Phospholipid inhibitors
State of the Art

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Summary
The antiphospholipid syndrome (APS) is defined by the association of arterial and/or venous thrombosis and/or pregnancy complications with the presence of at least one among the main antiphospholipid antibodies (aPL) (i.e., Lupus anticoagulants, LA, IgG and/or IgM anticardiolipin antibodies, aCL, IgG and/or IgM anti-β2-glycoprotein I antibodies, aβ2-GPI). Several clinical studies have consistently reported that LA is a stronger risk factor for both arterial and venous thrombosis compared to aCL and aβ2-GPI. In particular, LA activity dependent on the first domain of β2-GPI and triple aPL positivity are associated with the risk of thrombosis and obstetrical complications. Asymptomatic aPL-positive subjects do not require primary thromboprophylaxis. Venous thromboembolism is the most common initial clinical manifestation of APS. To prevent its recurrence indefinite anticoagulation is recommended. Long duration treatment with warfarin or aspirin is used after a first cerebral arterial thrombosis. Low molecular weight heparin (LMWH) with or without aspirin is recommended to reduce the rate of obstetrical complications of APS pregnant women.

Zusammenfassung

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The antiphospholipid syndrome (APS) is defined by the association of arterial and/or venous thrombosis and/or pregnancy complications with the presence of at least one among the main antiphospholipid antibodies (aPL), i.e.,

- lupus anticoagulants, LA,
- IgG and/or IgM anticardiolipin antibodies, aCL,
- antiβ2-glycoprotein I antibodies, aβ2-GPI.

Since the clinical events are relatively common in the general population and they do not have distinctive features, the presence of aPL antibodies is an absolute requirement for the correct diagnosis of APS. APS may occur in the setting of an autoimmune disease, most commonly systemic lupus erythematosus (SLE), the so-called secondary APS, or in the absence of an underlying disease. In this case, the term primary APS is commonly used.

In 2006 the clinical and laboratory criteria of APS have been updated (1). Details have been provided regarding isotypes and titres of aCL and aβ2-GPI antibodies as well as timing of antibody determination. In 2009 also the criteria for LA determination have been updated (2).

Clinical criteria and epidemiology
According to the revised so-called Sydney criteria, thrombosis may occur in arteries, veins or small vessels of any tissue or organ (1). The events must be confirmed by objectively validated methods. Superficial venous thrombosis has been excluded from the clinical criteria. The accepted pregnancy criteria include at least three consecutive early abortions (within the 10th gestational week), one or more late pregnancy losses (occurring after the 10th gestational week) or one or more premature births (before
the 34th gestational week) (1). Obviously, all other causes of pregnancy complications must be excluded. The Sydney criteria underline that at least 12 weeks and less than five years must separate the clinical event and the aPL positivity.

The retrospective analysis of the APS qualifying event in 1000 patients enrolled in the Euro-Phospholipid Project confirmed that thrombosis may virtually occur in any district (3), although deep vein thrombosis (DVT) of the lower limbs, pulmonary embolism (PE) and superficial thrombophlebitis of the legs were the most common venous thrombosis, which accounted for about two thirds of the events. Approximately 30% of the patients suffered from arterial thrombosis, mainly cerebral stroke and transient ischaemic attacks (TIAs). During a 5-year follow-up of this cohort (4), 200 patients developed APS-related clinical manifestations. Recurrent thrombosis were experienced by 166 patients, who suffered from cerebral stroke, TIAs, DVT and PE, in decreasing order of incidence.

- Thrombosis occurred despite treatment with oral anticoagulation in 90 patients or aspirin in other 49 cases.
- Of note: 230 patients did not receive any immunosuppressive treatment.

Approximately 10% of the women enrolled in the Euro-Phospholipid Project suffered from obstetrical complications. Almost 10% of women became pregnant during the five years study period: more than 80% of them succeeded in having one or more live births. No differences in the occurrence of the APS manifestations were observed depending on the underlying disease (primary versus secondary APS), gender, age, comorbidities or immunosuppressive treatment.

Thrombocytopenia and livedo reticularis were two other common clinical manifestations developed during follow-up. Neither of them is included among APS criteria.

