Review

Seronegative antiphospholipid syndrome

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Abstract

APS is an autoimmune disease that leads to arterial and/or venous thrombosis, recurrent pregnancy loss and persistently positive aPLs. Patients with clinical manifestations highly suggestive of APS but persistently negative conventional aPLs are classified as having seronegative APS. Ongoing research has revealed the existence of non-criteria antibodies proposed to be relevant to APS and that can be potentially included in the disease’s classification criteria. We present a literature review on the most promising antibodies of this heterogeneous aPL family, which includes antibodies to a zwitterionic phospholipid, namely phosphatidylethanolamine, phospholipid-binding plasma proteins, phospholipid–protein complexes and anionic phospholipids other than cardiolipin. Although these molecules can increase the diagnostic yield of APS, their clinical relevance is still debatable and needs to be confirmed by interlaboratory efforts toward standardizing diagnostic tools, in addition to experimental data and larger longitudinal studies.

Key words: phosphatidylethanolamine, antiphospholipid antibodies, thrombosis, pregnancy morbidity, antiphospholipid syndrome, seronegative antiphospholipid syndrome, anti-β2 glycoprotein I antibodies.

Introduction

APS is characterized by diffuse arterial and/or venous thrombosis, recurrent pregnancy loss and persistently positive aPLs [1]. The latest classification criteria for diagnosing APS are the 2006 reviewed Sapporo criteria that require the presence of at least one clinical manifestation and one positive laboratory criteria (Table 1) [1]. Following the application of the Sapporo criteria, controversy arose because those criteria identify a more homogeneous group of APS patients at the expense of excluding another, a group collectively referred to as seronegative APS (SNAPS) [2].

SNAPS was first introduced in 2003 by Hughes and Khamashta [3] to describe patients with clinical manifestations highly suggestive of APS but with persistently negative LA, aCL and anti-β2 glycoprotein I (anti-β2GPI) antibodies. Like classical APS, SNAPS can have an accelerated progression resulting in multi-organ failure, a life-threatening medical condition known as catastrophic APS [4, 5].

Studies reported the involvement of several other antigens besides those mentioned in the Sapporo criteria, ranging from anionic phospholipids to phospholipid–protein complexes and plasma proteins [1, 6, 7]. This study presents a review of the literature for the most relevant antibodies soon to follow on the footsteps of anti-β2GP1 antibody, which was recently introduced to the reviewed Sapporo criteria as one of the diagnostic laboratory markers for APS, along with LA and aCL [5] (Table 2).

Methods

A comprehensive literature review of English-language full-text articles was done using PudMed and Medline. The following search entries were used: seronegative [All Fields] AND (antibodies, antiphospholipid [MeSH Terms] AND antiphospholipid antibodies [All Fields]) AND (antiphospholipid syndrome [MeSH Terms] OR antiphospholipid syndrome [All Fields] OR antiphospholipid antibody [All Fields]). The combined search revealed 27 articles, which were reviewed, and relevant data were extracted from them as well as other articles cited in the reference section of the retrieved articles.
Among the multiple aPLs supported by increasing evidence as having a relationship with the clinical manifestations of APS are those directed against a zwitterionic phospholipid, phosphatidylethanolamine (PE) [8]. PE is mainly found in the inner leaflets of mammalian plasma membranes and contributes 20–50% of total phospholipids. Furthermore, PE works as an anticoagulant, enhancing activated protein C (APC) activity in blood coagulation reactions. PE’s dramatic effect on enhancing APC’s inactivation of activated factor Va (FVa) and consequently downregulating its procoagulant function was reported by Smirnov et al. [9]. Alternatively, Tsuda et al. [10] showed that PE’s anticoagulant effect is achieved by inhibiting the factor Xa–prothrombin (PT) system.

