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INVITED REVIEW

Lipid-binding antiphospholipid antibodies: significance for pathophysiology and diagnosis of the antiphospholipid syndrome

Nadine Müller-Calleja[a,b], Wolfram Ruf[b,c] and Karl J. Lackner[a]

[a]Institute of Clinical Chemistry and Laboratory Medicine, University Medical Center of the Johannes Gutenberg-University, Mainz, Germany; [b]Center for Thrombosis and Hemostasis (CTH), University Medical Center of the Johannes Gutenberg-University, Mainz, Germany; [c]Department of Immunology and Microbiology, Scripps Research, La Jolla, CA, USA

ABSTRACT

The antiphospholipid syndrome (APS) is an autoimmune disease characterized by the presence of pathogenic antiphospholipid antibodies (aPL). Since approximately 30 years ago, lipid-binding aPL, which do not require a protein cofactor, have been regarded as irrelevant for APS pathogenesis even though anticardiolipin are a diagnostic criterion of APS. In this review, we will summarize the available evidence from *in vitro* studies, animal models, and epidemiologic studies, which suggest that this concept is no longer tenable. Accordingly, we will only briefly touch on the role of other aPL in APS. This topic has been amply reviewed in detail elsewhere. We will discuss the consequences for laboratory diagnostics and future research required to resolve open questions related to the pathogenic role of different aPL specificities.

Abbreviations: Acl: anticardiolipin; anti-D1: antibodies against domain 1 of β2GPI; anti-PS/PT: antibodies to phosphatidylserine/prothrombin; anti-β2GPI: anti-β2 glycoprotein I; aPL: antiphospholipid antibody; apoER2: apolipoprotein E-receptor 2; APS: antiphospholipid syndrome; ASM: acid sphingomyelinase; CAPS: catastrophic antiphospholipid syndrome; EPCR: endothelial protein C receptor; HCQ: hydroxychloroquine; LA: lupus anticoagulant; LBPA: lysobisphosphatidic acid; PAR: protease activated receptor; SLE: systemic lupus erythematosus; TF: tissue factor; TFPI: tissue factor pathway inhibitor; TLR: toll like receptor.; β2GPI: β2 glycoprotein I

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1. Introduction

The antiphospholipid syndrome (APS) is an autoimmune disease characterized by thrombo-embolic events and/or pregnancy morbidity combined with the persistent presence of antiphospholipid antibodies (aPL) [1,2]. APS is a chronic, moderately rare disease with an estimated prevalence of 40-50 cases per 100,000 and an annual incidence of 1-2 cases per 100,000 [3,4]. In rare cases, APS can present as an acute life-threatening disease with wide-spread thrombosis in the (micro)circulation and multi-organ failure, which is known as catastrophic antiphospholipid syndrome (CAPS). CAPS is considered an extreme course of APS, which is preceded by a triggering event such as an infection, surgical procedure, or malignancy [5]. APS was recognized as a disease entity only approximately 40 years ago, when the first immunoassays for antibodies against cardiolipin (aCL) were developed [6,7]. Further research showed that APS patients had not only aCL but also antibodies against other phospholipids, antibodies against phospholipid binding proteins, and in particular antibodies reactive with the serum protein β2-glycoprotein I (β2GPI) [8–11]. In addition to these autoantibodies there is a group of aPL which inhibit phospholipid-dependent clotting tests. Since they were initially observed in patients with systemic lupus erythematosus (SLE), they were called lupus anticoagulant (LA) [12]. While the antibodies associated with APS were apparently quite heterogeneous the term antiphospholipid antibodies (aPL) was used for all of them, even though the antigen might be a protein, e.g. β2GPI, or even incompletely characterized as is the case with LA. Historical aspects of early research leading to the discovery of APS as a disease entity have been excellently reviewed in an earlier article by Vlachoyiannopoulos et al. in this journal [13].

A major milestone in APS research was the observation in the early 1990s that the two most important disease manifestations, thrombo-embolism and fetal

CONTACT Karl J. Lackner karl.lackner@unimedizin-mainz.de Institute of Clinical Chemistry and Laboratory Medicine, University Medical Center Mainz, Langenbeckstrasse 1, D-55131 Mainz, Germany.

loss, were inducible in experimental animals by transfer of sera or immunoglobulins from APS patients [14–17]. These data were later amply confirmed and extended. Animal studies became more sophisticated by the inclusion of genetically modified mice and pharmacologic means to prevent the effects of aPL [18–33]. Most animal studies were performed in mice, but some studies with rats were also performed with equivalent results [34–37]. Even though the overall results of animal studies on thrombosis induction by aPL are very similar, the experimental approaches are highly heterogeneous. For more details, readers are referred to a recent comprehensive review [38]. Currently there is consensus that aPL are not only a diagnostic marker of an underlying autoimmune disease, but are the major pathogenic cause of the clinical manifestations of APS. This is also reflected in the consensus criteria for the classification of APS which were first published by an expert group in 1999 [39] and updated and extended in 2006 (Table 1) [40].

In 2023, an international working group of experts supported by the American College of Rheumatology and the European Alliance of Associations for Rheumatology (EULAR) published a new classification system for APS which takes account of scientific progress since 2006. [41]. Six clinical domains and two laboratory domains are considered. As the classification criteria are supposed to provide guidance for clinical studies on APS, their focus is on specificity. This implies that failure to fulfill the criteria does not necessarily rule out APS. However, in this case a patient should not be included in an APS study. The new classification criteria provide for a two-stage procedure (Table 2). Only patients who fulfill entry criteria similar to the 2006 classification criteria may be considered for definitive classification. The entry criteria were expanded to include cardiac valve disease and thrombocytopenia. In addition, relevant microvascular pathology was described in more detail. Persistent aPL positivity is not required here. In the second step, clinical domains are analyzed in more detail and assigned a specific weight. The same applies to the laboratory domains. For more detail, the reader is referred to [41]. Only patients with at least 3 weight points each from clinical domains and laboratory domains should be classified as APS.

The major novel aspects relate to the addition of new clinical criteria from microvascular disease, cardiac valve disease, and thrombocytopenia. As in the 2006 criteria, only persistent LA, moderate to high titer aCL and anti-β2GPI of the IgG isotype are considered sufficient laboratory criteria for APS. However, aPL of the IgM isotype have been assigned low weight so that they alone are no longer sufficient for APS classification. Further autoantibodies have been detected in APS patients, but these are currently not considered diagnostic criteria (Table 3).

Obviously, the question arises if all aPL or only specific aPL are pathogenic. Over the last three decades a concept has evolved based on *in vitro* and *in vivo* data stating that pathogenic aPL do not bind to lipids alone but require the presence of so-called cofactor proteins, in particular β2GPI, or bind to these cofactor proteins directly. According to this concept, aPL, which bind to lipids in the absence of any cofactor proteins are associated with infections and considered irrelevant for the pathogenesis of APS. This concept has been the topic of several recent reviews on APS which focused on the pathogenetic role of antibodies to β2GPI and other lipid-binding proteins [1,2,42–46]. In this review, we will therefore focus on the emerging role of lipid-binding aPL in APS, and discuss the implications for laboratory diagnostics and epidemiologic studies.

Table 1. Consensus criteria for APS (Sidney 2006) condensed from [40].

Clinical criteria
Vascular thrombosis
• One or more clinical episodes of arterial, venous, or small vessel thrombosis in any tissue or organ
Pregnancy morbidity[a]
• One or more unexplained deaths of a morphological normal fetus at or beyond the 10th week of gestation.
• One or more premature births of a morphological normal neonate before the 34th week of gestation due to (pre)eclampsia or placental insufficiency.
• Three or more unexplained consecutive abortions before the 10th week of gestation
Laboratory criteria
Lupus anticoagulant[b]
Anticardiolipin IgG and/or IgM present in medium or high titer[b]
Anti-β2GPI IgG and/or IgM present in medium or high titer[b]

[a]Alternative causes for any pregnancy morbidity listed must be ruled out.
[b]Positive aPL must be shown to persist for at least 12 weeks.

Table 2. Classification criteria for APS 2023 condensed from [41].

Step 1 – Entry Criteria
Only patients fulfilling one clinical and one laboratory entry criterion should be considered for further, definitive classification as APS (step 2)
Clinical entry criteria (Domains 1-6)
• 1. Macrovascular venous thromboembolism
• 2. Macrovascular arterial thrombosis
• 3. Microvascular disease
• 4. Obstetric morbidity
• 5. Cardiac valve disease
• 6. Thrombocytopenia
Laboratory criteria (Domains 7-8)
• 7. Lupus anticoagulans
• 8. aCL and/or anti-β2GPI moderate or high positive IgG or IgM
Step 2 – Additive Clinical and Laboratory Criteria
Criteria from all eight domains are specified and assigned a specific weight between 1 and 7
APS should be classified for research purposes if ≥3 weight-points each from clinical domains **and** laboratory domains are attained.

Table 3. Autoantibody targets identified in APS patients.

Lipid antigens
*Cardiolipin**
Phosphatidylserine
Lysobisphosphatidic acid
Phosphatidylethanolamine
Phosphatidylinositol
Phosphatidylcholine
Sphingomyelin
Protein Antigens
*β2 glycoprotein-I**
β2 glycoprotein-I/domain 1
Prothrombin
Phosphatidylserine/Prothrombin
Annexin A2
Annexin A5
Vimentin
Lupus anticoagulant*

*Only antibodies against cardiolipin and β2GPI of the IgG and IgM isotype and the lupus anticoagulant are considered laboratory criteria of APS [40,41].

2. Lipid-binding antiphospholipid antibodies (aPL)

In order to discuss the role of lipid-binding aPL it is mandatory to define the term. As the term suggests these aPL bind to (phospho)lipid antigens in the absence of other proteins which might mediate or modify binding. It should be kept in mind that when patient serum is analyzed in routine diagnostics, the whole spectrum of serum proteins is present in the assay (see also below). To detect lipid-binding aPL lipids are usually coated to a solid phase but they can also be present in the form of liposomes in solution.

Obviously, lipid-binding aPL represent only one subgroup within the heterogeneous group of aPL. Table 3 summarizes autoantibodies commonly included in the term aPL without claim to completeness. It should be noted that there is a distinction between criteria and non-criteria aPL, i.e. aPL which are part of the classification criteria and those which are not. In this section, we will focus on the definition of lipid-binding aPL and the development and validity of the concept of "cofactor dependency" of aPL.

The earliest description of antibodies against phospholipids dates back to the development of a complement binding assay to diagnose syphilis by August von Wassermann in 1906 [47] and the discovery by Mary Pangborn that the relevant antigen in this assay was a phospholipid, later called cardiolipin [48]. In the early 1950s, reports had appeared that biologically false positive syphilis tests were observed in patients with autoimmune disease [49,50]. In 1983, a specific radioimmunoassay was developed which detected antibodies against cardiolipin (aCL) [6]. Since aCL were found to be associated with thrombosis and pregnancy morbidity, the term anticardiolipin syndrome was introduced [7]. It became clear quite soon that other anionic phospholipids were also recognized by aPL. The term anticardiolipin syndrome was replaced by antiphospholipid syndrome. Later, studies with monoclonal aPL showed that lipid-binding aPL can cross react with several structurally related mostly anionic phospholipids [51–53].

2.1. Cofactors for lipid-binding aPL

In 1990 three independent studies were published reporting that IgG fractions from APS patients only bound to cardiolipin-coated ELISA plates when a serum factor was present in the assay which was identified as β2GPI [8–10]. At this time, the term "cofactor-dependent" aPL was coined which introduced the concept that there are lipid-binding aPL, and aPL which bind to lipids only in the presence of a protein cofactor which mediates binding. This concept had profound consequences for the laboratory diagnosis of APS until now [54], as it implies that only anticardiolipin, which require a cofactor, are relevant for APS. Therefore, the experimental basis of this concept needs to be considered in more detail.

