The future of treatment for antiphospholipid syndrome

Recurrent thrombosis and pregnancy morbidity are the clinical hallmarks of the antiphospholipid syndrome (APS) and occur in the presence of persistent pathogenic antiphospholipid antibodies (aPL). The current approach to the treatment of APS is focused on anticoagulation – as it has been for the past three decades. While some APS patients derive benefit from this approach, there are still a great number who do not and worse, some who suffer severe complications as a result of treatment. As such, there has been much research in the last 30 years aimed at elucidating the pathophysiological mechanisms at play in the development of clinical manifestations in APS. These studies have identified putative molecular targets for aPL action and concordantly enabled the development of therapeutic agents with specific inhibitory activity against these targets. These new targeted therapies are discussed, as well as the role they may play in the future treatment of APS patients.

KEYWORDS: anti-β2 glycoprotein I ▪ anticoagulation ▪ antiphospholipid ▪ antiphospholipid antibody ▪ complement ▪ hydroxychloroquine ▪ rituximab ▪ statin ▪ targeted therapy ▪ treatment

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Antiphospholipid syndrome (APS) is an autoimmune multisystemic disorder in which patients develop recurrent thrombosis and pregnancy morbidity in the presence of antiphospholipid antibodies (aPL), including anticardiolipin, anti-β2 glycoprotein I (anti-β_2GPI) and lupus anticoagulant [1,2]. Secondary APS is the designation given to patients who have the disorder as well as a related connective tissue disease, while in primary APS (PAPS) the disease occurs in isolation. It is unclear whether manifestations of APS differ between both patient groups [3].

There is overwhelming evidence that at least a subgroup of aPL are the pathogenic elements driving disease pathophysiology. This may occur through a number of mechanisms. aPL probably provide a propensity for thrombosis in affected patients, which has been referred to by some authors as the 'first hit', but thrombosis most likely occurs only when some triggering event, termed the 'second hit', takes place. The pathophysiological role of aPL antibodies has been variously attributed to the activation and disruption of cellular and humoral components of the vascular system [4–8]. Inflammation appears to be a key player in disease pathophysiology, perhaps being the inciting factor linking the procoagulant propensity to thrombus formation and an important component of placental damage seen in patients with pregnancy morbidity [9,10]. Perturbation of cell proliferation and function has recently been highlighted as a mediator of aPL-induced obstetric morbidity and probably plays a role in the evolution of prothrombotic manifestations of the disease [11,12].

The occurrence of aPL is associated with the development of venous and arterial thrombosis, as well as fetal loss. PAPS is recognized as the commonest cause of acquired thrombophilia. APS-related venous thrombosis occurs in approximately 0.3–1% of the general population since deep vein thrombosis prevalence is estimated at 2–5% in the general population and 15–20% of these are associated with PAPS [13]. APS is also the most frequent acquired risk for a treatable cause of recurrent pregnancy loss and for pregnancy complications in the general population as well as in systemic lupus erythematosus (SLE) patients [14]. Up to 40% of SLE patients also test positive for aPL and approximately a third of these patients have clinical manifestations of APS [15].

Interestingly, there has been little advancement in the approach to the management of APS patients although the syndrome was first described over three decades ago. It is widely accepted that the treatment and prevention of thrombosis in APS has its basis in conventional anticoagulant therapy involving agents such as heparin, low-dose aspirin or warfarin singly or in combination, depending on the clinical scenario [16]. However, despite compliance, some patients still experience thrombotic manifestations, while many others experience bleeding as

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a complication. Additionally, warfarin therapy requires frequent blood monitoring, involves significant dietary and lifestyle alterations, and is not suitable for treating pregnant patients. Similarly, the management of APS patients with a history of pregnancy morbidity involves the use of some combination of heparin and low-dose aspirin [17]. An additional issue is the management of patients who persistently test positive for aPL in the absence of clinical manifestations of the disease. This remains a controversial issue, with some physicians recommending low-dose aspirin despite there being no evidence-based data demonstrating that it is sufficient alone for primary thrombo-prophylaxis [18].

Although there is universal agreement that the current recommendations for treating APS patients are appropriate with respect to the clinical evidence available, there is also a recognized need for the development of novel targeted therapies. The motivating principle for developing novel therapeutic approaches to any disease is improvement of patient outcomes coupled with reduction of the adverse effects that plague current approaches. There is currently a vast pool of data from *in vitro* and *in vivo* animal studies highlighting several putative molecular targets for therapeutic action in APS and an important consideration is that these targets represent an 'early' phase in the pathology of the disease. Theoretically, blocking the 'early' activity of pathogenic aPL on target cells could result in less harmful and more efficacious therapy, reducing the risk for thrombosis in the event of an inciting 'secondary' event as well as pregnancy morbidity.

The therapeutic agents that are currently being investigated for their potential in APS treatment can be grouped broadly by their mechanism of action. The first group comprises immunomodulatory agents such as hydroxychloroquine (HCQ), statins, B-cell inhibitors and complement inhibitors that target various immune and inflammatory pathways of importance in the pathophysiology of APS. The second group is composed of agents that target specific antigen–antibody interactions, cell antigens and/or intracellular signaling pathways important in aPL-induced pathology. The third comprises novel anticoagulants with unique pharmacokinetic and pharmacodynamic properties, which translate into minimization of adverse effects while maintaining optimal therapeutic action (Table 1).

Given the promising preliminary data available for new therapeutic agents for APS – why has there been no new agent available for clinical use for over 30 years? Unfortunately, the reasons are manifold and perhaps the most dispiriting is that the underlying mechanisms responsible for the aPL-induced pathogenic effects seen in the disease remain incompletely understood. There are some researchers who believe the many mechanisms identified using *in vitro* and *in vivo* animal studies are of varying importance in actual APS patients and it is likely that different mechanisms play key roles in distinct patient subgroups [19]. As such, the identification of biomarkers that can define these subgroups would be an initial necessary step for the stratification of patients with respect to risk of a particular disease manifestation and by extension an appropriate treatment approach. Additionally, there is still debate concerning the strength of association of various aPL with the development of thrombosis and pregnancy morbidity, as well as optimal standardization of methods for antibody identification [20]. Perhaps the most significant roadblock is the inability to mount adequate clinical research trials due to insufficient funding and poorly defined patient cohorts to study the disease prospectively. The fact that the major clinical event in the disease, thrombosis, is relatively rare makes it necessary to enroll a large number of patients to adequately power any trial. In this review, we plan to outline the currently available data that give us an understanding of the pathophysiology of APS, as well as the data that demonstrates the effectiveness of the various novel therapeutic approaches under investigation by researchers around the world. Additionally, we focus our attention on global efforts to overcome prohibitive factors in mounting adequately powered clinical research trials in APS and how this impacts the future of treatment for the disease.

