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Extracellular microvesicles from patients with antiphospholipid syndrome carry antigenic targets and promote endothelial cell activation *in vitro*

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Disclosure

No conflicts of interest to disclose. The authors do not have financial relationships with commercial entities during the 36 months prior to publication.

Contributions

FC and ST designed the study; AC, GR and SR performed the experiments and validated the data; VM and ST analyzed the data; AL and MS wrote the manuscript; SM and CA selected the patients; MS, RM and TG supervised the study. All authors read, edited, participated in the revision, and approved the manuscript.

Data-sharing statement

To obtain raw data and protocols, please contact the corresponding author Maurizio Sorice (maurizio.sorice@uniroma1.it).

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Abstract

Antiphospholipid syndrome (APS) is characterised by thrombosis, recurrent miscarriages and the stable presence of antiphospholipid antibodies (aPLs). Circulating aPLs can activate molecular mechanisms that may promote the activation of various cell types, particularly endothelial cells. Recently, endothelial activation has been associated with the release of procoagulant and proinflammatory extracellular microvesicles (EMVs). This study analysed the presence of EMVs in the plasma of APS patients, their correlation with clinical manifestations, and their role in carrying antigenic targets and promoting endothelial cell activation. Nanosight analysis revealed that EMV concentrations were significantly elevated in APS patients than in healthy donors. Moreover, we observed the presence of the main autoantigenic targets, β2-GPI and CL, on the surface of EMVs from APS patients' plasma, by both biochemical and flow cytometry analysis. It also revealed significantly higher CL levels on EMVs from patients with obstetrical APS compared to those without pregnancy morbidity, specifically in women with a history of foetal death.

To analyze the effect of the EMVs from APS patients on endothelial cell activation and signaling, we then incubated *in vitro* human microvascular endothelial cells (HMEC-1) with patient-derived EMVs, demonstrating a significant phosphorylation of IRAK, NF-κB, as well as increased expression and release of TF.

These findings suggest that circulating EMVs may act as platforms for aPL binding and propagation of pathogenic immune complexes, introducing a new task to explain the immunoreactivity of the main antigenic targets of APS patients. Moreover, the presence of EMVs may reflect disease features, but also actively participate in the pathogenesis, by triggering intracellular signaling pathways that sustain vascular inflammation and thrombosis.

Introduction

Antiphospholipid syndrome (APS) is an autoimmune disease characterized by thrombosis, repeated miscarriages and stable presence of antiphospholipid antibodies (aPLs), including anti-cardiolipin antibodies (aCL), anti-β2-glycoprotein I (anti- β2-GPI) and Lupus Anticoagulant (LA).[1-3] Circulating aPLs in the serum can activate molecular mechanisms, which involve not only endothelial cells, but also cells of the immune system, both of the innate and specific response. In addition, platelets and possibly trophoblast cells also participate in the damage, which are central to the development of APS clinical manifestations.[4,5] aPLs interact with phospholipids or phospholipid-binding proteins; among these, antibodies specific for the plasma protein β2-GPI have been shown to promote the activation of monocytes, neutrophils, endothelial cells, platelets and trophoblasts, triggering phosphorylation of the myeloid differentiation factor 88 (MyD88), activation downstream of IRAK, mitogen-activated protein kinases (MAP kinases) and nuclear factor kappa B (NF-κB).[6-8] The activation of this signal transduction pathway leads to increased release of Tissue Factor (TF) as well as an excessive expression and release of proinflammatory cytokines and chemokines, which are closely related with inflammatory responses and coagulation disorders in APS.[9,10] Furthermore, the impairment of the endothelial barrier results in increased expression of adhesive molecules (ICAM, VCAM, E-Selectin and P-selectin) resulting in endothelial cell activation and enhanced leukocyte adhesion, subsequently triggering an exacerbated inflammatory cascade that underlies the pathogenesis of thrombosis.[11,12]

Endothelial dysfunction is also associated with the release of procoagulant and proinflammatory extracellular vesicles (EVs).[13,14] Recently, the greatest attention to the complexity of inter- and intracellular communication has led to growing research on the immunostimulatory and immunosuppressive roles of EVs, in different pathologies also in the field of autoimmunity.

EVs are lipid bilayer–bounded particles, characterized by different sizes and released from nearly all cell types. They are present in different biological fluids, including blood plasma and serum, synovial fluid and urine, under both physiological and pathological conditions.

The International Society of Extracellular Vesicles (ISEV) classifies the main EV subpopulations into exosomes, microvesicles and apoptotic vesicles. Exosomes, which are approximately 50–100 nm in diameter, originate inside the cell, within the endocytic

pathway regulated mainly by the ESCRT complex, Extracellular microvesicles (EMVs), ranging from 100 nm up to 1 μm in diameter, are formed by direct budding outwards or by pinching of the cell plasma membrane. Apoptotic bodies are generated by plasma membrane blebbing of cells undergoing apoptosis.[15]

In this study we focused on EMVs, which are secreted from plasma membrane during physiological cellular processes; indeed, EMVs release increases in response to stimuli such as inflammatory activation.[16] This event is also related to a higher cytoplasmic Ca^{++} concentration, which triggers calcium-dependent enzymes, altering the asymmetric distribution of phospholipids with the consequent exposure of phosphatidylserine (PS) and phosphatidylethanolamine, normally confined in the inner leaflet of the membrane bilayer.[17,18]

Physiological release of EMVs is usually increased during pregnancy, after intensive physical activity, as well as in individuals with obesity and smokers, but during pathology, their levels and composition are significantly changed. EMVs can be derived from monocytes, endothelial cells and platelets, which are key players in the maintenance of vascular hemostasis; their increase alters the hemostatic balance, leading to inflammation, coagulation and endothelial dysfunction, contributing to the development of several pathologies, including arterial and venous thrombosis, pulmonary embolism and other cardiovascular disorders.[13,14,19,20] Several data have demonstrated that both plasma or polyclonal IgG derived from APS patients can induce EMV release from endothelial cells, linking EMV generation to aPL-mediated pathogenic effects on endothelial dysfunction.[21,22] EMVs migrate through the bloodstream and their molecular cargo reflects the characteristics of the origin's cell, they can transfer a wide variety of molecules including heat shock proteins (HSP-90, HSP-70), cytokines, enzymes, growth factors, RNA and DNA, exerting pleiotropic effects across multiple tissues in the body.[23] Platelet EMVs induce endothelial cell activation also increasing their adhesion to monocytes.[24,25] Instead, EMVs released from endothelial cells and monocytes induce a procoagulant and proadhesive profile on both cells of origin.[26-29] In the same way, EMVs from patients with obstetric complications or from cultured trophoblasts have been shown to promote inflammation, coagulation, and endothelial dysfunction.[30,31] Starting from these premises, EMVs are considered markers of cellular activation, but they also express bioactive lipids, proteins and nucleic acids. Therefore, EMVs can transport processed antigen to the cell surface, becoming a source of antigen and autoadjuvants in generating an autoimmune response.[32] Since some clinical features of APS may be also attributed to

an underlying autoimmunity or disruptions in intercellular signaling, EMVs-related mechanisms may play a role not only in the thrombosis and obstetric manifestations, but also in neurological, hematological, cutaneous, nephropathic, and cardiac valvular complications consistent with the clinical criteria of APS.[3,33]

Our work aims to investigate the presence of EMVs in the plasma of patients affected by APS and any correlations with clinical manifestations. We will evaluate their role in antigen transport and endothelial cell activation to better clarify their potential contribution to the pathogenesis of the disease.

Methods

Patients

The study included consecutive patients with a diagnosis of obstetric and thrombotic APS, according to the classification criteria,[3] referred to the Sapienza Lupus Clinic of Rome. The presence of other systemic autoimmune diseases represented an exclusion criterion.

Healthy donors (HDs), without a medical history of thrombosis, obstetric morbidity or autoimmune diseases, were selected and matched by age and sex.

All participants provided written informed consent, in accordance with the Helsinki Declaration. The local ethics committees approved the research protocol.

Anti-phospholipid antibodies detection

aCL and anti-β2-GPI (IgG/IgM) antibodies were measured in patient sera using QUANTA Lite ELISA and confirmed via QUANTA Flash chemiluminescence assay (Inova Diagnostic Inc.). Lupus anticoagulant (LAC) was assessed through aPTT and dRVVT, with confirmation testing (Hemoliance Instrumentation Laboratory).

Isolation of EMVs

Fasting blood was collected in sodium citrate tubes. Platelet-poor plasma (PPP) enriched in extracellular vesicles (EMVs) was obtained by double centrifugation at 2500×g for 15 min. EMVs were isolated via centrifugation at 14000×g for 30 min at 4°C and resuspended in filtered PBS.[34] Alternatively, EMVs released from platelets, endothelial cells, and leukocytes were isolated from PPP, using CD61, CD31 or CD45 MicroBeads (Miltenyi Biotec).

