Chapter

Extracellular Vesicles: Intercellular Communication Mediators in Antiphospholipid Syndrome

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Abstract

Antiphospholipid syndrome (APS) is a systemic autoimmune disease characterized by thrombosis, obstetric complications and the presence of antiphospholipid antibodies (aPL) that cause endothelial injury and thrombophilia. Extracellular vesicles are involved in endothelial and thrombotic pathologies and may therefore have an influence on the prothrombotic status of APS patients. Intercellular communication and connectivity are important mechanisms of interaction between healthy and pathologically altered cells. Despite well-characterized *in vitro* and *in vivo* models of APS pathology, the field of extracellular vesicles is still largely unexplored and could therefore provide an insight into the APS mechanism and possibly serve as a biomarker to identify patients at increased risk. The analysis of EVs poses a challenge due to the lack of standardized technology for their isolation and characterization. Recent findings in the field of EVs offer promising aspects that may explain their role in the pathogenesis of various diseases, including APS.

Keywords: Extracellular vesicles, Antiphospholipid syndrome, Antiphospholipid antibodies, Thrombosis, Extracellular vesicles, Endothelial cells, Monocytes, Platelets

1. Introduction

Antiphospholipid syndrome (APS) is a systemic autoimmune disease characterized by thrombosis and/or obstetric complications and persistent presence of antiphospholipid antibodies (aPL) [1]. aPL cause the activation of cells involved in the vasculature (endothelial cells, platelets, monocytes) and the release of extracellular vesicles (EVs). EVs are submicron particles that are constitutively released from nearly all cell types [2] and circulate in plasma of healthy individuals in concentrations of approximately 10^{10} EVs/ml [3]. In response to stimuli, such as cell activation due to inflammation and/or apoptosis, increased amounts of EVs are released. The frequencies of plasma EVs, which originate from different cellular origins, can be altered in disease states [4]. Over the last decade, the number of scientific publications describing physiological and pathological functions of EVs has increased significantly. The term"extracellular vesicles" is a collective term that encompasses various subtypes of cell-releasing membranous structures called exosomes, microvesicles, microparticles, ectosomes, oncosomes, apoptotic bodies, and many others. The International Society for Extracellular Vesicles (ISEV) proposed Minimal Information for Studies of Extracellular Vesicles

("MISEV") guidelines for accurate isolation and characterizations of EVs [5]. MISEV2018 proposes the classification of EVs according to their physical properties (size and density), biochemical composition (protein marker positivity), cells of origin or based on the description of the conditions that induce their release. The heterogeneity of EVs research is, apart from nomenclature, also a reflection of poorly standardized methods of isolation and downstream analysis. Complex biological samples containing non-EV contaminants pose a challenge for both the isolation and characterization of EVs. Usually a combination of different methods is used to obtain good data quality. The most common EVs are of platelet or megakaryocyte origin (> 50%) [6], while about 5-15% of EVs are of endothelial origin [7]. An increase in circulating EVs, especially endothelial EVs, is considered a hallmark of vascular dysfunction and cardiovascular disease. Increased EVs are found particularly in patients with hypertension [8], diabetes [9], acute coronary syndromes [10] and cardiovascular disease [11]. EVs, especially medium to large endothelial EVs, have been studied in patients with APS, who had significantly higher levels of circulating endothelial and platelet EVs compared with healthy controls [12]. One study also reported increased levels of small EVs (sEVs), which are less than 200 nm in size, in the plasma of patients with APS [13]. In addition, they reported on an altered protein profile of sEVs, indicating platelet and endothelial activation. These results show that a complex systemic network that exists in the form of cell–cell communication via sEVs is altered in APS patients.

2. Extracellular vesicles

Extracellular vesicles are small particles composed of a phospholipid bilayer that encloses soluble cytosolic or endosomal material and nuclear components and, unlike a cell, are unable to replicate. EVs can be as small as the smallest physically possible unilamellar liposome (about 20-30 nm) or as large as 1 μm or more [14]. EVs serve as regulators of the transfer of biological information (proteins, nucleic acids, lipids and metabolites), which act both locally and remotely [15]. EVs are found in a variety of human biofluids including serum, plasma, urine, saliva, breast milk, amniotic fluid, ascites fluid, cerebrospinal fluid and even bile [16]. Under normal physiological conditions, they are continuously secreted into the extracellular environment, however, the amount of EVs is increased by activated and apoptotic cells and is associated with different pathologies, including thrombosis [7]. EVs are probably the most extensively studied in cancer and were also found to play a significant role in cancer-associated thrombosis [17]. Over the last decade, EVs have been extensively studied in the field of biomedical research to determine their biological role in normal physiology and in disease state as well as to exploit potential clinical applications in the diagnosis and prognosis of disease. EVs are considered a promising source of biomarkers since they carry different biological materials that reflect the status of the cell of origin. Nevertheless, EVs have also been considered as a therapeutic agent, as an alternative to their synthetic counterparts, such as liposomes [18].

2.1 Classification of EVs

The classification and nomenclature of EVs is complicated and could be confusing due to overlapping definitions. The most common classification of EVs currently used in the literature is the classification of different EVs into subtypes, such as endosomal derived exosomes, membrane derived (microparticles, microvesicles or ectosomes) and apoptotic bodies. This classification is based on the

assignment of a specific EV to a particular biogenesis pathway, which remains very difficult to assess [19]. Unless biogenesis is investigated directly, EVs are classified according to their a) physical characteristics such as size: "small EVs" (sEVs; size <100 nm or < 200 nm) and "medium/large" (m/lEVs; size >200 nm), and density; low, medium, high, with defined range, b) biochemical composition (surface expression or by the presence of a specific molecule within EVs), or c) description of a specific condition or cell of origin (**Figure 1**) [19].

2.2 Biological role of EVs

The key biological function of EVs is cell to cell communication and the transfer of biological materials that act closely, but also, and more importantly, remotely. Cargo within the EVs is protected from degradation in the bloodstream and can be successfully transferred to specific cells of interest, affecting several biological functions of these cells. EVs can transfer a wide variety of molecules: heat shock proteins (HSP-90, HSP-70), interleukins (IL), such as tumor necrosis factor-alpha (TNFα), acute phase proteins, such as serum amyloid A [20], enzymes, peptides, growth factors [14]. Therefore, EVs have a wide range of biological functions including immune response, antigen presentation, and the transfer of RNA, including micro RNA (miR) and DNA. Given the fact that EVs migrate through the bloodstream they can have pleiotropic effects that are likely to affect every tissue in the body [14]. In immunity, they modulate immune cells, cell–cell interactions, and transfer of cytokines and chemokines. In the heart and vessels, they stimulate

