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

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Abstract

Antiphospholipid syndrome (APS) is characterized by thrombosis, recurrent miscarriages and the stable presence of antiphospholipid antibodies (aPL). Circulating aPL 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 pro-coagulant and pro-inflammatory extracellular microvesicles (EMV). This study analyzed the presence of EMV 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 more elevated in APS patients than in healthy donors. Moreover, we observed the presence of the main autoantigenic targets, β2-GPI and cardiolipin, on the surface of EMV from APS patients' plasma, by both biochemical and flow cytometry analysis. It also revealed significantly higher cardiolipin levels on EMV from patients with obstetrical APS compared to those without pregnancy morbidity, specifically in women with a history of fetal death. To analyze the effect of the EMV from APS patients on endothelial cell activation and signaling, we then incubated *in vitro* human microvascular endothelial cells with patient-derived EMV, demonstrating a significant phosphorylation of IRAK, NF-κB, as well as increased expression and release of tissue factor. These findings suggest that circulating EMV 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 EMV 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 (aPL), including anti-cardiolipin antibodies (aCL), anti-β2-glycoprotein I (anti-β2-GPI) and Lupus anticoagulant (LAC).[1-3] Circulating aPL 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] aPL 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 κ 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 pro-inflammatory 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 pro-coagulant and pro-inflammatory extracellular vesicles (EV).[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 EV, in different pathologies also in the field of autoimmunity.

EV 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 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 (EMV), 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 EMV, which are secreted from plasma membrane during physiological cellular processes; indeed, EMV 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 EMV 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. EMV 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] EMV 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 EMV induce endothelial cell activation also increasing their adhesion to monocytes.[24,25] Instead, EMV released from endothelial cells and monocytes induce a pro-coagulant and proadhesive profile on both cells of origin.[26-29] In the same way, EMV 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, EMV are considered markers of cellular activation, but they also express bioactive lipids, proteins and nucleic acids. Therefore, EMV 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, EMV-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 EMV in the plasma of patients affected by APS and any correlations with clinical manifestations. We 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 (HD), 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 Declaration of Helsinki. The local ethics committees approved the research protocol.

Antiphospholipid antibody 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.). LAC was assessed through aPTT and dRVVT, with confirmation testing (Hemoliance Instrumentation Laboratory).

Isolation of extracellular vesicles

Fasting blood was collected in sodium citrate tubes. Platelet-poor plasma (PPP) enriched in EMV was obtained by double centrifugation at 2,500×g for 15 minutes (min). EMV were isolated via centrifugation at 14,000×g for 30 min at 4°C and resuspended in filtered phosphate-buffered saline (PBS).[34] Alternatively, EMV released from platelets, endothelial cells, and leukocytes were isolated from PPP, using CD61, CD31 or CD45 MicroBeads (Miltenyi Biotec).

Nanoparticle tracking analysis

EMV from APS patients and HD 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

Phospholipids from EMV 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 blotting

For western blotting (WB), EMV 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 horseradish peroxidase-conjugated secondary antibodies. Signals were detected with chemiluminescence (Clarity ECL, Bio-Rad) and quantified with NIH ImageJ software.

Flow cytometric analysis of extracellular vesicles

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 (forward scatter [FSC]-A *vs*. side scatter [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) (*Online Supplementary Figure S1*). EMV were stained with anti-CD41a, -CD45, and -CD31 to identify platelet, leukocyte and endothelial origins.[34] For surface CL and β2-GPI detection, EMV were incubated with specific primary antibodies,[36,37] followed by FITC-conjugated secondary antibodies. Labeled EMV were analyzed after 10^7 acquired events.

Cells, treatments and western blotting

HMEC-1 ($5x10^5$/mL) were seeded into 6-well cell culture and incubated at 37°C with EMV from APS patients ($2x10^8$/mL), EMV from HD ($2x10^8$/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

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

Characteristics	N (%)
Female sex	25 (89)
Median age, years[1]	52 (48, 60)
Caucasian	28 (100)
Obstetric APS	11/25 (44)
Recurrent miscarriage	3/25 (12)
Fetal 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)
aCL[2]	20 (71)
anti-β2-GPI antibodies[2]	14 (50)
LAC[2]	3 (11)
High risk profile[2]	15 (54)

[1]Median (Q1, Q3). [2]Anti-cardiolipin antibodies (aCL) and anti-β2-glycoprotein I (β2-GPI) antibodies and Lupus anticoagulant (LAC) positivity refers to findings previously documented in the patient's clinical history. APS: antiphosholipd syndrome.

