dysfunction, valvular heart disease with presence of evidence of other systemic autoimmune diseases, aPL-associated nephropathy
  - Patients with undifferentiated connective tissue diseases (mainly SLE-like) to identify asymptomatic carriers and to characterize them in order to prevent vascular events [84]
  - Patients of younger age (<50 years) following provoked VTE when the provoking environmental factor is disproportionately mild
  - Patients with unexplained prolonged aPTT as an incidental finding

may hopefully benefit harmonization in LA testing and comparability between laboratories [14]. Table 2 summarizes the test procedure for LA.

Inherent to the methodology of functional assays based on clotting tests, LA testing is influenced by anticoagulant therapy. Although discouraged to test during anticoagulant therapy, in daily practice testing for LA happens frequently in these patients [14,27]. Heparins, vitamin K antagonists and direct oral anticoagulants (DOAC) can result in false LA conclusions. There are some tools to overcome the interference, for instance by removal of DOAC or sampling at distance from the administration of the last dose of low molecular weight heparin, or the

**Table 2**

Test procedure for lupus anticoagulant (LA) [14].

### Blood collection/pre-analytical factors

- incorporation of information on the patient's anticoagulation in the request is mandatory
- be aware that elevated CRP may give false positive LA results

### Choice of test/test procedure

- Two tests based on different principles
- dRVVT should be the first test considered
- The second test should be a sensitive aPTT (suitable PL composition and low concentration) and preferably silica as activator)
- LA should be considered positive if one of the two test systems gives a positive result in the three steps (screen-mix-confirm)
- Screening tests are performed with dRVVT and aPTT, and regarded to be positive if the normalized clotting time is prolonged beyond the locally established cut-off
- Mixing test in a 1:1 proportion of patient: PNP should be used, without pre-incubation
- A mixing test with screening reagent is performed if the screening test(s) on undiluted sample is prolonged
- Results of mixing test are suggestive of LA when the normalized clotting time is greater than the local cut-off value
- Confirmatory test(s) must be performed by increasing the concentration of PL used in the screening test(s). Bilayer or hexagonal (II) phase PL should be used to increase the concentration of PL.
- Confirmatory test to be performed if the screening test suggests LA presence, irrespective of the result of the mixing test with screening reagent
- Confirmatory test is performed on a mix of 1:1 PP and PNP if the confirmatory clotting time is prolonged

### Interpretation

- For paired test LA ratio (screen/confirm) expressed as normalized ratio is calculated
- Or the percentage correction [(screen-confirm)/screen x 100]
- Results are suggestive for LA if LA ratio (screen/confirm or screen mix/confirm mix) or percentage correction is above the 99th centile
- Some of the integrated tests are designed to measure a difference in clotting times on a mixture of plasma

### Expression of results

Results should be expressed as ratio of patient-to-PNP run in parallel with the test plasma for all procedures (screening, mixing and confirm)

### Cut-off values

- Use in-house cut-off values, do not use cut-off values established elsewhere
- Calculate 99th centile on at least 120 normal samples with outlier detection for all normalized ratios
- Alternatively, transference of the manufacturer's cut-off values after verification is possible, if manufacturers provide cut-off values established in accordance with guidelines and by appropriate statistical models using a sufficiently large donor population

### Post-analytical issues

- It is imperative that testing be repeated after an initial positive result on a second occasion after 12 weeks

### Report of results

- LA is reported with a final conclusion as positive/negative
- Comments such as borderline or dubious LA are highly discouraged and in these cases the comment should be "suggest re-testing after one week or more", without suggesting positive or negative LA
- Along with the analytical results for the three steps, local cut-off values must be reported
- A report with an explanation of the results should be given
- Results should always be related to the results of aCL and  $\text{a}\beta_2\text{GPI}$  to assess the risk profile
- Results should be interpreted in a clinical context and knowledge of ongoing treatment

Information provided in the request on the patient's anticoagulation status should also be incorporated into the report

- A close interaction between the laboratory and the clinician is essential

use of alternative tests such as taipan snake venom-ecarin clotting time, but all have their limitations. Information on the patient's anticoagulant status should be provided by the clinician and integrated in the report on LA. If any doubt exists on dubious LA results, the laboratory should include a comment on the result and warn for potential interferences [14,21,27].

