Microparticles: An Alternative Explanation to the Behavior of Vascular Antiphospholipid Syndrome

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Abstract

Antiphospholipid syndrome is an autoimmune disease characterized by the persistent presence of antiphospholipid antibodies, along with occurrence of vascular thrombosis and pregnancy morbidity. The variety of antiphospholipid antibodies and their related mechanisms, as well as the behavior of disease in wide groups of patients, have led some authors to propose a differentiation of this syndrome into two independent entities: vascular and obstetric antiphospholipid syndrome. Thus, previous studies have discussed whether specific autoantibodies may be responsible for this differentiation or, in contrast, how the same antibodies are able to generate two different clinical presentations. This discussion is yet to be settled. The capability of serum IgG from patients with vascular thrombosis to trigger the biogenesis of endothelial cell-derived microparticles in vitro is one of the previously discussed differences between the clinical entities of antiphospholipid syndrome. These vesicles constitute a prothrombotic mechanism as they can directly lead to clot activation in murine models and recalcified human plasma. Nevertheless, other indirect mechanisms by which microparticles can spread a procoagulant phenotype could be critical to understanding their role in antiphospholipid syndrome. For this reason, questions regarding the cargo of microparticles, and the signaling pathways involved in their biogenesis, are of interest in attempting to explain the behavior of this autoimmune disease.

Keywords

- antiphospholipid syndrome
- antiphospholipid antibodies
- cell-derived microparticles
- thrombosis
- blood coagulation disorders

Microparticles (MPs) were first described in 1946 by the Columbia University scientists Erwin Chargaff and Randolph West, who noticed that the high centrifugation of platelet-poor plasma resulted in proportional extension of its clot activation time. Therefore, something necessary to the coagulation process must have been precipitated from the normal plasma content, at elevated centrifugal forces.[1] In 1967, Wolf postulated that the plasma fraction with the *thromboplastic agent* (previously described by Chargaff and West) must have been composed of cell fragments, or in Wolf's terms, "*platelet dust*," according to its sudanophilic nature, sedimentation coefficient, and other properties.[2] Like Chargaff and West[1] as well as Wolf,[2] other early authors described the procoagulant nature of these vesicles. For example, in 1981, Dvorak et al noted that the connection between tumors and procoagulant state can be explained, at least partially, by the tendency of transformed cells to produce MPs with procoagulant nature.[3] This connection has been used to try to explain the pathogenic mechanisms of cardiovascular, metabolic, and autoimmune diseases, in which there is a chronic proinflammatory and procoagulant state, such as in obesity, diabetes, metabolic syndrome,[4] rheumatoid arthritis,[5,6] systemic lupus erythematosus,[6,7] and antiphospholipid syndrome (APS).[8] In relation to the latter, evidence not only shows that endothelial MPs are

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increased in APS patients but also that these vesicles could play an important role in the procoagulant state and other pathogenic mechanisms that are yet to be clarified.[9]

In APS, the antibodies to which the disease is attributed may induce two different clinical presentations, which can behave as independent entities: vascular APS and obstetric APS.[10,11] The exact pathogenic mechanisms related to this differentiation are not fully clear, as, despite the results of in vitro assays suggesting antibodies could be the cause of one or another clinical manifestation,[12,13] in vivo experiments suggest the participation of other factors such as the presence of a previous disturbance, for example, an infection (*second hit* theory).[14] In development of the aforementioned hypothesis, the MPs, clearly related to thrombosis risk in other cases,[15–18] could offer an alternative explanation for vascular APS procoagulant state and the related endothelial dysfunction. Nevertheless, studies comparing MP production between clinical groups of APS patients have had contradictory results.[9,19–22] On the other hand, related aspects, like signaling pathways involved in MP production process, and influence of these pathways in vesicles procoagulant activity, have not been extensively researched as yet. These topics will be reviewed in this publication.

Antiphospholipid syndrome is a complex disease whose behavior points to the existence of at least two independent entities.

According to the clinical classification definition, APS is an autoimmune disease whose basic features are the persistent presence of seropositivity toward anticardiolipin (aCL) antibodies, anti-β2 glycoprotein I (aβ2GPI) antibodies, and/or lupus anticoagulant (LA), in addition to the occurrence of at least one of the classical clinical manifestations (thrombosis or pregnancy morbidity).[23] The traditional description of this disease involves a noninflammatory procoagulant state, in which the therapeutic core (as of the time of writing) has been the use of antiplatelet, anticoagulant, and thromboprophylactic drugs.[24]

In contrast with these defining features, the physiopathology of APS reveals a more complex landscape. On one hand, it is known that criteria antibodies (LA, aβ2GPI, and aCL) are not those implicated only in the development of disease. In fact, this syndrome could be attributed to a wide set of antibodies with immunological activity toward phospholipids,[25] phospholipid-binding proteins, and protein–phospholipid complexes.[26] Thus, antiphospholipid antibodies (aPL) can include other immunoglobulins (Ig), such as anti-phosphatidylethanolamine, anti-phosphatidylglycerol, anti-phosphatidylcholine, anti-phosphatidylserine/thrombin, anti-annexin V, antiphosphatidic acid, and anti-vimentin/cardiolipin. All these, despite not being traditionally included in the current classification criteria, have been suggested to be important antibodies in the diagnosis and prognosis of APS.[27–31]

Another important consideration is that, against the noninflammatory procoagulant state included in the classification definition of disease,[23] new insights into relevant pathogenic mechanisms have revealed the participation of inflammatory actors such as complement proteins, leukocytes, and β2GPI itself,[32] which is the main antigenic protein to aPL. Indeed, besides the variety of aPL, multiple pathogenic mechanisms have been described in different in vivo and in vitro models.

The heterogeneity of aPL and APS pathogenic mechanisms has led some authors to hypothesize that some antibodies which recognize different epitopes may be responsible for specific clinical manifestations. This idea is supported by two types of in vitro evidence,[12,13,33,34] and epidemiological observation that is uncommon for thrombotic manifestations to be present in patients with pregnancy morbidity.[10,35] These findings suggest that gestational manifestations cannot be explained by placenta infarction, as previously proposed,[36] and instead it can be described as a complement-dependent inflammatory injury.[37,38] Vascular APS and obstetric APS do not seemingly share pathological mechanisms and, thus, the syndrome manifests itself in two different ways (►**Table 1**).

