

An update on laboratory detection and interpretation of antiphospholipid antibodies for diagnosis of antiphospholipid syndrome: guidance from the ISTH-SSC Subcommittee on Lupus Anticoagulant/Antiphospholipid Antibodies

Katrien M. J. Devreese¹  | Maria Laura Bertolaccini²  | D. Ware Branch³  |
Bas de Laat⁴  | Doruk Erkan⁵  | Emmanuel J. Favaloro⁶  | Vittorio Pengo⁷  |
Thomas L. Ortel⁸  | Denis Wahl⁹  | Hannah Cohen^{10,11} 

¹Coagulation Laboratory, Department of Laboratory Medicine, Ghent University Hospital, Department of Diagnostic Sciences, Ghent University, Ghent, Belgium

²Academic Department of Vascular Surgery, School of Cardiovascular and Metabolic Medicine & Sciences, King's College London, London, United Kingdom

³Department of Obstetrics and Gynecology, University of Utah, Salt Lake City, Utah, USA

⁴Synapse Research Institute, Maastricht, the Netherlands

⁵Barbara Volcker Center for Women and Rheumatic Diseases, Hospital for Special Surgery, Weill Cornell Medicine, New York, New York, USA

⁶Department of Haematology, Sydney Centres for Thrombosis and Haemostasis, Institute of Clinical Pathology and Medical Research, NSW Health Pathology, Westmead Hospital, Westmead, New South Wales, Australia

⁷Thrombosis Research Laboratory, Department of Cardio-Thoracic-Vascular Sciences and Public Health, University of Padova, Padova, Italy

⁸Division of Hematology, Departments of Medicine and Pathology, Duke University School of Medicine, Durham, North Carolina, USA

⁹Vascular Medicine Department, Reference Center for Rare Systemic Autoimmune and Autoinflammatory Diseases, Nancy University Hospital, Institut national de la santé et de la recherche médicale, University of Lorraine, Nancy, France

¹⁰Department of Haematology, Cancer Institute, University College London, London, United Kingdom

¹¹Department of Haematology, University College London Hospitals NHS Foundation Trust, London, United Kingdom

Correspondence

Katrien M. J. Devreese, Coagulation Laboratory, Ghent University Hospital, Corneel Heymanslaan 10, 9000 Gent, Belgium.

Email: Katrien.devreese@ugent.be

Abstract

Antiphospholipid syndrome (APS) diagnosis is dependent on the accurate detection and interpretation of antiphospholipid antibodies (aPL). Lupus anticoagulant (LA), anti-cardiolipin antibodies (aCL), and anti-beta2 glycoprotein I antibodies (aβ2GPI) remain the cornerstone of the laboratory part of APS diagnosis. In the 2023 American College of Rheumatology (ACR)/European Alliance of Associations for Rheumatology (EULAR) APS classification criteria, the type of laboratory parameters remain essentially unchanged compared with the updated Sapporo classification criteria, and aCL and aβ2GPI measurement are still restricted to enzyme-linked immunosorbent assays (ELISAs) with moderate and high titer aPL thresholds defined as 40 and 80 Units, respectively, and a cutoff calculated by the 99th percentile has been abandoned. We

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must differentiate between classification criteria and assessment of aPL in clinical care. Classification criteria are strict and meant for participant inclusion in studies and trials to study homogeneous populations of patients. In contrast, laboratory detection for APS diagnosis in daily practice is broader, meant to diagnose each APS patient to optimize their management. Nowadays, there is increasing use of measurement of aPL by methods other than ELISAs, the semiquantitative reporting of titers is a matter of debate, as well as the role of the isotypes immunoglobulin (Ig)M and IgA, and the role of other aPL, such as antiphosphatidylserine (aPS)/prothrombin (PT) antibodies. Patients diagnosed with the disease may or may not fulfill the classification criteria, and inappropriate use of classification criteria may lead to mis(under)diagnosis. The aim of this guidance, based on literature and expert opinion, is to provide guidance recommendations for laboratory workers and clinicians on routine diagnostic assessment of patients with suspected APS.

KEYWORDS

anti- β 2-glycoprotein I, anticardiolipin, antiphospholipid syndrome, diagnosis, lupus anticoagulant

1 | INTRODUCTION

Antiphospholipid syndrome (APS) is a thrombo-inflammatory disease driven by antiphospholipid antibodies (aPL). By recognizing phospholipid surfaces and phospholipid-binding proteins, aPL can induce thrombosis, pregnancy morbidity, and other autoimmune and inflammatory manifestations [1]. The clinical features are not specific for APS, and thus, APS diagnosis is dependent on accurate detection and interpretation of aPL.

Since the 2018 International Society on Thrombosis and Haemostasis (ISTH) Scientific and Standardization Committee on Lupus Anticoagulant/Antiphospholipid Antibodies (ISTH-SSC LA/aPL) Subcommittee guidance recommendations on APS laboratory criteria [2], more information and new insights have emerged [3]. These include studies on antiphosphatidylserine (aPS)/prothrombin (PT) and anti-domain I β 2-glycoprotein I (β 2GPI) antibodies (aDI) antibodies, increasing use of non-enzyme-linked immunosorbent assays (ELISAs), semiquantitative reporting of titers, and the role of immunoglobulin (Ig) M and IgA isotypes. Also, new 2023 American College of Rheumatology (ACR)/European Alliance of Associations for Rheumatology (EULAR) classification criteria for APS have been published [4] in which persistent lupus anticoagulant (LA), anticardiolipin (aCL), and anti- β 2GPI (a β 2GPI) remain the cornerstone of the laboratory criteria, as in the revised Sapporo (Sydney) classification criteria [5], and measurement of aCL and a β 2GPI remain restricted to ELISA. In addition, moderate and high titer thresholds are set at 40 Unit (U) and 80 U levels, respectively, with cutoffs calculated by the 99th percentile abandoned [4]. The 2018 ISTH-SSC LA/aPL guidance recommendations had advised against fixed cutoff values for non-ELISA assays [2].