C-reactive protein (CRP) interferes with PL in the reagents of the PL-dependent clotting assays used for LA. Elevated levels of CRP may prolong clotting times, resulting in false positive LA result [14]. This is often observed in inflammatory patients due to infection or autoimmune inflammatory rheumatic disorders [28,29].

The three-step procedure of LA including a screening step, a mixing step and a confirmation step is performed in two test systems, the activated partial thromboplastin time (aPTT) and the diluted Russell's viper venom time (dRVVT). Results of LA are expressed as positive or negative, since insufficient evidence exists whether the strength of the LA effect (the degree of prolongation of the clotting tests) is related to the risk for thrombosis and pregnancy morbidity. Isolated positivity of LA, in absence of aCL and  $\text{a}\beta 2\text{GPI}$ , is regarded as a lower risk factor [4]. We observed that LA positivity, expressed as normalized ratio, was weaker in isolated LA-positive patients compared with LA activity in triple-positive patients, suggesting that stronger LA better corresponds with a high risk profile [30]. Attempts have been made to quantify or measure LA by other methods such as thrombin generation assays [31–33]. It is too premature to use this method in daily practice by lack of standardization [34]. So far, LA is regarded positive if the three steps within at least one test system have a normalized ratio above the local cutoff value.

#### 4. Anticardiolipin and anti- $\beta 2$ glycoprotein I antibodies

aCL and  $\text{a}\beta 2\text{GPI}$  are detected by solid phase assays. In contrast to LA detecting all functional aPL, with solid phase assays one group of antibodies is detected, depending on the coating of the solid phase: antibodies binding to  $\beta 2\text{GPI}$  complexed with cardiolipin, or antibodies binding directly to the  $\beta 2\text{GPI}$  will be detected. aCL have been recognized to play a role in thrombosis and abortion, and led to the first description of the so-called anticardiolipin syndrome in 1985, later on called the APS with persistent positivity of LA and aCL as necessary conditions [35]. The  $\text{a}\beta 2\text{GPI}$  antibodies were introduced during the Sydney consensus conference on APS in 2006 [1].

Recommendations on how to measure aPL with solid phase assays emphasize on technical aspects and interpretation [13]. Inherent to the methodology of immunological assays, these assays are not prone to interference of anticoagulant therapy or acute phase proteins. Recommendations on how to perform the tests, cannot prevent that differences (e.g. in calibrators, type of solid phase, coating of the solid phase, source of  $\beta 2\text{GPI}$ ) exist amongst the large variety of commercial and in-house assays, leading to inter-assay variation. Reference materials have been developed over time such as Harris/Louisville standards and Koike monoclonal antibody standards in an attempt to introduce an international standard for the aCL assays. Patient-derived material is finite, and may have batch-to-batch variation. An alternative are monoclonal antibodies having indefinite production and reproducibility over time, but may not be representative for the reactivity of patient's polyclonal aPL, and are not commercially available anymore. Human monoclonal antibodies derived from APS patients can offer an alternative [3]. Recently, a patient derived reference material for  $\text{a}\beta 2\text{GPI}$  have been developed but not available yet [36]. As there is no "golden" standard or international reference material for measuring these antibodies, comparison of results between kits remains very difficult.

Besides differences in titers, also differences in semi-quantitative expression of results are observed, as illustrated in external quality control exercises by users ascribing a different classification into ranges of low-medium-high to an identical numerical test result [37,38]. Semi-quantitative reporting may harmonize inter-laboratory

interpretation. Although useful for the clinician and making positive and negative results interchangeable between different systems, semi-quantitative results are difficult to define. Awaiting recommendations how to define thresholds for low-medium-high positivity, each test result above the cut-off value should be regarded as positive [2].

Moreover, also sensitivity and specificity between solid phase assays differ [39,40]. 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.