Nanoparticle Tracking Analysis

EMVs from APS patients and HDs were analyzed by Nanoparticle Tracking Analysis (NTA) using NanoSight NS300 (Malvern Panalytical). Samples were diluted 1:100 in filtered PBS. Five 60-second videos were recorded per sample, and data analyzed using NTA 3.4 software.

Phospholipids extraction and analysis by High-performance thin-layer chromatography (HPTLC)

Phospholipids from EMVs were extracted using the Folch method and separated by high-performance thin-layer chromatography (HPTLC) on silica gel 60 plates. The mobile phase was chloroform:methanol:acetic acid:water (100:75:7:4), and phospholipids visualized by iodide vapor exposure.[35]

Analysis by western blot

For western blotting (WB), EMVs were lysed in RIPA buffer with protease inhibitors. Protein concentration was determined by Bradford assay and samples run on 10% SDS-PAGE, then transferred to PVDF membranes. After blocking, membranes were incubated with antibodies against Annexin A1, CD63, CD81, ALIX, β-tubulin, and β2-GPI, followed by HRP-conjugated secondary antibodies. Signals were detected with chemiluminescence (Clarity ECL, Bio-Rad) and quantified with NIH ImageJ software.

Flow cytometric analysis of EMVs

Flow cytometry was performed using a CytoFLEX cytometer (Beckman Coulter), optimized for small particles (equipped with a Cristal Violet Laser, 405 nm). The gating strategy (FSC-A versus SSC-A plot) was defined using FITC fluorescence intensity on the x-axis and Violet SSC-A on the y-axis, with the threshold set on the Violet SSC channel, and including the individual gates established with calibration beads. Size gates were set with a mixture of fluorescent Megamix-Plus SSC and Megamix-Plus FSC beads (100–900 nm) (Figure S1). EMVs were stained with anti-CD41a, -CD45, and -CD31 to identify platelet, leukocyte and endothelial origins.[34] For surface CL and β2-GPI detection, EMVs were incubated with specific primary antibodies,[36,37] followed by FITC-conjugated secondary antibodies. Labeled EMVs were analyzed after 10^7 acquired events.

Cells, treatments and western blot

HMEC-1 ($5x10^5$/mL) were seeded into 6-well cell culture and incubated at 37°C with EMVs from APS patients (2 x 10^8/mL), EMVs from HDs (2 x 10^8/mL) or LPS (0.1 μg/ml). Whole extracts were prepared and analyzed by WB, using antibodies against phospho-IRAK1, TF and, as loading control, anti-IRAK1, β-actin. Nuclear extracts were analyzed by WB, using anti-phospho-NF-κB-p65 and as loading control anti-Histone H1 antibodies.[38] In parallel experiments, HMEC-1 were incubated with APS plasma $CD61^+$, $CD31^+$ or $CD45^+$ EMVs and then analyzed by WB to evaluate IRAK phosphorylation and TF expression.

Statistical Analysis

Data were expressed as mean ± SD or median (IQR). Comparisons used Student's t-test or Mann–Whitney U test; correlations were assessed by Pearson or Spearman methods. Significance was set at $p < 0.05$. Analyses were performed using R (v4.3.3) or GraphPad Prism 8.0.

Methods section has been extended in the Supplementary data.

Results

Patients

A total of 28 Caucasian patients with a diagnosis of Primary APS (PAPS) were enrolled, with a median age of 52 years (48–60) and a female-to-male ratio of about 8:1. Among the 25 female patients, 11 (44%) experienced obstetrical complications, while 18 out of the 28 total patients (64%) had a clinical history of thrombotic events. All clinical, demographic, and laboratory characteristics of patients are reported in Table 1. As controls, 16 age- and sex-matched HDs were included.

Quantitative analysis and characterization of EMVs from plasma of patients with APS

The presence of EMVs in plasma samples from patients with APS and HDs was initially assessed using NTA (NanoSight), which enables quantitative measurement. The analysis revealed that EMV concentrations were significantly elevated in APS patients (4.72×10^9

EMVs/ml, standard deviation: 2.86×10^8), compared to 2.81×10^9 EMVs/ml (standard deviation: 7.29×10^7) in HDs (Figure 1A).

To evaluate the purity of the EMV preparations, WB analysis was performed. The results confirmed the expression of characteristic EMV markers derived from the plasma membrane, including Annexin A1 and β-tubulin, while exosomal markers, such as CD63, CD81 and Alix were not detected (Figure 1B).

Moreover, the presence of the main antigenic targets of APS, β2-GPI and phospholipids, was investigated.

The presence of β2-GPI on EMVs was detected using specific anti-β2-GPI antibodies. Western blot data demonstrated that β2-GPI levels were markedly higher in EMVs isolated from APS patients, compared to those from HDs (Figure 1B). β2-GPI association with EMVs may actively contribute to the prothrombotic state observed in these patients.

Since anti-β2-GPI antibodies bind β2-GPI only when associated to anionic phospholipids, such as CL and PS, we investigated whether these phospholipids were present on EMVs released from APS patients. Phospholipids were extracted from EMVs derived from APS patients and HDs and then analyzed by HPTLC. The analysis revealed the presence of two main iodide vapors-positive bands, comigrating with CL and PS, respectively (Figure 1C, left panel). Quantitative densitometric analysis revealed that CL and, to a lesser extent, PS levels were markedly higher in EMVs isolated from APS patients, compared to those from HDs (Figure 1C, right panel).

Flow cytometric analysis of CL and β2-GPI in EMVs from plasma of patients with APS and clinical associations

Preliminary analysis revealed that EMVs derived from platelet, endothelial and leukocyte origin, as detected by anti-CD41a, anti-CD45 and anti-CD31, respectively (Figure S2).

To analyze the presence of the main APS autoantigens on EMVs, isolated from plasma of APS patients, we used flow cytometry to measure the expression of β2-GPI and CL.

As showed in Figure 2 A-B, the analysis demonstrated a higher β2-GPI mean fluorescence intensity (MFI) on EMVs from APS patients compared to HDs (16789 vs 2932; $p = 0.005$), while, although the trend is similar, CL MFI levels on EMVs did not differ significantly between the two groups (4999 vs 3416; $p = 0.401$).

In the APS cohort, EMVs from patients with obstetric complications showed significantly higher CL MFI than those from patients without pregnancy morbidity (40021 vs 1950, p=0.001), specifically in women with a history of foetal death (82117 vs 2300, p=0.005,

Figure 3A). Consequently, patients with a history of thrombosis showed significantly lower CL MFI levels on EMVs compared to those without thrombotic events (1950 vs 27331; p = 0.004), with a significant reduction as compared with patients with arterial thrombosis (1873 vs 11580; p = 0.022; Figure 3B). EMVs from patients with obstetric complications showed higher β2-GPI MFI than those from patients without pregnancy morbidity, without any significant clinical association. Antigens expression (β2-GPI, CL) and clinical manifestations, such as thrombosis and obstetric complications, are summarized in Table S1.

EMVs from plasma of patients with APS induce endothelial cell activation signaling and Tissue Factor expression in endothelial cells

Given the pivotal role of endothelial cells in the pathogenic mechanisms underlying APS clinical manifestations, we evaluated the effects of APS patients' EMVs on HMEC-1 cells, studying signaling that potentially plays a role in promoting a procoagulant and proinflammatory microenvironment.

In particular, WB analysis of HMEC-1 cell lysates demonstrated that APS patient EMVs, as well as LPS, induced a significant increase of IRAK (Figure 4A) and NF-kB p65 (Figure 4B) phosphorylation, compared to both untreated and HD EMVs treated cells. Quantitative analysis confirmed these data (see histograms, right panels, Figure 4).

Furthermore, in line with this signaling pathway, we also explored the expression of TF as a procoagulant factor following endothelial cells activation. Therefore, lysates and supernatants of HMEC-1 cells were analyzed by WB. Obtained results, from the study of both types of samples, indicated a significant TF expression increase, in lysate (Figure 5A) and supernatant (Figure 5B) samples, treated with APS patient EMVs or LPS. Conversely, a weak reactivity was observed in untreated cells or in cells stimulated with HD EMVs (Figure 5).

Moreover, we isolated, from APS plasma, EMVs released by platelets ($CD61^+$), endothelial cells ($CD31^+$) or leucocytes ($CD45^+$) and then we analyzed their individual contribution to endothelial activation (IRAK phosphorylation) and procoagulant signaling (TF levels). Obtained results showed that all three types of EMVs, mainly $CD31^+$ EMVs, significantly induced both IRAK phosphorylation (Figure S3 A) and increase of TF (Figure S3 B).