The distinction between the detection and interpretation of aPL for classification vs use in routine clinical care is of critical importance. Classification criteria enhance the specificity of APS diagnosis and

improve the quality of APS research by identifying homogeneous populations of patients for inclusion in studies. Classification criteria are distinct from the detection and interpretation of aPL in the day-to-day clinical setting aimed at optimizing APS diagnosis and patient care [4]. The goal is to provide guidance recommendations for laboratory workers and clinicians on routine diagnostic assessment of patients with suspected APS.

2 | LUPUS ANTICOAGULANT

LA positivity is mandatory to diagnose and identify high-risk triple aPL-positive patients [2] and informs the prognosis and management of thrombotic and obstetric APS [6,7]. Persistent single-positive LA has lower thrombotic risk than triple aPL positivity [8–11]. Current ISTH guidance for LA detection and interpretation addresses methodology, choice of assays, cutoff values, calculation and interpretation of results, and timing of testing in relation to thrombosis and pregnancy [12]. Key points are highlighted below.

2.1 | Methodology

Guidance recommendations on how to test for LA are summarized in a guidance document of the ISTH-SSC LA/aPL Subcommittee [12]. ISTH guidance recommends 2 parallel tests, with a dilute Russell's viper venom time (dRVVT) and an LA-sensitive, activated partial thromboplastin time (APTT) as assays of choice. The corresponding test-specific mixing and confirmatory test is performed in response to prolonged screening tests to confirm or refute the presence of a phospholipid-dependent inhibitor [12]. A situation of positive screening, negative mixing, and positive confirm step can only be

picked up by performing the screen followed by simultaneous mix and confirm in all samples with a prolonged screening test, as advised in the 2020 LA guidance [12]. LA should be interpreted as absent, with a comment on repeat testing and potential false negatives when tested during vitamin K antagonist (VKA) therapy or other reasons for coagulation factor deficiency.

The combination of APTT and dRVVT has been proven to have high LA detection rates and is widely used in most diagnostic laboratories [13]. A recent study showed that omitting either the APTT or dRVVT increases the risk of underdiagnosis in up to 55% of triple aPL-positive samples (APS and aPL carriers) and 31% of APS patients [14]. Furthermore, many differences in test results among dRVVT and APTT reagents are observed [15]. There is also a paucity of paired reagents for APTT-based testing; thus, the majority of laboratories use an APTT screening assay combined with an alternative method for APTT as confirmation [15]. Whether single (dRVVT or APTT) or double (dRVVT and APTT) LA test positivity in triple aPL-positive patients contributes to a higher risk should be further investigated [16].

Other tests, such as Taipan snake venom time/ecarin time (TSVT/ET), exhibit high sensitivity to LA in triple aPL-positive non-anticoagulated patients [17]. The added value of TSVT/ET in patients negative for APTT or dRVVT merits further investigation [17].

2.2 | LA detection during anticoagulant therapy

A major challenge in LA testing is the interference of anticoagulation, resulting in false positives or false negatives [12,18]. Therefore, it is discouraged to test for LA during anticoagulation. However, in APS, stopping anticoagulation for LA testing may not be achievable, while establishing the aPL profile may be desirable for risk stratification and treatment guidance [19]. Strategies to circumvent these issues include the removal of anti-Xa or anti-IIa direct oral anticoagulant (DOAC) [12]. Commercial DOAC removal agent availability might be limited in some parts of the world, with activated charcoal an inexpensive and easily accessible alternative, albeit possibly with more heterogeneous effects [20,21]. As sample pretreatment might influence clotting times of APTT and dRVVT tests, and DOAC removal may be incomplete, LA testing pre-/postprocedure can inform the interpretation of results [21,22]. In case of persisting positive clotting test results after treatment with a DOAC removal agent, residual DOAC interference cannot be ruled out. DOAC measurement can be limited to these samples with persistent, prolonged clotting times (LA positive) measured on the sample that underwent the DOAC removal procedure. Comparing clotting test results before and after DOAC adsorption can prevent misinterpretation due to effects on baseline clotting times by the DOAC removal agent. Minor changes in LA assay results after adding DOAC removal agents have been observed and can lead to altered interpretation in both directions (negative to positive and *vice versa*), especially in samples with clotting times around the

cutoff value. Also, the use of DOAC removal agents should be limited to samples from patients known to be on a DOAC.

Sampling during the trough period of low-molecular-weight heparin (LMWH) or DOAC is an alternative [12]. However, even with low DOAC levels, there is no guarantee of a lack of DOAC effect on LA testing [21]. The use of TSVT/ET in patients on warfarin/other VKAs or anti-Xa DOACs is another alternative [12,23].

Heparin (unfractionated or LMWH) affects LA tests, especially the APTT, as the dRVVT reagents contain heparin neutralizers, quenching heparins up to 1 anti-Xa IU/mL. Applying the 3-step method (screening, mixing, and confirmation steps) makes LA testing by APTT and dRVVT reliable in the presence of unfractionated heparin and LMWH if the anti-Xa activity level is within the therapeutic range [12,24].

LA testing during VKA therapy remains the foremost challenge because removal agents cannot overcome the effect of VKAs. VKAs are prone to false negatives due to a negative mixing step and false positives due to a positive mixing test at high anticoagulation intensity for both dRVVT and APTT [25,26]. Mixing tests in the confirmation step improves diagnostic efficacy in situations with prolonged clotting times due to factor deficiencies as met in VKAs [12]. An ISTH multicenter study confirmed no interference of the TSVT/ET in VKA or anti-Xa DOAC-treated patients [23]. However, the TSVT/ET assay is not widely used [27], with the low availability of commercial kits being a limitation. The TSVT/ET test panel has a large but incomplete overlap with the recommended dRVVT and APTT test panels, with a positive result being diagnostic and a negative result not excluding LA [17,23]. This means TSVT/ET could be an additional assay to dRVVT and APTT, specifically for patients during VKA once standardized reagents are more widely available.

