PART 11 Antiphospholipid Antibody Syndrome (APS) Lab Investigation

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TOPICS COVERED

- ✓ Classification and Diagnostic Criteria for APS
- ✓ Anti-Phospholipid Antibodies
- ✓ Lupus Anticoagulant

A heterogeneous group of antibodies that can cause prolongation of the APTT test are antiphospholipid antibodies, which generally react with epitopes on proteins that are complexed with negatively charged phospholipids. Many of these antibodies require beta-2-glycoprotein 1, a protein that binds to phospholipids. Others can be directed against prothrombin. Proper identification of these antibodies will allow antiphospholipid antibody syndrome (APS) to be characterized (Ruiz-Irastorza et al, 2010; Schreiber et al, 2018). It is important to note that these antibodies can interfere with coagulation reactions in the laboratory, prolonging phospholipid-dependent tests such as the APTT and occasionally the PT, but they are not associated with bleeding, except in some rare cases where there is a significant acquired prothrombin deficiency. Paradoxically, these antibodies are clearly associated with venous and arterial thrombosis by mechanisms that are not well understood. In diagnostic centers for bleeding disorders, it is necessary to be able to detect these antibodies using specific tests for the investigation of patients with prolonged APTT (Barbosa et al, 2019). There are currently specific guidelines for the correct performance of the tests used for the laboratory diagnosis of APS are available and can be used to update lab information, see below.

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Classification and Diagnostic Criteria for APS: Since it became clear that antiphospholipid antibodies were significantly associated with vascular thrombosis and pregnancy morbidity, the need for consensus criteria for APS resulted in the Sydiney criteria, Table 38 (Miyakis et al, 2006). Patients are classified as having APS when a clinical event occurs together with at least one positive laboratory criterion. The laboratory criteria for defining APS are the presence of lupus anticoagulant, aCL IgG/IgM, or a2GPI IgG/IgM, persistently present for at least 12 weeks. Currently, a novel international initiative is being carried out to develop new criteria for classifying APS. The proposed laboratory criteria include only the antibodies from the current criteria (lupus anticoagulant, aCL IgG/IgM, and a2GPI IgG/IgM).

Table 38. Sydney 2006 criteria for classifying APS

Clinical Criteria	Laboratory Criteria
1. Vascular thrombosis	**1. Lupus anticoagulant**
Venous, arterial or microvascular;	≥2 positive results
Confirmed by objective validated criteria;	At least 12 weeks apart
No evidence of inflammation in vessel wall	
And/or	
2. Pregnancy morbidity	**2. Anticardiolipin antibody IgG and/or IgM**
≥1 unexplained fetal death _10th week of gestation or;	Serum and plasma
≥1 premature birth <34th week of gestation because of: Eclampsia or severe pre-eclampsia, placental insufficiency;	Medium or high titer (>40 GPL or MPL, or >99th percentile),
≥3 unexplained consecutive abortions <10th week of gestation.	Measured by ELISA Standardized
	≥2 positive results
	At least 12 weeks apart
And/or	
	3. Anti-β2 glycoprotein I antibody IgG and/or IgM
	Serum and plasma
	Medium or high titer (>40 GPL or MPL, or >99th percentile),
	Measured by ELISA Standardized
	≥2 positive results
	At least 12 weeks apart

Lupus anticoagulant

How to choose the test? Lupus anticoagulant can be detected by different phospholipid-dependent coagulation tests. The most recent update of the ISTH guidelines on lupus anticoagulant detection recommends using two tests in parallel, the diluted Russell viper venom time (dRVVT) and the APTT (Devreese et al, 2020). The dRVVT is more specific, while the APTT is more sensitive for lupus anticoagulant (depending greatly on the reagent used). The two assays are complementary because the antibodies do not always react in both assays. The dRVVT assay is based on the direct activation of FX by an enzyme present in the venom of Russell's vipers. The APTT assay is based on the activation of the contact (intrinsic) pathway of the coagulation cascade. The selection of appropriate reagents for lupus anticoagulant testing purposes is important, as there are several reagents available, especially for APTT (Favaloro et al, 2019). Two topics in the selection of the APTT reagent need to be addressed, the choice of activating agent and the composition and concentration of phospholipids. As an alternative to the APTT, the silica clotting time (SCT) test, can be used for lupus anticoagulant testing. The performance of lupus anticoagulant assays must be validated or verified before implementation in clinical practice. Part of the verification process should include testing samples with known lupus anticoagulant, and mean values well characterized (Gardiner et al, 2021a; Gardiner et al, 2021b).

How is the test carried out? Lupus anticoagulant assessment consists of a three-step procedure: screening, mixing, and confirmation (Devreese et al, 2020). PPP is necessary to avoid false-negative results due to the interaction of phospholipids and platelets. The screening stage includes tests with dRVVT and APTT reagents at low phospholipid concentrations. Coagulation factor deficiency or inhibitors other than lupus anticoagulant can cause a positive screening test, so mixing and a confirmation test step are necessary. The stepwise procedure can reduce costs, as it avoids carrying out the mixing and confirmation step unnecessarily if the screening step is negative. In the confirmation step, an excess of anionic phospholipid

is added to the test reagent, and the excess of phospholipid can reduce or neutralize the antibodies. In dRVVT tests, the screening and confirmation assays are carried out in parallel, and the result of the confirmation step is expressed as a normalized ratio according to the calculation: [(screening patient result)/(screening pool result)]/[(confirmation patient result)/(confirmation pool result)]. In the mixing step, the screening test is performed on a mixture of 1:1 patient plasma and normal plasma pool. The mixing test is expressed as the normalized ratio [(screen mix)/(screen pool of normal plasma)]. When the clotting time in the confirmation assay is prolonged, an additional mixing step with the confirmatory reagents (confirm mix) can be performed, and the ratio is more robust and less affected by interference from congenital or acquired factor deficiencies. There are integrated assays that carry out all three stages in a single procedure. In these assays, screening and confirmatory tests are performed parallel on the patient's plasma mixed with PNP and the results are mostly expressed as the difference between the two tests.