McNeil [8] purified aPL from two patients by an affinity procedure, based on a mixture of cardiolipin, cholesterol, and the surfactant dicetyl phosphate trapped in an acrylamide matrix. Affinity purified aPL did not bind in a cardiolipin ELISA that was blocked with milk powder rather than bovine serum, whereas the blocking procedure had no effect on the usual 1:50 dilution of patient serum. Thus, it was apparent that a serum factor which was absent in the affinity purified aPL was required for binding. The authors succeeded to identify β2GPI as this serum factor. Galli [9] also affinity purified aPL from two APS patients. Affinity purification from patient serum was performed with cardiolipin liposomes. ELISA plates were blocked with bovine serum albumin (BSA). Again, affinity purified aPL did not bind under these conditions. The addition of human serum restored binding of aPL. These two studies were the basis of the current view that aPL detected in APS patients are not lipid-binding aPL, but bind to phospholipids only in the presence of an appropriate cofactor. In fact, cofactor dependence is considered a distinguishing feature of APS associated aPL versus infection associated aPL.

There are two aspects of these two studies which are noteworthy: First, both used affinity purified aPL rather than whole IgG. Accordingly, the purified aPL may not be representative for the whole spectrum of aPL of the patients. In fact, McNeil et al. [8] describe that a relevant proportion of the aCL-activity did not

absorb to the affinity column. Even in the presence of plasma or β2GPI two thirds or one quarter, respectively, of aCL activity was detected in the fall through of the column. Notably, the material bound to the affinity matrix contained large amounts of β2GPI which co-eluted with the immunoglobulins. This indicates that β2GPI bound to the affinity matrix where it served as antigen for anti-β2GPI. No attempt was made to analyze the anticardiolipin which did not bind to the affinity matrix. It remains unknown if similar results would have been obtained with unbiased IgG-fractions isolated by protein G-affinity. Second, these two highly cited papers and their far-reaching conclusions are based on only four patients.

Matsuura [55] later partly confirmed these data. However, two out of three IgG fractions isolated from SLE patients bound partially independent of the presence of cofactor proteins to cardiolipin. This is early evidence that a considerable percentage of aPL from APS patients bind to phospholipids in the absence of cofactors. Pierangeli [56] reported that the addition of β2GPI only moderately enhanced binding of APS-IgG to cardiolipin. This study again shows very convincingly that it is likely that the majority of APS patients harbor cofactor independent lipid-binding aPL. In 1996, Forastiero [57] presented data from 35 patients with APS and SLE. aPL-binding was not enhanced by addition of cofactor in 14 out of 35 aPL-positive serum samples. In fact, two out of 10 IgG fractions isolated from these patients were fully cofactor independent.

Shortly after the observation that some aPL do not bind to cardiolipin-coated surfaces in the absence of proteins such as β2GPI, it was shown that sera from APS patients contain antibodies directed against β2GPI [11]. Further autoantibodies against protein antigens have been described since then (see Table 3), but their potential role in APS is less well established (see sections below).

2.2. β2GPI as cofactor

The cofactor protein β2GPI is an approximately 50 kDa serum glycoprotein which binds to phospholipid surfaces with high affinity [58–60]. The protein consists of 5 domains. The first four domains are of the complement control protein (CCP) module type, also known as sushi domains. The fifth domain contains a lysine rich domain which confers a positive charge to this part of β2GPI and mediates binding to negatively charged lipid surfaces. Due to its lipid binding property β2GPI is also found in association with lipoprotein particles, mainly very low-density lipoproteins and high-density lipoproteins [61]. The physiologic functions of β2GPI are still not well defined. Besides potential roles in lipoprotein metabolism, it has been implicated in modulation of platelet function, plasmatic coagulation and fibrinolysis as well as complement regulation [62]. Surprisingly, genetic deficiency of β2GPI in humans has no obvious clinical consequences [63]. However, in a lupus-prone mouse strain, deficiency of β2GPI aggravated disease development with more severe glomerulonephritis, splenomegaly, and lymphadenopathy [64].

How might β2GPI serve as "cofactor" for aPL? The term cofactor implies that cofactor proteins and in particular β2GPI are not the relevant antigen but somehow enable aPL to bind to cardiolipin or other lipids. Alternatively, cofactor binding to lipids might generate an epitope combined of lipid and protein. To date neither biochemical evidence for a combined lipid-protein epitope has been found nor a mechanism has been identified by which β2GPI enables aPL to bind to lipids. Due to its high affinity to phospholipids the most likely explanation of the observation of the proposed "cofactor activity" is binding of β2GPI to the lipid coated solid phase where it serves as antigen for autoantibodies directed against β2GPI (Figure 1) [13,65].

This concept is also supported by a study using line blots with phospholipids and phospholipid-binding proteins spotted to a polyvinylidene difluoride (PVDF) membrane [66]. In this assay two monoclonal anti-β2GPI bound only to the β2GPI-line as expected but not to any phospholipid-line. However, when the blots were pre- or co-incubated with excess β2GPI this enabled additional binding of anti-β2GPI to the same lines as a human monoclonal lipid-binding aPL, i.e. cardiolipin, phosphatidylserine, phosphatidylinositol, and phosphatidylglycerol. This shows that β2GPI binds to immobilized phospholipids where it serves as an antigen for anti-β2GPI. There is no reason to believe that this is different between line blots and ELISA formats. To further analyze whether APS patient sera contain lipid-binding aPL we studied purified IgG-fractions from 20 APS patients [67]. IgG isolated from patients who tested positive for aCL IgG but negative for anti-β2GPI IgG in commercial diagnostic assays always bound fully independent of other proteins to cardiolipin coated ELISA plates. IgG from patients who tested positive for both aCL and anti-β2GPI bound to cardiolipin but showed enhanced binding to cardiolipin coated plates when β2GPI was added. IgG from two patients who tested positive for anti-β2GPI only, but negative for aCL did not bind to cardiolipin in the absence of added β2GPI.

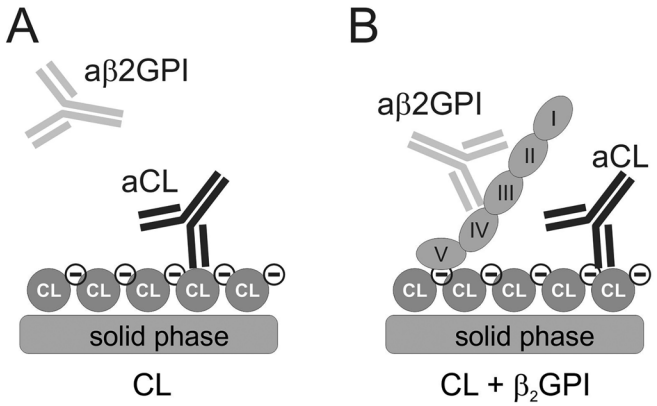

Figure 1. Schematic representation of anticardiolipin assays with the solid phase coated with cardiolipin only (A) and coated with cardiolipin and β2GPI (B). While addition of β2GPI to the assay format enables binding of anti-β2GPI it does not prevent binding of anticardiolipin. Note that the situation shown in panel A only occurs, when the sample to be tested does not contain β2GPI. This is the case with IgG-fractions.

In summary, current evidence indicates that there are true lipid-binding aPL, defined as antibodies which bind to phospholipids in the absence of other proteins, and antibodies which bind to proteins with high affinity to phospholipids. In addition, we have also identified an aPL with dual reactivity, i.e. an antibody which reacts both with cardiolipin in the absence of protein and with β2GPI in the absence of added phospholipids [53]. While all these types of aPL are present in APS patients, the prevalence of dual reactivity aPL remains unknown due to obvious technical difficulties to identify them unequivocally from a mixture of aPL. In our opinion, the terms "cofactor-dependent" or "β2GPI-dependent" aPL are misleading and should be abandoned, because these antibodies are in fact anti-β2GPI or antibodies with phospholipid cross-reactivity. In this review we will therefore use these terms only in the context of previous work in which they were still used.

2.3. Consequences for laboratory assays

Anticardiolipin IgG and IgM should be routinely tested in patients with suspected APS, because they belong to the criteria aPL. The Sidney criteria from 2006 state that aCL should be measured "by a standardized ELISA" [40]. There is no reference to the topic of cofactor dependence. In addition, the literature on standardization of aCL assays cited in this consensus paper does not deal with cofactor dependence. In addition, the 2023 classification criteria do not use the term cofactor dependence when aCL are discussed [41]. In 2018 the Scientific and Standardization Committee for lupus anticoagulant/antiphospholipid antibodies of the International Society on Thrombosis and Haemostasis published recommendations on laboratory diagnostics of APS stating that "it is mandatory to avoid detection of non-cofactor-related aCL associated with infections or several drugs" [54]. While the statement implies that this goal can be achieved using appropriate assays, the committee does not provide any clues which assays would be suitable for this purpose and acknowledges that current aCL assays detect "cofactor-dependent and cofactor-independent" aPL [68]. Attempts to devise assays for aCL which permit distinction between these different aPL types in serum have not been successful. The major difficulty arises from the fact that normal serum contains sufficient β2GPI which binds with high affinity to phospholipid-coated surfaces and serves as antigen for anti-β2GP, or may interfere with aPL binding by blocking phospholipid. Levy et al. [69] described an assay designed to differentiate β2GPI-dependent aCL from cofactor-independent aCL. The assay principle was based on ELISA plates coated with cardiolipin which were blocked with chicken ovalbumin. Accordingly, the solid phase did not contain any potential cofactor protein. For testing, serum samples were highly diluted to reduce endogenous β2GPI. Diluted samples were assayed with and without the addition of purified β2GPI. If β2GPI increased optical density-readings by more than 30%, a sample was classified as β2GPI-dependent. Apparently, addition of β2GPI only enhanced binding which was observed in the absence of cofactor. The authors did not provide any rationale for their definition of cofactor dependence, which remains arbitrary. In fact, the data presented are fully compatible with the presence of lipid-binding aPL, anti-β2GPI, and dual reactivity aPL. In any case, it is obvious that this assay format is not suited to differentiate "cofactor-dependent" aCL from anti-β2GPI. An additional drawback of this ELISA which precluded its widespread use in clinical routine was the

lower sensitivity caused by serum dilution. This limited its use to samples with at least "moderate" GPL or MPL units [69]. To date the only reliable method to distinguish lipid-binding from protein-binding aPL requires isolation of immunoglobulins and analysis in an assay which contains only proteins, which do not bind to phospholipids e.g. bovine serum albumin and are not targeted by aPL [9]. As outlined above, affinity purification of aPL on β2GPI rather than isolation of the whole immunoglobulin fractions cannot be regarded as an unbiased approach and should therefore be avoided.

This widely neglected limitation of current assays for aCL is a fundamental problem, because all available clinical and epidemiologic data are based on aCL assays which are not suitable to distinguish lipid-binding (i.e. "cofactor independent") aPL from protein-binding (i.e. "cofactor dependent") aPL. The consequences of this limitation will be discussed below.

2.4. Lupus anticoagulant (LA) and lipid-binding aPL

The term lupus anticoagulant (LA) describes an activity in human plasma that prolongs clotting times in phospholipid dependent assays of plasmatic coagulation [70,71]. This effect can be overcome by addition of surplus phospholipids. The name LA was coined because it was initially detected in patients with SLE. It was discovered relatively quickly that this activity was found in the immunoglobulin fraction of plasma. Due to its phospholipid-dependency and its common coincidence with other aPL, LA is considered to belong to the group of aPL.