2.3 | Result reporting and interpretation

Interpretation of LA positivity or negativity depends on the cutoff values, which depend on the assay and the local laboratory conditions. Cutoffs should be calculated in-house and by a nonparametric method set at the 99th percentile to optimize specificity [12]. Results on cutoff values are variable [28–30]; thus, further efforts are needed to establish cutoff values per reagent/instrument combination in a multicenter setting.

LA is reported qualitatively (positive/present or negative/absent) without grading, based on the combination of the normalized results of the screening, mixing, and confirmatory steps [12]. Previous studies indicated that quantification of LA, for instance, by thrombin generation (TG), is only partially informative in the prediction or exclusion of thrombosis in APS patients [31]. The normalized ratio in the screening APTT and dRVVT test was weaker in isolated LA-positive APS patients compared with triple-positive patients, suggesting that stronger LA correlates better with a high-risk profile [8]. Further studies are needed on the potential utility of LA strength in risk assessment.

3 | ANTICARDIOLIPIN AND ANTI-BETA2-GLYCOPROTEIN I ANTIBODIES

aCL and a β 2GPI are measured with immunologic solid phase assays in which cardiolipin or β 2GPI is presented on a solid phase. Both tests recognize β 2GPI-dependent aPL, with aCL assays detecting antibodies against cardiolipin immobilized in the solid phase carrier in the presence of β 2GPI and a β 2GPI assays with β 2GPI immobilized on the solid phase. However, aCL immunoassays detect an array of autoantibodies, including cofactor-dependent and cofactor-independent antibodies [32]. Available data generally show significant overlap between the 2 assays with comparable sensitivity and specificity [33,34]. Pathophysiological mechanisms of APS warrant the detection of β 2GPI-dependent antibodies [1]. A meta-analysis confirmed the role of aCL as a marker for an increased risk of thrombosis [35]. Conversely, a systematic review found little added diagnostic value for a β 2GPI, with a weak association with thrombosis and an inconsistent association with obstetric morbidity [36]. The major limitation of this review was the different methodologies and study populations throughout the studies, indicating that the value of a β 2GPI as a risk factor should be reinvestigated using a standardized approach. This ISTH-SSC LA/aPL Subcommittee believes that the result of each assay reinforces that of the other and allows for the consideration of the aPL profile.

3.1 | Methodology

Guidance recommendations on how to test for aCL and a β 2GPI by ELISA and non-ELISA solid phase assays are summarized in a guidance document of the ISTH-SSC LA/aPL Subcommittee [37]. In many laboratories, ELISA testing has been replaced by different platforms measuring aCL and a β 2GPI [38,39]. Fluorescence enzyme immunoassay (FEIA), chemiluminescent immunoassay (CLIA), and multiplex flow immunoassay (MFI) are the most frequently used non-ELISA methods [38]. Automated systems have the advantages of performance simplicity and protocol harmonization, which reduce inter-laboratory variation [40,41]. Automated systems also perform aCL and a β 2GPI assays more rapidly, are less prone to human error, and are less labor-intensive compared with running multiple ELISAs. Providing all results simultaneously by one test system increases the diagnostic value [34].

3.2 | Agreement between assays

There is variability in the results of immunologic assays, ELISAs and non-ELISAs, as well as in classifying samples as positive or negative and by antibody levels. Clinical studies, external quality assessment exercises, surveys, and experiments using human monoclonal antibodies with different reactivity toward β 2GPI demonstrate differences in ELISAs as well as automated systems [34,41–45]. Lack of standardization remains

a limitation as no “gold standard” or universal international reference material for measuring aCL and a β 2GPI is available. Various reference materials have been developed, with none universally adopted [46]. Patient-derived material may exhibit batch-to-batch variation, and monoclonal antibodies may not be representative of the reactivity of the patient’s polyclonal aPL or be positive in all aPL assays. Patient-derived reference material for a β 2GPI has shown a good correlation between results when measured with 7 different ELISAs [47].

Qualitative agreement (positive/negative) is suboptimal [34], and daily practice shows that a sample positive in one assay does not automatically test positive with another assay or if tested in another laboratory using the same assay. In patients with high clinical suspicion of APS and when aPL results are not what is expected, testing with another platform may be useful, as it might be when discrepant results are obtained between aCL and a β 2GPI. Pathologic aCL are β 2GPI-dependent, thus, the combination of positive aCL and negative a β 2GPI can indicate that none or other cofactors are involved [32,48] or a lower a β 2GPI titer that is not detected. Conversely, positive a β 2GPI with negative aCL may represent a β 2GPI directed against domain 4/5 of the protein regarded as nonpathogenic in contrast to the antibodies directed against domain I of β 2GPI (aDI) that correlate with thrombosis and obstetric complications [49,50].

3.3 | Titers and cutoff values

Reported titers are derived from the calibration curves in each assay system and differ between systems [37,51]. This results in dissimilar titers obtained by different systems, with much higher antibody titers in newer automated systems compared with ELISA [34,52]. This raises the issue of how to categorize results into the semiquantitative sets of low, moderate, or high positive. Note that a single threshold for moderate-high positive ELISA titer is used as a formal classification criterion in the Sydney (>40 U or >99th percentile) classification, while the 2023 ACR/EULAR criteria propose a threshold for moderate and high ELISA positivity (40/80 U, respectively) [4,5]. The 2018 ISTH guidance discouraged semiquantitative grouping while assessing aCL and a β 2GPI results for diagnostic reasons because one numerical value cannot be recommended as a general criterion for moderate or high positive as this threshold is dependent on the method used [2]. The 2023 ACR/EULAR classification criteria regard levels below 40 U but higher than the cutoff value as low or weakly positive and thus insufficient for APS classification, even with an appropriate clinical profile. We strongly advise that each laboratory uses the 99th percentile of a local normal population to determine the cutoff value for both aCL and a β 2GPI [37], and we acknowledge that high-risk patients with definite APS usually have values of aCL/a β 2GPI far exceeding 40 U, measured with any method. Of note, lower levels of aCL/a β 2GPI (>95th and <99th percentile) have been reported in patients with pregnancy morbidity [53], and their clinical relevance needs further study, especially using the updated pregnancy morbidity definitions included in the 2023 ACR/EULAR APS classification criteria.

