

Guidance from the Scientific and Standardization Committee for lupus anticoagulant/antiphospholipid antibodies of the International Society on Thrombosis and Haemostasis

Update of the guidelines for lupus anticoagulant detection and interpretation

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Abstract

This guidance focuses on methodological aspects of lupus anticoagulant (LA) testing, as well as interpretation of results for clinicians. The main changes in how to test for LA compared with the International Society on Thrombosis and Haemostasis Scientific and Standardization Committee 2009 guidelines, in the preanalytical phase are more detailed recommendations on how to handle testing in anticoagulated patients, and the timing of testing. Also, routine coagulation tests are advised to obtain

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more information on the coagulation background of the patient, and when necessary, anti-Xa activity measurement for heparins or specific assays for direct oral anticoagulants should be performed. The three-step procedure with two test systems (diluted Russell's viper venom time and activated partial thromboplastin time [aPTT]) is essentially not changed. Silica remains the preferable activator in the aPTT assays, but ellagic acid is not excluded. We advise simultaneous performance of the mixing and confirmatory step, in each sample with a prolonged screening test. The confirmatory step can also be performed on a mixture of patient plasma and normal pooled plasma. Cutoff values should be established in-house on at least 120 normals, with transference of the manufacturer's cutoffs as an alternative. Reporting of results has not been changed, although more attention is focused on what clinicians should know. Patient selection for LA testing has been expanded.

KEYWORDS

antiphospholipid syndrome, confirmatory testing, cut-off values, guidance, laboratory diagnosis, lupus anticoagulant, pre-analytical

1 | INTRODUCTION

Lupus anticoagulant (LA) is one of the three laboratory criteria for the identification of the antiphospholipid syndrome (APS).^{1,2} LA detection is based on phospholipid (PL)-dependent coagulation tests, which complicate the methodology and hamper its interpretation because of interference, for instance by anticoagulant therapy and some acute phase response proteins.^{3,4} LA is the best-established risk factor for APS-related clinical manifestations.⁵⁻⁹ Therefore, accurate assessment of LA is essential for diagnosis and management of APS patients. External quality assessment exercises still show high rates of false-positive or false-negative results.^{10,11} The analysis of LA therefore remains complex, with many pitfalls in the implemented procedure, including the preanalytical conditions and interpretation.³

The last update of the guidelines for LA detection by the International Society on Thrombosis and Haemostasis Scientific and Standardization Subcommittee (ISTH-SSC) in 2009¹² has proven useful in achieving greater uniformity in performance and interpretation of LA testing. Equally, the British Society for Haematology¹³ and the Clinical and Laboratory Standards Institute (CLSI) guidelines¹⁴ have contributed to increased uniformity. Following an LA/antiphospholipid antibodies (LA/aPL) SSC decision that for some issues these recommendations required a further update, a survey¹⁵ was undertaken with a panel discussion during the ISTH-SSC meeting in 2018 in Dublin. The aim was to capture the spectrum of clinical and laboratory practice in LA detection, with particular focus on issues where there is variability in practice. The responses forced the formulation of further ISTH-SSC recommendations. There was good agreement on several key elements addressed in the 2009 guidelines, such as sample preparation, choice of assays, repeat testing, and the use of interpretative comments. However, on other points, including testing in patients on anticoagulation, cutoff values, and calculation and interpretation of results, there was uncertainty or lack of agreement.^{12,15}

The laboratory aspects of testing for LA in anticoagulated patients are addressed in separate guidance of the ISTH-SSC LA/aPL.¹⁶ The guidance herein will focus on aspects of methodology, choice of assays, cutoff values, calculation and interpretation of results, and timing of testing in relation to thrombosis and pregnancy, with the emphasis on practical guidance for laboratory scientists and clinicians. The LA/aPL SSC invited the authors of the 2009 guidelines still active in the field, and additionally, other expert scientists and clinicians, to contribute to this guidance. We aim for "the best fit" and what seems appropriate, based on the results of the questionnaire¹⁵ and a current review of the literature. Here, we summarize the consensus on optimal LA testing, with attention to what has changed since the 2009 guidelines.¹²

2 | PATIENT SELECTION AND TIMING OF LA TESTING

2.1 | Patient selection

Testing for LA should focus on patients who are likely to have APS.¹² Indiscriminate testing for LA is discouraged to avoid incidental findings.

See Table 1 for information for clinicians on LA testing and other points addressed in the following sections.