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⁺ EMV and then analyzed by WB to evaluate IRAK phosphorylation and TF expression.

Statistical analysis

Data were expressed as mean ± standard deviation (SD) or median (interquartile range [IQR]). Comparisons used Student's *t* test or Mann-Whitney U test; correlations were assessed by Pearson or Spearman methods. Significance

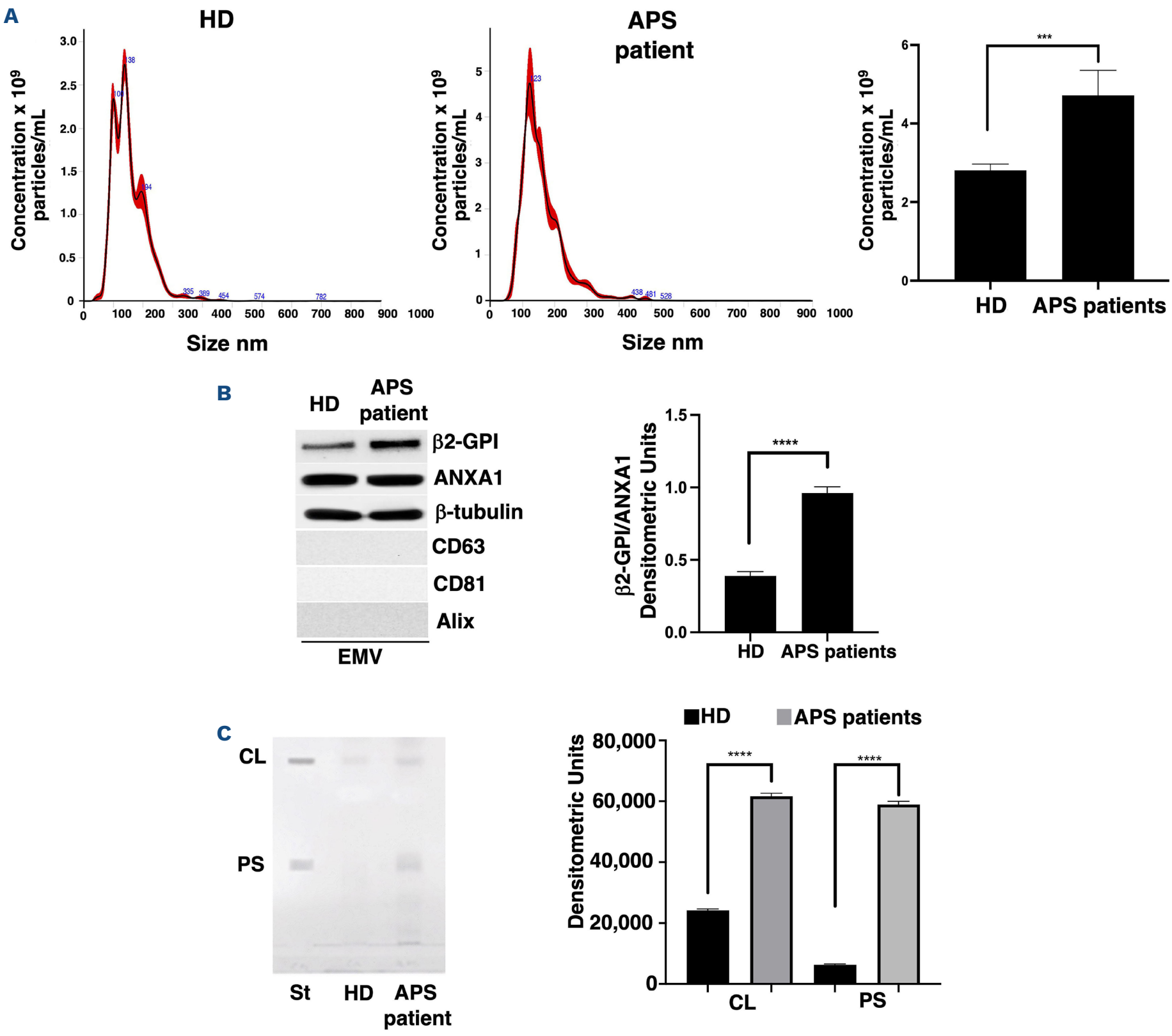

Figure 1. Quantitative analysis and biochemical characterization of extracellular microvesicles from the plasma of antiphospholipid syndrome patients. (A) Quantitative analysis of extracellular microvesicles (EMV) from 1 representative healthy donor (HD) and from 1 representative antiphospholipid syndrome (APS) patient by NanoSight (Nanoparticle Tracking Analysis). The number of EMV in APS patients was $4.72x10^9$ EMV/mL (standard deviation [SD] $2.86x10^8$), compared to $2.81x10^9$ EMV/mL (SD $7.29x10^7$) detected in HD. ***P<0.001 *versus* HD. (B) Characterization of the EMV from 1 representative HD and from 1 representative APS patient by western blotting (WB) analysis, using anti-β2-GPI, anti-Annexin A1 (ANXA1), anti-β-tubulin, anti-CD63, anti-CD81 and anti-Alix antibodies. (C) High-performance thin-layer chromotography analysis of phospholipids extracted from EMV, 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 vapors (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 co-migrating bands. Results represent the mean ± SD. ****P<0.0001.

was set at $P<0.05$. Analyses were performed using R (v4.3.3) or GraphPad Prism 8.0.
The methods section has been extended in the *Online Supplementary Appendix*.

Results

Patients

A total of 28 Caucasian patients with a diagnosis of Primary APS (PAPS) were enrolled, with a median age of 52 years (IQR, 48-60) and a female-to-male ratio of about 8:1. Among the 25 female patients, 11 (44%) experienced obstetrical complications, while 18 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 HD were included.

Quantitative analysis and characterization of extracellular vesicles from plasma of patients with antiphospholipid syndrome

The presence of EMV in plasma samples from patients with APS and HD 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 EMV/mL, standard deviation: 2.86×10^8), compared to 2.81×10^9 EMV/mL (standard devia-

