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Extracellular vesicles released upon stimulation with antiphospholipid antibodies: An actual direct procoagulant mechanism or a new factor in the lupus anticoagulant paradox?

Daniel Álvarez [a,*], Carolina Rúa [b], Manuela Velásquez Berrío [a], John Ubeimar Cataño [b,c], Carlos Escudero [d], Ángela P. Cadavid J [a,b]

[a] Grupo Reproducción, Departamento Microbiología y Parasitología, Facultad de Medicina, Universidad de Antioquia UdeA, Calle 70 No. 52-21, Medellín, Colombia
[b] Grupo de Investigación en Trombosis, Departamento Medicina Interna, Facultad de Medicina, Universidad de Antioquia UdeA, Medellín, Colombia
[c] Hospital San Vicente Fundación, Medellín, Colombia
[d] Vascular Physiology Laboratory, Group of Research and Innovation in Vascular Health (GRIVAS Health), Basic Sciences Department, Faculty of Sciences, Universidad del Bio-Bio, Chillán, Chile

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ABSTRACT

Antiphospholipid antibodies (aPL) lead to a hypercoagulable state *in vivo*. Paradoxically, some of these autoantibodies perform as inhibitors of the coagulation cascade *in vitro* (a phenomenon referred to as "lupus anticoagulant"). The presence of lupus anticoagulant has been related to an increased quantity of plasma extracellular vesicles, which may constitute a direct procoagulant mechanism in antiphospholipid syndrome. This study investigates whether or not endothelial cell-derived extracellular vesicles released upon stimulation with aPL (aPL-EDEVs) are related to a higher direct coagulation activity. Using an *in vitro* model of endothelium, flow cytometry and a recalcified plasma-based assay, we found that the coagulation activity of aPL-EDEVs is mainly conditioned by the lupus anticoagulant-like activity of autoantibodies. Nevertheless, in the presence of β2 glycoprotein I, a cofactor of aPL during the stimulation of endothelial cells, the coagulation activity of EDEVs is restored in a mitogen-activated protein kinase kinases 1 and 2 (MEK1/2)-dependent manner. This phenomenon was especially evident when using immunoglobulins G from patients with vascular and obstetric primary antiphospholipid syndrome who manifest refractoriness to treatment. Our findings suggest that the role of aPL-EDEVs in the antiphospholipid syndrome-related hypercoagulable state may not rely on their capacity to enhance clotting directly. While β2 glycoprotein I performs as a procoagulant cofactor and restores the coagulation activity of extracellular vesicles via MEK1/2 pathway, proportionally, autoantibodies interact with aPL-EDEVs and exhaust their coagulation properties. Further analysis is required to establish whether lupus anticoagulant-like autoantibodies opsonise extracellular vesicles and whether opsonised vesicles may lead to thrombosis by indirect means.

1. Introduction

Antiphospholipid antibodies (aPL) are a broad and heterogeneous group of autoantibodies targeting anionic phospholipids [1] and phospholipid-binding proteins [2,3]. These antibodies are known to lead to vascular thrombosis and pregnancy-related morbidity from clinical data [4] and assays using murine models [5,6]. Given the well-established relationship between the presence of aPL and the hypercoagulable state that characterises antiphospholipid syndrome (APS), it is a paradox that some of these autoantibodies share the functional capacity to extend clotting times *in vitro*, behaving as inhibitors of reactions that rely on phospholipids as cofactors (lupus anticoagulant - LA) [7–9]. This paradox is puzzling in the clinical setting since recognising the LA phenomenon constitutes a better predictor of thrombosis risk than the isolated assessment of any single antibody with a given antigenic specificity [10,11]. Furthermore, the precise identity

Abbreviations: aPL, Antiphospholipid antibodies; EVs, Extracellular vesicles; EDEVs, Endothelial cell-derived medium/large EVs; aPL-EDEVs, Endothelial cell-derived medium/large EVs released upon stimulation with aPL.

* Corresponding author. Calle 70 No. 52-21, Universidad de Antioquia, Medellín, Colombia.
E-mail address: daniel.alvarezj1@udea.edu.co (D. Álvarez).

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of aPL responsible for LA and the mechanism(s) involved in this phenomenon are still unclear.