Pathophysiology of APS

Thrombosis

Vascular cell activation (platelets, monocytes & endothelial cells)

Many *in vitro* and *in vivo* animal studies have demonstrated the important role of endothelial cells (ECs), monocytes and platelets in the pathophysiology of APS, and have also identified putative molecular targets for therapeutic action (Figure 1) [21,22]. The importance of proinflammatory cytokines to the pathophysiology of disease has been highlighted by these studies, but the central importance of tissue factor (TF) has been clearly demonstrated. The key role played by TF in the development of thrombosis in APS has been revealed by the upregulation of TF mRNA, protein expression and microparticle formation,

Table 1. Novel therapeutic strategies for the treatment of antiphospholipid syndrome.

Therapy	Supportive evidence	Ref.
Immunomodulatory agents		
Hydroxychloroquine	Decreased platelet activation induced by aPL *in vitro* and inhibited aPL-mediated thrombosis in mice *in vivo*	[84]
	Relative protection against primary thrombotic event in cross-sectional study	[85]
Statins	Fluvastatin reversed EC activation and TF upregulation by aPL *in vitro* and inhibited thrombus formation *in vivo* in mice	[86]
	Fluvastatin reduced proinflammatory cytokines in mechanistic studies in aPL-positive patients	[89,90]
Rituximab (anti-CD20)	Case reports of successful treatment in primary, secondary and catastrophic APS patients and those resistant to conventional anticoagulant therapy	[94]
	Open-label Phase II clinical trial of rituximab in APS showed improvement in procriteria manifestations	[95]
Complement inhibitors	Various C3 and C5 inhibitors ameliorated aPL-induced thrombus formation and pregnancy morbidity	[96,98]
	Case report of improvement in CAPS patient using anti-C5 MoAb (eculizumab)	
Inhibition of target cell interactions & intracellular pathways		
β_2GPI/anti-β_2GPI interaction	Domain I peptides abrogated *in vitro* EC activation and *in vivo* thrombus formation by aPL	[102]
β_2GPI/target cell interaction	TIFI peptide abrogated *in vitro* EC, macrophage and trophoblast activation and *in vivo* thrombosis and pregnancy morbidity mediated by aPL	[103,104]
ApoER2′ inhibitors	Specific inhibitors reduced EC activation *in vitro* and thrombosis *in vivo*	[106]
Dilazep, dipyridamole	Inhibit monocyte TF expression induced by aPL	[30,80]
SB203580 (p38 MAPK inhibitor)	Evidence for reduction of aPL-mediated *in vitro* EC activation and *in vivo* thrombosis, TF upregulation and platelet activation	[30]
MG-132 (NF-κB inhibitor)	Reduction of aPL-mediated *in vitro* EC activation and TF production and thrombosis *in vivo*	[30]
Novel anticoagulants		
Direct anti-Xa inhibitors	Ongoing trial of rivaroxaban in APS (RAPS), which will evaluate the anticoagulant effect of rivaroxaban compared with warfarin	[201]

Abs: Antibodies; aPL: Antiphospholipid antibodies; APS: Antiphospholipid syndrome; CAPS: Catastrophic antiphospholipid syndrome; EC: Endothelial cell; TF: Tissue factor.

with associated increases in IL-6 and IL-8 secretion in ECs and monocytes treated with aPL [23]. Similarly, increased plasma levels of the related cytokine VEGF and surface expression of both VEGF and the Flt-1 tyrosine kinase receptor (receptor for VEGF) on monocytes have been demonstrated in APS patients and this may result in further upregulation of TF in monocytes [24]. The first *in vivo* evidence that TF mediates thrombosis in APS was provided by Romay-Penabad *et al.* recently using a model of induced thrombosis in TF-deficient mice passively immunized with polyclonal IgG from an APS patient [25]. Several *in vitro* studies have shown that ECs also express significantly higher amounts of VCAM-1, ICAM-1 and E-selectin after incubation with aPL [5,26]. ICAM-1, E-selectin, P-selectin and VCAM-1 allow for leukocyte adhesion and thrombus formation once induced by human monoclonal and polyclonal aPL as demonstrated by the utilization of knockout mice for these mediators by Pierangeli *et al.* [27].

Vega-Ostertag *et al.* have described the intracellular mechanisms responsible for TF activation in ECs, reporting that aPL-induced activation and TF upregulation was dependent on p38 MAPK activation and NF-κB [28]. Similarly, the fact that aPL induces TF expression in monocytes from APS patients by activating MEK-1/ERK phosphorylatin and the p38 MAPK-dependent nuclear translocation and activation of NF-kB/Rel proteins was delineated by López-Pedrera *et al.* [29]. Pierangeli *et al.* subsequently showed that activation of p38 MAPK and subsequent phosphorylation of cytosolic phospholipase A2 (cPLA2) was the main pathway activated in platelets by aPL to produce TXB2. Accessory pathways that play secondary roles after initial p38 MAPK pathway activation are the ERK-1 (p44 MAPK) and ERK-2 (p42 MAPK) pathways [30].

There is substantial evidence to show that β_2GPI, a main antigen for aPL activity, is able to bind to platelets, ECs and monocytes, providing suitable epitopes for aPL binding. Several putative target cell receptors that bind β_2GPI/anti-β_2GPI complexes have been identified, but the intricacies of these interactions have not been fully elucidated. Annexin A2 (AnnA2) is able