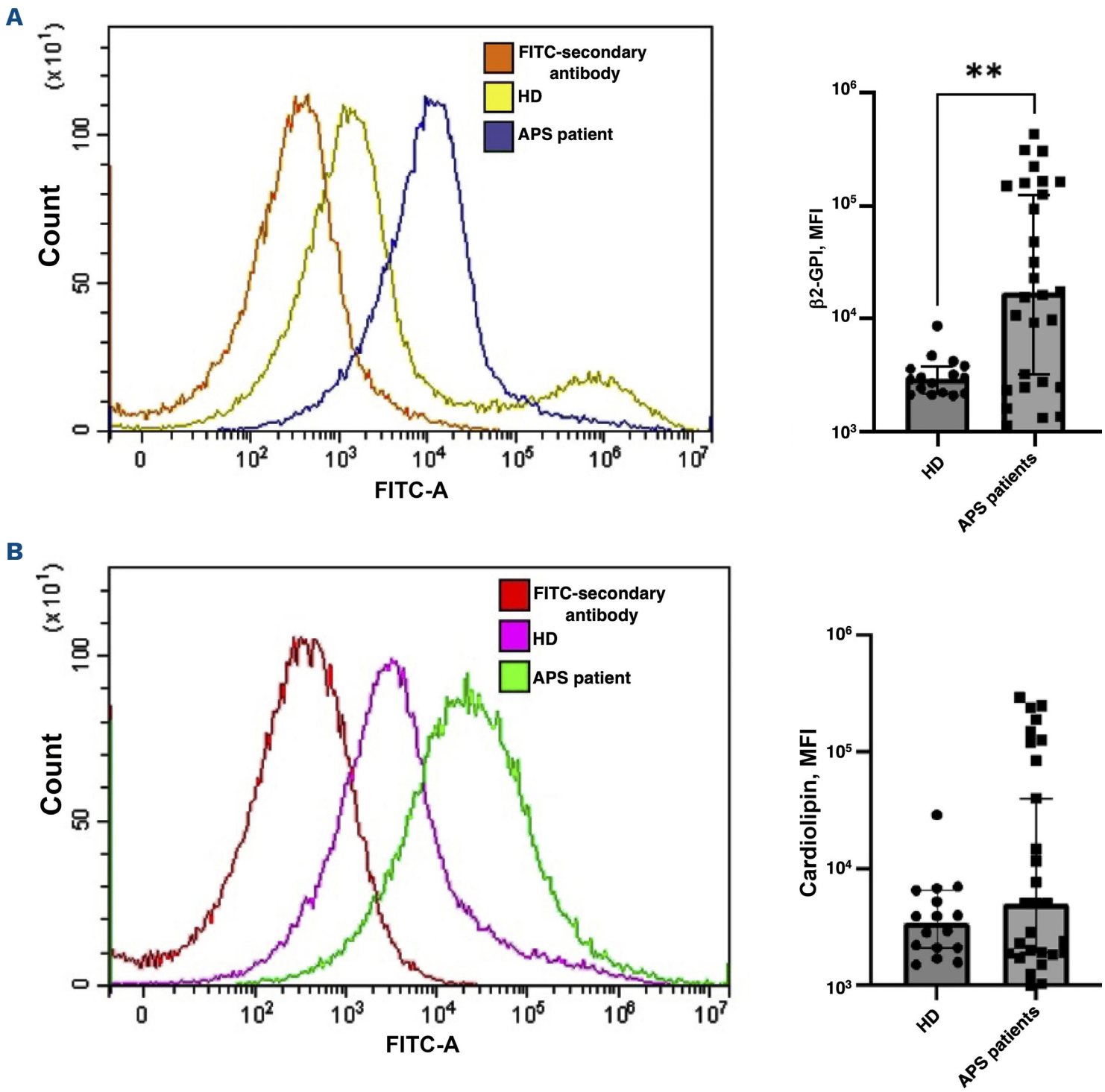

Figure 2. Flow cytometric analysis of cardiolipin and anti-β2-glycoprotein I in extracellular microvesicles from antiphospholipid syndrome patients and healthy donors. (A) Representative semiquantitative flow cytometry histogram overlay of cardiolipin (CL) and β2-glycoprotein I (β2-GPI) expressed on the surface of extracellular microvesicles (EMV) isolated from antiphospholipid syndrome (APS) patient and healthy donor (HD). The mean fluorescence intensity (MFI) on the FITC channel is shown. (B) Histograms show the MFI of CL and β2-GPI expression on EMV from plasma of APS patients and HD. Data are expressed as mean (standard deviation). Statistical analysis indicated: $^{**}P<0.01$.

tion: 7.29×10^7) in HD (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 EMV was detected using specific anti-β2-GPI antibodies. Western blotting data demonstrated that β2-GPI levels were markedly higher in EMV isolated from APS patients, compared to those from HD (Figure 1B). β2-GPI association with EMV may actively contribute to the pro-thrombotic state observed in these patients.
Since anti-β2-GPI antibodies may bind β2-GPI when associated to anionic phospholipids, such as CL and PS, we investigated whether these phospholipids were present on EMV released from APS patients. Phospholipids were extracted from EMV derived from APS patients and HD and then analyzed by HPTLC. The analysis revealed the presence of two main iodide vapor-positive bands, co-migrating 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 EMV isolated from APS patients, compared to those from HD (Figure 1C, right panel).