2.2 | Timing of LA testing in relation to thrombotic events and pregnancy

Testing during the acute phase, such as soon after a thrombotic event, should be interpreted with caution^{12,17} because of possible interferences with the test result because of raised levels of FVIII shortening the activated partial thromboplastin time (APTT)¹⁸ or C-reactive protein by interference with PL in the reagent,

TABLE 1 Information for clinicians on lupus anticoagulant testingPatient Selection for LA Testing^{1,2,12,13,82-88}

- LA testing should be performed, together with testing for aCL, and a β 2GPI, to assess the risk profile, in patients who are likely to have APS:
 - younger patients (<50 years) with unprovoked venous thromboembolism (VTE)
 - VTE at unusual sites
 - younger patients (<50 years) with ischemic stroke, transient ischemic attack or other evidence of brain ischemia
 - arterial thrombosis in other sites in younger patients (<50 years)
 - microvascular thrombosis
 - recurrent VTE unexplained by subtherapeutic anticoagulation, patient nonadherence, 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 is part of the diagnostic criteria and contributes to risk assessment
- LA testing could be considered in the following situations:
 - immune thrombocytopenia, particularly with presence of arthralgias or arthritis, hair loss, sun sensitivity, mouth ulcers, rash, thromboembolism
 - livedo reticularis, particularly with presence of symptoms of other systemic autoimmune diseases or mild thrombocytopenia
 - younger patients (<50 years) with noncriteria clinical manifestations, ie those not included in the Sydney criteria, eg cognitive dysfunction, valvular heart disease with presence of evidence of other systemic autoimmune diseases
 - 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

Consider the following before ordering LA testing

- Results of LA testing during an acute phase response (eg, in the setting of an acute thrombotic event) should be interpreted with caution, as false positive and negative results can occur
- Ideally, LA testing should be performed in patients not receiving any anticoagulant treatment as false positives and false negatives can occur
- LA testing may be clinically desirable in anticoagulated patients; the following points should be noted:
 - In VKA-treated patients, the interpretation of LA results is challenging because of the prolonged basal clotting time. If feasible, perform LA testing 1 to 2 weeks after discontinuation of the VKA, with consideration of LMWH bridging
 - If patients are tested during treatment with LMWH, samples should be taken, when feasible, at least 12 hours after the last dose of LMWH was administered and as near as possible to the next dose, with anti-Xa activity levels checked alongside the LA test
 - If feasible to temporarily interrupt DOAC anticoagulation (on a pragmatic, empirical basis at least 48 hours after the last dose, and longer in patients with renal impairment), LA testing can be performed, with the DOAC level checked alongside the LA test
- Incorporation of information on the patient's anticoagulation status in the request is mandatory
- Results of LA testing during pregnancy should be interpreted with caution as false positive and negative results can occur. Repeat testing should be considered at an appropriate time post-delivery to obtain reliable LA results

(Continues)

TABLE 1 (Continued)

Interpretation of results

- Results to be interpreted according to the local cutoff values stated in the report. The cutoff value may impact upon diagnosis (eg, weak positive LA) as it will determine whether a patient is classified to be LA positive or not.
- LA is reported with a final conclusion as positive or negative
- Results should always be related to the results of aCL and a β 2GPI to assess the risk profile
- Comments on the final conclusion should be provided if relevant (eg on possible interferences)
- Results should be interpreted in a clinical context, with knowledge of anticoagulation
- 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

Repeat testing

- Repeat testing is required after an initial positive result on a second occasion after at least 12 weeks to confirm persistent positivity

prolonging PL-dependent clotting tests¹⁹; the first may mask LA and the latter may yield false-positive results. Nevertheless, testing during the acute phase may be needed for instance in patients suspected having catastrophic APS.²⁰ LA testing may also be useful in new stroke patients in whom APS is clinically suspected, to decide whether the patient would benefit from an anticoagulant rather than antiplatelet therapy to potentially prevent APS-related recurrent stroke.^{13,21}

Little agreement exists on the timing of testing in relation to the thrombotic event or pregnancy,¹⁵ excluding considerations related to the effects of anticoagulant therapy or acute phase. LA positivity may fluctuate over time with or without the presence of systemic lupus erythematosus^{22,23} and during pregnancy, either decreasing or increasing.^{22,24,25} If aPL testing is done during pregnancy, there should be caution as regards interpretation of the result, as negative or positive LA (or anticardiolipin [aCL]/ anti- β 2glycoprotein I [a β 2GPI]) during pregnancy does not exclude or confirm a diagnosis of APS. One study suggested that LA testing in the first trimester is reliable and sufficient to assess the risk.²³ Repeat LA testing should be undertaken postdelivery to establish true aPL status, although the optimal timing of testing postdelivery is not established. Pregnancy-induced increases in levels of blood coagulation factors have been reported to resolve by 6 weeks postpartum,²⁶ and LA results returned to baseline status by 3 months postdelivery in one study.²³

3 | PREANALYTICAL, ANALYTICAL AND POSTANALYTICAL FACTORS IN LA TESTING

How to test for LA is summarized in Table 2, indicating the changes compared with the 2009 guidelines, what remains unchanged, and the current recommendations.