Discussion

In this study we characterized plasma derived EMVs in patients with APS, focusing on the presence of the main autoantigenic targets, β2-GPI and CL. We preliminary demonstrated a significant increase of EMV concentration in plasma of patients with APS, compared to HD, extending upon previous evidence showing elevated levels of platelet- and endothelial-derived extracellular vesicles in APS patients.[33] In particular, elevated plasma levels of endothelial EMVs were found in patients with primary or secondary APS since 2004,[21] demonstrating the capacity of plasma from these patients to induce vesiculation of cultured endothelial cells. In our analysis we have minimized any potentially confusing pre-analytical variable, respecting the methodological indications proposed in D MISEV2023.[15]

Moreover, in the present study, specifically, we demonstrated for the first time the presence of key aPL targets, β2-GPI and CL, but also PS, on the surface of EMVs isolated from APS patients' plasma. This observation suggests that circulating EMVs may act as platforms for aPL binding and propagation of pathogenic immune complexes in APS. This finding introduces a new task, explaining the immunoreactivity of the main antigenic targets of APS patients, β2-GPI and phospholipids, including CL and PS.

The expression of the main APS autoantigens, β2-GPI and CL on EMVs, isolated from plasma of APS patients, was confirmed by flow cytometry. Interestingly, the analysis revealed significantly higher CL MFI levels on EMVs from patients with obstetrical APS, specifically women with a history of foetal death, compared to those without pregnancy morbidity. This finding is not fully surprising, since higher levels of EVs in women with recurrent miscarriage have been reported.[33] It is consistent with a mechanism in which EMVs released through anti-β2-GPI-induced cellular activation may be implicated in, or result from, uteroplacental inflammation. Indeed, obstetric APS is associated with inflammation at the maternal-foetal interface, and poor placentation correlates with reduced trophoblast invasion and limited uterine spiral artery remodeling.[39,40] However, the main limitations of this observation are the relatively small and ethnically homogeneous patient cohort and the high variability in antigen expression among patients' samples.

Moreover, the presence of EMVs carrying β2-GPI, possibly complexed with phospholipid molecules, may have important functional implications. Indeed, it is well known that endothelial dysfunction is associated with the release of EVs,[39,41] which are linked to increased cardiovascular risk and may contribute to the pathogenesis of thrombosis and obstetric complications.[13,14,19,20] Thus, in this study, we focused on the

effect of the EMVs obtained from APS patients on endothelial cell activation and signaling. Functionally, we showed that APS-derived EMVs exert potent activating effects on endothelial cells. Exposure of human microvascular endothelial cells (HMEC-1) to patient-derived EMVs led to phosphorylation of IRAK and NF-κB, as well as increased TF expression and release. These results confirm and extend the observation that EMVs increase ICAM-1 and VCAM-1 expression in endothelial cells,[42] pointing toward TLR-related inflammatory signaling and procoagulant activation driven by circulating vesicles, linking immune recognition pathways with vascular thrombogenicity.

Importantly, this suggests that EMVs may not only reflect disease activity, but also actively participate in the pathogenesis by triggering intracellular signaling pathways that sustain vascular inflammation and thrombosis.

Taken together, our findings support the hypothesis that plasma EMVs in APS are not only biomarkers of endothelial, leukocyte or platelet activation, but also active participants in disease pathogenesis. By harboring CL and β2-GPI, these vesicles may facilitate the formation of pathogenic aPL complexes directly on their membrane surface. In turn, their interaction with endothelial cells promotes a cascade of signaling events that culminate in endothelial activation/dysfunction and TF-mediated coagulation, thus providing a mechanistic link between autoantibody presence and thrombosis. It could play a key role in pathophysiology of gestational complications, including preeclampsia, foetal growth restrictions and foetal loss, by proinflammatory signaling triggering, vascular remodeling and complement activation.[30,43]

Further investigations are warranted to elucidate the molecular mechanisms underlying EMV-induced endothelial activation. Subgroup observations—such as the higher levels of CL-positive EMVs in patients with obstetric APS—should be considered preliminary and hypothesis-generating. Larger studies will be needed to confirm these findings and to explore the potential of EMVs as therapeutic targets or biomarkers of clinical complications in APS.

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Table 1. Clinical and demographic characteristics of APS patients (N = 28)

Characteristics	N (%)
Female sex	25 (89%)
Median age in years[1]	52 (48, 60)
Caucasian	28 (100%)
Obstetric APS	11/25 (44%)
Recurrent miscarriage	3/25 (12%)
Foetal deaths	8/25 (32%)
Pre-eclampsia	2/25 (8%)
Premature placental abruption	1/25 (4%)
Intrauterine growth restriction	2/25 (8%)
Thrombosis	18 (64%)
Arterial thrombosis	9 (32%)
Stroke	7 (25%)
Myocardial infarction	1 (3.6%)
Venous thrombosis	9 (32%)
Deep vein thrombosis	8 (29%)
Pulmonary embolism	5 (18%)
Recurrent thrombosis	2 (7.1%)
Thrombocytopenia	7 (25%)
Livedo reticularis	7 (25%)
Raynaud's Phenomenon	3 (11%)
Migraine	9 (32%)
Renal thrombotic microangiopathy	1 (3.6%)
Cerebral white matter lesions	2 (7.1%)
Seizures	2 (7.1%)
Smoke	2 (7.1%)
Hypertension	7 (25%)
Hypercholesterolemia	6 (21%)
Hormonal replacement therapy	2 (7.1%)
Hyperhomocysteinemia	4 (14%)
Obesity	3 (11%)
anti-CL antibodies[2]	20 (71%)
anti-β2GP1 antibodies[2]	14 (50%)
LAC[2]	3 (11%)
High risk profile[2]	15 (54%)

[1] Median (Q1, Q3)
[2] anti-CL, anti-β2-GPI and LAC positivity refers to findings previously documented in the patient's clinical history

Legends for the Figures

Figure 1. Quantitative analysis and biochemical characterization of EMVs from the plasma of APS patients.

A) Quantitative analysis of EMVs from one representative healthy donor (HD) and from one representative APS patient by NanoSight (Nanoparticle Tracking Analysis). The number of EMVs in APS patients was 4.72 x 10^9 EMVs/ml (S.D. 2.86 x 10^8), compared to those detected in 2.81 x 10^9 EMVs/ml (S.D. 7.29 x 10^7) in HDs. ***p < 0.001 vs HDs.

B) Characterization of the EMVs from one representative HD and from one representative APS patient by WB analysis, using anti-β2-GPI, anti-Annexin A1 (ANXA1), anti- β-tubulin, anti-CD63, anti-CD81 and anti-Alix antibodies.

C) HPTLC analysis of phospholipids extracted from EMVs, according to the technique described by Folch, from one representative HD and from one representative APS patient. Phospholipids were separated by thin layer chromatography in chloroform:methanol:acetic acid:water (100:75:7:4) (v:v:v:v). The plate was stained with iodide vapours (phospholipid-specific stain). Cardiolipin (CL) and Phosphatidylserine (PS) are used as standards (St). On the right, bar graph shows densitometric scanning analysis of the CL and PS comigrating bands. Results represent the mean ± SD. **** *p* <0.0001.

Figure 2. Flow cytometric analysis of CL and β2-GPI in EMVs from APS patients and healthy donors. (A) Representative semiquantitative flow cytometry histogram overlay of CL and β2-GPI expressed on the surface of EMVs isolated from APS patient and HD. The mean fluorescence intensity (MFI) on the FITC channel is showed (B) Histograms show the MFI of CL and β2-GPI expression on EMVs from plasma of APS patients and HDs. Data are expressed as mean (S.D.). Statistical analysis indicated: **p < 0.01.

Figure 3. Clinical associations with CL and β2-GPI levels detected on EMVs from APS patients. (A) Comparison between CL MFI levels on EMVs in female patients, based on history of obstetric APS and foetal deaths. (B) Comparison between CL MFI levels on EMVs in patients, based on the history of thrombosis and arterial thrombosis. Statistical analysis indicated: *p < 0.05; **p < 0.01; ***p < 0.001.

Figure 4. Analysis of IRAK and NF-kB activation in HMEC stimulated with APS patient EMVs. HMEC-1 cells untreated or stimulated with LPS, APS patient EMVs and HD EMVs were lysed and analyzed by WB to evaluate: (A) Phospho-IRAK1 expression using rabbit anti-phospho-IRAK1 antibody in whole cell lysates. (B) phospho-NF-kB-p65 expression using rabbit anti-phospho-NF-kB-p65 antibody in nuclear cell lysates. Anti-β-tubulin or anti-Histone1 antibodies were used to evaluate loading controls. Densitometric phospho-IRAK1/total IRAK or phospho-NF-kB-p65/Histone 1 ratios are evaluated. Western blot and densitometric analysis from four representative APS patients and HDs are shown. Densitometric values, calculated in all APS patients and HDs, are represented by dot-plot. Data are reported as mean (S.D.). Statistical analysis indicated: ****$p < 0.0001$.

Figure 5. Analysis of Tissue Factor in HMEC cells stimulated with APS patient EMVs. HMEC-1 cells untreated or stimulated with LPS, APS patient EMVs and HD EMVs were lysed and analyzed by WB to evaluate Tissue Factor levels using rabbit anti-Tissue Factor antibody. (A) Analysis of whole cell lysates, anti-β-Actin was used as loading control. Densitometric Tissue Factor/β-Actin ratios are evaluated. (B) Analysis of cell supernatants, samples fold changes/untreated ratios were calculated. Western blot and densitometric analysis from four representative APS patients and HDs are shown. Densitometric values, calculated in all APS patients and HDs, are represented by dot-plot. Data are reported as mean (S.D.). Statistical analysis indicated: ****$p < 0.0001$.