Multiple pathogenic mechanisms have been described to explain the relationship between aPL and thrombosis in APS patients (recently reviewed in Ref. [12]). For example, it is known that aPL trigger a proinflammatory and procoagulant state in endothelial cells [13–15] via mitogen-activated protein kinase (MAPK) pathways [16]. In turn, this endothelial activation/dysfunction includes releasing medium/large extracellular vesicles [17].

Extracellular vesicles (EVs) are micron-sized fragments of the cell membrane that perform as an effective intercellular signalling mechanism due to their capacity to carry different biological molecules such as proteins [18], nucleic acids [19] and lipids [20]. Besides their function as messengers, EVs (particularly medium/large EVs, also known as microvesicles or cell-derived microparticles) are involved in the coagulation process. In this regard, while the outer surface of EVs brings anionic phospholipids that enable the formation of coagulation complexes [21], some EVs also carry procoagulant proteins such as tissue factor, thus increasing their coagulation potential [22].

A high number of plasma medium/large EVs from monocytes, platelets and endothelial cells has been described in aPL-positive patients (especially individuals with LA) [23–25]. Indeed, based on the procoagulant properties of medium/large EVs (consistently reported in other pathological conditions such as venous thromboembolism [26], cancer [22], and other autoimmune disorders [27]), it can be hypothesised that these structures could constitute a direct procoagulant mechanism in APS. Moreover, aPL are known to induce the expression of a procoagulant phenotype in endothelial cells [14,16], which may be reflected in the cargo of their daughter EVs [22].

Despite the above considerations, research showing clotting-related features of EVs derived from plasma of aPL carriers has failed to find an increased procoagulant activity [23,27,28]. This fact suggests that other variables such as the conditions of endothelial activation/dysfunction, or the interaction between EVs and autoantibodies, are required to explain the influence of EVs on clotting in the context of APS.

In this work, we used an *in vitro* model to directly assess how the interaction between recalcified plasma, endothelial cell-derived medium/large EVs released upon stimulation with aPL (aPL-EDEVs), and the same autoantibodies that induce and condition the release of aPL-EDEVs, modifies clot activation.

2. Material and methods

2.1. Patients

Serum samples of fifty-one patients, previously recruited from the Recurrent Miscarriage Program of Universidad de Antioquia and the Anticoagulation Clinic of Hospital San Vicente Fundación, Medellín, Colombia, were employed for this work. Execution of this project follows the principles stated in the Helsinki Declaration of Ethics as reviewed and approved by the Bioethics Committees of both institutions, which certify to this in the official letters 006-2018 and 35-2018 respectively. Informed consent was taken in written form in all cases.

All APS patients were assessed for meeting the updated Sapporo classification criteria [4]. First, a commercial ELISA (Biosystem, Barcelona, Spain) was used to identify criteria aPL (anti-β2 glycoprotein I and anti-cardiolipin antibodies). In addition, some non-criteria aPL were screened through an in-house ELISA based on the technique published by Kwak et al. (anti-phosphatidylserine, anti-phosphatidylinositol, anti-phosphatidylethanolamine, and anti-phosphatidylglycerol antibodies) [29,30]. Regarding LA, a preliminary test based on partial thromboplastin time (aPTT-SP) was used to meet the recommendations of the Clinical and Laboratory Standards Institute [8], then we performed screening and confirmatory tests based on dilute Russell's viper venom time (dRVVT Screen – dRVVT Confirm, HemosIL, Bedford MA, USA).

According to laboratory results and clinical manifestations, patients and controls were arranged into different groups as described in Table 1. Individuals with secondary APS were diagnosed when APS patients exhibited an additional autoimmune disorder (systemic lupus erythematosus). APS patients who manifest a new episode of pregnancy-related morbidity despite optimal pharmacological treatment with heparin and aspirin were classified as refractory.

