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How I treat

How we diagnose the antiphospholipid syndrome

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The antiphospholipid syndrome (APS) is an acquired thrombophilia, characterized by the occurrence of venous and arterial events. This article examines the laboratory and key clinical aspects of APS. Particular focus is given to anti-beta 2-glycoprotein I (β_2 GPI) antibodies in view of their recent inclusion in the APS classification criteria. The clinical utility of us-

ing the β_2 GPI enzyme-linked immunosorbent assay, in conjunction with the established lupus anticoagulant assays and cardiolipin enzyme-linked immunosorbent assay, for diagnosing and risk stratifying patients suspected of having APS is discussed. The relative importance of the various assays in diagnosing obstetric APS (early and late gestation

miscarriages) is explored. The implications of recent epidemiologic findings for possibly understanding the underlying pathophysiologic mechanisms of obstetric APS are highlighted. Insights into which patients with obstetric APS may be at most risk of thrombotic complications are presented. (Blood. 2009;113:985-994)

Introduction

The antiphospholipid syndrome (APS) is an important cause of acquired thrombophilia and recurrent miscarriages. This narrative-style review discusses the key laboratory and clinical aspects of APS. Particular focus is given to antibodies against beta 2-glycoprotein I (β_2 GPI), in view of their recent inclusion in the APS laboratory classification criteria¹ (Figure 1). The evidence for assessing antiprotease and antiphosphatidylethanolamine antibodies to diagnose APS is also examined.

The utility of the β_2 GPI enzyme-linked immunosorbent assay (ELISA), when used in conjunction with the cardiolipin (CL) ELISA and the lupus anticoagulant (LA) assays, in risk-stratifying APS patients is explored. Work undertaken by many groups over the years, ours included, in delineating the key characteristics of anti- β_2 GPI antibodies that associate with APS is presented.

Miscarriages are a major feature of APS. The relative importance of the LA assays, the CL-ELISA, and the β_2 GPI-ELISA in diagnosing APS in the context of early and late gestation miscarriages is assessed. The value of recent epidemiologic and basic science insights in refining our understanding of obstetric APS pathophysiology is examined, particularly with regards to considering the possibility that distinct mechanisms may be responsible for early and late miscarriages. The clinical implications arising from these observations are discussed.

Clarification of the nomenclature

Antiphospholipid antibodies is a term applied to antibodies detected traditionally by 2 types of assays, the CL-ELISA, which stems from the earlier work of Harris et al,² and the LA assays.³

The first report of a cofactor requirement for antibodies to bind cardiolipin (an anionic phospholipid) from patients with APS was by McNeil et al in 1989.⁴ This was subsequently confirmed by Galli et al⁵ and Matsuura et al⁶ in 1990. Purification and sequencing of the cofactor as β_2 GPI was reported by McNeil et al in 1990.⁷ It was noted that anionic phospholipid is not an absolute requirement for antibodies to bind β_2 GPI,⁸ negating the notion that β_2 GPI is a "cofactor" for antibody binding. Antibodies from these patients can

bind to β_2 GPI immobilized on an irradiated plate in the absence of anionic phospholipids.⁹ A negatively charged surface serves a 2-fold role. It enables β_2 GPI clustering, allowing divalent binding by the low affinity antibodies.^{10,11} It also enables the β_2 GPI molecule to undergo a conformational change,⁹ exposing a cryptic epitope on the first domain.¹²

Positivity on the CL-ELISA is not directed against β_2 GPI in the context of a number of infections.¹³ However, this is not true for all types of infections, as elevated anti- β_2 GPI antibodies in patients with leishmaniasis, leptospirosis,¹⁴ and leprosy¹⁵ have been noted. A key distinguishing feature between anti- β_2 GPI antibodies occurring in the context of leprosy and those that strongly associate with thrombosis is that in the former instance they are directed against an epitope on domain V of β_2 GPI,¹⁵ whereas evidence generated by a number of independent groups suggests in the context of thrombosis they target a specific epitope on domain I¹⁶⁻²⁰ (Figure 2). In addition, anti- β_2 GPI antibodies that associate with the APS clinical phenotype are predominantly the immunoglobulin G (IgG) isotype, particularly the IgG₂ subclass,²¹ whereas those occurring in association with infections such as leprosy are of the IgM isotype.²² The LA assay detects either anti- β_2 GPI²³⁻²⁵ or antiprotease antibodies,^{26,27} with the latter also occurring in association with infections, such as leprosy.²²

Hence, even though it is a misnomer, the term antiphospholipid antibody as a generic term persists when referring to antibodies that specifically target β_2 GPI and prothrombin.