Interestingly, PE was found to have procoagulant characteristics in certain conditions, particularly through activating factor X [11] and PT [12] and decreasing the amount of phosphatidylserine needed for optimal activation. A recent report also mentioned the major contribution cell surface-exposed PE makes in translocating protein C inhibitor proteins across the plasma membrane [11]. The procoagulant effect of PE is further evidenced by experiments proving that its hexagonal transition phase is capable of neutralizing certain LAs in the presence of PT [12].
Certain antibodies to PE (aPEs) were reported not to bind to PE alone but instead to some PE-binding plasma proteins involved in the coagulation cascade. Among these are the high-molecular-weight kininogens (HKs) [13]. Other proposed plasma proteins are factor XI and prekallikrein [14].

Clinical significance of aPE

Because patients persistently testing negative for aPLs but having clinical features highly suggestive of APS cannot be labelled as having APS, some studies focused on aPE as a potential alternative laboratory criterion for diagnosing this syndrome. They reported that these antibodies are significantly associated with major clinical events (fetal loss and/or thrombosis) and are currently present in the absence of the laboratory criteria of APS [15–21]. These articles discussed the clinical significance of aPEs, where in many instances were the sole players in patients complaining of symptoms highly suggestive of APS.

Regarding obstetric complications, in a case–control study on 1554 women not known to have previous autoimmune diseases, aPE IgM was found to be significantly more frequent in women complaining of unexplained early recurring pregnancy loss (RPL) as compared with healthy controls or those with explained RPLs. Also, aPE was the sole aPL present in 73% of aPE-positive patients [17]. Furthermore, in their study on 139 women with early RPL, aPE IgG, rather than IgM, was seen significantly more often in women with RPL than in the control group (20.1%, P = 0.01). Moreover, aPE was the most frequent aPL in infertile women (67.5% of all aPL-positive sera) [17]. The importance of aPE in the pathogenesis of RPLs was further elucidated in a recent study of a murine model [22]. It was shown that mice with inactivated ethanolamine kinase (Etnk) gene (Etnk2−/− mice), which catalyses the first step of PE biosynthesis, had significant reductions in litter size, frequent perinatal death and placental thrombosis. Moreover, other studies showed that passive immunization of anti-PE or anti-LDC27 (antigen site on HK) in pregnant mice increased fetal resorption significantly, correlating with increases in placental apoptosis [23]. Such study results bring about the importance of PE in maintaining placental homeostasis and supports the role aPE has in human fetal loss [24].

Several articles were published that studied the relationship between aPE and thrombosis, another key clinical feature of APS. An initial report showed that aPE was the only detectable aPL by ELISA in a severely thrombotic patient with positive LA [25]. Consequently, a report was published on aPE being the only aPL detected in 6 of 34 thrombotic patients with negative LA antibodies [15]. Hirmerova et al. later measured criteria and non-criteria aPLs in 140 patients with venous thrombosis and 136 controls, in an attempt to define the aPL profile in thrombotic patients. Of the different non-criteria aPLs tested, only IgM-aPE was significantly more prevalent in patients with venous thrombosis than in controls (14.3%; P = 0.035). Interestingly, IgM-aPE was present in most cases without criteria aPLs [26]. In another study investigating the significance of aPEs in 240 patients with thrombotic events, thrombosis was unexplained in 98 cases (UT) and explained in 142 cases, of them 67 patients with APS and 75 with hereditary haemostatic defects (HHDs). When compared with 100 controls, aPEs were found to be significantly higher in APS and UT, and not in HHDs. Also, aPEs did not overlap with the laboratory criteria for APS [18].

Furthermore, in a multicentre study conducted by the European Forum on aPLs, aPEs were compared with the conventional aPLs in a large cohort of patients, where 317 suffered from venous thrombosis and 52 from arterial thrombosis, with or without the main known clinical and biological risk factors for thrombosis. Anti-PE antibody happened to be present in 15% of the thrombotic patients, in comparison with 3% among the controls (P < 0.001), being alone in 67% of positive cases [20].