As the foundations of an aPL-dependent hypothesis to differentiation between clinical presentations of APS, there are findings that associate some monoclonal antibodies, with specific effects in monocytes and platelets. Müller-Calleja et al have proposed the use of ex vivo monocytes obtained from knockout mice for investigating the signaling pathways activated by aβ2GPI or aCL antibodies. According to their results, while effects of aCL antibodies occur through activation of endosomal NADPH oxidase (NOX), the effects of aβ2GPI antibodies depend on apolipoprotein E receptor 2 (ApoER2), as well as the mammalian target of rapamycin (mTOR).[34] In another study, Hollerbach et al explored the effect of monoclonal aPL in platelets of healthy donors. Using flow cytometry, the authors found that, in comparison with aCL antibodies, only aβ2GPI antibodies have the capability to induce P-selectin and GPIIb/IIIa expression in platelets. Furthermore, if they used the sera of APS patients, only those which had aβ2GPI activity could induce platelet activation.[33] To corroborate monoclonal aPL evidence, some authors have used cellular models with polyclonal antibodies, purified from patients with obstetric or vascular manifestations, whether simultaneous or not. In these cases, although antibodies from obstetric patients were effective in decreasing the migration of trophoblast human cells,[12] those from vascular APS patients increased the expression of tissue factor (TF) in monocytes.[13]

In spite of aforementioned relationship between antibodies from APS patients with specific clinical manifestations and observed effects in different cellular models, there have been no settled antibody profiles to accurately differentiate between vascular APS and obstetric APS patients at the time of this writing. Conversely, it has been found that a single antibody that recognizes one single epitope can be responsible for the development of both main clinical manifestations in a murine model (MBB2 antibody).[39] This, combined with other in vivo observations, suggests that other factors may be involved in differentiating manifestations of thrombosis and pregnancy morbidity besides antibodies' specificities. One example of this is the failure of aPL to trigger thrombosis in murine models, unless intraperitoneal pretreatment of animals with lipopolysaccharide (LPS) is performed (*second hit* theory).[14]

Considering these two apparently contradictory points, Meroni et al suggested that aspects such as tissue

Table 1 Summary of the evidence suggesting differences between obstetric APS and vascular APS

a. APS seems to behave as two different entities, when it is studied in wide patient groups (obstetric APS and vascular APS)	
Alijotas-Reig et al[10]	Record of 1,000 females with obstetric APS. Only 3.1% developed maternal thrombosis
Drozdinsky et al[35]	Tracing of 115 females with obstetric APS. Only 10.4% developed vascular manifestations
b. In vitro experimentation suggests that specific antibodies can explain the differences between APS manifestations	
Hollerbach et al[33]	aβ2GPI monoclonal antibodies trigger platelet activation, while aCL monoclonal antibodies fail to do so
Müller-Calleja et al[34]	aCL and aβ2GPI monoclonal antibodies stimulate monocytes through different pathways (NOX and ApoER2)
Lambrianides et al[13]	Polyclonal antibodies from vascular APS patients increase TF expression in monocytes, in a p38MAPK-dependent manner. Polyclonal antibodies from obstetric APS patients fail to do likewise
Poulton et al[12]	Polyclonal antibodies from obstetric APS patients decrease HTR8 cell invasion capability. Polyclonal antibodies from vascular APS patients do not
c. In vivo evidence suggests that other factors, in addition to antibodies, define the differences between clinical manifestations	
Fischetti et al[14]	aβ2GPI antibodies do not trigger mesenteric thrombosis in a murine model unless the animal is pretreated with intraperitoneal LPS
Agostinis et al[39]	A recombinant antibody, whose antigenic specificity is the β2GPI domain I, can trigger both manifestations
Durigutto et al[26]	aβ2GPI domain I antibodies trigger mesenteric thrombosis in a murine model treated with intraperitoneal LPS. Untreated rats, or rats with aβ2GPI domain V antibodies stimulation, do not develop thrombosis

Abbreviations: aCL, anticardiolipin; ApoER2, apolipoprotein E receptor 2; APS, antiphospholipid syndrome; aβ2GPI, anti-β2 glycoprotein I; LPS, lipopolysaccharide; NOX, NADPH oxidase; p38MAPK, p38 mitogen-activated protein kinase; TF, tissue factor.

distribution of β2GPI, serum antibodies titers, and *second hit* are variables that could explain differences between clinical presentations of APS, while both, thrombosis and pregnancy morbidity, could be attributed to the same antibodies.[11] According to this postulate, given the preferential in vivo distribution of β2GPI in implantation sites of uterus,[40] this tissue represents the main aPL target. In this manner, obstetric manifestations can be seen even in patients with low aPL titers.[41] In contrast, endothelial cells, monocytes, and platelets, which are the main causatives of vascular manifestations, could be considered secondary targets. For that reason, their activation requires the occurrence of a previous proinflammatory stimulus.[14]

Nevertheless, this model does not completely exclude the possibility that some antibodies could have specific effects on particular cell types, in the same way that in vitro assays have demonstrated before, and which the existence of aPL without dependence on β2GPI as a cofactor also suggests. It is hence possible that both aPL-independent factors and aPL specificities–dependent factors could be implicated in the occurrence of different clinical manifestations. An example of how those two types of variables could converge in vivo can be appreciated through comparing two aβ2GPI antibodies with antigenic specificities toward two different domains. While the antidomain I antibodies are able to trigger thrombosis in mesenteric vessels of Wistar rats, the antidomain V antibodies do not generate these effects. Nevertheless, the effects of antidomain I antibodies depend, first, on the conformational state of its antigenic protein (which must be linearized to allow an effective binding of the Ig) and, second, on the LPS pretreatment of animal model.[26]

The Procoagulant State of APS Has Been Explained by Different Pathogenic Mechanisms, Some of Which Have Been Associated with Specific aPL

Hemostasis physiology includes a group of procoagulant and anticoagulant processes that can be summarized in three main physiological phenomena: platelet aggregation (primary hemostasis), coagulation cascade (secondary hemostasis), and fibrinolysis.[42] It has been noted that these three processes are disturbed in APS.[33,43,44] Moreover, aPL can lead to changes in monocytes,[13] platelets,[33] and endothelial cells,[45] all of which are main constituents of hemostatic balance (►**Fig. 1**). In addition to aPL's effects on activation of blood cell components, antibodies can also interfere with the anticoagulant function of plasma and surface molecules such as annexin A5,[46] heparan sulfate,[47] protein C,[48] and β2GPI itself[43] (►**Table 2**).

