

Publication of the Month

September 09/11: Pathogenesis of the Antiphospholipid Syndrome

Key messages:

- Antiphospholipid antibodies (aPL) are both diagnostic markers and pathogenic drivers for the antiphospholipid syndrome.
- β 2 glycoprotein I-dependent autoantibodies seem to be the main pathogenic subpopulation of aPL.

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Pathogenesis of antiphospholipid syndrome: understanding the antibodies

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Background:

Antiphospholipid syndrome (APS) is characterized by vascular thrombosis and/or pregnancy morbidity, in association with antiphospholipid antibodies (aPL). Detectable by anticardiolipin, anti- β 2 glycoprotein I and lupus anticoagulant assays, aPL are not only diagnostic of APS but are also believed to have a pathogenic role, mediating several clinical manifestations of the syndrome.

Key points:

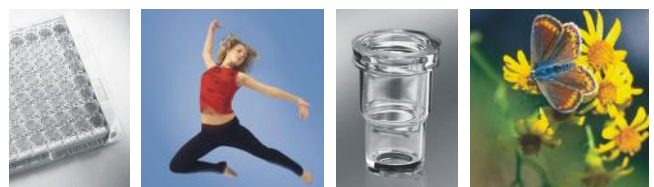
- Antiphospholipid antibodies (aPL) are autoantibodies that are diagnostic and pathogenic for APS.
- aPL mediate several procoagulant mechanisms that can explain their thrombogenic effect in animal models, and their epidemiological association with APS in clinical studies.
- Whereas evidence shows that a second hit (usually an inflammatory event) is required for thrombus formation in APS, this requirement is less clear for fetal loss.
- In addition to placental thrombosis, other mechanisms for direct effects of aPL on placental tissues have been proposed.
- β 2 glycoprotein I (β 2GPI)-dependent autoantibodies seem to be the main pathogenic subpopulation of aPL.
- More information about the epitope specificity of anti- β 2GPI aPL, as well as about the tissue expression of the target molecule, might help to better understand the pathogenesis of APS.

Conclusions:

Although APS is considered as a single disease, there seem to be slightly different mechanisms for the two clinical manifestations of APS, thrombosis and pregnancy morbidity. Thrombosis does not seem to have sole responsibility for the obstetrical complications. The three aPL subtypes (anticardiolipin, anti- β 2GPI and lupus anticoagulant) detect slightly different populations. Whether different subpopulations of autoantibodies, detected by the same diagnostic assays, are responsible for the different clinical manifestations remains an open question.

Comment:

Pier Luigi Meroni et al. published this review on the pathogenesis of the antiphospholipid syndrome (APS) in Nature Reviews / Rheumatology in June this year. This article is the most comprehensive and summarizing review on this matter in the last years and is worth to read for everybody interested in autoimmunity and in particular in APS. It is available with free access online (<http://www.nature.com/nrrheum/journal/v7/n6/full/nrrheum.2011.52.html>).



Pathogenesis of antiphospholipid syndrome: understanding the antibodies

Pier Luigi Meroni, M. Orietta Borghi, Elena Raschi and Francesco Tedesco

Abstract | Antiphospholipid antibodies (aPL) are both diagnostic markers for, and pathogenic drivers of, antiphospholipid syndrome (APS). Although the presence of aPL is a necessary pre-condition, APS-associated clotting is seemingly triggered by an additional 'second hit', frequently related to innate inflammatory immune responses. β_2 glycoprotein I (β_2 GPI)-dependent aPL, the most important subset of these antibodies, mediate several—not necessarily alternative—thrombogenic mechanisms, mainly on the basis of their reactivity with β_2 GPI expressed on the membrane of cells that participate in the coagulation cascade. Recurrent pregnancy complications associated with aPL cannot be explained solely by thrombosis, and alternative pathogenic mechanisms have been reported. Although one *in vivo* model of fetal loss suggests a mechanism of aPL-mediated acute placental inflammation, other models and the histopathological examination of APS placentae do not support a widespread inflammatory signature. β_2 GPI-dependent aPL are thought to recognize their antigen on placental tissues, inhibit the growth and differentiation of trophoblasts, and eventually cause defective placentation. Why antibodies with similar antigen specificity produce different clinical manifestations is not clear. Characterization of the molecular basis of the pathogenic mechanisms involved, including the putative second hits and the role of complement activation, might offer an answer to this question.

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Introduction

Antiphospholipid syndrome (APS) is characterized by vascular thrombosis and/or pregnancy morbidity (Box 1),¹ in association with antiphospholipid antibodies (aPL). Detectable by anticardiolipin (aCL), anti- β_2 glycoprotein I (β_2 GPI) and lupus anticoagulant (LA) assays,¹ aPL are not only diagnostic of APS but are also believed to have a pathogenic role, mediating several clinical manifestations of the syndrome (Boxes 2 and 3).

Although APS is currently considered as a single disease, the clinical and biological characteristics of the vascular involvement (Figure 1) are quite different from those associated with the obstetrical problems. For example, thrombosis—which appears to be the most common denominator of the vascular events—does not seem to have sole responsibility for the obstetrical complications, for which involvement of additional pathogenic mechanisms has been reported (Figure 2).^{2–4} Thrombosis itself also seems to be the eventual result of several different pathogenic pathways, involving clot formation, inhibition of natural anticoagulant mechanisms and impairment of fibrinolysis.⁵ (Box 2, Figure 1).