Therefore, based on published studies, aPEs may hold promise in labelling persistently seronegative patients as having APS, although a standardized and valid ELISA is needed for detection. In addition, well-designed prospective studies need to be initiated on diagnosed APS patients in order to confirm the clinical relevance and diagnostic value of aPEs [27]. Furthermore, in a recent review by Staub et al., the group presented clinical studies concerned with aPE and its impact on pregnancy morbidity and thromboembolic disease. Also, they supported the idea that prospective, well-controlled studies are needed in the future to solidify the relationship between aPE and thrombotic disease [28].

aPLs to negatively charged phospholipids other than cardiolipin

The panel of non-criteria aPLs being explored for improving early identification of SNAPS expands to include antibodies against phosphatidic acid (PA), phosphatidylserine (PS) and phosphatidylinositol (PI), which fall under the category of anionic phospholipids. These are found, with varying proportions, on the inner and outer membranes of almost every cell. However, taking this group of antibodies into consideration when diagnosing APS is still controversial [27]. In a study on 866 women with RPL, 150 (17.3%) were positive for IgG and/or IgM aCL compared with 12 of 288 control women without any prior obstetrical complications (P < 0.001). Eighty-seven of 866 women with RPL who tested negative for aCL were positive for one of the other aPLs [29]. In another study on a group of 872 women with RPL, 49 of 872 (3.6%) were negative for both aCL and LA but positive for aPS. Also, the prevalence of aPS had a positive correlation with the number of consecutive losses [30]. These two studies suggest that a significant number of women with RPL would have been missed if anionic phospholipids other than aCL were not included in the diagnostic process. Yet, an obstacle often faced in trying to incorporate
other aPLs is the lack of standardization between laboratories.

The significant role of aPS, aPI and aPA in obstetric APS is supported by basic research. In an in vitro model system, aPS antibodies inhibited the development and invasion of the trophoblast [31], decreased hCG levels and retarded the formation of syncytiotrophoblast. This study showed that low-molecular-weight and unfraccionated heparin help reduce the binding of both aPS and aCL in vitro [32]. Moreover, published clinical studies suggested that treatments that benefited women with RPL and aCL positivity in delivering healthy offspring could also be applied to women with RPL and other aPL positivity [33].

Since the laboratory criteria for APS were revised and published in 2006, not many studies were carried out regarding the aforementioned anionic aPLs. Gharavi et al. [34] found in their early investigations wide cross-reactivity of aCLs to both aPS and aPI antibodies. Furthermore, aPS, in particular, appears to be more specific for APS than aCL, as the latter is frequently positive in infectious diseases, among others [35, 36]. Despite the fact that aPS has been the most extensively investigated negatively charged aPL in APS [33, 37–41], the optimal conditions for its best clinical and analytical performance are to be determined. Based on the current evidence, the 13th International Congress on Antiphospholipid Antibodies did not recommend testing for aPA, aPI and aPS, as these antibodies appeared to overlap with the accepted diagnostic markers of APS. The congress further encouraged the development of a standardized method for testing for aPS, as it proved to be the most promising among the other anionic phospholipids, especially in the area of RPL [27].

**Antibodies to domains of 2GPI**

It was not until 2006, when the modified Sapporo criteria were published, that IgG and IgM anti-2GPI antibodies were added to the definition of APS [1, 42]. Among the heterogeneous population of aPLs, 2GPI is regarded as the most important antigen in APS [43]. Anti-2GPI antibodies also comprise a diverse family of antibodies recognizing different epitopes on 2GPI. Throughout the past decade, accumulated data showed that domain I, out of the five homologous domains of 2GPI, is the primary epitope for aPLs. Reactivity to domain I, specifically glycine40-arginine43, was first presented by Iverson et al. [44, 45]. However, other studies reported that the epitope may comprise a larger region on domains I and II [27, 46].

2GPI is a glycoprotein synthesized mainly by hepatocytes but present in high concentrations in plasma. 2GPI mRNA is also detected in fetal astrocytes, neurons, lymphocytes, intestinal, placental and endothelial cells [47]. Through flow cytometry, 2GPI was found to be expressed on the cell surface of human peripheral blood monocytes. It was found to be present in the cell lysate on western blotting. Its expression was significantly increased in APS and SLE patients compared with healthy blood donors and correlates with tissue factor expression on monocytes [48].