Of the 1000 patients enrolled 35 died during the five years of follow up (4). The most common causes of death were bacterial infections, myocardial infections, cerebral stroke, haemorrhage, malignancy, PE and catastrophic APS (CAPS).

CAPS Registry

The CAPS Registry was created in 2000 by the European Forum on aPL antibodies, to document the clinical, laboratory and therapeutic data of this rare manifestation, which accounts for less than 1% of APS. Approximately 300 patients have been enrolled so far (5). According to the preliminary criteria (6), diagnosis requires the evidence of involvement of three or more organs, systems and/or tissues, the simultaneous occurrence of the clinical events (or in less than one week) and their histopathologic confirmation. Of course, the presence of aPL antibodies is mandatory, although sometimes it is difficult to obtain or to confirm it in time.

Laboratory criteria of APS

The presence of at least one among LA, IgG and/or aCL and IgG and/or β2-GPI antibodies is a sufficient laboratory criterion for APS (1). The 2006 Sydney update of the original criteria clarified some relevant issues:

- APL antibodies must be confirmed over time by two consecutive positive determinations at least 12 weeks apart.
- The G and M isotypes have the same diagnostic value.
- Only medium to high titres of aCL (i.e., above 40 units or above the 99th percentile) and aβ2-GPI (i.e., above the 99th percentile) antibodies are accepted.

These changes reduced the possibility to wrongly diagnose APS in patients with thrombosis or pregnancy morbidity who happened to have transient or low titre aPL antibodies. Nevertheless, some unsolved problems remain, such as the referral to standardized ELISA methods to measure both aCL and β2-GPI antibodies. Actually, standardization is far from being reached for both types of tests, as recently underlined by Tincani et al. (7).

In the last two decades, however, several standardization efforts have been made through various international workshops, which have reduced, but not yet abrogated, the inter- and intra-laboratory variability of ELISA tests measurement (8–12).

The A isotype and other antibodies (such as antiprothrombin or antiphosphatidylserine antibodies), although frequently reported in patients with APS, were excluded from the updated Sydney criteria.

The update of the guidelines for LA detection have clarified some relevant technical issues (2). Details were provided about the cut-off values and the interpretation of screening, mixing and confirmatory coagulation tests. The guidelines also provided useful information about:

- selection of patients in whom LA testing is appropriated,
- pre-analytical handling of blood samples,
- choice of the test,
- how to perform mixing and confirmatory tests,
- expression and transmission of the results.

The updated guidelines underlined the necessity to measure both ELISAs and coagulation tests, thus allowing the generation of aPL profiles and the categorization of aPL positivity as single or multiple. This helps to define the patients risk of thrombosis and pregnancy morbidity.

LA, aCL and aβ2-GPI antibodies: The same or different antibodies?

The observation that β2-GPI allows the binding of aCL antibodies to cardiolipin-coated ELISA plates dates back to 1990 (13–15). Although true aCL antibodies may exist, such as those that develop in the course of some infectious diseases – aCL are polyspecific antibodies which recognize different epitopes on each of the five globular “sushi” domains that constitute the β2-GPI molecule. This glycoprotein circulates in plasma at a relatively high concentration (approximately, 200 mg/l) as a globular molecule, which is not recognized by aCL and aβ2-GPI antibodies in free solution. Immune recognition requires the binding of β2-GPI to an anionic (phospholipid) surface via a large positively-charged residue patch present in the fifth domain (16). Upon this binding, β2-GPI undergoes a conformational change to a “J” or “hook”-like shaped molecule, which un-
covers otherwise hidden epitopes. Among them, the Gly40-Arg43 peptide sequence in the first domain is particularly relevant, because it is recognized by a substantial proportion of $\alpha_2$-GPI antibodies, which are, obviously, aCL-positive, and cause LA activity (17, 18). In other words, the same antibody is recognized by three different assay systems.

ELISAs with the first domain of $\beta_2$-GPI coated onto hydrophobic and hydrophilic plates have been developed to characterize the $\alpha_2$-GPI antibody reactivity (16). Antibodies that recognized the first domain of $\beta_2$-GPI only when coated on hydrophobic plates were directed against the Gly40-Arg43 peptide sequence, and displayed LA activity. Conversely, antibodies that recognized the first domain of $\beta_2$-GPI coated on both hydrophobic and hydrophilic plates were polyspecific and only a minority of them had LA activity.