Several aPL subtypes are known, and the three formal diagnostic assays (aCL, anti- β_2 GPI and LA) detect slightly different populations. In spite of the reported presence of small subpopulations of aPL with different antigenic specificities, it is generally agreed that reactivity with the LA assay is mainly mediated by antibodies directed

against prothrombin and β_2 GPI, whereas aCL positivity is primarily caused by β_2 GPI-dependent aPL.^{2–4}

The clinical spectrum of APS is becoming more complex as additional symptoms are attributed to the syndrome. Some manifestations, such as APS nephropathy and central nervous system (CNS) symptoms (epilepsy and cognitive abnormalities), cannot be fully explained by the occurrence of ischemic events. For these symptoms, direct effects of aPL on glomerular microcirculation or on neuronal cells have been suggested.^{6,7} Whether different subpopulations of autoantibodies, detected by the same diagnostic assays, are responsible for the different clinical manifestations (and the different pathogenic events that mediate them) remains an open question. Additional local factors that depend on characteristics of APS target tissues (that is, different expression of autoantigens) might affect clinical aspects of the syndrome.

As mentioned above, there is now a general consensus that different mechanisms are responsible for the vascular and the obstetrical manifestations of APS (Boxes 2 and 3). In this Review, we focus on the roles of the main aPL subpopulations in these two areas of pathology, and discuss the aspects of APS pathogenesis that are still a matter of debate.

Pathogenic mechanisms mediated by aPL Thrombosis

Evidence for aPL involvement

The association between aPL and both venous and arterial thrombosis is widely accepted, and clot formation is the

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Competing interests

The authors declare no competing interests.

key event underpinning the vascular manifestations.^{1–5} Most of the pathogenic mechanisms that are potentially responsible for thrombus formation have been demonstrated using *in vitro* models only. Nevertheless, *in vivo* models of thrombosis—induced in mice and hamsters by mechanical, chemical or photochemical trauma—have confirmed that aPL are able to increase thrombus formation in the venous and arterial trees.^{8–10} Furthermore, passive infusion of human aPL IgG together with a small amount of lipopolysaccharide (LPS) was shown to trigger clotting in the rat mesenteric microcirculation.¹¹ After infusion of aPL with or without dimeric β_2 GPI, altered expression of endothelial adhesion molecules, and upregulation of nitric oxide and tissue factor (TF) expression have been reported in arterial endothelia, supporting a key role for aPL in causing vascular abnormalities.^{10,12–14} Platelets were found to be involved in arterial photochemical-induced thrombus formation,⁹ and upregulated TF expression was reported on monocytes of mice passively injected with aPL.^{13,14} However, more detailed studies are needed to further define the roles of the different steps of the coagulation cascade, and the cell types involved, in contributing to clot formation.

Pathogenicity of aPL subtypes

In considering which subpopulations of aPL drive thrombotic mechanisms, it should be pointed out that aPL reacting with human β_2 GPI, and crossreacting with the mouse, rat and hamster molecules, were shown to be pathogenic in all the corresponding *in vivo* models. Specifically, the *in vivo* thrombotic effects were produced using affinity-purified anti- β_2 GPI IgG, and were inhibited by specific absorption of the anti- β_2 GPI activity.^{11,15} In view of these experimental findings, β_2 GPI-dependent aPL should be considered the antibody subpopulation responsible for the thrombotic manifestations of APS. In agreement with this assessment, β_2 GPI-dependent LA is reported to correlate better with thrombosis than LA in general.¹⁶

Besides β_2 GPI, however, aPL are also known to react with other phospholipid-binding proteins, in particular with prothrombin.^{1–5} The manner by which anti-prothrombin antibodies exert their procoagulant effect is still a matter of research, but *in vitro* studies have suggested that they might perturb endothelial cell function by reacting with the target molecule expressed on the cell surface.¹⁷ *In vivo* studies using purified antiprothrombin polyclonal antibodies are lacking, however, and the pathogenic activity of these autoantibodies is mainly supported by experiments performed with monoclonal preparations, and by their epidemiological association with thrombosis.^{18–20} The most convincing link between antiprothrombin antibodies and thrombosis was reported for antibodies detected by the phosphatidylserine–prothrombin assay, suggesting that the pathogenic antibodies might recognize a conformational epitope or epitopes expressed by prothrombin when in complex with anionic phospholipids in the presence of calcium ions.^{19,20}

In contrast with what is known for β_2 GPI, information on the crossreactivity of antiprothrombin antibodies with prothrombin from different species is lacking, making it

Key points

- Antiphospholipid antibodies (aPL) are autoantibodies that are diagnostic of, and pathogenic in, antiphospholipid syndrome (APS)
- aPL mediate several procoagulant mechanisms that can explain their thrombogenic effect in animal models, and their epidemiological association with APS in clinical studies
- Whereas evidence shows that a second hit (usually an inflammatory event) is required for thrombus formation in APS, this requirement is less clear for fetal loss
- In addition to placental thrombosis, other mechanisms for direct effects of aPL on placental tissues have been proposed
- β_2 glycoprotein I (β_2 GPI)-dependent autoantibodies seem to be the main pathogenic subpopulation of aPL
- More information about the epitope specificity of anti- β_2 GPI aPL, as well as about the tissue expression of the target molecule, might help to better understand the pathogenesis of APS

Box 1 | Classification criteria for antiphospholipid syndrome*

Clinical criteria[†]

Vascular thrombosis

- ≥ 1 clinical episodes of arterial, venous, or small vessel thrombosis, in any tissue or organ
- Thrombosis must be confirmed by appropriate imaging studies or histopathology
- Thrombosis should be present without significant evidence of inflammation in the vessel wall

Pregnancy morbidity

- ≥ 1 unexplained deaths of a morphologically normal fetus at or beyond the 10th week of gestation, with normal fetal morphology documented by ultrasound or by direct examination of the fetus
- ≥ 1 premature births of a morphologically normal neonate before the 34th week of gestation due to eclampsia and severe preeclampsia, or to recognized features of placental insufficiency
- ≥ 3 unexplained consecutive spontaneous abortions before the 10th week of gestation, with maternal anatomic or hormonal abnormalities, and paternal and maternal chromosomal causes excluded

Laboratory criteria[†]