2.2. Purification of immunoglobulin G

Serum samples from patients with the same clinical features were pooled. Then, the immunoglobulin G (IgG) fraction from each pool was purified through affinity chromatography based on protein G-Sepharose (MAbTrap kit, GE Healthcare, Uppsala, Sweden). IgG samples were tested for endotoxin contamination using a Limulus Amebocyte Lysate (LAL)-based assay (Pierce LAL chromogenic endotoxin quantitation kit, Thermo Scientific, Rockford IL, USA). For the samples that required it, endotoxin removal was carried out employing polymyxin B columns (Detoxi-Gel Endotoxin Removing Gel, Thermo Scientific, Rockford IL, USA). The absence of endotoxins above 0.06 EU/mL was subsequently confirmed. Electrophoresis of proteins, and protein quantification, were also performed, as well as a new ELISA tests in order to confirm or reject the presence of aPL in pooled IgG samples (Aeskulisa Phospholipid-8Pro-GM, Aesku diagnostics, Wendelsheim, Germany; Anti-cardiolipin antibodies ACA-IgG, Byosistems, Barcelola, Spain; IMTEC-β2-Glycoprotein I – Antibodies IgG, Magdeburg, Germany).

2.3. Endothelial cell cultures

Human umbilical vein endothelial cells (HUVECs) were isolated from umbilical cords of healthy donors whose normal vaginal deliveries took place in local obstetric services at Hospital San Vicente Fundación and Unidad Hospitalaria Manrique, E.S.E. Metrosalud, Medellín, Colombia. Each woman signed a form of informed consent, previously approved by the Ethical Committees of the same Hospitals.

A standardised protocol [31] based on the technique published by Jaffe et al. [32] was used to isolate endothelial cells by mechanical and enzymatic methods. HUVECs were cultured in surface-treated culture flasks (Nunc EasYFlask, Thermo Scientific, Rockford IL, USA) with endothelial cell growth medium (Growth Medium kit, PromoCell, Heidelberg, Germany) supplemented with 2% fetal bovine serum (same kit) at 37 °C and under a 5% CO_2 atmosphere. A solution with 5 mg/mL gentamicin (Genfar/Sanofi, Gentilly, France), 1000 IU/mL penicillin G (Sigma-Aldrich, St. Louis MO, USA), and 25 μg/mL amphotericin B (Fungizone, Gibco, Grand Island NY, USA), at a final concentration of 1% v/v, was added to the medium. Between culture passages 1 to 3, 7×10^4 HUVECs per well were seeded in 24-well microplates (Corning, Corning NY, USA) to perform the corresponding stimuli. In this regard, 24 h after seeding, once a 100% confluence was reached, 250 μg/mL IgG

Table 1
Patient and control groups.

APS groups		
Obstetric APS	PM	n = 10
Vascular APS (primary)	VTI	n = 6
Vascular APS (secondary)	VTII	n = 4
Vascular and obstetric APS (primary and refractory)	RI	n = 3
Vascular and obstetric APS (secondary and refractory)	RII	n = 2
Vascular and obstetric APS (Non-refractory)	NR	n = 6
Control groups		
aPL-non-related pregnancy morbidity	PM/aPL–	n = 10
aPL-non-related primary vascular thrombosis	VTI/aPL–	n = 5
aPL-non-related secondary vascular thrombosis	VTII/aPL–	n = 5
Healthy women with proven pregnancy success	NHS	n = 10

or 8 μg/mL lipopolysaccharide (as a positive control) (LPS from *Escherichia coli* O111:B4, Sigma-Aldrich, St. Louis MO, USA) were added to cell cultures. All stimuli were performed at 37 °C and in 250 μL of Opti-MEM without any serum supplementation (Gibco, Grand Island NY, USA). Opti-mem was previously filtered to avoid the presence of foreign medium/large EVs employing 0.2 μm-pore sized filters (Advantec, Tokyo, Japan). In selected experiments, IgG stimuli were performed in the presence of 5 μg/mL of commercial purified human β2GPI (LAPL-GP1-100L, Louisville APL Diagnostics, Texas city TX, USA).

In some final verifications, along with IgG stimulus, we perform a 1-h pretreatment plus a concomitant treatment of HUVECs with selective inhibitors of MAPK pathways or pharmacological agents commonly used to treat APS. Thus, we used 20 μM SB203580 (selective inhibitor of p38MAPK, Sigma-Aldrich, St. Louis MO, USA) and 10 μM U0126 (selective inhibitor of MEK1/2, Abcam, Cambridge, UK), both dissolved in dimethyl sulfoxide (DMSO, Sigma-Aldrich, St. Louis MO, USA), 1 μg/mL hydroxychloroquine (Sigma-Aldrich, St. Louis MO, USA), 50 IU/mL enoxaparin (Clenox, Pharmayect S.A., Bogotá, Colombia), and 10 mM acetylsalicylic acid (Sigma-Aldrich, St. Louis MO, USA).

