Role of the complement in pregnancy with antiphospholipid syndrome: mechanisms of pathogenesis and clinical aspects

Antiphospholipid syndrome is a disease that was discovered just 25 years ago. Although clinical manifestations have been clearly described, pathogenetic mechanisms are still barely understood. A recent hypothesis involves inflammation in the setting of antiphospholipid syndrome morbidity and experimental data support the activation of a complement cascade as a pivotal event in its physiopathology. In this review, the authors will analyze the recent literature, focusing on contemporary and emerging aspects of complement-mediated disease pathogenesis, and pinpoint the clinical significance of this novel finding.

KEYWORDS: adverse pregnancy outcome ■ antiphospholipid syndrome ■ complement pathway ■ hypocomplementemia

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Antiphospholipid syndrome (APS) is an acquired thrombophilic disease. According to the most recent international consensus statement on an update of the classification criteria for definite APS of Sydney 2004, the disease is diagnosed by the persistent presence of antiphospholipid (aPL) antibodies, such as lupus anticoagulant, anticardiolipin antibodies and anti-β2 GPI antibodies, in patients with vascular thrombosis and/or pregnancy morbidity. Thrombotic events include arterial and venous thrombosis, both in large or small vessels, confirmed by imaging or histopathology in the absence of significant evidence of inflammation in the vessel wall. Adverse obstetric outcome is a hallmark of the syndrome, and includes:

- Three or more consecutive spontaneous miscarriages before week 10 of gestation, and/or;

- One or more unexplained fetal death at or beyond week 10 of gestation, and/or;

- One or more preterm delivery at or before week 34 of pregnancy owing to severe pre-eclampsia, HELLP syndrome (hemolytic anemia, elevated liver enzymes, low platelet count) or placental insufficiency, which includes abnormal or nonreassuring fetal surveillance tests, abnormal Doppler flow velocimetry waveforms analysis suggestive of fetal hypoxemia, oligohydramnios and a postnatal birth weight less than the tenth percentile [1].

In clinical practice, APS is usually distinguished in primary APS (PAPS) or APS associated with coexistent autoimmune diseases.

aPL antibodies were linked to obstetric complications more than 20 years ago. To explain APS-associated obstetrical manifestations, thrombosis has been generally accepted as the key pathogenetic mechanism underlying pregnancy morbidity. This hypothesis fits with the classical interpretation of the disease as a prothrombotic condition rather than an inflammatory process.

aPL antibodies promote thrombotic events through two possible ways: a direct effect on platelets, endothelial cells, monocytes and trophoblastic cells or an indirect interaction with components of the coagulation cascade and fibrinolytic proteins.

APS-associated antibodies can directly bind to platelets and promote their activation and aggregation, enhancing the expression of membrane glycoproteins GPIIb/IIIa [2]; aPL antibodies also promote an overexpression of the adhesion molecules in endotelial cells and an upregulation of tissue factors and cytokines in endothelial cells and monocytes. Another investigation reported that aPL antibodies can displace annexin V from the trophoblast surface, where this protein has a potent anticoagulant activity based on its high binding affinity to anionic phospholipid [3].

aPL antibodies may also interfere *in vivo* with the normal functions of phospholipid-binding proteins crucial for the regulation of coagulation cascades, such as thrombomodulin, protein C, protein S, annexin V and β2 GPI, causing a reduction of antithrombotic mechanisms; they also cause accelerated thrombin formation and induce a prothrombotic state [4]. Other authors speculated that aPL antibodies can react with oxidized low-density lipoproteins, participating in oxidant-mediated endothelial injury [5].

Despite the persistent presence of aPL antibodies in the circulation, thrombotic events in

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patients with APS only occur occasionally, suggesting that the presence of these antibodies is necessary, but not sufficient, for clot formation *in vivo*. For this reason, the 'two-hit hypothesis' has been proposed recently, according to which aPL antibodies provide the 'first hit' that increases the risk of thrombotic events and potentiates the procoagulant effect of a later thrombophilic condition that acts as a 'second hit' [6].