Lupus anticoagulant

- Present in plasma, on ≥ 2 occasions at least 12 weeks apart, detected according to the guidelines of the International Society on Thrombosis and Hemostasis (Scientific Subcommittee on LAs/phospholipid-dependent antibodies)

Anticardiolipin antibody of IgG and/or IgM isotype

- Present in serum or plasma at medium or high titer (>40 GPL or MPL, or $>99^{\text{th}}$ percentile), on >2 occasions, at least 12 weeks apart, measured by a standardized enzyme-linked immunosorbent assay (ELISA)

Anti- β_2 GPI antibody of IgG and/or IgM isotype

- Present in serum or plasma (titer $>99^{\text{th}}$ percentile), ≥ 2 occasions, at least 12 weeks apart, measured by a standardized ELISA, according to recommended procedures

*Adapted from Miyakis, S. et al. (2006).¹ [†]The syndrome is present if ≥ 1 clinical criteria plus 1 laboratory criteria) are met. Abbreviations: β_2 GPI, β_2 glycoprotein I; ELISA, enzyme-linked immunosorbent assay; GPL, IgG phospholipid units; GPM, IgM phospholipid units.

difficult to evaluate the *in vivo* pathogenicity of this type of aPL. Nevertheless, the suggestion that anti- β_2 GPI antibodies are the only pathogenic aPL for thrombosis is in seeming contrast with the widely-accepted view that LA positivity is strongly associated with thrombotic risk;²¹

Box 2 | Thrombotic mechanisms mediated by aPL**Disruption of fluid phase coagulation**

- Interference with natural anticoagulants
- Interference with activation of anticoagulation protein C (also known as vitamin K-dependent protein C)*
- Interference with annexin A5*
- Inhibition of fibrinolysis

Disruption of coagulation cascade cell functions

- Endothelial cell perturbation*
- Induction of tissue factor expression on circulating monocytes*
- Platelet activation*

Complement activation*

*Involves anti- β_2 GPI autoantibodies. Abbreviations: aPL, antiphospholipid antibodies; β_2 GPI, β_2 glycoprotein I.

Box 3 | aPL-mediated mechanisms of fetal loss**Mechanisms thought to involve anti- β_2 GPI autoantibodies**

- Placental tissue thrombosis
- Acute inflammation
- Inhibition of syncytium-trophoblast differentiation
- Induction of decidual cell inflammatory phenotype
- Complement activation

Other mechanisms

- Embryo and/or placental apoptosis

Abbreviations: aPL, antiphospholipid antibodies; β_2 GPI, β_2 glycoprotein I.

the finding that LA activity is more frequently linked to antiprothrombin than to anti- β_2 GPI antibodies thus seems to support pathogenic involvement of antiprothrombin antibodies in thrombosis in patients with APS.^{21,22}

Consequences of aPL binding

The procoagulant mechanisms mediated by aPL are related to their ability to react with phospholipid-binding proteins expressed on the cell membranes of different cell types. The antibody is thought to form a complex with the corresponding antigen, leading to cell membrane perturbation and eventually to signaling to the nucleus (Figure 3).²³ Depending on their biological functions, perturbed cells will mediate different responses, which might contribute to the variety of APS clinical manifestations.

It is not yet clear whether aPL react substantially with phospholipid-binding proteins (in particular β_2 GPI and prothrombin) in the fluid phase. In this regard, all aPL are characterized by low avidity, suggesting that complex formation in the fluid phase requires stoichiometric antigen-antibody ratios that are not commonly present in patients.²⁴ This point further supports the hypothesis that the main pathogenic mechanisms mediated by aPL are those related to their reactivity with the target molecules expressed on cell membranes. In particular, β_2 GPI is expressed on cell membranes at high antigenic density, and

so is more easily recognized by low avidity autoantibodies than more sparsely expressed molecules would be.

The question of whether aPL are able to affect the biological function of target proteins in the fluid phase is another matter for investigation. For example, autoantibodies directed against enzymes may increase the enzymatic activity of the target molecules.²⁵ There is some evidence that antiprothrombin antibodies might induce a 'gain of function' of prothrombin leading, for example, to increased fibrin production.²⁶ Assessing whether anti- β_2 GPI can alter the function of their target protein is, however, more difficult, as we still do not know what the true physiological role of β_2 GPI is in the coagulation cascade. A mild natural anticoagulant activity and a powerful effect in protecting against cell death induced by endothelial oxidative stress have been described for β_2 GPI,^{2,27} but whether anti- β_2 GPI antibodies increase these putative physiological activities remains to be investigated.

Fetal loss

The presence of aPL represents the most frequent acquired risk factor for a treatable cause of recurrent pregnancy loss, and for pregnancy complications (early and severe pre-eclampsia).¹ Such an association is supported by several epidemiological studies and by experimental models showing that passive transfer of aPL IgG induces fetal loss and growth retardation in pregnant naive mice.²⁸

Placental thrombosis

In line with the thrombophilic effect of aPL, intraplacental thrombosis, with impairment of maternal-fetal blood exchange, was initially suggested to be the main pathogenic mechanism of fetal loss. Placental thrombosis and infarction were reported, and *in vitro* studies showed that aPL might induce a procoagulant state at the placental level through several mechanisms,^{29,30} including the ability of the aPL antibodies (specifically, anti- β_2 GPI antibodies) to disrupt the anticoagulant annexin A5 shield on trophoblast and endothelial cell monolayers.³¹ Supporting the *in vitro* findings, the distribution of annexin A5 covering intervillous surfaces was found to be considerably more sparse in the placentas of aPL-positive women, in comparison with those lacking the autoantibodies.³² Nevertheless, these observations were not confirmed by other studies, which have failed to show intravascular or intervillous blood clots. Indeed, histopathological findings suggestive of thrombosis cannot be detected in most samples from miscarried fetuses and placentas from women with APS.³³