2.4. Enrichment of endothelial cell-derived extracellular vesicles from supernatants

Following a protocol previously published by Pericleous et al. [17], endothelial cell-derived medium/large EVs (EDEVs) were enriched from 200 μL of supernatant from each well of treated HUVECs. Normalisation criteria were cell counts and a constant volume for the stimuli and the enrichment process. Debris and dead cells of supernatants were excluded in a pellet after washing with 200 μL of fresh and filtered Opti-MEM without any serum supplementation (3000×*g* for 5 min). Subsequently, EDEVs were spun down in a 25 μL pellet (15,000×*g* for 60 min). In selected experiments, EDEV-poor supernatants were used as a negative control.

2.5. Flow cytometry of endothelial extracellular vesicles

Once EDEVs were spun down in a 25 μL pellet, each sample was resuspended and incubated at room temperature for 1 h with 300 μL of a mixture with FITC-conjugated anti-CD31 antibody (1:100 dilution, Life Technologies, Carlsbad CA, USA) and APC-conjugated Annexin V (1:100 dilution, BD Pharmingen, San Jose CA, USA), both diluted in 1X annexin V binding buffer (BD Pharmingen, San Jose CA, USA) previously filtered by employing 0.2 μm pore-sized filters (Advantec, Tokyo, Japan). In some cases, EDEVs were also stained with PE-conjugated anti-CD106 (1:100 dilution, R&D systems, Minneapolis MN, USA) and AF700-conjugated anti-CD62E/P (1:100 dilution, SouthernBiotech, Birmingham AL, USA). Then, EDEVs were analysed using flow cytometry (LSRFortessa, BD, Franklin Lakes NJ, USA).

The threshold was set according to the electronic noise recorded in an ultrafiltered sheath fluid tube. Commercial polystyrene microspheres of known size between 0.5 and 2 μm (Flow Cytometry Sub-Micron Particle Size Reference Kit, Invitrogen, Waltham MA, USA) were used to test the FSC resolution and set gates for rough sizing EDEVs. Fluorescence-minus-one (FMO), unstained and single stained controls were used to correct fluorescence spill-over and set gates. The cytometer acquired all samples at a medium flow rate, and events were recorded consistently for 120 s. Flow cytometry files were managed using FlowJo (BD, Ashland OR, USA) 10.7.1 version.

Considering that other authors have found that when aPL are present, phosphatidylserine (PS) expression by medium/large EVs is abnormally low, leading to underestimating the vesicle quantity [25,33,34], we operatively define as EDEVs those events with a size between 0.5 and 2 μm positive for CD31, PS, or both markers simultaneously. In some assays, we optimised the number of detected EDEVs by including particles positive for at least one of both CD106 and CD62E/P markers. In terms of size, authors have proposed that medium/large EVs can range in diameter from 0.1 to 1 μm [17] or 0.1–2 μm [35]. Nevertheless, particles smaller than 0.5 μm were confused with electronic noise in our experimental setting. Early standardisation tests (data not shown) showed that even under basal conditions, endothelial cells release particles close to 2 μm, so we included EVs of this size in our analysis.

2.6. Recalcified plasma-based assay

In order to assess the coagulation activity of EDEVs released upon IgG stimulus, a recalcified plasma-based assay adapted from the protocols published by Combes et al. [36] and Antova et al. [37] was performed. The basis of this test is to incubate each sample of EDEVs with a given volume of decalcified normal human plasma. Controlled recalcification of the plasma/EDEV mixture induces activation of coagulation pathways, which is indirectly recorded in time by the absorbance of light (optical clot detection). The coagulation properties of EDEVs influence features of kinetic records. A graphical overview of the protocol for this assay can be found in the supplementary material (Fig. S1).

