Diagnosing antiphospholipid syndrome: 'extra-criteria' manifestations and technical advances

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Abstract

First described in the early 1980s,antiphospholipid syndrome (APS) represents a unique form of acquired autoimmune thrombophilia, with clinical features of recurrent thrombosis and pregnancy morbidity, in patients persistently positive for antiphospholipid antibodies (aPL).At least one clinical (vascular thrombosis or pregnancy morbidity) and one laboratory (lupus anticoagulant, anticardiolipin and/or anti-β2-glycoprotein I antibodies) criterion have to be met for the classification of APS. However, the clinical spectrum of the disease encompasses additional manifestations which may affect every organ and cannot be explained exclusively by a prothrombotic state; clinical features not listed in the classification criteriainclude neurologic manifestations (chorea, myelitis and migraine), haematologic manifestations (thrombocytopenia and haemolytic anaemia), *livedo reticularis*, nephropathy and heart valve disease. Similarly, increasing interest has focused upon the development of novel assays that may be more specific for APS. This review focuses on lights and shadows of the current classification criteria for APS, analyzing the role of extra-clinical criteria and laboratory features. The diagnostic approach todifficult cases, including the so-called "seronegative APS" and the role of promising novel techniques for aPL testing (chemiluminescence or multiline dot assays, thin layer chromatography immunostaining) are also discussed.

Introduction

Antiphospholipid syndrome (APS) was initially described in 1980s[1] and the term was first created to describe subjects with recurrent thrombosis or pregnancy complications who persistently tested positive for antibodies directed against phospholipids or protein/phospholipid complexes (named antiphospholipid antibodies, aPL). Although APS was first described in patients with systemic lupus erythematosus (SLE), subsequently it became obvious that SLE was not a necessary condition for its occurrence[2]. Over the past 30 years, understanding of the disease has witnessed a remarkable evolution and the diagnostic approach has changed accordingly. In 1999, consensus criteria on the clinical and laboratory criteria for "definite APS" (that became then known as the Sapporo criteria) were developed by international experts[3]. In 2006, the Sapporo criteria were updated duringa workshop heldin Sydney, Australia, before the Eleventh International Congress on Antiphospholipid Antibodies[4]. (Box 1)

Diagnostic Criteria Vs Classification Criteria

Classification criteria are described as a set of disorder characteristics used to group subjects into a well-defined homogenous population with similar clinical disease features[5,6]. In autoimmune diseases, classification criteria have been designed to mainly categorize well-defined homogenous cohorts for clinical research. Although their use is common in clinical practice, they may not be appropriate for routine use in diagnosis. Classification criteria are adjusted with improved methodology and further understanding of disease pathophysiology, but they still may not encompass all unique clinical and laboratory features to be applied for diagnosis of heterogeneous, rare, evolving autoimmune conditions.Conversely, diagnostic criteria development is challenging primarily due to the difficulty for universal application

given significant differences in the prevalence of rheumatic diseases based on geographical area and clinic settings[7].Overall, while classification criteria increase the specificity for underlying disease by creating a homogenous population, at times they may result in losing sensitivity on the receiver operating characteristic (ROC) curve continuum. As previously mentioned for APS, in 1999, a preliminary classification criteria set was established after an expert workshop held in Sapporo, Japan[3]. During a subsequent workshop (2004 in Sydney, Australia) experts proposed some modifications to the previous criteria, the main was the inclusion of anti-β2GPI antibodies among the laboratory features[4]. Although no new clinical criteria were added, some particular manifestations were remarked on, such as associated APS features, including cardiac valve involvement, *livedo reticularis*, thrombocytopenia, APS nephropathy, and non-thrombotic central nervous system manifestations (i.e. cognitive dysfunction)[4].

By emphasizing risk stratification, the Sydney APS classification criteria provide a more homogeneous basis for selecting patients for APS research. Investigating coexisting inherited and acquired risk factors for thrombosis in patients with APS, especially in those who are included in clinical studies, was strongly recommended. A validation exercise of the 2006 revised APS classification criteria has shown that only 59% of the patients meeting the 1999 APS Sapporo classification criteria met the revised criteria[8]. The Sydney criteria were meant to potentially limit facilitate the inclusion of heterogeneous groups of patients and also promote a risk-stratified approach. Now, though the APS classification criteria were not designed for clinical purposes, they represent the best available tool to limit over-diagnosis of APS in clinical practice. However, one should bear in mind that they still may not encompass all unique clinical manifestations to be applied for diagnosis of APS and that the final diagnostic decision relies on the treating physician's judgment.

Clinical manifestations of APS: Criteria and Extra Criteria clinical Manifestations

APS is commonly considered as the major acquired thrombophilia condition, potentially affecting any vascular bed (arterial, venous and the microvasculature) and explaining the heterogeneity of clinical manifestations described in patients with APS. While most of the clinical manifestations can be attributed to underlying thrombosis, other mechanisms (including inflammation, complement and platelet activation) have been shown to play crucial roles in the pathophysiology of the syndrome[9–11].

In a European cohort of 1,000 APS patients, deep vein thrombosis and pulmonary embolism were the most frequent clinical manifestations of the syndrome, whereas the most frequent arterial manifestations were neurological, such as stroke and transient ischemic attacks[12].

Pregnancy morbidity includes unexplained fetal death, premature birth before 34 weeks of gestation due to severe pre-eclampsia, eclampsia or placental insufficiency or recurrent (more than 3) first trimester miscarriages. Pre-eclampsia, premature birth or fetal loss are the most common manifestations and occur in 10–20% of APS pregnancies[12].