Inflammatory responses

Fine-tuning of the maternal immune response takes place during embryo implantation, to allow normal progression of pregnancy. There is evidence for a dynamic balance between proinflammatory and anti-inflammatory mediators in normal pregnancy, with fluctuations between which signals predominate occurring during gestation.³⁴ Acute inflammatory events are widely accepted to be generally responsible for a negative pregnancy outcome, and

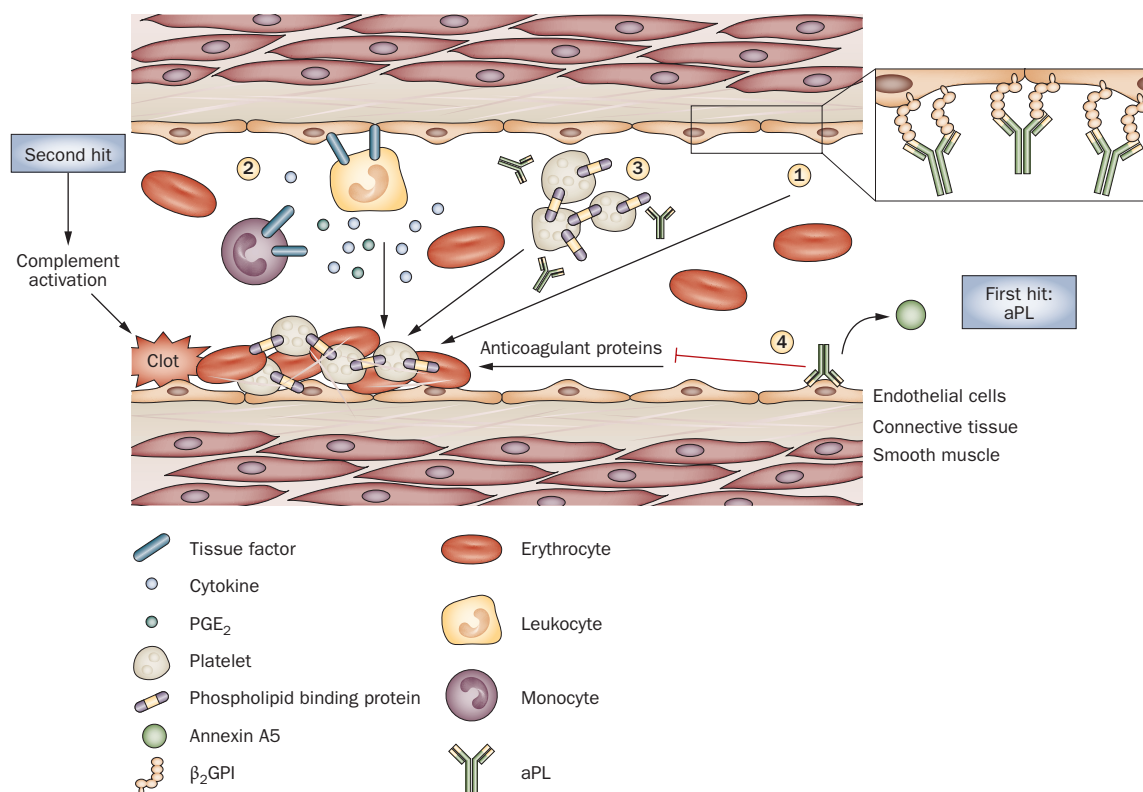


Figure 1 | Pathogenic clotting mechanisms mediated by aPL. aPL actions favor clot formation through several routes. (1) aPL interact with endothelial cells, primarily through binding of β_2 GPI on the cell surface, and induce a procoagulant and proinflammatory endothelial phenotype. (2) aPL upregulate tissue factor expression on endothelial cells and blood monocytes, and promote endothelial leukocyte adhesion, cytokine secretion and PGE₂ synthesis. (3) aPL recognize phospholipid-binding proteins expressed on platelets—aPL binding potentiates platelet aggregation induced by another agonist. (4) aPL interfere with plasma components of the coagulation cascade, by inhibiting anticoagulant activity, by affecting fibrinolysis, and by displacing the binding of the natural anticoagulant annexin A5 to anionic structures. These mechanisms all contribute to a procoagulant state that is necessary but not sufficient for clotting. Clot formation seems to require two steps: the presence of aPL provides the ‘first hit’, which produces clotting when accompanied by another procoagulant condition, a ‘second hit’. Complement activation seems to be necessary for clot formation *in vivo*. Abbreviations: aPL, anti-phospholipid autoantibodies; β_2 GPI, β_2 glycoprotein I; PGE₂, prostaglandin E₂.

proinflammatory mediators (such as complement, tumor necrosis factor [TNF], and CC chemokines) have been shown to have a role in animal models of aPL-induced fetal loss.²⁸

Repeated intraperitoneal injections of large amounts of human IgG with aPL activity (10 mg/mouse per injection) to pregnant naive mice after embryo implantation induces considerable placental inflammatory damage that results in fetal resorption and growth retardation at day 15 of pregnancy. Immunohistochemical and histological examination of decidua showed deposition of human IgG and mouse complement, neutrophil infiltration and local TNF secretion, in association with a transient but significant increase in blood TNF levels.^{35–37} Several lines of evidence support involvement of the complement system in inducing aPL-mediated fetal loss in this mouse model, as suggested by the protection that deficiency in complement components confers on the animals, or that follows from *in vivo* inhibition of complement.^{38,39} The cleavage product C5a of the complement component C5 is the key effector in this model, and acts through the upregulated expression of TF on neutrophils infiltrating placental tissues.^{40–42} The hypothesis that complement is involved

in the fetal loss induced by aPL is further supported by the demonstration that the protective effect of heparin in the mouse model is linked to the anticomplement, rather than to the anticoagulant, activity.⁴³ However, the amount of IgG injected during these studies was much larger than the levels of antibodies that spontaneously occur in aPL patients, and the β_2 GPI specificity of the IgG preparation was not investigated; these points need to be further addressed.