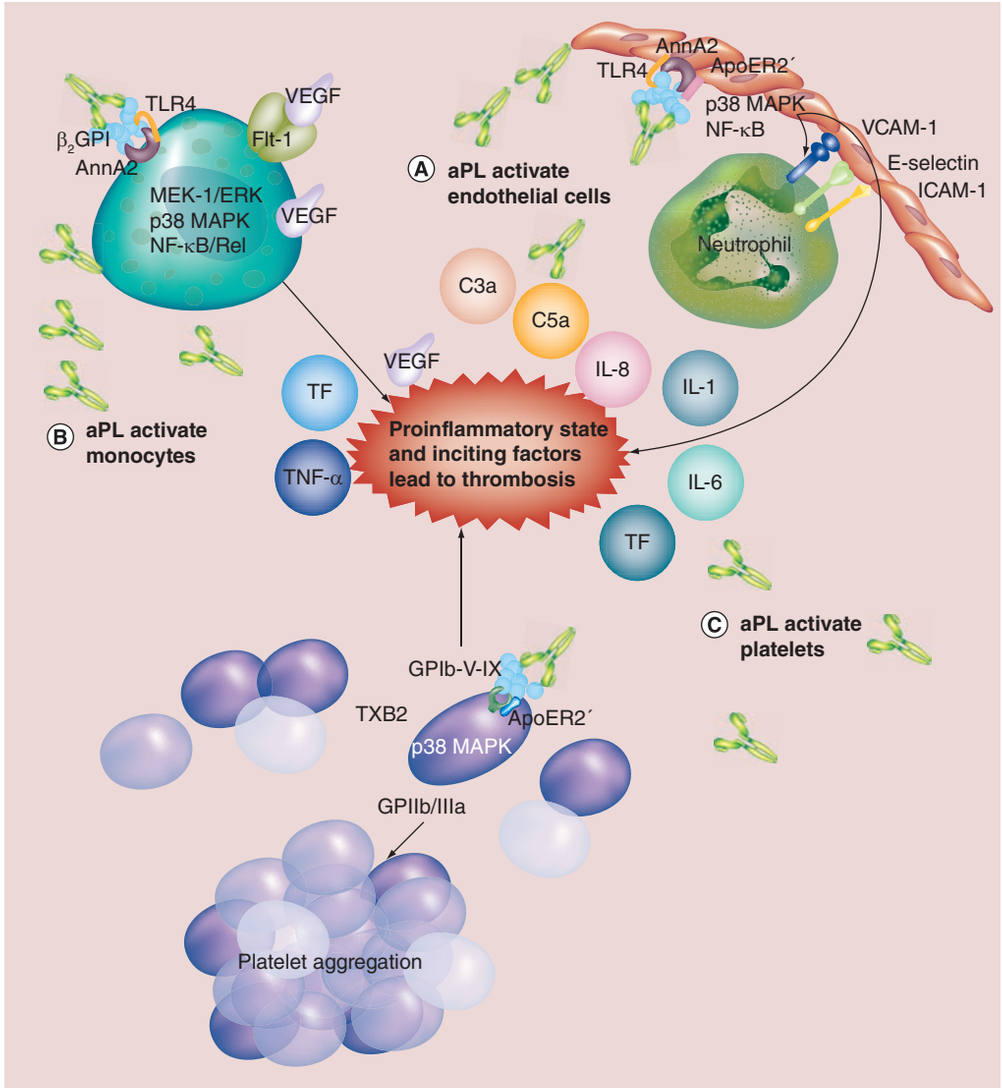

Figure 1. Thrombogenic mechanisms of antiphospholipid antibodies affecting cellular components of vasculature. (A) Pathogenic aPL activate endothelial cells via p38 MAPK and NF-κB to produce several proinflammatory cytokines and adhesion molecules. **(B)** Pathogenic aPL activate monocytes via MEK-1/ERK, p38 MAPK and NF-κB/Rel to also produce proinflammatory cytokines, most importantly VEGF and TF. **(C)** Pathogenic aPL activate platelets via p38 MAPK and NF-κB to upregulate GPIIb/IIIa to induce platelet aggregation.
AnnA2: Annexin A2; aPL: Antiphospholipid antibodies; TF: Tissue factor.

to bind anti-β_2GPI/β_2GPI complexes on both ECs and monocytes leading to activation and expression of a procoagulant phenotype [31]. Recent *in vivo* evidence provided by Romay-Penabad *et al.* in AnnA2 deficient (-/-) mice has demonstrated a significant reduction in thrombus formation and TF production induced by polyclonal IgG aPL, an anti-β_2GPI monoclonal Ab (4C5) and an anti-A2 monoclonal Ab compared with wild-type controls, highlighting the role of this receptor in disease pathophysiology [31]. However, since AnnA2 on the cell surface lacks an intracellular tail, a coreceptor would be required for intracellular signal transduction and subsequent cell activation as a result of anti-β_2GPI/β_2GPI complex binding [32].

The possibility of TLR4 being a coreceptor for aPL activation on ECs was introduced by Raschi *et al.* who highlighted the importance of myeloid differentiation factor 88 (MyD88) signaling in this process [33]; a finding that was confirmed in a subsequent *in vivo* study by Pierangeli *et al.* utilizing lipopolysaccharide (LPS) nonresponsive mice (LPS -/-) [34]. These findings are indicative of TLR4 serving as a coreceptor for AnnA2 in EC activation and there is also evidence for these two molecules acting as coreceptors for anti-β_2GPI/β_2GPI complexes on human

monocytes [35]. Quite recently, Allen *et al.* showed that the assembly of AnnA2, TLR4, calreticulin and nucleolin on the EC surface led to signaling through TLR4 in anti-β_2GPI-mediated EC activation, which was dependent on the presence of all four molecules [36]. Interestingly, there is recent evidence outlining the potential role of endogenous TLRs (namely TLR7 and TLR8) as well in the initiation and subsequent propagation of the disease process in APS patients [37,38].

The presence of dimeric β_2GPI and the exposure of anionic phospholipids, especially phospatidylserine (PS), seem to be essential for the interaction of aPL with platelet membranes [39]. GPIIb/IIIa is a major fibrinogen receptor present on platelets and Pierangeli *et al.* showed that aPL-mediated platelet activation resulted in the increased expression of this receptor and subsequently that aPL-induced thrombus formation was significantly reduced in GPIIb/IIIa deficient (β_3-null) mice [30,40]. Researchers have demonstrated that the ApoER2′ receptor and the GPIb-α subunit of the GPIb-V-IX receptor are able to form a complex on the platelet membrane and that anti-β_2GPI/dimeric β_2GPI complex-mediated signaling through both is required for platelet activation [39,41]. A putative role for platelet factor 4 (PF4) in the stabilization of dimeric β_2GPI and subsequent binding to ant-β_2GPI Abs, exposed phospholipids and receptors on the platelet surface has been delineated [42].

Humoral vascular components (coagulation & fibrinolytic systems)

Antiprothrombin antibodies (aPTs) that display lupus anticoagulant activity have been isolated from APS patients and these antibodies are able to upregulate TF expression as well as the binding of prothrombin (PT) to the EC surface and have thrombogenic properties *in vivo* [43,44]. These antibodies seem to exhibit this pathogenic capacity when aPT is complexed to PS rather than when aPT occurs in isolation [45]. Chen *et al.* also showed that several aPT could indeed bind thrombin and in turn promote thrombosis by preventng its inactivation by antithrombin (AT) [46]. The fact that unregulated thrombin activation can induce platelet activation also contributed to the induction of a thrombogenic state by these antibodies [47].

When blood samples from 38 patients were analyzed for IgG antibodies to FIXa, they were found in 11 patients (28.9%) [48]. Functionally, four FIXa-reactive monoclonal aPL impaired AT inactivation of FIXa. Moreover, IgG from two positive plasma samples were also found to hinder AT inactivation of FIXa [48]. The result is a hypercoagulant state and since FIXa is more upstream than thrombin, unchecked FIXa may contribute more toward thrombosis than unchecked thrombin. Dr Chen's group similarly reported that several monoclonal aPL reacted with FXa and significantly inhibited its inactivation by AT [49].

Activated protein C (APC), a serine protease sharing a homologous enzymatic domain with thrombin, was also shown to bind with several thrombin-reactive aPL. Some of these APC-reactive monoclonal aPL were able to hinder the natural anticoagulant function of APC [50]. The natural anticoagulant action of AT has also been shown to be inhibited by up to 80% by aPL, which could potentiate a thrombogenic state [51].

Plasmin, also being a serine protease, was tested against six thrombin-reactive monoclonal aPL and all displayed good reactivity [52]. One of these antibodies was found to impair plasmin-mediated fibrinolysis and this finding was consistent with a report that IgG from APS patients impaired fibrin dissolution with plasmin [52,53]. Furthermore, a small pilot analysis of the plasma samples from 25 APS patients showed that seven of these patients (28%) had IgG antiplasmin antibodies [52].