The aCL and  $\text{a}\beta 2\text{GPI}$  are most commonly detected by ELISA. Recently, solid phase assays with various detection systems have been introduced using a variety of solid phase (magnetic particles, microbeads, membranes, and coated polystyrene cups), and various detection systems (chemiluminescence, flowcytometry, and multiplex systems). These latter have the advantage offering more harmonized working conditions compared to manually performed ELISA [41]. Newer automated systems and ELISA show comparable sensitivity, although antibody titers in chemiluminescent techniques are much higher compared to ELISA [37,39]. This raises the issue of classification into low-medium-high positive, especially for aCL where a medium high titer (40 GPL/MPL) is used as formal classification criterion in the Sydney criteria [1].

Classification of samples into positive or negative depends on the cutoff values. In the Sydney criteria cutoff values for  $\text{a}\beta 2\text{GPI}$  positivity at a medium titer was set at the 99<sup>th</sup> percentile for normal subjects determined by standardized ELISA [1]. For aCL cutoff value was set at 40 GPL or MPL or the 99<sup>th</sup> percentile. But as described above, because of the inconsistency in titers between assays in various commercial kits it is advisable that each laboratory uses the 99<sup>th</sup> percentile of a local normal population to determine the cutoff value, also for aCL [13]. High-risk patients with definite APS usually have values of aCL far exceeding 40 GPL [6]. However, lower levels of antibodies are observed in pregnancy morbidity [42–44]. The clinical relevance of titers below the 99<sup>th</sup> percentile needs to be further studied.

Both aPL, aCL and  $\text{a}\beta 2\text{GPI}$  have their diagnostic value. With methodological correct aCL assays, meaning  $\beta 2\text{GPI}$ -dependent, a high correlation between aCL and  $\text{a}\beta 2\text{GPI}$  antibodies is observed [2,39,45,46]. In this context, single aCL positivity should be repeated or checked by another type of assay. If  $\text{a}\beta 2\text{GPI}$  antibodies are negative, then aCL antibodies recognize other binding proteins different from  $\beta 2\text{GPI}$  whose clinical significance is unknown [47]. Positive  $\text{a}\beta 2\text{GPI}$  with a negative aCL may include  $\text{a}\beta 2\text{GPI}$  directed against domain 4/5 of the protein and are usually positive in the  $\text{a}\beta 2\text{GPI}$  assays (containing the whole  $\beta 2\text{GPI}$  molecule) but negative for aCL [48]. Antibodies against domain 4/5 of the  $\beta 2\text{GPI}$  are regarded non-pathogenic, compared to the aDI that are well correlated with thrombosis and obstetric complications [9,49].

In the current criteria the isotypes IgG and IgM are included [1]. IgG aCL/ $\text{a}\beta 2\text{GPI}$  show a stronger association with clinical events and is often associated with IgM positivity [50–52]. Interestingly, a multicenter study comparing four platforms, illustrated that this was independent of the solid phase platform used [50]. Isolated IgM is rare in thrombotic APS and more frequent in obstetric APS. Although, in contrast to thrombotic complications, in pregnancy morbidity IgM is an independent risk factor. Data of this multicenter study support testing for IgG and IgM, especially in women suspected for obstetric APS [50]. In thrombotic complications, IgM can be used in risk stratification since positivity of IgM, on top of IgG and LA, increases the risk [50]. For non-criteria clinical manifestations the association is mainly shown for LA and aCL IgG, except for thrombocytopenia with significant association with IgM [53–55].

Several studies confirmed that the presence of aCL and  $\text{a}\beta 2\text{GPI}$  of the same isotype reinforces the clinical probability of APS [6,40,56].

IgA aCL and  $\text{a}\beta 2\text{GPI}$  are not included in the current criteria [1,2]. Although many studies have illustrated the association between APS related clinical symptoms and the presence of aCL/ $\text{a}\beta 2\text{GPI}$  IgA, especially in SLE [57], there is no strong evidence of the added value of IgA

aPL [58,59]. IgA aPL are usually found in association with IgG and/or IgM. Isolated IgA is very rare and of unknown clinical significance since IgA aPL are in most of the cases linked to non-criteria clinical manifestations of APS [58–61]. Based on the data published until now, there is not enough evidence to recommend testing for IgA aCL and/or IgA a $\beta$ 2GPI to increase the diagnostic accuracy of the APS.