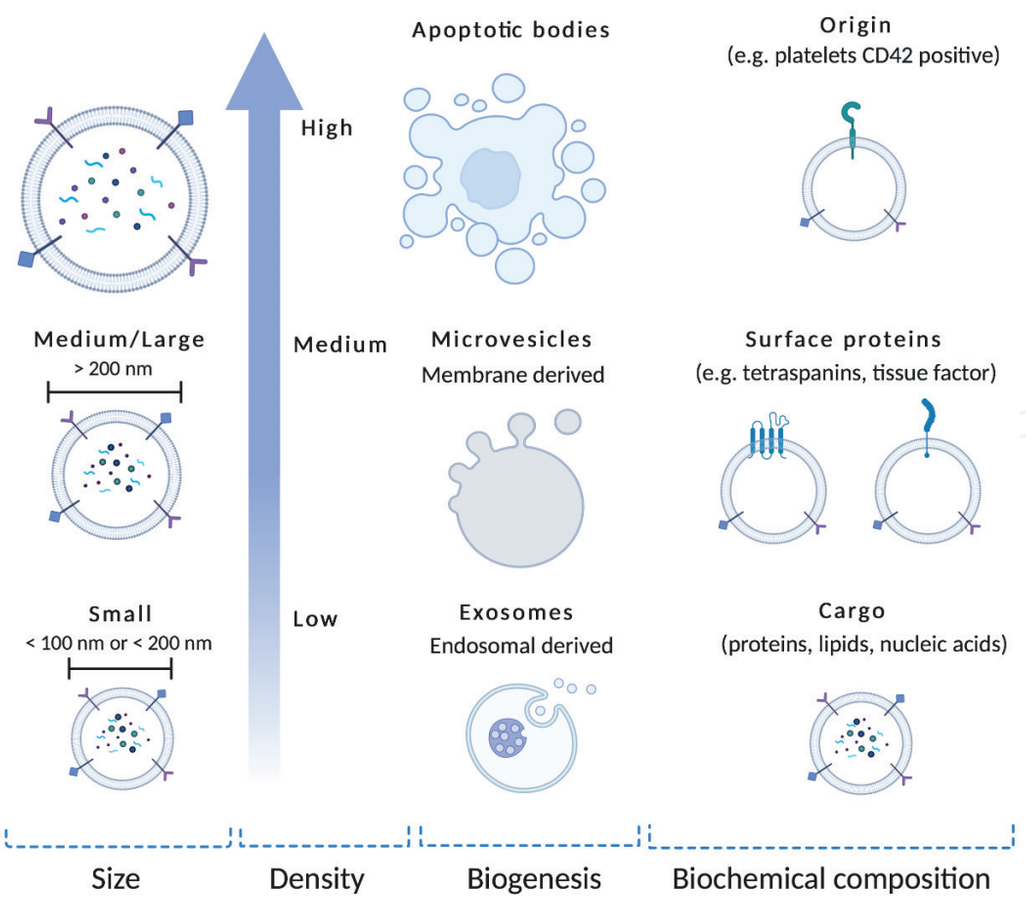

Figure 1.
Classification of EVs. EVs can be classified according to their size (Small <100 nm or < 200 nm, Medium/large >200 nm), density (Low, Medium, High) with a defined density range, biogenesis pathway (Exosomes; endosomal derived, Microvesicles; membrane derived and Apoptotic bodies; released upon cell apoptosis) or biochemical composition defining EVs origin, surface proteins or cargo. Created with BioRender.com.

coagulation and thrombosis, modulate angiogenesis, calcification and vascular repair. In the adipose tissue, they modulate angiogenesis, inflammation, cell differentiation and secretion of cytokines. In the bone marrow, they are involved in cell–cell cooperation, cell proliferation, differentiation and maturation. In the central nervous system, they are involved in the integration of neurons and various glial cells, modulate angiogenesis, neuronal plasticity and myelination. In the blood, they influence activation and aggregation of platelets, are directly involved in coagulation, as well as cargo transfer of procoagulant or anticoagulant molecules, cytokines and growth factors [14].

2.3 Methods for EVs isolation

Biological fluids containing EVs, which serve as potential minimally invasive liquid biopsies, have shifted its proteomic and genomic profiling research towards identification of biomarkers for disease diagnosis, prognosis and longitudinal monitoring. Studying EVs and their cargo typically requires separation from a biological matrix (such as a complex fluid or tissue) to analyze the unique EV components. However, isolating EVs from different sources presents certain challenges. For example, in serum and plasma the main challenge is to separate EVs from highly abundant non-EV proteins, such as albumin and globulins and non-EV lipid particles, such as lipoproteins and chylomicrons [21]. These co-purified contaminants pose a challenge for the isolation, analysis, and application of EVs. Correct interpretation and detailed reporting of the nature of EV samples and sample handling including storage, isolation, and analytical procedures for the analysis of EVs is required [18]. Many approaches have been used, including differential ultracentrifugation, density gradient ultracentrifugation, size exclusion chromatography, and affinity/immunoaffinity capture methods. All these approaches have their limitations and advantages, which are challenged by both the source and quantity of starting material and the downstream application [21]. Serial centrifugation enables the separation of EVs from cells, cell debris and larger vesicles such as apoptotic bodies. Ultracentrifugation (UC) exploits high centrifugal speed (100.000 x g) for a sufficient time to allow EVs to pellet. It separates particles based on their size, shape, and flotation density and is less efficient for smaller and less dense particles. Repeated centrifugation can reduce the amount of non-EVs particles, but also reduces the yield and may damage the EVs [21]. Density centrifugation or density ultracentrifugation uses a density gradient medium or cushion of denser solution (e.g. sucrose cushion; sUC) [22] to separate particles of a similar density. This technique takes advantage of the fact that particles denser than the solvent sediment in the suspension, while particles less dense float up. This increases the purity of samples and reduces the potential of mechanical damage to the vesicles [23]. Density gradient ultracentrifugation is successful in separating chylomicrons, very low-density, low-density and intermediate density lipoproteins present in plasma. However, particles of similar density, such as high-density lipoproteins, are co-isolated with the EVs [21]. Size exclusion chromatography (SEC) is a chromatographic method that allows vesicles of a particular size to be separated where EVs retain their structure and physiological function [24]. When performing SEC protein contaminants and aggregates of similar size, are often still present. In addition, the sample has to be further concentrated because of the different pooled fractions, decreasing the yield of isolation. Holcar et al. have investigated the purity of the samples by comparing sUC and SEC; the two most commonly used methods for the isolation of EVs. Transmission electron microscopy (TEM) of EVs isolated with SEC showed increased levels of lipoproteins. This was further confirmed by determining a significant increase of ApoA1 (found in high-density lipoproteins) and ApoB100

(found in very low-density, low-density and intermediate-density lipoproteins) [22]. Based on their results, the presence of lipoproteins in SEC isolates could have a significant impact on downstream analysis. Polymer-based precipitation uses volume-excluding polymers to lower the solubility of EVs and similarly sized non-EV particles which are isolated via low speed centrifugation. The main problem using this method is that protein removal kits must be used [21]. The highest purity of isolated EVs is achieved by using different immunopurification methods, such as immunomagnetic isolation. This method separates EVs on the basis of an antigen–antibody interactions where the antibodies linked to the matrix (e.g. magnets) are directed against a specific antigen of interest on EVs [25]. Using this methodology, a specific EV subpopulation is investigated, however, the information about the general vesicle population is lost. In addition, when using the immunopurification method, EVs stay bound to the matrix, which makes them incompatible with certain downstream analyses (**Table 1**).

2.4 Methods for EVs analysis

The analysis of EVs is greatly hampered by their heterogeneity (size, different populations etc.) and by the complex nature of any biological or clinical sample (the presence of non-EVs contaminants). The characteristics of EVs can be determined by biochemical analysis (immunoblotting, immunosorbent EV assays and flow cytometry) or with physical analysis (electron microscopy (EM), atomic force microscopy (AFM), dynamic light scattering (DLS), nanoparticle tracking analysis (NTA), tunable restrictive pulse sensing (tRPS) and flow cytometry) as well as novel, optical based, technologies (fluorescence-based techniques, surface plasmon resonance, interferometric imaging and electrochemical sensing) [18] (**Table 1**). Due to challenges in EVs analysis, a combination of different methods is very common.

2.4.1 Physical analysis

The physical analyses of EVs involve determining a size range, shape and concentration. The size of EVs can be determined directly by high-resolution imaging, or indirectly, by using optical or electrical readouts. Direct high-resolution imaging includes microscopy methods, such as EM or AFM, to obtain an accurate estimate of individual EVs in nanoscale resolution [18]. EM is used to determine the size and morphology of individual EVs. This method employs an electron beam instead of light to obtain high-resolution images in nanoscale. The most commonly used EM techniques are scanning (SEM) and transmission (TEM). Scanning electron microscopy image will explore the topography of the EVs surface. Since electrons pass through the sample in TEM, a 2D image of EVs will be obtained, which will

Type of EVs	Isolation of EVs	Characterization of EVs
Small	Ultracentrifugation +/− density gradient, SEC, polymer-based approaches, immunopurification	AFM, EM, ELISA, NTA, RPS, DLS, WB
Medium/Large	Centrifugation +/− density gradient	AFM, EM, NTA, IF, ELISA, flow cytometry

Abbreviations: AFM: atomic-force microscopy; DLS: dynamic light scattering; ELISA: enzyme-linked immunosorbent assay; EM: electron microscopy; IF: immunofluorescence microscopy; NTA: nanoparticle tracking analysis; RPS; restrictive pulse sensing; SEC: size exclusion chromatography: EM: transmission electron microscopy WB: western blotting.