The evidence that thrombosis cannot completely explain APS clinical manifestations has shifted the focus of research to other pathogenetic mechanisms, such as inflammation. Emerging evidence shows that an important role in mediating clinical events in APS can be played by the complement pathway.

The complement pathway

The complement pathway is a strategic component of immune systems in close collaboration with other components of innate and adaptive immunity. It acts as an efficient surveillance system discriminating between healthy host tissue, cellular debris, apoptotic cells and microbial cells.

The complement consists of more than 20 plasma and membrane proteins circulating as inactive enzyme precursors. The activation of one component triggers a stepwise cascade that results in the deposition of complement fragments on pathologic targets and the release of fragments that promote inflammation [7]. Three different activation pathways of the complement system have been described, according to the difference in activation elements involved.

The classical pathway starts with the binding of immunoglobulin IgM or IgG to an antigenic target (bacterial, viral or autoantigen); the Fc portion of the antibody is then bouund by the C1q subcomponent of the C1 complex and this event causes the cleavage of C1 by autoactivation, leading to C1r formation. Activated C1r then cleaves and activates C1s, which in turn cleaves C4 and C2. Resultant fragments C4b and C2a form the C3 convertase C4b2a.

The lectin pathway is activated by the binding of a mannose-binding lectin to repetitive sugar sequences on the pathogen's surface. Notably, lectins do not bind sugar found on mammalian cell-surface glycoproteins and, thus, distinguish pathogen surfaces from host cellular surfaces. After this binding, MASP cleaves C4 and C2, releasing C4b and C2a to form the C3 convertase C4bC2a.

The alternative pathway is capable of recognizing and destroying foreign pathogens in the absence of antibody binding. It is initiated by the low rate of spontaneous hydrolysis of C3 ('C3 tick-over'), which is abundant in the blood plasma. Hydrolyzed C3 binds the plasma protein factor B, which allows factor D to cleave factor B into Ba and Bb. C3bBb is the C3 convertase of the alternative pathway and is stabilized by properdin, forming C3bBbP, which cleaves C3 to deposit C3b on the target surface. Some C3b also forms the C5 convertase C3bBC3bP.

These three pathways converge in the formation of a C3 convertase that activates C3, forming the C3b fragment. The following step is the activation of C5 by a C5 convertase; the C5b fragment then forms a multimolecular unit consisting of C5b, C6, C7, C8 and C9, known as the membrane attack complex (MAC). The MAC forms transmembrane channels in targeted cells that result in cell membrane disturbance and ultimately cell lysis. Activation of the complement components also leads to the production of C3a and C5a, called 'anaphilatoxins'; these are strong proinflammatory molecules that recruit inflammatory cells, such as neutrophils and monocytes.

Other complement proteins, such as DAF, CD59, MCP, CFI and CFH, work as regulatory or control proteins to prevent complement damage to normal host tissues [8].

Complement activation in healthy & APS pregnancy

The placenta represents an important site of complement system function. The complement protects the fetal–maternal interface against invading pathogens and promotes the removal of immune complex and apoptotic cells [9]. A pivotal role of some complement components in placental development was suggested by several recent reports.

In 2010, Agostinis *et al.* showed that the complement fraction C1q plays an important role in promoting trophoblast invasiveness; after analyzing human placental tissue, deposits of C1q in maternal decidua were found mainly in areas of trophoblast invasion, suggesting that C1q may have been synthesized locally and used for some special functions, such as binding avidly to the cell surface and making a physical link between endovascular trophoblasts and decidual endothelial cells. It was also seen that defective local production of C1q may be involved in several pregnancy disorders, characterized by poor trophoblast invasion, such as pre-eclampsia. Moreover, the same authors demonstrated that mice deficient in C1q (C1q$^{-/-}$) had increased frequency

of fetal resorption, reduced fetal weight and smaller litter sizes when compared with wild-type mice [10].

Another recent paper showed that C3 has a physiological role in early phases of pregnancy; C3-knockout mice have smaller blastocysts, higher resorption rates and smaller sizes of placentae [11].