For this set of experiments, samples from 20 healthy donors (males and females) were used to make a pool of normal human plasma. Blood was collected in tubes with 3.2% sodium citrate. Within 2 h of sample collection, platelet-poor plasma was extracted by double centrifugation (2000×*g* for 15 min, and then 2500×*g* for 15 min). Prothrombin time and integrated LA detection through screening and confirmatory tests of dilute Russell's viper venom time (dRVVT) were assessed on each volunteer. Gaussian distribution of coagulation times and normal ranges were calculated using mean ± 2 SD. Healthy volunteers with coagulation times out of the calculated range were considered outliers, and their samples were excluded from the pool of normal human plasma. The resulting normal pooled plasma was aliquoted, stored at −70 °C, and used within six months. Immediately before use, plasma was thawed in a water bath by a complete submersion (37 °C for 5 min). Then, native medium/large EVs were removed from the plasma by centrifugation (15,000×*g* for 60 min).

To evaluate the coagulation properties of EDEVs, after sample centrifugation, a 25 μL pellet containing the EDEV-rich supernatant of each well was diluted in 50 μL of medium/large EV-depleted normal pooled plasma. This mixture was incubated for 5 min in a water bath at 37 °C, and with intermittent stirring at 300 rpm. All samples were then seeded into 96-well microplates and simultaneously recalcified with 75 μL of 0.025 M $CaCl_2$. Records of absorbance at $\lambda = 405$ nm were taken every 3 min during 5 h employing a microplate spectrophotometer (Multiskan FC, Thermo Scientific, Ratastie, Finland). In selected experiments, instead of using samples with EDEVs and $CaCl_2$, clotting was triggered by adding commercial total-thromboplastin as a positive control (PT-Fibrinogen HS PLUS, HemosIL, Bedford MA, USA) or diluted Russell's viper venom to detect LA autoantibodies (dRVVT Screen – dRVVT Confirm, HemosIL, Bedford MA, USA).

In order to assess the direct effect of IgG on EDEVs, a modified version of the above protocol was carried out. In this case, 2.1×10^5 HUVECs were seeded, equally divided into three wells of a 24-well microplate, and stimulated with 8 μg/mL LPS for 2 h. The resulting LPS-derived supernatants were collected, pooled and used in normalised amounts (volume) for subsequent assays. 200 μL of pooled supernatant from LPS-stimulated HUVECs were incubated at 37 °C in the presence of IgG from NHS, IgG from RI patients, or pre-filtered medium without any serum supplementation as control (volume was adjusted to maintain the IgG concentration at 250 μg/mL and mimic conditions of previous assays). After 2 h, EDEVs were washed, spun down and tested in the recalcified plasma-based assay.

2.7. Analysis of coagulation curves

Kinetic records of absorbance from the recalcified plasma-based assay constitute coagulation curves with a sigmoidal shape. Briefly,

from the time of recalcification, there is a time-lapse of no increase in plasma absorbance, extending to the clot activation onset (lag time). This lag time represents the facility (if shortened) or difficulty (if extended) with which plasma initiates clotting. According to the analysis proposed by Shustova et al. [38], we define the clot onset as the point in time at which 5% of the total change in the absorbance (ΔA405) is reached. In summary,

$$A_c = A_0 + (\Delta A405 * 0.05)$$

Where A_C is the absorbance at the clot onset, A_0 is the absorbance at the record starting point, and ΔA405 is the total change in the absorbance.

To find the time point at which absorbance equals A_C in each coagulation curve (lag time), we fit the five-parameter (5 PL) nonlinear regression model (based on Richard's equation [39]) to each data set, thus getting an equation of absorbance as a function of time. Then, we interpolate the previously calculated value of *Ac* in the inverse function, i.e., the equation of time as a function of absorbance (Fig. S2). To complete the analysis of coagulation curves, we also calculate the overall coagulation potential as the area under the curve (absorbance vs time), as reported previously by Zong et al. [40].

Table 2
General features of study groups.