Table 1.
Most commonly used methods for isolation and characterization of EVs.

also provide the information on the inner structure [26]. These electron microscopy methods require fixation or drying of the sample which complicates the translation of observed structures to the native morphology of the EVs. To avoid sample dehydration variations of electron microscopy techniques, such as cryogenic TEM, have been evolved [27]. In the AFM, an extremely sharp tip scans the area and its deflection is translated into topology information. It provides additional information about mechanical properties, such as stiffness and elasticity of the vesicles. In most cases, AFM is performed on dry, immobilized surfaces, which in turn may damage the EVs [28]. This can be prevented by analyzing EVs in a solution [29]. Indirect methods estimate the size and concentration based on the interaction of EVs with light (DLS and flow cytometry), their diffusion trajectories (NTA or their effect on the electrical current (tRPS). DLS is based on the analysis of temporal intensity fluctuation of laser light scattered by a dispersion of freely diffusing EVs. Unlike EM and AFM it measures the collective mobility (diffusion coefficient) of scattering EVs that are present in the measured volume. Flow cytometry is often used to analyze the number of cells and their biochemical composition. EVs are much smaller than cells and are usually not detected due to the low sensitivity of the method. However, adapted protocols have been developed to enable the analysis of EVs [30]. In flow cytometry, the flow of cells is hydrodynamically focused in a flow chamber and enables the illumination of a single cell by several different lasers. The forward light scatter on the cell will allow information on the cells' sizes while the side scatter will gave information on the granularity and composition [31]. Because the EVs are very small and have a low refractive index, flow cytometers can more accurately determine the EVs larger than 500 nm. Smaller EVs are detected in the background signal and collectively due to the swarm effect, which happens when multiple EVs are simultaneously and not separately illuminated by the laser, creating a swarm [32]. The recent advances in the field of flow cytometry enable to detect also populations as small as 100 nm [33]. NTA measures how fast a particle diffuses in a static solution due to the principle of Brownian particle motion. By analyzing its motion trajectories, it determines the size distribution of vesicles. tRPS is a technique that measures changes in electrical current as each particle passes through an adjustable nanopore [18]. The heterogeneity of the samples is a major problem with all indirect methods. Compared to direct methods the number of EVs that can be analyzed is typically higher, which allows a better estimate of the concentration. This is also due to the fact that these vesicles are in their original state. However, these methods are not able to provide information on the presence of contaminants, such as lipoproteins.

2.4.2 Biochemical analysis

The characterization of EVs to determine the surface markers, markers of origin and proteins they carry allows to infer the functional role of these vesicles in health and disease. Methods might be divided to more conventional ones; the immunoblotting assays or the methods that will employ the capture of the vesicle; immunosorbent methods. Immunoblotting methods are based on the lysis of a vesicle and the analysis of its contents either by direct spotting on a membrane (dot blot) or separation of proteins using SDS PAGE combined with western blotting, in which specific proteins of interest are determined with labeled antibodies. Immunoblotting methods are often used to determine the presence of EVs in a sample. These methods can also be used to determine the purity of samples [18]. Immunosorbent assays are based on the detection of EVs using specific antibodies directed against surface proteins of EVs. Derived from the classical enzyme linked immunosorbent protein assay (ELISA), EVs are captured on a solid surface coated with antibodies that are typically present on the EVs. EVs capture results in a

strong enrichment. Analysis of EVs surface proteins is afterwards performed with antibodies directed to a protein of interest on the surface of the EVs. These detection antibodies are conjugated to an enzyme enabling the conversion of a fluorescent/colored substrate that can be quantified with a spectrophotometer [18].

3. Extracellular vesicles in vascular pathologies

The main cell types involved in vascular hemostasis are endothelial cells, platelets and monocytes. All these cells release EVs, which leads to a complex interplay between different vesicles and different cells. EVs are continuously released in low concentrations from the cells into the intercellular environment, but this is greatly increased during cellular activation and apoptosis. EVs transmit various biological information (in the form of proteins, lipids and nucleic acids). Travelling through the bloodstream, EVs serve as local or distant messengers that transmit information to a variety of cells and tissues. Hemostasis is a very strictly regulated process that maintains normal function of vasculature despite the presence of triggers, such as injury and/or infection. One of the consequences of an altered hemostatic balance is the formation of thrombi, a process in which EVs play an important role [15]. EVs coming from activated cells have been shown to have both procoagulant and proinflammatory effects. Procoagulant effects are related to the fact that some EVs contain anionic phospholipids, mainly phosphatidylserine (PS), on their surface, which contributes to the assembly and activation of the prothrombinase complexes, thus promoting thrombin formation [34]. However, not all EVs carry PS on their surface, suggesting the involvement of other mechanisms contributing to the procoagulant state [35], including other important coagulation factors, such as tissue factor (TF), Factor XII [36], and reduced activity of tissue factor pathway inhibitor (TFPI) and thrombomodulin on endothelial cells [37]. In addition, EVs also induce the expression of adhesion molecules; integrins and selectins on the recipient cells causing platelets, monocytes, and endothelial cells to interact more intensively with each another. Finally, EVs also contribute significantly to the proinflammatory state in the vascular microenvironment by delivering or inducing certain cytokines and chemokines and by transferring nucleic acids and lipids [38]. The effects that these EVs have on different cell types disrupt the normal functioning of the vascular system, leading to the development of different pathologies, including deep vein thrombosis or pulmonary embolism [7] and cardiovascular diseases (atherosclerosis [39], hypertension [8], myocardial infarction [40] and stroke [41]).

3.1 Platelet-derived EVs

EVs from activated platelets can have different effects on endothelial cells, monocytes and other platelets (**Figure 2A**). Namely, increased levels of intracellular adhesion molecule-1 (ICAM-1), a well-known activator of endothelium was observed on endothelial cells upon stimulation with platelet EVs [42, 43], an effect later ascribed to miR-320b transfer [42]. Increased expression of lymphocyte function-associated antigen-1 LFA-1 (CD11a/CD18) and macrophage antigen-1 Mac-1 (CD11b/CD18); both important in mediating monocyte-endothelium interactions, were observed on monocytes upon stimulation with platelet EVs. These effects are induced by the transfer of arachidonic acid from platelet EVs and appear to be dependent on the activation of protein kinase C [44]. Platelet EVs therefore significantly modulate adhesion of monocytes to endothelial cells. It has also been shown that platelet EVs increase the deposition of platelets on damaged arteries and increase platelet aggregation and adhesion to collagen [45]. By influencing