TABLE 2 Recommendations for the optimal laboratory detection of lupus anticoagulant (LA) and comparison with the ISTH-SSC 2009 guidelines

Recommendations of the ISTH-SSC 2009 Guidelines ¹²	Recommendations Added/Changed Compared to 2009 Guidelines
Blood collection/pre-analytical factors	
<i>Blood collection before the start of any anticoagulant drug or a sufficient period after its discontinuation</i>	Whenever possible, blood for LA detection should be collected in patients not receiving any anticoagulant treatment
<i>The effect of DOAC is unknown</i>	If feasible to briefly interrupt DOAC anticoagulation, LA testing can be performed after checking the level of DOAC DOAC adsorption may be considered in DOAC treated patients Incorporation of information on the patient's anticoagulation in the request is mandatory
<i>Acute phase reactants such as FVIII may be increased during acute events</i>	Be aware that acute phase reactants (FVIII and CRP) may give false-negative/false-positive LA results
<i>LA detection during acute thromboembolic events should be interpreted with caution as patients may be treated with full doses of UFH, LMWH, and/or VKA</i>	Testing during pregnancy may result in false-positive/false-negative results and should be interpreted with caution, and repeated at an appropriate time postdelivery, after at least 6 weeks and ideally after 3 months, to obtain reliable LA results Avoid LA testing on hemolyzed samples
Venous blood collected into 0.109 mol/L sodium citrate 9:1	
Double centrifugation at 2000g for 15 minutes at room temperature to obtain platelet-poor plasma	
<i>Quickly frozen plasma is required if LA detection is postponed</i>	Freeze plasma within 4 h of collection if LA detection is postponed
Frozen plasma must be thawed at 37°C for 5 minutes in water bath at 37°C by total immersion and then mixed before testing	
Avoid freeze/thawing cycles	
Choice of test/test procedure	
<i>Thrombin time (TT) will help to identify heparin</i>	Prothrombin time (PT)/INR, activated partial thromboplastin time (aPTT), TT, and fibrinogen should be performed for background information on anticoagulation status or coagulopathy
<i>Textarin (Taipan)/Ecarin clotting times or integrated tests (ie %correction for APTT, SCT, and dRVVT at low and high phospholipid concentration) are not currently recommended as they require further critical evaluation</i>	Measure anti-FXa activity together with LA testing in patients who are known to be on LMWH or UFH
<i>In VKA-treated patients: if the INR is between 1.5 and <3.0, a 1:1 dilution of patient plasma and PNP can be considered</i>	Taipan/Ecarin tests are less affected by VKAs and anti-FXa DOACs. Recommendations for their general use awaits the provision of independent evidence from collaborative studies with standardized kits. Dilution of patient plasma into PNP is not a reliable solution in patients on VKA (false-negative or false-positive LA results may occur) Although in VKA treated patients (INR < 3.0) LA testing is discouraged, if attempted, results should be interpreted with care
Test principle:	
<ul style="list-style-type: none"> • Two tests based on different principles • dRVVT should be the first test considered 	
<i>The second test should be a sensitive aPTT (low phospholipids and silica as activator)</i>	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 test on dRVVT and aPTT	
Screening tests are performed with dRVVT and aPTT, and regarded to be positive if the normalized clotting time is prolonged beyond the locally established cutoff	
Mixing test on dRVVT and aPTT	
Procedure:	
<ul style="list-style-type: none"> • PNP for mixing studies should ideally be prepared in house. Adequate (specifically prepared) commercial lyophilized or frozen PNP can alternatively be used <ul style="list-style-type: none"> • a 1:1 proportion of patient: PNP should be used, without preincubation, within 30 minutes • a mixing test with screening reagent is performed if the screening test on undiluted sample is prolonged 	

(Continues)

TABLE 2 (Continued)