Numerous attempts to identify a unique specific antigen responsible for LA activity have failed, because apparently several different immunoglobulins can interfere with phospholipid dependent clotting reactions. From several clinical studies it is known that a relevant proportion of LA positive individuals have isolated LA, i.e. no other detectable aPL [72–75]. In the most detailed analysis of patients with isolated LA, it is shown that these patients are not only negative for the criteria aPL (aCL and anti-β2GPI) but they are also negative for the non-criteria aPL, such as antibodies against domain 1 of β2GPI (anti-D1), anti-phosphatidylserine/prothrombin (anti-PS/PT), or IgA isotypes of aCL/anti-β2GPI [75]. LA activity of lipid-binding aPL has not been systematically investigated. One study of seven human monoclonal aPL concluded that only one of these antibodies had weak LA activity [76]. This antibody bound also to β2GPI. Nakamura et al. generated 6 monoclonal antibodies with LA activity from a patient with SLE [77]. Two IgM antibodies bound to cardiolipin independent of β2GPI while binding of three IgG antibodies to cardiolipin was enhanced by addition of β2GPI. We studied two lipid-binding monoclonal aPL and found that one of them prolonged aPTT while the other had no effect [78]. Overall, data regarding LA-activity of lipid-binding aPL is scant [79]. The available data suggest that some lipid-binding aPL have LA activity, but their contribution to LA positivity in patients appears to be minor compared to other aPL species, e.g. anti-D1 and anti-PS/PT [80–85] which have been amply shown to have LA-activity.

3. Pathogenic mechanisms of lipid-binding aPL

After the discovery that aPL induce APS manifestations in experimental animals, *in vitro* and animal studies have been the main pillar of research on the pathogenesis of APS. While *in vitro* assays provide mechanistic insights into specific humoral and/or cellular aPL effects, these experiments do not answer the question if the observed effects are also disease-causing. Their potential role in the pathogenesis of APS can only be assessed in appropriate animal models, which integrate aPL effects on the level of the intact organism. In *in vivo* models, thrombus development and pregnancy failure can be studied. Notably all animal models of thrombus formation require preconditioning of the experimental animals to enable or to accelerate thrombus formation. This is achieved either by chemical or physical injury to the vessel wall, severe reduction of blood flow, or induction of a systemic procoagulant state by infusion of appropriate mediators, e.g. bacterial lipopolysaccharide [38,86]. This suggests that aPL-induced thrombophilia is moderate in the absence of preconditioning. This fits well with the human situation where aPL increase the risk for thrombosis but do not lead inevitably to thrombo-embolic events. Pregnancy models on the other hand do not require additional injury beyond the exposure to aPL. However, in mice there is usually only a reduction of live births and surviving pups have a lower average weight. A major challenge of animal models is the validation of specific aPL effects observed *in vitro*, e.g. activation of a signaling pathway, in the different *in vivo* models. The two most common approaches are genetically modified animals and/or targeted pharmacologic intervention. Translation of the results into human APS is even more complex and requires clinical and epidemiologic studies.

As mentioned above, the question whether all aPL or only certain aPL species are pathogenic and if there

are differences between types of aPL has never been decisively answered. The available evidence for the pathogenic role of anti-β2GPI from *in vitro* and *in vivo* studies has been amply reviewed in recent years [1,2,42–46]. Therefore, the following section will concentrate on *in vitro* and *in vivo* data analyzing the pathogenic role of lipid-binding aPL and apparent differences to anti-β2GPI.

3.1. Lipid-binding aPL

Obviously, the role of lipid-binding aPL can only be studied when pure lipid-binding aPL without contaminating other aPL species are available. In particular, early studies oftentimes used APS immunoglobulins which were not well characterized. Pierangeli et al. developed an *in vivo* thrombosis model in the femoral vein subject to pinch injury and showed that IgG obtained from APS patients increased thrombus size and persistence [17]. The fine specificity of aPL was not addressed in this study. These researchers performed further studies in the same model, but aPL were not injected but generated by immunization of mice with human β2GPI or IgG from APS-patients [87]. Mice developed aPL within two weeks after immunization. This approach had been shown previously to induce aPL and APS manifestations in mice [15]. Both immunization schemes yielded high titers of aCL, while only immunization with β2GPI generated anti-β2GPI. Thrombus formation was indistinguishable between the two immunization schemes. Thus, this study is already suggestive of a pathogenic effect of lipid-binding aPL as mice negative for anti-β2GPI developed thrombi similar to mice positive for both anticardiolipin and anti-β2GPI. However, it cannot provide proof, because the specificities of aPL induced in the experimental animals remained poorly characterized.

Firm evidence that lipid-binding aPL are pathogenic can best be obtained with monoclonal antibodies generated from APS patients. While such monoclonal antibodies may not represent the full spectrum of aPL of a given patient, they are a unique tool to corroborate or refute their role in APS pathogenesis. Using this approach, Ikematsu et al. showed in 1998 that two lipid-binding human monoclonal aPL (Mab 516 and Mab 519) induced fetal growth retardation and resorption in mice [88]. Both antibodies were thoroughly analyzed to exclude dependence on β2GPI or binding to β2GPI. These data were later confirmed and extended [18]. In this study aPL were shown to induce complement activation. This was observed with IgG isolated from APS patients as well as with Mab 519. Complement C5-deficient mice were protected from the detrimental effects of APS-IgG and the monoclonal lipid-binding aPL. In further experiments it was shown that C5a generated in response to aPL attracted neutrophils *via* the C5a-receptor. Prevention of complement activation as well as blockade of C5a-receptors again were protective [18]. These data established the important contribution of neutrophils in pregnancy failure. The role of complement activation was further supported by the observation that heparin was protective in this model including protection against the monoclonal aPL Mab 519 [31]. Notably, the effect of heparin was not related to its anticoagulant properties, but rather to its complement inhibitory properties, as hirudin or fondaparinux had no effect. These studies represent perhaps the first undisputable evidence that lipid-binding aPL are pathogenic *in vivo*, in this case pregnancy failure. A follow-up study established that upon aPL exposure the complex of tissue factor and factor VIIa induced neutrophil activation by protease activated receptor (PAR)2 signaling [33]. The complex role of proteases and receptors of the coagulation system in signaling of lipid-binding aPL was later confirmed and extended (see below).

We showed that two monoclonal aPL of the IgG isotype isolated from two patients with APS bound to cardiolipin in the absence of any other protein [78]. The monoclonal aPL induced procoagulant activity in monocytes [89]. Later it was shown that they were internalized by monocytes and plasmacytoid dendritic cells into the endosomal route and activated endosomal NADPH-oxidase, followed by translocation of TLR7 and TLR8 to the endosome [90]. As a consequence, cells were sensitized to ligands for TLR7/8, i.e. single stranded RNA. Interestingly, the production of several cytokines like TNFα was induced indirectly by activation of TLR7/8. On the other hand, tissue factor expression was induced *via* NFκB, which was activated by ROS produced by endosomal NADPH-oxidase [90]. In further studies the binding site for lipid-binding aPL on the cell surface was identified as lysobisphosphatidic acid (LBPA) presented by the endothelial protein C-receptor (EPCR) unraveling the complete signaling pathway from the cell surface to endosomal NADPH-oxidase (Figure 2) [91,92]. These studies also substantiated the role of thrombin and PAR1/PAR2 heterodimer activation in signaling induced by lipid-binding aPL (Figure 3). This confirmed and extended previous data suggesting that TF/factor VIIa/PAR2 mediate pregnancy failure in experimental APS in mice [33]. In addition, lipid-binding aPL activate encrypted tissue factor on the cell surface [93]. This also depends on EPCR/LBPA and activation of acid sphingomyelinase, but not on activation of endosomal NADPH-oxidase.

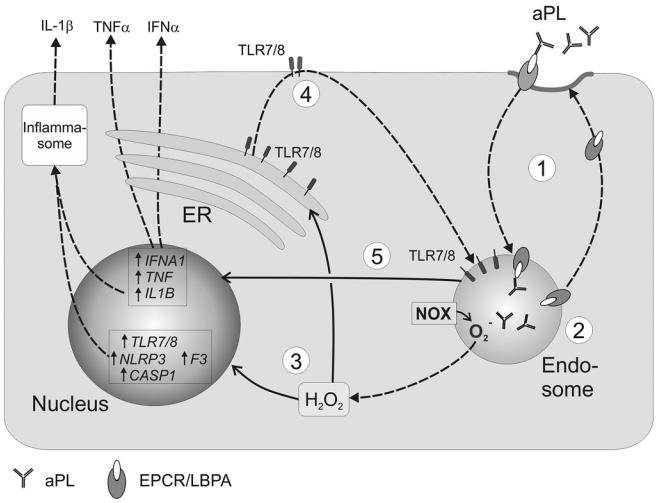

Figure 2. Schematic representation of endosomal signaling of lipid-binding aPL (adapted from [94]). ① aPL bind to EPCR/LBPA and are internalized into endosomes; ② aPL activate endosomal NADPH oxidase (NOX) which generates O_2^-; ③ O_2^- is released from the endosome and reacts with water to generate hydrogen peroxide which induces NFκB-dependent genes and ④ induces translocation of TLR7/8 to the endosome; ⑤ this latter effect leads to increased TLR7/8 signaling.

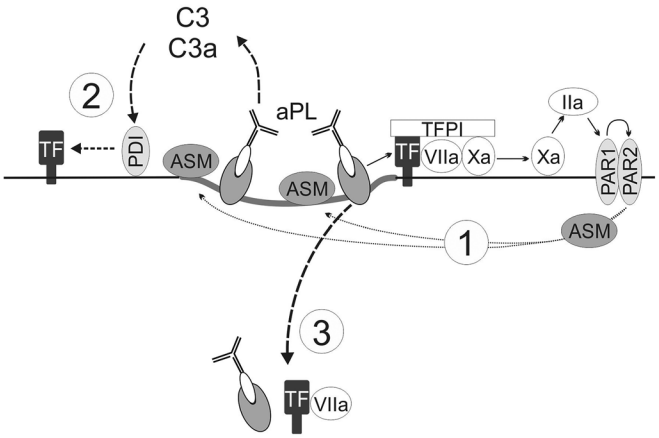

Figure 3. Cell surface events induced by lipid-binding aPL. ① binding of aPL to EPCR/LBPA induces release of tissue factor pathway inhibitor (TFPI) from the ternary complex of TF-FVIIa-FXa, followed by activation of thrombin by FXa with subsequent PAR1/2 signaling and acid sphingomyelinase (ASM)-translocation and activation by EPCR-LBPA [91,92]. ② activation of complement with subsequent decryption of TF. This requires ASM induced phosphatidylserine exchange to the outer membrane leaflet [93]. ③ internalization of the complex of EPCR/LBPA-aPL and TF/FVIIa into the endosomal route which does not occur when TF decryption is prevented [91].

It is important to note that lipid-binding aPL recognize cell surface expressed EPCR only when modified with a stereospecific isomer of LBPA. EPCR has many similarities to the structurally related non-classical major histocompatibility complex class-I like CD1 molecules, which present bacterial lipids to the immune system [91,95]. Interaction of lipid-binding aPL with the EPCR-phospholipid complex depends on the exchange of phosphatidylcholine which typically occupies the lipid-binding groove of EPCR with LBPA typically present in the endo-lysosome. For this exchange, endosomal cycling of EPCR is required. Thus, lipid-binding aPL could not bind to cells with an EPCR-mutant defective in endosomal cycling [91]. However, when LBPA is added to the cell culture media it readily binds to the lipid binding groove of cycling defective EPCR and thereby restores binding of aPL. Additional specificity of this process is apparently obtained by the structure of the lipid-binding groove of EPCR which can only accommodate certain phospholipids. This is illustrated by the fact that only the (S,R) stereoisomer of LBPA, but not the (S,S) isomer or hemi-LBPA could rescue aPL-binding [91].