In another experimental model of fetal loss, mice deficient in chemokine-binding protein D6 (also known as chemokine-binding protein 2), a placental receptor that recognizes the majority of inflammatory CC chemokines and targets them for degradation, are more susceptible to fetal loss when passively infused with a small amount of human aPL IgG than wild-type mice or mice infused with normal IgG.⁴⁴ Altogether, these findings suggest that a local acute inflammatory response might have a role in experimental aPL-mediated fetal loss.

From a clinical point of view, the contribution of acute inflammation to aPL-associated recurrent fetal loss would support a beneficial effect of corticosteroids in treating or preventing such complications. Although

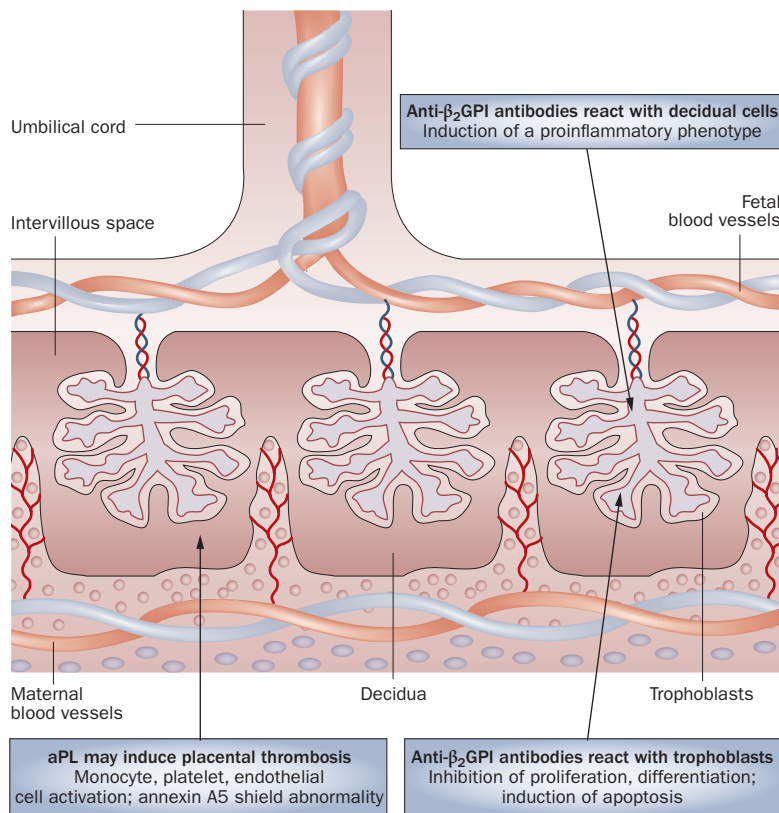


Figure 2 | Main effects of aPL on placenta. Several pathogenic mechanisms mediate aPL-associated fetal loss. Placental thrombosis might be induced by aPL binding to monocytes, endothelial cells, platelets and plasma components of the coagulation cascade. Non-thrombotic mechanisms are thought to involve direct effects of anti- β_2 GPI autoantibodies on the placenta: binding of aPL to β_2 GPI expressed on trophoblast membranes might trigger membrane perturbation, resulting in modulation of several cell biological functions, decrease of trophoblast proliferation and growth, and eventually defective placentation; preliminary evidence suggests that aPL might also affect the maternal side by reacting with endometrial cells in the decidua, inducing a pro-inflammatory phenotype that might interfere with physiological implantation. Abbreviations: aPL, anti-phospholipid autoantibodies; β_2 GPI, β_2 glycoprotein I.

low corticosteroid doses (<20 mg/day) are occasionally used, particularly in women unresponsive to the standard therapy (low-dose aspirin and heparin), there is no sound evidence to support the routine use of corticosteroids.^{45,46} Furthermore, immunohistological analysis of abortive material or full-term placentae from women with APS has not provided conclusive information about the pathogenic contributions of acute local inflammatory events and complement deposition.^{33,47–49} Finally, an inflammatory process does not seem to participate in another model of fetal resorption and growth retardation, elicited by intravenous injection of a small amount of human aPL IgG (10–50 μ g/mouse) into mice before implantation, as indicated by a lack of histological evidence of inflammation in the placentae.⁵⁰

Defective placentation

Besides thrombosis, evidence indicates that alternative aPL-mediated pathogenic mechanisms impede placentation, involving direct targeting of the maternal decidua and the invading trophoblast (Figure 2). On the fetal

side, aPL (in particular β_2 GPI-dependent antibodies) bind to human trophoblasts and affect several cell functions *in vitro* (Figure 4), inducing cell injury and apoptosis, inhibition of proliferation and syncytia formation, decreased production of human chorionic gonadotrophin, defective secretion of growth factors and impaired invasiveness. All of these aPL-mediated effects might participate in causing defective placentation.^{3,28}

Data have shown that aPL also cause abnormalities at the maternal side of the placenta. In fact, impaired endometrial differentiation and reduced expression of complement decay-accelerating factor (also known as CD55) were found on endometrial biopsies. These alterations before conception might compromise implantation and predispose to complement-mediated pregnancy failure.⁵¹ In addition, β_2 GPI-dependent aPL are able to react with human stromal decidual cells *in vitro*, inducing a proinflammatory phenotype.⁵²

As a whole, these findings suggest that APS-associated pregnancy complications can be mediated by several distinct pathogenic events that are not necessarily related to the procoagulant or proinflammatory effects of aPL (Box 3). On the other hand, data from *in vivo* animal models are biased by the fact that findings are restricted to the period of the pregnancy when the investigation was performed, or are dependent on the timing of passive infusion of the putative pathogenic autoantibodies. For example, examination of animals immediately after the administration of large amounts of autoantibodies mid-gestation might show an inflammatory signature that can go undetected if smaller amounts of the same autoantibodies are infused soon after mating. For the same reason, histological examination of full-term human placentae might not show a clear picture of events that take place at the beginning of the pregnancy, showing only the resulting damage.