Recommendations of the ISTH-SSC 2009 Guidelines ¹²	Recommendations Added/Changed Compared to 2009 Guidelines
	PNP should be constituted from at least 40 normal donors
<i>Interpretation: Results of mixing test are suggestive of LA when their clotting times or the index of circulating anticoagulant are greater than the local cutoff value</i>	Interpretation: Results of mixing test are suggestive of LA when the normalized clotting time is greater than the local cutoff value See also interpretation of confirmatory test
	Confirmatory test on dRVVT and aPTT
	Procedure:
	<ul style="list-style-type: none"> Confirmatory test(s) must be performed by increasing the concentration of PL used in the screening test(s) <ul style="list-style-type: none"> Bilayer or hexagonal (II) phase PL should be used to increase the concentration of PL.
<i>Confirmatory test is performed if the screening test and the mixing test with screening reagent suggest LA presence</i>	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
	<ul style="list-style-type: none"> for paired test LA ratio (screen/confirm) expressed as normalized ratio is calculated <ul style="list-style-type: none"> or the percentage correction $[(\text{screen} - \text{confirm})/\text{screen} \times 100]$ some of the integrated tests are designed to measure a difference in clotting times on a mixture of plasma
<i>Mean value of the percentage correction for the confirmation step on 40 normals</i>	Results are suggestive for LA if LA ratio (screen/confirm) or percentage correction is above the 99th centile.
	If the confirmatory test on (screen/confirm) neat plasma is prolonged, normalized LA ratio should be calculated by screen and confirmatory test results on a mixture of 1:1 NPP and patient plasma (screen mix/confirm mix) and regarded positive if above the 99th centile
	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)
	Cutoff values
	Do not use cutoff values established elsewhere
<i>99th percentile on 40 normal donors for screening and mixing test</i>	Use in-house cutoff values Calculate 99th centile on at least 120 normal samples with outlier detection for all normalized ratios Alternatively, transference of the manufacturer's cutoff values after verification is possible, if manufacturers provide cutoff values established in accordance with guidelines and by appropriate statistical models using a sufficiently large donor population
	Postanalytical issues
	It is imperative that testing is repeated after an initial positive result on a second occasion after 12 weeks
	Report of results
	<ul style="list-style-type: none"> 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 <ul style="list-style-type: none"> Along with the analytical results for the three steps, local cutoff values must be reported <ul style="list-style-type: none"> A report with an explanation of the results should be given Results should always be related to the results of aCL and aβ2GPI to assess the risk profile <ul style="list-style-type: none"> 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 <ul style="list-style-type: none"> A close interaction between the laboratory and the clinician is essential

Note: This table provides a complete comparison with the 2009 ISTH guidelines on LA testing. The left column written in italics shows what is stated in the 2009 guidelines and what is replaced in the current update of the guidelines; the right column shows the current guidance recommendations; the overlapping lines over the two columns shows what is unchanged compared with the 2009 guidelines.

Abbreviations: aCL, anticardiolipin antibodies; aPTT, activated partial thromboplastin time; a β 2GPI, anti-beta2 glycoprotein I antibodies; CRP, C-reactive protein; DOAC, direct oral anticoagulants; dRVVT, diluted Russell's viper venom time; FVIII, factor VIII; INR, international normalized ratio; ISTH-SSC, International Society on Thrombosis and Haemostasis-Scientific and Standardization Committee for lupus anticoagulant/antiphospholipid antibodies; LA, lupus anticoagulant; LMWH, low molecular weight heparin; M, molar; PL, phospholipids; PNP, pooled normal plasma; PT, prothrombin time; SCT, silica clotting time; TT, thrombin time; UFH, unfractionated heparin; VKA, vitamin K antagonists.

3.1 | Sample preparation and quality

Sample collection and processing as described in the ISTH-SSC 2009 guidelines is widely applied.¹⁵ A total of 51% of the participants of the survey indicated plasma should ideally be frozen within 4 hours, in accordance with general guidelines for coagulation assays.²⁷ The effect of freeze-thawing on diluted Russell's viper venom time (dRVVT) ratios leading to false-negative results was confirmed in a more recent study.²⁸ Many participants of the survey expressed some concern about sample quality. Hemolysis, icterus, and lipemia may impact routine coagulation results, with their influence dependent on the detection method (mechanical end-point clotting vs photometric), the concentration of interfering substances and the reagents used. Hemolysis, icterus, and lipemia mainly affects the APTT, resulting in a falsely normal APTT in hemolyzed samples.²⁹⁻³¹ Preanalytical conditions potentially influencing test results should be mentioned on the report.

3.2 | Interferences

One of the main confounding factors in LA testing is anticoagulant therapy, prolonging the clotting times in the PL-dependent assays used for LA detection. Ideally, LA testing should be deferred until anticoagulation is discontinued, but requests for LA testing during therapy occur frequently in routine clinical practice, resulting in potentially false-positive or false-negative results.^{3,32} LA testing may become important, for instance when deciding on extended duration versus discontinuation of therapy, or regarding the choice of anticoagulant.¹⁶

Heparins interfere with LA clotting assays; however, a recent study³³ showed unfractionated heparin (UFH) and enoxaparin affect the dRVVT at supra-therapeutic anti-Xa levels but did not lead to false-positive LA in the three step procedure. In contrast, enoxaparin caused false-positive aPTT-based LA detection only at supra-therapeutic anti-Xa activity levels. Some reagents, such as dRVVT reagents and some LA-specific aPTT reagents contain heparin neutralizers, but it is important to verify the levels of heparins that are quenched in these reagents.³³ Checking anti-Xa activity alongside LA testing can ensure that results are reliable if anti-Xa activity levels are within the therapeutic range. Therefore, samples should be taken, when feasible, at least 12 hours after the last dose of low molecular weight heparin (LMWH) was administered and as near as possible to the next dose. If anti-Xa levels are supra-therapeutic, positive LA results should be interpreted with care since false positives occur.³³ It should be considered that all the above interferences are likely dependent on the composition of reagents (APTT or dRVVT) used for testing.