Tissue plasminogen activator (tPA) was subsequently shown to bind to several plasmin-reactive aPL, which were able to reduce tPA activity [54]. Similarly, there are reports of anti-tPA antibody prevalence rates up to 15% in APS patients, these levels being inversely correlated with tPA activity [54,55]. Decreased tPA release and elevated plasminogen activator inhibitor-1 levels after venous occlusion in APS patients has also been reported [56]. A summary of the effect of aPL on humoral vascular components is shown in Figure 2.

Inflammatory mediators

In murine models of thrombus formation due to aPL action, mice deficient in C3 and C5 were found to be resistant to thrombosis and EC activation. The important role of the membrane attack complex (MAC) and C5a–C5aR interactions in aPL-mediated pathogenic processes were demonstrated by our group when C5aR-deficient and C6-deficient mice were similarly protected from aPL-induced EC activation and thrombosis [57–59]. Upregulation of TF expression occurs as a result of the binding of C5a and MAC to receptors on ECs and so contributes to thrombus formation [23].

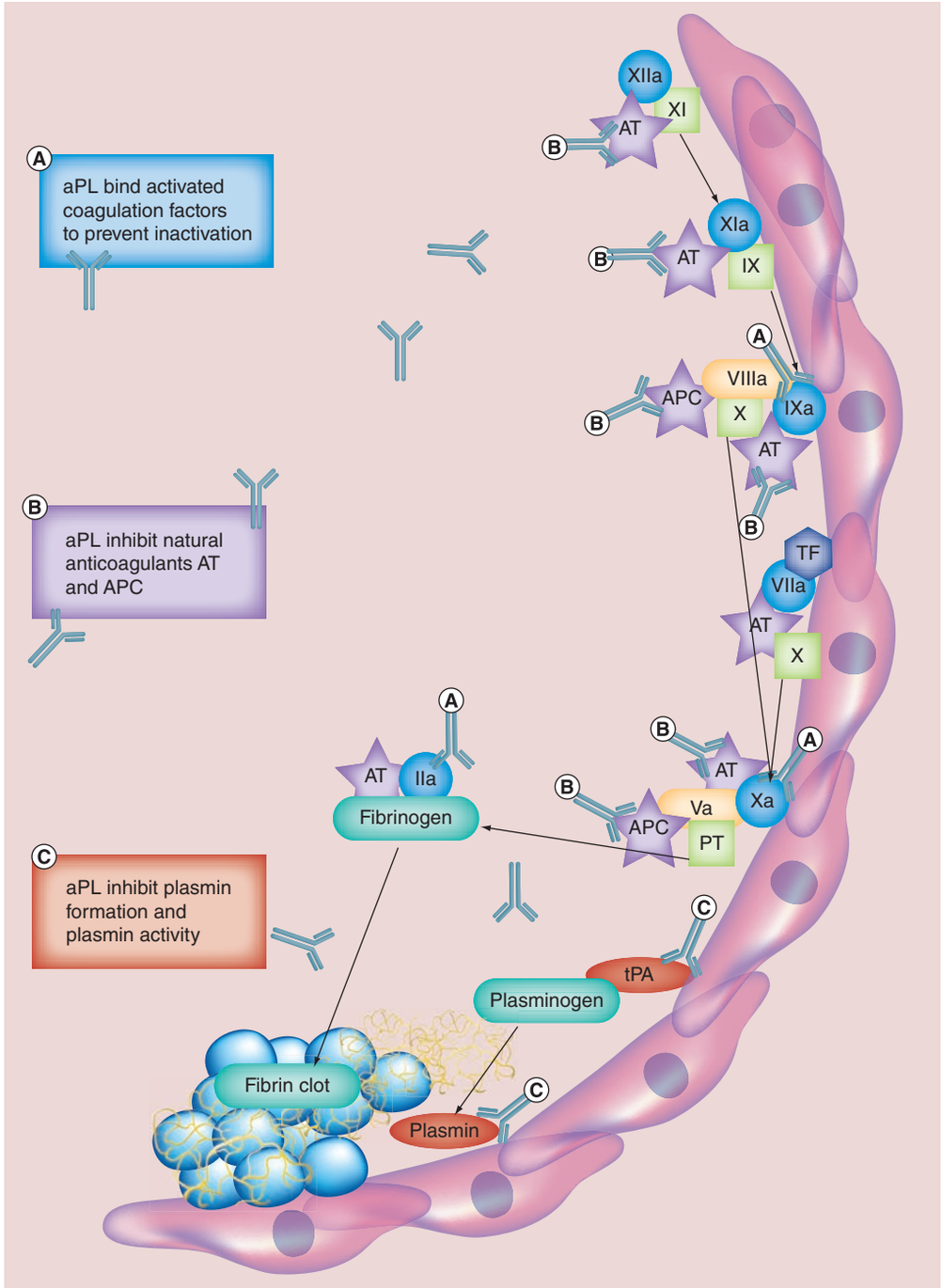

Figure 2. Thrombogenic mechanisms of antiphospholipid antibodies affecting humoral components of vasculature. (A) Pathogenic aPL also bind several coagulation factors and **(B)** their regulators: APC and AT to prevent inactivation of activated coagulation factors. **(C)** Fibrinolysis is also inhibited by inactivation of tPA and plasmin.
APC: Activated protein C; aPL: Antiphospholipid antibodies; AT: Antithrombin; PT: Prothrombin; tPA: Tissue plasminogen activator.

The activation of complement and the resulting generation of vasoactive mediators is likely to be an essential intermediary step linking EC, monocyte and platelet activation by aPL with thrombosis. In fact, there have been reports of primary APS patients who have hypocomplementemia occurring as the result of complement activation rather than deficiency. However, there was no correlation between reduced complement levels and thrombotic or obstetric manifestations in these patients [60].

Pregnancy morbidity

Thrombosis

Placental thrombosis and infarction on histological examination being demonstrated in APS patients with first and second trimester abortions provided evidence that thrombosis was an important factor in aPL-mediated pregnancy morbidity [61,62]. Rand *et al.* have reported evidence that annexin A5 (Ann A5), an important anticoagulant during pregnancy acting as a shield on potentially thrombogenic anionic membrane surfaces in the placenta, is important in maintaining placental integrity and is displaced by aPL *in vitro* and also occurs at significantly lower levels on the intervillous surfaces of placentas in women with aPL compared with controls [63,64]. Anti-AnnA5 antibodies have been reported in APS patients at frequencies up to 30% and several studies have demonstrated the association of these antibodies with recurrent fetal loss in APS patients [65,66].

Initial research in APS-related pregnancy morbidity was focused on the role of aPL-mediated thrombosis, but while experimental models provided evidence for its importance in the pathophysiology of these manifestations, epidemiological studies failed to demonstrate this consistently. In fact, there was a lack of histological evidence of thrombotic processes in placental vasculature in most APS patients with obstetric sequelae, and morever this finding was not specific for patients with APS-related pregnancy complications [67]. Further research has highlighted several unique pathophysiological processes that may play a part in APS-related pregnancy morbidity as outlined below (FIGURE 3).