Overview of the assays

Lupus anticoagulant

The LA is an assay that detects immunoglobulins that, although prevent coagulation in vitro, associate with thrombosis (Figure 3).²⁸ It is an important tool for diagnosing APS.¹ The LA assay system chosen has to comply with the 3-step strategy defined in the International Society of Thrombosis and Hemostasis criteria²⁹: (1) screening test: demonstration

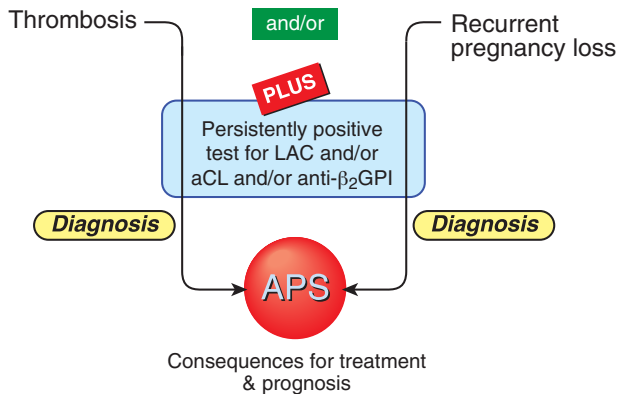


Figure 1. The updated classification criteria for APS.

of the prolongation of a phospholipid-dependent clotting time beyond the upper limit of the reference interval; (2) mixing test: confirmation of the presence of an inhibitor and the exclusion of a coagulation factor deficiency; and (3) confirmation that the inhibitor is phospholipid-dependent and not directed against a specific coagulation factor. Hence, evidence for the presence of LA is provided by performing a sequential series of investigations, which are demarcated by the terms screening tests and confirmatory tests, the latter divided into mixing studies and tests assessing for phospholipid dependence.

There are different assays that can be used to screen for LA, although a number of surveys performed in different countries³⁰⁻³² suggest that some of the commonly used ones are the activated partial thromboplastin time (aPTT), the dilute Russell's viper venom time (dRVVT), and the kaolin clotting time. A description of the various assays and the methodology for their performance can be found in published guidelines.^{33,34} In view of the fact that no single screening test is 100% sensitive to detect all LA, at least one additional screening test is recommended.³⁵ One of the screening tests undertaken should use activation of the intrinsic pathway of coagulation (eg, aPTT or kaolin clotting time) and the other the direct activation of factor X (eg, dRVVT).³⁶ Local reference ranges should be established for each LA method used and for each coagulometer.³⁷

The mixing studies involve combining the patient's plasma with normal plasma (1:1) and assessing the influence of this procedure on clotting time, the theoretical underpinning being that if prolongation of clotting time is the result of a coagulation factor deficiency, it will correct to normal, whereas with LA, correction requires larger volumes of normal plasma.³³ A number of methods have been proposed by which this step can be interpreted,³⁶ the most robust being the calculation of the index of circulating anticoagulant, initially proposed by Rosner et al.³⁸

To determine whether the inhibitor is phospholipid-dependent, the platelet-neutralization procedure, which uses either washed platelets activated with calcium ionophore or platelets lysed by repeated freeze-thawing (leading to the exposure of anionic phospholipid surfaces), is commonly used.³³ Correction of the clotting time will occur in the presence of LA. Alternate reagents to assess for this property include a modified aPTT reagent, which contains hexagonal (II) phase phospholipids, which specifically bind LA.³⁹ With regard to the dRVVT confirmatory tests, alternatives to the platelet-neutralization procedure that can be considered include reagents with a high concentration of phospholipid or an LA-insensitive phospholipid.³³

It is pertinent to note that there is considerable interlaboratory variation with the performance of the LA assays. National

Domain I

- AA G40, R43 dominant epitope
- AA K19 minor epitope

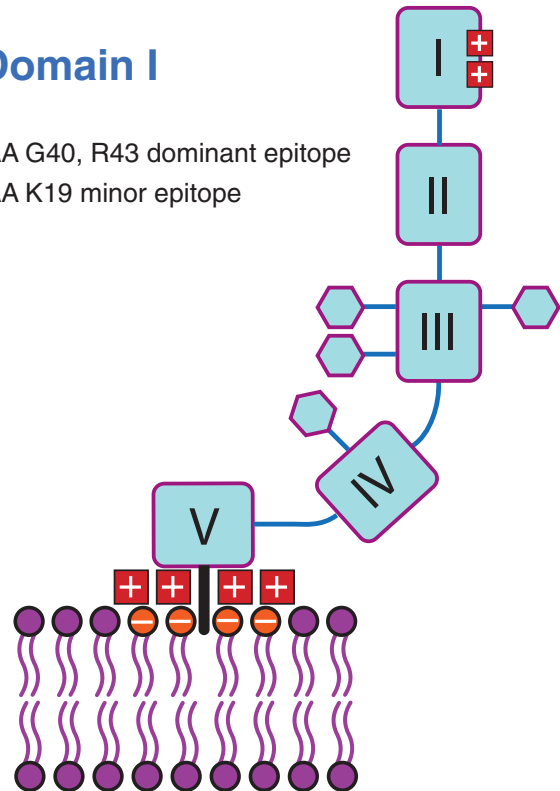


Figure 2. The β_2 GPI B-cell epitope in patients with APS is on domain I of the molecule. AA indicates amino acids; G40, glycine at position 40; R43, arginine at position 43; K19, lysine at position 19; I-V, domains I through to V. From Passam and Krlis¹⁰⁸ with permission.