In addition, there are multiple potential sites activating the complement in healthy placenta. Fetal trophoblast and maternal deciduas form a strict vascular connection in the placenta; in particular, the outer layer of fetal trophoblast (the multinucleated syncitiotrophoblast), coating the villi, is directly exposed to maternal blood and could potentially be attacked by maternal complement, especially in the presence of paternal alloantigens.

Furthermore, extravillous trophoblasts invading the decidua are another likely factor contributing to complement activation: during the deep vascular changes of spiral arteries, extravillous trophoblast cells can migrate through cell junctions reaching a subendothelial position; this process can favor local complement activation [12].

These observations suggest that, to avoid pathological complement activation during placentation, the complement pathway at the fetal–maternal interface has to be tightly regulated [9]. This is assured by the local expression of complement-regulating proteins, such as DAF, CD59 and MCP, which inhibit enhanced complement activation at the syncitiotrophoblast level, protecting the fetus from potential damage [13]. In addition, cytotrophoblast and extravillous trophoblast cells express MCP and CD59, although these layers are not directly in contact with maternal blood flow [14]. The importance of complement regulatory proteins has recently been investigated in a prospective, multicenter, observational study (PROMISSE) [15]. The genes encoding three complement regulatory proteins (MCP, CFI and CFH) were sequenced in women with systemic lupus erythematosus (SLE) or aPL antibodies who developed pre-eclampsia. Mutations of these regulatory proteins were found in 18% of autoimmune patients with pre-eclampsia and in the 8% of nonauto-immune pregnancy-patients. Results showed that an uninhibited complement activation can predispose to pre-eclampsia.

In human pregnancy, as shown in a recent paper by Cohen *et al.* that related the obstetric outcome to staining patterns in placental tissue, the placental deposition of complement components, such as C4d, has not been found in healthy pregnancies [16]; conversely, a diffuse C4d staining was observed at fetal–maternal interface in a setting of autoimmune diseases, such as SLE and APS, and was related to intra-uterine fetal death. These data support the concept that a severe autoantibody-mediated immune response at the fetal–maternal interface could lead to impaired fetal outcome [16].

As previously mentioned, deregulated activation of the complement pathway could represent the 'second hit' required to trigger clinical events in the context where aPL antibodies have exerted their prothrombotic influence [6]. Recent findings in animal models deficient in specific complement components or complement receptors and in mice overexpressing complement inhibitors suggest that complement activation is involved in pregnancy loss.

First, Xu and coworkers investigated the role of complement activation in fetal–maternal tolerance. Crry is a complement regulatory protein in rodents, similar to DAF and MCP in humans, that inhibits C3 convertase activity, thus inhibiting complement activation on cell surface membranes; complete Crry deficiency ($Crry^{-/-}$) in mice results in embryonic developmental arrest and embryonic loss owing to complement deposition and placental inflammation. If $Crry^{-/-}$ embryos are also C3 deficient or factor B deficient, no intrauterine growth restriction or fetal loss is observed. This strongly suggests that embryonic deaths in $Crry^{-/-}$ mice were caused by uncontrolled complement activation [17].

To investigate the role of complement in APS, Holers *et al.* used a mouse model in which passive transfer of human IgG containing aPL antibodies (aPL–IgG) induced fetal loss; when the complement cascade is inhibited using C3 convertase inhibitor, aPL antibody-induced fetal injury, growth retardation and aPL antibody-mediated thrombosis are prevented. In addition, $C3^{-/-}$ mice were resistant to fetal injury caused by aPL antibodies [18]. Moreover, the same authors highlighted the involvement of complement fraction C5 activation as a critical phase in the pathogenesis of thrombosis and fetal loss associated with aPL antibodies, but they could not locate the site of abnormal activation since in their model, both the classical and the alternative pathways were inhibited.