It should be noted that the majority of APS patients harbor aPL which bind to EPCR/LBPA and activate endosomal NADPH-oxidase [67]. Other cell types are

also activated *via* the EPCR/LBPA pathway. These include endothelial cells and trophoblast cells. On the other hand, lipid-binding aPL do not activate platelets *in vitro* [89,96]. Anti-β2GPI without CL binding activity do not bind to EPCR/LBPA and do not induce the cellular responses observed with lipid-binding aPL [67].

There are only few data on humoral effects of lipid binding aPL. The two monoclonal aPL described above have lupus anticoagulant activity which is probably weak [78]. Other reports confirming lupus anticoagulant activity of lipid-binding aPL have been published [79]. This is important to keep in mind, when discussing the epitope specificity of lupus anticoagulant which has been the topic of active research (see above).

The observed *in vitro* effects described above are supposedly procoagulant and proinflammatory. The obvious question is whether these lipid-binding aPL are pathogenic *in vivo*. This was shown in a mouse model of venous thrombosis in 2016 [22]. In this study mice deficient in NADPH-oxidase 2 were protected from the prothrombotic effects of aPL. This indicated that aPL signaling through endosomal NADPH-oxidase was involved. Furthermore, it was shown that monocyte tissue factor played a critical role in mediating the procoagulant effects of lipid-binding aPL. The observations by Ikematsu [88] and Girardi et al. [18,31–33] that lipid-binding aPL induce pregnancy failure in mice were confirmed with another human monoclonal lipid-binding aPL [91]. Further studies with genetically modified mice showed that the EPCR/LBPA pathway identified *in vitro* was responsible for the pathogenic effects observed *in vivo* in mice [91–93]. In fact, all interventions blocking this pathway anywhere from the cell surface downstream to NADPH-oxidase protected animals from the procoagulant effects of lipid-binding aPL.

Interestingly, this also includes hydroxychloroquine (HCQ), a drug which has been considered for the treatment and prevention of thrombotic and in particular obstetric APS [97–99]. HCQ inhibits activation of endosomal NADPH-oxidase by lipid-binding aPL and protects mice from thrombosis induction by these aPL [100]. There are only few other *in vivo* studies analyzing HCQ in experimental APS. Edwards et al. showed in the femoral vein pinch model that HCQ reduces thrombus size in mice injected with aPL obtained from an APS patient [101]. Unfortunately, there is no conclusive information regarding the specificity of aPL injected. Miranda et al. investigated the effects of HCQ *in vivo* in a mouse model of APS and *in vitro* [102]. aPL-IgG were obtained by protein G adsorption from patients with high levels of anti-β2GPI and aCL. In this study niflumic acid, an inhibitor of endosomal NADPH-oxidase [90,103] prevented several *in vitro* effects of aPL-IgG. This is strong evidence that at least part of the observed effects are mediated by lipid-binding aPL *via* EPCR/LBPA, because rapid activation (within 2 to 4 h) of procoagulant and proinflammatory mediators by endosomal NADPH-oxidase has only been observed with lipid-binding aPL. Nevertheless, it cannot be ruled out that HCQ also affects anti-β2GPI and thereby further reduces thrombus formation in this *in vivo* model which was assessed 7 days after injection of aPL-IgG. Several *in vitro* effects might include target anti-β2GPI, e.g. inhibition of platelet activation [104], or protection of the annexin A5 anticoagulant shield [105,106]. Further work on the mechanisms underlying the apparent clinical benefit of HCQ in APS patients is needed.

Another important effect of lipid-binding aPL is the induction of massive IFNα secretion by plasmacytoid dendritic cells [90,91]. *In vitro* this is sufficient to stimulate B1-lymphocytes in coculture to secrete large amounts of aPL without T-cell help [91]. *In vivo* immunization with lipid-binding aPL induces aPL production within 1-2 weeks. This phenomenon has been described already in 1992 and was proposed as an animal model for APS, because mice immunized with aPL developed manifestations of APS [16]. At this time, it was erroneously interpreted as consequence of the idiotypic network [107,108]. The observation that lipid-binding aPL are secreted by B1-lymphocytes *in vivo* and also *in vitro* rather suggests that they belong to the repertoire of natural antibodies even though they are of the IgG-isotype [91,109]. Thus lipid-binding aPL can induce their own production in a positive feed-back loop. This effect depends on secretion of IFNα by plasmacytoid dendritic cells after activation *via* EPCR/LBPA and further stimulation *via* TLR7. In lupus prone Mrl/lpr mice blockade of the EPCR/LBPA pathway reduces the production of aPL and also the progression of renal pathology [91]. Recently, it was shown that viral infections, e.g. Covid-19 induces production of lipid-binding aPL and circulating B1-lymphocytes with phospholipid reactivity were detected in hospitalized patients. Contrary to common belief, these infections associated aPL accelerate thrombus formation in an *in vivo* mouse model underscoring the pathogenic potential of lipid-binding aPL [110].

3.2. Differences of lipid-binding aPL to anti-β2GPI

According to the available data lipid-binding aPL target other cell surface structures than anti-β2GPI, and induce different signaling, and cellular effects. With respect to the latter, several molecular targets on the cell surface for anti-β2GPI have been described. These

are apolipoprotein E-receptor 2 (apoER2, also called low-density lipoprotein receptor related protein 8–lrp8) [111,112], toll-like receptors (TLR) 4 [113,114], 2 [115,116], and annexin A2 [117] with sometimes conflicting data between different studies. The reasons for different results are not well established but are probably related to the more complex nature of interactions. Several lines of evidence suggest that rather than anti-β2GPI alone, the complex of β2GPI bound to the cell surface and anti-β2GPI is the relevant agonist. Cellular responses appear to require the interaction of several proteins. Accordingly, not only the source of the autoantibodies but also of β2GPI may affect results. A major conceptual difficulty for studies analyzing the role of cell membrane TLRs like TLR2 and TLR4 is the high affinity of β2GPI to LPS and perhaps other bacterial lipid mediators [118].

The best evidence indicates that apoER2 is a cell surface receptor involved in signal induction by anti-β2GPI. The first observation that apoER2 may mediate anti-β2GPI signaling was obtained in platelets. Lutters et al. showed that platelet adhesion and deposition to collagen under flow conditions was increased by anti-β2GPI [111]. They showed that dimeric β2GPI bound to apoER2 and induced platelet adhesion. This could be prevented by receptor associated protein, an inhibitor of ligand binding to members of the LDL-receptor family. The authors hypothesized that anti-β2GPI promotes dimerization of β2GPI and thereby platelet activation. The interaction of anti-β2GPI with apoER2 in platelets was further elucidated in a series of seminal studies by this group in the following years [112,119–122]. As mentioned above, currently available data show that lipid-binding aPL do not activate platelets [96]. This difference is perhaps of clinical relevance and deserves further attention.

In 2011 it was shown by two groups that apoER2$^{-/-}$ mice were protected from the prothrombotic effects of anti-β2GPI [21,26]. The latter study showed that anti-β2GPI inhibited endothelial nitric oxide synthase (eNOS). This effect was not observed in apoER2$^{-/-}$ cells. It should be mentioned here that another group found no effect of apoER2 knock-down on activation of HUVEC by anti-β2GPI [117] underscoring the complexity of interaction of cell surface proteins with β2GPI and anti-β2GPI. Further investigation of downstream signaling from apoER2 showed that activation of protein phosphatase A2 (PPA2) lead to dephosphorylation and inactivation of Akt and eNOS which ultimately lead to increased thrombophilia [123]. In any case, it is obvious that signal transduction processes induced by anti-β2GPI and lipid-binding aPL are fundamentally different.

Involvement of apoER2 in pregnancy failure induced by anti-β2GPI also appeared to depend on apoER2 mediated signaling [124]. However, this study could not differentiate between the role of apoER2 in induction of proinflammatory cytokines, e.g. TNFα in the dam [32] on the one hand and interference with trophoblast function on the other. In a further study from this group, it was conclusively shown that selective deletion of apoER2 from trophoblast cells prevented fetal resorption in mice treated with aPL/anti-β2GPI [125]. aPL-induced hypertension was also prevented in these mice. Similar to the thrombosis model discussed above, PPA2 was activated *via* apoER2. This study provided new evidence that the interaction of anti-β2GPI with β2GPI and apoER2 on the trophoblast can cause pregnancy failure including preeclampsia in mice. It does not conclusively answer the question why apoER2± embryos are protected in apoER2$^{-/-}$ mice. Further work to delineate the role of maternal and embryonic mechanisms of the pathogenicity of anti-β2GPI is required.

3.3. Summary comments on the pathogenesis of APS

As described in the previous sections, there are a plethora of effects of different aPL on cells and the clotting system. At first glance it may appear as if each aPL-effect leading to manifestations of APS in experimental animals stands for itself. This oftentimes leads to the conclusion that targeting a specific pathway might prevent APS in humans. However, one should keep in mind that the experimental systems are highly artificial and designed to lead to much more rapid thrombus formation than observed clinically. Thus, all thrombosis models require an additional trigger, e.g. vascular injury or induction of a systemic procoagulant state [38]. Depending on the model, thrombus formation follows significantly different time courses and thrombi may be transient or permanent. aPL used in animal models are oftentimes selected, e.g. affinity purified or monoclonal aPL and do not represent the whole spectrum of aPL found in APS patients. Under these conditions, it is impossible to rule out the possibility that pathways other than those investigated in the respective study may contribute to pathology.

In summary, it is evident that both lipid-binding aPL and anti-β2GPI mediate a prothrombotic state and pregnancy complications in mouse models. They do this by activation of different cellular signaling pathways. Since many patients harbor both types of aPL including most likely dual reactivity aPL, this raises the question, if the antibodies have additive effects or

even amplify their respective effects. To our knowledge there have been no *in vivo* studies which specifically addressed this question.

4. Epidemiology and clinical manifestations

As outlined in the section on APS pathogenesis, lipid-binding aPL induce potentially pathogenic responses in leukocytes, endothelial cells, and trophoblasts. *In vivo* they cause fetal resorption in mice and accelerate thrombus formation. These are the generally accepted criteria for considering an aPL as likely pathogenic in APS. In other words, experimental data do not lend support to the concept that lipid-binding aPL are irrelevant for APS. Thus, the only evidence for this concept could come from human studies, such as clinical or epidemiological studies.

As discussed above, the current laboratory tests for aCL cannot distinguish between lipid-binding aPL and anti-β2GPI simply for methodological reasons (Figure 1) [65,68]. Therefore, it is impossible to interpret epidemiologic data on aCL obtained in the past with regard to the concept of "cofactor-dependence". We have shown previously that all IgG fractions isolated from APS patients positive for aCL IgG but negative for anti-β2GPI react with cardiolipin in the absence of any other proteins [67]. This indicates that most if not all patients which are positive only for aCL IgG have true lipid-binding aPL. In addition, our data suggest that the majority of patients positive for both aCL and anti-β2GPI have lipid-binding aPL besides anti-β2GPI. While this obviously requires confirmation in a larger cohort, it proves that the presence of lipid-binding aPL in APS patients is not a rare event. In the APS Action clinical database [126] 4.1% of 660 patients with complete aPL data are positive for aCL only and another 13.8% are positive for aCL and LA. These patients most likely have lipid-binding aPL. Another 8.2% have only anti-β2GPI with or without LA, i.e. they most likely do not have lipid-binding aPL. Taking our experience described above into account, the remaining almost three quarters of patients likely also have lipid-binding aPL. In EUROAPS, a cohort of patients with obstetric APS, the percentage of patients with isolated aCL is 22.4% (14.9% IgG and/or IgM) and with isolated anti-β2GPI is 12.6% (8.1% IgG and/or IgM) [127].