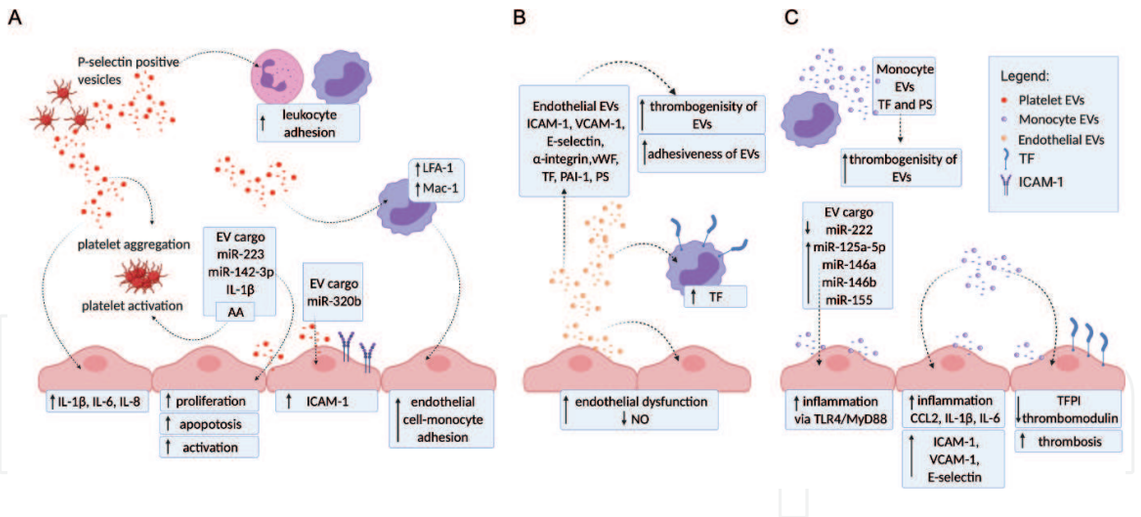

Figure 2.
Activation of platelets, monocytes and endothelial cells by EVs deriving from different cells. Schematic representation of the potential in vitro mechanisms focusing on vascular function, inflammation and thrombosis. (A) Platelet EVs can stimulate endothelial cells and monocytes via direct interaction or cargo delivery (miR and lipids). Furthermore, platelets EVs can also act via a feedback loop causing platelet aggregation and activation. Platelet EVs induce endothelial cell activation, proliferation and apoptosis by the transfer or miR-223 and miR-142-3p while ICAM-1 expression is induced by the delivery of miR-320b. Increased adhesion between endothelial cells and monocytes as well as between leukocytes in mediated by platelet EVs. (B) EVs released form endothelial cells were found to have a procoagulant profile expressing vWF, TF, PAI-1, PS as well as increased adhesive properties expressing VCAM-1, ICAM-1, E-selectin, and α-integrin. Endothelial EVs promote procoagulant profile of monocytes by induction of the TF expression on these cells. Endothelial EVs induce endothelial dysfunction by attenuating the production of nitric oxide from endothelial cells (C) Monocytes release procoagulant EVs that carry TF and PS. Furthermore, monocyte EVs interact with endothelial cells causing increased expression of adhesion molecules (ICAM-1, VCAM-1 and E-selectin), increased inflammation and procoagulant profile by reducing the expression of anticoagulant molecules (TFPI and Trombomodulin). Monocyte EVs transfer miR cargo (miR125a-5p, miR-222, miR-146a, miR-146b, miR-155) and induce inflammation in endothelial cells. CCL2, C-C motif chemokine ligand 2; ICAM-1, intercellular adhesion molecule 1; IL, interleukin; LFA1; lymphocyte function-associated antigen 1; Mac-1, Macrophage antigen-1; mIR; micro RNA; MyD88, myeloid differentiation primary response gene 88; NO, nitric oxide; PAI-1, plasminogen activator inhibitor-1; PS, phosphatidylserine; TF, tissue factor; TLR4, tool like receptor 4; VCAM-1, vascular cell adhesion molecule 1; vWF, von Willebrand factor. Created with BioRender.com.

cell adhesiveness, EVs also modulate interactions between leukocytes. Platelet EVs use P-selectin to bridge leukocytes, increase leukocyte-leukocyte interactions and enhance leukocyte accumulation on a P-selectin surface [46, 47]. Platelet EVs can therefore contribute to increased adhesion and aggregation of platelets and leukocytes on blood vessel walls during pathology. In addition, platelet EVs influence the production of cytokines (IL-1β, IL-6, IL-8) [43] and the transfer of miRNA (miRs 142-3p and 223), affecting the activation, proliferation and apoptosis of endothelial cells [48, 49]. In addition, platelet activation by the transfer of arachidonic acid from platelet EVs to other platelets, was observed [50]. Importantly, the role of platelet EVs in hemostasis is not entirely clear, as there is evidence that these EVs can also have anticoagulant effects [51, 52]. Further research is needed to determine, which key stimuli are responsible for determining the final effect of platelet EVs.

3.2 Endothelial-derived EVs

Endothelial cell activation and damage play an important role in vascular pathologies, with endothelial EVs being proposed as one of the causative agents in vascular pathologies (**Figure 2B**). Many proinflammatory factors (e.g. TNF-α, lipopolysaccharide, C-reactive protein and reactive oxygen species) and coagulation stimuli (thrombin, plasminogen activator inhibitor-1 (PAI-1)) can increase the

levels of endothelial EVs. These vesicles carry adhesion molecules; ICAM-1, vascular cell adhesion protein 1 (VCAM-1), E-selectin, VE-cadherin, α-integrin, growth factors; endoglin, CD146, vascular endothelial growth factor (VEGF) receptor and molecules involved in coagulation, such as von Willebrand factor (vWF), TF, PAI-1 [53–55]. The expression of anionic phospholipids; such as PS, together with coagulation molecules, contribute to their procoagulant role. In addition, endothelial EVs may interact with other cells such as monocytes and induce the expression of TF on these cells [56]. Endothelial EVs induce endothelial dysfunction by attenuating the production of nitric oxide from endothelial cells [57]. Conversely, endothelial EVs may also have anticoagulant and antiinflammatory potential [38]. Although they exert different effects that are mostly dependent on the environment they originate from, endothelial EVs are generally believed to impair vascular function [58].

3.3 Monocyte-derived EVs

Leukocytes play an important role in the maintenance of vascular homeostasis. The activation of monocytes leads to increased release of monocyte EVs, which contribute to the disturbance of the hemostatic balance (**Figure 2C**). Monocyte EVs adhere to endothelial cells via LFA-1-ICAM-1 adhesion, as shown by the blocking of LFA-1 [37]. Once internalized, EVs were able to induce extracellular signal-regulated protein kinase (ERK1/2) and nuclear factor-κB (NF-κB) signaling pathways that increase the expression of the adhesion molecules VCAM-1, ICAM-1, and E-selectin on endothelial cells [59]. On the other hand, Tang et al. suggested that monocyte EVs induce *de novo* synthesis of ICAM-1, chemokine C-C motif ligand 2 (CCL2) and IL-1β in endothelial cells. This occurs via the activation of toll like receptor 4 (TLR4)/Myeloid differentiation primary response gene 88 (MyD88)/NF-κB [60]. An increase in the adhesion profile of endothelial cells makes them more susceptible to interactions with platelets and monocytes and increase the prothrombotic state of the vasculature. Monocyte EVs trigger immune dysfunction related proinflammatory pathways also by the transfer of different miRs to the recipient cells. Levels of miR-125a-5p, miR-146a, miR-146b, miR-155 were significantly increased and miR-222 levels were decreased in INFα and lipopolysaccharide stimulated monocyte EVs compared to unstimulated monocyte EVs. Monocyte EVs transfer functional EVs to endothelial cells and activate the TLR4/MyD88/NF-κB signaling leading to differential expression of immunomodulatory miR in endothelial cells [61]. Both monocytes and monocyte EVs are positive for TF [37], a primary cellular initiator of blood coagulation. In vascular injury, TF forms a complex with factor VIIa, which activates the coagulation protease cascade and eventually leads to fibrin deposition and platelet activation [62]. In addition, monocyte EVs reduce the expression of the anticoagulant TFPI and of thrombomodulin on endothelial cells [37].