The expression of β_2 GPI on trophoblast cell membranes explains the placental tropism of anti- β_2 GPI antibodies. Being a cationic plasma protein, β_2 GPI has been suggested to bind to exposed phosphatidylserine on the external cell membranes of trophoblasts undergoing syncytium formation (Figure 4), but additional receptors might also be involved.^{3,28} β_2 GPI was shown to bind human trophoblasts through the phospholipid-binding site in the fifth domain of the molecule.⁵³ The synthetic peptide TIFI spans Thr101–Thr120 of ULB0–HCMVA from human cytomegalovirus, and shares similarity with the β_2 GPI phospholipid-binding site. The peptide prevents aPL-mediated thrombosis *in vivo*, and inhibits the *in vitro* binding of β_2 GPI conjugated with fluorescein isothiocyanate to human endothelial cells and mouse monocytes.⁵⁴ As aPL do not react with TIFI, the inhibitory effect of the peptide was thought to result from its ability to compete with the phospholipid-binding site of β_2 GPI, displacing the molecule from the cell surfaces and thus inhibiting binding of aPL to the target tissues.⁵⁴ We reported preliminary data showing that TIFI—but not an irrelevant peptide—inhibits the reactivity of anti- β_2 GPI monoclonal antibodies with human trophoblast monolayers, suggesting an effect comparable to that reported

in experiments with mouse cells.²⁸ Repeated infusions of TIFI also protect pregnant naive mice from fetal loss induced by human aPL IgG.⁵⁰ We speculate that the protective effect of TIFI infusion in pregnant mice treated with aPL IgG provides further indirect evidence for the effect of β_2 GPI-anti- β_2 GPI complexes on trophoblasts in this experimental model of aPL-induced fetal loss.²⁸

β_2 GPI-dependent aPL seem, therefore, to represent the main pathogenic autoantibodies in obstetrical APS. Accordingly, it has been hypothesized that most of these potentially pathogenic autoantibodies should be absorbed at the placental level (where β_2 GPI is expressed) and should not be transferred to the fetus. This mechanism would explain why thrombotic events are rarely reported in babies born to aPL-positive mothers, in spite of the high thrombophilia profile of neonates.⁵⁵

Open questions

Several issues with regard to the mechanisms that precipitate the various clinical manifestations of APS are still subject to debate, and are under active investigation. Here, we briefly discuss three of these issues, and their potential diagnostic and therapeutic implications.

Receptors and signaling pathways

The main candidate receptors for β_2 GPI on cell membranes are listed in Table 1 and depicted with their intracellular signaling pathways in Figure 3.⁵⁶⁻⁷⁵ Their involvement in β_2 GPI binding is mainly supported by *in vitro* findings, but annexin A2, Toll-like receptor (TLR)-4 and apolipoprotein E receptor 2 (also known as low-density lipoprotein receptor-related protein 8) have also been investigated *in vivo*. Animals deficient in any one of these molecules are protected from the thrombogenic effect of aPL only in part, suggesting that none of the receptors is essential and that all are semi-redundant.^{14,15,76,77}

Annexin A2 was clearly shown to behave as a receptor for β_2 GPI, but it fails to signal by itself because it lacks a cytoplasmic tail. After the existence of a signaling co-receptor was suggested, TLR4 was identified as a potential candidate.^{77,78} Several groups showed that TLR2 and TLR4 are involved in aPL-mediated cell activation, and two reports have been published on the direct binding of β_2 GPI to TLR2 and TLR4.^{57,77}

There is agreement that nuclear factor κ B (NF κ B) and p38 mitogen-activated protein kinase (MAPK) are involved downstream from β_2 GPI in signaling routes that are shared by the different cell types activated by aPL.³ However, whether separate pathways mediate different clinical manifestations, or are differentially triggered by different aPL subpopulations, are still open questions. Recently, IgG from APS patients with thrombosis, but not with pregnancy morbidity, were found to cause phosphorylation of NF κ B and p38 MAPK, and upregulation of TF activity through activation of TLR4.⁶¹ How and why β_2 GPI-dependent aPL might react with the same molecule on the cell membrane and yet trigger different signaling responses is not clear.

Analysis of the fine specificity of β_2 GPI-dependent aPL is still a matter of research. A preliminary study showed

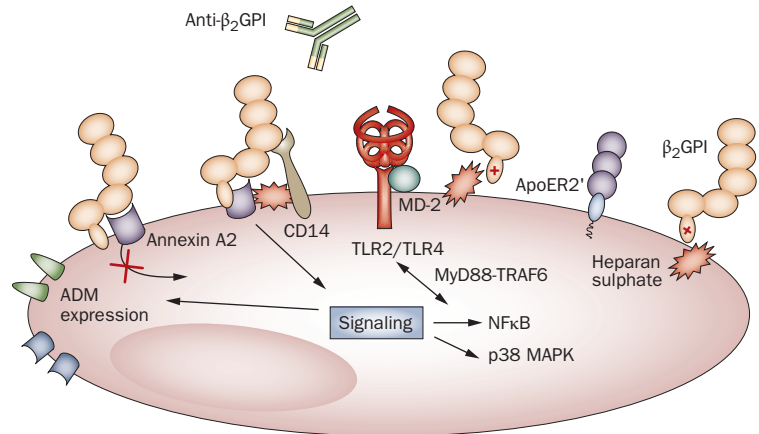


Figure 3 | Endothelial cell activation by anti- β_2 GPI autoantibodies. aPL react with β_2 GPI expressed on the endothelial cell membrane and induce cell signaling. β_2 GPI adheres to endothelial cell membranes: (i) through the electrostatic interaction between the cationic phospholipid-binding site (located in the fifth domain of the molecule) and anionic structures, such as heparan sulfate, on the cell membrane; or (ii) as a ligand for annexin A2. Binding of anti- β_2 GPI antibody induces clustering of β_2 GPI with its potential receptors, and induces cell signaling, resulting in activation of NF κ B or p38 MAPK or both. The interaction of the β_2 GPI clusters with TLR2/TLR4 might be responsible for MyD88 and TRAF6-dependent signaling. Abbreviations: aPL, anti-phospholipid autoantibodies; ApoER2', apolipoprotein E receptor 2'; β_2 GPI, β_2 glycoprotein I; MyD88, myeloid differentiation primary response protein MyD88; NF κ B, nuclear factor κ B; TLR, Toll-like receptor; TRAF6, TNF receptor-associated factor 6.