As previously mentioned, aPL can interact with monocytes in two main ways to induce a proinflammatory/procoagulant phenotype.[34] The first of those pathways was described by using aCL monoclonal antibodies,[49,50] and given the results obtained with knockout mice monocytes, this seems the main aCL-dependent activation pathway.[34] According to findings obtained through fluorescence microscopy, after MonoMac1 cells are exposed to aPL, aCL antibodies are internalized into acidic compartments, probably endosomes and lysosomes.[50]

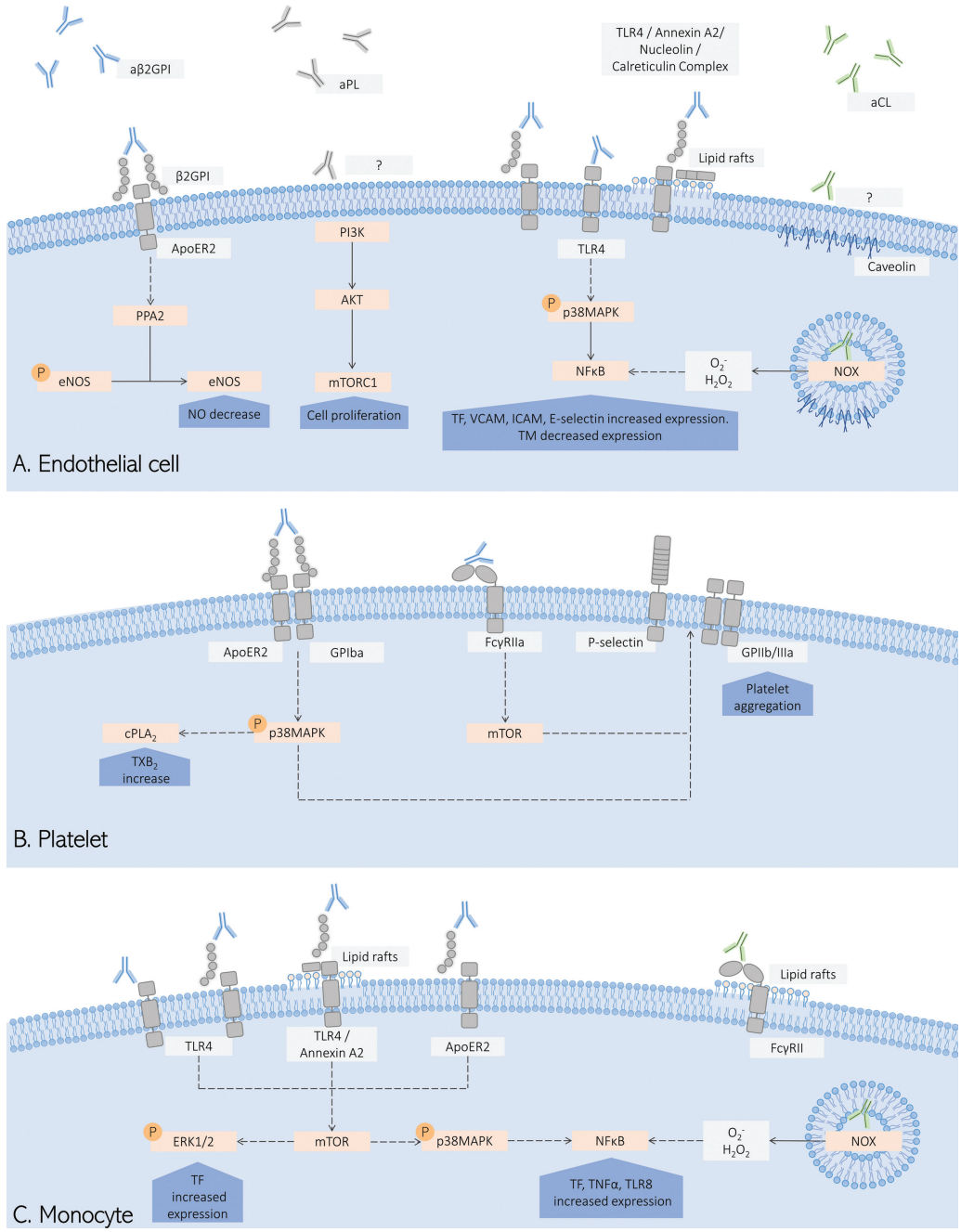

Fig. 1 aPL-related pathogenic mechanisms involved in the procoagulant state of APS. (**A**) In endothelial cells, aβ2GPI antibodies activate ApoER2, which in turn decreases NO production. These antibodies can also activate TLR4 by different means, including a lipid rafts-dependent mechanism. Then, the expression of TF, E-selectin, VCAM-1, and ICAM-1, is increased. aCL antibodies are internalized in a caveolin-dependent manner and, once inside the endothelial cell, they activate NOX. NOX favors NFκB activation and the expression of a procoagulant phenotype. In addition, aPL can trigger endothelial cell proliferation through mTOR activation. (**B**) In platelets, aβ2GPI antibodies increase the membrane expression of P-selectin and GPIIb/IIIa, as well as TXB_2 production, which favors platelet aggregation. These mechanisms involve the activation of ApoER2, GPIba, and FcγRII. (**C**) Finally, monocytes can be stimulated by aβ2GPI antibodies in a TLR4- and ApoER2-dependent manner, which implies the activation of MEK1/2 and p38MAPK pathways. These pathways upregulate the expression of TF and TNFα. aCL antibodies also favor these effects by means of NOX activation. Again, lipid rafts seem to play an important role in the aPL to monocyte interaction, both for aβ2GPI and aCL antibodies.[13,45,50,51,53–55,58,60,62,63] aβ2GPI, anti-β2 glycoprotein I antibodies; aCL, anticardiolipin antibodies; aPL, antiphospholipid antibodies; NOX, NADPH oxidase; ApoER2, apolipoprotein E receptor 2; TLR4, toll-like receptor 4; PP2A, protein phosphatase 2A; eNOS, endothelial nitric oxide synthase; NO, nitric oxide; PI3K, phosphatidyl-inositol 3 kinase; AKT, protein kinase B; mTOR, mammalian target of rapamycin; p38MAPK, p38 mitogen-activated protein kinase; ERK, extracellular signal-regulated kinase; NFκB, nuclear factor kappa-light-chain enhancer of activated B cells; TF, tissue factor; VCAM-1, vascular cell adhesion protein 1; ICAM-1, intercellular adhesion molecule 1; TM, thrombomodulin; $cPLA_2$, cytosolic phospholipase A_2; TXB_2, thromboxane B_2; GPIba, platelet glycoprotein Ib; FcγRII, Fc gamma receptor II; TNFα, tumor necrosis factor-α; TLR8, toll-like receptor 8.