4. Pathological mechanisms of the Antiphospholipid syndrome

Antiphospholipid syndrome (APS) is a systemic autoimmune disorder characterized by venous and/or arterial thrombosis and pregnancy complications in the presence of antiphospholipid antibodies (aPL). aPL are a heterogeneous group of autoantibodies, of which anti-cardiolipin (anti-aCL), anti-β2 glycoprotein I (anti-β2GPI) and lupus anticoagulant (LA), are in the laboratory criteria for the diagnosis of APS [63]. In addition to criteria aPL other, non-criteria aPL, such as antibodies against phosphatidylserine/prothrombin complex, were found to play an important role in APS [64, 65]. These antibodies are, in some patients, the only

elevated aPL. Although aPL are persistent in APS patients, thrombosis occurs only occasionally, suggesting the involvement of other triggers that, together with aPL, turn the hemostatic balance in favor of thrombosis. In the development of APS, a two hit theory has been proposed in which the continuous presence of aPL as the first hit and inflammation, trauma, or surgery as a second hit together lead to thrombus formation [66, 67]. APS pathogenesis clearly involves both inflammatory and coagulation pathways in endothelial cells, monocytes, neutrophils, and platelets. Frequently identified prothrombotic mechanism is inhibition of the natural anticoagulant pathways [68]. It has been shown that aPL inhibit the activation of protein C [69] and its ability to inactivate factors V and VIII [70]. In addition, aPL inhibit the activity of TFPI [71] and activation of antithrombin [72]. They have also been found to be involved in fibrinolysis by neutralizing the ability of anti-β2GPI to stimulate tissue-type plasminogen activator [73]. Furthermore, aPL impair the ability of Annexin A5 to form a network on procoagulant anionic phospholipids [74]. aPL also directly bind to vascular cells and trigger their activation, which in response, release prothrombotic molecules and thus contribute significantly to the pathogenesis of APS. The activation of endothelial cells leads to a disruption of the normally anticoagulant endothelial surface [68]. This is achieved by upregulating adhesion molecules (E-selectin, ICAM-1, VCAM-1) [75], molecules involved in coagulation (TF) [76] and by the decrease in endothelial cell derived nitric oxide [77]. The biochemical pathways are not fully defined, but research has suggested several receptor-mediated mechanisms including, annexin A2, TLR4/NF-κB, TLR2, TLR7 and low-density lipoprotein receptor-related protein 8 [68]. In addition to endothelial cells, aPL also act on platelets. Increased production of thromboxane B2, increased platelet adhesion to collagen type I and III and increased platelet activation have been described [66]. Among immune cells, monocytes are the most extensively studied in APS. In APS patients, monocytes have been shown to have a proinflammatory and procoagulant phenotype that is mediated by upregulation of NF-κB, MEK-1/ERK, and p38 MAP kinase pathways [78]. The main player of the procoagulant phenotype is increased surface expression, production and activity of TF on monocytes [79]. Stimulation of monocytes with aPL influences the release of IL-1β [80] and TNFα [81], probably by the activation of NLR family pyrin domain containing 3 inflammasome [82]. Monocyte-endothelial interactions are increased by upregulation of adhesion molecules on both cell types, as well as expression of other molecules, such as monocyte chemoattractant protein-1 by the endothelium, which in turn promotes the synthesis of TF by monocytes [83].

5. Extracellular vesicles in antiphospholipid syndrome: literature review and discussion

The role of EVs as communicators between different types of cells involved in the pathology of APS have been studied *in vivo* by analyzing the characteristics of EVs from plasma of APS patients and *in vitro* after stimulation of cells with aPL. As discussed above, EVs can carry characteristic proteins that determine their origin (**Figure 3**, upper panel) and their prothrombotic profile (e.g. by the presence of TF, PS) (**Figure 3**, lower panel). However, all EVs carry also different receptors, adhesion molecules and cargo (nucleic acids, lipids and proteins), which together influence the interaction between different cells, as well as information transfer. Larger vesicles (microvesicles) usually carry surface TF, PS and annexins while smaller EVs (exosomes) carry surface tetraspanins (CD9, CD63, CD81) and flotillin and alix, clathrin and TSG101 proteins as their cargo (**Figure 3**, lower panel).

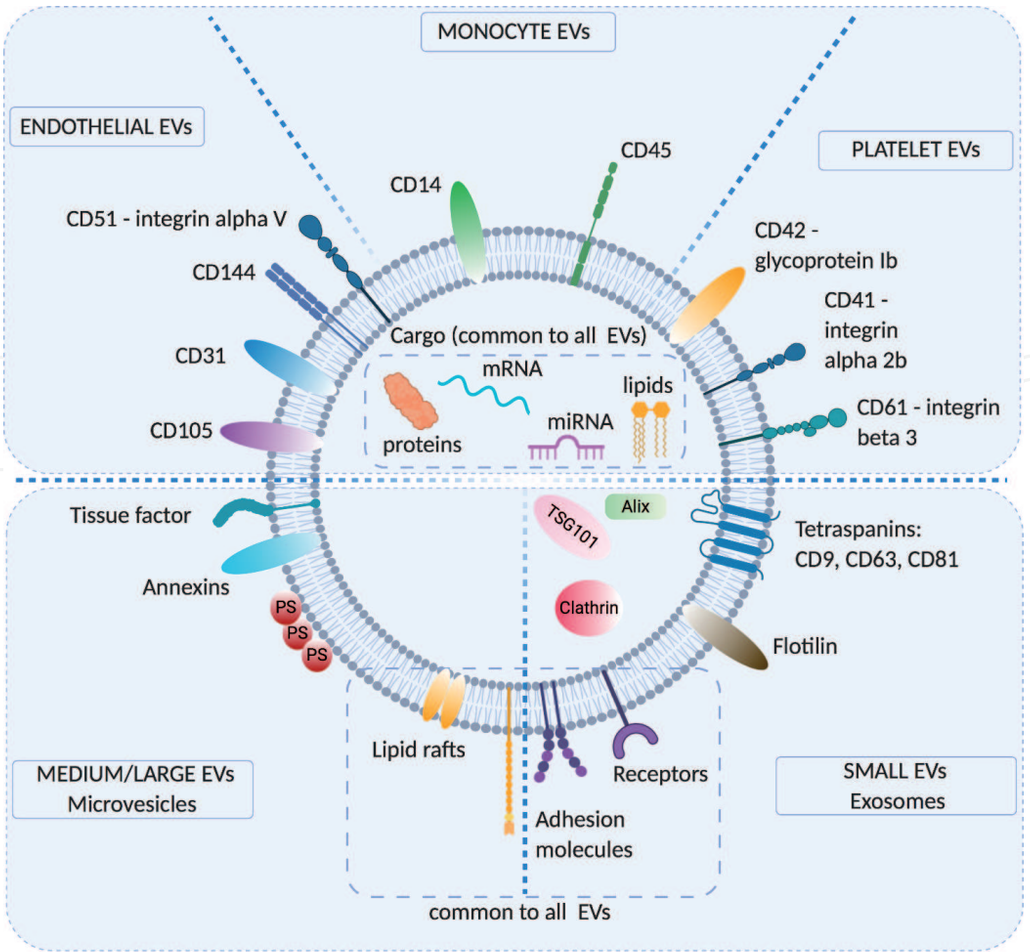

Figure 3.
Characterization of endothelial, monocyte and platelet EVs. Schematic representation of commonly expressed surface protein markers of endothelial cells, monocytes and platelets, as well as markers currently associated with small and medium/large EVs. Endothelial EVs usually express CD51 (Integrin alpha V) which is a part of a complex that binds extracellular matrix proteins, CD144 (Vascular endothelial cadherin), an important cell adhesion molecule in the formation of adherent junctions, CD31 (PECAM-1; platelet endothelial cell adhesion molecule) mediates leukocyte- and platelet-endothelial cell adhesion, CD105 (Endoglin) is a type I membrane glycoprotein and a part of transforming growth factor β receptor complex. Monocyte EVs commonly express CD14 (Cluster of differentiation 14) a known monocyte marker and CD45 (PTPRC; protein tyrosine phosphatase receptor type C) that is leukocyte specific cell surface glycoprotein involved in various cellular processes. Platelet EVs usually express different glycoproteins (CD42; glycoprotein IX, CD41; glycoprotein IIb, CD61; glycoprotein IIIa) that are integrin complex proteins involved in platelet aggregation. All EVs carry adhesion molecules, receptors and lipids that are involved in interaction of EVs with different cells. Furthermore, they carry proteins, nucleic acids and lipids that can be transferred to a target cell. Membrane derived vesicles-microvesicles, are usually larger and express procoagulant molecules, such as TF (Tissue factor), annexins and PS (Phosphatidylserine), whereas tetraspanins (CD9, CD63, CD81) and specific luminal proteins (Clathrin, TSG101 and Alix) are specific for smaller vesicles of endosomal origin-exosomes. Created with BioRender.com.