3.4 | Result reporting

The recommendation in this guidance is to report aCL and $\text{a}\beta 2\text{GPI}$ results with their numerical value along with the local cutoff value calculated by the 99th percentile [2]. Indicating whether this titer can be considered as low, moderate, or high positive would be informative for the clinician. However, external quality assessment programs show that semiquantitative classification into ranges of low-moderate-high differs between platforms and users [42], with efforts to harmonize semiquantitative reporting ongoing [54,55].

The Harris 40/80 GPL/MPL (IgG phospholipid units/IgM phospholipid units) threshold for moderate and high aCL IgG/IgM positivity for ELISA [56] was critically heightened by calculating likelihood ratios (LRs) in a large multicenter cohort [55], with a positive LR of a test result representing how many times more likely it is that the result occurs in a patient with rather than without that disease. The 40/80 U threshold, vs 20 to 40 U, was confirmed as being useful for the semiquantitative interpretation of aCL and $\text{a}\beta 2\text{GPI}$ IgG ELISA in thrombotic and obstetric APS [55]. However, this phenomenon was not observed for the 40/80 U IgM ELISA threshold in this cohort [55]. Even higher LRs were observed when using a threshold of 80 U for aCL IgG. The same trend was observed in the thrombotic, but not obstetric, test population for $\text{a}\beta 2\text{GPI}$ IgG [55]. aCL IgM and $\text{a}\beta 2\text{GPI}$ IgM LRs did not increase with higher thresholds, suggesting that semiquantitative reporting for IgM has less impact on the increasing probability of APS [55].

Different approaches can identify corresponding thresholds for low, moderate, and high positivity for non-ELISA platforms. A clinical approach applying receiver operating characteristic analysis can be used, but this requires a large patient cohort of APS and non-APS patients [55,57]. The ELISA thresholds (40/80 U) were translated to an MFI and CLIA platform by determining the thresholds with equal clinical sensitivity for APS. Receiver operating characteristic sensitivity-based thresholds for CLIA and MFI were from 5 up to 64 times higher compared with 40 and 80 U with ELISA. Thresholds were different for aCL and $\text{a}\beta 2\text{GPI}$ and for IgG and IgM isotypes. For aCL IgM and $\text{a}\beta 2\text{GPI}$ IgM, comparable values for ELISA and non-ELISA CLIA and MFI were observed [55]. With these adapted thresholds, increasing LRs were confirmed for aCL and $\text{a}\beta 2\text{GPI}$ IgG in thrombotic and obstetric APS. Better harmonization of semiquantitative interpretation of aCL and $\text{a}\beta 2\text{GPI}$ between ELISA and other analytical platforms could be achieved via this clinical approach [55]. Besides sensitivity-based LRs [55], specificity-based thresholds or LRs at predefined intervals can be calculated [58]. Although specificity and sensitivity at the cutoff value varied between CLIA, ELISA, FEIA, and MFI aPL assays, interval-specific LRs for identical aPL were comparable across the 4 systems. With test result-specific LRs, relevant differences in clinical significance between low antibody levels and higher antibody levels were confirmed, again more obvious for IgG and in thrombotic APS [58].

Another more accessible approach to define platform-specific thresholds is analyzing a dilution series of reference material in parallel by ELISA and a non-ELISA assay, calculating corresponding thresholds through linear regression analysis [55]. In a multicenter cohort, better agreement in semiquantitative classification was

obtained by applying the corresponding thresholds derived from the linear regression analysis by monoclonal standards compared with the patient-derived reference material [55].

Assay manufacturers could help by providing the thresholds for moderate/high levels of aPL for their system on the condition that besides the reference material, a large representative patient population has been tested with ELISA and their method in parallel to illustrate that the adapted thresholds correlate with a higher positive LR. Further studies are needed to explore whether a transfer of thresholds can be applied in analogy with the transfer of reference values.

3.5 | Isotype

aCL and $\text{a}\beta 2\text{GPI}$ IgG and IgM isotypes are included in the classification criteria [4,5]. In the 2023 ACR/EULAR APS classification criteria, a low weight is attributed to isolated aCL IgM or $\text{a}\beta 2\text{GPI}$ IgM, and further research was suggested on the value of single IgM in patients with clinical symptoms of APS [4]. IgG aCL/ $\text{a}\beta 2\text{GPI}$ show a stronger association with clinical events and are often associated with IgM positivity [59–61]. The role of IgM may differ in thrombotic and obstetric APS [53]. Isolated aCL or $\text{a}\beta 2\text{GPI}$ IgM is rare in thrombotic APS and more frequent in obstetric APS, and there is evidence that aCL and $\text{a}\beta 2\text{GPI}$ IgM are independent risk factors [59]. Thus, in women with suspected obstetric APS, aCL and $\text{a}\beta 2\text{GPI}$ IgM and IgG should be tested. In suspected thrombotic APS, first-line testing for IgM is not strictly necessary but may be useful in risk stratification since positivity for IgM, in addition to positivity for IgG and LA, increases the thrombotic risk [59]. Further studies are needed in specific patient populations, such as patients with stroke, where isolated IgM might be more frequent [62]. Thrombocytopenia, included in the ACR/EULAR classification criteria, is significantly associated with IgM [63]. 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 [45,64]. A multicenter study confirmed that odds ratios for 3 out of the 4 tested platforms are higher for triple aPL positivity with concordance of isotype compared with triple aPL positivity, including combinations of aCL and $\text{a}\beta 2\text{GPI}$ irrespective of isotype [45]. Future studies should address the role of isolated IgM positivity in patients meeting the clinical 2023 ACR/EULAR classification criteria.