Figure 1

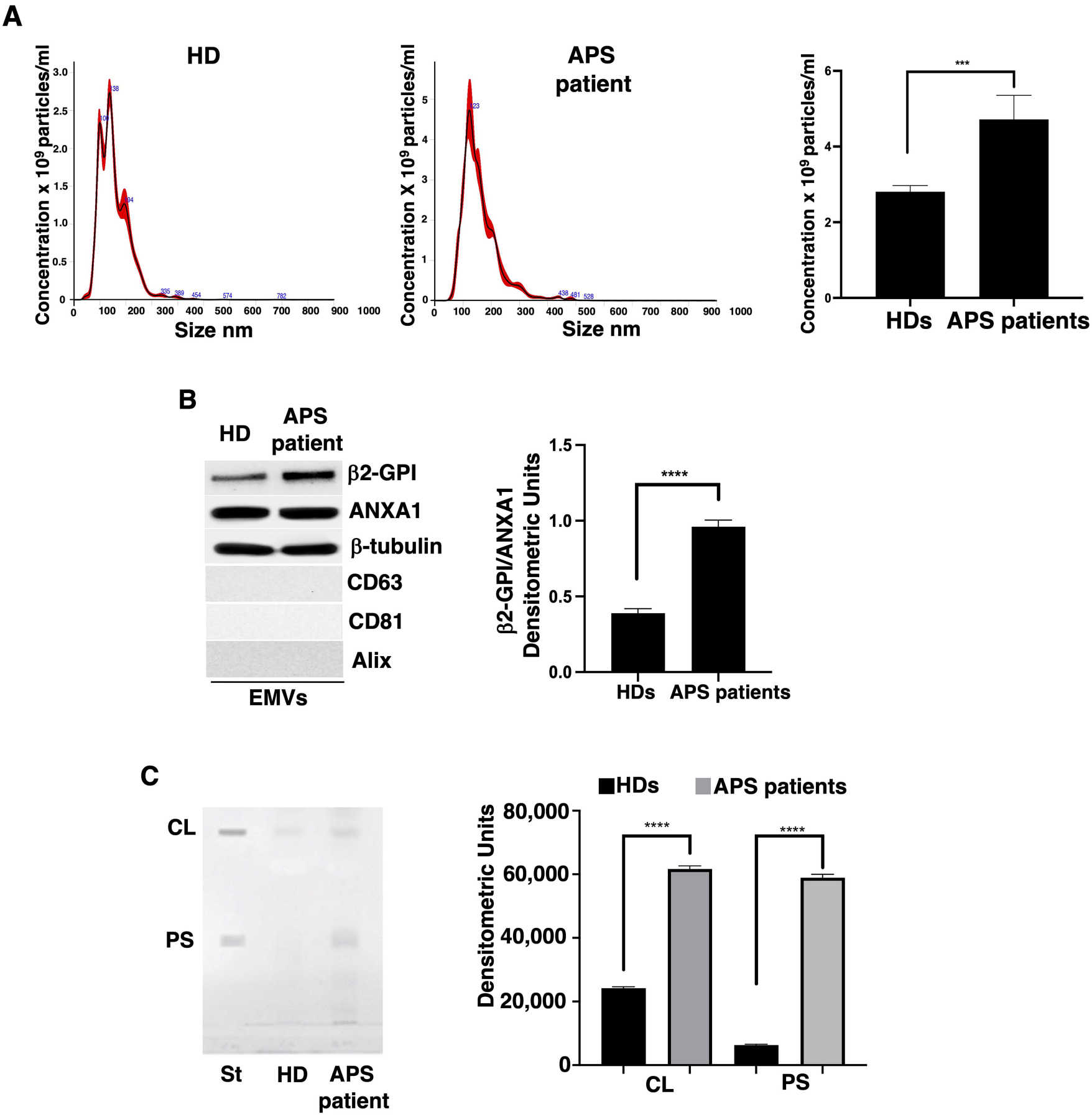
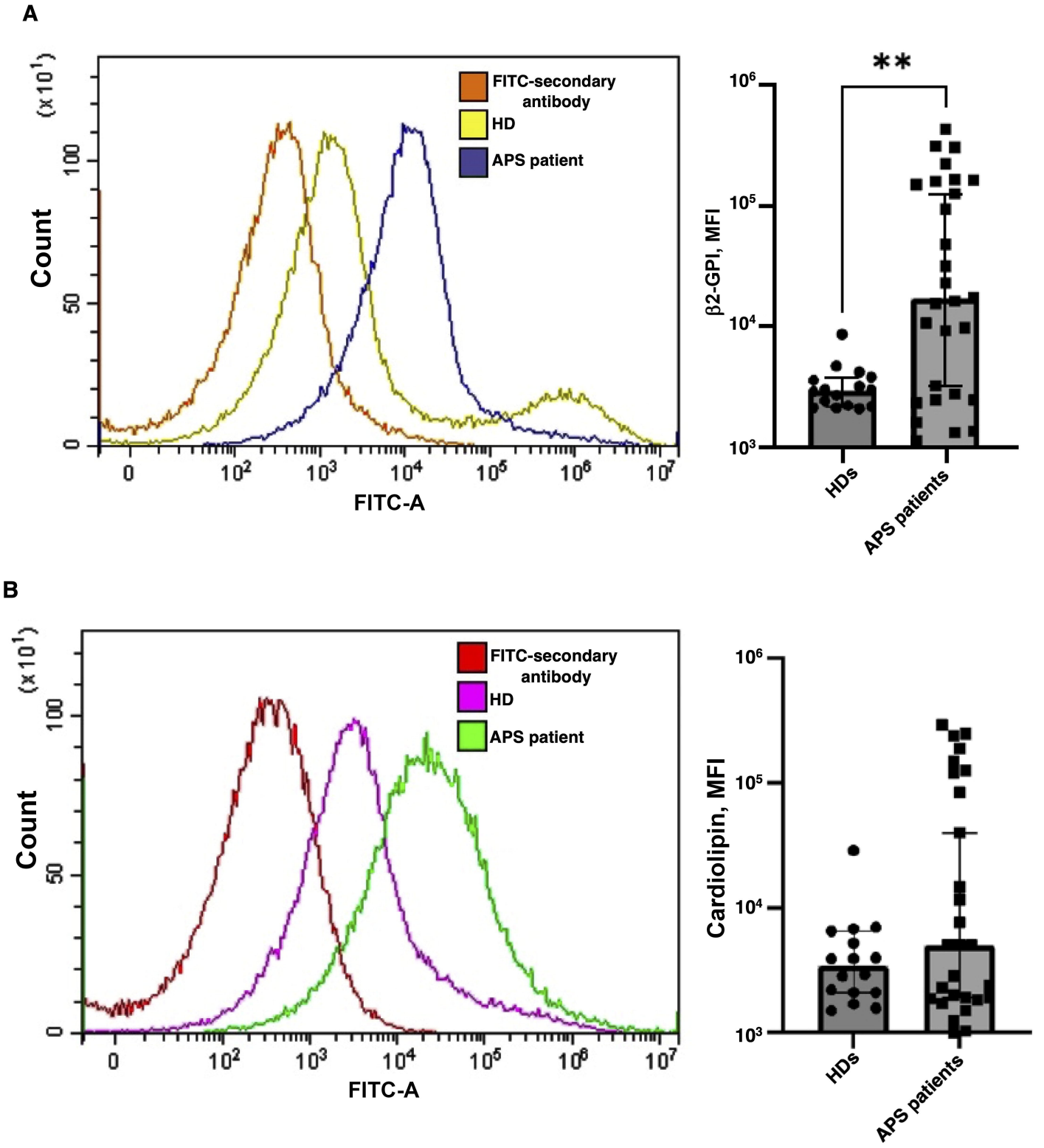

A
HD
APS patient
Concentration x 10^9 particles/ml
Concentration X 10^9 particles/ml
Size nm
***
HDs
APS patients
B
HD
APS patient
β2-GPI
ANXA1
β-tubulin
CD63
CD81
Alix
EMVs
β2-GPI/ANXA1 Densitometric Units
****
HDs
APS patients
C
CL
PS
St
HD
APS patient
HDs
APS patients
Densitometric Units
****
****
CL
PS