5.1 *In vivo* studies (characterization of EVs from plasma of APS patients)

The role of EVs has been studied in many vascular pathologies, including deep vein thrombosis [7] and cardiovascular disease [38], whose common denominator is endothelial dysfunction. In addition, platelet EVs have been proposed as a useful biomarker for long-term follow-up after myocardial infarction [84], whereas increases in the number of endothelial EVs play a role in many inflammatory diseases, such as atherosclerosis [39]. Studies investigating EVs in patients with APS are limited and heterogeneous (**Table 2**). To date and to our knowledge, there have been 13 studies investigating EVs in thrombotic APS patients. With one exception, all of them have focused on medium/large EVs. Furthermore, the results of these studies are not completely comparable because the methods for isolating

Reference	Patients	Controls	Isolation protocol	Method of quantification	Type of EVs	Main findings
Combes et al., 1999 [53]	5 APS, 8 APS + SLE	17 asympt. aPL+ (6 autoimmune, 4 infections, 5 malignancy, 2 undefined) 30 HBDs	2 x 1,500 × g (15″) 13,000 × g (1″)	AnxV+ or CD51+ < 1.5 μm (latex beads)	endothelial (CD51+)	↑ endothelial EVs in aPL+ pts. vs. HBDs. ↑ endothelial EVs in thrombotic aPL+ pts. vs. asympt. aPL+ pts. Levels of SLE aPL- pts. were similar to HBDs.
Joseph et al., 2001 [85]	20 APS 14 APS + SLE	16 SLE 20 HBDs	2 x 1,500 × g (15″) 13,000 × g (1″)	GPIIb-IIIa+ < 0.8 μm	platelet (GPIIb-IIIa+)	No difference in platelet EVs between APS pts., SLE pts. and HBDs.
Nagahama et al., 2003 [86]	24 APS 13 SLE + APS	30 HBDs	200 x g (10″, RT), 1000 x g (15″, RT)	AnxV+, CD42a+, CD14+	platelet (CD42a+) monocyte (AnxV+/ CD14+)	↑ monocyte EVs in APS pts. vs. APS + SLE pts. and vs. HBDs. ↑ P-selectin+ platelets and platelet EVs in APS pts. vs. HBDs.
Dignat-George et al., 2004 [87]	23 APS 14 APS + SLE	28 SLE aPL+ no thrombosis 23 SLE aPL- no thrombosis 25 aPL- with thrombosis 25 HBDs	2 x 1,500 × g (15″) 13,000 × g (2″)	CD51+ < 0.8 μm (latex beads)	endothelial (CD51+)	↑ endothelial EVs in APS pts. vs. HBDs and vs. non aPL related thrombotic pts. ↑ endothelial EVs in SLE aPL+ pts. vs. HBDs. No difference between SLE aPL- pts. and non aPL related thrombotic pts. vs. HBDs. ↑ endothelial EVs in aPL+ pts. vs. aPL- pts. and vs. HBDs. No difference between primary or secondary APS.
Jy et al., 2007 [88]	60 APS	28 asympt. aPL+ 39 HBDs	160 × g (10″) 1500 × g (6″)	CD31+ or CD42+ < 1.5 μm	endothelial (CD31+/ CD42-) platelet (CD31+/ CD42+)	↑ platelet and endothelial EVs in APS pts. vs. HBDs. ↑ endothelial EVs in asympt. aPL+ pts. vs. HBDs. No difference in levels of endothelial EVs in APS pts. vs. asympt. aPL+ pts. ↑ platelet EVs in APS pts. vs. asympt. aPL+ pts. No difference in levels of platelet EVs in asympt. aPL+ pts. vs. HBDs.

Reference	Patients	Controls	Isolation protocol	Method of quantification	Type of EVs	Main findings
Flores-Nascimento et al., 2009 [89]	11 APS	9 DVT pts. at diagnosis 10 DVT pts. After 1-3 years of warfarin withdrawal 7 FVL pts. 37 HBDs	3000 x g (20″) 13,000 x g (30″)	AnxV+, CD14+, CD31+, CD45+, CD61+, CD142+, CD235+	total (AnxV+) platelet (CD61+) erythrocyte (CD235+) monocyte (CD14+) endothelial (CD31+) leukocyte (CD45+)	No difference in total EVs in DVT pts. at diagnosis, FVL pts., APS pts. and HBDs. ↑ total EVs in DVT 1-3 years and HBDs. No difference in platelet, erythrocyte, monocyte, endothelial and leukocyte EVs in all pts. groups vs. HBDs.
Vikerfors et al., 2012 [90]	40 APS, 12 secondary APS	52 HBDs	Isolation not described	phalloidin-, lacadherin+ or CD14+, CD42a+, CD142+, CD144+ < 1 μm (MegaMix beads)	total (lacadherin+) endothelial (CD144+) platelet (CD42a+) monocyte (CD14+) endothelial (CD144+/CD142+)	↑ total EVs in APS pts. vs. HBDs. ↑ endothelial, endothelial TF+ and monocyte EVs in APS pts. vs. HBDs. No difference in levels of platelet EVs in APS pts. vs. HBDs.
Willemze et al., 2014 [91]	11 APS 19 APS + SLE	72 asympt. aPL+	1,500 x g (10″, 4 °C) 2,000 x g (5″, 4°C) 20,000 x g (30″, 4°C)	not studied	TF+ EVs by a functional assay (TF activity)	↑ EV-TF activity in APS pts. vs. asympt. aPL+. No difference in EV-TF activity in the presence or absence of underlying SLE. No difference between different APS clinical complications. No correlation between EV-TF activity and aPL subtype.
Chaturvedi et al., 2015 [92]	47 aPL+ pts. (38 APS, 2 APS + SLE, 6 asympt. aPL+, 1 aPL+ migraine)	144 HBDs	2 x 1,500 × g (15″) 13,000 × g (2″)	AnxV+ or CD14+, CD41+, CD105+, CD142+, CD144+ < 1 μm (latex beads)	total (AnxV+) endothelial (CD105+/CD144+) platelet (CD41+) monocyte (CD14+) TF (CD142+)	↑ total EVs in aPL+ vs. HBDs. endothelial, platelet, and TF+ EVs in aPL+ vs. HBDs. No difference in levels of monocyte EVs in aPL+ vs. HBDs.