Flow cytometric analysis of cardiolipin and β2-glycoprotein I in extracellular microvesicles from plasma of patients with antiphospholipid syndrome and clinical associations

Preliminary analysis revealed that EMV derived from platelet, endothelial and leukocyte origin, as detected by anti-CD41a, anti-CD45 and anti-CD31, respectively (*Online Supplementary Figure S2*).
To analyze the presence of the main APS autoantigens on EMV, isolated from plasma of APS patients, we used flow cytometry to measure the expression of β2-GPI and CL.
As showed in Figure 2A, B, the analysis demonstrated a higher β2-GPI mean fluorescence intensity (MFI) on EMV from APS patients compared to HD (16,789 *vs*. 2,932; *P*=0.005), while, although the trend is similar, CL MFI levels on EMV did not differ significantly between the two groups (4,999 *vs*. 3416; *P*=0.401).
In the APS cohort, EMV from patients with obstetric complications showed significantly higher CL MFI than those from patients without pregnancy morbidity (40,021 *vs*. 1,950; *P*=0.001), specifically in women with a history of fetal death (82,117 *vs*. 2,300; *P*=0.005; Figure 3A). Consequently, patients with a history of thrombosis showed significantly lower CL MFI levels on EMV compared to those without thrombotic events (1,950 *vs*. 27,331; *P*=0.004), with a significant reduction as compared with patients with arterial thrombosis (1,873 *vs*. 11,580; *P*=0.022; Figure 3B). EMV 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 *Online Supplementary Table S1*.
Extracellular microvesicles from plasma of patients with antiphospholid syndrome 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' EMV on HMEC-1 cells, studying signaling that potentially plays a role in promoting a pro-coagulant and pro-inflammatory microenvironment. In particular, WB analysis of HMEC-1 cell lysates demonstrated that APS patient EMV, as well as LPS, induced a significant increase of IRAK (Figure 4A) and NF-κB p65 (Figure 4B) phosphorylation, compared to both untreated