Table 2 Summary of the APS-related procoagulant mechanisms

a. aPL activate a procoagulant/proinflammatory phenotype in monocytes	
Prinz et al[49]	aCL antibodies are internalized by means of interaction with FcγRII in lipid rafts, and once inside cell, NOX is activated. The generated ROS due to the NOX function sensitizes monocytes to TLR8 activation
Sorice et al[54] Xia et al[55] López-Pedrera et al[56] Lambrianides et al[13]	aβ2GPI antibodies trigger the expression of TF and TNFα through mTOR, ERK1/2, and p38MAPK pathways. Activation of ApoER2 and TLR4 by different means is involved in this mechanism
b. aPL activate a procoagulant phenotype in platelets	
Hollerbach et al[33]	aβ2GPI antibodies activate mTOR pathway in a FcγRII-dependent manner. Then, the membrane expression of P-selectin and GPIIb/IIIa is increased
Pennings et al[57] Zhang et al[58] Vega-Ostertag et al[59]	ApoER2/GPIba membrane complexes are activated by aβ2GPI/β2GPI complexes, which in turn lead to p38MAPK phosphorylation. Production of TXB_2 and membrane expression of P-selectin and GPIIb/IIIa are increased
c. aPL activate a procoagulant/proinflammatory phenotype in endothelial cells	
Ramesh et al[45] Miranda et al[64]	aβ2GPI antibodies activate ApoER2, which leads to eNOS inactivation. NO production is affected
Canaud et al[65]	aPL-stimulated endothelial cells show PI3K, AKT, and mTOR pathway activation. In account of this signal, cell proliferation increases, leading to intima hyperplasia
Allen et al[60] Miranda et al[64] Prinz et al[50] Wang et al[61]	The expression of TF, E-selectin, VCAM-1, and ICAM-1 is increased in aPL-stimulated endothelial cells, while the expression of TM is decreased. These effects are mediated by TLR4 and NOX activation through shared mechanisms with aPL-stimulated monocytes
Pericleous et al[9]	By means of unexplored pathways, aPL trigger MP production in endothelial cells. These MPs exert a procoagulant effect by different direct and indirect means
d. aPL interfere with anticoagulant functions of plasma and surface molecules	
Bu et al[43]	aβ2GPI antibodies interfere with profibrinolytic function of β2GPI
de Laat et al[48]	aPL with LA activity seem to prevent the anticoagulant effect of APC
Shibata et al[47]	Antiheparin/heparan sulfate antibodies interfere with generation of thrombin–antithrombin complexes, inhibiting heparin anticoagulant activity
Rand et al[46]	aPL disrupt annexin A5 anticoagulant shield on anionic lipid surfaces

Abbreviations: aCL, anticardiolipin; AKT, protein kinase B; APC, activated protein C; aPL, antiphospholipid antibodies; ApoER2, apolipoprotein E receptor 2; APS, antiphospholipid syndrome; aβ2GPI, anti-β2 glycoprotein I; eNOS, endothelial nitric oxide synthase; ERK, extracellular signal-regulated kinase; FcγRII, Fc gamma receptor II; GPIIb/IIIa, glycoprotein IIb/IIIa; ICAM-1, intercellular adhesion molecule 1; LA, lupus anticoagulant; MPs, microparticles; mTOR, mammalian target of rapamycin; NO, nitric oxide; NOX, NADPH oxidase; p38MAPK, p38 mitogen-activated protein kinase; PI3K, phosphatidyl-inositol 3 kinase; ROS, reactive oxygen species; TF, tissue factor; TLR4, toll-like receptor 4; TLR8, toll-like receptor 8; TM, thrombomodulin; TNFα, tumor necrosis factor-α; TXB_2, thromboxane B_2; VCAM-1, vascular cell adhesion protein 1.

Despite the presumptive role of Fc gamma receptor II (FcγRII) in the antibody intake process, the antigen-binding site of aCL antibodies seems to be necessary too, inasmuch as the blockade of this domain with a cognate peptide results in the inhibition of aPL internalization.[50] In addition, lipid raft structures seem to be important to the successful monocyte–aCL antibody interaction.[49,50] Following the activation pathway, once inside the endosome, aPL induce NOX activation, which in time enhances superoxide anion production. Then, superoxide suffers dismutation into a more stable reactive oxygen species (ROS), hydrogen peroxide.[50] Monocyte sensitization to intracellular toll-like receptor 8 (TLR8) activation is the final effect of this increase in ROS intracellular levels. It is known that this sensitization effect is not related to direct receptor agonism by the antibodies, but depends on enhanced receptor expression, and its translocation to endosomes. Both these mechanisms are mediated by nuclear factor kappa-light-chain enhancer of activated B cells (NFκB).[50]

The second pathway described in monocytes is associated with aβ2GPI antibodies, and depends on TLR4 and ApoER2 activation.[34] Different members of low-density lipoprotein (LDL) receptor family are known to act as clearance receptors to β2GPI. Specifically, in APS, aPL join β2GPI and form dimers of these molecules, which in time are recognized through their domain V by ApoER2 and other similar receptors.[51] In contrast, there are different proposals to explain the way in which aβ2GPI antibodies interact with TLR4. First, using confocal microscopy, it has been said that fluorescence-tagged β2GPI can bind specifically to TLR4, so that aβ2GPI/β2GPI complexes activate these receptors.[52] Nevertheless, comparisons between the amino acidic sequences of β2GPI domain I, and TLR4, have shown that there is a shared

antigenic fragment with 88% similarity. Moreover, cross-reaction of aβ2GPI antibodies with specificity toward the aforementioned-shared sequence with TLR4 has been confirmed. These antibodies can hence directly activate monocytes and endothelial cells.[53] In spite of this, co-immunoprecipitation assays show annexin A2 as main β2GPI receptor in monocyte surface. Accordingly, it seems that β2GPI/annexin A2 complexes are present in lipid rafts, and later in the presence of aPL, TLR4 is recruited and activated in these membrane structures.[54]