It is against this background that epidemiologic studies on the relationship of specific aPL and APS manifestations should be evaluated. Epidemiologic studies regarding the role of aPL for thrombosis and pregnancy morbidity have been systematically reviewed in the past [128–131]. A major shortcoming discussed in all these reviews is the paucity of high quality prospective studies. The first systematic review was published in 2003 by Galli et al. [128]. They combined 25 studies from 1988 through 2000 with 4,184 patients and 3,151 controls. There were 9 prospective studies with 1,976 (range 21-412) patients. Studies were heterogeneous with three studies analyzing recurrent VTE in patients after discontinuation of anticoagulation ($n = 586$). All three studies showed an association of recurrence with aCL and/or LA. Another three studies were performed in SLE patients ($n = 817$) which showed no association of aCL with thrombosis, while LA was significantly associated with future events in one study and prolonged aPTT in another. Two studies on stroke patients suggested an increased risk of recurrent stroke with aCL. One study in unselected patients positive for aCL showed an increased risk for thrombosis but not pregnancy failure. Thus, the studies suggest that the relevance of aCL may be higher with respect to recurrent than first events. In any case it should be kept in mind that selection of patients and endpoints heavily influence the results. In addition, these early studies do not provide information on the presence of anti-β2GPI. Notably, this early review points already toward a higher clinical relevance of positive LA compared to aCL.

A second systematic review on the role of anti-β2GPI and anti-prothrombin published in 2003 did not identify any prospective study among 28 studies on this topic [129]. Accordingly, the authors concluded that it was difficult to establish the value of anti-β2GPI and anti-prothrombin as independent risk factors. As described above, the available studies were again heterogeneous with respect to patient and endpoint selection.

In 2014, a third systematic review was published which summarized thrombosis risk in persons without SLE [130]. The authors identified 28 case-control and two cohort studies dating from 1990 to 2010. Twenty-two studies were prospective. The combined data suggest that there is a substantial risk for venous thrombosis in persons with LA, and a slightly increased risk for persons with aCL, which may be overestimated by inclusion of low quality studies. The presence of anti-β2GPI led to a higher odds ratio than aCL (1.61 vs. 1.46). However, this was not significant due to a wide 95% confidence interval. Also, other aPL subtypes had no significant association with venous thrombosis. The authors could not exclude that this negative result was related to the smaller number of studies and available data for these aPL. Interestingly the association with arterial events is similar for all aPL subtypes tested, i.e. LA, aCL, anti-β2GPI, aPT, and aPS. The major limitation

of this meta-analysis was again heterogeneity in study design, and particularly aPL-assays and cutoffs.

Recently, a further systematic review of the role of anti-β2GPI was published [131]. The authors limited their meta-analysis to prospective studies devoted to APS manifestations in patients with anti-β2GPI and identified only six studies before 2020, four of these dealt with pregnancy outcomes and two with thrombosis. The authors conclude that there was only a weak association of anti-β2GPI with thrombosis and inconsistent results for pregnancy outcomes with an overall very low quality of evidence.

The overarching theme of all systematic reviews is the paucity of large prospective studies of the role of aPL as risk factors for thrombosis and/or pregnancy failure. For the subgroup of lipid-binding aPL the data are even less satisfactory, because the available anticardiolipin assays cannot differentiate between lipid-binding aPL and anti-β2GPI and therefore cannot provide quantitative data on lipid-binding aPL.

The two large register studies APS ACTION and EUROAPS [126,127] mostly provided relevant cross-sectional data but will hopefully generate prospective data in the future. In particular, in APS ACTION there are approximately 20% of aPL positive patients without clinical manifestations of APS which will permit an estimate of the risk conferred by different aPL patterns. The same applies for recurrent events in the 80% patients with APS. An initial analysis of pregnancy outcomes has already been performed [132]. However, due to the small number of APS-related pregnancy morbidity ($n = 9$) no firm conclusions regarding the relevance of different aPL could be drawn, even though the highest frequency of endpoints was observed in patients which were positive for LA only.

Regarding the pathogenic potential of lipid-binding aPL, a case-control study in Tanzania by de Mast et al. [133] found that besides LA, only anticardiolipin IgG were significantly more common in the stroke group. While the odds ratio for LA was calculated with 20.8 (95% confidence interval 7.2-60.5), it was 2.1 (95% CI 1.0-4.3) for aCL IgG. Intriguingly, the association of aCL with stroke disappeared when data were adjusted for treponemal antibodies. As the authors state, it is well established that anticardiolipin are commonly present in patients with treponemal antibodies. The authors argue that these anticardiolipin are not considered pathogenic aPL and therefore adjusted for the presence of treponemal antibodies. After adjustment the residual association of anticardiolipin and stroke was no longer significant. Strictly speaking, this procedure is based on a classic circular argument. Rather, this study indicates that lipid-binding aPL which were long considered as infection associated and benign, might be pathogenically active in APS. Along these lines, it should be kept in mind that aPL are commonly found in the context of viral and bacterial infections. Interestingly, several infections known to be accompanied by aPL have an increased thrombosis risk [134–140]. Currently, it remains an unanswered question whether the association of aPL and thrombotic events in certain infectious diseases is coincidental or causal. There are only two investigations of the potential pathogenicity of aPL induced by infections in Covid-19. These studies show that aPL which occur in the course of Covid-19 are pathogenic *in vitro* and in mouse models [110,141]. Interestingly, in one study lipid-binding aPL were identified by analysis of IgG-fractions in a cardiolipin specific ELISA [110], while in the other study pathogenic anti-PS/PT were found [141].

In summary, current data show that the presence of aPL is associated with an increased risk of (recurrent) thrombosis or pregnancy complications. A positive result in more than one aPL species appears to increase the relative risk [142–146], even though this has not been undisputed [147]. The reason for this latter observation remains speculative. One rather simple explanation might be that positivity in more than one assay raises specificity of the laboratory result and thus, increases the observed relative risk. Another attractive hypothesis is based on the available *in vitro* data which show that lipid-binding aPL and anti-β2GPI induce different cellular responses which may be additive *in vivo*. In particular, the activation of platelets by anti-β2GPI may increase thrombosis risk considerably, because this cell type is not affected by lipid-binding aPL. Further work is clearly required to better understand the combined effects of different aPL.

5. Conclusions

Based on the observation that some aPL of APS patients do not bind to cardiolipin in the absence of β2GPI or other protein cofactors, lipid-binding aPL were considered to be irrelevant for the pathogenesis of APS, but rather a corollary of infection. However, to date there is ample evidence that APS patients harbor lipid-binding aPL and that these aPL induce prothrombotic and proinflammatory pathways in several cell types. Furthermore, lipid-binding aPL have been shown to accelerate thrombus formation and induce pregnancy failure in mouse models. Genetically modified mice with defects in the signaling pathway of lipid-binding aPL were consistently protected from their detrimental effects. Furthermore, in the case of Covid-19, lipid-binding aPL have been isolated from

patients which induce thrombosis in mice, calling into question the concept that infection associated aPL are harmless. The interaction and perhaps cooperation of lipid-binding and protein-binding aPL in the pathogenesis of APS should be a topic of APS research in the future.

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ORCID

Nadine Müller-Calleja http://orcid.org/0000-0002-1985-7931
Wolfram Ruf http://orcid.org/0000-0002-0409-7474
Karl J. Lackner http://orcid.org/0000-0002-6064-2166

References

[1] Schreiber K, Sciascia S, de Groot PG, et al. Antiphospholipid syndrome. Nat Rev Dis Primers. 2018;4(1):17103. doi: 10.1038/nrdp.2017.103.
[2] Garcia D, Erkan D. Diagnosis and management of the antiphospholipid syndrome. N Engl J Med. 2018;378(21):2010–2021. doi: 10.1056/NEJMra1705454.
[3] Duarte-García A, Pham MM, Crowson CS, et al. The epidemiology of antiphospholipid syndrome: a population-based study. Arthritis Rheumatol. 2019;71(9):1545–1552. doi: 10.1002/art.40901.
[4] Dabit JY, Valenzuela-Almada MO, Vallejo-Ramos S, et al. Epidemiology of antiphospholipid syndrome in the general population. Curr Rheumatol Rep. 2022;23(12):85. doi: 10.1007/s11926-021-01038-2.
[5] Cervera R, Rodríguez-Pintó I, Espinosa G. The diagnosis and clinical management of the catastrophic antiphospholipid syndrome: a comprehensive review. J Autoimmun. 2018;92:1–11. doi: 10.1016/j.jaut.2018.05.007.
[6] Harris EN, Gharavi AE, Boey ML, et al. Anticardiolipin antibodies: detection by radioimmunoassay and association with thrombosis in systemic lupus erythematosus. Lancet. 1983;2(8361):1211–1214. doi: 10.1016/s0140-6736(83)91267-9.
[7] Hughes GR. The anticardiolipin syndrome. Clin Exp Rheumatol. 1985;3(4):285–286.
[8] McNeil HP, Simpson RJ, Chesterman CN, et al. Anti-phospholipid antibodies are directed against a complex antigen that includes a lipid-binding inhibitor of coagulation: β2-glycoprotein I (apolipoprotein H). Proc Natl Acad Sci U S A. 1990;87(11):4120–4124. doi: 10.1073/pnas.87.11.4120.
[9] Galli M, Comfurius P, Maassen C, et al. Anticardiolipin antibodies (ACA) directed not to cardiolipin but to a plasma protein cofactor. Lancet. 1990;335(8705):1544–1547. doi: 10.1016/0140-6736(90)91374-j.
[10] Matsuura E, Igarashi Y, Fujimoto M, et al. Anticardiolipin cofactor(s) and differential diagnosis of autoimmune disease. Lancet. 1990;336(8708):177–178. doi: 10.1016/0140-6736(90)91697-9.
[11] Arvieux J, Roussel B, Jacob MC, et al. Measurement of anti-phospholipid antibodies by ELISA using β2-glycoprotein I as an antigen. J Immunol Methods. 1991;143(2):223–229. doi: 10.1016/0022-1759(91)90047-j.
[12] Lee SL, Sanders M. A disorder of blood coagulation in systemic lupus erythematosus. J Clin Invest. 1955;34(12):1814–1822. doi: 10.1172/JCI103237.
[13] Vlachoyiannopoulos PG, Samarkos M, Sikara M, et al. Antiphospholipid antibodies: laboratory and pathogenetic aspects. Crit Rev Clin Lab Sci. 2007;44(3):271–338. doi: 10.1080/10408360601079549.
[14] Branch DW, Dudley DJ, Mitchell MD, et al. Immunoglobulin G fractions from patients with antiphospholipid antibodies cause fetal death in BALB/c mice: a model for autoimmune fetal loss. Am J Obstet Gynecol. 1990;163(1 Pt 1):210–216. doi: 10.1016/s0002-9378(11)90700-5.
[15] Blank M, Cohen J, Toder V, et al. Induction of anti-phospholipid syndrome in naive mice with mouse lupus monoclonal and human polyclonal anti-cardiolipin antibodies. Proc Natl Acad Sci U S A. 1991;88(8):3069–3073. doi: 10.1073/pnas.88.8.3069.
[16] Bakimer R, Fishman P, Blank M, et al. Induction of primary antiphospholipid syndrome in mice by immunization with a human monoclonal anticardiolipin antibody (H-3). J Clin Invest. 1992;89(5):1558–1563. doi: 10.1172/JCI115749.
[17] Pierangeli SS, Barker JH, Stikovac D, et al. Effect of human IgG antiphospholipid antibodies on an in vivo thrombosis model in mice. Thromb Haemost. 1994;71(05):670–674. doi: 10.1055/s-0038-1642501.
[18] Girardi G, Berman J, Redecha P, et al. Complement C5a receptors and neutrophils mediate fetal injury in the antiphospholipid syndrome. J Clin Invest. 2003;112(11):1644–1654. doi: 10.1172/JCI200318817.
[19] Romay-Penabad Z, Montiel-Manzano MG, Shilagard T, et al. Annexin A2 is involved in antiphospholipid antibody-mediated pathogenic effects in vitro and in vivo. Blood. 2009;114(14):3074–3083. doi: 10.1182/blood-2008-11-188698.
[20] Nishimura M, Nii T, Trimova G, et al. The NF-κB specific inhibitor DHMEQ prevents thrombus formation in a mouse model of antiphospholipid syndrome. J Nephropathol. 2013;2(2):114–121. doi: 10.5812/nephropathol.10112.
[21] Romay-Penabad Z, Aguilar-Valenzuela R, Urbanus RT, et al. Apolipoprotein E receptor 2 is involved in the thrombotic complications in a murine model of the antiphospholipid syndrome. Blood. 2011;117(4):1408–1414. doi: 10.1182/blood-2010-07-299099.
[22] Manukyan D, Müller-Calleja N, Jäckel S, et al. Cofactor-independent human antiphospholipid antibodies induce venous thrombosis in mice. J Thromb Haemost. 2016;14(5):1011–1020. doi: 10.1111/jth.13263.
[23] Pierangeli SS, Girardi G, Vega-Ostertag M, et al. Requirement of activation of complement C3 and C5

for antiphospholipid antibody-mediated thrombophilia. Arthritis Rheum. 2005;52(7):2120–2124. doi: 10.1002/art.21157.