Several non-thrombotic clinical manifestations associated with aPL are not included in the revised classification criteria[4]. Over the last decade since Sydney criteria were detailed, a significant body of basic research and clinical studies on APS has emerged, potentially paving the way for a further update of clinical as well as laboratory manifestations included in the current classification criteria. In fact, a series of clinical features (detailed in Table 1), that are not currently comprised in the classification criteria, but are recognized to be related to the presence of aPL have been named non-classical or extra-criteria clinical manifestations.These clinical characteristics are not exclusively related to thrombosis, and they can occur also when thrombosis is not evident. Addressing the value of these extra-criteria manifestations is crucial. They may add prognostic and morbidity correlations, provide a fertile field for research, impact on treatment and improve patient outcome.

In this context, the Antiphospholipid Antibodies Task Force on Clinical Manifestations was carried out from January to September 2013 to examine data, the quality of evidence and to develop recommendations in order to carefully suggest a revision of the current classification criteria[13]. A workshop held in Rio de Janeiro at the 14th International Antiphospholipid Congress discussed these findings[13,14].(The main conclusions are presented in Box 2).

Thrombocytopenia is critical for decision-making. Once the most common causes are ruled out, aPL should be checked even in the absence of other features that are characteristic of APS. However, despite thrombocytopenia being commonly found in patients with APS in the clinical practice, overall, the evidence does not support its inclusion as a main clinical feature of the syndrome[13]. In general, aPL-related thrombocytopenia is mild or moderate and the risk of bleeding is minimal even with very low counts[14].

The kidneys are major targets in APS and thrombotic events can occur at any level within the renal vasculature (renal arteries, intrarenal arteries, glomerular capillaries and renal veins); clinical manifestations depend on the site and size of the involved vessels. A prompt recognition of renal involvement in APS might impact on the patient's outcome and it is critical for decision-making. All calibers of renal arteries and veins and capillaries can be involved in APS. Pathology is dominated by fibrin or organizing vascular thrombi with focal recanalization. Acute lesions present as thrombotic microangiopathy. Chronic lesions include arteriosclerosis, fibrous intimal hyperplasia, fibrous obliteration of arteries and arterioles, and focal cortical atrophy. These lesions have been described both in primary APS patients as well as SLE patients with aPL, including secondary APS patients and non-APS lupus patients with aPL. Inflammation, if any, is rare in APS. This allows a clear-cut distinction, especially in secondary APS, from renal involvement mainly due to immune complex deposition, like in lupus nephritis.

The prevalence of heart valve disease (HVD) assessed by transthoracic echocardiography is very high in APS (65%) and even though the pathogenic mechanisms are not known, there is evidence that aPL have a role in these lesions[15]. HVD in APS is defined by a) valve thickness >3mm b) localized thickening involving the proximal or middle portion of the leaflets; or c) irregular nodules on the atrial face of the mitral valve and/or the vascular face of the aortic valve. There is a clinical association between HVD and central nervous system events (ischaemia, migraine, epilepsy) in APS patients .Patient-important outcomes linked to HVD related to aPL, including heart failure, heart valve replacement or death from heart failure or valve replacement, were categorized as very critical for decision making.[13]

The recognition of *livedo reticularis* is important, as it can be associated with the occurrence of thrombosis. Similarly, migraine can be associated with thrombotic events in patients with aPL, but migraine is of little importance for decision-making in patients with APS when compared to the general population[13]. Prompt identification of other neurological manifestations, including myelitis, seizures and chorea in patients with aPLis considered very critical for decision making[13]. Indeed, the central nervous system in APS is a complex hot topic. Stroke or transient ischemic attack on the arterial side, and cerebral venous thrombosis on the venous side are the most characterized CNS manifestations of the syndrome. However, beside these criteria manifestations, some non-vascular neurological manifestations of aPL are progressively emerging, being associated with a wide range of polymorphic neurological, psychological and psychiatric manifestations. These includes headache, migraine, bipolar disorder, transverse myelitis, dementia, chorea, epileptic seizures, multiple sclerosis-like lesions, psychosis, cognitive impairment, Tourette's syndrome, parkinsonism, dystonia, transient global amnesia, obsessive compulsive disorder and leukoencephalopathy[16–18]. In vitro experimental and animal models data support an immune-mediated pathogenesis, with direct binding and effect of aPL on neurons and glial cells which are thought to occur through

different mechanisms, including the disruption or a permeability alteration of the blood-brain barrier[19,20] The magnitude of the association, cellular and molecular mechanisms involved and potential new therapeutic strategies (e.g. the use of hydroxycloroquine to prevent fetal brain abnormalities[21]) are currently under investigation[21–23]. Recently, the role of aPL in inducing cognitive dysfunction has been extensively investigated. Among others, Kozora et al. showed that compared to controls, aPL-positive patients had elevated cortical activation, primarily in the frontal lobes, during tasks involving working memory and executive function, findings consistent with cortical over-activation as a compensatory mechanism for early white matter neuropathology. Future researches are highly needed to confirm these observations; decisional algorithms helping in attribution of these manifestations to the presence aPL will be critical for decision making[13]

Recent advances in the pathogenesis potentially impacting on future classification system

Although the full pathogenesis of APS is not clear yet, key discoveries were described recently. Thrombosis is one of the major disease mechanisms, mediated mainly by activation of endothelial cells, monocytes, platelets, coagulation and complement pathways, in addition to inhibition of fibrinolytic and anticoagulation pathways[11]. Figure 1 summarizes the main pathogenetic mechanisms leading to thrombosis in APS. Recent evidence shows that vasculopathy driven mainly by severe intimal hyperplasia can also play a role in arterial vascular occlusions (mainly due to stenotic lesions) and pregnancy morbidity[28,29].