Figure 2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100
50
0
Count
FITC-secondary antibody
HD
APS patient
0
10²
10³
10⁴
10⁵
10⁶
10⁷
FITC-A
**
10⁶
10⁵
10⁴
10³
β2-GPI, MFI
HDs
APS patients
B
(x10¹)
100
50
0
Count
FITC-secondary antibody
HD
APS patient
0
10²
10³
10⁴
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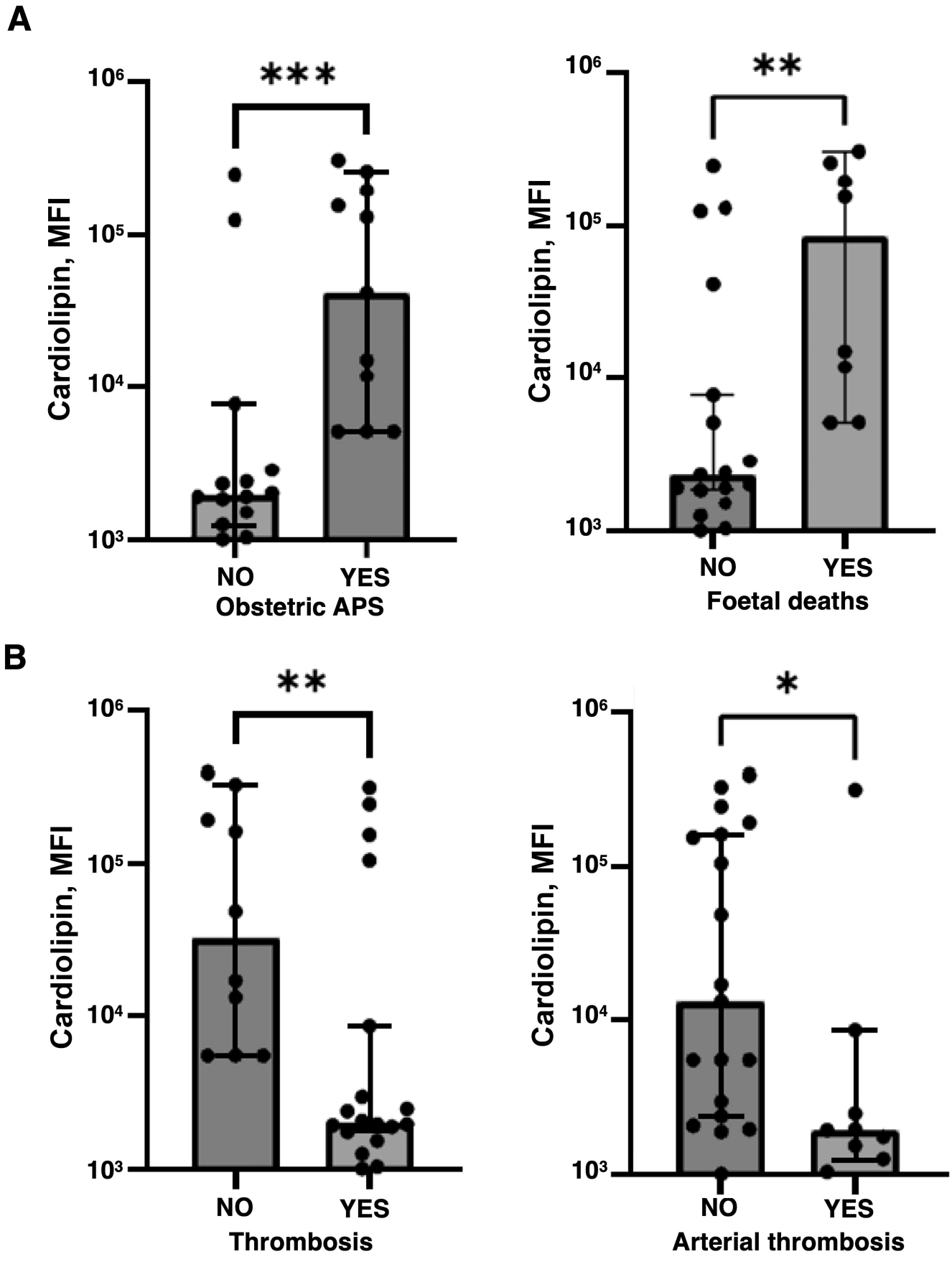
10⁴
10³
Cardiolipin, MFI
HDs
APS patients

Figure 3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***
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10⁵
10⁴
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NO
YES
Obstetric APS
**
Cardiolipin, MFI
10⁶
10⁵
10⁴
10³
NO
YES
Foetal deaths
B
**
Cardiolipin, MFI
10⁶
10⁵
10⁴
10³
NO
YES
Thrombosis
*
Cardiolipin, MFI
10⁶
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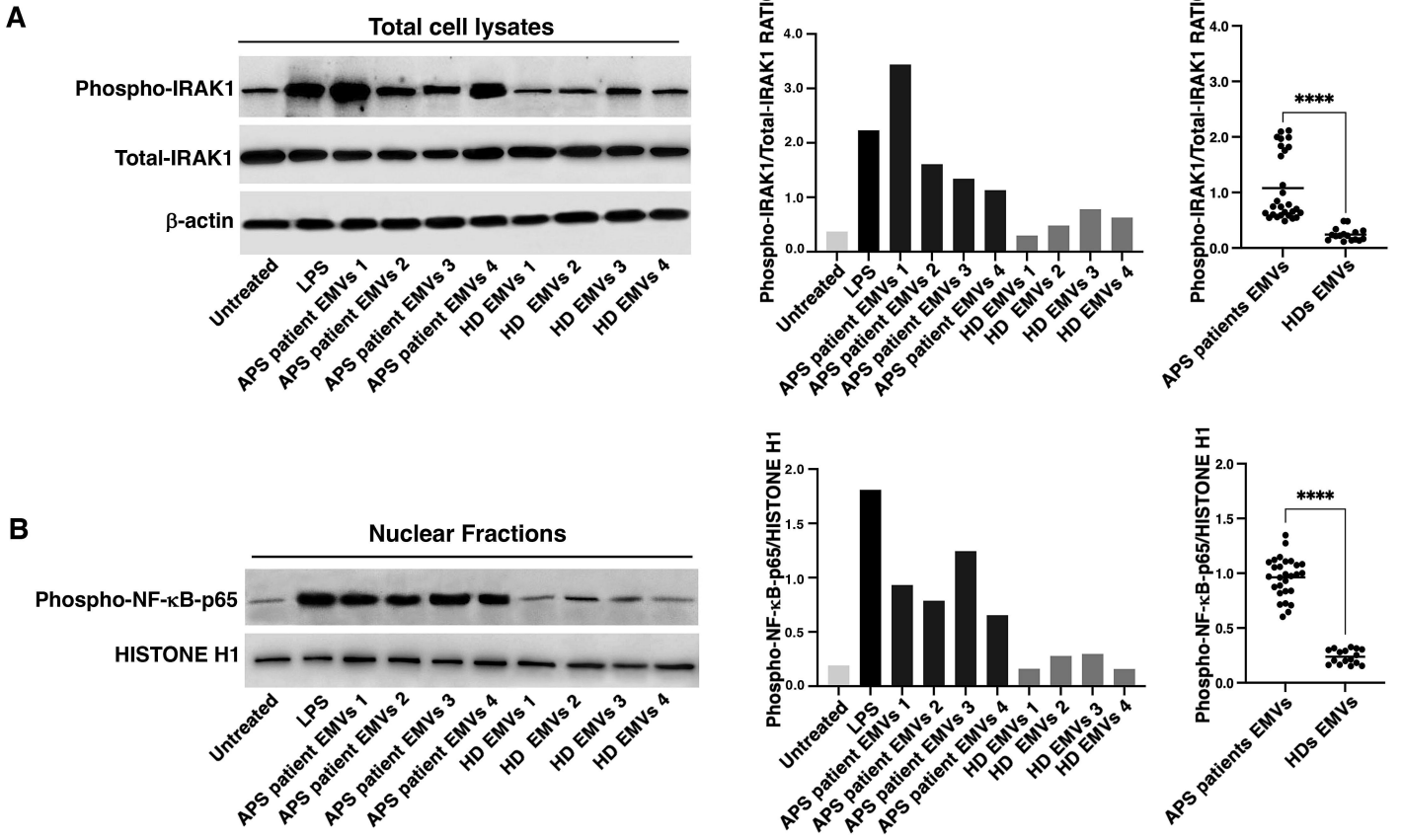
10⁴
10³
NO
YES
Arterial thrombosis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Total cell lysates
Phospho-IRAK1
Total-IRAK1
β-actin
Untreated
LPS
APS patient EMVs 1
APS patient EMVs 2
APS patient EMVs 3
APS patient EMVs 4
HD EMVs 1
HD EMVs 2
HD EMVs 3
HD EMVs 4
Phospho-IRAK1/Total-IRAK1 RATIO
4.0
3.0
2.0
1.0
0.0
****
APS patients EMVs
HDs EMVs
B
Nuclear Fractions
Phospho-NF-κB-p65
HISTONE H1
Phospho-NF-κB-p65/HISTONE H1
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1.5
1.0
0.5
0.0