surveys carried out in Europe,^{30,32,40} the United States,⁴¹ and Australia³¹ emphasize this issue. In recent surveys there was a false-positive detection of LA in 24% of samples³² and a false-negative result from 18.5% of participating centers.⁴² One of the factors that might contribute to a false-positive result is heparin contamination.³² This possibility may be assessed for by measuring the thrombin time and, if prolonged, determining anti-FXa activity.³² Other reasons for false-positive results include the presence of specific anticoagulation factor antibodies.^{29,43} The patient's history, assessing for a bleeding diathesis, is relevant in this setting. Preanalytical variables, such as improper plasma preparation, may lead to false-negative results because of contamination with platelets.⁴⁴ The diluting effect of mixing studies may also lead to false negatives in instances in which the LA is weak.^{33,36} In one externally conducted quality control study, half of the weak LAs were not detected.³⁰ There is evidence to suggest that adherence to published guidelines may improve the performance of LA testing.⁴²

It is recommended that an assessment for LA not be undertaken while the patient is anticoagulated.¹ However, if it is necessary to do so, LA can be detected by such methods as assessing the taipan snake venom/ecarin clotting times because they are relatively insensitive to oral anticoagulation.⁴⁵ Alternatively, the performance of the dRVVT on equal volume mixtures of normal and test plasma can be considered, with a similar procedure for the confirmation steps. A positive result is useful, although a negative result may be unreliable.³³

Regardless of the method chosen to test for LA, internal laboratory quality controls are important, particularly the utilization of known positive and negative LA controls with each batch of testing.³³ An attempt in the direction of standardization of the LA test has also been made by the development of monoclonal

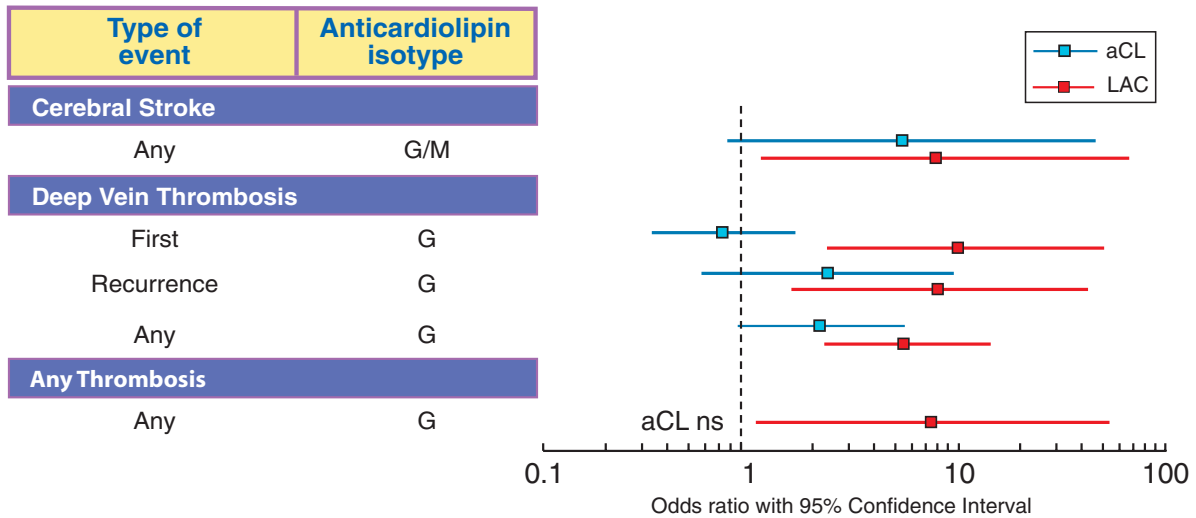


Figure 3. The lupus anticoagulant (LAC) correlates better with thrombosis than aCL. From Galli et al²⁸ with permission.

antibodies with LA activity for spiking normal plasma to overcome restrictions in the availability of patients samples.^{46,47} In addition, developing reference standards from lyophilized LA plasmas of known potency is being considered (International Society of Thrombosis and Hemostasis Scientific Subcommittee Session 2007). An update of the criteria for the optimal performance of LA is anticipated in the near future.

To refine the clinical utility of LA testing, investigators have been interested in differentiating the LA immunoglobulin according to target antigen^{48,49} given reports that β_2 GPI-dependent LA may more strongly associate with thrombosis than non- β_2 GPI-dependent LA.⁵⁰ These novel assays take advantage of the distinct properties of β_2 GPI-dependent LA to bind cardiolipin⁴⁸ and to display enhanced phospho-

lipid binding in the presence of calcium chloride, compared with non- β_2 GPI-dependent LA.⁴⁹ The value of these tests awaits further clinical trials.

The cardiolipin ELISA

This ELISA involves the assessment of diluted patient serum to binding a CL-coated plate in the presence of bovine serum. It will detect antibodies that bind CL alone and those that bind CL-bound bovine β_2 GPI. Both types are termed anti-CL antibodies (α CL; Figure 4). An inherent weakness of this assay is that there remains the potential to miss patients with antibodies that bind human but not bovine β_2 GPI. Hence, some assays now use human β_2 GPI in the CL-ELISA.