Very recently Canaud and co-workers demonstrated that the vascular endothelium of proliferating intrarenal vessels from patients with APS nephropathy showed indications of activation of the mammalian target of rapamycin (mTORC) pathway. In cultured vascular endothelial cells, IgG antibodies from patients with APS stimulated mTORC through the phosphatidylinositol 3-kinase (PI3K)-AKT pathway. Patients with APS nephropathy who required transplantation and were

receiving sirolimus had no recurrence of vascular lesions and had decreased vascular proliferation on biopsy as compared with patients with aPL who were not receiving sirolimus. Among 10 patients treated with sirolimus, 7 (70%) had a functioning renal allograft 144 months after transplantation versus 3 of 27 untreated patients (11%). Activation of mTORC was also found in the vessels of autopsy specimens from patients with catastrophic APS[30].

A recent study performed on patients with primary and secondary APS nephropathy, which is mainly mediated by vasculopathy rather than thrombosis, revealed that the activation of the mTORC enzyme stimulates intimal hyperplasia, leading to the formation of the chronic vascular lesions as seen in APS[31].Confirmatory observation and data investigating all the steps that lead to the mTORC pathway recruitment and the molecular consequences of its activation is still needed. However, there is growing evidence that these enzymes can induce prothrombogenic phenotypes leading to thrombosis[32]. This might represent an obstacle to further testing of these drugs in patients with APS.

An exhaustive analysis of novel therapeutic approaches in APS is out of the scope of this review and has been discussed elsewhere[33,34]. However, it is likely that the evolving scenario of our understanding of the mechanisms underlying the different clinical manifestations in APS will lead in the very near future to more targeted therapeutic approaches (Figure 2).

Complement and aPL

The involvement of complement activation in the pathophysiology of APS was first investigated in murine models of aPL-related pregnancy morbidities [35,36]. Despite it is out of the scope of this manuscript to review the obstetric manifestations of the syndrome, it is worth noting that complement-derived inflammatory mediators such as C4a, C3a, and C5a have been shown to contribute to the pathophysiology of complement-induced placentae inflammation. They increase vascular permeability, activate platelets or neutrophils[37], and induce the release of pro-inflammatory cytokines from monocytes. An emerging body of evidence is supporting the role of the complement proteins in the pathogenesis of aPL-related thrombosis[38]. Among others, Atsumi usmi and co-workers showed that patients with primary APS had lower serum levels of C3, C4, and CH50 compared to healthy volunteers and patients with non-lupus connective tissue disease. However, in their cohort,

C5 levels in APS patients were within normal range[39]A role for complement activation in thrombotic APS was supported by Peerschke E at al., who showed that enhanced complement fixation, especially C4d deposition on heterologous platelets, was positively associated with arterial thrombotic events in patients with SLE and aPL[40]. More recently, Gropp K and coworkers showed that β2GPI functions as a complement regulator [41], located on the surface of apoptotic cells, changes its conformation to an elongated form that acquires C3/C3b binding activities. β2GPI seems to mediate complement activation by changing the conformation of C3 to facilitate the degradation of C3 and mediates further cleavage of C3/C3b compared to factor H alone and strengthening the function of factor H, an inhibitor of complement activation. In line with these observations, a recent report showed that autoantibodies against factor H are prevalent in patients with APS and are related to recurrent venous thrombosis[42] Similarly, very recently, Meroni and co-workers reported for the first time complement activation by antiphospholipid antibodies in arterial thrombosis in the circulation and, more importantly, in the arterial wall.[43]The most recent hypothesis suggests that the classic pathway is persistently activated in APS but generally until C3[38]. However, when complement activation is accelerated, usually by as second-hit triggers, it overcomes the inhibitory functions and C5a is produced. However, this model does not answer to the following question: whether or not the immune complex consisting of aPL is the initiator of complement activation. Further researches are needed to elucidate the role of the complement activation in thrombotic APS, potentially paving the way to new therapeutic strategies including complement inhibitors[33].

aPL testing: the emerging role for extra-criteria aPL

It has been suggested that several autoantibodies besides aCL, LA and anti-β2GPI are relevant to APS[11]. These antibodies specificities target other plasma proteins from the coagulation cascade (i.e. prothrombin (PT) and/or phosphatidylserine/prothrombin (PS/PT) complexes), to specific domains of β2GPI, or interfere with the anticoagulant activity of annexin A5

(A5)[16].The clinical utility of these newly developed assays and their clinical value in assessing thrombotic risk are currently under debate. A schematic flow-chart for APS diagnosis taking into account the role of criteria and extra-criteria aPLis presented in Figure 3.

aCL and anti-β2GPI IgA Isotypes

The diagnostic accuracy for APS of IgA aPL and whether this isotype should be included in the routine diagnostic algorithm has been a subject of intense debate[52–58]. The current laboratory criteria for APS do not recommend the inclusion of IgA isotypes for both aCL and anti-β2GPI tests[4]. Despite some available data support testing for IgA aPL, the evidence mainly relies on retrospective studies, case-report and case-series, making it challenging to provide sound recommendations[14]. Besides, comparison among these studies is difficult due to heterogeneity in design, population studied, the assays used and cut-off chosen for the definition of positivity. One should also consider that some studies also failed to prove the usefulness of IgA aCL and IgA anti-β2GPI testing, mainly because of failure to enhance the diagnostic accuracy of routine testing[58], either because of low prevalence of these antibodies or because they are found along with other aPL in most cases.

Recent evidence[59] suggested that isolated IgA anti-β2GPI may be useful in identifying additional patients with clinical features of APS but tested negative for IgG and IgM isotypes, and hence, recommended testing for these antibodies when other aPL are negative and APS is strongly suspected on a clinical basis. However, it is important to note that of the 5,892 samples tested in this study, only 57 (<1%) were positive for IgA anti-β2GPI alone, limiting the application of these recommendations to a very selected population of patients.