Work by Girardi *et al.* highlighted the critical role of complement proteins in the pathogenesis of fetal loss in APS using mice genetically deficient in C5, C5a receptor, C4 and factor B. As a first approach, the transfer of aPL–IgG or the

transfer with the $F(ab')_2$ fragments of the same antibodies were compared in pregnant mice: the second case did not result in fetal growth restriction or fetal loss, suggesting that although the Fc portion of antibodies is required for fetal damage, it was not mediated through Fc–Fc receptor binding. In C4- and C5-deficient mice, no fetal loss was observed, suggesting a role of both the classical and lectin pathways in aPL antibody-mediated pathology. Furthermore, the authors showed that mice treated with C5-specific monoclonal antibodies were protected from fetal loss; similarly, C5a receptor-deficient mice as well as mice given C5a receptor antagonist peptide were protected from obstetric complications. Finally, factor B-deficient mice were protected from pregnancy morbidity, suggesting the role of the alternative pathway in aPL antibody-mediated fetal injury [19].

Despite this evidence, the exact mechanism through which complement mediates APS complications remains to be unknown.

To explain aPL antibody-related placental and fetal damage, the following pathogenetic cascade has been proposed. In accordance with the classical pathway, aPL antibodies should activate the complement cascade and should produce potent mediators of platelet and endothelial activation, including C3a, C5a and C5b-MAC. In particular, the engagement of C5a–C5a receptor complex was reported to play a central role in increasing inflammation through the release of TNF-α and TF [20,21]. TNFα could cause a direct toxic effect on trophoblast cells and maintain the activation of immune cells in the placenta [20]. TF participates in both coagulation and inflammation; the physical contact of TF and factor VII triggers coagulation cascade. Moreover, the protrombinase complex (TF–factor VIIa–factor Xa) could also induce neutrophil activation through its interaction with protease-activated receptors exposed on these cells, with consequent oxidative burst and trophoblast injury (FIGURE 1) [21].

Recently, complement activation through the alternative pathway has been proposed as one of the mechanisms responsible for aPL antibody-mediated fetal injury. Deficit of factor B or the use of a factor B-specific monoclonal antibody in mice protects from pregnancy morbidity [22]. A role of the alternative pathway has also been demonstrated in human pregnancies; women with high circulating Bb fragment in early pregnancy had a 3.8-fold higher risk of developing pre-eclampsia than women with normal levels of this protein [23].

Moreover, the complement system has also been identified as having an effect on the coagulation cascade itself. For example, activated factor XII, an initiator of the intrinsic coagulation pathway, degrades and activates C1 [24]; thrombin directly degrades C5 in the absence of C3 to produce the anaphylatoxin C5a [25]; in addition, C5a increases the expression of TF [21] and the MAC degrades prothrombin to thrombin [26].

The involvement of complement activation in adverse pregnancy outcome in patients with APS has been studied by, first, analyzing placental tissue and searching for complement fragment deposition.

An interesting retrospective study documented a novel finding in the placentas from APS patients demonstrating an increased deposition of complement products, such as C4d and C3b, compared with those of normal control subjects [27]. This placental alteration was evident even when the presence of aPL antibodies was clinically silent. On the contrary, Cavazzana *et al.* failed to demonstrate C4d and C3c deposition in placentas from two APS women who experienced late abortions [28].

More recently, Cohen *et al.* have shown that deposition of C4d in murine and human placentae is strongly related to adverse fetal outcome in SLE and APS pregnancies [16]. As a result, hypocomplementemia has also been investigated as an indicator of complement activation in APS patients owing to its easy detection from a clinical point of view.

Outside of pregnancy, hypocomplementemia was reported in a small series of APS patients with CNS involvement; low circulating complement levels were associated with livedo reticularis, hemolytic anemia and thrombocytopenia, and with high Lupus anticoagulant activity [29].

In a recent retrospective report, hypocomplementemia was found in a significant proportion of patients with PAPS compared with non-SLE connective tissue diseases patients or healthy subjects. No healthy subjects showed hypocomplementemia. Most of the patients with hypocomplementemia showed high serum levels of C3a and C5a, suggesting that hypocomplementemia was due to complement activation rather than complement deficiency; none of them had low serum complement regulatory factors, indicating that complement activation is not caused by deficiency of these factors, but presumably by enhanced immune complex formation [30].