The prevalence of $\beta_2$-GPI-dependent LA activity is about 45%, as shown by the original article by de Laat et al. (18) and by a cumulative analysis of almost 200 LA-positive patients (Tab. 1) (19). Tests have been developed to identify $\beta_2$-GPI-dependent LA activity (20–22). Simmelink et al. (20) showed that the addition of cardiolipin vesicles shortened the clotting time of $\beta_2$-GPI-dependent LA activity, but had no effect on prothrombin-dependent LA activity. Pengo et al. (21) and Devreeze (22) reported that the reduction of calcium concentration increased the ratios of clotting time tests only in the presence of $\beta_2$-GPI-dependent LA.

The prevalence of antiprothrombin antibodies in aPL-positive patients is high, ranging from 58% to 93%, according to the type of ELISA assay used (23). These antibodies may display LA activity in vitro (24). However, Horbach et al. (25) showed that they are the only responsible of the $\beta_2$-GPI-dependent LA activity in a minority of patients, 4 out of 28 well characterized cases. Thus, the existence of other, not yet described aPL antibodies that cause $\beta_2$-GPI-independent LA activity may be postulated.

### Prognostic significance of aPL antibodies

At present, the three aPL antibodies have the same role and dignity as laboratory criteria for APS. In order to define the risk profile for developing APS-related clinical events aPL antibody detection has been used, too. Systematic reviews have consistently reported that LA is a stronger risk factor for both arterial and venous thrombosis and obstetric complications compared to aCL and $\alpha_2$-GPI antibodies (26–28). The latter two antibodies show some significant association only at high titre.

The group of experts that determined the Sydney criteria advised on how to classify patients according to their positivity for multiple or single aPL antibodies (1), thus underlying the concept that the aPL profile rather than the individual test is useful to define the risk to develop APS-related events. In line with this is the observation that – independent of LA and aCL antibodies – neither $\alpha_2$-GPI nor antiprothrombin antibodies are associated with arterial or venous thrombosis (29).

#### Triple aPL positivity

Triple aPL positivity is defined by the presence of LA together with high titres of aCL and $\alpha_2$-GPI antibodies. Several retrospective and prospective studies have shown that triple positivity correlates with both thrombosis and pregnancy morbidity more strongly than single or double positivities (30–34). With respect to thrombosis, it confers an odds ratio (OR) with 95% confidence intervals (95% CI) ranging from 5.24 (95% CI 1.5–18.28) to 33.3 (95% CI 7–156.7). None of the other combinations reached statistical significance.

A large, prospective multicentre study on triple aPL-positive patients reported a cumulative incidence of thrombosis of 12.2%, 26.1% and 44.2% after 1, 5 and 10 years of follow-up, respectively (32). The prospective study by Forastiero et al. (35) showed that the triple positivity for LA, IgG $\alpha_2$-GPI and IgG antiprothrombin antibodies gave the highest annual rate of thrombosis (8.4%), which was significant in multivariate analysis (OR 2.6, 95% CI 1.35–5.01). Regarding obstetric complications, triple aPL positivity gave an OR of 16.2 (95%CI 0.9–292) for late pregnancy loss and an OR of 34.4 (95%CI 3.5–335) for a new pregnancy loss (31).

Triple aPL positivity is likely caused by IgG directed against the Gly40-Arg43 epitope in the first domain of $\beta_2$-GPI, which cause LA activity and give positive ELISAs for aCL and $\alpha_2$-GPI antibodies. Notably, the presence of antibodies to the Gly40-Arg43 epitope in the first domain of $\beta_2$-GPI highly correlated with the history of thrombosis of a large group of patients with autoimmune diseases (17, 18).

When measured by means of a coagulation test-based assay able to discriminate between $\alpha_2$-GPI-dependent and $\beta_2$-GPI-independent LA activity, these antibodies gave an OR for thrombosis of 42.3 (95% CI 9.9–194.3) (18). When the antibodies were measured by an ELISA with the first domain of $\beta_2$-GPI coated on hydrophobic plates, the OR for thrombosis was 18.9 (95% CI 3.4–13.5) in the same population of patients (17). A large retrospective, international, multicentre study on 442 $\alpha_2$-GPI-positive patients reported a 55% prevalence of IgG antibodies directed against the first domain (36). The presence of these antibodies was associated with thrombosis (OR 3.5, 95%CI 2.3–5.4) and obstetric complications (OR 2.4, 95%CI 1.4–4.3).