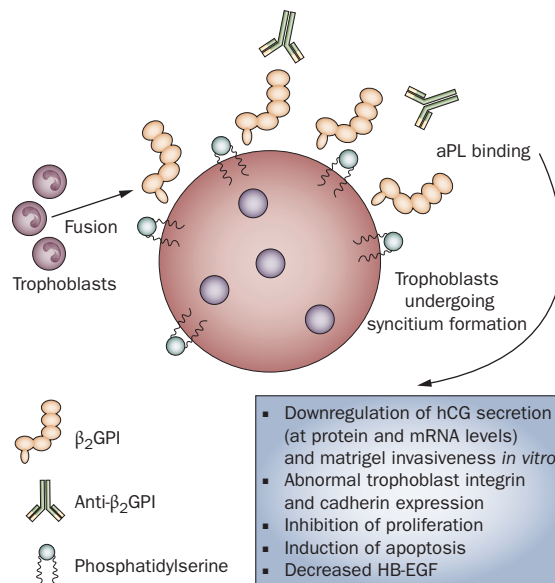


Figure 4 | aPL effects on trophoblasts. aPL might affect trophoblast cells directly. Mononuclear trophoblasts fuse during syncytium formation, and phosphatidylserine molecules flip to the outer layer of the cell membrane. β_2 GPI can be expressed on the cell membrane of syncytium-trophoblast, perhaps due to binding to phosphatidylserine, and be recognized by circulating anti- β_2 GPI antibodies. Once bound, the antibodies induce cell membrane perturbation resulting in modulation of several biological functions. Abbreviations: aPL, anti-phospholipid autoantibodies; β_2 GPI, β_2 glycoprotein I; hCG, human chorionic gonadotrophin; HB-EGF, heparin-binding EGF-like growth factor. Adapted from Meroni, P. *et al. Clin. Rev. Allergy Immunol.* **34**, 332-337 (2008), with kind permission from Springer Science+Business Media B. V.

Table 1 | Expression, and evidence for β_2 GPI binding, of candidate β_2 GPI receptors on the cell types involved in APS

Cell type	Candidate receptors for β_2 GPI				
	Heparan sulfate	TLR2 or TLR4	ApoER2	Annexin II	GPIIb α
Endothelial cells	Protein expressed β_2 GPI binding indirectly demonstrated ⁵⁶	Protein expressed β_2 GPI binding directly ⁵⁷ and indirectly ⁵⁸ demonstrated	Protein expressed β_2 GPI binding indirectly demonstrated ¹⁰	Protein expressed β_2 GPI binding demonstrated ⁵⁹	No protein expression
Monocytes	Expressed, but evidence for binding lacking ⁶⁰	Protein expressed β_2 GPI binding indirectly demonstrated ⁶¹	Expressed, but evidence for binding lacking ⁶²	Protein expressed β_2 GPI binding demonstrated ⁶³	No protein expression
Platelets	Expressed, but evidence for binding lacking ⁶⁴	Expressed, but evidence for binding lacking ⁶⁵	Protein expressed β_2 GPI binding demonstrated ⁶⁶	No protein expression	Protein expressed β_2 GPI binding demonstrated ^{2,66}
Trophoblasts	Expressed, but evidence for binding lacking ⁶⁷	Protein expressed β_2 GPI binding indirectly demonstrated ^{68,69}	No protein expression	Expressed, but evidence for binding lacking ⁷⁰	No protein expression
Decidual cells	Expressed, but evidence for binding lacking ⁶⁷	Protein expressed β_2 GPI binding indirectly demonstrated ⁵²	No protein expression	No protein expression	No protein expression
Neurons	Expressed, but evidence for binding lacking ⁷¹	Expressed, but evidence for binding lacking ⁷²	Expressed, but evidence for binding lacking ⁷³	No protein expression	No protein expression
Fibroblasts	Expressed, but evidence for binding lacking ⁷⁴	Expressed, β_2 GPI binding indirectly demonstrated ⁷⁵	No protein expression	No protein expression	No protein expression

Abbreviations: ApoER2, apolipoprotein E receptor 2; APS, antiphospholipid syndrome; β_2 GPI, β_2 glycoprotein I; GPIIb α , platelet glycoprotein Ib α chain; TLR, Toll-like receptor.

heterogeneous activity of anti- β_2 GPI autoantibodies in patients with APS, which was directed, often concurrently, against various (mostly linear) epitopes of the molecule.⁷⁹ More recently, IgG antibodies that recognize the epitope Gly40–Arg43 in domain I of β_2 GPI were found to be closely associated with LA activity and their presence strongly correlated with thrombosis and obstetrical complications.⁸⁰ Furthermore, the specific target of these antibodies was identified as a conformational epitope localized to residues Arg39–Gly43 and involving other residues within domain I, such as Asp8 and Asp98.^{80,81} The ability of domain I to protect mice from the thrombogenic effect of aPL was considered a proof-of-concept for the key pathogenic role of anti-domain I antibodies.⁸² However the amount of the peptide injected into the animals was below the β_2 GPI concentration in their circulation, raising some concern about the final message of the study.