Abnormal placentation

The increased production of β_2GPI and expression of anionic PLs on placental membranes as a result of extensive tissue remodeling during placentation facilitates trophoblast binding to β_2GPI [68]. This allows for the direct reactivity of aPL on these cells and also explains the placental tropism of aPL as demonstrated in several *in vitro* studies utilizing monoclonal and polyclonal aPL [69,70]. Anti-β_2GPI monoclonal antibodies have been shown to inhibit trophoblast proliferation and prevent intertrophoblast fusion, trophoblast invasiveness and hCG secretion [69,71,72]. Several aPL have also been shown to induce apoptosis in rat embryos and placental explant cultures [73,74].

It has been shown that aPL, particularly anti-β_2GPI, also react with human stromal decidual cells and induce a proinflammatory phenotype characterized by increased ICAM-1 expression and TNF-α secretion, thus affecting the maternal side of the placenta [75]. Inhibition of endometrial angiogenesis by aPL antibodies occurring through VEGF and matrix metalloprotease production and NF-κB DNA binding has been demonstrated recently in both *in vitro* and *in vivo* experimental models [76].

The negative effect of aPL on trophoblast survival was shown to be dependent on signaling through the TLR4/MyD88 pathway [77]. Evidence provided by inhibitory studies have shown that cell surface TLR4, AnnA2 and ApoER2′ play roles in the interaction between β_2GPI and cells of the decidua and trophoblast [75].

Inflammation

Collective evidence indicates that placental damage in APS patients is characterized by recruitment of neutrophils, upregulated TF and TNF-α secretion, decidual focal necrosis and apoptosis, loss of fetal membrane elements and complement deposition [78]. In particular, components of both the classical and alternate complement pathways (C3, C4, C5 and factor B) have been highlighted as having important roles in mediating pregnancy morbidity in murine APS models [79]. Interestingly, current evidence suggests that the MAC (C5b-9) plays no role in APS-mediated pregnancy complications [79,80].

Novel therapeutic strategies for APS patients

Immunomodulatory approaches

Hydroxychloroquine

HCQ initially found its clinical usefulness as an antimalarial drug, but since then has been proven to possess myriad anti-inflammatory and immunomodulatory effects including modulation of endosomal function, antigen processing and T- and B-cell signaling [81]. Interestingly, HCQ also has antithrombotic properties through its effect on inhibiting platelet activation and aggregation, and has been used historically as a prophylactic agent against deep vein thrombosis and pulmonary embolism after hip replacement surgeries [82]. Given its many immunomodulatory properties, HCQ has been repurposed for use in the management of SLE patients with proven clinical efficacy in decreasing risk of flares and damage accrual, as well as protecting against vascular events, including arterial thrombosis [83]. However, there still remains a lack of clinical data that substantiates the utilization of HCQ therapy in treating APS patients.

HCQ is able to limit aPL-induced thrombus formation and platelet GPIIb/IIIa receptor

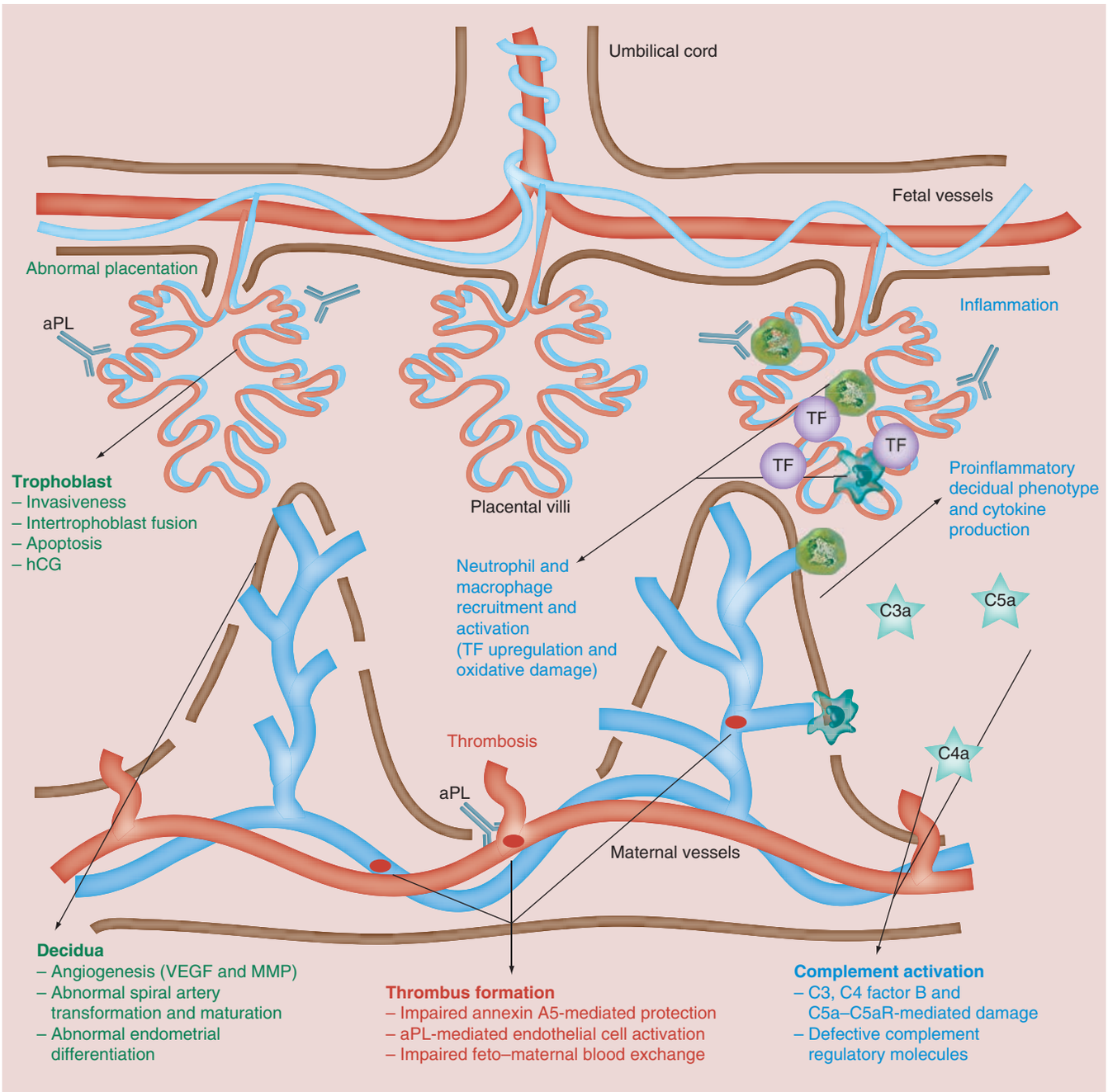

Figure 3. Pathogenic mechanisms of antiphospholipid antibodies leading to obstetric pathology in antiphospholipid syndrome. aPL induce thrombosis, as well as the recruitment of inflammatory cells and the release of proinflammatory mediators such as TF, which lead to placental damage and pregnancy morbidity. aPL also induce abnormal decidual and trophoblast cell function, proliferation and differentiation, further contributing to the obstetric complications seen in antiphospholipid syndrome.
aPL: Antiphospholipid antibodies; hCG: Human chorionic gonadotrophin; MMP: Matrix metalloproteinase; TF: Tissue factor.