Vitamin K antagonists (VKAs) may cause false-positive or false-negative results.^{34,35} Dilution of the patient sample into a pooled normal plasma was proposed as an option in the 2009 guidelines. However, this may reduce LA potency, the degree of correction

of the acquired factor deficiency induced by VKA is reagent dependent and interpretation remains difficult.¹⁶ The Taipan snake venom/Ecarin clotting time may help in LA detection in VKA- and rivaroxaban-treated patients,³⁶ but its diagnostic efficacy (sensitivity) is not well documented in the literature. We await larger studies to assess its use in anticoagulated patients.¹⁶

Direct oral anticoagulants (DOACs) have emerged as a major challenge, not discussed in the ISTH-SSC 2009 guidelines¹² (when they were not yet licensed for treatment and secondary thromboprophylaxis). Since the introduction of DOACs, many studies illustrated the effect on LA testing.³⁷ DOACs give false-positive results in APTT and dRVVT test systems, even at low concentrations.³⁸ Use of antidotes or neutralizers have been investigated to eliminate and overcome the effect of DOACs.³⁹⁻⁴² Several studies indicated that pretreatment with adsorbents may affect clotting times resulting in false-positive or false-negative LA results, or incomplete removal of DOACs.^{33,41-43} Consequently, pretreatment of plasma with adsorbents is only advised in DOAC-treated patients.¹⁶ Clinical and laboratory experience will guide whether the use of DOAC adsorbents should become standard practice, but this will make the methodology for LA even more complex. If feasible, LA testing should progress after a brief interruption of DOACs—on a pragmatic, empirical basis at least 48 hours after the last dose, and longer in patients with renal impairment. DOAC levels should also be checked.³⁷

3.3 | Choice of assays

The type and number of test systems was restricted in the 2009 ISTH-SSC guidelines to dRVVT and an APTT with a low concentration of PL. The combination of APTT and dRVVT has been proven to have high detection rates of LA,^{44,45} and is supported by an overwhelming majority of participants (94.5%) of the survey reflecting daily practice.¹⁵ dRVVT is recommended for its specificity and robustness, and APTT for its sensitivity, although the choice of APTT reagents should be carefully considered because some aPTT reagents are much more sensitive than others.⁴⁶ Compliance with the choice of assays in the 2009 guidelines has risen markedly¹⁵ compared with a 2010 survey that showed compliance of 48% of laboratories.⁴⁴

In contrast to other guidelines, other LA tests were not recommended in the 2009 ISTH-SSC guidelines because of variability in reagents (dilute prothrombin time), poorer reproducibility (kaolin clotting time), or nonavailability of standardized commercial assays (Taipan/Ecarin snake venom based assays).¹²⁻¹⁴ Although other tests may perform with comparable diagnostic strength, for instance dilute prothrombin time,⁴⁷ we adhere to the two-test principle inspired by the fact that a restriction in the choice of assays reduces the interlaboratory and interassay variability, as well as the number of false positives.² Based on unanimous agreement of the 2009 expert panel and available literature at that time, the choice of activator for APTT was restricted to silica.^{12,48,49} It has subsequently been demonstrated that ellagic acid as APTT

activator may show acceptable sensitivity at least in some APTT reagents, although with lower normalized ratios for the screening step.^{50,51} LA sensitivity of commercial APTT reagents also depends on the combination of activator and PL composition.⁵⁰ Silica clotting time (SCT), an APTT-based test employing silica as activator, can also be used.^{52,53} Laboratories should evaluate the sensitivity of their APTT reagents using well-characterized LA-positive samples.⁵⁴

For optimal patient management, ideally, functional diagnostic assays should discriminate between thrombosis-related and non-thrombosis-related aPL.⁵⁵ These tests have been described but are not sufficiently robust to use in routine practice.⁵⁶ Thrombin generation assays may help in the diagnosis or follow-up of APS patients,⁵⁷ but are difficult to use in daily practice. The three-step procedure for LA testing is still applied.⁵⁵

Before starting with the LA specific tests, routine coagulation tests including the PT, APTT, thrombin time, and fibrinogen help to identify the presence of anticoagulant therapy, clotting factor deficiencies or specific coagulation factor inhibitors and acute phase reactants. For routine APTT testing, a reagent with minimal response to LA should be used, to avoid unnecessary evaluations for aPL in otherwise asymptomatic individuals.¹⁴ If interference is suspected or information on use of anticoagulant is lacking, specific tests for DOACs or LMWH can also be applied to identify/exclude interference.