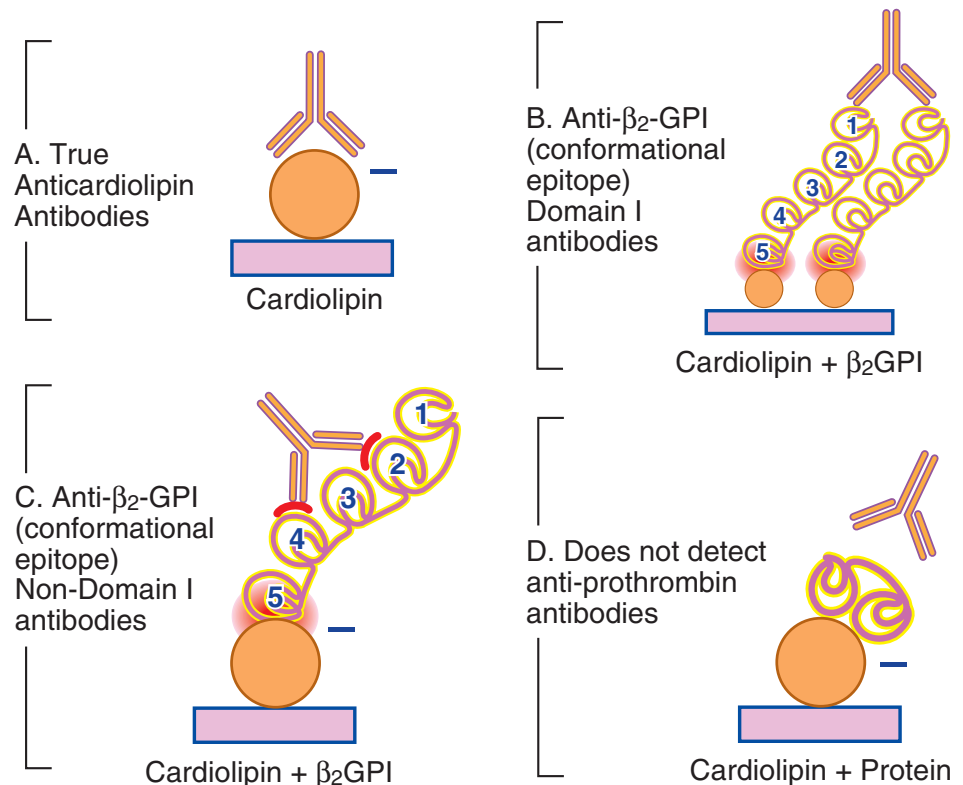


Figure 4. Schematic representation of the cardiolipin ELISA, which detects a number of antibody specificities, including β_2 GPI. From Passam and Krilis¹⁰⁸ with permission.

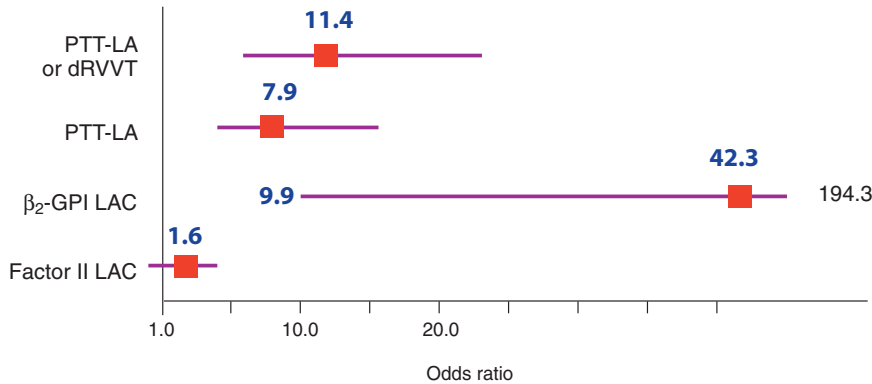


Figure 5. β_2 GPI-dependent LAC strongly associates with thrombosis, compared with factor II (prothrombin)-dependent LAC. From de Laat et al⁵⁰ with permission.

IgG and IgM α CL are expressed in international standardized GPL and MPL units, respectively. These units should be derived using standardized IgG and IgM α CL calibrators.⁵¹ GPL and MPL units are defined as binding observed with 1 μ g of affinity-purified polyclonal IgG and IgM α CL that were distributed to participating laboratories.⁵² Secondary standards were ultimately calibrated against the primary standards. Hence, different batches of calibrators will contain heterogeneous polyclonal α CL from different patients. This has led to suggestions that these calibrators may not necessarily behave in a homogeneous fashion when assayed at different dilutions or using different kits.⁵³ This is underlined by the observation of considerable interlaboratory variability,⁵¹ even when using the same batch of calibrators. There is also a high degree of variability between different commercial kits for α CL detection when assessed within the same laboratory, with lower variability seen with different commercial anti- β_2 GPI kits.⁵⁴ Methodologic insights pertaining to the optimal performance of the CL-ELISA have recently been published by Pierangeli and Harris.⁵⁵

A positive result is defined as a medium or high titer (ie, either > 99th percentile, or > 40 GPL or MPL).¹ To satisfy the APS laboratory classification criteria, a patient has to be persistently positive on either one of the assays (CL-ELISA, LA, or β_2 GPI-ELISA) for at least 12 weeks.