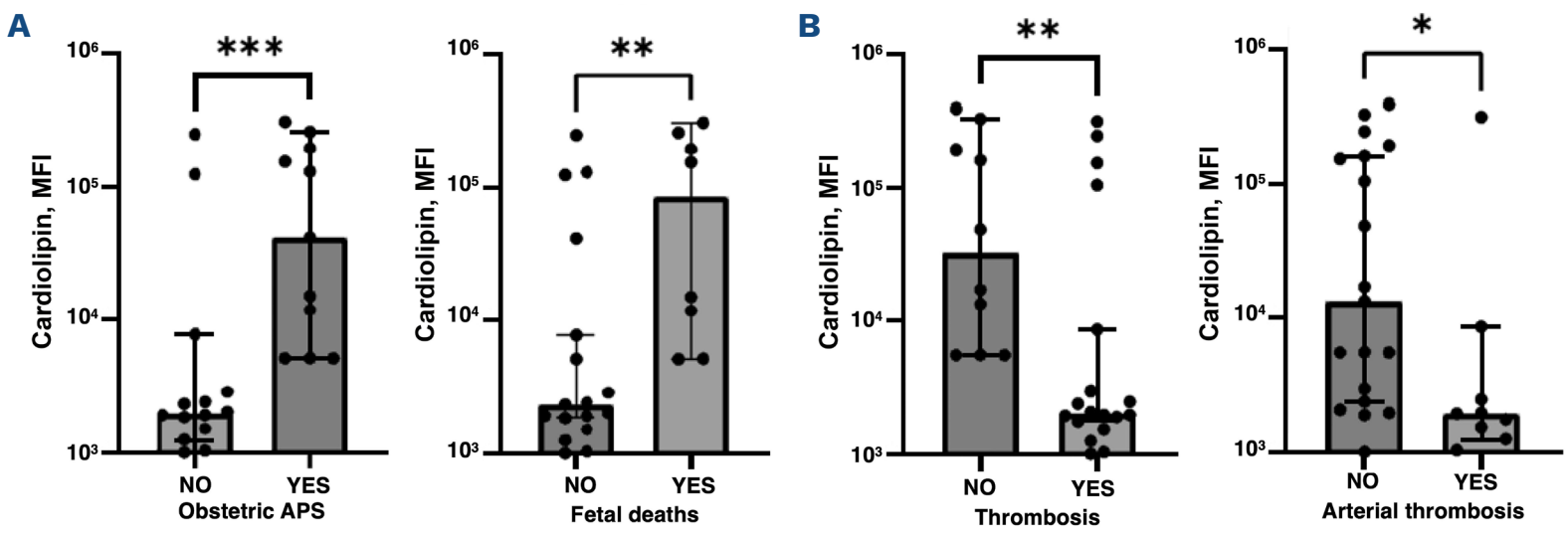

Figure 3. Clinical associations with cardiolipin and anti-β2-glycoprotein I levels detected on extracellular microvesicles from antiphospholipid syndrome patients. (A) Comparison between cardiolipin (CL) mean fluorescense intensity (MFI) levels on extracellular microvesicles (EMV) in female patients, based on history of obstetric antiphospholipid syndrome (APS) and fetal deaths. (B) Comparison between CL MFI levels on EMV in patients, based on the history of thrombosis and arterial thrombosis. Statistical analysis indicated: **P*<0.05; ***P*<0.01; ****P*<0.001. anti-β2-GPI: anti-β2-glycoprotein I.

and HD EMV 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 pro-coagulant 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 EMV or LPS. Conversely, a weak reactivity was observed in untreated cells or in cells stimulated with HD EMV (Figure 5).

Moreover, we isolated, from APS plasma, EMV released by platelets ($CD61^+$), endothelial cells ($CD31^+$) or leukocytes ($CD45^+$) and then we analyzed their individual contribution to endothelial activation (IRAK phosphorylation) and pro-coagulant signaling (TF levels). Obtained results showed that all three types of EMV, mainly $CD31^+$ EMV, significantly induced both IRAK phosphorylation (*Online Supplementary Figure S3A*) and increase of TF (*Online Supplementary Figure S3B*).

Discussion

In this study we characterized plasma derived EMV 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 EMV 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