There remains insufficient evidence to include aCL and $\text{a}\beta 2\text{GPI}$ IgA for diagnostic testing in APS [4,5]. There is an association between APS-related clinical manifestations and aCL/ $\text{a}\beta 2\text{GPI}$ IgA, especially in systemic lupus erythematosus (SLE) [65]. However, the added value of IgA is unclear, particularly because isolated IgA is rare [65,66].

4 | ANTI-PHOSPHATIDYLSERINE/PROTHROMBIN ANTIBODIES

It is widely accepted that antiprothrombin and $\text{a}\beta 2\text{GPI}$ are the major autoantibodies responsible for LA activity [67,68]. Antiprothrombin antibodies have low diagnostic or predictive value for thrombotic or

obstetric APS manifestations compared with aPS/PT [69]. A systematic review confirmed that aPS/PT represents a strong risk factor for thrombosis in patients with previous thrombosis or SLE [70].

A meta-analysis including 1853 APS patients (thrombotic and obstetric) showed a pooled prevalence of aPS/PT IgG, aPS/PT IgM, and aPS/PT IgG and/or IgM of 50.0%, 45.0%, and 65.0%, respectively [71]. LA-positive APS patients showed an aPS/PT prevalence of 85%, with a comparable prevalence of 84% in triple aPL-positive patients. There was a significant association with thrombosis [71], also found in a multicenter study on thrombotic APS patients [10]. Existing studies are contradictory about aPS/PT as being dependent/independent of LA as a risk factor for thrombosis [10,72]. Two studies included in the meta-analysis reporting on obstetric APS showed significant odds ratios that were comparable for IgG and IgM [71], a finding that was confirmed in a multicenter study [11] but not in another meta-analysis, where no significant association could be found for IgM [70]. aPS/PT was more prevalent and showed higher titers in patients with late pregnancy loss than early pregnancy loss [11]. However, in contrast to thrombotic APS, higher aPS/PT titers did not increase the likelihood of having obstetric APS [11]. The added value of aPS/PT testing in the current diagnostic workup of obstetric APS seems limited. However, aPS/PT might be useful in specific subsets of patients with obstetric complications. Future multicenter studies are needed to elucidate the risk of less frequent, severe obstetric manifestations [11]. A study in 230 SLE patients showed that aPL positivity for aPS/PT, LA, and β 2GPI was more strongly associated with clinical events (thrombosis and/or pregnancy loss) compared with double or single aPL positivity for these aPL [73].

To assess the added value of aPS/PT to the current aPL panel, aPS/PT prevalence should be evaluated in aPL-negative (LA, aCL, and β 2GPI) patients meeting the 2023 ACR/EULAR APS clinical classification criteria. A systematic review found a low prevalence of aPS/PT (1.8% and 4.6% aPS/PT IgM and IgG positive, respectively) in a heterogeneous group of disease-control patients [74]. In aPL-negative patients with autoimmune disease without APS clinical manifestations, one study found a prevalence of 25.5% aPS/PT IgG and/or IgM, slightly higher compared with the prevalence of 12.4% for aPS/PT IgM in another cohort [10,75]. Patients with aPS/PT positivity also had a higher prevalence of APS associated with other autoimmune diseases compared with primary APS, supporting the link to autoimmune diseases [10,71]. This may question the specificity of aPS/PT for the diagnosis of APS patients and implicates potential overdiagnosis of APS when aPS/PT is included in a first-line diagnostic workup. Few studies evaluated aPS/PT in seronegative patients where there is suspicion of APS. In a multicenter study, the prevalence of aPS/PT IgG and IgM in non-APS thrombotic patients was 9% and 5%, respectively, comparable with the prevalence in a healthy population [10]. In small study populations with neurologic manifestations and cerebrovascular events, single aPS/PT positivity ranged from 1% to 15%, although with an even smaller proportion persistently positive [75,76].

Because LA testing is prone to interferences by anticoagulation, aPS/PT measured by solid phase assays has been suggested as a surrogate for LA testing. Although a large overlap exists between aPS/PT positivity and LA, available data show that this overlap is

incomplete, and therefore, LA can be missed if replaced by aPS/PT on a one-by-one basis [10,71,77]. In some conditions, aPS/PT can be useful in diagnostic testing when LA testing is unreliable or the result is uncertain [78,79]. When the aPL profile shows double aPL positivity, positive aPS/PT may suggest a false negative LA, and when aPS/PT are negative, this may suggest a lower thrombotic risk [80].

A distinction should be made between aPS/PT use as a diagnostic biomarker or as a marker for thrombotic risk assessment. aPS/PT is highly associated with and shows higher titer in triple aPL-positive thrombotic APS patients [71]. In second-line testing, aPS/PT can consolidate the APS diagnosis in high-risk patients. aPS/PT association was independent of aCL/ β 2GPI status, suggesting that aPS/PT may also have added value in double-positive LA-negative patients [10]. aPS/PT closely correlates with activated protein C resistance and might be useful to identify patients at higher risk of thrombosis [81]. Additionally, the association of LA and aPS/PT might confer an increased risk for cerebrovascular events, even when β 2GPI-dependent tests (β 2GPI and aCL) are negative [76].

4.1 | Methodology

aPS/PT is commonly measured by ELISA (non-ELISA systems are not yet available). In these ELISAs, prothrombin is bound, in the presence of calcium ions, to plastic wells precoated with phosphatidylserine, thus facilitating the detection of aPS/PT antibodies. Detection of aPS/PT has proven difficult to standardize, and reference material is lacking [82]. A comparison of an in-house ELISA method to a commercial ELISA that is used in many studies [71] showed reproducible and accurate results with the advantage of shorter running times for the commercial assay, which uses a simpler protocol with a reduction of analytical variables [83].