Cut-off values: To interpret the lupus anticoagulant results, it is necessary to determine cut-off values to define positivity in all stages. First, laboratories should determine the cut-off values using a population of healthy individuals with at least 120 people, determining the cut-off point as the 99th percentile after rejecting outliers (Devreese et al, 2020). However, the number of 120 normal individuals to calculate cut-off values can be difficult to obtain for many laboratories. An approach that requires fewer volunteers is the transfer of cut-off values recommended by the manufacturer. This assumes that the manufacture cut-off points are based on a large healthy reference population with adequate demographic data, a correct statistical method and a correct reagent-instrument combination (Castellone, 2017). When these conditions are satisfied, the manufacturer's cut-off values should be verified before transfer by testing 20 healthy volunteers representing the demographics of the local population. After rejecting outliers and replacing them with new results from healthy volunteers, the results (outlier-free population) should be compared with the suggested cut-off value.

Interferences and limitations: The C-reactive protein interferes in vitro with the APTT test through its affinity for phospholipids, leading to false-positive results. Although this effect has not been observed for the dRVVT assay, this can vary between reagents. In addition, the increased coagulant activity of FVIII is linked with a shorter APTT giving false-negative results. Elevated FVIII levels can be seen during pregnancy, surgery, inflammation, malignancy, and other conditions. Lupus anticoagulant testing during the thrombotic event or during anticoagulation treatment is not recommended (Devreese et al, 2020). The most recent ISTH guidelines do not advise pre-diluting samples for lupus anticoagulant testing in the presence of AVKs (Devreese et al, 2020). DOACs directly inhibit thrombin (e.g. dabigatran) or FXa (e.g. apixaban, betrixaban, edoxaban, and rivaroxaban), with various effects on coagulation tests, leading to the interpretation of false-negative and false-positive results. The APTT and PT should be performed before starting the lupus anticoagulant test in order to have more information about the sample, but this does not exclude the presence of DOACs or LMWH.

Anti-Phospholipid Antibodies:

How do I choose the test? The anti-cardiolipin and anti-beta-2-glycoprotein1 antibodies are identified by solid phase immunoassays. The SAF classification criteria indicate the measurement of these antibodies by standardized ELISA. However, alternative detection techniques for antibodies testing, such as chemiluminescence, fluorescence enzyme, and multiplex flow immunoassays have become available (Devreese et al, 2014). Compared to traditional manual ELISA methods, the newest techniques are easier to use and show better precision. Assays differ in terms of solid phase, detection principle, coating, source of antigens and antibodies, blocking agents to prevent non-specific binding, dilution protocol, calibration, and units (Devreese et al, 2014). It is recommended to carry out the patient's follow-up tests in the same laboratory, as the platforms cannot be used interchangeably.

How to carry out the test? Serum or PPP can be used for aCL and a2GPI testing (Devreese et al, 2018). The need to perform the test in duplicate depends on the performance characteristics of the assay. Duplicate testing is especially recommended for manual ELISAs or if the inter- and intra-run imprecision of the

assay is >10% (Devreese et al, 2014). In each run, internal quality control material needs to be analyzed at relevant titer levels. Calibration curves need to be determined in every single ELISA run or for each reagent lot in automated systems. Each calibration should be evaluated and rejected when it does not meet the manufacturer's requirements or when the correlation coefficient between test values and target values is less than 0.90 (Devreese et al, 2014). Unfortunately, there is no uniformity in reference material for test calibration. Efforts are being made to develop new monoclonal and polyclonal standards for aCL and a2GPI with the aim of creating WHO standards with IU/ml as the universal unit.

Cut-off values and antibody profile: The 40 GPL/MPL as the aCL cut-off point was based on studies showing better correlation of this point with APS (Levine et al, 1997). However, there may be a marked difference between 40 GPL/MPL and the 99th percentile for aCL (Vandevelde et al, 2024). And the ISTH-SSC does not recommend using 40 GPL/MPL as a cut-off point. It is recommended to calculate a laboratory-specific cut-off value for positivity based on a non-parametric 99th percentile of at least 120 reference subjects. Outlier rejection with the Dixon/Reed method is recommended to escape overestimation of cut-off values. Transferring the manufacturer's cut-off points after verification on 20 or more reference subjects is a valid alternative if the manufacturer's cut-off point is calculated on a sufficiently large reference population and an appropriate statistical methodology has been applied. Each aCL and a2GPI result above the cut-off point should be reported as positive, accompanied by the numerical value and the internal cut-off value (Vandevelde et al, 2024). Positivity in one of the tests (lupus anticoagulant, aCL IgG, aCL IgM, a2GPI IgG, or a2GPI IgM) is sufficient to diagnose APS. The combined interpretation of different aPL as antibody profiles has been suggested to identify high-risk patients, compared to individual assessment. In asymptomatic aPL carriers, double and triple positivity was a risk factor for the development of thrombotic events, but single positivity of aCL or a2GPI was not (Mustonen et al, 2014).

Interferences: The presence of rheumatoid factor can cause false-positive aCL IgM and a2GPI IgM results (Devreese et al, 2014; Forastiero et al, 2014). Unlike lupus anticoagulant assays, antibodies testing with solid phase immunoassays is not subject to analytical interference from acute phase reactor reagents or anticoagulation therapy. However, a transitory increase in aCL and a2GPI is observed in inflammatory conditions (Exner et al, 2020; Laureano and Crowthe, 2018).

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