expression in a dose-dependent manner and to reverse binding of aPL–β_2GPI complexes to phospholipid bilayers [84]. It also has proven benefit in preventing a primary thrombotic event in asymptomatic aPL-positive patients [85]. There is still, however, insufficient data to recommend HCQ for primary or secondary thrombosis prevention, but it might be an appropriate adjunctive therapeutic agent in APS patients who develop recurrent thrombosis despite optimum anticoagulation. Fortunately, there is an ongoing HCQ trial in APS patients being performed under the auspices of AntiPhospholipid Syndrome Alliance For Clinical Trials and InternatiOnal Networking (APS ACTION), which was highlighted at the recent International Congress on Antiphospholipid Antibodies held in Rio de Janeiro (Brazil) in September 2013.

This highly anticipated study will most likely provide meaningful clinical data outlining the usefulness of HCQ in preventing thrombosis in APS and cultivate guidelines regarding its use in these patients.

Statins

Both *in vitro* studies utilizing ECs and murine studies have demonstrated the beneficial effects of fluvastatin in abrogating the thrombogenic and proinflammatory effects of aPL antibodies, independent of the drug's cholesterol-lowering effects [86]. Rosuvastatin also demonstrated this protective effect in an *in vitro* model utilizing ECs [87]. Murine models of pregnancy loss have also been used to demonstrate the capacity of simvastatin and pravastatin in reducing fetal death [80]. These *in vitro* and animal studies were subsequently corroborated by mechanistic studies in human APS patients, the first demonstrating variable, but significant, reductions in VEGF, TF and TNF-α following a month long course of fluvastatin [88]. Interestingly, by employing a proteomic analytical approach, Cuadrado *et al.* were able to show that aPL-induced inflammatory proteins in monocytes can also be reversed following a month long course of fluvastatin therapy in APS patients [89]. Quite recently, Erkan *et al.* reported data from a prospective open-label pilot study examining the effect of fluvastatin therapy in persistently aPL-positive patients with or without SLE. The data shows that a 3-month course of 40-mg fluvastatin significantly reduced the proinflammatory and prothrombotic biomarkers IL-6, IL-1b, sTF, sICAM-1, sVCAM-1 and E-selectin [90]. Of particular interest in that study was a patient who experienced a lupus flare with concomitant and significant elevation of selected proinflammatory and prothrombotic markers, indicating that these biomarkers are sensitive to fluctuations in disease activity despite statin treatment. This observation indicates that the beneficial effects of statins in aPL-positive patients can be mitigated in the setting of a lupus flare.

It is important to note, however, that no conclusive evidence exists for a beneficial effect of statins in reducing thrombosis risk in APS patients, but it is conceivable that in reducing the upregulation of prothrombotic/proinflammatory cytokines in ECs and monocytes, a reduction in thrombosis risk would be the result. The use of statin therapy is especially attractive for primary prevention of thrombosis in persistently aPL-positive patients without a history of thrombosis. Further mechanistic and clinical studies need to be carried out to delineate the role that statin therapy will play in treating APS patients.

Rituximab

Rituximab is an anti-CD20 chimeric monoclonal antibody that inhibits B-cell activity and is approved for the treatment of rheumatoid arthritis [91]. There has not been extensive research on the role that B cells play in the pathophysiology of APS, but there has been a growing awareness of the possible role they may play in autoantibody production [92]. A recent meta-analysis evaluating the efficacy and safety of rituximab in SLE (a related autoimmune disease) found that rituximab did have favorable effects on refractory lupus despite some clinical studies that contradicted this finding. The researchers did, however, call for further studies to clarify the long-term efficacy and safety of the drug in treating SLE patients [93].

A systematic review of published data regarding the use of rituximab therapy in APS revealed several case reports of successful treatment in primary, secondary and catastrophic APS patients and in patients with aPL and autoimmune-mediated thrombocytopenia and hemolytic anemia [94]. The only clinical trial to date evaluating the effect of rituximab in APS was conducted by Erkan *et al.*, who conducted an open-label Phase II clinical trial using rituximab to treat aPL-positive patients resistant to conventional anticoagulation (RITAPS). Preliminary data from this clinical trial was first reported at the 2011 ACR meeting and was published recently, and the data suggest that the safety of rituximab therapy in APS patients is consistent with the safety profile of the drug [95]. Despite a lack of an overall decrease in aPL titres, rituximab therapy resulted in decreased $CD19^+$ B cells with an improvement in some noncriteria manifestations, namely thrombocytopenia and resolution of skin ulceration [95].

Complement inhibition

There is an abundance of preclinical data demonstrating the importance of complement activation in the progression of thrombotic and obstetric pathological processes in APS. The inhibition of complement components C3 and C5 have been shown to limit the adverse effects of aPL, both thrombotic and obstetric complications, using *in vitro* and *in vivo* murine models [58,96]. Concurrent treatment with anti-C5 monoclonal antibodies was able to reduce thrombus formation and EC activation in CD1 mice injected intraperitoneally with IgG

aPL [97]. Mice treated with a C5aR antagonist were similarly protected from aPL-induced EC activation and thrombosis [57–59]. Similar results were also obtained using a naturally occurring C5 inhibitor isolated from the saliva of the tick *Ornithodoros moubata* (Coversin [rEV576]) [98].

Utilizing an inhibitor of classical and alternative pathway complement C3 convertases (Crry-Ig), researchers were able to decrease fetal resorption and limit placental lesions and growth restriction in surviving fetuses [96]. Additionally, pregnancy complications induced by aPL were reduced in C5 or C5a receptor (C5aR)-deficient mice and in mice treated with monoclonal anti-C5 antibodies or a highly specific peptide antagonist of C5aR (C5aR-AP) [79].

To date, there are no trials evaluating the efficacy of complement inhibition in APS patients. There was, however, a recent case report of eculizumab (an anti-C5 monoclonal antibody) being used to treat a patient who had recurrent catastrophic APS characterized by multiple arterial thromboses in large and small vessels despite maximal anticoagulation, immunosuppression and plasma exchange therapy [99]. The researchers administered eculizumab at doses that blocked activation of terminal complement components and this resulted in resolution of the acute thrombotic events, reversed thrombocytopenia and was associated with no further clinical episodes of thrombosis during more than 3 years of therapy. This study demonstrates the benefit of complement inactivation in APS and highlights the need for clinical trials that will hopefully define the potential role these agents will play in treating the disease.