Reference	Patients	Controls	Isolation protocol	Method of quantification	Type of EVs	Main findings
Breen et al., 2015 [93]	66 aPL+ pts. (37 thrombotic APS, 11 obstetric APS, 18 asympt. aPL+).	18 HBD	2x 2,000 x g (15″, 4°C), 12,000 x g (2″, 4°C)	CD41+, CD51+, CD61+ or CD105+	endothelial (CD51+/ CD105+) platelet (CD41+/ CD61+)	↑ endothelial and platelet EVs in aPL+ pts. vs. HBDs. ↑ endothelial and platelet EVs in thrombotic APS pts. vs. HBDs. No difference in levels of endothelial and platelet EVs in obstetric APS pts. vs. HBDs. No difference in levels of endothelial and platelet EVs in asympt. aPL+ pts. vs. HBDs.
Niccolai et al., 2015 [94]	16 APS	16 asympt. aPL+ 16 HBDs	1,500 x g (15″) 3,000 x g (3″)	VPD450+ or CD31+, CD41a+, CD45+ < 0,9 μm (Megamix beads)	total (VPD450+ 7AAD-) endothelial (CD31+) platelet (CD41a+) leukocyte (CD45+)	↑ total, endothelial, platelet, and leukocyte EVs in APS pts. vs. HBDs, APS pts. vs. asympt. aPL+ pts. and asympt. aPL+ pts. vs. HBDs. ↑ total EVs in APS double and triple positivity vs. single positivity. Different EVs populations (endothelial, platelet and monocyte) did not correlate with aPL positivity. ↑ endothelial EVs in asympt. aPL+ pts. triple positivity vs. single positivity. Total, leukocyte and platelet EVs did not correlate with aPL positivity.
Hell et al., 2018 [95]	64 APS 18 APS + SLE 12 APS + LLD	30 HBDs	2,500 x g (15″, 15 °C)	not studied	TF+ EVs by a functional assay (TF activity)	No difference in EV-TF activity in LA+ pts. with thrombosis vs. HBDs. No difference in EV-TF activity in single, double or triple aPL+ patients. No difference in EV-TF activity in LA+ pts. with AT vs. VT vs. combination of both. No difference in EV-TF activity and the number of events (thromboses).

Reference	Patients	Controls	Isolation protocol	Method of quantification	Type of EVs	Main findings
Štok et al., 2020 [13]	14 APS	5 aPL- with thrombosis 7 HBD	820 x g (10″, RT) 2,500 x g (10″, RT) 10,000 x g, (45″, RT) 100,000 x g (2 h15”, 4°C)	NTA	< 200 nm. Multiplex flow cytometry for 38 markers (detection via tetraspanins)	↑ sEVs in APS pts. vs. HBDs. Platelet (CD41b+, CD42a+), lymphocyte (CD8+), leukocyte (CD45+) and endothelial (CD31+) sEVs were detected. ↑ P-selectin on sEVs from APS pts. vs. HBDs. ↑ CD133/1 on sEVs from APS pts. vs. aPL- pts. with thrombosis.

Abbreviations: Anx V, annexin V; APS, antiphospholipid syndrome; AT, arterial thrombosis; aPL, antiphospholipid antibodies; asympt., asymptomatic; DVT, deep vein thrombosis; EVs, extracellular vesicles; FVL, factor V Leiden; HBDs, healthy blood donors; LLD, lupus like disease; NTA; nanoparticle tracking analysis; pts., patients; sEVs, small extracellular vesicles; SLE, systemic lupus erythematosus; TF, tissue factor; VT, venous thrombosis; ↑, elevated levels.

Table 2.
Isolation, quantification and characterization of EVs in plasma of APS patients.

and characterizing EVs are not standardized, the sample sizes in some studies are small and the patient population studied is very heterogeneous (e.g. patients with concomitant autoimmune or other disease). Overall, the studies investigated EVs from the three major cell types involved in the pathogenesis of APS: endothelium, platelets, and monocytes. Studies in the field of cardiovascular diseases and EVs have shown that both platelet and endothelial EVs are elevated in patients with hypertension, compared to healthy blood donors [8], therefore it is important to note that certain proportion of EVs detected in plasma of APS patients might be associated with hypertension. Correlations between the levels of EVs and systolic and diastolic blood pressure needs to be evaluated when investigating EVs in APS patients.

5.1.1 Medium and large extracellular vesicles

5.1.1.1 Endothelial-derived EVs

The endothelium is the major player in APS pathogenesis, so it is not surprising that endothelial EVs have been the most extensively studied (**Table 2**). Combes et al. published in 1999 the first study investigating endothelial EVs in APS using flow cytometry to detect endothelial marker integrin CD51+ EVs. They showed increased levels of endothelial EVs in LA+ patients compared to HBDs [53]. In addition, they have also showed a significant increase in endothelial EVs in LA+ patients with a history of thrombosis compared to asymptomatic LA+ patients. On the other hand, Jy et al. found no difference in endothelial EVs (CD31+/CD42-) between aPL+ thrombotic patients and asymptomatic aPL+ group, suggesting that the release of EVs might be related to the autoimmune process involving the presence of aPL [88]. Dignat-George et al. in 2004, showed increased levels of CD51+ endothelial EVs in APS patients and in aPL+ SLE patients compared to HBDs [87]. Increased levels of endothelial EVs were observed in aPL+ patients vs. HBDs as well as in aPL+ patients vs. aPL- patients. Increased levels of endothelial EVs in the plasma of APS patients compared to HBDs were later confirmed also in several other studies [90, 93, 94] (**Table 2**), in which different endothelial surface markers (CD31+, CD51+, CD105+, CD144+) were examined. Levels of endothelial EVs were shown to be increased in APS patients with exception of one study where the increase was not observed [89]. Chaturvedi et al., on the other hand investigated levels of TF+ endothelial EVs, and found them to be elevated in aPL+ patients, compared to HBDs [92]. A higher TF activity was also observed when comparing APS patients with asymptomatic aPL+ patients [91]. Contrarily, Hell et al. could not observe increased TF activity of endothelial EVs in APS patients vs. HBDs.

5.1.1.2 Monocyte- and Platelet-derived EVs

Platelet-derived EVs are the most numerous type of vesicles found in the circulation of healthy individuals [96], and their levels are further increased in disease [38]. They are known to play key roles in coagulation, thrombosis, vascular senescence and permeability. It has been suggested that platelet EVs induce vascular dysfunction and influence immune modulation, leading to vascular remodeling. Monocytes contribute to APS pathogenesis also by being the main source of tissue factor, which is one of the key initiators of the coagulation cascade. Similar to platelet EVs, it has been suggested that monocyte EVs cooperate in coagulation and vascular inflammation [38]. However, in APS, monocyte EVs (**Table 2**) have been less extensively studied compared to endothelial EVs. Joseph et al., showed no difference in plasma levels of

CD41+ platelet EVs between APS patients and HBDs [85]. This is consistent with the study by Vikenfors et al. (CD42a+) [97] and by Nascimento et al. (CD61+) [89]. On the other hand, increased levels of platelet EVs (CD41+, CD41a+, CD42+, CD42a+) were found in five other studies [86, 88, 92–94]. Jy et al. have shown an increase in platelet EVs in APS patients vs. asymptomatic aPL+ suggesting thrombosis rather than aPL may play a role in platelet EVs release [88]. An increase in monocyte EVs in APS patients compared to HBDs was observed by Nagahama et al. and Vikenfors et al. which is in contrast to two other studies where the authors could not see an increase [89, 92]. There is no consensus on whether platelet and monocyte EVs are elevated in APS patients and there is too little data to conclude on the effects of these EVs in APS patients.

5.1.2 Small extracellular vesicles

To date, only a study by Stok et al. has investigated the presence of sEVs in plasma of APS patients (**Table 2**). Compared to HBDs, significantly increased levels of sEVs were observed in APS patients. In addition, sEVs from different cellular origin: platelet (CD41b+, CD42a+), lymphocyte (CD8+), leukocyte (CD45+) and endothelial (CD31+) were detected. Flow cytometric characterization of sEVs defined a subpopulation of vesicles that were positive for P-selectin (CD62P) and the endothelial progenitor cell marker (CD133/1). sEVs from APS patients were enriched in surface expression of P-selectin, suggesting endothelial and platelet activation in APS. In addition, APS patients showed increased CD133/1 expression compared to aPL- patients with thrombosis, suggesting endothelial damage in APS [13]. The authors of this study suggest that increased levels of sEVs with distinct biological properties circulate in patients with thrombotic APS.