On the other hand, during pregnancy there is considerable evidence of complement activation in patients with SLE and its relationship with the grade of disease activity [31–34]; however, studies investigating the clinical correlation between
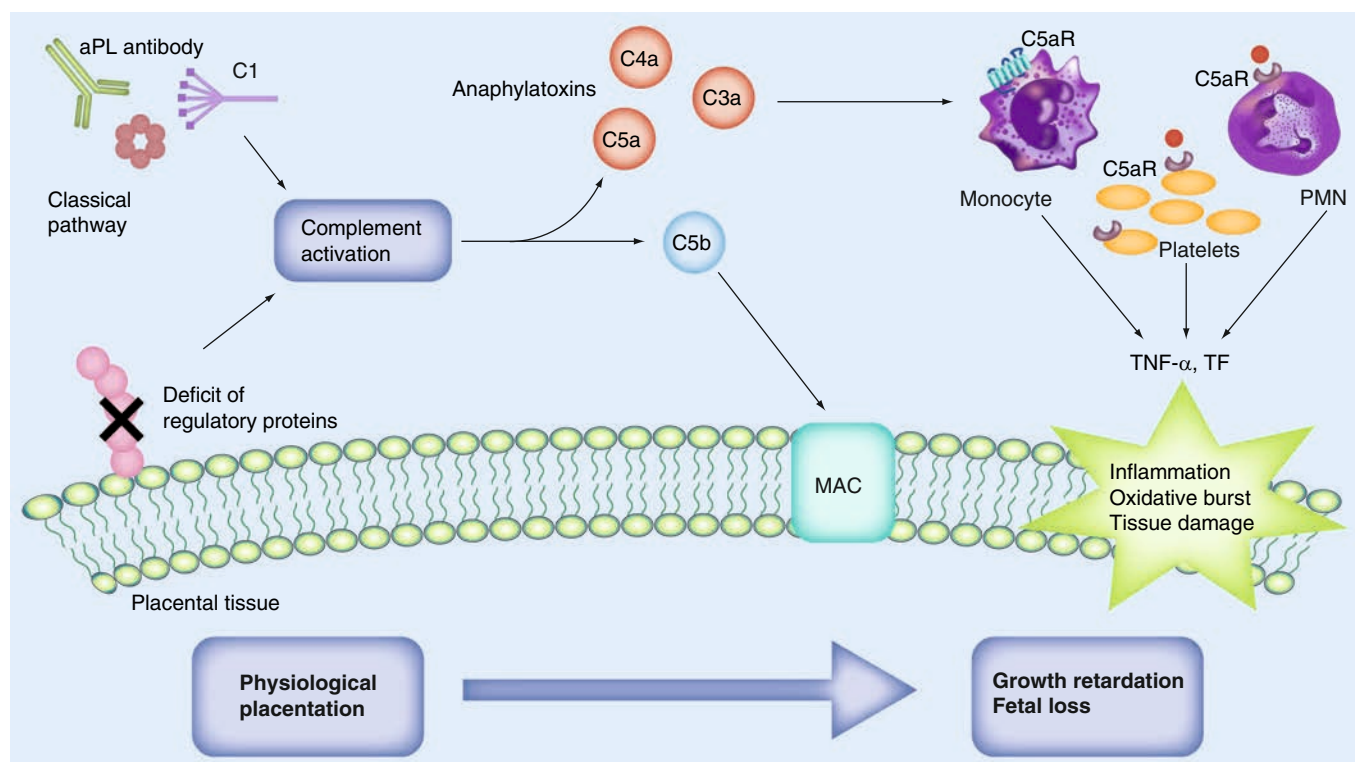
Figure 1. Pathogenetic mechanisms of complement activation in antiphospholipid syndrome.
aPL: Antiphospholipid; C5aR: C5a receptor; MAC: Membrane attack complex; PMN: Polymorphonuclear cell; TF: Tissue factor.

obstetric outcome and complement activation in pregnancies affected by APS are still lacking.

Our contribution to this research is demonstrated in a recent published report in which we searched for the role of complement activation in 47 pregnancies complicated by either PAPS or APS associated with other autoimmune diseases [35]. The correlation of hypocomplementemia, defined as C3 <90 mg/dl and/or C4 <10mg/dl, with adverse obstetric outcome was highlighted.