Setting aside the exact way in which aβ2GPI antibodies interact with TLR4 or ApoER2, different authors converge in describing the consequent activation of mTOR,[34,55] which is then responsible for extracellular signal-regulated kinase 1/2 (ERK1/2) and p38MAPK phosphorylation.[55] Using specific MAPK inhibitors and electrophoretic mobility shift assay, López et al concluded that only p38MAPK, and not ERK1/2, is involved in the improvement of binding NFκB to DNA,[56] which finally implies an increase in the expression of TF,[13,54,55] tumoral necrosis factor-α (TNFα),[53,54] and interleukin 8 (IL8).[55] It is important to note that even if ERK1/2 does not activate NFκB, and acts in an independent pathway, its inhibition also blocks TF expression in aPL-stimulated monocytes.[56]

In relation to mechanisms involved in aPL-triggered platelet aggregation, it seems that, according to findings obtained with monoclonal antibodies and IgG of APS patients, aβ2GPI antibodies are more closely related to platelet activation, at least in comparison with aCL antibodies. It is known that the effect of aPL in platelets depends on mTOR as well as on FcγRII, inasmuch as platelets pretreated with rapamycin or with anti-CD32 antibodies are not susceptible to stimulation with aβ2GPI antibodies. Moreover, using antigen-binding fragments $F(ab)_2$, or IgM isotype of a monoclonal aβ2GPI antibody that had previously demonstrated the ability to increase platelet expression of P-selectin and GPIIb/IIIa, results in failure to activate platelets.[33]

However, Fc receptors are not the only way through which aPL may interact with platelets. Pennings et al brought platelets into flow conditions, to demonstrate that these cells can adhere to a surface coated with aβ2GPI antibodies, as long as the coat has been incubated with β2GPI.[57] These and other authors concluded that the interaction between platelets, β2GPI dimers, and aβ2GPI antibodies is mediated by ApoER2 and GPIbα.[57,58] Furthermore, co-immunoprecipitation assays suggest that these two receptors form complexes in platelet membrane.[57] The consequence of platelet activation through these receptors seems to be p38MAPK phosphorylation, while the MEK1/2 pathway remains unaffected.[58,59] Finally, there is an increased expression of P-selectin and GPIIb/IIIa,[58] in addition to activation of cytosolic phospholipase A_2, which in turn increases thromboxane B_2 production.[59]

The third way by which aPL can interfere with hemostasis in favor of a procoagulant state is the interaction of these antibodies with plasma and surface proteins that exert natural anticoagulant functions. The clearest example is the inhibitory effect of aPL on profibrinolytic activity of β2GPI. As a fibrin gel assay shows, native β2GPI and recombinant domain V of this protein upregulate the catalytic activity of tissue plasminogen activator (tPA), with consequent increase in available plasmin and enhancement of clot lysis. Instead, aβ2GPI monoclonal antibodies, or IgG of APS patients, significantly reduce these effects.[43] Another protein presumptively involved in procoagulant activity of aPL is activated protein C (APC), a proteolytic inhibitor of activated coagulation factors V (FVa) and VIII (FVIIIa). This arises from the noted relationship between the presence of LA and APC resistance in APS patients. It seems that β2GPI can bind directly to, and inactivate, APC, and that this junction is enhanced by an aβ2GPI monoclonal antibody.[48]

In 1994, Shibata et al identified the presence of antiheparin/heparan sulfate antibodies in sera of a group of APS patients. These authors proved that the aforementioned antibodies bind to a disaccharide present in the pentasaccharide sequence of heparin glycosaminoglycan, a structure which specifically recognizes antithrombin. In this way, antiheparin/heparan sulfate antibodies can interfere with generation of thrombin–antithrombin complexes, and lead to inhibition of heparin anticoagulant activity.[47] Finally, it has been described that aβ2GPI antibodies, in complex with β2GPI, may disrupt annexin A5 anticoagulant shield on anionic lipid surfaces of in vitro–prepared lipid bilayers.[46]

The fourth group of procoagulant mechanisms is attributable to the interaction between aPL and endothelial cells. Some of the pathogenic mechanisms in this group are shared with aPL-stimulated monocytes. For example, aCL antibodies are internalized through caveolae-dependent endocytosis, and then upregulate NOX and NFκB activation.[49] The interaction between aβ2GPI antibodies and TLR4 is another event also noted in endothelial cells, either through cross-reactivity between a β2GPI antigenic peptide and TLR4,[53] or mediated through aβ2GPI/β2GPI complexes.[52,60] Meanwhile, even before aPL stimulation, endothelial cell surface expresses complexes integrated by annexin A2, nucleolin, calreticulin, and TLR4. The inhibition of any of those four proteins decreases cell sensitivity to the stimulus aβ2GPI antibodies.[60] Following the signaling pathway, TLR4 activation leads to p38MAPK phosphorylation, which in turn results in activation of NFκB.[61] Either due to TLR4 stimulation or NOX activation, the increase in NFκB activity involves an upregulated expression of TF,[60] vascular cell adhesion protein 1 (VCAM-1),[53,62] intercellular adhesion molecule 1 (ICAM-1), and E-selectin,[61,63] as well as downregulation of thrombomodulin expression[64] (the physiological activator of protein C). These phenomena explain concomitantly the procoagulant and proinflammatory phenotype of aPL-stimulated endothelial cells.[62,63]

In addition to increased expression of adhesion molecules and TF, another three phenomena could contribute to explain the procoagulant state associated with aPL-stimulated endothelial cells. First, APS patients can develop intimal hyperplasia, which is related to large artery occlusion. The postulated mechanism involves aPL-mediated activation of PI3K, AKT, and mTOR pathway, which finally leads to

enhanced cell proliferation.[65] Some authors have also described a pathological vascular resistance to acetylcholine vasodilatation, as part of endothelial dysfunction accompanying APS.[45,64] The mechanism involved in this resistance is not insensitivity to nitric oxide (NO) itself, as treatment with direct NO donors (e.g., sodium nitroprusside) restores vessel dilatation capability. Instead, it has been described as a decrease in NO production, mediated by endothelial NO synthase dephosphorylation. This effect has been triggered in vitro by β2GPI dimers, and seems mediated by ApoER2 and protein phosphatase 2A.[45]

Finally, increased levels of plasma MPs have been identified in APS patients compared with healthy controls.[19–22] The causal relationship between aPL and this elevation of blood MPs has been confirmed by means of in vitro stimulation of endothelial cells with IgG from APS patients.[9] Thus, the ability of aPL to trigger MP production in endothelial cells is clear, though the underlying mechanisms are yet to be clarified, as well as the presumptive role of aPL in the setting process of MP procoagulant potential.