[24] Pierangeli SS, Vega-Ostertag ME, Raschi E, et al. Toll-like receptor and antiphospholipid mediated thrombosis: in vivo studies. Ann Rheum Dis. 2007;66(10):1327–1333. doi: 10.1136/ard.2006.065037.

[25] Romay-Penabad Z, Liu XX, Montiel-Manzano G, et al. C5a receptor-deficient mice are protected from thrombophilia and endothelial cell activation induced by some antiphospholipid antibodies. Ann N Y Acad Sci. 2007;1108(1):554–566. doi: 10.1196/annals.1422.058.

[26] Ramesh S, Morrell CN, Tarango C, et al. Antiphospholipid antibodies promote leukocyte-endothelial cell adhesion and thrombosis in mice by antagonizing eNOS via β2GPI and apoER2. J Clin Invest. 2011;121(1):120–131. doi: 10.1172/JCI39828.

[27] Carrera-Marín A, Romay-Penabad Z, Papalardo E, et al. C6 knockout mice are protected from thrombophilia mediated by antiphospholipid antibodies. Lupus. 2012;21(14):1497–1505. doi: 10.1177/0961203312458839.

[28] Proulle V, Furie RA, Merrill-Skoloff G, et al. Platelets are required for enhanced activation of the endothelium and fibrinogen in a mouse thrombosis model of APS. Blood. 2014;124(4):611–622. doi: 10.1182/blood-2014-02-554980.

[29] Kolyada A, Porter A, Beglova N. Inhibition of thrombotic properties of persistent autoimmune anti-β2GPI antibodies in the mouse model of antiphospholipid syndrome. Blood. 2014;123(7):1090–1097. doi: 10.1182/blood-2013-08-520882.

[30] Holers VM, Girardi G, Mo L, et al. Complement C3 activation is required for antiphospholipid antibody-induced fetal loss. J Exp Med. 2002;195(2):211–220. doi: 10.1084/jem.200116116.

[31] Girardi G, Redecha P, Salmon JE. Heparin prevents antiphospholipid antibody-induced fetal loss by inhibiting complement activation. Nat Med. 2004;10(11):1222–1226. doi: 10.1038/nm1121.

[32] Berman J, Girardi G, Salmon JE. TNF-alpha is a critical effector and a target for therapy in antiphospholipid antibody-induced pregnancy loss. J Immunol. 2005;174(1):485–490. doi: 10.4049/jimmunol.174.1.485.

[33] Redecha P, Franzke CW, Ruf W, et al. Neutrophil activation by the tissue factor/factor VIIa/PAR2 axis mediates fetal death in a mouse model of antiphospholipid syndrome. J Clin Invest. 2008;118(10):3453–3461. doi: 10.1172/JCI36089.

[34] Matalon ST, Shoenfeld Y, Blank M, et al. Antiphosphatidylserine antibodies affect rat yolk sacs in culture: a mechanism for fetal loss in antiphospholipid syndrome. Am J Reprod Immunol. 2004;51(2):144–151. doi: 10.1046/j.8755-8920.2003.00140.x.

[35] Fischetti F, Durigutto P, Pellis V, et al. Thrombus formation induced by antibodies to β2-glycoprotein I is complement dependent and requires a priming factor. Blood. 2005;106(7):2340–2346. doi: 10.1182/blood-2005-03-1319.

[36] Halperin R, Elhayany A, Ben-Hur H, et al. Pathomorphologic and immunohistochemical study on the devastation of rat embryos by antiphospholipid antibody positive serum. Am J Reprod Immunol. 2008;60(6):523–528. doi: 10.1111/j.1600-0897.2008.00650.x.

[37] Durigutto P, Grossi C, Borghi MO, et al. New insight into antiphospholipid syndrome: antibodies to β2glycoprotein I-domain 5 fail to induce thrombi in rats. Haematologica. 2019;104(4):819–826. doi: 10.3324/haematol.2018.198119.

[38] Gandhi AA, Estes SK, Rysenga CE, et al. Understanding the pathophysiology of thrombotic APS through animal models. Int J Mol Sci. 2021;22(5):2588. doi: 10.3390/ijms22052588.

[39] Wilson WA, Gharavi AE, Koike T, et al. International consensus statement on preliminary classification criteria for definite antiphospholipid syndrome: report of an international workshop. Arthritis Rheum. 1999;42(7):1309–1311. doi: 10.1002/1529-0131(199907)42:7<1309::AID-ANR1>3.0.CO;2-F.

[40] Miyakis S, Lockshin MD, Atsumi T, et al. International consensus statement on an update of the classification criteria for definite antiphospholipid syndrome (APS). J Thromb Haemost. 2006;4(2):295–306. doi: 10.1111/j.1538-7836.2006.01753.x.

[41] Barbhaiya M, Zuily S, Naden R, et al. 2023 ACR/EULAR antiphospholipid syndrome classification criteria. Ann Rheum Dis. 2023;82(10):1258–1270. doi: 10.1136/ard-2023-224609.

[42] De Groot PG, Urbanus RT. The significance of autoantibodies against β2-glycoprotein I. Blood. 2012;120(2):266–274. doi: 10.1182/blood-2012-03-378646.

[43] Sammaritano LR. Antiphospholipid syndrome. Best Pract Res Clin Rheumatol. 2020;34(1):101463. doi: 10.1016/j.berh.2019.101463.

[44] Arreola-Diaz R, Majluf-Cruz A, Sanchez-Torres LE, et al. The pathophysiology of the antiphospholipid syndrome: a perspective from the blood coagulation system. Clin Appl Thromb Hemost. 2022;28:10760296221088576. doi: 10.1177/10760296221088576.

[45] Knight JS, Kanthi Y. Mechanisms of immunothrombosis and vasculopathy in antiphospholipid syndrome. Semin Immunopathol. 2022;44(3):347–362. doi: 10.1007/s00281-022-00916-w.

[46] Grossi C, Capitani N, Benagiano M, et al. Beta 2 glycoprotein I and neutrophil extracellular traps: potential bridge between innate and adaptive immunity in antiphospholipid syndrome. Front Immunol. 2022;13:1076167. doi: 10.3389/fimmu.2022.1076167.

[47] Wassermann A, Neisser A, Bruck C. Eine serodiagnostische reaktion bei syphilis. Dtsch Med Wochenschr. 1906;32(19):745–746. doi: 10.1055/s-0028-1142018.

[48] Pangborn M. Isolation and purification of a serologically active phospholipid from beef heart. J Biol Chem. 1942;143(1):247–256. doi: 10.1016/S0021-9258(18)72683-5.

[49] Moore JE, Mohr CF. Biologically false positive serologic tests for syphilis; type, incidence, and cause. J Am Med Assoc. 1952;150(5):467–473. doi: 10.1001/jama.1952.03680050033010.

[50] Harvey AM, Shulman LE, Tumulty PA, et al. Systemic lupus erythematosus: review of the literature and clinical analysis of 138 cases. Medicine (Baltimore). 1954;33(4):291–437. doi: 10.1097/00005792-195412000-00001.

[51] Harmer IJ, Loizou S, Thompson KM, et al. A human monoclonal antiphospholipid antibody that is representative of serum antibodies and is germline