3.4 | Three step procedure

In the 2009 guidelines, integrated test systems, with “paired” performance of the screening and confirmation step with reagents with low and high phospholipid content, were mentioned separately because they are widely used in routine practice.¹² A misinterpretation that these paired tests do not require the mixing step resulted in omission of the mixing test, mainly in the dRVVT, in many laboratories.⁵² Discussion on the mixing test ensued,^{52,58,59} but subsequently, the CSLI guidelines emphasized that all three steps (screening, mixing, and confirmation) are essential.^{12,14} More recent studies illustrate that omitting the mixing step may give false-positive and false-negative results.^{35,58,60} The need for mixing tests is reflected in the affirmative response of majority of the ISTH-SSC survey respondents (84.1%) and recent literature.^{15,61,62}

Mixing tests are commonly performed on screening assays. A mixing test in the confirmatory step can be performed if the confirmatory clotting time on undiluted plasma is prolonged. This increases the diagnostic efficacy,^{14,35} when there are coexisting reasons for clotting time prolongation (factor deficiency or VKA) or a strong LA sufficiently potent to overcome the excess of PL added in the confirmatory step.⁵²

Both in-house and commercially available pooled normal plasma (PNP) can be used, fulfilling the specifications of minimal residual platelets and approximately 100% (>80%) activity of clotting

factors.¹⁴ However, these specifications are often not included in the manufacturer's product insert.^{12,63} Specifications on the minimal number of donors to constitute the PNP are difficult to define. The majority of survey participants indicated that the number should be at least 40, which seems reasonable to guarantee an overall normal clotting factor activity.¹²

The 2009 ISTH-SSC guidelines advocate performing the mixing step after finding a prolonged screening test, and if the mixing test suggests LA presence, performance of a confirmatory test.¹² However, information on the confirmatory test, even if the mixing step is negative, can help in the interpretation.^{34,35,58} On the other hand, mixing tests add information on the results of screening and confirmatory testing, especially in anticoagulated patient samples.³⁵ We recommend that a final conclusion on possible interferences and the potential need for retesting (see more at “Interpretation of Results and Report”) is provided. Therefore, performing the two next steps, the mixing step and the confirmatory test, at the same time can be helpful. CLSI guidelines prioritize the confirmatory test over the mixing test, omitting the mixing test only where there is no evidence of other causes of elevated clotting times or the PL-dependency is already demonstrated by the combination of screening and confirmatory test.¹⁴ Because of these arguments, and to have maximal information, we advise performance of screen followed by simultaneous mix and confirm in all samples with a prolonged screening test. Results are suggestive for LA presence when the screening step result and the mixing step result is higher than the local cutoff value, and the confirmatory step shows correction. If the screening and confirmatory steps are positive, with a mixing step negative, and presence of anticoagulants are excluded, measurement of coagulation factor levels can help in identifying the reason for prolonged screening times and correction in the mixing test clotting time. If coagulation factors are normal, it is recommended to comment on the LA result, repeat LA testing, and interpret LA results along with aCL and a β 2GPI results, to inform a final conclusion on the LA result.

Automatically reflex testing can facilitate the performance of the multistep procedure for LA testing, and may assist laboratories in assessment of LA status and interpretation according to the guidelines.⁶⁴

3.5 | Interpretation of the mixing test

Final conclusions on LA positivity differ regarding the way the mixing test is interpreted.⁶⁴ A more uniform way of interpretation may contribute to more standardized performance of LA tests. The current recommendation gives two acceptable alternatives: a local cutoff value of the clotting time (not clearly indicated that the value should be expressed as ratio) or the index of circulating anticoagulant, known as the Rosner index.¹²

We recommend that mixing tests should be interpreted with a mixing test-specific cutoff expressed as normalized ratio because recent studies suggest it has better sensitivity than the alternatively recommended index of circulating anticoagulant.^{65,66}

3.6 | Normalization of results

PNP is used for LA testing in mixing studies and for normalization of results to reduce intra-laboratory variability. Normalized ratios calculated by dividing the clotting time of the patient by the clotting time of the PNP measured in the same run, as advised in the 2009 guidelines, is applied by the majority of laboratories.¹⁵ This procedure helps to diminish variation depending on reagent, instrument and operator, for each batch of samples. The alternative, as described in the CSLI guidelines, the mean of a reference interval determined per lot of reagent used in the denominator of the formula, is less applied.^{14,67} We discourage this procedure because it does not compensate for the day-to-day variation.