Inhibition of specific aPL: antigen interactions, target cell receptors & intracellular signaling pathways

Disruption of β_2GPI interaction with anti-β_2GPI & target receptors

Variants of β_2GPI lacking domain I or with point mutations in domain I have reduced ability to bind aPL derived from patients with APS, while the same is not true for changes in the other domains [100]. Similarly, anti-β_2GPI antibodies that have the greatest affinity for domain I of β_2GPI have been shown in several studies to be more closely associated with the development of thrombosis in APS patients than anti-β_2GPI antibodies with reactivity to other domains [101]. Recently, Ioannou *et al.* developed a system for creating site-directed mutations in domain I of β_2GPI and produced several domain I variants, some with an increased affinity for binding aPL. They subsequently showed that soluble recombinant domain I of β_2GPI (especially variants with increased affinity for aPL) abrogated in a dose-dependent fashion the *in vitro* and *in vivo* effects of anti-β_2GPI [102].

Ostertag *et al.* demonstrated that a 20 amino acid peptide (TIFI), a structural analogue of the phospholipid-binding region in domain V of β_2GPI, significantly decreased thrombus size in IgG-aPL injected mice by displacing the binding of β_2GPI to target cells. TIFI also demonstrated dose-dependent inhibition of the binding of β_2GPI to ECs and macrophages *in vitro* [103]. Similar experiments have demonstrated TIFI's ability to cause dose-dependent inhibition of aPL binding to trophoblasts *in vitro* [104]. In the same experiment, TIFI was able to significantly reduce aPL-induced growth retardation and fetal loss in pregnant C57BL/6 mice.

Theoretically, blocking anti-β_2GPI binding to β_2GPI or β_2GPI binding to target cells may be the most specific approach to ameliorate the pathopgenic effects of aPL while minimizing potential adverse effects since there would be no interference with important physiologic mechanisms. However, clinical studies are needed to establish the safety and evaluate the efficacy of such treatments.

Inhibition of target cell antigens

Another approach to the amelioration of aPL activity in APS patients is the inhibition of the target cell receptors responsible for cell activation. Studies have indicated the importance of cell-surface receptors ApoER2′, TLR4, AnnA2 and GPIIb/IIIa in aPL-induced activation of ECs, monocytes and platelets in APS.

ApoER2′ is expressed on ECs and *in vitro* studies utilizing anti-ApoER2′ antibodies have shown partial inhibition of β_2GPI-dependent aPL binding to and subsequent activation of ECs [105]. Romay-Penabad *et al.* reported a significant reduction in aPL-induced thrombus formation and TF production in wild-type mice treated with soluble binding domain I of ApoER2′ (sBD1), an inhibitor of ApoER2′ [106]. Similarly, Pierangeli *et al.* showed that aPL-mediated thrombus formation was significantly reduced in mice treated with a monoclonal anti-GPIIb/IIIa antibody [30,40].

To date, there are no human studies investigating the potential benefit of this approach in treating APS patients. Despite the potential benefits, the inhibition of cellular receptors that trigger intracellular signaling pathways as a part of normal physiological processes could potentially

have undesirable adverse effects. Careful analysis of the potential side effects of such therapy would be essential before any serious undertaking in mounting clinical studies evaluating these agents.

Intracellular signaling pathways

Specific inhibition of TF with agents such as dilazep and inhibition of NF-κB and p38 MAPK activity, with agents MG132 and SB203580, respectively, are potentially promising therapeutic approaches in APS management. TF plays a central role in aPL-induced thrombosis and the activation of ECs, monocytes and platelets by aPL results in signaling mainly through the p38 MAPK pathway and NF-κB activation. Specific inhibition of these targets has been shown to reduce aPL-induced TF upregulation in monocytes and ECs and aPL-enhanced thrombosis in mice [30]. In addition, murine studies utilizing mice treated with monoclonal anti-TF Abs or TF-deficient mice were similarly protected from aPL-induced obstetric complications [80]. Again, there are no clinical trials evaluating these agents in APS patients, but careful analysis of the potential adverse effects would be a necessary precursor to mounting these studies.

Novel anticoagulants

As noted previously, the mainstay of treatment in APS patients for over 30 years has been anticoagulation with heparin and/or warfarin – an approach that has many side effects. Quite recently, novel oral anticoagulants that directly inhibit activated factor Xa have been developed that possess improved pharmacokinetic and pharmacodynamic properties enabling fixed dosing. Additionally, since their anticoagulant effect is predictable and there are very few drug and dietary interactions that could affect anticoagulant intensity, the frequent monitoring that has been necessary for warfarin therapy is not a requirement for these agents [107].

At the recent International Congress on Antiphospholipid Antibodies held in Rio de Janeiro, Dr Cohen presented preliminary data from the ongoing RAPS trial. This is an open-label prospective randomized controlled trial evaluating the anticoagulant effect of rivaroxaban compared with warfarin in APS patients with a history of thrombosis. Dr Cohen highlighted that the trial is still in the recruitment phase, but that if the trial does demonstrate that anticoagulation with rivaroxaban is not inferior to warfarin and induces no serious adverse effects, it would provide sufficient supportive evidence for making rivaroxaban the standard of care for patients with thrombotic APS.

Conclusion

Approaches to overcoming prohibitive factors for clinical trials

Characterization of biomarkers that define APS patient subgroups

Many potential pathogenic mechanisms for aPL inducing clinical manifestations in APS have been described. However, it is unlikely that all identified mechanisms are responsible for the thrombotic and obstetric manifestations that characterize the disease. The extent to which any of these mechanisms contribute individually or collectively to the development of clinical sequelae in APS *in vivo* is unknown. However, the myriad number of cell surface receptors that bind β_2GPI in different tissues possibly indicates that selective signaling through different receptors could result in distinct clinical sequelae [108,109]. Furthermore, there is some presumptive evidence that functional or structural differences in anti-β_2GPI antibodies could determine the intracellular signaling cascades that are activated, quite possibly by selective signaling through the different cell surface receptors for β_2GPI, and so determine clinical phenotypes. Interestingly, polyclonal IgG from APS patients with a thrombotic phenotype were shown to upregulate TF activity in monocytes through TLR4-mediated phosphorylation of NF-κB and p38 MAPK, while IgG from patients with an obstetric pathology phenotype induced the release of sEndoglin from trophoblasts instead [110,111]. Further characterization of the distinct signaling pathways and concomitant molecular markers activated by subgroups of aPL is a necessary step towards the stratification of patients for tailored treatment regimens.

