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

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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

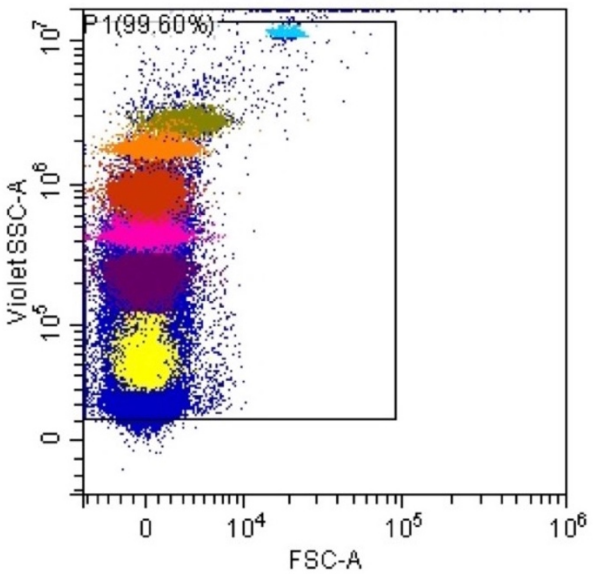

Figure S1

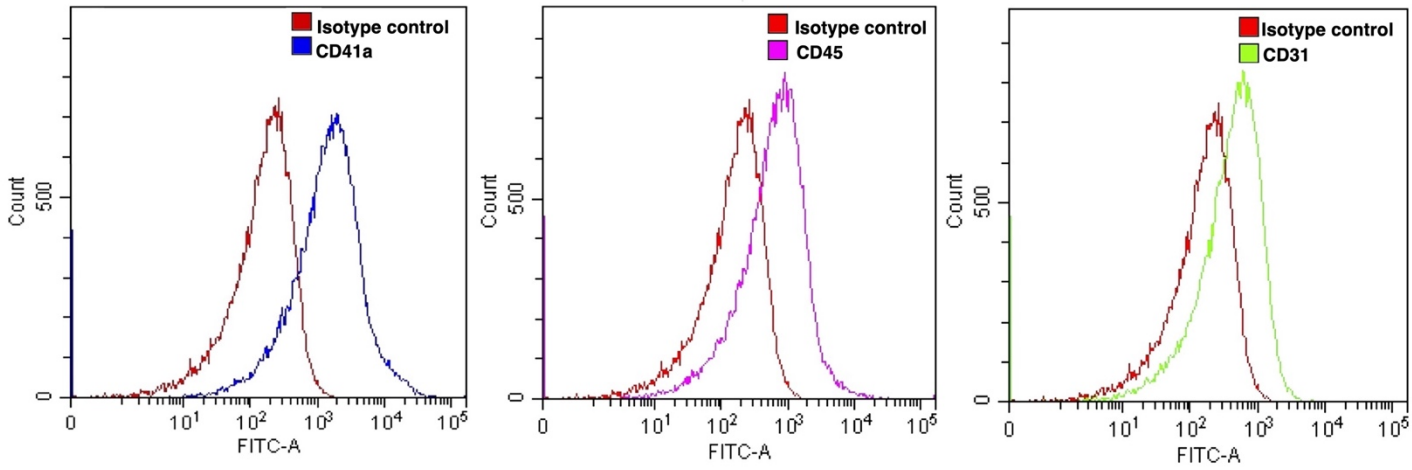

Figure S2

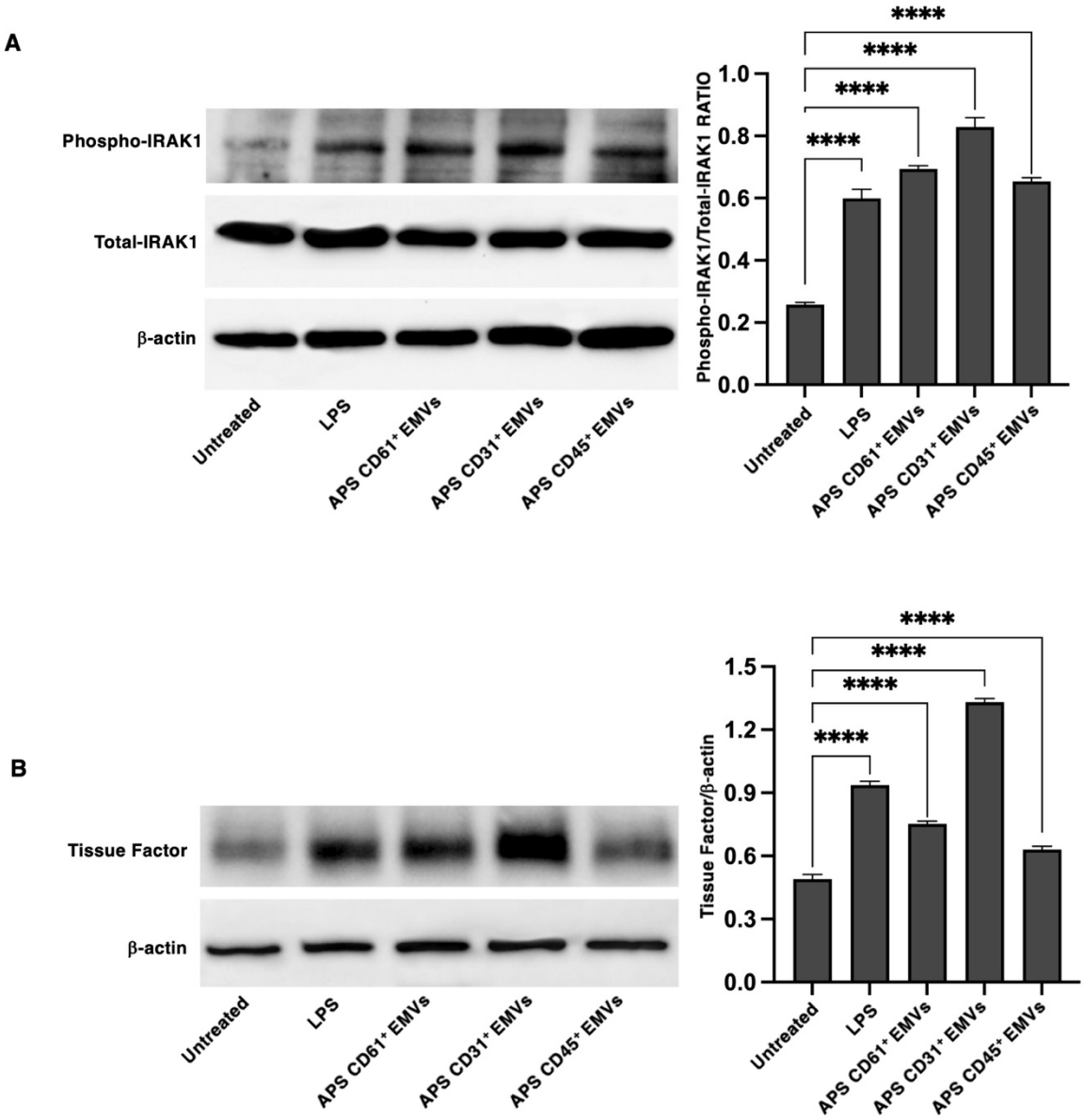
A
Phospho-IRAK1
Total-IRAK1
β-actin
Untreated
LPS
APS CD61+ EMVs
APS CD31+ EMVs
APS CD45+ EMVs
Phospho-IRAK1/Total-IRAK1 RATIO
0.0
0.2
0.4
0.6
0.8
1.0
****
B
Tissue Factor
β-actin
Tissue Factor/β-actin
0.0
0.3
0.6
0.9
1.2
1.5

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: ****$p < 0.0001$.