Some of the functions of 2GPI are the inhibition of prothrombinase and tenase and factor XI–factor XI activation, in its role as a natural anticoagulant regulator. Besides the fact that it can inhibit anticoagulant activity of activated protein C [48], 2GPI further contributes to the in vivo generation of thrombin as was deduced from knockout mice. The binding of antibodies to 2GPI forms a complex that increases the affinity of 2GPI to a continuously growing list of cell-surface-binding sites, thereby activating endothelial cells and monocytes through a series of signal transduction events [49].

Recently, Ramesh et al. [50] showed that in mice, the binding of aPL to 2GPI led to endothelial cell–leucocyte adhesion and thrombus formation through the inhibition of endothelial nitric oxide.

**Clinical significance of anti-domain I antibodies of 2GPI**

In the attempt to demonstrate the clinical significance of anti-domain I antibodies of 2GPI in APS patients, two studies have been conducted. In the first study, anti-domain I antibodies were shown to be associated with thrombosis (predominantly venous) more than their counterparts targeted to other domains of 2GPI [51]. This finding was recently confirmed in 2009, where 442 patients, positive for anti-2GPI antibodies, were enrolled in a double-blinded multicentre study. Two hundred and forty three of 442 (55%) had anti-domain I antibodies, of which 83% had a history of thrombosis with an odds ratio of 3.5:1 (95% CI 2.3, 5.4) for thrombosis. Moreover, anti-domain I antibodies were found to be associated with pregnancy morbidity [52]. Recently, in a study conducted on mice, these were injected with IgG purified from APS patients. After standardized vessel injury, mice injected with aPL-related IgG had increased thrombus size that could be inhibited by domain I of 2GPI [53]. After presenting these data at the 13th International Congress on Antiphospholipid Antibodies, the clinical data on anti-domain I antibodies were found to be encouraging. Yet, additional prospective clinical studies and in vivo data on the causality of anti-domain I on APS are needed, along with a standardized consensus protocol for anti-domain I assay, before this antibody can be added to the established diagnostic guidelines [27].

In a recent review written by Mahler et al. [54], a significantly lower likelihood for thrombosis was reported, whether the 2GPI (whole molecule) chemiluminescence assay or ELISA was used. Authors called for the necessity of additional studies to completely analyse and confirm the reported clinical associations of anti-2GPI-D1 antibodies with thrombosis and/or pregnancy complications.

**Antibodies to vimentin/cardiolipin complex**

Vimentin is the most abundant type III intermediate filament of the cytoskeletal system. Vimentin was recently
localized on the surface of apoptotic neutrophils and T cells [55, 56], activated macrophages [57], platelets [58], vascular endothelial cells [59] and others.

In search for new antigenic targets for the diagnosis of APS, a proteomic approach identified vimentin as the main endothelial molecule recognized by aPLs. This discovery was followed with an in vitro binding of positively charged vimentin to negatively charged cardiolipin consistent with the literature [60]. Based on these findings, 191 patients were tested for the presence of anti-vimentin/cardioli- pin complex antibodies. Results showed persistent presence of IgG and IgM anti-vimentin/cardioli- pin complex antibodies in almost all patients with APS and a large portion of patients diagnosed with SNAPS [60]. These data ascertain the importance of anti-vimentin/cardioli pin antibodies as sensitive markers for APS, especially in SNAPS patients. Unfortunately, the overlapping presence of anti-vimentin/cardioli pin antibodies in SLE and RA patients weakens the specificity of such a diagnostic marker [60].