### Pathogenic mechanisms of thrombosis and fetal loss

The epidemiological association between aPL antibodies and APS-related clinical events is still not fully understood. The group of experts that determined the Sydney criteria advised on how to classify patients according to their positivity for multiple or single aPL antibodies (1), thus underlying the concept that the aPL profile rather than the individual test is useful to define the risk to develop APS-related events. In line with this is the observation that – independent of LA and aCL antibodies – neither $\alpha_2$-GPI nor antiprothrombin antibodies are associated with arterial or venous thrombosis (29).

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events does not necessarily imply a patho-
genetic link, as these antibodies could only be a useful marker of thrombosis and/or obstetric complications. A large body of studies, however, provides evidence that aPL antibodies are pathogenic. It has, in fact, been shown:
1. evidence of an imbalance of pro- and antithrombotic mechanisms towards a hypercoagulable state in patients suffering from APS (37–39) such as
   – increased resistance to annexin V,
   – increased resistance to activated protein C pathway,
   – increased concentration of activated von Willebrand factor,
   – upregulation of tissue factor and tissue factor pathway inhibitor;
2. in vitro activation of target cells upon exposure to human IgG containing aPL antibodies or to purified human αβ2-GPI antibodies (40–42) such as neutrophils, monocytes, endothelial cells and platelets,
3. enhancement of venous and arterial thrombosis and development of obstetric complications upon passive injection of human IgG containing aPL antibodies or purified human aPL antibodies in animal models of thrombosis (43–46);
4. increased rate of fetal resorption in mouse models of SLE and APS (47, 48).

This abundance of evidence, however, does not answer some relevant questions:
- How is aPL antibody production triggered?
- How can APS-related clinical manifestations be so diverse?
- Can β2-GPI be used as a pharmacologic target?

Native β2-GPI is a monomeric molecule with a low affinity to phosphatidylserine (PS)-containing membranes, which increases approximately 1000-fold in the presence of αβ2-GPI antibodies (49). Multimeric β2-GPI exists in circulation, which has a higher affinity for PS-containing membranes (50). Hence, a role for multimeric β2-GPI as an opsonin for apoptotic cells cannot be excluded. It is also possible that debris of apoptotic cells may express components able to bind monomeric β2-GPI (51). Impaired clearance of apoptotic cells has been suggested to occur in SLE and other autoimmune diseases and to serve as a common mechanism leading to the generation of autoantibodies (51). Data have been provided that, upon binding to apoptotic (but not viable) cells, prothrombin and β2-GPI may induce the generation of aPL antibodies (52–54).

Molecular mimicry is another mechanism possibly leading to the generation of aPL antibodies. This hypothesis has been proposed following the observation that mice immunized with the bacteria Bacillus subtilis, Haemophilus influenzae and Neisseria gonorrhoeae or tetanus toxoid may develop aβ2-GPI antibodies, which induce fetal resorption upon passive infusion into pregnant mice (55). Viral and bacterial peptides, in fact, have been shown to share homology with the phospholipid binding site of the fifth domain of β2-GPI (56).

The dual role of innate immunity

Recently, the group of Rauch (57) proposed the model of a dual role of innate immunity, which may rationalize the generation of aPL antibodies, their persistence in time and why some, but not all, aPL-positive subjects develop thrombosis.

According to this model, during the so-called initiation phase the generation of apoptotic cells and debris allows the binding of β2-GPI, prothrombin or other proteins with affinity for anionic phospholipids, which may elicit the production of long-lived aPL antibodies. By itself, this is insufficient to cause thrombosis. In case a subsequent local or systemic trigger, such as organ injury, infection, inflammation or ischaemia, activates endothelial cells, monocytes, neutrophils and/or platelets, exposure of PS or tissue factor on cell membranes occurs (effector phase). The binding of β2-GPI, prothrombin or other phospholipid-binding proteins to these surfaces induces the intervention of aPL antibodies, which amplifies the coagulation cascade, finally causing thrombosis.