The direct β_2 GPI ELISA

This type of ELISA involves coating-purified, native β_2 GPI directly onto an irradiated plate. In theory, this type of assay should detect a greater proportion of clinically relevant antibodies than the CL assay. However, observations that there exist “nonpathogenic” antibodies that bind β_2 GPI within this assay¹² suggest that human β_2 GPI binding an artificial plastic surface may expose additional neoepitopes, thus reducing the specificity of this assay in detecting clinically relevant antibodies. This may in part explain why some studies have found no association of thrombosis with positivity on the direct β_2 GPI-ELISA.⁵⁶

There remains a lack of a formal, universally accepted method for performing this ELISA, coupled with a lack of standardized calibrators. The commercial kits are calibrated by the manufacturers' own calibrators and expressed in arbitrary units. This has prompted calls to use universally standardized calibrators, such as the humanized monoclonal antibodies HCL (IgG) and EY2C9 (IgM).^{57,58} Despite this, there generally seems to be better interlaboratory consensus with this ELISA compared with the CL assay.^{59,60} In the revised APS classification criteria, a positive result on the direct β_2 GPI-ELISA is defined as a titer greater than the 99th percentile.¹

Characteristics of anti- β_2 GPI antibodies that associate with thrombosis

The β_2 GPI molecule is divided into 5 domains (DI-DV).⁶¹ Anti- β_2 GPI antibodies may predominantly target domain I in APS patients.¹⁶ There is some controversy, as a number of other epitopes on β_2 GPI have been reported.⁶² This area has been extensively reviewed by Giles et al,⁶² who concluded that the major APS epitope is probably on domain I. The major β_2 GPI domain I epitope includes the surface-exposed residues Gly40-Arg43.^{17,18} It is a complex conformational epitope, with evidence suggesting it may include Arg39, the domain I-II interlinker, and Asp8 and Asp9.²⁰ Nondomain I anti- β_2 GPI antibodies (which target domain V) may be detected in patients with leprosy¹⁵ and childhood atopic dermatitis.⁶³

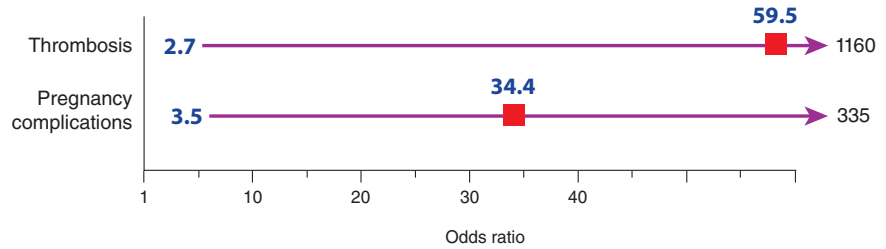
de Laat et al⁵⁰ analyzed patients with thrombosis who were positive on the LA assay and satisfied the classification criteria for APS. The predominant autoantibodies were targeting β_2 GPI,⁵⁰ involving the domain I residues Gly40-Arg43.¹⁹ Non- β_2 GPI antibodies with LA activity did not independently associate with thrombosis⁵⁰ (Figure 5). The importance of anti- β_2 GPI, relative to non- β_2 GPI antibodies with LA activity, in the context of thrombosis has been suggested by others.^{64,65} Antiprothrombin and anti- β_2 GPI antibodies may occur concurrently.⁶⁶

Anti- β_2 GPI antibodies may be divided into high and low avidity, and it is the former that tend to associate with thrombosis.⁶⁷⁻⁷¹ The 2 types may be distinguished by their ability to dissociate from immobilized β_2 GPI in the presence of increasing concentrations of urea⁶⁷ or ionic buffer.⁷⁰ Both types have been detected in patients with systemic lupus erythematosus (SLE).^{67,68}

The epitopes to which the low- and high-avidity antibodies bind has not been determined. de Laat et al¹⁹ divided anti- β_2 GPI antibodies into type A (those targeting the epitope on domain I) and type B (those targeting nondomain I epitopes) in patients with SLE, lupus-like illness, and APS. They found that it was the type A antibodies that associated with thrombosis. It should be noted this association has not been confirmed in rigorous prospective studies. It seems reasonable to hypothesize that high-avidity antibodies probably target the epitope containing the residues Gly40-Arg43, whereas the low-avidity antibodies may be representative of the type B, nondomain I antibodies.

The corollaries to these investigations would seem to be that (1) a patient who tests positive on the LA assay and the direct β_2 GPI-ELISA may be likely to have a greater proportion of the high-avidity, domain I-targeting anti- β_2 GPI antibodies than a

Figure 6. Positivity on multiple assays (LAC/CL-ELISA/direct β_2 GPI-ELISA) is associated with an increased risk of thrombosis and pregnancy complications. From Ruffatti et al⁷⁵ with permission.



patient who tests positive on the direct β_2 GPI-ELISA alone, and (2) the LA in this context is likely to be related to anti- β_2 GPI antibodies, compared with a patient who tests positive on the LA assay alone. Whether these possibilities explain the greater association with thrombosis described in patients positive on the direct β_2 GPI-ELISA and the LA assay, compared with positivity on either assay alone,⁷² needs further analysis.

High-risk profile of patients who test positive on the CL-ELISA, the direct- β_2 GPI ELISA, and LA

A strong association in a number of retrospective analyses has been noted between positivity on multiple assays (LA, CL, and β_2 GPI-ELISA) and thrombosis and miscarriages, compared with patients positive on one or 2 assays⁷²⁻⁷⁶ (Figure 6). What do these findings imply? Patients positive on 3 assays (CL, direct β_2 GPI-ELISA, and LA) tend to have higher levels of anti- β_2 GPI antibodies than patients positive on fewer assays⁷⁵ (Figure 7). Hence, this may be a factor. Potentially, they may also be the patients who have high-avidity, domain I binding anti- β_2 GPI antibodies. Certainly in the future, should assays that specifically detect antibodies with β_2 GPI-dependent LA activity become widely available,^{19,48,49} then these possibilities can be assessed.