5.2 *In vitro* studies (characterization of EVs released by aPL stimulated cells)

One mechanism by which aPL promote thromboses is through their binding to endothelial cells causing the activation of endothelial cells [98, 99] which in response, release EVs that might modulate the activation of other adjacent cells [87, 100]. These effects were investigated on endothelial cells [87, 100–102] and placental explants [103] involving both small EVs and medium/large EVs (**Table 3**). A study by Dignat-George et al., showed a significant 4-fold increase in endothelial EVs with procoagulant activity after stimulation of human umbilical vein endothelial cells (HUVEC) with plasma of APS patients [87]. Only a moderate, non-significant increase was observed after HUVEC stimulation with the plasma from HBDs. In addition, endothelial EVs released after HUVEC stimulation with APS plasma, significantly reduced the normalized clotting time ratio. Wu et al. showed data where stimulation of HUVEC with anti-β2GPI caused the formation of an endothelial cell inflammasome and the release of EVs that were enriched in mature IL-1β, with a distinct mIR profile and caused endothelial activation [101]. However, activation of HUVEC does not appear to involve IL-1β receptor, but most likely follows the TLR/myd88-IRAK4 signaling pathway. Pericleous et al. [102] investigated the effect of purified polyclonal IgG from patients with APS (APS-IgG) and healthy controls (HC-IgG) on HUVEC [102]. HUVEC exposed to APS-IgG, produced significantly more endothelial EVs than those exposed to HC-IgG and a larger proportion of these EVs carried surface E-selectin. Levels of ICAM-1+, endoglin+ and VE-cadherin+ EVs did not differ from the ones stimulated with HC-IgG. VCAM-1+ and TF+ endothelial EVs could not be detected. Later Betapudi et al., also observed a 2-fold increase in levels of endothelial EVs released from HUVEC stimulated with anti-β2GPI [100]. EVs in obstetric APS patients

Reference	Cell type	Stimulation	Isolation protocol	Method of quantification of EVs	Levels	Other major findings
Dignat-George et al., 2004 [87]	HUVEC	plasma from APS pts. or HBDs	direct use of cell culture supernatant	AnxV+ (total EVs) < 0.8 μm (latex beads)	↑	↑ endothelial EVs with procoagulant activity.
Wu et al., 2015 [101]	HUVEC	anti-β2GPI purified from APS pts. plasma and from rabbits immunized with β2GPI	2,500 × g (15 “) 13,000 × g (2 “) 100,000 × g (90″)	qPCR, immunoblotting, inflammasome staining	NA	Anti-β2GPI caused formation of an endothelial cell inflammasome and the release of EVs that were enriched in mature IL-1β, had a distinct miR profile, and caused endothelial activation.
Pericleous et al., 2013 [102]	HUVEC	purified IgG from APS pts. and HBDs	3,000 × g (5″) 12,000 × g (60″)	AnxV+ (total EVs) CD62E+ (E-selectin), CD106+ (VCAM-1), CD54+ (ICAM-1), CD142+ (TF), CD105+ (endoglin), CD144+ (VE-cadherin) < 1 μm (latex beads)	↑ AnxV+ and E-selectin+	No difference in levels of ICAM-1+, endoglin+, and VE-cadherin+ EVs after APS IgG stimulation vs. HBD IgG. VCAM-1+ and TF+ EVs could not be detected.
Betapudi et al., 2013 [100]	HUVEC	anti-β2GPI purified from 3 APS pts., HBDs and rabbits immunized with β2GPI	1,500 × g, (30″) 13,000 × g (2″)	CD144+ < 1 μm (latex beads)	↑	Anti-β2GPI antibodies stimulate endothelial EVs release via nonmuscle myosin motor protein-dependent pathway.
Tong et al., 2017 [103]	1st trimester human placenta explants, HMEC-1	murine monoclonal anti-β2GPI, purified IgG from 5 APS pts. and HBDs	2,000 × g, (5″) 20,000 × g (60″) 100,000 × g (60″)	NTA	Not increased	↑ mean and modal size of EV after aPL stimulation. ↑ of mtDNA in EVs after aPL stimulation. EVs from placental explants activated HMEC-1 through TLR-9 receptor signaling.

Abbreviations: Anx V, annexin V; APS, antiphospholipid syndrome; aPL, antiphospholipid antibodies; β2GPI, β2-glycoprotein I; EVs, extracellular vesicles; HBDs, healthy blood donors; HUVEC, human umbilical vein endothelial cells; HMEC-1, human dermal microvascular endothelial cells; ICAM-1, intercellular adhesion molecule 1; IgG, immunoglobulin G; IL, interleukin; mtDNA, mitochondrial DNA; NA, not applicable; NTA, nanoparticle tracking analysis; pts., patients; TF, tissue factor; TLR, toll-like receptor; VCAM-1, vascular cell adhesion molecule 1; ↑, elevated levels.

Table 3.
Isolation, quantification and characterization of EVs derived from endothelial cells after stimulation with aPL.

were studied by Tong et al. [103], whereby exposure of first trimester human placental explants to monoclonal anti-β2GPI and IgG fractions from five anti-β2GPI positive APS patients did not affect the number or size of EVs. However, an increase in levels of mitochondrial DNA was observed in these vesicles that activated endothelial cells through a TLR-9-mediated pathway. This is supporting the idea that EV-associated mitochondrial DNA could be pathological in pregnant women with aPL.

6. Conclusions

Extracellular vesicles are small phospholipid bilayer particles that carry various biologically active molecules, such as proteins, lipids and nucleic acids. Their key biological function is cell–cell communication and the transfer of cargo. EVs normally circulate in the bloodstream of healthy individuals, but their levels are elevated in various pathological conditions, including APS. The classification, isolation and characterization of EVs has been developing in an accelerated manner over the last 20 years. Nevertheless, terms such as exosomes and microparticles are still present in the literature, but it is important to note that this classification is based on biogenesis, which is rather difficult to assess. It is therefore more optimal to classify EVs based on their other characteristics, such as size, density, origin etc. Each isolation and characterization techniques have their advantages and disadvantages and influences the properties of the EVs studied. Choosing the best combination, albeit of different isolation techniques, along with the characterization of EVs, is of utmost importance to achieve good data quality. In addition, the limitations of the methods used in both isolation and characterization must be considered. In the rapidly developing field of EVs research, variations of existing methods, as well as new technologies, are emerging that enable more precise isolation and characterization of EVs. EVs from platelets, monocytes and endothelial cells play a crucial role in vascular dysfunction, which is a causal factor in the disturbance of hemostasis and the development of thrombosis. Platelet and monocyte EVs are involved in the increased adhesiveness of endothelial cells and the increased interaction of leukocytes with the endothelium. Platelet, monocyte and endothelial EVs carry procoagulant molecules, such as TF, and modulate the expression of coagulation molecules in endothelial cells. Research on EVs in APS is very heterogeneous, due to the lack of standardization of isolation and characterization methods, all of which limits solid findings and conclusions. In addition to the technological challenges, EVs in APS are difficult to study because of the puzzling nature of APS. It is a chronic disease with a complex clinical spectrum due to many different features and symptoms (e.g. hypertension, thrombocytopenia). Patients with APS receive lifelong treatment with anticoagulants, and the actual acute phase is practically impossible to monitor. However, in view of the data on EVs in APS, a trend towards elevated total endothelial and platelet EV levels can be observed, suggesting an activated endothelium, even in the absence of an acute event. The results of the study of sEVs suggest that smaller vesicle populations may also play a role in the pathogenesis of APS. It appears that in patients with APS, levels of sEVs and different medium/large EVs are elevated. Further research is needed to confirm this in a larger number of patients as well as determine their functionality in APS. Data on increased levels of endothelial EVs in APS is supported by *in vitro* studies showing elevated levels of endothelial EVs following stimulation of endothelial cells with aPL. Studies investigating the role of aPL in vesicular release and its effects on the original cells also suggest that both small and medium/large EVs may play an important role in endothelial dysfunction in APS. However, future studies are needed to obtain a clearer picture of the signaling pathways and key molecules involved in interactions of EVs with the target cells.

Conflict of interest

The authors declare no conflict of interest.

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