Figure 4

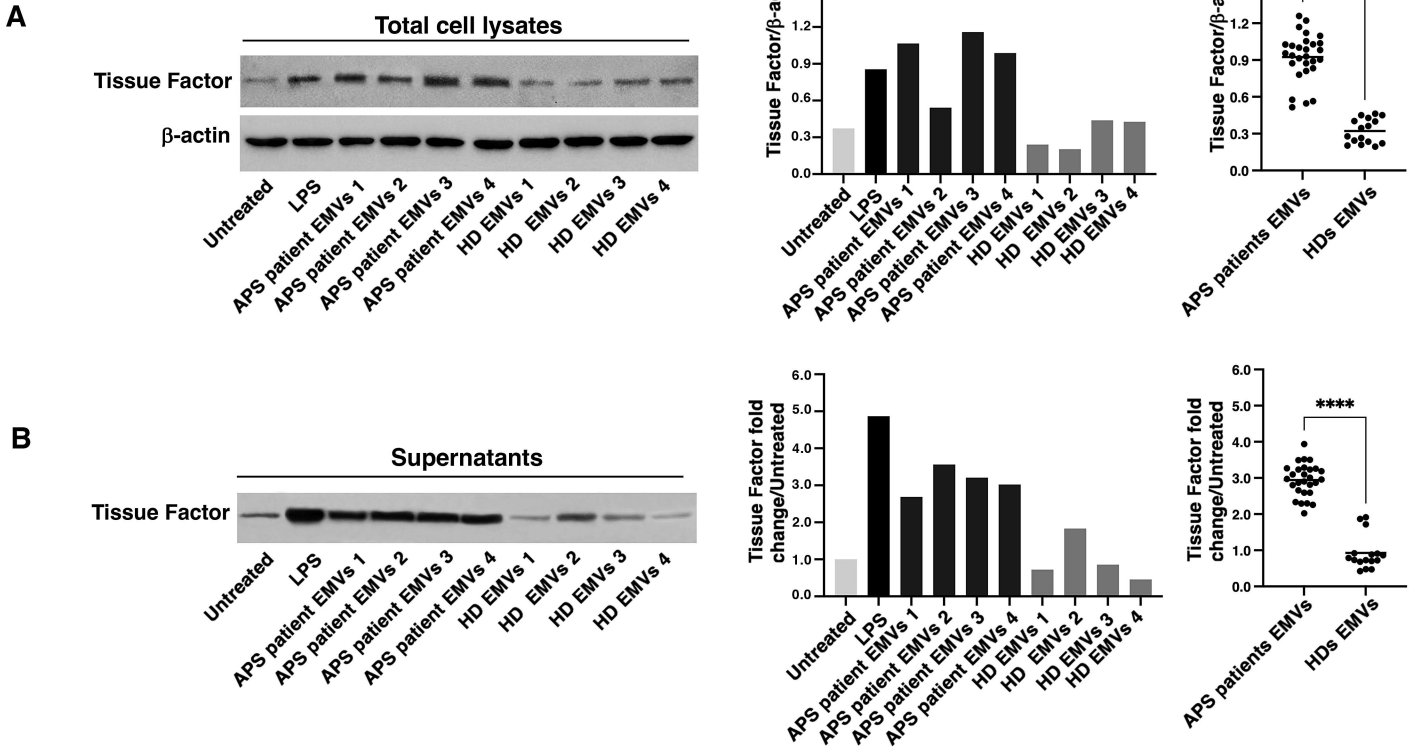

A
Total cell lysates
Tissue Factor
β-actin
Untreated
LPS
APS patient EMVs 1
APS patient EMVs 2
APS patient EMVs 3
APS patient EMVs 4
HD EMVs 1
HD EMVs 2
HD EMVs 3
HD EMVs 4
Tissue Factor/β-actin
1.8
1.5
1.2
0.9
0.6
0.3
0.0
****
APS patients EMVs
HDs EMVs
B
Supernatants
Tissue Factor
Tissue Factor fold change/Untreated
6.0
5.0
4.0
3.0
2.0
1.0
0.0

Figure 5

Supplementary Methods

Anti-phospholipid antibodies detection

aCL and anti-β2-GPI (IgG/IgM) antibodies were detected in patients' sera by the QUANTA Lite detection kit, using a commercial Enzyme-linked immunosorbent assay (Inova Diagnostic Inc., San Diego, California, USA), according to the manufacturer. All the results were also confirmed by chemiluminescence assay by QUANTA Flash detection kit (Inova Diagnostic Inc.).

Lupus anticoagulant (LAC) was evaluated by a dilute sensitized activated partial thromboplastin time (aPTT) and a dilute Russell's viper venom time (dRVVT), followed by a confirmation test (Hemoliance Instrumentation Laboratory, Lexington, MA, USA).

Isolation of EMVs

Fasting blood samples were collected from APS patients and HDs via venipuncture into 10 mL tubes containing sodium citrate as anticoagulant.

Platelet poor plasma (PPP) enriched in EVs was obtained by centrifuging samples twice at 2500 x g for 15 minutes at room temperature to separate the cellular component and cellular debris from EVs. The supernatant was transferred in tubes and filled up with particle-free phosphate buffer saline (PBS) (filtered at 0.02 mm) to prevent collapse during the centrifugation procedure. EMVs were then isolated by centrifugation twice at 14000 x g for 30 minutes at 4°C. The pellet was resuspended in particle-free PBS.[34]

Isolation of EMVs was performed minimizing any potentially confusing pre-analytical variable respecting the methodological indications proposed in MISEV guidelines ranging from donor characteristics to conditions of blood draw, handling, centrifugation, and storage.[15]

PPP of APS patients was also used to isolate EMVs released by platelets, endothelial cells and leucocytes using CD61, CD31 and CD45 MicroBeads (Miltenyi Biotec, Bergisch Gladbach, Germany). In brief, 1 mL of PPP was diluted with an equal amount of PBS. After vortexing, 40 µl of CD61, CD31 or CD45 MicroBeads were added to the suspension and incubated at 4° C in the dark for 2 hours. After incubation, the mixture was loaded onto the µ column (Miltenyi Biotec) to allow separation. After three washes with 500 µl PBS, the column was removed from the magnetic support and $CD61^+$, $CD31^+$ and $CD45^+$ EMVs were eluted in 1 mL of PBS with the help of a plunger. For use of $CD61^+$, $CD31^+$ and $CD45^+$ EMVs for *in vitro* experiments, these were first placed on the magnetic stand for 2

min to detach from beads avoiding nonspecific toxicity and allow proper EMV delivery to recipient cells. Next, PBS is removed and 500 µl of Na_2CO_3/$NaHCO_3$ solution pH 11.3 was added and neutralized immediately with the addition of 50 µL of 1 M HCl.

Then, samples are placed on the magnetic stand for removal of the supernatant containing eluted EMVs, without disturbing the bead pellet. Finally, EMVs were pelleted at 14000 x g for 30 minutes at 4°C and resuspended in particle-free PBS.

Nanoparticle Tracking Analysis

The analysis of EMVs isolated from APS patients and HDs were performed by Nanoparticle Tracking Analysis (NTA), using the NanoSight NS300 (Malvern Panalytical, Ltd, Malvern, UK) to determine their size and concentration.

To obtain an appropriate concentration for measurement, the samples were diluted at a 1:100 in particle-free PBS. The instrument was provided with a 488 nm laser (blue), a high sensitivity sCMOS camera and a syringe pump set to a flow rate of 30 arbitrary units, representing the relative dispensing speed. The system recorded five 60-second videos under automated script control, and the data were analyzed using NTA 3.4 Build 3.4.4 software.

Phospholipids extraction and analysis by High-performance thin-layer chromatography (HPTLC)

Phospholipids were extracted from EMVs derived from a HD and an APS patient according to the technique described by Folch.[35] Briefly, both samples were treated with 1 mL of methanol, followed by 2 mL of chloroform, and vigorously mixed for 5 minutes. After adding 0.5 mL of 0.15 M NaCl, the mixture was centrifuged to separate the organic phase. The organic layer was then dried under nitrogen, re-dissolved in chloroform, and centrifuged again to eliminate any insoluble material. The final extract containing phospholipids was separated by high-performance thin-layer chromatography (HPTLC) by using silica gel 60 (10 × 10) plates (Merck, Darmstadt, Germany). Chromatography was performed in chloroform:methanol:acetic acid:water [100:75:7:4] (v:v:v:v). Phospholipids were stained by exposure to iodide vapours.

Analysis by western blot

EMVs from APS patients and HDs were lysed in RIPA buffer (100 mM NaCl, 1 mM EDTA, 1% Triton X-100, 10 mM Tris-HCl pH 7.4, 0.5% Na-deoxycholate, 0.1% SDS,

Na_3VO_4) with a protease inhibitor cocktail (Sigma, Milan, Italy). The EMV lysates were analyzed by the Bradford assay, to obtain the protein concentration (Bio-Rad, Segrate, MI, Italy), and equal sample amount were analyzed by western blot. They were subjected to 10% SDS-PAGE gel and then the proteins transferred onto polyvinylidene difluoride (PVDF) membranes (Bio-Rad).