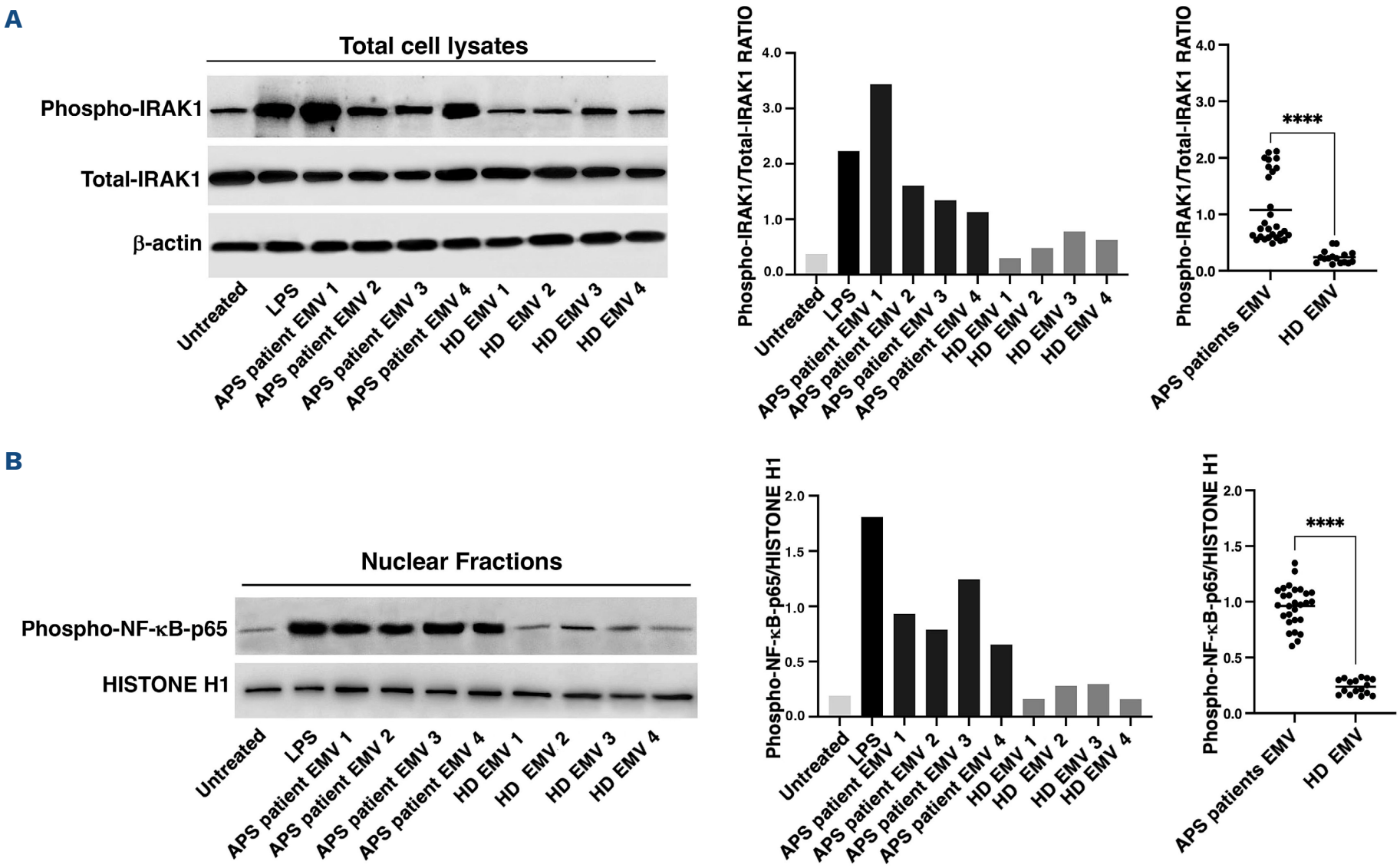

Figure 4. Analysis of IRAK and NF-κB activation in human microvascular endothelial cells stimulated with antiphospholipid syndrome patient extracellular microvesicles. Human microvascular endothelial cells (HMEC-1) cells untreated or stimulated with lipopolysaccharide (LPS), antiphospholipid syndrome (APS) patient extracellular microvesicles (EMV) and healthy donor (HD) EMV were lysed and analyzed by western blotting to evaluate: (A) phospho-IRAK1 expression using rabbit anti-phospho-IRAK1 antibody in whole cell lysates. (B) Phospho-NF-κB-p65 expression using rabbit anti-phospho-NF-κB-p65 antibody in nuclear cell lysates. Anti-β-tubulin or anti-Histone H1 antibodies were used to evaluate loading controls. Densitometric phospho-IRAK1/total IRAK or phospho-NF-κB-p65/Histone H1 ratios are evaluated. Western blotting and densitometric analysis from four representative APS patients and HD are shown. Densitometric values, calculated in all APS patients and HD, are represented by dot-plot. Data are reported as mean (standard deviation). Statistical analysis indicated: ****P<0.0001.

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 EMV isolated from APS patients' plasma. This observation suggests that circulating EMV 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 EMV, isolated from plasma of APS patients, was confirmed by flow cytometry. Interestingly, the analysis revealed significantly higher CL MFI levels on EMV from patients with obstetrical APS, specifically women with a history of fetal death, compared to those without pregnancy morbidity. This finding is not fully surprising, since higher levels of EV in women with recurrent miscarriage have been reported.[33] It is consistent with a mechanism in which EMV 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-fetal 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 EMV 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 EV,[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 EMV obtained from APS patients on endothelial cell activation and signaling. Functionally, we showed that APS-derived EMV exert potent activating effects on endothelial cells. Exposure of HMEC-1 to patient-derived EMV led to phosphorylation of IRAK and NF-κB, as well as increased TF expression and release. These results confirm and extend the observation that EMV increase ICAM-1 and