More recently, Pericleous *et al.* performed an observational, multicenter cohort study to evaluate the utility of IgG, IgM and IgA assays to each of aCL, anti-β2GPI and autoantibodies to

domain 1 of β2GPI (anti-β2GPI-D1) analyzing serum from patients with APS (n = 111), SLE but not APS (n = 119), and 200 healthy controls. They showed that although all assays displayed good specificity for APS, testing positive for IgA anti-β2GPI resulted in a higher hazard ratio for APS compared to positivity for IgM anti-β2GPI[60].

One should also bear in mind that variability between kits may also account for some degrees for the discrepancy in results obtained in the different studies and for the lack of consensus concerning their clinical significance IgA testing[53,61]. As a result, and in the absence of well-designed prospective studies, the controversy over the usefulness of IgA aPL testing in assessing the thrombotic risk in APS continues. IgA aPL testing should only be considered for thrombotic risk assessment in selected cases, in the presence of clinical signs and symptoms of APS, mainly associated with SLE, and, particularly, when other aPL tests are negative[14]

Anti-prothrombin Antibodies

Anti-prothrombin antibodies have been proposed as novel potential new biomarkers for thrombosis and/or pregnancy morbidity when APS is suspected. Anti-prothrombin antibodies are usually tested by ELISA, using prothrombin-coated irradiated plates (aPT), or using prothrombin in complex with phosphatidylserine (aPS/PT), as antigen. Although these antibodies can both be detected in the same patient, aPT and aPS/PT appear to belong to different populations of autoantibodies[62–64].

The diagnostic accuracy for APS of antiprothrombin antibodies, tested either as aPT or aPS/PT, has been evaluated with conflicting results[70–72]. Most of the studies support an association between antibodies directed to prothrombin, particularly aPS/PT, and clinical manifestations of APS. Our team performed a systematic review including data from more than 7,000 patients and controls, concluding that while both aPT and aPS/PT are associated

with an increased risk of thrombosis, aPS/PT appear to represent a stronger risk factor for both arterial and/or venous thrombosis when compared to aPT[63]. An active debate is ongoing about the possibility of antiprothrombin antibodies, particularly aPS/PT, being an additional tool for risk stratification, particularly when trying to improve the identification of APS patients negative for criteria aPL.

Autoantibodies to domain 1 of β2GPI

An intriguing subject of research is focusing on the epitope distribution of anti-β2GPI antibodies with the aim of identifying the pathogenic specificities[74–77].The β2GPI molecule has five homologous domains: D1 to D5. Most of the antibodies have been described to target epitopes located in domains β2GPI-D1, D4, and D5. It is has been proposed that these antibodies may have dissimilar clinical interpretations[76].The principal epitope associated with APS has been described to be cryptic and a conformation-dependent structure that includes different regions of D1[78,79]. In details, recent studies have identified the main pathogenic DI epitope in the arginine 39-arginine 43, aspartic acid 8-asparticacid 9, and possibly the interlinking region between DI and DII, with R39 being the most important residue[80,81]The epitope in the circular and the S-shape forms of the molecule is not available for autoantibody binding but can be exposed in the open J configuration. The conformational-dependent binding of anti-β2GPI antibodies to DI might explain whyβ2GPI/anti-β2GPI immunecomplexes are not easily detected in APS sera because the epitope is covered by the interaction between DI and DV in the circular form of the molecule that represents the main variant present in the circulation. lackingAlong with in vivo models[85], immuno-histopathological findings[43,86], the pathogenic role of the DI epitope has been recently supported in patients with APS. In an international, multicenter evaluation, an association between anti-β2GPI-D1 antibodies and history of (mostly venous) thrombosis was found[87].

Recent studies have demonstrated that patients with multiple positive test results(so-called 'triple-positive' patients), who are usually considered at a higher risk for developing clinical complications, seem to have higher prevalence and higher titers of anti-β2GPI-D1 antibodies[88]. Conversely, a very recent study by De Craemer et al. failed to demonstrate an added diagnostic value to the formal aPL panel for anti-β2GPI-D1 antibodies, since anti-β2GPI IgG was nearly as specific but more sensitive for APS, and the agreement between IgG anti-β2GPI-D1 antibodies and anti-β2GPI IgG was high (positive and negative agreement 91.7% and 98.4%, respectively)[77]. Consistently, the hypothesis of antibodies specifically binding the domain I of the β2GPI molecule as a promising biomarker with a better diagnostic accuracy when compared to anti-β2GPI is scientifically sound, but needs further verification.

Anti-vimentin antibodies

Vimentin is a type III intermediate filament ubiquitous protein part of the cytoskeleton structure. Anti-vimentin antibodies were initially observed in patients with SLE, in whom they are strongly associated with the presence of aCL[89].In vitro evidence supports the ability of anti-vimentin antibodies to activate leukocytes and platelets and to induce an increased expression of tissue factor (TF), P-selectin and fibrinogen[90]. However, their diagnostic accuracyin the context of APS is still largely debated. Ortona et al.[91]showed that vimentin binds cardiolipin in vitro, possibly as a result of electrostatic interactions with negatively charged amino acids of cardiolipin. Antibodies binding the antivimentin/cardiolipin complexes were observed in a large proportion of patients with thrombosis and pregnancy morbidity without criteria aPL tests (aCL, LA, anti-β2GPI)and in almost all those with APS[91]. These observations led the authors to suggest vimentin as a potential antigenic target for aPL and to consider the antibodies targeting the vimentin/cardiolipin complex as new biomarker

in patients with suspected APS. It is worth noting, however, that antibodies against the antivimentin/cardiolipin complex have also been found in several other autoimmune conditions, including SLE and rheumatoid arthritis. Overall, despite their high sensitivity, antibodies targeting the vimentin/cardiolipin complex do not seem to be specific in identifying patients at higher risk for thrombosis or pregnancy morbidity. To date, their role as potential biomarker for APS is still largely undefined.