The PVDF membranes, once blocking with 5% non-fat dried milk in Tris-buffered saline (TBS) containing 0.05% Tween-20, were incubated with the following primary antibodies: mouse anti-Annexin A1 (Santa Cruz Biotechnology, Dallas, TX, USA), rabbit anti- β-tubulin, rabbit anti-CD63 (Abcam, Cambridge, UK), rabbit anti-CD81 (Abcam), rabbit anti-ALG-2 interacting protein X (ALIX) (Abcam) and goat anti-β2-GPI (Affinity Biologicals). Subsequently, an incubation with horseradish peroxidase–conjugated anti-rabbit or anti-goat IgG antibodies (Sigma) was performed. Immunoreactivity was assessed by the chemiluminescence reaction, using the Clarity Western ECL substrate detection system (Bio-Rad). National Institutes of Health ImageJ 1.62 software, by Mac OS X (Apple Computer International, Cupertino, CA, USA), was employed to perform densitometric scanning analysis, allowing the assessment of the absolute value density of each band on the same gel.

Flow cytometric analysis of EMVs

EMVs from all APS patients and HDs were analyzed using a CytoFLEX flow cytometer (Beckman Coulter), equipped with a violet laser (405 nm) and configured for Forward Scatter (FSC) detection in logarithmic mode to optimize the analysis of small particles. Detection thresholds were set to exclude background noise while retaining relevant events. The gating strategy (FSC-A versus SSC-A plot) was defined using FITC fluorescence intensity on the x-axis and Violet SSC-A on the y-axis, with the threshold set on the Violet SSC channel, and including the individual gates established with calibration beads.

The gating region for EMVs was defined by Megamix-Plus SSC Megamix-Plus FSC fluorescent beads (Beckman Coulter, Brea, USA) which have different sizes (0.1 μm, 0.16 μm, 0.2 μm, 0.24 μm, 0.3 μm, 0.5 μm and 0.9 μm) (Figure S1).

Anti-CD41a–FITC, anti-CD45–FITC and anti-CD31–FITC (Beckman Coulter) were used to identify specific subsets of EMVs: platelet, endothelial and leukocyte, respectively.[34]

To evaluate the surface expression of cardiolipin and β2-GPI, EMV suspension was incubated with unconjugated antibodies, separately, affinity purified anti-human-cardiolipin[36,37] and goat anti-β2-GPI (Affinity Biologicals, Ancaster, Ontario, Canada) at

room temperature for 45 minutes in Annexin V buffer (10 mM Hepes, 150 mM NaCl, 5 mM KCL, 1 mM $MgCl_2$ 1.8 mM $CaCl_2 + 2H_2O$, pH 7.4). After incubation, samples were centrifuged with Annexin V buffer at 14000 x g for 30 minutes at 4°C.

FITC-conjugated anti-human (Sigma, Milan, Italy) and FITC-conjugated anti-goat (Invitrogen, Waltham, USA) antibodies were added for 30 minutes at room temperature, followed by a second centrifugation with Annexin V buffer at 14000 x g for 30 minutes at 4°C. The pellets were resuspended in an appropriate volume of Annexin V buffer for flow cytometric analysis.

Labeled EMVs were acquired maintaining the parameters established with the calibration beads. To ensure an appropriate statistical representation, a minimum of 10^7 acquired events were collected per sample. Gating was performed on the population of events defined by the beads, excluding aggregates and debris. Data analysis was performed using CytoFLEX software, Beckman Coulter, quantifying the fluorescence intensity within gate corresponding to their expected size range (100–1000 nm).

Cells and treatments

Human microvascular endothelial cells (HMEC-1, American Type Culture Collection, ATCC, Manassas, VA, USA) were maintained in MCDB13 medium supplemented with 10 ng/mL Epidermal Growth Factor (EGF, Thermofisher, Waltham, MA, USA), 1 μg/mL Hydrocortisone (Sigma-Aldrich), 10 mM Glutamine (ATCC) and 10% Fetal Bovine Serum (FBS, ATCC).

HMEC-1 ($5x10^5$/mL) were seeded into 6-well cell culture and incubated at 37°C with EMVs from APS patients (2 x 10^8/mL), EMVs from HDs (2 x 10^8/mL) or LPS (0.1 μg/mL), for different incubation times (45 minutes for IRAK1 and NF-kB-p65 activation, 4 hours for TF expression).[38] After treatments, whole-cell extracts were prepared in lysis buffer, containing 20 mM HEPES, pH 7.2; 1% Nonidet P-40, 10% glycerol, 50 mM NaF, 1 mM Na_3VO_4 and protease inhibitors cocktail (Sigma-Aldrich). Alternatively, nuclear-cell extracts were obtained as previously described.[38] Cells were resuspended in buffer A (20 mM HEPES, pH 7.2; 0.1% Nonidet P-40, 20 mM KCl, 3.0 mM $MgCl_2$, 1 mM Na_3VO_4, 5 mM DTT and protease inhibitors cocktail), and after 30 minutes on ice, samples were centrifuged for 30 minutes at 10000 g at 4°C and pellets were resuspended in buffer B (40 mM HEPES, pH 7.2; 0.84 M NaCl, 0.4 mM EDTA, 50% glycerol, 1 mM Na_3VO_4, 5 mM DTT and protease inhibitors cocktail). After 1 hour on ice, samples were centrifuged at 10000 g for 1 hour at 4°C and supernatants were harvested as nuclear extracts.

In parallel experiments, HMEC-1 cells were incubated with APS plasma EMVs derived from platelets ($CD61^+$), endothelial cells ($CD31^+$) or leucocytes ($CD45^+$) and lysed under the same experimental conditions previously described.

Western blot analysis of IRAK phosphorylation, NF-κB activation and Tissue factor expression

Equal amounts of whole and nuclear extracts or supernatants proteins of HMEC-1 cells untreated or treated with APS EMVs, HD EMVs or LPS were separated in 7.5% SDS-PAGE. Proteins were electrophoretically transferred to PVDF membranes (Bio-Rad) and then blocked with TBS-T 3% BSA. Subsequently, whole and nuclear extracts membranes were incubated with rabbit anti-phospho-IRAK1 (Cell Signaling, Inc., Danvers, MA, USA) or rabbit anti-phospho-NF-kB-p65 antibodies (Cell Signaling, Inc.), respectively. Alternatively, whole extracts and supernatants membranes were incubated with rabbit anti-TF (Abcam, Cambridge, UK). Antibody reactions were visualized by HRP-conjugated anti-rabbit IgG (Sigma-Aldrich) and immunoreactivity was assessed by the chemiluminescence reaction, using the Clarity Western ECL substrate detection system (Bio-Rad). As loading control phospho-IRAK1-blotted membranes were stripped and reprobed with polyclonal anti-IRAK1 antibody (MBL, Woburn, MA, USA) or anti-β-actin mAb (Sigma-Aldrich), whereas TF-blotted membranes with anti-β-actin mAb (Sigma-Aldrich). In addition, to check purity of nuclear extracts, phospho-NF-kB-p65-blotted membranes were reprobed with anti-Histone H1 antibodies (Abcam, Cambridge, UK). Densitometric scanning analysis was performed by NIH Image 1.62 software (by Mac OS X, Apple Computer International) analyzing the density of each band (absolute value) in the same gel. Alternatively, equal amounts of whole protein extracts of HMEC-1 cells untreated or treated with $CD61^+$, $CD31^+$, $CD45^+$ EMVs isolated from APS plasma or LPS were analyzed by western blot as previously described, to assess IRAK phosphorylation and TF expression.

Statistical Analysis

Continuous variables were reported as mean ± standard deviation for normally distributed data, or as median and interquartile range (IQR) for non-normally distributed data. Normality was assessed using the Shapiro-Wilk test. Group comparisons were performed using the Student's t-test for normally distributed variables and the Mann-Whitney U test for variables with non-normal distribution. Correlations between continuous variables were

assessed using Pearson's correlation coefficient when normality was confirmed or Spearman's rank correlation coefficient otherwise. A p-value < 0.05 was considered statistically significant. All statistical analyses were performed using R software (version 4.3.3) via the RStudio graphical interface (version 0.98) or GraphPad Prism 8.0 software (San Diego, CA, USA).

Table S1. EMVs autoantigens expression according to the clinical manifestations.