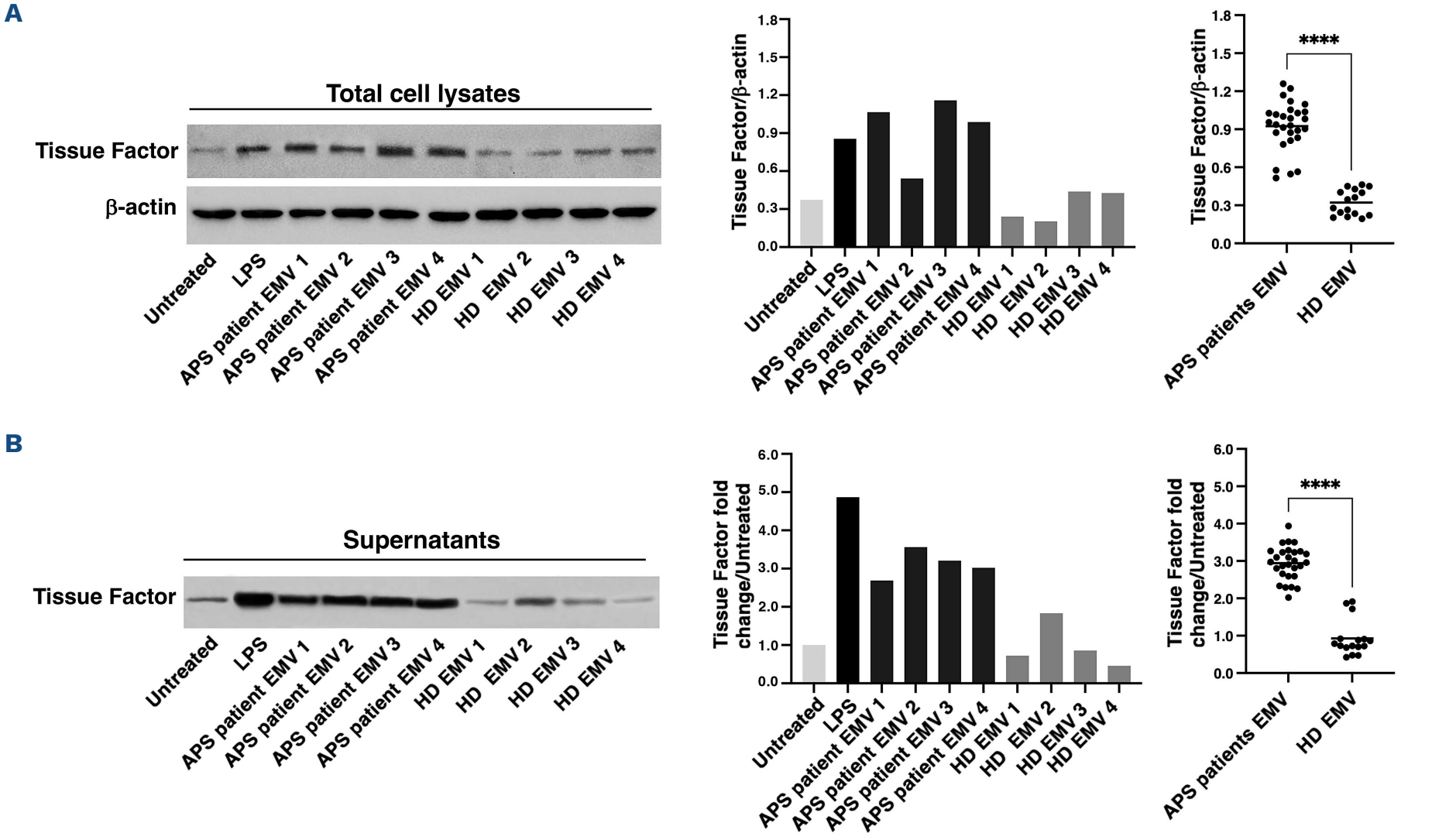

Figure 5. Analysis of tissue factor in HMEC cells stimulated with antiphospholipid syndrome patient extracellular microvesicles. Human microvascular endothelial cells (HMEC-1) cells untreated or stimulated with lipopolysaccharide (LPS), antiphospholipid syndrome (APS) patient extracellular microvesicles (EMV) and healthy donors (HD). EMV were lysed and analyzed by western blotting 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 blotting and densitometric analysis from 4 representative APS patients and HD are shown. Densitometric values, calculated in all APS patients and HD, are represented by dot-plot. Data are reported as mean (standard deviation). Statistical analysis indicated: ****P<0.0001.

VCAM-1 expression in endothelial cells,[42] pointing toward TLR-related inflammatory signaling and pro-coagulant activation driven by circulating vesicles, linking immune recognition pathways with vascular thrombogenicity.
Importantly, this suggests that EMV 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 EMV 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 pre-eclampsia, fetal growth restrictions and fetal loss, by pro-inflammatory 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 EMV 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 EMV as therapeutic targets or biomarkers of clinical complications in APS.

Disclosures
No conflicts of interest to disclose.

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.

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Data-sharing statement
To obtain raw data and protocols, please contact the corresponding author.

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