3.7 | Cutoff values

The ISTH-SSC 2009 guidelines recommend application of the 99th percentile to determine the cutoff value on at least 40 donors for the screening and mixing procedures and the mean percentage of correction for the confirmation procedure.¹² This recommendation raises the following considerations.

A minimum of 120 healthy donors is needed for deriving a 99th centile.⁶⁸ From a statistical viewpoint, as illustrated for aCL and $\alpha 2$ GPI antibodies, the minimum sample size for an appropriate estimation of the 99th centile is at least 300.⁶⁹ In-house calculated cutoff values may be significantly different from those recommended by the manufacturers.⁷⁰⁻⁷² The value may depend on the performance characteristics, the statistical method and the reference population used to establish cutoff values. The ISTH-SSC survey showed a considerable lack of agreement on calculation of cutoff values, although 79% stated that they calculate their in-house cutoff values, but only 12% indicated use of >120 normals.¹⁵ This is not unexpected because these large numbers of healthy volunteers are not feasible for most laboratories, given as one of the reasons for not calculating in-house cutoff values, besides the high cost and the labor intensity.¹⁵

The 99th centile, chosen to avoid false positives, was applied by 50% of survey participants.¹⁵ A recent study showed that clotting times or ratios in LA testing were not normally distributed, which confirms that cutoff values should be determined by a non-parametric method based on centiles.⁷³ This study illustrated a greater detection rate with the 95th centile compared with the 99th centile (without exclusion of outliers), although this was evaluated with international reference standard material of the National Institute for Biological Standards and Controls and not with patient samples.⁷³ Based on the same data of this multicenter study, it was illustrated that variable numbers of outliers were identified depending on the algorithm used for identification and that their elimination showed no appreciable effects on the inter-laboratory variability of cutoff values, indicating that outliers are not the main cause of the inter-laboratory variability of cutoffs for LA detection. This notwithstanding, the authors recommended

the identification of outliers before cutoff calculation by using one of the current available algorithms.⁷⁴

Despite the fact that higher specificity is accompanied by lower sensitivity, we would maintain the 99th centile as the appropriate cutoff value. If laboratories decide to calculate in-house cutoff values, standardized criteria concerning the statistical analysis to define the 99th centile should be applied. Based on a study for aCL and $\alpha 2$ GPI antibodies, we recommend the use of a nonparametric procedure with identification of outlier data, by the use of the Reed method, given its simplicity.⁶⁹

More practical, is the transference of the manufacturer's cutoff values after verification using a small number (20 or 40) of normal donors,⁶⁸ as often applied for the solid phase assays,⁷⁵ and found reliable by more than 80% of the survey participants.¹⁵ However, this assumes that manufacturers' cutoffs are established by appropriate statistical models using a sufficiently large donor population.

An alternative to establish cutoff values is a multicenter approach. Previously, it was demonstrated that a multicenter approach can determine the cutoff values of aCL and $\alpha 2$ GPI antibodies with a higher accuracy by increasing the number of healthy donors.⁷⁶ Recently, the concordance of cutoff value values by users of three widely used commercial platforms for LA testing following the ISTH-SSC guidelines was evaluated. In that study, each laboratory included 120 normal samples collected locally.⁷³ Differences were observed between platforms for clotting times as well as results expressed as ratio, but also between participants using the same platform. These differences were observed even after detection and exclusion of outliers before cutoff calculation.⁷⁴ Probably, they might be explained by the variation in reagent lots, as confirmed in another multicenter study with the same reagent lot used by all participating centers.⁷⁷ A more recent study showed poor interlaboratory agreement of cutoff value even when a common set of normal donors were investigated by different platforms.⁷⁸ These observations illustrate that because of many variables (eg, sample collection system, sample tubes, demographics of the normal population, variation in PNP, lot of reagents), cutoff values determined elsewhere cannot be transferred irrefutably from one laboratory to another. In the meantime, it is still advised to determine in-house cutoff values⁷³ but joint efforts to calculate universal cutoff values per test/instrument combination should continue, to find a solution for this challenging issue.