Although there is evidence to encourage the diagnostic utility of aPS/PT, this remains debated. aPS/PT has not been considered as an additional laboratory criterion for the 2023 ACR/EULAR classification criteria for APS [4] but could be useful in the clinical setting, in particular cases, as part of the laboratory workup for APS.

5 | β 2GPI DOMAIN I ANTIBODIES

A subset of anti- β 2GPI antibodies, aDI, bind to the first domain of β 2GPI [84] that contains a cryptic epitope Glycine40-Arginine43, exposed when β 2GPI is bound to negatively charged phospholipids [85]. Several groups have reported that aDI are strongly associated with APS-related thrombosis [86]. Murine studies have shown that immunization with unfolded β 2GPI induces aDI and a prothrombotic phenotype [87]. The high correlation between aDI and triple aPL positivity in obstetric and thrombotic patients confirms the higher risk for clinical events in APS [49,88,89]. Therefore, their detection may aid risk stratification [49,80,90]. The added value in diagnosis is unclear as no higher risk association for thrombosis was found by adding aDI to the aPL panel or by replacing β 2GPI with aDI [49,91]. One recent study indicated aDI as an independent risk factor [92]. Although not necessary in first-line

diagnosis, aDI can be used as a confirmatory test and is useful for proving the specificity of the $\alpha\beta 2\text{GPI}$ antibodies [80]. $\alpha\beta 2\text{GPI}$ antibodies directed against other domains are not associated with APS clinical features [46]. Most studies report IgG aDI, with little data on IgM aDI. Unpublished data showed that all IgM aDI had an affinity for domain I (B de Laat, personal communication).

5.1 | Methodology

There are several methods to measure aDI antibodies. As of today, there is 1 dominant commercial assay available and several in-house assays [90]. It was indirectly shown that the commercial assays do not expose all epitopes correctly, especially Glycine40-Arginine43, which might lead to inaccurate measurements [90].

6 | OTHER ANTIPHOSPHOLIPID ANTIBODIES

Assays for other autoantibodies may have promise with regard to aPL detection. These include antibodies against phosphatidylethanolamine, phosphatidic acid, phosphatidylserine, phosphatidylinositol, annexin V, aCL/vimentin, protein S/protein C, or neutrophil extracellular traps [93]. All are often detected by alternative nonstandardized solid phase assays such as multiline dot assays, line immunoassays, and thin layer chromatography. Study results on these aPL tests are inconsistent and show no definite added value [94].

7 | ANTIBODY PROFILES

The 3 groups of criteria aPL (LA, aCL, and $\alpha\beta 2\text{GPI}$) have different predictive values for thrombotic and obstetric risk. All 3 aPL assays should be performed concurrently, preferably on the same sample, and results of LA, aCL, and $\alpha\beta 2\text{GPI}$ should be correlated to assess the risk profile [12,37]. High clinical suspicion might warrant testing in another laboratory that uses different reagents/methods since triple positivity depends on the methods that are used [45].

Triple aPL positivity (LA, aCL, and $\alpha\beta 2\text{GPI}$ positive) is associated with a high risk of a first thrombotic event and recurrence [45,73,95–97]. Some studies specifically define triple positivity regarding the isotype of aCL and $\alpha\beta 2\text{GPI}$ and illustrate that a concordant isotype includes a higher risk profile [45,98–100]. The risk profile of double aPL-positive patients is less well studied, with various definitions of double positivity or studies including low numbers of patients [96,97,101,102]. One study found that 90% of 54 triple-positive patients presented with clinical criteria for APS, compared with 86% of 50 double-positive patients, defined as LA negative and aCL and $\alpha\beta 2\text{GPI}$ positive with a concordance of isotype [99]. Double aPL-positives defined as such seem to have the highest significance in APS [99]. The thrombotic risk in carriers of single aCL is contradictory depending on the study [16,96]. In the 2023 ACR/EULAR classification criteria, a lower weight is attributed to a single aCL or

$\alpha\beta 2\text{GPI}$, even if it is a high titer [4]. Isolated aCL might reflect antibodies directed against cardiolipin itself or to cardiolipin-binding proteins different from $\beta 2\text{GPI}$ [48]. The clinical relevance of noncofactor-related aCL is disputed since the data in humans are limited and related to infections and remains a matter of debate [32,103]. Equally, isolated $\alpha\beta 2\text{GPI}$ show no association with thrombosis and are directed against epitopes not determining LA activity [104,105].

Persistent positive single LA has a high weight in the 2023 ACR/EULAR classification criteria [4]. The role of single LA has been debated as it has been variously described as showing a poor or strong predictive value for a first thrombotic event [96,106] and being predictive for adverse pregnancy outcomes [107]. A prospective observational study found that in an LA-positive population, the association between thrombosis and increased mortality was independent of the detection of aCL and $\alpha\beta 2\text{GPI}$ [108]. A retrospective study illustrated that isolated LA proved to be strongly associated with thrombosis even though it had a weaker LA activity compared with LA activity in triple aPL-positive patients [8]. In obstetric APS, LA was confirmed to be the strongest risk factor for pregnancy morbidity, but an association was shown for every single aPL. This study clearly emphasized the clinical significance of concomitant positivity in LA and $\alpha\beta 2\text{GPI}$ as the profile with the highest predictive value for pregnancy morbidity [53].