Anti-annexin antibodies

Annexins are a heterogeneous group of 12 highly conserved proteins with several regulatory functions on cell homeostasis. In vitro models have shown that annexin V has a calcium-dependent binding affinity for anionic phospholipids and activated platelets, and prevents prothrombinase activity[81]. Among other immune conditions, anti-AnxA5 antibodies (aAnxA5) have been observed in patients with APS. However, , despite some observations showed that aAnxA5 were frequently found in patients with arterial or venous thrombosis, especially in those with autoimmune rheumatic diseases such as SLE or systemic sclerosis[92], among others, de Laat et al.[93] failed to confirm any strong association between aAnxA5 and a history of thrombotic events.

Standardization of aPL testing

The general issue if standardization of autoantibody testing in rheumatic diseases has been addressed elsewhere in this Journal[94]. In this paragraph, we aimed to highlights progresses and ongoing problems related to the Standardization of aPL testing. aPLbelong to a heterogeneous family of antibodies directed against phospholipids or protein/phospholipid complexes.aPL are currently tested using either “solid-phase” assays that identify aCLand anti-β2GPIantibodies, or “liquid-phase/coagulation assays” that identify LA[4]. “Solid-phase” assays include ELISAs to detect aCL and anti-β2GPIand results are usually reported using

arbitrary units or as GPL or MPL units, reflecting respectively the level of reactive IgG or IgM based on a monoclonal/polyclonal antibody reference calibrator. The lack of agreement on standard materials and procedures has led (especially in the past) to a high interlaboratory or interassay variability.

This high interassay variability could explain ,at least in part, the relative low clinical utility of aCL testing when assessed on a global basis and in terms of association with thrombosis in the general population[95]. One of the explanations for why aCL assays have such a relatively low clinical utility relies on the fact that aCL assays suffer from over-sensitivity, leading to detection of both clinically relevant and clinically non-relevant aPL. Similarly, low titers of aPL may be detected during infections: usually such positivity is transient and not associated with clinical features ofAPS[96]. Therefore, while the currently available assays for the detection of aCL maintain diagnostic validity, especially for moderate to high titers, they may lack prognostic value, especially when the clinical utility of each test (aCL, LA and anti-β2GPI) is evaluated separately. Overall, aPL testing may still have a limited role as indicators for preventive therapies because currently used assays used for LA, aCL and anti-β2GPIdetection do not always recognize pathogenic antibodies, but they measure a mixture of clinically relevant and non-relevant antibodies. New avenues of research into laboratory testing for APS aim not only to develop assays to detect novel antibody specificities, but also to design approaches that assess the risk of clinical manifestation recurrence[4,14] (refer to "New approaches in risk stratification").

Guidelines for the evaluation of potential new biomarkers for cardiovascular disorders have been issued by the American Heart Association (AHA).[97] In detail, the AHA guidelines suggest standard procedures for the critical assessment of potential new risk biomarkers that are developed for clinical use[97] and state that assays to provide a risk-stratified approach should meet the following criteria: a) fulfillment of standardization criteria, including the availability

of standard materials and procedures; b) the test results should correlate with clinical symptoms and should have value for predicting the risk of future clinical events;c) the test should improve diagnostic accuracy, especially in terms of its predictive value when compared to already established biomarkers. These recommendations could similarly be applied to aPL testing. To date, the currently available aPL assays do not entirely fulfill the above-mentioned principles, and so it seems appropriate to continue with research aiming to improve the diagnostic accuracy of aPL testing: this goal might be achieved by introducing novel assays able to overcome the current methodological limitations (e.g. by advances in biotechnology).

It is worth mentioning, however, that considerable effort has being put into developing international standards for aPL testing. Among others, Meroni, and co-workers evaluated the suitability of polyclonal/monoclonal candidate reference materials for anti-β2GPI testing, showing promising results[98]. More recently, Willis et al. investigated the performance characteristics and impact of newly developed reference calibrators on the commutability between anti-β2GPI immunoassays in APS and/or SLE. They evaluated the diagnostic accuracy, correlation between kits, and specific clinical manifestations linked to four immunoassays for IgG and IgM anti-β2GPI in serum samples from 269 patients. When expressing results in kit-specific arbitrary units and in calibrator reference units (RUs, based on 99th percentile cutoff values), they showed that although qualitative agreements between immunoassays for both antibody isotypes were acceptable (almost perfect interassay reliability, as expressed by Cohen κ ranging from 0.69 to 0.98), correlations with APS clinical manifestations were kit-dependent. Besides, only the use of IgG reference material improved quantitative correlations between assays[99].Theymight be crucial to refine our understanding of the aPL antigen specificities, sinceall these novel systems differ from standard ELISA sin terms of antigen presentation and/or phospholipid–protein complexes.

Chemiluminescence Assay

Automated chemiluminescence immunoassay (CLIA) has been proposed as an alternative method to ELISA. The strength of CLIA mainly relies on high level of automatization, potentially leading to better reproducibility together with reduced interlaboratory variation[103].Besides, CLIA systems reduce hands-on time compared to labour-intensive ELISAs. CLIA testing is based on a two-phase immunoassay method. First, the specific antibodies present in the sample bind to a solid phase represented by magnetic particles coated with the antigen. Subsequently, after the addition of reagents triggering the chemiluminescent reaction, emitted light is detectable by an optical system. Results are usually reported in relative light units (RLUs). RLUs are directly proportional to the concentration of aPL antibodies in the sample[104,105].