encoded. Arthritis Rheum. 1995;38(8):1068–1076. doi: 10.1002/art.1780380808.
[52] Buschmann C, Fischer C, Ochsenhirt V, et al. Generation and characterization of three monoclonal IgM antiphospholipid antibodies recognizing different phospholipid antigens. Ann N Y Acad Sci. 2005;1051(1):240–254. doi: 10.1196/annals.1361.065.
[53] Prinz N, Häuser F, Lorenz M, et al. Structural and functional characterization of a human IgG monoclonal antiphospholipid antibody. Immunobiology. 2011;216(1-2):145–151. doi: 10.1016/j.imbio.2010.05.001.
[54] Devreese KMJ, Ortel TL, Pengo V, et al. Laboratory criteria for antiphospholipid syndrome: communication from the SSC of the ISTH. J Thromb Haemost. 2018;16(4):809–813. doi: 10.1111/jth.13976.
[55] Matsuura E, Igarashi Y, Fujimoto M, et al. Heterogeneity of anticardiolipin antibodies defined by the anticardiolipin cofactor. J Immunol. 1992;148(12):3885–3891. doi: 10.4049/jimmunol.148.12.3885.
[56] Pierangeli SS, Harris EN, Davis SA, et al. Beta 2-glycoprotein 1 (beta 2GP1) enhances cardiolipin binding activity but is not the antigen for antiphospholipid antibodies. Br J Haematol. 1992;82(3):565–570. doi: 10.1111/j.1365-2141.1992.tb06468.x.
[57] Forastiero RR, Martinuzzo ME, Kordich LC, et al. Reactivity to beta2 glycoprotein I clearly differentiates anticardiolipin antibodies from antiphospholipid syndrome and syphilis. Thromb Haemost. 1996;75(05):717–720. doi: 10.1055/s-0038-1650354.
[58] Schwarzenbacher R, Zeth K, Diederichs K, et al. Crystal structure of human beta2-glycoprotein I: implications for phospholipid binding and the antiphospholipid syndrome. Embo J. 1999;18(22):6228–6239. doi: 10.1093/emboj/18.22.6228.
[59] Hammel M, Schwarzenbacher R, Gries A, et al. Mechanism of the interaction of beta(2)-glycoprotein I with negatively charged phospholipid membranes. Biochemistry. 2001;40(47):14173–14181. doi: 10.1021/bi0114372.
[60] Hammel M, Kriechbaum M, Gries A, et al. Solution structure of human and bovine beta(2)-glycoprotein I revealed by small-angle X-ray scattering. J Mol Biol. 2002;321(1):85–97. doi: 10.1016/s0022-2836(02)00621-6.
[61] Polz E, Kostner GM. The binding of β2-glycoprotein-I to human serum lipoproteins: distribution among density fractions. FEBS Lett. 1979;102(1):183–186. doi: 10.1016/0014-5793(79)80955-2.
[62] McDonnell T, Wincup C, Buchholz I, et al. The role of beta-2-glycoprotein I in health and disease associating structure with function: more than just APS. Blood Rev. 2020;39:100610. doi: 10.1016/j.blre.2019.100610.
[63] Yasuda S, Tsutsumi A, Chiba H, et al. β2-glycoprotein I deficiency: prevalence, genetic background and effects on plasma lipoprotein metabolism and hemostasis. Atherosclerosis. 2000;152(2):337–346. doi: 10.1016/s0021-9150(99)00496-7.
[64] Giannakopoulos B, Mirarabshahi P, Qi M, et al. Deletion of the antiphospholipid syndrome autoantigen β2 -glycoprotein I potentiates the lupus autoimmune phenotype in a toll-like receptor 7-mediated murine model. Arthritis Rheumatol. 2014;66(8):2270–2280. doi: 10.1002/art.38646.
[65] Lackner KJ, Müller-Calleja N. Pathogenesis of antiphospholipid syndrome: recent insights and emerging concepts. Expert Rev Clin Immunol. 2019;15(2):199–209. doi: 10.1080/1744666X.2019.1546578.
[66] Roggenbuck D, Borghi MO, Somma V, et al. Antiphospholipid antibodies detected by line immunoassay differentiate among patients with antiphospholipid syndrome, with infections and asymptomatic carriers. Arthritis Res Ther. 2016;18(1):111. doi: 10.1186/s13075-016-1018-x.
[67] Müller-Calleja N, Hollerbach A, Häuser F, et al. Antiphospholipid antibody-induced cellular responses depend on epitope specificity: implications for treatment of antiphospholipid syndrome. J Thromb Haemost. 2017;15(12):2367–2376. doi: 10.1111/jth.13865.
[68] Devreese KMJ, Ortel TL, Pengo V, et al. Laboratory criteria for antiphospholipid syndrome: reply. J Thromb Haemost. 2018;16(10):2117–2119. doi: 10.1111/jth.14238.
[69] Levy RA, de Meis E, Pierangeli S. An adapted ELISA method for differentiating pathogenic from nonpathogenic aPL by beta 2 glycoprotein dependency anticardiolipin assay. Thromb Res. 2004;114(5-6):573–577. doi: 10.1016/j.thromres.2004.06.032.
[70] Rapaport SI, Ames SB, Duvall BJ. A plasma coagulation defect in systemic lupus erythematosus arising from hypoprothrombinemia combined with antiprothrombinase activity. Blood. 1960;15(2):212–227. doi: 10.1182/blood.V15.2.212.212.
[71] Bajaj SP, Rapaport SI, Fierer DS, et al. A mechanism for the hypoprothrombinemia of the acquired hypoprothrombinemia–lupus anticoagulant syndrome. Blood. 1983;61(4):684–692. doi: 10.1182/blood.V61.4.684.684.
[72] Urbanus RT, Siegerink B, Roest M, et al. Antiphospholipid antibodies and risk of myocardial infarction and ischaemic stroke in young women in the RATIO study: a case control study. Lancet Neurol. 2009;8(11):998–1005. doi: 10.1016/S1474-4422(09)70239-X.
[73] Fabris M, Giacomello R, Poz A, et al. The introduction of anti-phosphatidylserine/prothrombin autoantibodies in the laboratory diagnostic process of anti-phospholipid antibody syndrome: 6 months of observation. Auto Immun Highlights. 2014;5(2):63–67. doi: 10.1007/s13317-014-0061-3.
[74] Pengo V, Testa S, Martinelli I, et al. Incidence of a first thromboembolic event in carriers of isolated lupus anticoagulant. Thromb Res. 2015;135(1):46–49. doi: 10.1016/j.thromres.2014.10.013.
[75] Yin D, de Groot PG, Ninivaggi M, et al. Clinical relevance of isolated lupus anticoagulant positivity in patients with thrombotic antiphospholipid syndrome. Thromb Haemost. 2021;121(9):1220–1227. doi: 10.1055/a-1344-4271.
[76] Zhu M, Olee T, Le DT, et al. Characterization of IgG monoclonal anti-cardiolipin/anti-b2GPI antibodies from two patients with antiphospholipid syndrome reveals three species of antibodies. Br J Haematol. 1999;105(1):102–109.
[77] Nakamura N, Azuma C, Akamizu T, et al. Heterogeneity and diversity of IgM and IgG lupus anticoagulants in an individual with system lupus erythematosus. Biochem Biophys Res Commun. 1994;203(3):1789–1794. doi: 10.1006/bbrc.1994.2394.
[78] von Landenberg C, Lackner KJ, von Landenberg P, et al. Isolation and characterization of two human

monoclonal anti-phospholipid IgG from patients with autoimmune disease. J Autoimmun. 1999;13(2):215–223. doi: 10.1006/jaut.1999.0316.

[79] Gallart T, Benito C, Reverter JC, et al. True anti-anionic phospholipid immunoglobulin M antibodies can exert lupus anticoagulant activity. Br J Haematol. 2002;116(4):875–886. doi: 10.1046/j.0007-1048.2002.03335.x.

[80] Molhoek JE, de Groot PG, Urbanus RT. The lupus anticoagulant paradox. Semin Thromb Hemost. 2018;44(5):445–452. doi: 10.1055/s-0037-1606190.

[81] Arnout J, Wittevrongel C, Vanrusselt M, et al. Beta-2-glycoprotein I dependent lupus anticoagulants form stable bivalent antibody beta-2-glycoprotein I complexes on phospholipid surfaces. Thromb Haemost. 1998;79(1):79–86. doi: 10.1055/s-0037-1614224.

[82] Simmelink MJ, Horbach DA, Derksen RH, et al. Complexes of anti-prothrombin antibodies and prothrombin cause lupus anticoagulant activity by competing with the binding of clotting factors for catalytic phospholipid surfaces. Br J Haematol. 2001;113(3):621–629. doi: 10.1046/j.1365-2141.2001.02755.x.

[83] Simmelink MJ, Derksen RH, Arnout J, et al. A simple method to discriminate between beta2-glycoprotein I- and prothrombin-dependent lupus anticoagulants. J Thromb Haemost. 2003;1(4):740–747. doi: 10.1046/j.1538-7836.2003.00146.x.

[84] Pengo V, Biasiolo A, Pegoraro C, et al. A two-step coagulation test to identify antibeta-glycoprotein I lupus anticoagulants. J Thromb Haemost. 2004;2(5):702–707. doi: 10.1111/j.1538-7836.2004.00712.x.

[85] de Laat HB, Derksen R, Urbanus R, et al. β2-glycoprotein I dependent lupus anticoagulant highly correlates with thrombosis in the antiphospholipid syndrome. Blood. 2004;104(12):3598–3602. doi: 10.1182/blood-2004-03-1107.

[86] Müller-Calleja N, Lackner KJ. Mechanisms of cellular activation in the antiphospholipid syndrome. Semin Thromb Hemost. 2018;44(5):483–492. doi: 10.1055/s-0036-1597290.

[87] Pierangeli SS, Liu SW, Anderson G, et al. Thrombogenic properties of murine anti-cardiolipin antibodies induced by β2 glycoprotein 1 and human immunoglobulin G antiphospholipid antibodies. Circulation. 1996;94(7):1746–1751. doi: 10.1161/01.cir.94.7.1746.

[88] Ikematsu W, Luan FL, La Rosa L, et al. Human anticardiolipin monoclonal autoantibodies cause placental necrosis and fetal loss in BALB/c mice. Arthritis Rheum. 1998;41(6):1026–1039. doi: 10.1002/1529-0131(199806)41:6<1026::AID-ART9>3.0.CO;2-1.

[89] Lackner KJ, von Landenberg C, Barlage S, et al. Analysis of prothrombotic effects of two human monoclonal IgG antiphospholipid antibodies of apparently similar specificity. Thromb Haemost. 2000;83(4):583–588. doi: 10.1055/s-0037-1613867.

[90] Prinz N, Clemens N, Strand D, et al. Antiphospholipid antibodies induce translocation of TLR7 and TLR8 to the endosome in human monocytes and plasmacytoid dendritic cells. Blood. 2011;118(8):2322–2332. doi: 10.1182/blood-2011-01-330639.

[91] Müller-Calleja N, Hollerbach A, Royce J, et al. Lipid presentation by the protein C receptor links coagulation with autoimmunity. Science. 2021;371(6534):eabc0956. doi: 10.1126/science.abc0956.

[92] Müller-Calleja N, Hollerbach A, Ritter S, et al. Tissue factor pathway inhibitor primes monocytes for antiphospholipid antibody-induced thrombosis. Blood. 2019;134(14):1119–1131. doi: 10.1182/blood.2019001530.

[93] Müller-Calleja N, Ritter S, Hollerbach A, et al. Complement C5 but not C3 is expendable for tissue factor activation by cofactor-independent antiphospholipid antibodies. Blood Adv. 2018;2(9):979–986. doi: 10.1182/bloodadvances.2018017095.

[94] Lackner KJ, Manukyan D, Müller-Calleja N. Endosomal redox signaling in the antiphospholipid syndrome. Curr Rheumatol Rep. 2017;19(4):20. doi: 10.1007/s11926-017-0647-7.

[95] Chancellor A, Gadola SD, Mansour S. The versatility of the CD1 lipid antigen presentation pathway. Immunology. 2018;154(2):196–203. doi: 10.1111/imm.12912.

[96] Hollerbach A, Müller-Calleja N, Ritter S, et al. Platelet activation by antiphospholipid antibodies depends on epitope specificity and is prevented by mTOR inhibitors. Thromb Haemost. 2019;119(7):1147–1153. doi: 10.1055/s-0039-1685453.

[97] Tektonidou MG, Andreoli L, Limper M, et al. EULAR recommendations for the management of antiphospholipid syndrome in adults. Ann Rheum Dis. 2019;78(10):1296–1304. doi: 10.1136/annrheumdis-2019-215213.

[98] Sciascia S, Hunt BJ, Talavera-Garcia E, et al. The impact of hydroxychloroquine treatment on pregnancy outcome in women with antiphospholipid antibodies. Am J Obstet Gynecol. 2016;214(2):273.e1-8–273.e8. doi: 10.1016/j.ajog.2015.09.078.

[99] Schmidt-Tanguy A, Voswinkel J, Henrion D, et al. Anti-thrombotic effects of hydroxychloroquine in primary antiphospholipid syndrome patients. J Thromb Haemost. 2013;11(10):1927–1929. doi: 10.1111/jth.12363.

[100] Müller-Calleja N, Manukyan D, Canisius A, et al. Hydroxychloroquine inhibits proinflammatory signalling pathways by targeting endosomal NADPH oxidase. Ann Rheum Dis. 2017;76(5):891–897. doi: 10.1136/annrheumdis-2016-210012.

[101] Edwards MH, Pierangeli S, Liu X, et al. Hydroxychloroquine reverses thrombogenic properties of antiphospholipid antibodies in mice. Circulation. 1997;96(12):4380–4384. doi: 10.1161/01.cir.96.12.4380.

[102] Miranda S, Billoir P, Damian L, et al. Hydroxychloroquine reverses the prothrombotic state in a mouse model of antiphospholipid syndrome: role of reduced inflammation and endothelial dysfunction. PLoS One. 2019;14(3):e0212614. doi: 10.1371/journal.pone.0212614.

[103] Miller FJ, Jr, Filali M, Huss GJ, et al. Cytokine activation of nuclear factor kappa B in vascular smooth muscle cells requires signaling endosomes containing Nox1 and ClC-3. Circ Res. 2007;101(7):663–671. doi: 10.1161/CIRCRESAHA.107.151076.

[104] Espinola RG, Pierangeli SS, Gharavi AE, et al. Hydroxychloroquine reverses platelet activation induced by human IgG antiphospholipid antibodies. Thromb Haemost. 2002;87(3):518–522. doi: 10.1055/s-0037-1613033.