The mechanism behind the antigenicity of vimentin is still unknown, but it is assumed that vimentin reaches the surface of plasma membranes through a caspase-dependent pathway. On the surface of apoptotic cells, vimentin can bind cardiolipin to form a highly immunogenic particle [61, 62].

aPTs: aPT-A and aPS-PT

Prothrombin is a plasma glycoprotein converted to thrombin by extrinsic thromboplastin during the second stage of blood clotting [63]. The detection of aPT antibodies varies amply from one ELISA kit to another. Some investigators use irradiated plates and buffers containing detergent (Tween20), while others use non-gamma-irradiated plates in addition to the different blocking solutions [64–68]. Facing this diversity, a comparative study of the multiple identification kits was pursued to optimize the aPT testing. Findings showed that the combination of gamma-irradiated plates, phosphate-buffered saline buffer and a coating antigen of 10 mg/ml prothrombin is the optimal choice [69, 70]. Later, a collaborative study showed better concordance of diverse in-home and commercial kits for IgG aPT compared with the IgM aPT assays [71].

Clinical significance of aPT

Since aPT was first recognized in 1995, conflicting conclusions were obtained from various, mainly retrospective, studies concerning its clinical significance [65, 69, 70, 72–74]. However, in recent years, at least two prospective studies validated the role of aPT in predicting the first or recurrent risk of thrombosis in patients with APS [75, 76]. Needless to say, other studies stressed on the increased risk of thrombosis with more types of aPLs (the quadruple positivity of LA, aCL, a2GPI and aPT being the most effective) [77]. It is noteworthy to bring up the 15-year longitudinal study by Bizzaro et al. [76] that identified IgG aPT antibody as the most useful thrombosis predictor in SLE patients.

aPT is also capable of binding to PT/PS complex giving rise to a new group of antibody involved in APS-related clinical manifestations [66]. Again, many conflicting studies concerning the role of aPT/PS were pursued, until Bertolaccini et al. [78] demonstrated the positive association between aPS/PT (IgG and/or IgM isotype) and arterial and/or venous thrombosis. Furthermore, aPS/PT has higher sensitivity and specificity than aCL, and in view of its solid correlation with LA, it can be used as a confirmatory test for APS [27, 74, 79].

Based on the above studies, aPT and aPS/PT are promising antibodies that can be potentially used as confirmatory diagnostic markers and as indicators of the risk of thrombosis. Nonetheless, further collaborative studies and better standardization efforts must be undertaken in order to include these antibodies in the diagnostic criteria of APS.

Annexin A5 resistance assay and annexin A5 antibody (aAnxA5)

In reviewing the antibodies involved in APS and SNAPS, we agree with Rand on the fact that most of them are empirically derived and not based on the disease’s thrombogenic mechanism. Accordingly, Rand laboratory developed a novel assay to follow on the physiology of APS and further established the annexin A5 resistance assay [27].

Annexin A5 is an anticoagulant protein mainly found in trophoblasts and vascular endothelial cells. After binding to anionic phospholipids, it undergoes oligomerization to form a protective shield against coagulation enzymes [80–82].

The annexin A5 resistance assay consists of two phases similar to the identified mechanism of action [83–85]. The first phase consists of exposing patient serum to phospholipids followed by centrifugation and washing out the substrate. For the second phase, the same phospholipids substrate is used to coagulate normal pooled plasma. Patients with coagulation time lower than the reference are considered annexin-A5-resistant. To follow on his findings, Rand [27] collected data from five studies and showed that 52% of APS patients by current consensus criteria were found Annexin-A5-resistant, in comparison with 2–5% of disease-free (control) and SNAPS patients.

Clinical significance of the annexin A5 antibody

The clinical correlation of aAnxA5 is disappointing in relation to pregnancy-related morbidity, due to inconsistent results among studies [17, 86, 87]. In addition, de Laat et al. [88] found no association between aAnxA5 and a history of thrombosis. On the other hand, aPL antibodies were shown to interfere with the protective binding of annexinA5 to the endothelium, hence leading to thrombosis by promoting competitive a2GPI binding to exposed anionic phospholipids [82]. This mechanism is manifested by myocardial infarction and stroke [89].
Accordingly, the task force of the 13th International Congress on Antiphospholipid Antibodies requested additional clinical data and encouraged other centres to implement the annexinA5 resistance assay before recommending the assay as part of the standard aPL testing panels [27].

We are very optimistic about the application of a mechanistic approach in identifying the optimal antibodies for diagnosing APS.