Microparticles Constitute a Versatile Signaling Mechanism which Is also Related to Procoagulant States

Extracellular vesicles could be described as lipid bilayer fragments carrying different macromolecules and metabolites, from mRNA to cytokines.[66–68] Far from being simple cell pieces with a random sample of cellular content as cargo, which could be recognized by any receptor cell, extracellular vesicles are capable of performing very specific messaging tasks between cells. Observations were on the existence of different vesicle populations with exclusive cargos originated from one single cell type.[69] It has been also noted the accuracy with which these particles are addressed to a specific cell type,[70] as well as the influence of signaling pathways in cargo of vesicles, thus leading to changes in the sent message.[71] In conclusion, wrapping different messenger molecules in membranes guarantees, among other things, integrity of their content, and accuracy with which the message is sent. Thanks to these properties, extracellular vesicles can be seen mediating many physiological processes, such as sperm capacitation,[72] antigen presentation,[73] inflammation,[74] and iron metabolism.[75]

There are three different types of extracellular vesicles according to their origin, all of which can interact with their addressee cell in different ways. Initially, it was proposed that extracellular vesicles were exclusively the product of cytoplasmic membrane blebbing. Nevertheless, the discovery of particles originated from multivesicular bodies[76] led to the establishment of a nomenclature, which would allow for differentiation between those two types of vesicles. Vesicles from cytoplasmic membranes, identified by a diameter between 0.1 and 1 µm, are known as "microparticles." Vesicles from multivesicular bodies, measuring between 0.04 and 0.1 µm in diameter, came to be termed "exosomes."[77] Finally, there are apoptotic bodies, the remains of membranes and other contents released during apoptosis or cell injury, which are considered a type of extracellular vesicle as well. The way by which these three types of vesicles can interact with, and influence a response in an addressee cell, can vary, and it may involve fusion of the vesicle with the cell membrane,[78] a ligand to receptor interaction,[79] endocytic assimilation of the vesicle,[73] or transference of functional receptors.[80]

Once the function of extracellular vesicles as very specific messengers is understood, it is important to notice that all of these vesicles, as membrane fragments in plasma, have an intrinsic procoagulant nature.[81] In this way, no matter the origin or cargo, every vesicle increases the total available anionic phospholipid surface, which favors the reaction between coagulation factors.[82] Despite exosomes, apoptotic bodies, and MPs share this feature, this review focuses on MPs, as the amounts of this specific type of extracellular vesicle in plasma have been empirically related to thrombotic manifestation in human patients.[15,17] Furthermore, MPs obtained in vitro have been used to enhance thrombotic events in mice,[18] as well as to trigger clot activation in normal recalcified plasma.[83]

Regarding the procoagulant properties of MPs, there are two main factors that can interfere with the direct influence of these vesicles in human plasma, that is, blockade of phosphatidylserine (sometimes abbreviated PS) and depletion or inhibition of TF.[18,82] Phosphatidylserine is an anionic phospholipid which normally prevails in the inner monolayer of cell membrane. As a consequence of stimulation and increase in intracellular amounts of calcium (which usually accompany the activation of MP production), phosphatidylserine is exteriorized to the outer monolayer of cell membrane.[84] This phenomenon is directed by a calcium-mediated scramblase.[85] The reason why phosphatidylserine (among other anionic phospholipids) enhances the coagulation process is the function that it performs as a confluent platform to coagulation factors VII, X, IX, and II (prothrombin). The structure of these proteins includes cationic domains of modified amino acid γ-carboxy-glutamic acid, so that they join through electrostatic interactions with anionic phospholipid surface.[81]

In relation to TF, this integral membrane protein functions as a FVII receptor. TF/FVII complexes lead to FX activation, which finally triggers clot formation through the extrinsic and common coagulation pathways.[86] Even if this protein is primarily expressed by cells that are not in direct contact with blood, its expression is inducible in monocytes and endothelial cells.[87] In this last case, minutes after cell activation by means of a proinflammatory stimulus, the intracellular domain of TF is phosphorylated in the aminoacyl residue serine 253. This event, in turn, leads to TF loading and its release in MPs.[88] The role of this protein in the procoagulant activity of extracellular vesicles has been demonstrated in vivo, in as much as using MPs from TF-silenced cells has a significantly smaller effect in murine models compared with normal MPs.[18]

In addition to their role as direct clot activators, MPs also exhibit indirect procoagulant activity by interaction with different cell types (►**Fig. 2**). Some cargo molecules in the