4 | CONFIRMATION OF PERSISTENT LA POSITIVITY

The guidelines advise that confirmation of a positive result after 12 weeks is necessary to exclude transient LA induced by infection or drugs.^{2,12} The interval of 12 weeks, defined in the Sydney criteria (previously 6 weeks in the Sapporo criteria), was based on expert opinion and little is known on persistence of aPL beyond 3 months.¹ A recent long-term follow-up study reported persistence of aPL over time to be associated with the highest risk of thrombosis in triple-positive

patients.⁷⁹ A retrospective study evaluated extended persistence and demonstrated persistence in 96% in a median follow-up of 56 weeks, with no significant lower persistence in the single positive patients compared with the double and triple positives.⁸⁰ These results support the choice of extending the retest interval beyond 12 weeks, which would identify patients who remain persistently positive even beyond this period.⁸⁰ Confirmation of triple-positive patients (same isotype⁸¹ or independent of isotype of aCL and $\alpha 2$ GPI⁸⁰) seems to be predictable without the need to retest after 12 weeks because triple-positivity persists in the majority of patients.^{80,81}

A confirmatory positive test after 12 weeks renders the initial test result more reliable and increases assurance of a test result in the context of poor standardization and interferences affecting the test result.^{2,3} We conclude that retesting for confirmation after 12 weeks is still recommended.

TABLE 3 Research agenda to further optimize and harmonize LA testing

The optimal timing of initial and subsequent aPL testing after a first unprovoked venous thromboembolism and for stroke is not established. An ISTH-supported SSC-aPL observational study with serial LA/aPL testing ("Successive follow up of antiphospholipid antibodies fluctuations in patients with clinical Sydney criteria for APS: Long Term impact for diagnosis and outcome - SKYLARK Study", see www.isth.org/resource/resmgr/subcommittees/final_skylark_project_ssc_ap.pdf) should provide information on optimal timing of aPL testing in these situations and fluctuations in aPL titers.

Investigation of the use of lower cutoff values (95th centile instead of 99th centile) in pregnancy morbidity may be useful, as such patients might benefit from the standard treatment during pregnancy. Further studies are needed, comparing the 99th versus 95th centile in women with pregnancy associated clinical manifestations of APS. Data on changes in aPL titers during pregnancy and their influence on pregnancy outcomes are controversial. An ISTH-supported SSC-aPL observational study with serial LA/aPL testing pre- and during pregnancy, with 24-month follow-up, is being set up (part of the SKYLARK Study).

There is little information on the value or therapeutic consequences of retesting persistently positive patients annually, for LA, aCL and $\alpha 2$ GPI. This could be potentially useful as part of risk assessment (fluctuation of titers, change of antibody profile over long-term period) follow-up assessment visits. A prospective study could help to define the utility of annual aPL testing. Also, in systemic lupus erythematosus patients who have initially tested negative for aPL, optimal intervals for aPL retesting have not been defined. Little evidence is available regarding the optimal timing of testing in stroke/TIA patients in relation to initiating treatment. Early testing for LA (and aPL) may benefit management of these patients, however so far, the influence of immediate versus delayed initiation of anticoagulation on outcome in acute stroke is unknown.

A multicenter evaluation of TSVT/ET testing for LA in anticoagulated and non-anticoagulated patients is ongoing within the frame of an ISTH-SSC Project to validate the assays, to endorse recommendations and wider adoption.

Efforts to calculate universal cutoff values per test/instrument combination in a multicenter setting should continue.

Further research on elucidating the biochemical or biophysical basis of LA is encouraged as this may lead to improved standardization of the assays with significant clinical impacts.

5 | INTERPRETATION OF RESULTS AND REPORT

LA is finally reported as positive/negative.¹² Furthermore, the laboratory can report the detailed quantitative results (normalized ratios) for each step to allow clinicians to make the interpretation according to the local cutoff values and comparison of results over time. If LA is found positive, a comment for retesting should be included to confirm persistence if not previously established. Other comments informative for the interpretation should go in an interpretative comment added to the final LA conclusion, for instance a warning about possible interference of anticoagulants (VKA, heparins, DOAC) or acute phase proteins. Information provided in the request on the patient's anticoagulation status should also be incorporated into the report. If DOAC adsorbents are used, this should be mentioned in a comment (for instance, "LA negative after adsorption of DOAC" or "LA remains positive after adsorption of DOAC").

The results should always be related to the results of aCL and $\alpha 2$ GPI to assess the risk profile in the clinical context, and close collaboration between the laboratory and the clinician is essential.^{2,12,75}

6 | RESEARCH AGENDA

In this guidance, clarifications and adaptations of procedures are given based on published data and consensus of experts in the field. Some points need more evidence before being recommended. Therefore, we suggest a research agenda: see Table 3.

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CONFLICTS OF INTEREST

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AUTHOR CONTRIBUTIONS

Katrien M.J. Devreese and Hannah Cohen conceived the guidance and reviewed the literature. Katrien M.J. Devreese wrote the first draft of the manuscript. All the authors revised and accepted the manuscript, which was then accepted 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 ISTH.

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