**IgA aCLs**

As previously mentioned, the diagnosis of APS requires the presence of at least one positive LA, aCL IgG or IgM or anti-β2GPI antibodies. Numerous studies recently studied the role of the IgA isotypes of the aforementioned tests. The prevalence of IgA aCL in unselected patients with SLE varied significantly from as low as 1% [90] to as high as 44% [91].

It is well known that aCL isotype distribution is relatively ethnicity-dependent and IgA aCL is no exception. IgA aCL prevalence in SLE was shown in African American, Afro-Caribbean and Hispanics to be 16%, 21% and 14%, respectively [92]. Furthermore, in 82% of aCL-positive Afro-Caribbeans, IgA aCL was the only isotype detected. In another study, only 4.4% of Chinese patients with SLE expressed the IgA isotype [93].

**Clinical significance of IgA aCL**

In order to assess the pathogenicity of IgA aCL, mice were injected with IgA aCLS from patients with APS. Later on, this same group of mice developed thrombosis [94].

In addition, a study involving 100 African Americans with SLE found an association between elevated levels of IgA aCL and β2GPI and thrombosis. However, only 5% of the 100 African American patients developed thrombosis [95]. Also, a study showed a positive relation between high IgA aCL levels and thrombocytopenia in patients with SLE or other collagen vascular diseases [96]. In view of these studies, it is recommended to check IgA aCL assay in cases of high clinical suspicion for APS with negative IgG and IgM aCLs [27].

**IgA anti-β2GPI antibodies**

IgA anti-β2GPI was found in various studies to be an independent marker for the development of various atherosclerotic disease manifestations such as acute myocardial infarction and acute cerebral ischemia [97–100]. Likewise, various studies described the association between the exclusive expression of IgA anti-β2GPI antibody and the clinical manifestations of APS [101–104]. For instance, women with unexplained recurrent spontaneous abortions and fetal death solely expressed the IgA isotype of anti-β2GPI antibody and negative for LA [104].

In a recent large, multi-ethnic, multicentre cohort study, out of 588 patients with SLE, 149 sera were found positive for IgA anti-β2GPI antibody, where 75 patients were exclusively positive for this isotype [27]. From these subjects obtained from three different groups, a considerable number displayed at least one clinical manifestation consistent with APS (70%, 100% and 80%) [27].

Mehrani [105] also presented data confirming the association between IgA anti-β2GPI antibody and thrombosis in SLE patients. Moreover, Mehrani [105] demonstrated IgA anti-β2GPI antibody to be more involved with deep venous thrombosis than the IgM isotype.

In concordance with the recent published studies, it is agreed that patients should get tested for IgA anti-β2GPI antibody in cases of SLE and/or APS symptoms, especially when other aPL tests are negative. Nevertheless, in order to be included in the approved classification criteria for APS, more studies comparing multiple commercially available assays in larger and well-characterized populations are needed [27].

**Conclusion**

The focus of this review has been to highlight the effectiveness of testing for non-criteria aPLs in an attempt to increase the diagnostic yield in APS, particularly in patients who present with clinical manifestations of the syndrome but persistently test negative for its accepted laboratory markers, namely aCL, LA and anti-β2GPI antibodies. Continuously expanding evidence has shown higher prevalence of antibodies to a number of other antigens in clinical APS; interestingly, these are often found in isolation. The most promising of this heterogeneous aPL family includes antibodies to PE, phospholipid-binding plasma proteins (PT, protein C, protein S, annexin V and domains of β2GPI), phospholipid-protein complexes (vimentin/cardiolipin complex) and anionic phospholipids other than cardiolipin (PS, PI and PA). Although these molecules may allow early detection of APS, their clinical relevance is still debatable and needs to be confirmed by interlaboratory efforts toward standardizing diagnostic tools, in addition to experimental data and longitudinal studies on large number of patients.

**Rheumatology key messages**

- Testing for non-criteria aPLs can increase the diagnostic yield in SNAPS patients.
- Standardized tools and longitudinal studies are needed to confirm the clinical relevance of non-criteria antibodies in SNAPS.

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