	Total thrombosis n= 18	Arterial thrombosis n= 9	Venous thrombosis n= 9	Obstetric complications n= 11	Recurrent miscarriage n= 3	Foetal deaths n=8
EMVs-CL MFI median (IQR)	1950 (1733-6454)	1873 (1500-2375)	2300 (1900-83422)	40021 (8301-168595)	40021 (22511-83088)	82117 (9940-202446)
EMVs-β_2GPI MFI median (IQR)	9254 (2347-116686)	15325 (2467-124461)	3182 (2308-93359)	31268 (10184-160753)	47742 (28452-102814)	27079 (10407-200674)

CL: Cardiolipin; **β_2GPI**: Beta2Glycoprotein I; **EMVs**: Extracellular microvesicles; **MFI:** mean fluorescence ıtensity

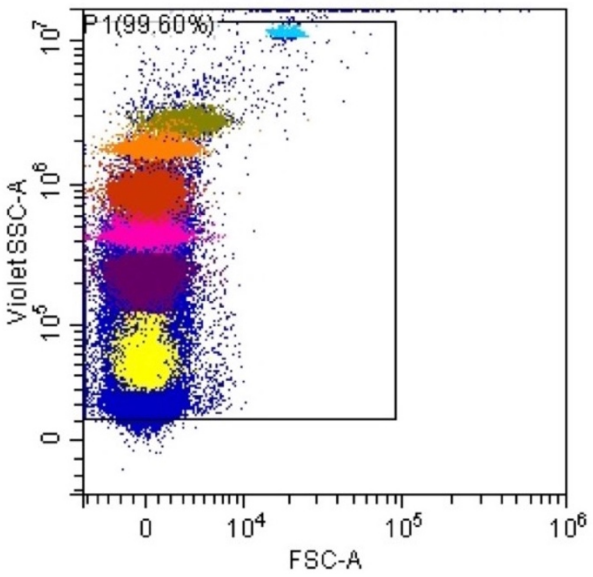


Figure S1

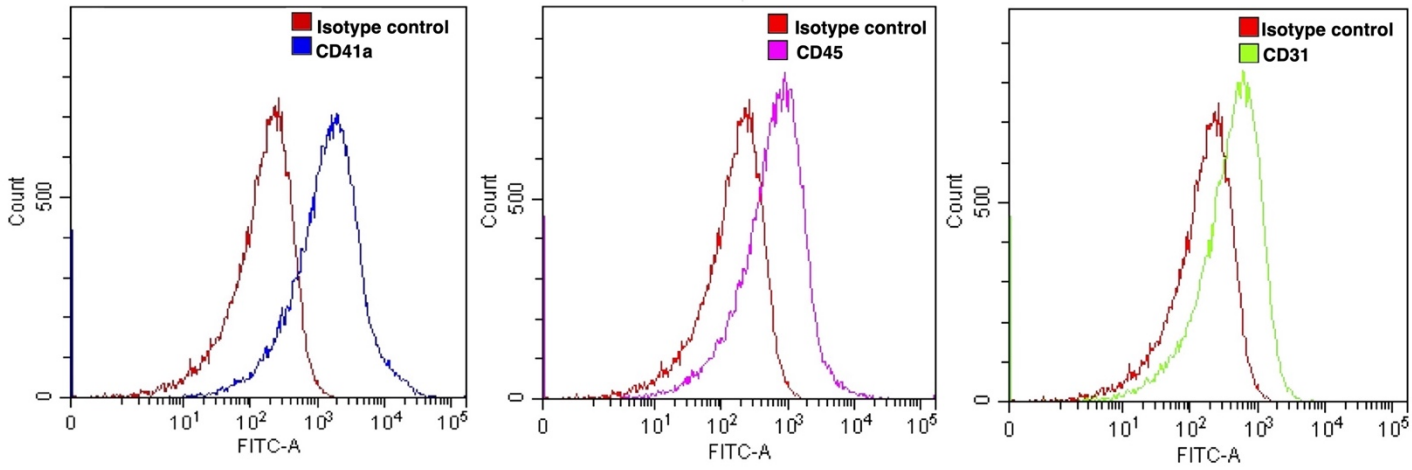
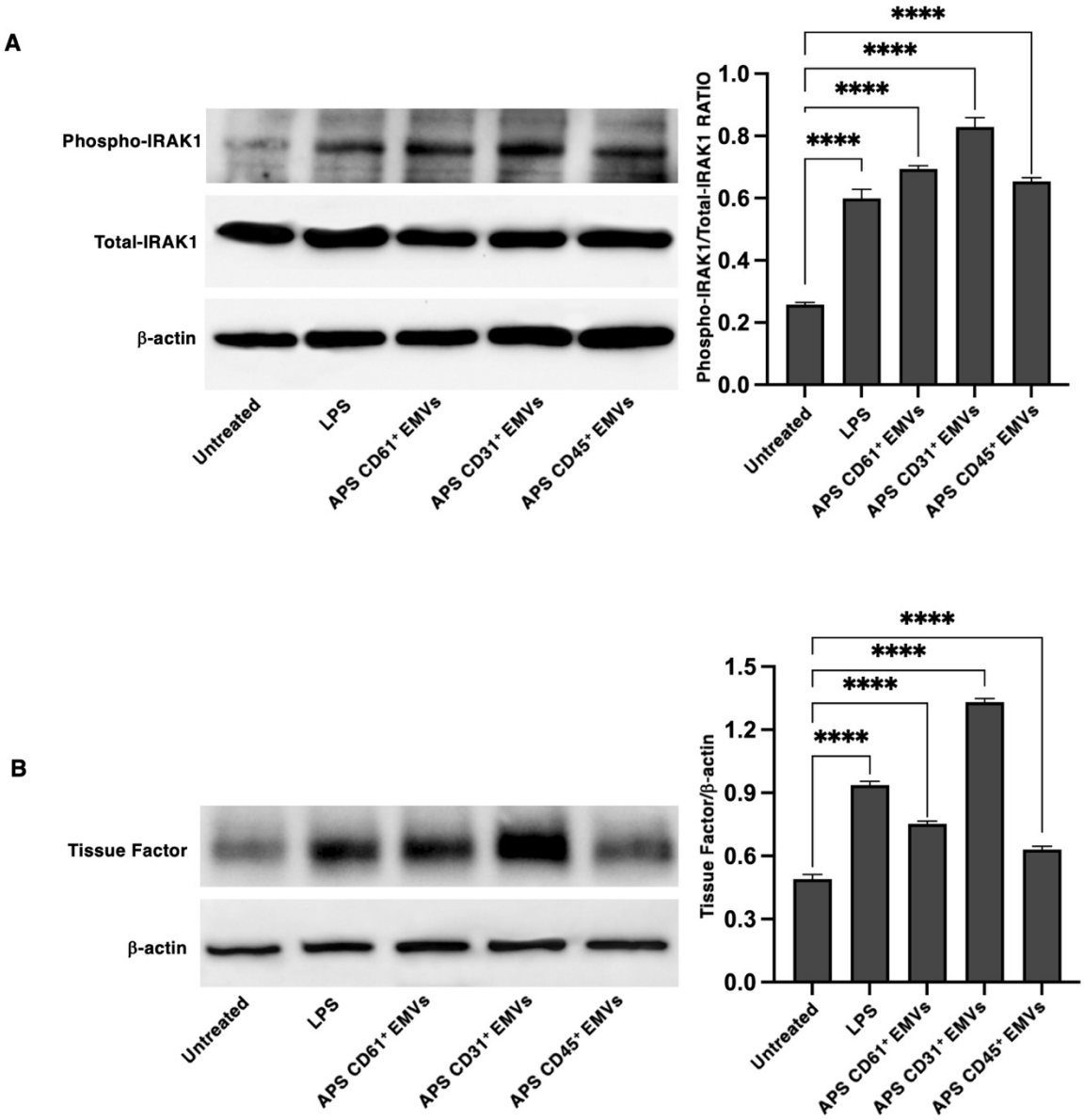


Figure S2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Phospho-IRAK1
Total-IRAK1
β-actin
Untreated
LPS
APS CD61+ EMVs
APS CD31+ EMVs
APS CD45+ EMVs
Phospho-IRAK1/Total-IRAK1 RATIO
0.0
0.2
0.4
0.6
0.8
1.0
****
B
Tissue Factor
β-actin
Tissue Factor/β-actin
0.0
0.3
0.6
0.9
1.2
1.5

Figure S3

Figure S1. EMV gating strategy.

FSC and SSC were resulting from 488 nm laser line excitation while vSSC (violet SSC) was resulting from 405 nm laser line excitation. Gating strategy have been performed using a combination of Megamix-Plus SSC Megamix-Plus FSC fluorescent beads (Beckman Coulter), containing beads of sizes 100 nm, 160 nm, 200 nm, 240 nm, 300 nm, 500 nm, 900 nm.

Figure S2. Flow cytometry characterization of EMVs from APS patients.

Representative semiquantitative flow cytometry histogram overlay of CD41a, CD45 and CD31, expressed on the surface of APS patient EMVs, derived from platelets, leukocytes and endothelial cells.

Figure S3. Analysis of IRAK activation and Tissue Factor expression in HMEC stimulated with APS patient $CD61^+$, $CD31^+$ or $CD45^+$ EMVs.

HMEC-1 cells untreated or stimulated with LPS, APS patient platelet ($CD61^+$), endothelial cells $CD31^+$ or leucocytes derived ($CD45^+$) EMVs were lysed and analyzed by western blot to evaluate: (A) Phospho-IRAK1 expression using rabbit anti-phospho-IRAK1 antibody. (B) TF expression using rabbit anti-TF antibody. Densitometric phospho-IRAK1/total IRAK or TF/β-Actin ratios are evaluated. Data are reported as mean (S.D.). Statistical analysis indicated: ****$p < 0.0001$.