## 5. Antibody profiles

Supporting the idea that the antibody profile rather than the individual test findings defines the risk to develop thrombosis or pregnancy complications, guidelines strongly advise to classify APS patients into categories according to type and number of tests positive [1,2]. Therefore, all three tests should be performed, preferable on the same sample, and results of LA, should always be related to the results of aCL and a $\beta$ 2GPI to assess the risk profile [2,13,14].

Combined positivity for LA, aCL, and a $\beta$ 2GPI antibodies (ie, triple positivity) has been shown to be associated with a high risk of both a first event and recurrence, and for a first event in asymptomatic carriers [4, 40,62,63]. Also, in a long-term follow-up study (median of 13 years follow-up) the highest risk of first thrombosis in aPL carriers was observed in triple positive patients [64]. Double or triple positivity for aPL is a risk factor for future thrombotic events, especially in individuals with an underlying autoimmune disease, whereas single positivity does not seem to carry an elevated risk of thrombosis [63]. Compared to triple positives, the risk in double positives (aCL and a $\beta$ 2GPI) is slightly lower, and single positivity does not seem to carry a risk to develop thrombosis [63].

Triple positive patients usually have a persistent antibody profile on follow-up testing after twelve weeks [65,66]. However, double and single positive patients may be also persistent positive, as illustrated in a retrospective study illustrating no significant lower persistence in the single positive patients (93.3%) compared to the double and triple positive patients (96.8% and 97.3%, respectively) [66].

Although LA is a well-established risk factor for thrombosis in APS, the relevance of isolated LA positivity, in the absence of aCL and a $\beta$ 2GPI antibodies, has been debated [67]. In literature there is inconsistency, as some studies showed a poor predictive value for a first thrombotic event for isolated LA, and other studies suggested a strong predictive value [63,68,69]. Also, isolated LA is predictive for adverse pregnancy outcome [70,71]. Recently, a prospective observational study found that in a LA positive population the association between occurrence of thrombosis and inferior survival was independent of the detection of aCL and a $\beta$ 2GPI [72]. A retrospective multicenter study illustrated that isolated LA proved to be strongly associated with vascular thrombosis even though with a weaker LA activity compared with LA activity in triple positive patients [30]. The majority of these patients was negative for aPS/PT (see also further on). Isolated LA positivity implies that these patients are negative for aPL binding through  $\beta$ 2GPI or prothrombin as cofactor, and further studies are needed to identify the target antigen for the antibodies responsible for isolated LA [30,73].

Isolated aCL reflect antibodies either different from those binding through  $\beta$ 2GPI or directed against cardiolipin itself. Non-cofactor related aCL should be avoided since the clinical relevance in humans is very limited and related to infections or drugs [46,73]. Equally, isolated a $\beta$ 2GPI show no association with thrombosis and are directed against epitopes not determining LA activity [48,73].

For the interpretation of the antibody profile, we should be aware of the methodological shortcomings of the solid phase assays as well as the clotting tests used for LA [21,22]. Retesting with another type of solid phase assay may be helpful when clinical suspicion is high for APS, and results of aPL are not in line with what is expected. Non-criteria aPL (see next paragraph) may be useful especially in patients with incomplete antibody profiles (double or single positives) [47].

## 6. Non-criteria antiphospholipid antibodies: anti-domain I and ant phosphatidyl serine/prothrombin antibodies

Lately, among the 'non criteria' aPL anti-phosphatidylserine/prothrombin antibodies (aPS/PT) and antibodies towards the domain I of  $\beta$ 2GPI (aDI) have been frequently studied. These antibodies are not included in the current classification criteria, but can be valuable in risk stratification.