Several authors have compared the diagnostic accuracy of aPL testing using CLIA automated systems versus ELISA, aimin gto assess whether their diagnostic performance is equivalent with heterogeneous results[104,106]. .As previously mentioned, aCL tests have shortcomings in terms of standardization and reproducibility, which deeply impact on their clinical relevance; on the other hand, anti-β2GPI and LA tests are more specific, but may lack sensitivity[103]. Although CLIA has been reported to have inferior sensitivity to ELISA, overall CLIA appears to be more accurate than ELISA for identifying patients with APS. These differences are not unexpected, as automated CLIA systems differ from ELISA in having antigen and phospholipid/protein complex (mainly CL/β2GPI) presentation on magnetic particles, rather than on the surface of microtitre wells. Binding of β2GPI to the solid-phase is critical ,as it impacts both antigen density and orientation or conformational change of the protein[107]. The new coating systems used in CLIA are a crucial difference when comparing to ELISA, and

these might explain, together with the amplification reaction of the chemiluminescent principle, the significantly higher titres for aPL (especially for aCL) detected with automated systems. Therefore, CLIA shows good diagnostic performance[108], showing in some studies a sensitivity up to 100% and specificity above 70% for patients with APS[109]. Recently, Mahler et al. investigated the role of anti-β2GPI-D1 determined using a novel CLIA. Analyzing sera collected from 106 patients with primary or secondary APS they showed that anti-β2GPI-D1 IgG titers were significantly higher in patients with thrombosis, demonstrating an association with thrombosis in patients with APS[110].Thus, CLIA may represent a useful tool to detect mostly relevant aPL for the diagnosis of APS.

Multiline Dot Assay.

Multiline dot assays (MLDA) are part of a heterogeneous group of multiplex assay techniques able to test for several aPL antibodies simultaneously, using different solid phases for binding of different antigensThe potential cost and time effectiveness of multiplex autoantibody profiling (as reported in other autoimmune diseases such as SLE and rheumatoid arthritis)makes MLDA an appealing candidate for aPL testing[111,112].Available studies from different research teams showed that MLDA results have good agreement with ELISA data, with no statistical difference in the diagnostic capacity for identifying patients with APS[113,114] In some cases, however, the rate of false-positive aPL detected by MLDA was higher when compared to ELISA. [113]Despit ethis technique is a readily available, single-step, sensitive diagnostic tool that might be useful to identify patients with APS, future researches are needed to evaluate its diagnostic accurancy in the very settings of aPL testing, where assay standardization remains a challenge[115].

TLC Immunostaining

Thin layer chromatography (TLC) is a non-quantitative assay method firstly described in 1994[116]. Some studies report its use for detection of aPL. In brief, TLC is based on three main phases: antigen separation, immunostaining with patients' sera, and detection of immunoreactivity[97]. For the first step, using an adequate eluent system, phospholipids are run on thin layer high-performance liquid chromatography (HPLC) plates. Chromatograms are then incubated with samples from the patients. Finally, immunoreactivity is detected by a chemiluminescence reaction. TLC is capable of simultaneously revealing reactivity of autoantibodies directed against various purified antigens, with different specificities as compared to ELISA[117]. In TLC immunostaining, antigens run on aluminium-backed silica gel HPLC plates, mimicking the exposure of phospholipid to binding proteins. These features result in TLC being a less sensitive but more specific test than ELISA in both autoimmune and infectious diseases[118,119], and represent a further technical useful tool for aPL testing[120,121].

New approaches to risk stratification

Risk factors other than aPL

The association between traditional cardiovascular risk factors and the occurrence of clinical events (especially thrombosis)in patients with APS has been extensively investigated. Patients with aPL presenting with thrombosis frequently have one or more additional cardiovascular risk factors such as hypertension, smoking, hypercholesterolemia or estrogen use[122].

When focusing on arterial events, Matyja-Bednarczyk *et al.* recently showed that the presence of livedo reticularis and hypertension or hypercholesterolemia are associated with an increased risk of arterial thrombosis in APS[123]. Moreover, the interaction between aPL and smoking and oral contraceptives has been elucidated in a case-control study by Urbanus *et al.*[124]. The authors showed that the risk for stroke doubled among LA-positive women who smoked, as compared with non-smokers, and the risk of stroke among oral contraceptive

users multiplied more than 7-fold. All LA-positive women who suffered a myocardial infarction were also smokers.

Patients with SLE are at higher risk of vascular events, which often can occur also in the absence of traditional vascular risk factors[125]. Besides, the combination of SLE and aPL has been shown to further increase the risk of thrombosis and a diagnosis of SLE appears to further enhance the likelihood of vascular events in patients with aPL[126].Indeed, in patients with aPL and coexisting SLE, the annual risk of first thrombosis is higher than in healthy aPL-positive individuals without other cardiovascular risk factors (4% vs <1%)[125].

Observational studies support the association between thrombosis and manifestations of APS other than those listed in the clinical classification criteria, including heart valve lesions[127], livedo reticularis and thrombocytopenia[123]; however, these associations have been considered not strong enough to guide clinical decisions.

Thrombotic risk assessment should also be considered in patients with previous pregnancy morbidity due to aPL. Lefèvre *et al.* showed that patients with obstetric APS have a higher thrombotic event rate than healthy women (3.3 vs. 0–0.5 per 100 patient-years), even when treated with low dose aspirin[126]. In a 10-year observational study of 1,592women with no history of thrombosis who had experienced 3 consecutive spontaneous abortions before the 10th week of gestation or 1 fetal death at or beyond the 10th week of gestation, Gris *et al.*[128] reported that LA was a risk factor for unprovoked proximal and distal deep and superficial vein thrombosis. More recently, a case control study including 57 women with primary APS and recurrent early pregnancy loss (REPL) confirmed these results, indicating that a history of REPL associated with aPL was a risk factor for subsequent thrombosis in the long term[129].

One of the unsolved questions is why some aPL carriers never develop any APS manifestation, some develop thrombosis while others present with morbidity in pregnancy and a minority develop a catastrophic form of APS. Therefore, assessing the risk of developing APS manifestations for an individual with aPL is very important for physicians.