8 | REPEAT TESTING

Positive LA, aCL, and $\alpha\beta 2\text{GPI}$ should be confirmed on repeat testing as transient antibodies have been described with infections and drugs [51], with APS diagnosis requiring persistently positive aPL [2]. Retesting also confirms the aPL profile. The time frame for retesting is no less than 12 weeks, based on expert opinion [5]. Persistence of aPL beyond 3 months was evaluated in a retrospective study that illustrated extended persistence of aPL in 96% of patients in a median follow-up of 56 weeks, irrespective of the aPL profile [109]. A decrease in aCL levels has been described in association with belimumab (anti-B-cell therapy) and a decrease in aCL and $\alpha\beta 2\text{GPI}$ levels in association with hydroxy-chloroquine [51]. There is little information on the value or therapeutic consequences of annual retesting of persistently positive patients. This could be potentially useful as part of risk assessment related to fluctuation of titers or change of antibody profile over time.

aPL positivity may fluctuate over time with or without SLE [110,111] and during pregnancy, either decreasing or increasing [110–112]. Results of a prospective observational study of 152 aPL-positive pregnant patients enrolled in the late first or early second trimesters found that 25% of LA-positive patients became negative in the second or third trimester [110]. Factor VIII increases gradually during pregnancy, which might mask the presence of LA by shortening the APTT [113].

IgG aPL levels were also significantly lower during the second and third trimesters, but among patients testing positive, IgG aCL and $\alpha\beta 2\text{GPI}$ results remained positive through pregnancy in 93% and 85% of patients, respectively.

Levels of $\alpha\beta 2\text{GPI}$ may vary in aPL-positive patients during thrombosis, with $\alpha\beta 2\text{GPI}$ titers decreasing around the time of thrombosis due

to the deposit of these pathogenic aPL at the thrombotic site with a subsequent increase in antibody titer after thrombosis [114]. Based on small patient cohorts and limited follow-up time points, the optimal timing of testing postthrombosis cannot be established [114]. Nevertheless, test results obtained during pregnancy or in the early post-thrombotic phase should be repeated postdelivery or at a distance from the acute thrombotic event [12].

Retesting of an equivocal result should be performed to confirm positivity or negativity. Low positive results around the cutoff value of aCL and a β 2GPI should be interpreted with care. Imprecision of the solid phase method should be considered with a maximal acceptable imprecision of 10%. This implies that a small difference ($\leq 10\%$) around a value near the cutoff value may influence the classification as positive or negative [37,51]. Retesting of LA if a potentially unreliable

result was obtained during VKA treatment to check the similarity of results, or in double-positive patients (aCL and a β 2GPI positive) or when tested in the acute phase might overcome false positive or false negative results [12]. Ideally, LA is assessed 1 to 2 weeks after discontinuation of VKA (whether or not with bridging to LMWH), but this may not be feasible or preferred by the clinician and should be assessed case by case.

9 | ALTERNATIVE APPROACHES

TG has been widely studied in APS, being a phospholipid-dependent assay exploring the overall coagulation process with 1 assay instead of the multitude of phospholipid-dependent clotting assays used for

TABLE 1 Guidance recommendations for the optimal laboratory detection of antiphospholipid antibodies in the diagnosis of anti-phospholipid syndrome.

Antiphospholipid antibody tests -which ones to test for?

We recommend:

- Concurrent testing for LA, aCL IgG and IgM, and a β 2GPI IgG and IgM
 - Measurement of LA in citrated plasma according to the ISTH-SSC recommendations by a 3-step methodology (screening, mixing, and confirmation) with parallel testing in APTT and dRVVT as first-choice clotting tests [12]
 - Pretest DOAC removal (with LA testing pre-/post-DOAC removal procedure) can be used in patients during DOAC therapy
 - LA testing during VKA is reliable in some situations but should always be interpreted with care
 - TSVT/ET can be used in patients during anti-Xa DOAC or VKA therapy, keeping in mind that TSVT/ET does not have 100% sensitivity
 - Measurement of aCL of IgG/IgM isotype and a β 2GPI of IgG/IgM isotype in plasma or serum by solid phase assays (ELISA or non-ELISA systems), according to the ISTH-SSC recommendations [37]

We do not recommend:

- aPS/PT IgG/IgM or aDI in the first-line diagnostic tests
- To replace LA with aPS/PT

We recommend using aPS/PT and aDI in specific situations:

- aPS/PT can be useful if LA testing is unreliable or uncertain
- aDI can be used to confirm the specificity and pathogenicity of a β 2GPI

Antiphospholipid antibody tests -interpretation of results

We recommend:

- Interpretation of results according to the local cutoff values stated in the report
- Along with the detailed results, LA should be reported with a final conclusion as positive or negative and warning for potential interferences (anti-coagulant therapy, acute phase proteins). Results should be interpreted in a clinical context, with the knowledge of anticoagulation and a close interaction between the laboratory and the clinician
- Reporting aCL and a β 2GPI with their level; each level of aCL and β 2GPI higher than the cutoff value for positivity set at >99th percentile of normal controls is considered positive
- Against grading positivity of aCL and a β 2GPI into low-medium-high positivity since it is method-dependent and should be harmonized between methods with adapted level intervals
- Two consecutive aPL tests to be positive at least 12 weeks apart to rule out transient aPL positivity, which is not sufficient for APS diagnosis. In the repeat testing, the same aPL need to be positive
- Relating LA, aCL and a β 2GPI results to assess the aPL profile to include in the diagnostic assessment. Triple and double (if aCL and a β 2GPI, with concordant isotype) aPL positivity increases the confidence in APS diagnosis. In patients positive for aCL/a β 2GPI, the IgG isotype is regarded as clinically more relevant rather than IgM. In diagnosis, 1 aPL can be sufficient, although a single IgM is regarded as less clinically relevant, and a persistent medium/high titer (>99th percentile) is of utmost importance

The method endorsed by the ISTH Guidance and Guidelines Committee Panel on writing guidance is applied. Accordingly, the wording “recommend” indicates a strong consensus among the coauthors, “suggest” indicates a moderate consensus, and “propose” indicates the areas where there is limited knowledge.

a β 2GPI, anti-beta2 glycoprotein I antibodies; aCL, anticardiolipin antibodies; aDI, anti-domain I antibodies; aPL, antiphospholipid antibodies; aPS, antiphosphatidylserine; APS, antiphospholipid syndrome; APTT, activated partial thromboplastin time; DOAC, direct oral anticoagulant; dRVVT, dilute Russell's viper venom time; ELISA, enzyme-linked immunosorbent assay; ET, ecarin time; Ig, immunoglobulin; ISTH-SSC, International Society on Thrombosis and Haemostasis Scientific and Standardization Committee; LA, lupus anticoagulant; PT, prothrombin; TSVT, Taipan snake venom time; VKA, vitamin K antagonists.