aDI antibodies are a subgroup of a $\beta$ 2GPI antibodies, directed against the domain one of the five domains containing protein. Antibodies recognizing a specific epitope (G40-R43) within the domain I have been illustrated to have a high association with thrombosis. However, the different methods to detect aDI IgG differ in specificity for this subgroup of antibodies. A commercial chemiluminescence based assay (only available for IgG) have been evaluated in many studies with various results regarding association with thrombosis and pregnancy morbidity [74]. The original in-house ELISA test measured a more specific population of aDI directed against the G40-R43 epitope, compared the commercial aDI probably measuring all aDI antibodies against any epitope on domain I of  $\beta$ 2GPI [74]. Studies looking into detail to the added value of aDI by adding the aDI, or replacing the a $\beta$ 2GPI in the current aPL panel, could not illustrate higher odd ratios or area under the curve for thrombosis, nor could indicate aDI as an independent risk factor [9,75], except in one prospective study [12].

aDI antibodies are frequently detected in the high-risk patient populations, defined as triple positive patients with positivity for LA, aCL and a $\beta$ 2GPI. Also, titers of aDI in these patient groups are higher [9,11, 49,76,77]. The high correlation between aDI and triple positivity in obstetric and thrombotic patient populations, confirms the patients at higher risk for clinical events in APS [9,77]. aDI are significantly associated not only with thrombosis, but also with late pregnancy morbidity, while positive anti-domain 4/5 antibodies are not predictive of thrombosis or pregnancy morbidity [11].

aDI are useful to prove the specificity of the a $\beta$ 2GPI antibodies in particular in patients with an incomplete antibody profile defined as double positive patients (aCL and a $\beta$ 2GPI positive) or single LA or single a $\beta$ 2GPI positive patients. Testing for aDI in these patients could confirm or exclude the association of pathogenic a $\beta$ 2GPI autoantibodies [47].

A review on the role of aPS/PT antibodies in thrombotic APS patients summarizes their role in addition to the criteria aPL. aPS/PT increased the risk of thrombosis and seemed to represent a strong risk factor for thrombosis, both arterial and/or venous. Measurement of aPS/PT might be useful in establishing the thrombotic risk of patients with previous thrombosis and/or systemic lupus erythematosus [78]. Patients and asymptomatic carriers with triple positivity, a marker of clinical severity, show persistently positivity for aPS/PT, making these antibodies effective as part of risk scoring [79]. Equal as for aDI, in triple-positive patients, titers of aPS/PT are higher than in double or single-positive patients [79,80]. Since aPS/PT antibodies are strongly correlated with LA [81,82], these antibodies might be a surrogate for LA, in conditions where LA assays show methodological shortcomings as in anticoagulated patients [47,83]. However, not all single LA positive patients are aPS/PT positive [30].

Similarly, as for aCL and a $\beta$ 2GPI antibodies, a standardized ELISA for aPS/PT is not available and reference sera are lacking. Automated systems are not available yet, and a limit number of commercial ELISA's for aPS/PT is available. Search for aPS/PT in daily practice is not recommended yet. However, in some conditions aPS/PT might help, especially to confirm the risk. Adding aPS/PT to the triple positive profile, further consolidates the diagnosis of APS. When the aPL profile shows double positivity, positive aPS/PT may suggest a false negative LA, and when aDI and aPS/PT are negative, this indicates a lower risk for thromboembolic events [47].

## 7. Conclusion

The aPL play a crucial role in the diagnosis of APS. However, the laboratory diagnosis of APS remains challenging. Progress has been made to address some of the methodological challenges of all tests currently available, but an international reference material to overcome the inter-assay and inter-laboratory variation is still lacking. To obtain optimal performance, all assays have to be performed according to the guidelines. All three assays, LA,  $\beta$ 2GPI-dependent aCL, a $\beta$ 2GPI IgG, and IgM should be performed at the same time to increase diagnostic utility, with an integrated interpretation of all results and an interpretative comment. Making antibody profiles including LA, aCL and a $\beta$ 2GPI help identify patients at risk. Confirmation of a positive result after 12 weeks is required since only persistently positive results are clinically relevant in the context of APS. Results should be interpreted in a clinical context and knowledge of the patient's anticoagulation status. A report with an explanation of the results should be given with warning for interferences. Other aPL, such as aDI and aPS/PT are not included in the current classification criteria, but may help to identify the patients at risk. For optimal interpretation of results a close interaction between the laboratory and the clinician is mandatory.

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The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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