There are other possibilities to be considered as to why testing positive on multiple assays may be associated with

greater risk. There may be an additive pathogenic effect between multiple distinct antibodies. For this latter notion to be firmly supported, one of the requirements would be the demonstration of an association between the non- β_2 GPI-reactive autoantibodies detected by the relevant assays with thrombosis, independent of the presence of anti- β_2 GPI antibodies. Studies in this regard pertaining to antiprothrombin antibodies are not consistent, with some suggesting they may associate independently⁷⁷ and others suggesting they do not.^{50,65}

Antiprothrombin and other antibodies

It was noted by Galli et al,⁵⁶ in their systematic review of the literature, that positivity on the prothrombin ELISA did not consistently associate with thrombosis. Furthermore, a recent study has suggested that this assay does not provide additional utility for diagnosing APS.⁷⁸ However, it has been proposed that positivity on an ELISA that detects antibodies targeting the prothrombin-phosphatidylserine complex may more consistently associate with thrombosis⁷⁹; hence, this is an area of ongoing research. At the moment, testing positive for antiprothrombin and antiprothrombin/phosphatidylserine antibodies is not a laboratory criterion for APS classification.¹

In recent studies, it was noted that a positive result on an assay that specifically detects antibodies targeting phosphatidylethanolamine associates with thrombosis⁸⁰ and recurrent miscarriages.⁸¹ However, the utility of this assay and other assays that detect antibodies that directly target various types of phospholipids needs further validation, as there are reports, assessing commercially available assays, which question their ability to improve the yield of APS diagnosis.⁸²

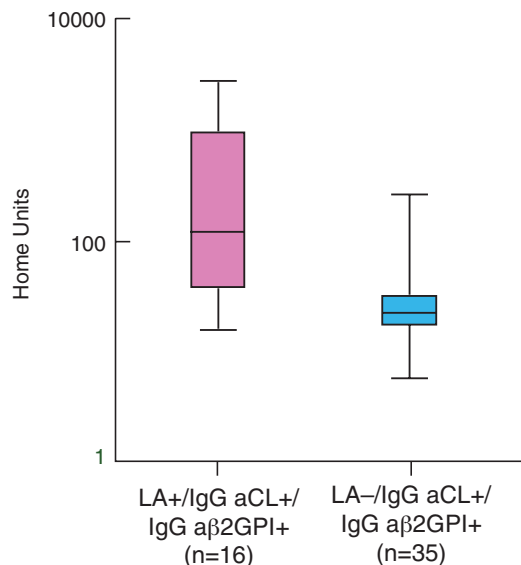


Figure 7. Positivity on multiple assays (LAC/CL-ELISA/direct β_2 GPI-ELISA) is associated with higher levels of anti- β_2 GPI Abs than patients who are positive only on the CL-ELISA and the direct β_2 GPI-ELISA. From Ruffatti et al⁷⁵ with permission.

Considerations arising at the laboratory-clinical interface of APS diagnosis

Venous thrombosis

There is evidence derived both from the SLE^{83,84} and the general population^{85,86} that LA associates with venous thrombosis. These observations support the notion that LA may be a potential risk factor for venous thromboembolism (VTE). As a point of contrast, in recent longitudinal prospective studies,^{87,88} which analyzed the general population, positivity on the CL-ELISA did not associate with the occurrence of a first VTE. There are data, however, to suggest that the CL-ELISA may have utility in the prognostic setting. Schulman et al⁸⁹ noted that, on cessation of warfarin after 6 months of treatment for a VTE, patients who were positive on the CL-ELISA, compared with patients who were negative, had a higher risk of recurrence. The interplay between antibody profile, the type of initial VTE event (below knee, above knee, nonlower limb deep venous thrombosis, or pulmonary embolus), and the presence or absence of concurrent risk factors for VTE (transient, eg, pregnancy; or permanent,

eg, genetic thrombophilia) in modulating the risk of VTE recurrence is an area warranting further delineation in prospective studies. These types of studies may allow the development of a risk-stratified approach in guiding the optimal duration of anticoagulation in APS patients who have sustained a VTE.

Arterial thrombosis

LA has been found to associate with stroke in the SLE and the general population settings.⁹⁰ The association may be greater in young adult stroke patients.⁹⁰ In view of these findings, it is highly relevant for a physician who orders these tests after a patient has sustained an initial stroke, to know whether a patient's risk for stroke recurrence or other thrombotic events is different relative to a patient who has had a stroke and does not have these antibodies. The importance of knowing this information is worth considering. If the risk for recurrence is the same, all else being equal (ie, the type of stroke: cardioembolic or noncardioembolic, the type of treatment and its duration), then the implication is that patients who are antibody positive do not have a worse prognosis and hence do not need to be treated differently to those who have had a stroke and do not have the antibodies. It also implies that information obtained from large prospective, randomized controlled trials assessing stroke treatment, which involve the general population, may be directly relevant to those who are antibody positive. On the other hand, if the risk for thrombotic recurrence is greater in positive patients, then the implication is that trial data from the general stroke population cannot be confidently extrapolated to antibody-positive stroke patients. The stroke population with antibody positivity would need to be assessed as an entity with distinct therapeutic implications.