TABLE 2 Research agenda to further optimize the laboratory detection of antiphospholipid antibodies in the diagnosis of antiphospholipid syndrome**Further research on lupus anticoagulant**

- Whether single (dRVVT or aPTT) or double (dRVVT and aPTT) LA test positivity in triple aPL-positive patients contributes to a higher risk
- Further studies on the use of TSVT/ET in VKA or anti-Xa DOAC-treated and non-anticoagulated patients
- The added value of TSVT/ET in patients negative for aPTT or dRVVT
- Results on cutoff values are variable between methods, further efforts are needed to establish cutoff values per reagent/instrument combination in a multicenter setting
- Further studies are needed on quantification of LA and the potential utility of LA strength in risk assessment

Further research on anticardiolipin and anti-beta2 glycoprotein I antibodies

- Initiatives for harmonization between various assays by comparing reference materials
- Investigation of the use of lower cutoff values (95th percentile) in pregnancy morbidity and their clinical relevance in updated pregnancy morbidity definitions included in the 2023 ACR/EULAR APS classification criteria
- Efforts to harmonize semiquantitative reporting into ranges of low-moderate-high for non-ELISA methods and whether these thresholds per method can be used inter-laboratory
- Further research on the value of isolated IgM in patients with clinical features of TAPS and OAPS
- Role of single aCL and a β 2GPI in TAPS and OAPS

Further research on antiphosphatidylserine/prothrombin and anti-domain I antibodies

- Further investigations on aPS/PT as being dependent/independent of LA as a risk factor for thrombosis since existing studies are contradictory
- Definition of role of aPS/PT isotype in TAPS and OAPS
- Role of the level in TAPS and OAPS
- Definition of role of aPS/PT according to the type of obstetric complication
- Evaluation of aPS/PT in patients negative for LA, aCL and a β 2GPI where there is suspicion of APS
- Epitope specificity of antibodies detected by aDI assays

Further research on repeat testing of aPL

- Value and potential therapeutic consequences of retesting persistently positive patients annually, for LA, aCL and a β 2GPI
- Evaluation of fluctuation of titers, change of antibody profile over long-term period

Further research on alternative methods

- Harmonization in TG methodology for use in APS patients
- Place of TG-based assessment of aPL-induced ACPr

a β 2GPI, anti-beta2 glycoprotein I antibodies; aCL, anticardiolipin antibodies; ACR, American College of Rheumatology; aDI, anti-domain I antibodies; APCr, activated protein C resistance; aPL, antiphospholipid antibodies; aPS, antiphosphatidylserine; APS, antiphospholipid syndrome; APTT, activated partial thromboplastin time; DOAC, direct oral anticoagulant; dRVVT, dilute Russell's viper venom time; ELISA, enzyme-linked immunosorbent assay; ET, ecarin time; Ig, immunoglobulin; EULAR, European Alliance of Associations for Rheumatology; LA, lupus anticoagulant; OAPS, obstetric APS; PT, prothrombin; TAPS, thrombotic APS; TG, thrombin generation; TSVT, Taipan snake venom time; VKA, vitamin K antagonists.

LA detection. A systematic review reported that the prevalence of activated protein C resistance, assessed using TG, was significantly increased in APS patients compared with healthy and thrombotic controls [115]. The use of TG in APS diagnosis awaits more research to identify the potential added value of TG parameters in APS diagnosis [115], and guidance is required to harmonize TG methodology for use in APS patients.

The combined use of TG and machine learning has been proposed as a possible diagnostic aid in APS [116–118]. Machine learning is a form of artificial intelligence that aims to build a prediction model by learning from existing data (eg, from a cohort of APS patients). A combined interpretation of laboratory parameters might have the potential to optimize the use of antibody profiles and alternative biomarkers, such as TG in APS.

10 | SUMMARY OF GUIDANCE RECOMMENDATIONS AND RESEARCH AGENDA

In this guidance, recommendations for routine diagnostic workup for APS are given based on published data and the consensus of experts in the field and summarized in Table 1. Some points need more evidence before forming guidance recommendations. Therefore, we suggest a research agenda (Table 2).

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AUTHOR CONTRIBUTIONS

K.M.J.D. conceived the guidance and reviewed the literature. K.M.J.D. wrote the first draft of the manuscript. All the authors contributed to the content and revised and accepted the manuscript, which was then approved by the Scientific and Standardization Committee for Lupus Anticoagulant/Antiphospholipid Antibodies of the International Society on Thrombosis and Haemostasis (ISTH). The manuscript was reviewed and approved by the Guidelines and Guidance Committee of the ISTH.

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ORCID

Katrien M.J. Devreese <https://orcid.org/0000-0002-7559-2579>

Maria Laura Bertolaccini <https://orcid.org/0000-0002-8623-139X>

D. Ware Branch <https://orcid.org/0000-0002-1718-5094>

Bas de Laat <https://orcid.org/0000-0001-9596-1944>

Doruk Erkan <https://orcid.org/0000-0001-7216-677X>

Emmanuel J. Favaloro <https://orcid.org/0000-0002-2103-1661>

Vittorio Pengo <https://orcid.org/0000-0003-2064-6071>

Thomas L. Ortel <https://orcid.org/0000-0001-6193-4585>

Denis Wahl <https://orcid.org/0000-0003-4846-6793>

Hannah Cohen <https://orcid.org/0000-0003-2032-390X>

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