A role for the toll-like Receptor 4, a transmembrane protein which is a key component of the innate immune response, in the cell activation signalling induced by β2-GPI/αβ2-GPI antibody binding has been reported (58).

Since the effector phase may start at any site of the body and activate blood cells either selectively or broadly, the aPL-driven clinical events may be very diverse in terms of site of occurrence and type of manifestation.

Therapeutic target: β2-GPI

Due to the central role of β2-GPI in APS, this protein is a potentially attractive target for a specific therapy. In this respect, mutants of the first domain with enhanced aPL-binding capacity have been developed (59), which may be used as inhibitors of aPL binding, thus inhibiting the aPL-related pathogenicity.

In a mouse model of femoral vein thrombosis, the pre-injection of one such mutant led to the complete abrogation of the enhancing effect on thrombus formation exerted by IgG purified from APS patients (60). Research in the coming years will show if this approach represents a new way to treat APS patients.

Treatment of APS

Asymptomatic subjects who are incidentally found to be aPL-positive do not require primary thromboprophylaxis, because their risk of first thrombosis is < 1% patients/year (61). They require, however, prophylaxis in all situations at increased risk of thrombosis, such as surgery or prolonged immobilization, and the abolishment/control of all the known acquired risk factors of thrombosis.

Venous thromboembolism is the most common initial clinical manifestation of APS.

Its treatment in patients with APS consists of initial unfractionated or low-molecular-weight heparin for at least five days, overlapped with warfarin therapy administered to achieve a target INR of 2.0 to 3.0 (62–65). The optimal duration of anticoagulation is uncertain but based on prospective data suggesting a high rate of recurrence after warfarin discontinuation:

Indefinite anticoagulation is recommended.
Patients who suffered from a first arterial thrombosis (mostly represented by cerebral ischaemia) have an annual rate of (venous or arterial) recurrence of approximately 11%, irrespective of treatment with warfarin (PT INR 1.4–2.8) or aspirin (325 mg/d) (66).

The risk of recurrent thrombosis among APS patients is based on limited retrospective studies of untreated patients or studies of patients followed up prospectively after their anticoagulant drugs have been discontinued. Recurrent thrombosis in APS tends to occur in the same vascular distribution as the original event: generally patients with

- venous thrombosis have recurrent venous events,
- arterial thrombosis have recurrent arterial events.

Treatment of patients with APS who have recurrent thrombotic events is uncertain (67). For patients not receiving anticoagulant drugs, immediate initiation of anticoagulation with heparin followed up by long-term warfarin is indicated. Patients with recurrent thrombotic events despite warfarin pose a challenge for clinicians. The INR (International Normalized Ratio) at the time of recurrence is important; an INR (International Normalized Ratio) at poses the risk of warfarin failure. These patients who had showed inadequate anticoagulation as opposed to warfarin failure. These patients may be treated in the same manner as a patient presenting with new thrombosis without warfarin.

Possible treatment options for recurrent thrombosis despite warfarin in the target INR range include

- increasing the intensity of warfarin anticoagulation to achieve a higher target INR (target INR, 2.5–3.5 or 3.0–4.0),
- switching from warfarin to therapeutic doses of unfractionated heparin or low-molecular-weight heparin, or
- adding an antiplatelet agent to warfarin.

Plasma exchange or intravenous immune globulin, particularly in patients with catastrophic APS, have also been suggested. Several randomized clinical studies have shown that low molecular weight heparin (LMWH) in combination with aspirin increases to approximately 80% the rate of successful pregnancy outcome (68). However, a recent randomized clinical trial did not show any difference in the outcome of pregnancy of a group of aPL-positive women randomized to receive either aspirin or LMWH in combination with aspirin (69).

Conclusions

- Research in the last decade has elucidated many facets of the pathogenicity and clinical significance of the various aPL antibodies.
- At the same time, the results of several randomized controlled clinical trials have defined the most rational therapy of thrombosis and pregnancy complications of APS patients.
- Finally, the definition at a molecular level of the central role β2-GPI in the generation of aβ2-GPI antibodies and in the development of thrombosis and pregnancy complications will help to establish new avenues for a more appropriate treatment of APS.

Conflicts of interest

The author declares that she has no conflicts of interest.

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