Risk of clinical sequelae associated with aPL

In recent years, there has been a call for the use of aPL, not simply for classification of APS patients, but for defining the risk for development of thrombotic or obstetric manifestations. However, there is a lack of prospective studies evaluating the risk for these manifestations associated with aPL. Most of the available studies are limited by a high incidence of comorbid conditions, such as SLE, a small number of patients, retrospective design and the absence of appropriate control populations [112,113]. The analysis for much of the data obtained has focused on assessing risk qualitatively, meaning association with a positive

versus negative result in a particular assay. This approach has masked the strong association of higher aPL titers with clinical manifestations (as is indicated by several studies), with the weaker association seen with low positive titers [114]. In moving forward, there needs to be collaborations with existing large, population-based, prospective cohorts with available thrombotic and obstetric morbidity data utilizing standardized statistical analytical techniques for risk assessment. Interestingly, there has been some evidence to suggest that combinations of aPL could also be related to the risk for clinical sequelae in addition to aPL titers [115]. There is, in particular, an abundance of data linking so-called 'triple positivity' (anticardiolipin, anti-β_2GPI and lupus anticoagulant positive) with an increased risk for thrombosis and pregnancy morbidity [115].

An additional consideration is the standardization of testing methods so that results from several prospective studies can be compared appropriately. Considerable inter-laboratory variation has always been a cause for concern in aPL assays and has been compounded by the development of newer platforms and detection technologies (some automated) for these tests [116,117]. The 'criteria' aPL testing task force was established at the 13th International Congress on Antiphospholipid Antibodies (APLA) to address these concerns and presented an update of their standardization activities at the 14th APLA meeting in Brazil recently. These include the publication of international consensus guidelines on anticardiolipin and anti-β_2GPI testing and a shorter technical document detailing suitable standards and reference materials for these tests [118,119]. Additionally, this task force has established universal standardized units for IgG and IgM anti-β_2GPI measurement and has performed initial validation of suitable reference material for both these assays.

Novel outcome measures & clinical research networks

A major roadblock to mounting clinical studies in APS is the relatively rare occurrence of major clinical events, thrombosis and pregnancy morbidity, making it difficult to enroll enough patients to achieve adequate power. An interesting approach to overcoming this problem is the selection of laboratory outcome measures that are easily achievable. This technique has been used in the RAPS trial in which the anticoagulant effect of rivaroxaban is compared with warfarin by evaluating thrombin generation 42 days after randomization into the two groups. This is assessed by the thrombin generation test, with the endogenous thrombin potential a key parameter [120]. Selection of this functional outcome allows for a large enough number of patients to be enrolled to compare study treatment with current standard of care. The identification of surrogate biomarkers that are representative of the risk for certain clinical manifestations in APS patients would be an important development in the use of this approach to study other potential therapeutic agents.

Another major step in overcoming this obstacle in clinical research efforts is the establishment of global collaborative research networks dedicated to mounting adequately powered clinical trials by pooling collective resources. APS ACTION is the first ever international research network that has been created specifically to design and conduct well-designed, large-scale, multicenter clinical trials in persistently aPL-positive patients [121]. This research network has already initiated two important collaborative international projects since the beginning of 2012. The first is a randomized controlled trial of HCQ assessing the drug's capacity to prevent primary thrombotic events in persistently aPL-positive patients in the absence of other systemic autoimmune diseases. The second is the development of a web-based registry of aPL-positive patients with or without systemic autoimmune diseases, which will serve as a resource for future basic science and clinical research initiatives. This network represents perhaps the most significant advance in the global research effort to investigate this disease in an effort to find improved therapeutic options for APS patients.

Future perspective

The use of immunomodulatory agents and targeted therapies has revolutionized the management of many autoimmune diseases, including rheumatoid arthritis and SLE. Despite the comparatively slow pace of implementing such therapeutic agents in the management of APS patients, the last few years have produced several excellent clinical studies highlighting the efficacy of these drugs as well as international collaborative initiatives dedicated to furthering APS research. This will ideally translate the very promising preclinical data we have into meaningful therapeutic guidelines for managing APS patients. The next few years, in addition to increasing knowledge of the pathophysiology of APS, will probably bring results of highly anticipated clinical trials in APS. These are namely the RAPS trial evaluating the anticoagulant effect of rivaroxaban in treating APS patients and the multicenter HCQ

trial being performed by APS ACTION. The results of these trials in particular could potentially have a huge impact on how APS patients are treated, since they have relatively favorable side-effect profiles compared with current therapeutic agents such as warfarin. The next few years will also hopefully bring data on the effect of some of the newer targeted therapies in actual APS patients, moving beyond *in vitro* studies and *in vivo* animal models. Many researchers have led the drive to improve understanding of the pathophysiology of APS in an effort to identify APS patients at risk for clinical sequelae, allowing for the appropriate use of effective treatment modalities for the disease and improved outcomes for APS patients. The long-term goal should be that patients with APS never have to suffer any thrombotic or obstetric complications and that treatment regimens should similarly not hinder quality of life. Clinicians and researchers should strive for nothing less.

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Executive summary

Thrombosis in antiphospholipid syndrome

- Antiphospholipid antibodies (aPL) activate endothelial cells, platelets and monocytes through several intracellular mechanisms including p38 MAPK, NF-κB and ERK 1/2 signaling pathways.
- Cellular activation results in the release of proinflammatory cytokines including tissue factor, VEGF and several cell adhesion molecules.
- $\beta_2$glycoprotein I (β_2GPI) is the major antigenic target for aPL and there are numerous cell surface receptors that bind β_2GPI allowing for interaction of aPL with cells; including annexin A2, ApoER2′, TLR4 and the GPIb-IX-V receptor.
- Humoral regulators of thrombosis and fibrinolysis such as antithrombin, activated protein C, tissue plasminogen activator and plasmin can also be affected by aPL, resulting in an increased propensity for thrombosis.

Pregnancy morbidity in antiphospholipid syndrome

- Induction of thrombosis, inflammation and abnormal trophoblast and decidual cell function, proliferation and differentiation are key factors in aPL-mediated obstetric complications.
- β_2GPI and its receptors are abundant in placental tissues, allowing direct reactivity of aPL with trophoblast and decidual cells.

Novel therapeutic approaches for antiphospholipid syndrome management

- Current treatment for antiphospholipid syndrome (APS) is focused on anticoagulation with warfarin, heparin and/or low-dose aspirin, which may be ineffective and result in serious complications.
- Immunomodulatory agents such as hydroxychloroquine and complement inhibitors have proven benefit in reducing aPL-mediated thrombosis in *in vitro* and *in vivo* animal models, while there have been promising human pilot studies showing the benefit of statins and rituximab in treating APS patients.
- Disruption of the interaction of β_2GPI with its target receptors and anti-β_2GPI antibodies using peptides displaying structural similarity with domains V and I of β_2GPI, respectively, have limited thrombosis and pregnancy losses in murine models.
- Clinical trials evaluating the effect of novel anticoagulants such as rivaroxaban and the immunomodulatory agent hydroxychloroquine are currently ongoing and are likely to greatly impact APS management.

Future research in evaluating novel therapeutic agents for antiphospholipid syndrome

- It has been difficult to achieve adequate power in APS clinical trials owing to the relatively rare occurrence of thrombotic and obstetric clinical events.
- The development of global collaborative research initiatives, most recently AntiPhospholipid Syndrome Alliance For Clinical Trials and InternatiOnal Networking, has allowed for the mounting of well-designed, large-scale multicenter clinical trials.

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