The large, prospective, observational, Antiphospholipid Antibodies and Stroke Study, nested within a much larger randomized controlled trial involving the general stroke population,⁹¹ addressed this question. It concluded that patients from the general population who have sustained an initial noncardioembolic stroke and are positive for LA and/or the CL-ELISA (low, medium, and high titers included), within 30 days of the stroke (patients were tested once), do not have a different prognosis than those who test negative. This result was consistent within the aspirin and warfarin (low to moderate range international normalized ratio) arms of the trial.

It remains to be assessed in future prospective studies, whether testing positive on multiple assays (CL-ELISA, direct β_2 GPI-ELISA, and LA) is associated with a greater risk of stroke recurrence relative to the risk of thrombotic recurrence in patients who are antibody negative. The prognostic value of antibody persistence (at least 12 weeks apart) and medium or high titers on the ELISAs also needs to be assessed in the prospective setting.¹

Distinct risk profile of persistent versus transient antibodies

Implications for the recruitment of APS patients for clinical trials

Danowski et al⁹² noted, in a cohort of SLE patients who had persistent positivity on the direct β_2 GPI-ELISA, on at least 2 occasions, at least 12 weeks apart, an association with thrombosis to a greater extent than transient positivity. They also demonstrated that positivity on the direct β_2 GPI-ELISA

correlates with LA positivity.⁹² Whether persistently elevated anti- β_2 GPI antibodies more strongly correlate with LA than transient ones was not addressed. This would seem a reasonable assumption, in view of the association between anti- β_2 GPI antibodies with LA activity and thrombosis.⁵⁰ The implications of this, if verified, would be that persistent and transient anti- β_2 GPI antibodies may be distinguished by the presence or absence of LA activity, respectively.

The possibility that patients who initially test positive on multiple assays (LA, CL, and β_2 GPI-ELISA) are more likely to remain positive on repeat testing at a future time interval is also suggested by the study of Nash et al.⁹³ They reported that the presence of persistently elevated IgG α CL, at levels more than 60 GPL, was a strong predictor of the presence of coexisting LA and positivity on the direct β_2 GPI-ELISA.⁹³

It was noted by Crowther et al,⁹⁴ in their randomized controlled trial assessing secondary prophylaxis in APS patients, that one of its limitations was that the first 3-month period after the initial thrombotic event was not included in the analysis because patients had to satisfy the APS laboratory criterion for persistence. This is a legitimate concern because there is currently no validated manner to predict who will remain persistently positive. Trial organizers risk enrolling patients who do not satisfy the classification criteria for the syndrome if they randomize patients after they initially test positive. This possibility is emphasized by a recent study⁹⁵ looking at young adults who had sustained a stroke and were noted to have a patent foramen ovale. Twelve had elevated titers (> 99th centile of normals) of IgG and IgM anti- β_2 GPI antibodies on initial testing, and yet on repeat testing at 12 weeks, only 1 of the 12 remained positive. Similar outcomes were seen using the CL-ELISA (IgG and IgM). None of the patients was initially positive for LA.

The importance of being able to analyze the first 3 months after an initial thrombotic event, in any future trials involving APS patients (perhaps when assessing the effectiveness of the newer generation of anticoagulants), can be inferred from the general population. It is not only a high-risk period during which thrombotic recurrences may occur,⁹⁶ but it is also a period of increased bleeding risk associated with anticoagulation.⁹⁷ Potentially, patients who initially test positive on all 3 assays (LA, CL, and β_2 GPI-ELISA) may enable the study of this critical period by allowing early randomization of patients who are likely, on subsequent testing, to satisfy the APS laboratory criterion of persistence.

Obstetric considerations

A meta-analysis has assessed the strength of association of positivity for LA, the β_2 GPI-ELISA, or the CL-ELISA, with recurrent miscarriages, focusing on women who did not have SLE (the period between 1975 and 2003 was analyzed).⁹⁸ It was noted that a positive LA strongly associated with late gestation miscarriages (defined by the authors of the study as < 24 weeks), although its association with early miscarriages (defined by the authors as < 13 weeks) was not assessed.⁹⁸ It should be noted that these definitions for early and late miscarriages are different from those in the APS classification criteria, in which early is defined as before 10 weeks' gestation and late as miscarriage at or beyond 10 weeks.¹ In the same analysis,⁹⁸ positivity on the β_2 GPI-ELISA did not associate with early recurrent miscarriage (< 13 weeks), although an assessment for an association with late miscarriages was not undertaken. In contrast, antibodies detected by the

CL-ELISA associate with both early (< 13 weeks) and late (< 24 weeks) recurrent miscarriages.⁹⁸

Ruffatti et al^{75,76} have noted in 2 retrospective studies that there is a stronger association with recurrent late fetal loss (> 10 weeks gestation) and thromboembolic events in women who are positive on all 3 assays (CL, direct β_2 GPI-ELISA, and LA), compared with women who are either dual or single assay positive. Furthermore, a recent study has noted that positivity on the β_2 GPI-ELISA associates with a positive LA in women with miscarriages.⁹⁹ Hence, one possibility warranting further consideration is whether anti- β_2 GPI antibodies with LA activity may specifically associate with late miscarriages.