Three models have been proposed to quantify the risk of thrombosis and obstetric events in APS[130–132]. The main aim of these models is to enable physicians to stratify patients according to their risk score, identifying those who have a higher likelihood of developing new events and therefore can benefit from preventive approaches. The first two models[131,132] mainly focused on aPL profiles, while the most recent one, the Global APS Score or GAPSS[130], also includes other variables such as cardiovascular risk factors and the patient's autoimmune profile at the time of formulating the risk model.

The Global APS Score

The Global APS Score (GAPSS) was first developed and validated in a large cohort of patients with SLE, divided into two statistically independent sets by a computer-generated randomized list[130].According to this model, risk assessment quantification was based on the computation of independent factors for thrombosis and pregnancy loss. The GAPSS was designed to incorporate the aPL profileas well as conventional cardiovascular risk factors. The variables identified by multivariate analysis to be independently related to thrombosis or pregnancy morbidity are listed in Table 2.

In the original development cohort including 106 patients with SLE, higher values of GAPSS were observed in patients who experienced thrombosis and/or pregnancy loss compared with those without clinical events. The GAPSS was then applied and validated in the second set of 105 patients with SLE. In this validation cohort the results were analogous, with statistically higher GAPSS values in patients with a clinical history of thrombosis and/or

pregnancy loss compared with those without events .The GAPSS score was subsequently prospectively validated in a separate cohort SLE patients followed up for a mean 32.94± 12.06 months[133], and in patients with primary APS[134]. 95% CI

GAPSS was also independently validated by Oku and co-workers,[135]and by , Zuily et al. , who evaluated the validity of the GAPPS to predict thrombosis in a prospective multicentre observational study[136], confirming that GAPSS may be a potential quantitative tool for APS related clinical manifestations[137].

So-called "seronegative" APS

Solid evidence supports a strong link between autoimmunity and autoantibodies. Nevertheless, some patients with autoimmune diseases (such as rheumatoid arthritis)might be persistently negative for all known disease-specific autoantibodies. In the past, these individuals have been described as having 'seronegative' disease, which may represent a challenge for physicians[138,139].As previously reported, the diagnosis of APS requires the co-existence of at least one clinical and one laboratory criterion. Nevertheless, occasionally it is possible to findpatients with a clinical profile suggestive of APS (thrombosis, pregnancy morbidity including recurrent miscarriages or fetal loss, and some extra-criteria manifestations), who are persistently negative on routine testing for aPL (aCL, anti-β2GPI, and LA). For these patients the term so called "seronegative APS"has been suggested[140–142].Several possible explanations for such seronegativity have been proposed: a) the diagnosis of APS is wrong; b)previously positive aPL tests have become negative; 3) the current range of tests is inadequate. The authors of this review feel that the latter is the most likely scenario. Seronegativity may result from limitations of the traditional technical approaches or on the existence of different antigenic targets. As some clinical manifestations of APS (such as myocardial infarction and stroke) are major causes of morbidity and mortality, due to the high

risk of recurrence, it is mandatory to identify among patients with so-called seronegative APS those who might benefit from long-term secondary thromboprophylaxis. Similarly, since APS is now known to be the one of the most common treatable causes of pregnancy morbidity, for women with a history of recurrent early abortions or fetal loss, a diagnosis of APS can direct them towards treatments that significantly improve the rate of live births.

Several studies have investigated novel methodological approaches to detect aPL and antigenic targets in seronegative APS[54,121,143]. In particular, as previously mentioned, anti-prothrombin antibodies have been described as the sole antibodies detected in few patients with SLE and a history of thrombosis who were persistently negative for aCL or LA[144]. Similarly, when using a proteomic approach, analyzing endothelial cell-surface membrane proteins, vimentin/cardiolipin complex was identified as a "novel" target antigen of seronegative APS[91]. Serum IgG anti-vimentin/cardiolipin antibodies, detected by ELISA, were found not only in a large proportion of patients with 'seronegative' APS patients (55%) but also in almost all APS patients. Repeating the test with a second sample obtained at least 12weeks after the initial one confirmed the same result in all seronegative APS patients.

As previously described, several new laboratory techniques capable of detecting aPLby methods other than ELISA have been proposed. TLC immunostaining was recently used for detection of aPL (CL, anti-lysobisphosphatidic acid, and anti-phosphatidylethanolamine antibodies) in a group of 36 patients with a clinical picture suggestive of APS (both criteria and extra-criteria APS features, including thrombocytopenia, livedo reticularis, migraine, cognitive dysfunctions, and seizures), who were persistently negative on routine aPL testing[120]. In about 60% of those 'seronegative' APS patients, the presence of aPL was identified using TLC. Interestingly, a strong correlation was observed among the three aPL specificities. In order to verify the possible pathogenic role of these autoantibodies, it was

shown that purified IgG from sera of seronegative APS patients induced serine phosphorylation of IRAK with consequent NF-κB activation[120].

In order to identify the best screening combination to detect aPL in 'seronegative' APS patients, sera from 24 such patients were analysed for aPL using TLC immuno staining, and tested for anti-vimentin/cardiolipin antibodies by ELISA, and for anti-annA5 and antiprothrombin antibodies by ELISA and dot blot[121]. In this cohort, the results obtained by TLC immunostaining showed the presence of aCL in 54.2% of patients. In addition, 45.8% of the patients showed serum antibodies (IgG) against vimentin/cardiolipin, 12.5% against prothrombin, and 4.2% against annA5. Despite the limitation of the small sample size, these observations showed that in 19 of 24 (79.2%) patients with 'seronegative' APS, at least one aPL/cofactor antibody was detected when expanding the laboratory panel beyond ELISA-based testing for criteria aPL. A combination of two of the tested methodological approaches (TLC immunostaining for aCL and ELISA for anti-vimentin/cardiolipin complex antibodies) was able to detect aPL/cofactor antibodies in about two-thirds of 'seronegative' APS patients with thrombosis or pregnancy morbidity, with a small additional gain when additionally performing ELISA for prothrombin and annA5 antibodies.