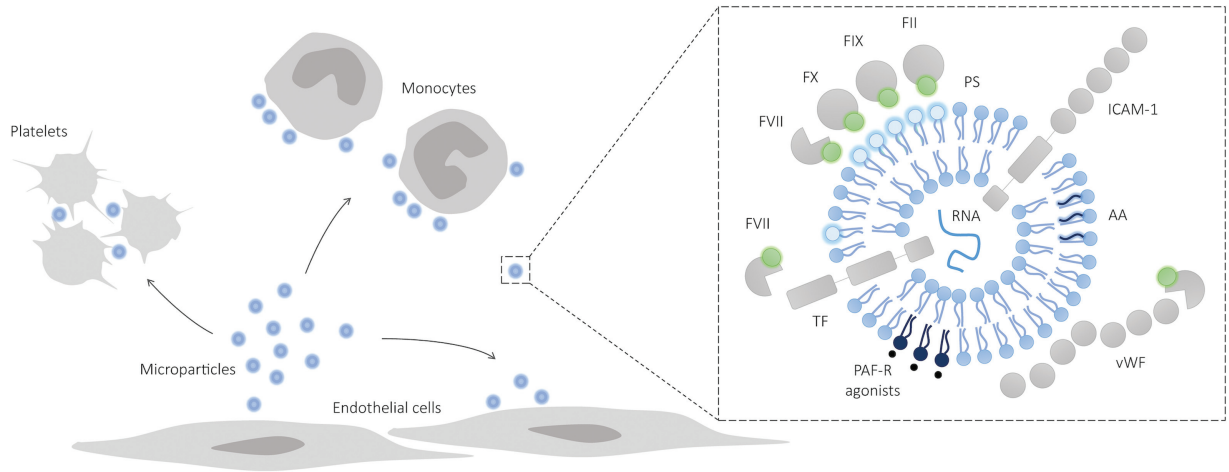

Fig. 2 Procoagulant mechanisms of microparticles. Microparticles can directly interact with coagulation factors and trigger the coagulation cascade by means of the anionic phospholipids on their surface, and the transmembrane glycoprotein TF. On the other hand, these vesicles can also carry adhesion molecules such as ICAM-1, through which monocytes can be activated to express TF. Arachidonic acid is another cargo that can induce changes in monocytes. In this case, it enhances cyclooxygenase 2 expression and thromboxane production. Microparticles triggered by prooxidative stimuli can also carry lipids with PAF-R agonist activity, which can induce other cells to release more of these vesicles. Interaction between platelets and microparticles has also been observed, forming complexes presumptively mediated by VWF. Finally, in APS, extracellular vesicles derived from aPL-stimulated endothelial cells can also carry RNA molecules which activate other naive endothelial cells, and therefore spread a proinflammatory/procoagulant phenotype.[79,82,90–93] FII, FVII, FIX, FX, factors II, VII, IX, X; PS, phosphatidylserine; TF, tissue factor; ICAM-1, intercellular adhesion molecule 1; AA, arachidonic acid; PAF-R, platelet-activating factor receptor; VWF, von Willebrand factor; RNA, ribonucleic acid.

vesicles can perform a signaling function through which a procoagulant phenotype is induced in addressee cell. Sabatier et al coincubated endothelial MPs with monocytes, showing interaction between ICAM-1 (expressed in MP surface) and β-integrins (present in the monocyte membrane). This interaction leads to induced expression of TF in monocytes, which then increases their ability to activate clot in recalcified plasma.[79] Blood sample analyses of different patients suggest that endothelial MP–monocytes complexes play an important role in hypercoagulable states, as the amounts of these complexes are elevated in patients with history of thromboembolism.[89] A second way by which MPs can activate monocytes involves arachidonic acid (AA), a lipid carried by these vesicles which upregulates cyclooxygenase 2 expression in the aforementioned cells. This effect is followed by an increase in prostaglandin E_2 and thromboxane B_2 synthesis. Furthermore, it seems to be that MP-delivered AA is directly used by monocytes as substrate for eicosanoids production.[90]

It is known that endothelial cell-derived MPs carry unusually large multimers of von Willebrand factor (VWF). The role of these MP-related multimers in platelet aggregation has been assessed under nonphysiological conditions, through a ristocetin-dependent assay.[91] Other physiologically relevant factors which are also able to activate VWF (e.g., flow conditions[92]) could be of interest in attempting to better describe this possible prothrombotic mechanism. MPs also operate as a kind of autocrine/paracrine signaling mechanism which functions by spreading a procoagulant phenotype among cells of a single tissue. This phenomenon has been observed in particular in APS. It is hence known that endothelial cells exposed to aβ2GPI antibodies increase their MP production and, in turn, these vesicles can be used to induce dysfunction in naive endothelial cells without aPL mediation.[93] Finally, it has been postulated that prooxidative stimuli, such as radiation or chemotherapeutic agents, may lead to oxidation of membrane phospholipids, and that these lipids are then released in MPs. The oxidized phospholipids perform as agonists of platelet-activating factor receptor (PAF-R), which promotes MP production in receiver cells.[94]

Focusing again on APS, it is known that aPL-mediated endothelial dysfunction is a principal feature of disease.[95] One of the consequences involved with this endothelial dysfunction is the increased production of endothelial MPs.[83] Accordingly, in the search for new biomarkers that could have prognostic value for APS, MP measurement in plasma has been proposed as a clinical risk indicator.[96] Supporting this proposal, many authors reported increased amounts of plasma MPs in APS patients.[19–22,97] The vesicles with increased numbers are consistently found to be of endothelial origin. Nevertheless, in some cases, increased amounts of platelet and monocyte MPs have also been found in APS patients compared with healthy controls.

Even if these results suggest that the number of plasma MPs can be useful as a clinical biomarker, there is evidence that points to another explanation. It seems to be that the amounts of plasma MPs are not related directly to the development of a given clinical manifestation, but to aPL titers themselves (which may be present even in healthy individuals). Some authors have hence compared aPL-negative individuals with aPL-positive individuals (even without clinical manifestations), finding differences in endothelial, platelet, and TF^+ MPs between those two groups.[98] Furthermore, a statistical correlation between MP levels and aβ2GPI antibodies titers,[98] or LA levels,[21,83] has been found.

A feasible explanation to this direct relationship between the amount of MPs and the presence of aPL is that aPL antibodies are not only involved in the induction of MP production in endothelial cells[9] but may also interfere with clearance of plasma vesicles. In this regard, β2GPI has a phosphatidylserine join domain through which β2GPI/MP complexes are formed. In healthy individuals, those complexes are recognized by means of LDL receptors, so that this serum glycoprotein facilitates MP clearance.[99] Instead, Mobarrez et al noted that the amount of $\beta 2GPI^{+}$ MPs is diminished in patients with aβ2GPI antibodies, while the mean fluorescence related to identification of IgG in MP surface is increased, thus suggesting that MP-related β2GPI is recognized, and remains encrypted, by aPL. In this way, aβ2GPI antibodies could disturb the normal MP clearance process, in favor of an Fc receptor–dependent recognition and assimilation.[99]

According to this idea, it could be that the role of MPs in the procoagulant state that characterizes the vascular clinical presentation of APS cannot be explained, at least in its entirety, by simple increment in plasma amounts of these vesicles. This view is also supported by various authors, who compared MP amounts between APS clinical groups, reporting no significant differences.[20–22] Instead, given the nature of these vesicles as very specific messengers, whose function depends on their cargo, and whose cargo can vary according to their production process, it could be postulated that MP influence on the hypercoagulable state related to APS is connected to the way in which they interact with different cell types and spread a procoagulant phenotype.[9,93] This relationship is still underexplored, but the different signaling pathways mediating MP production in aPL-stimulated endothelial cells could play an important role.