It is relevant to note that in *in vivo* studies¹⁰⁰⁻¹⁰² documenting the role of complement and inflammation in the pathogenesis of fetal loss, in which a murine model of APS was used, the specific human monoclonal antibody used (mAb 519) binds cardiolipin in the absence of β_2 GPI.¹⁰³ Polyclonal antibodies from 3 patients diagnosed with APS were also used, although the antigen specificity of the antibody fraction responsible for the experimental murine fetal resorption via a complement/inflammatory-mediated mechanism was not determined.¹⁰⁰ As a point of contrast, in a study conducted by Robertson et al,¹⁰⁴ a spontaneously occurring murine monoclonal anti- β_2 GPI antibody directed against domain I, and patient-derived polyclonal antibodies with β_2 GPI reactivity did not cause murine fetal resorption, nor did they affect fecundity in all mice.

Hence, a conceptual synthesis of the current epidemiologic and *in vivo* observations raises the possibility that non- β_2 GPI antibodies detected by the CL-ELISA may be particularly relevant in early miscarriages, perhaps via the induction of an inflammatory mechanism. Anti- β_2 GPI antibodies with LA activity may mediate a more prominent effect in late gestation miscarriages via distinct mechanism(s), perhaps by inducing intrauterine placental thrombosis (in view of the strong association of this class of antibodies with thrombosis^{19,50}). The pathogenic mechanisms operational in association with late miscarriages in women who have anti- β_2 GPI antibodies may not be reflected in murine APS models, perhaps because of the differences between human and murine placental development.¹⁰⁵ The development of obstetric APS animal models with closer homology to human placentation may be relevant to delineate the role of anti- β_2 GPI antibodies in the latter part of pregnancy.

It is important to confirm in future studies whether distinct antibodies are involved with different obstetric manifestations, as it may lay the foundation for exploring within clinical trials novel therapeutic approaches (eg, modulation of inflammation, such as targeting tumor necrosis factor- α ,¹⁰² vs further modulation of coagulation, perhaps with novel anticoagulants), based on antibody profile. The possibility is also raised that the risk of maternal thromboembolic events occurring in a patient with obstetric APS may vary according to whether they have anti- β_2 GPI antibodies with LA activity (perhaps higher risk), or non- β_2 GPI-dependent CL antibodies (perhaps lower risk). This possibility, if verified in prospective studies, may have implications when considering in which obstetric APS patients (without a history of previous thrombotic events), the administration of postpartum thromboembolic prophylaxis treatment may be most warranted.

et al.^{28,56} If a patient tests positive on all 3 assays (LA, β_2 GPI, and CL-ELISAs), confidence in the notion that the patient may have high titers of anti- β_2 GPI antibodies with LA activity is increased. The practical implication for initially testing with all 3 assays is as follows: Oral anticoagulants can potentially interfere with the performance of the LA assay,³² which has ramifications when considering repeating the LA test to assess for persistent positivity at 3 months to establish the diagnosis of APS. Knowing that the patient initially tested positive on all 3 assays suggests that the β_2 GPI and/or the CL-ELISAs can be performed as an alternative to LA at this later time point to enable a definitive diagnosis of APS to be made.

The type of thrombosis of particular relevance for considering APS, based on clinical association studies, appears to be venous thrombosis and perhaps stroke (particularly young adults). It is important when diagnosing thrombotic APS to also assess for concurrent prothrombotic factors, as they may be present in a significant proportion of patients, and some may be treatable in their own right (eg, traditional atherosclerotic risk factors).¹⁰⁶ We incorporate investigation with a trans-esophageal cardiac echo to thoroughly assess for a possible cardiac source of the arterial thrombus (cerebral and noncerebral events) in view of the relevance of this mechanism, which has been highlighted by Fulham et al.¹⁰⁷

In the obstetric APS setting, testing positive on all 3 assays (LA, β_2 GPI, and CL-ELISAs) seems to have implications for which patients may be at a greater risk for thrombosis.^{75,76} Hence, this may be one reason why it may be useful to perform all 3 assays when considering diagnosing obstetric APS, particularly in the context of late miscarriage (10 weeks and beyond). However, a case can also be made that, if a patient has only had recurrent early miscarriages (< 10 weeks) without a history of thrombosis, then the performance of the β_2 GPI-ELISA and perhaps LA assays, on the basis of current evidence,⁹⁸ cannot be confidently supported. In this situation, the CL-ELISA may have better utility in diagnosing APS.⁹⁸ The exclusion of other causes of miscarriages is necessary.¹

Some suggestions for future research

The relevant future research agenda, as can be inferred from this discussion, is broad and multifaceted. The development of novel assays that detect the clinically relevant anti- β_2 GPI antibodies and their subsequent validation will be of great utility. In addition, the validation of assays that assess for antibodies that specifically target prothrombin/phosphatidylserine and phosphatidylethanolamine is important.

Prospective, controlled epidemiologic analyses examining the relevance of issues, such as antibody profile in risk stratification, will be of value in informing decision making with regards to prognosis, laying the foundations for future therapeutic APS trials.

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Our perspective on diagnosing APS

When considering the importance of individual assays for diagnosing thrombotic APS, greater weighting would currently have to be given to a positive LA test, based on the systematic reviews of Galli

Authorship

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