APS patients with low circulating levels of complement components experienced a poor pregnancy outcome in terms of fetal loss, preterm delivery at ≤34 gestational weeks and neonatal birth weight <2500 g, in contrast with APS patients without hypocomplementemia. In our study population, the relationship between hypocomplementemia and pregnancy outcome, in terms of early delivery, low birth weight and low birth weight percentile, was statistically significant. Moreover, the predictive role of hypocomplementemia was better highlighted in the subgroup of PAPS patients, in whom low levels of complement components were negatively related to pregnancy outcome.

In our study, hypocomplementemia, incorporated into a logistic regression model, resulted as an independent predictive factor of worse pregnancy outcome in APS. In addition, low levels of circulating C3 complement components are strongly related to lower birth weight, suggesting

an intriguing crosstalk between inflammation and fetal growth.

Recently, a multicenter study has been published including 57 prospectively followed pregnancies in patients with PAPS, 49 pregnancies in patients with systemic sclerosis/undifferentiated connective tissue disease and 175 healthy pregnant women [36]. C3 and C4 levels, classified by a pregnancy-specific range obtained from healthy pregnant controls, were related to pregnancy outcome and complications. The authors found no difference in the prevalence of low complement levels between complicated and uncomplicated PAPS pregnancies, even if they anecdotally report low complement levels in pregnancies complicated by pre-eclampsia.

The discordance in these results demonstrates that a multicenter prospective study is necessary to better define the exact significance of complement levels in APS pregnancies in order to establish the role of hypocomplementemia as a prognostic factor of poor pregnancy outcome, in addition to well-known risk factors, such as triple positivity for aPL antibodies [37], hypertension at conception and presence of abnormal uterine artery Doppler findings [38,39].

Conclusion
APS is a complex disease whose pathogenesis is not yet completely clarified. As shown in this review,

a central role in APS adverse pregnancy outcome been has recently attributed to the complement pathway. In contrast with the high number of reports demonstrating the relationship between complement activation and pregnancy outcome in experimental models, there is lack of clinical findings regarding this correlation in APS patients. In particular, conclusive data concerning the usefulness of complement serum levels in monitoring APS pregnancies are not available. Our previous report detects hypocomplementemia as a novel prognostic factor of poor pregnancy outcome, but a large prospective and multicenter study is necessary to confirm the utility of this result in clinical practice, in order to identify patients at higher risk of obstetric complications and to define antepartum care and optimal timing of delivery.

Future perspective

In recent years, great improvements in the management of APS pregnancies have been reached, owing to accurate preconceptional counseling, the definition of appropriate therapeutic management and the individuation of risk factors related to adverse pregnancy outcome.

If the role of hypocomplementemia in predicting APS obstetric morbidity is confirmed in the future, this finding could provide an easily detectable method, useful to identify pregnancies potentially at risk of adverse outcome, which could be addressed by closer follow-up.

Furthermore, insights into the mechanisms of complement activation in the genesis of obstetric morbidity in APS should offer new therapeutic strategies. In particular, a complement-targeted therapy based on complement inhibition could represent a further treatment option compared with the current standard treatment of anticoagulation in the management of APS pregnancies.

Executive summary

Antiphospholipid syndrome pathogenesis
- Antiphospholipid syndrome (APS) pathogenesis has not been completely clarified.
- Thrombosis is the main APS pathogenetic mechanism; research has recently been focused on inflammation.

Complement cascade & pregnancy
- The complement cascade has a physiological role in placental development and represents a defense mechanism at the fetal–maternal interface.
- Excessive activation of the complement cascade at the fetal–maternal interface results in fetal damage and pregnancy complications.
- In APS, as shown in other autoimmune diseases, complement pathway activation is deregulated and results in placental damage and fetal loss.

Hypocomplementemia & pregnancy outcome
- Hypocomplementemia is the clinical result of an inappropriate activation of the complement cascade.
- Some authors have described hypocomplementemia as a novel prognostic factor of adverse pregnancy outcome in APS syndrome.

Conclusion
- Data regarding the relationship between hypocomplementemia in pregnancy and clinical outcome are uncertain; further studies are necessary.

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