Similarly, recent evidence showed that testing for anti-β2GPI-DI, IgA aCL, or IgA anti-β2GPI might be useful not only to improve risk stratification, but also to increase our ability to identify the proportion of patients with 'seronegative'APS[78]. In the near future, the combined use of different approaches could improve the diagnostic accuracy in patients suspected of having APS. However, although these approaches improve our diagnostic possibilities, we are still unable to detect autoantibodies in a percentage of seronegative APS patients. Thus, since other unidentified cofactors may be involved in sera reactivity, further studies could shed light on "new" antigenic specificities in seronegative APS.

Damage Index in APS (DIAPS)

Certain manifestations of APS carry a worse prognosis and permanent damage may occur in various organs. Moreover, a significant impact of the disease on long-term survival has been documented in large, prospective cohort studies of patients with different clinical subsets of APS. Recently a specific damage index for thrombotic APS (DIAPS) was developed[145]. The principal challenge of tools such as DIAPS is the accurate measurement of cumulative damage to predict disability, and even survival. The predictive value of DIAPS needs to be studied in multicenter surveys.

Conclusions

APS remains a significant diagnostic challenge for physicians[122-126]. On the one hand, we are assisting to an expanding range of reported clinical manifestations associated with the presence of aPL but not included in the current classification criteria (so called extra-criteria manifestations). On the other hand, current laboratory testing still suffers for technical limitations. Although it is the physician looking after the patient who ultimately makes the diagnosis, laboratory testing plays a critical role at many phases of the process. To date, it seems that the aPL profiling represents the most accurate risk stratification tool for associated adverse events such as thrombosis[131,146]. In details, while LA is the stronger predictor of risk when compared to either aCL or anti-β2GPI,the greatest risk appears to be associated with multiple positivities (LA, aCL, anti-β2GPI, the so called “triple positivity”) Despite the rapidly evolving scenario also including new insights in the pathogenic mechanisms of the syndrome, however, much remains to be done. For example, physicians have to be trained to avoid inappropriate test requests that are costly and potentially lead to

misdiagnosis or mistreatments. Developing better assays to improve standardization for APS diagnosis is the real goal.

Box 1: Updated Sapporo (or Sydney) classification criteria for APS

Clinical manifestations

Objectively confirmed venous, arterial or small vessel thrombosis, or pregnancy complications (including pregnancy loss, premature birth and features of placental insufficiency)

Laboratory features

A positive laboratory test for aPLs* found on 2 or more occasions at least 12 weeks apart

*aPLs recognized in the international criteria include: anticardiolipin (aCL) antibody (IgG or IgM) exceeding 40 IgG or IgMphospholipid units or anti-β2-glycoprotein I (β2-GPI) antibodies (IgG or IgM) at titers exceeding the 99th percentile and lupus anticoagulant (LA) detected according to guidelines published by International Society onThrombosis and Haemostasis (ISTH)[4,147].

Box 2:Main findings of the Antiphospholipid Antibodies Task Force on Clinical Manifestations

(Presented at the 14th International Antiphospholipid Congress in Rio de Janeiro[13,14].)

- Superficial vein thrombosis (SVT):Low overall quality of evidence to support the suggestion that SVT is due to aPL or APS, unless there are other features of APS[13].
- Thrombocytopenia: Low quality of evidence supporting its inclusion as a main clinical feature of APS. Data in the literature indicate that thrombosis risk is increased in those patients with lupus anticoagulant (LA)[13].
- aPL-related nephropathy:moderate quality of evidence for including biopsy-confirmed aPL-related nephropathy in the classification criteria[148,149]
- Heart valve disease: Moderate quality of evidence supporting its inclusion as part of APS classification criteria[13]
- *Livedo reticularis:* Moderate quality of evidence for inclusion in diagnostic criteria
- Neurological manifestations (including migraine, myelitis, seizures and chorea) were analysed separately.Moderate overall quality of evidence suggests that chorea and longitudinal myelitis, but not migraine or seizures, should be included in APS criteria, [13]

Figure Legends

Figure 1.Mechanisms of thrombogenesis induced by antiphospholipid antibodies, focusing on the role of monocytes, neutrophils, endothelial cells and platelets.

Figure 2.Mechanisms underlying the different clinical manifestations in APS and potential targeted therapies. [PLTs, platelets; VKA, vitamin K antagonists; DOAC; direct oral anticoagulants; ASA, aspirin or acetylsalicylic acid; HCQ, hydroxychloroquine; TMA, thrombotic microangiopathy.]

Table 1. Extra Criteria clinical features of the antiphospholipid syndrome

- Thrombocytopenia
- Leg ulcers
- Livedo reticularis
- thrombophlebitis and superficial veinthrombosis
- Budd–Chiari syndrome
- Heart valve lesions
- Transverse myelitis, chorea, and epilepsy
- Haemolytic anaemia, Coombs' positivity, and Evans' syndrome
- Pulmonary hypertension
- Cognitive impairment
- Chronic headache
- APS nephropathy
- Splinter haemorrhages
- Labile hypertension and accelerated atherosclerosis
- Ischaemic necrosis of bone
- Bone marrow necrosis
- Addison's disease
- Guillain–Barré syndrome and pseudo-multiple sclerosis
- Amaurosis fugax
- Sensorineural hearing loss

Table 2. The Global AntiPhospholipid Syndrome Score (GAPSS)

Factor	Point Value*
Anticardiolipin IgG/IgM	5
Anti-β2-glycoprotein IgG/IgM	4
Lupus anticoagulant	4
Anti-prothrombin/phosphatidylserine complex (aPS/PT) IgG/IgM	3
Hyperlipidemia	3
Arterial hypertension	1

*The GAPSS can be computed for each patient by adding the points corresponding to the different risk factors, weighted as shown.

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