Hence, both the fine epitope specificity of the anti- β_2 GPI autoantibodies and the different signaling pathways that can be induced may explain why seemingly similar aPL can be differentially responsible for vascular or obstetrical manifestations. Larger epidemiological studies are, however, necessary to confirm these findings before the anti-domain I antibody assay could be considered a more predictive diagnostic test than those currently used.

The second hit hypothesis

A ‘two hit hypothesis’ has been suggested to explain the clinical observation that thrombotic events occur only occasionally, in spite of the persistent presence of aPL. According to this principle, the antibody (representing

the first hit) induces a thrombophilic state, but clotting takes place only in the presence of another thrombophilic condition (the second hit).⁶ Providing support for this mechanism, the administration of a small amount of LPS was required for human β_2 GPI-specific aPL IgG to produce a thrombogenic effect in rat mesenteric microcirculation.¹¹ In line with this observation, it has been suggested that infectious processes might constitute the second hit, as they frequently precede full-blown APS and might be the initiator of the catastrophic subtype.⁸³ This hypothesis fits well with the potential involvement of pattern recognition receptors (such as TLRs) in sensing microbes and triggering an inflammatory response. As TLR2 and TLR4 have been reported to contribute to endothelial cell and monocyte activation by β_2 GPI-dependent aPL,^{58,61} one can speculate that the combination of the effect of infection plus the perturbation of TLR function mediated by the autoantibodies overcome the threshold for triggering thrombosis. Alternatively, infections or inflammation might increase the expression of the aPL target antigen or the expression of antigenic epitopes that are hidden in resting conditions.⁸⁴ In line with this hypothesis, preliminary data showed that LPS is able to upregulate β_2 GPI expression in mice.⁸⁵

The two hit hypothesis does not, however, fit well with the APS obstetrical manifestations, with the single exception of the increased risk of venous thromboembolism during pregnancy. In fact, passive infusion of IgG fractions with aPL activity induces fetal loss in naive pregnant mice without necessarily requiring a second hit. The expression of β_2 GPI seems to be restricted to placental tissues even in physiological conditions,^{86,87} and binding

of labeled exogenous β_2 GPI, infused into naive pregnant mice, to trophoblast and endothelial cells in the labyrinth was recently documented *in vivo* using the eXplore Optix™ imager.⁸⁵ The large availability at the placental level of the target antigen for potentially pathogenic aPL is in sharp contrast with the lack of comparable expression in other tissues of naive mice,⁸³ and even in highly vascularized human tissues such as kidney (P. L. Meroni, unpublished work). One might speculate that high expression of β_2 GPI at the placental level, together with the hormonal and vascular modifications that occur in pregnancy, are sufficient to favor the pathogenic activity of the autoantibodies without any additional factor.

Complement activation

Activation of complement seems to be a necessary step in *in vivo* models of aPL-mediated thrombosis and fetal loss. This conclusion is supported by the finding that animals deficient in complement components or complement receptors, or treated with inhibitors of complement activation, were protected from the thrombogenic effect of aPL.^{11,38,39,88} Likewise, pregnant mice deficient in complement C3 or C5, or C5a anaphylatoxin chemotactic receptor, or treated with an inhibitor of C3 convertase, did not experience fetal loss induced by aPL.^{35–40,43}

Human IgG aPL fractions that display a thrombogenic effect in the LPS-dependent rat model of APS, and sera from the majority of patients with APS, were found to fix complement *in vitro*;^{11,89} however, we do not see a clear decrease of complement levels *in vivo*. Mild hypocomplementemia has been reported in primary APS with no other underlying systemic autoimmune diseases, but only in two studies.^{90,91}

Complement deposition was found in placenta tissue from women positive for aPL, in a retrospective study.⁴⁷ By contrast, a case study reported no complement deposition in samples from fetuses miscarried by women with primary APS,⁴⁸ whereas a more recent prospective study on full-term placentas from 11 women with primary APS showed mild complement deposition but without any relationship with pregnancy outcome or therapy.⁴⁹

Although definite conclusions about the involvement of complement in APS-associated thrombosis and miscarriages can only be drawn from large prospective analyses, the potential role of complement in aPL-mediated clinical manifestations should not be neglected. In addition to causing cell lysis or acute inflammation, complement components are actually able to modulate cell functions.^{92,93} That being so, local activation and deposition of complement might affect the biological responses of cells involved in APS pathogenesis, such as endothelial cells or monocytes, or decidual or trophoblast cells.

Conclusions

The vascular manifestations of APS involve pathogenic mechanisms that only partially contribute to the obstetrical events. Such a difference fits well with the clinical differences between the two aspects of the syndrome. Although aPL comprise a heterogeneous family of autoantibodies, there is evidence that antibodies reacting with different epitopes of β_2 GPI are the main pathogenic subset.

We still do not know why, or how, a relatively homogeneous population of autoantibodies mediates different pathogenic mechanisms, presumably leading to the divergent clinical manifestations. Recently, attention has been paid to the epitope specificity of β_2 GPI-dependent aPL, in an effort to explain how the distinct functions arise. More information about the true physiological role of β_2 GPI, and about its tissue expression levels under different conditions, could provide the key to a better understanding of the pathogenesis of APS.

Review criteria

Original articles on APS pathogenesis were identified in the PubMed database using the search terms “antiphospholipid antibodies”, “beta2 glycoprotein I”, “prothrombin”, “thrombosis” and “recurrent fetal loss”, in various combinations. Only English-language, full-text papers were selected, with a focus on studies published after 2008. In addition, relevant publications were selected from the reference lists of other reviews on similar topics and from the authors' own bibliographic files.

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Author contributions

All authors contributed equally to researching data, discussing content and writing the article, and reviewing/editing of the manuscript before submission.