Evidence Suggests at Least Two Different Signaling Pathways Are Involved in Microparticle production: Details of the biogenesis Triggered by Antiphospholipid Antibodies Have Yet to Be Described

Because both main extracellular vesicles types (exosomes and MPs) have different origins, they also have different biogenesis mechanisms. Exosomes are mainly released as physiological way to export monoubiquitinated membrane proteins.[100] The observed biogenesis pathway in this case involves the endosomal sorting complex required for transport. Four complexes constitute this system, participating both in selection of proteins that will be loaded in exosomes (since three of those four complexes have ubiquitin-binding domains) and in conformation of intraluminal vesicles, using endosome as a membrane source.[101,102] After intraluminal vesicles are formed, multivesicular bodies tend to merge with lysosomes to enzymatic degradation of target protein and intraluminal membranes. Nevertheless, by means of a process presumptively mediated by small GTPase family members,[103] some multivesicular bodies are led to their merger with cytoplasmic membrane and, as a consequence, intraluminal vesicles are released in extracellular fluid (exosomes).[76]

On the other hand, MP production is less understood. Multiple stimuli perform as triggers of MP production process in different cell types. Most of these are mitogens, such as phorbol esters[83,94,104]; stressor stimuli, like chemotherapeutics agents,[94] agonists of platelet activating factor receptor,[94] benzoyl ATP,[105] TNFα,[83,106] IL1β, thrombin,[83] autoantibodies,[9,107] tobacco smoke extracts[108]; or agents involved in intracellular calcium balance, such as calcium ionophores or calcium ATPase inhibitors.[83,84,104] In all these cases, evidence points to MP production process involving p38MAPK[94,105–107] and/or MEK1/2,[94,104,108,109] and depending on increment of intracellular amounts of calcium,[83,84,104,105] cytoskeleton reorganization,[84,109] and changes in lipid bilayer composition.[94,105]

Early evidence suggests that increment of intracellular amounts of calcium may be a main inductor of membrane blebbing in healthy donor platelets.[84] There are two effects of this phenomenon that could explain the necessary changes in cytoplasmic membrane leading to MP production: redistribution of membrane phospholipids and activation of calcium-dependent protease (calpain), which in turn degrades the cytoskeleton. Nevertheless, even if there is exposure of phosphatidylserine to the outer monolayer of cell membrane, biogenesis of MPs is triggered only when calcium concentration is enough to activate calpain.[84]

While calcium seems to be a consistently present factor in MP production, activation of one, another, or both MAPK pathways has been described depending on stimulus and cell type involved. Surprisingly, in some cases, p38MAPK activation has been identified as mainly responsible for the blebbing process, while MEK1/2 inhibition has not had any such effect.[105,107] Nevertheless, in other cases, the opposite may occur (i.e., an MEK1/2-dependent and p38MAPK-independent MP production process).[104] This suggests that both pathways can guide membrane blebbing in an independent manner, or be simultaneously activated by a stimulus.[94] A third actor can be identified especially in cases where p38MAPK is involved, that is, acid sphingomyelin phosphodiesterase. This enzyme is moved to outer monolayer of cell membrane as a consequence of aforementioned kinase phosphorylation. Once there, it catabolizes sphingomyelin conversion into ceramide. This change favors membrane blebbing.[94,105]

In endothelium, TNFα and serum IgG from patients positive to angiotensin II receptor type 1 autoantibodies have been used to study possible related pathways in MP production. In both cases, p38MAPK has been involved. Remarkably, using a specific MEK1/2 inhibitor in TNFα-stimulated aortic endothelial cells, far from abrogating MP production, increases it. According to these results, and taking into account that p38MAPK has been involved in endothelium stimulation by aβ2GPI antibodies, as well as in the development of thrombotic events in murine models,[62] it is feasible to suggest that aPL-triggered MP production would depend on p38MAPK. Nevertheless, the limited evidence that points specifically to endothelial MP production in the context of APS alludes to the participation of MEK1/2 pathway. Betapudi et al used aβ2GPI antibodies to induce MP production in

human umbilical vein endothelial cells, finding that this process depends on rho-associated protein kinase (ROCK) and myosin light-chain kinase (MLCK), which in turn are responsible for myosin II regulatory light chain phosphorylation. Furthermore, they found that aβ2GPI antibodies lead to colocalization of myosin II with actin filaments around inner cell membrane. Myosin II contraction is hence probably responsible for membrane blebbing.[109] Interestingly, ROCK and MLCK are regulated by ERK1/2 in endothelial cells,[110] although the effect of the latter on MP production triggered by aβ2GPI antibodies remains unproven.

Considering these results, in addition to the fact that both MAPK pathways are activated in the presence of aPL (in monocytes and endothelial cells),[56,109] it could be hypothesized that both p38MAPK and MEK1/2 are independently involved in MP production triggered by aPL. Furthermore, given the variety of aPL, and multiple mechanisms by which they could activate endothelial cells, the relevance of different pathways in this process may vary according to aPL specificities.

Conclusions

APS is an autoimmune disease attributed to a wide set of antibodies directed toward phospholipids, plasma proteins, and phospholipid–protein complexes.[25] In vitro studies[12,13,33,34] and analysis of wide groups of APS patients[10,35] suggest that these antibodies may induce two different clinical presentations, which can behave as independent entities: vascular and obstetric APS. The nature of this differentiation could be associated with a combination of both specific antibodies, as well as with other variables such as serum antibodies titers, tissue distribution of β2GPI (main antigenic protein), or occurrence of a previous proinflammatory disturbance (*second hit*).[11,14]

Different pathogenic mechanisms have been proffered as an explanation for the procoagulant state that characterizes this syndrome. One of those mechanisms is the ability of some autoantibodies to enhance endothelial cell-derived MP production.[9] These MPs are a type of extracellular vesicle functioning as messenger in physiological contexts, but has also been connected with pathological procoagulant states given the capability to induce clot activation in vivo[18] and in vitro.[82]

In spite of in vitro results having described that IgG from vascular APS patients is related to higher endothelial MP production,[9] most authors who have directly analyzed the blood MP amounts in APS patients have not found any significant difference between clinical groups.[20–22] Nevertheless, MP quantities are not the only factor playing an important role in the procoagulant state of APS. Instead, it has been seen that these vesicles can perform an autocrine/paracrine function, by means of which they are capable to spread a procoagulant phenotype among endothelial cells[93] and monocytes.[79]

These indirect mechanisms largely depend on vesicles cargo, which in turn can be influenced by intracellular events accompanying the "blebbing" process.[88] In this regard, evidence suggests that both p38MAPK and MEK1/2 could be related to the aforementioned process,[106,107,109] even though the exact mechanism is yet to be clarified.

Authors' Contributions

The main topic of this review was proposed by Á.P.C.J. and D.Á. The draft copy of the manuscript was written by D.Á., and C.R. and Á.P.C.J. critically assessed and evaluated the writing process.

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Conflict of Interest

None declared.

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