[105] Rand JH, Wu XX, Quinn AS, et al. Hydroxychloroquine protects the annexin A5 anticoagulant shield from disruption by antiphospholipid antibodies: evidence for a novel effect for an old antimalarial drug. Blood.

2010;115(11):2292–2299. doi: 10.1182/blood-2009-04-213520.
[106] Wu XX, Guller S, Rand JH. Hydroxychloroquine reduces binding of antiphospholipid antibodies to syncytiotrophoblasts and restores annexin A5 expression. Am J Obstet Gynecol. 2011;205(6):576.e7–576.14. doi: 10.1016/j.ajog.2011.06.064.
[107] Jerne NK. Towards a network theory of the immune system. Ann Immunol (Paris). 1974;125C(1-2):373–389.
[108] Shoenfeld Y. Idiotypic induction of autoimmunity: a new aspect of the idiotypic network. Faseb J. 1994;8(15):1296–1301. doi: 10.1096/fasebj.8.15.8001742.
[109] Lackner KJ, Müller-Calleja N. Antiphospholipid antibodies: their origin and development. Antibodies (Basel). 2016;5(2):15. doi: 10.3390/antib5020015.
[110] Hollerbach A, Müller-Calleja N, Pedrosa D, et al. Pathogenic lipid-binding antiphospholipid antibodies are associated with severity of COVID-19. J Thromb Haemost. 2021;19(9):2335–2347. doi: 10.1111/jth.15455.
[111] Lutters BC, Derksen RH, Tekelenburg WL, et al. Dimers of beta 2-glycoprotein I increase platelet deposition to collagen via interaction with phospholipids and the apolipoprotein E receptor 2. J Biol Chem. 2003;278(36):33831–33838. doi: 10.1074/jbc.M212655200.
[112] van Lummel M, Pennings MT, Derksen RH, et al. The binding site in β2-glycoprotein I for ApoER2′ on platelets is located in domain V. J Biol Chem. 2005;280(44):36729–36736. doi: 10.1074/jbc.M504172200.
[113] Raschi E, Testoni C, Bosisio D, et al. Role of the MyD88 transduction signaling pathway in endothelial activation by antiphospholipid antibodies. Blood. 2003;101(9):3495–3500. doi: 10.1182/blood-2002-08-2349.
[114] Xie H, Kong X, Zhou H, et al. TLR4 is involved in the pathogenic effects observed in a murine model of antiphospholipid syndrome. Clin Immunol. 2015;160(2):198–210. doi: 10.1016/j.clim.2015.05.017.
[115] Satta N, Dunoyer-Geindre S, Reber G, et al. The role of TLR2 in the inflammatory activation of mouse fibroblasts by human antiphospholipid antibodies. Blood. 2007;109(4):1507–1514. doi: 10.1182/blood-2005-03-024463.
[116] Satta N, Kruithof EK, Fickentscher C, et al. Toll-like receptor 2 mediates the activation of human monocytes and endothelial cells by antiphospholipid antibodies. Blood. 2011;117(20):5523–5531. doi: 10.1182/blood-2010-11-316158.
[117] Allen KL, Fonseca FV, Betapudi V, et al. A novel pathway for human endothelial cell activation by antiphospholipid/anti-β2 glycoprotein I antibodies. Blood. 2012;119(3):884–893. doi: 10.1182/blood-2011-03-344671.
[118] Laplante P, Amireault P, Subang R, et al. Interaction of β2-glycoprotein I with lipopolysaccharide leads to toll-like receptor 4 (TLR4)-dependent activation of macrophages. J Biol Chem. 2011;286(49):42494–42503. doi: 10.1074/jbc.M111.230383.
[119] Pennings MT, van Lummel M, Derksen RH, et al. Interaction of beta2-glycoprotein I with members of the low density lipoprotein receptor family. J Thromb Haemost. 2006;4(8):1680–1690. doi: 10.1111/j.1538-7836.2006.02036.x.
[120] Pennings MT, Derksen RH, van Lummel M, et al. Platelet adhesion to dimeric beta-glycoprotein I under conditions of flow is mediated by at least two receptors: glycoprotein ibalpha and apolipoprotein E receptor 2. J Thromb Haemost. 2007;5(2):369–377. doi: 10.1111/j.1538-7836.2007.02310.x.
[121] Pennings MT, Derksen RH, Urbanus RT, et al. Platelets express three different splice variants of ApoER2 that are all involved in signaling. J Thromb Haemost. 2007;5(7):1538–1544. doi: 10.1111/j.1538-7836.2007.02605.x.
[122] Urbanus RT, Pennings MT, Derksen RH, et al. Platelet activation by dimeric beta2-glycoprotein I requires signaling via both glycoprotein ibalpha and apolipoprotein E receptor 2. J Thromb Haemost. 2008;6(8):1405–1412. doi: 10.1111/j.1538-7836.2008.03021.x.
[123] Sacharidou A, Chambliss KL, Ulrich V, et al. Antiphospholipid antibodies induce thrombosis by PP2A activation via apoER2-Dab2-SHC1 complex formation in endothelium. Blood. 2018;131(19):2097–2110. doi: 10.1182/blood-2017-11-814681.
[124] Ulrich V, Gelber SE, Vukelic M, et al. ApoE receptor 2 mediation of trophoblast dysfunction and pregnancy complications induced by antiphospholipid antibodies in mice. Arthritis Rheumatol. 2016;68(3):730–739. doi: 10.1002/art.39453.
[125] Chu H, Sacharidou A, Nguyen A, et al. Protein phosphatase 2A activation via apoER2 in trophoblasts drives preeclampsia in a mouse model of the antiphospholipid syndrome. Circ Res. 2021;129(7):735–750. doi: 10.1161/CIRCRESAHA.120.318941.
[126] Sevim E, Zisa D, Andrade D, et al. Characteristics of patients with antiphospholipid antibody positivity in the APS ACTION international clinical database and repository. Arthritis Care Res (Hoboken). 2022;74(2):324–335. doi: 10.1002/acr.24468.
[127] Alijotas-Reig J, Esteve-Valverde E, Ferrer-Oliveras R, et al. The european register on obstetric antiphospholipid syndrome (EUROAPS): a survey of 1000 consecutive cases. Autoimmun Rev. 2019;18(4):406–414. doi: 10.1016/j.autrev.2018.12.006.
[128] Galli M, Luciani D, Bertolini G, et al. Lupus anticoagulants are stronger risk factors for thrombosis than anticardiolipin antibodies in the antiphospholipid syndrome: a systematic review of the literature. Blood. 2003;101(5):1827–1832. doi: 10.1182/blood-2002-02-0441.
[129] Galli M, Luciani D, Bertolini G, et al. Anti-β2-glycoprotein I, antiprothrombin antibodies, and the risk of thrombosis in the antiphospholipid syndrome. Blood. 2003;102(8):2717–2723. doi: 10.1182/blood-2002-11-3334.
[130] Reynaud Q, Lega J-C, Mismetti P, et al. Risk of venous and arterial thrombosis according to type of antiphospholipid antibodies in adults without systemic lupus erythematosus: a systematic review and meta-analysis. Autoimmun Rev. 2014;13(6):595–608. doi: 10.1016/j.autrev.2013.11.004.
[131] Jiang D, Lim W, Crowther M, et al. A systematic review of the association between anti-b-2 glycoprotein I antibodies and APS manifestations. Blood Adv. 2021;5(20):3931–3936. doi: 10.1182/bloodadvances.2021005205.
[132] Erton ZB, Sevim E, de Jesús RG, et al. Pregnancy outcomes in antiphospholipid antibody positive patients:

prospective results from the AntiPhospholipid syndrome alliance for clinical trials and InternatiOnal networking (APS ACTION) clinical database and repository ('registry'). Lupus Sci Med. 2022;9(1):e000633. doi: 10.1136/lupus-2021-000633.

[133] de Mast Q, Molhoek JE, van der Ven AJ, et al. Antiphospholipid antibodies and the risk of stroke in urban and rural tanzania. Stroke. 2016;47(10):2589–2595. doi: 10.1161/STROKEAHA.116.013760.

[134] Asherson RA, Cervera R. Antiphospholipid antibodies and infections. Ann Rheum Dis. 2003;62(5):388–393. doi: 10.1136/ard.62.5.388.

[135] Justo D, Finn T, Atzmony L, et al. Thrombosis associated with acute cytomegalovirus infection: a meta-analysis. Eur J Intern Med. 2011;22(2):195–199. doi: 10.1016/j.ejim.2010.11.006.

[136] Kelkar AH, Loc BL, Tarantino MD, et al. Cytomegalovirus-associated venous and arterial thrombotic disease. Cureus. 2020;12(12):e12161. doi: 10.7759/cureus.12161.

[137] Wijarnpreecha K, Thongprayoon C, Panjawatanan P, et al. Hepatitis C virus infection and risk of venous thromboembolism: a systematic review and meta-analysis. Ann Hepatol. 2017;16(4):514–520. doi: 10.5604/01.3001.0010.0279.

[138] Abdel-Wahab N, Lopez-Olivo MA, Pinto-Patarroyo GP, et al. Systematic review of case reports of antiphospholipid syndrome following infection. Lupus. 2016;25(14):1520–1531. doi: 10.1177/0961203316640912.

[139] Ambrosino P, Tarantino L, Criscuolo L, et al. The risk of venous thromboembolism in patients with hepatitis C. A systematic review and meta-analysis. Thromb Haemost. 2016;116(5):958–966. doi: 10.1160/TH16-03-0185.

[140] Ambrosino P, Lupoli R, Tarantino P, et al. Viral hepatitis and anti-phospholipid antibodies positivity: a systematic review and meta-analysis. Dig Liver Dis. 2015;47(6):478–487. doi: 10.1016/j.dld.2015.03.006.

[141] Zuo Y, Estes SK, Ali RA, et al. Prothrombotic autoantibodies in serum from patients hospitalized with COVID-19. Sci Transl Med. 2020;12(570):eabd3876. doi: 10.1126/scitranslmed.abd3876.

[142] Pengo V, Biasiolo A, Pegoraro C, et al. Antibody profiles for the diagnosis of antiphospholipid syndrome. Thromb Haemost. 2005;93(6):1147–1152. doi: 10.1160/TH04-12-0839.

[143] Pengo V, Ruffatti A, Legnani C, et al. Incidence of a first thromboembolic event in asymptomatic carriers of high-risk antiphospholipid antibody profile: a multicenter prospective study. Blood. 2011;118(17):4714–4718. doi: 10.1182/blood-2011-03-340232.

[144] Farina N, Abdulsalam R, McDonnell T, et al. Antiphospholipid antibody positivity in early systemic lupus erythematosus is associated with subsequent vascular events. Rheumatology (Oxford). 2023;62(6):2252–2256. doi: 10.1093/rheumatology/keac596.

[145] Mustonen P, Lehtonen KV, Javela K, et al. Persistent antiphospholipid antibody (aPL) in asymtpomatic carriers as a risk factor for future thrombotic events: a nationwide prospective study. Lupus. 2014;23(14):1468–1476. doi: 10.1177/0961203314545410.

[146] Latino JO, Udry S, Aranda FM, et al. Pregnancy failure in patients with obstetric antiphospholipid syndrome with conventional treatment: the influence of a triple positive antibody profile. Lupus. 2017;26(9):983–988. doi: 10.1177/0961203317692432.

[147] Bertin D, Camoin-Jau L, Veit V, et al. Single or triple positivity for antiphospholipid antibodies in "carriers" or symptomatic patients: untangling the knot. J Thromb Haemost. 2021;19(12):3018–3030. doi: 10.1111/jth.15518.