Groups	APS patients						Controls			
	PM	VTI	VTII	RI	RII	NR	PM/ aPL−	VTI/ aPL−	VTII/ aPL−	NHS
Age (mean of years +/- SD)	32.5 +/- 4.6	30 +/- 11,21	35,25 +/- 11,95	34,33 +/- 6,81	46,5 +/- 3,54	38,83 +/- 7,22	30.9 +/- 5.5	38,8 +/- 15,61	42,8 +/- 12,09	35,5 +/- 5,46
Clinical features										
Pregnancy loss (mean and rank)										
≤ 10 weeks of pregnancy	1.2 (1-2)	0	0	2,33 (2-5)	1 (0-2)	1,2 (1-5)	1.9 (1-3)	0	0	0
> 10 weeks of pregnancy	0.9 (0-4)	0	0	2,33 (1-5)	3 (1-5)	0,6 (0-2)	0.7 (0-3)	0	0	0
Other pregnancy-related events										
Preeclampsia <34 weeks (n)	0	0	0	3	1	2	0	0	0	0
Intrauterine growth restriction (n)	0	0	0	2	0	1	0	1	0	0
Vascular thrombosis-related events										
Thrombosis (n)	0	6	4	3	2	6	0	5	5	0
DVT (n)	0	3	4	3	2	5	0	3	2	0
PTE (n)	0	3	2	1	0	1	0	1	5	0
AT (n)	0	2	1	0	0	0	0	2	0	0
Other autoimmune diseases										
SLE (n)	0	0	4	0	2	0	0	0	4	0
RA (n)	0	0	1	0	0	0	0	0	1	0
Antiphospholipid antibody profile										
Normalised ratio of dRVVT (mean +/- SD)	1.13 +/- 0.13	2,62* +/- 0,16	2,26* +/- 0,6	3,3* +/- 0,24	1,34* +/- 0,24	1,92* +/- 0,27	1.13 +/- 0.01	1,13 +/- 0,01	1,10 +/- 0,02	1,06 +/- 0,05
aβ2GPI †	0.72	2.82*	1.20*	2.93*	0.80	3.01*	0.67	0.46	0.41	0.49
aCL + β2GPI †	1.26*	3.71*	2.36*	6.06*	2.41*	5.25*	0.87	0.88	0.87	0.78
aCL †	0.95	1.57*	1.28*	1.46*	0.62	1.48*	1.09	1.07	0.99	1.08
aPI †	0.79	1.29*	1.19	1.00	0.48	0.95	0.86	0.66	0.65	0.77
aPS †	0.66	1.01	0.77	0.78	0.42	0.84	0.77	0.59	0.60	0.66
Quantitative values										
aβ2GPI #	3.0	40.3	13.3	120.1	10.4	85.3	0	2.0	0.4	0
aCL #	0.6	83.6	26.6	190.5	93.5	142.7	2.0	4.6	15.3	0
aβ2GPI ¥	0	80.37	5.37	133.2	24	118.8	0	0	0	6.7
aCL ¥	2.3	47.5	10	70.3	21.0	114.7	2.3	3.8	1.9	2.3

(n) Number of patients in each group with the corresponding clinical feature. † Semi-quantitative results from the evaluation of different antiphospholipid antibodies (all IgG isotype) obtained by testing the purified IgG samples at a final concentration of 250 μg/mL and using a commercial ELISA kit (details in the supplementary material). Quantitative values for aβ2GPI and aCL antibodies are also reported in U/mL and GPL respectively, both for purified IgG samples (#) and patient serum (¥). For all cases, red asterisks highlight positive values. The presence of aPC, aPE and aSM antibodies was also assessed with no positive results in any case. DVT, deep venous thrombosis; PTE, pulmonary thrombo-embolism; AT, arterial thrombosis; SLE, systemic lupus erythematosus; RA, rheumatoid arthritis; aβ2GPI, anti-β2 glycoprotein I antibodies; aCL, anti-cardiolipin antibodies; aPI, anti-phosphatidylinositol antibodies; aPC, anti-phosphatidylcholine antibodies; aPE, anti-phosphatidylethanolamine antibodies; anti-phosphatidylserine antibodies; aSM, anti-sphingomyelin antibodies; dRVVT, dilute Russell's viper venom time. Refer to Table 1 for abbreviations of all patient and control groups.

2.8. Statistical analysis

Statistical analyses were performed on Prism 6.0.0 (GraphPad Software, San Diego CA, USA). Normality assumption was tested using the Shapiro Wilk hypothesis test for all data sets. According to these results, comparisons between groups were carried out using the repeated-measures ANOVA test or Friedman's nonparametric test. Paired t-tests were used to perform some final analyses (as is indicated in the results, section 3). In all cases, differences were considered statistically significant when the p-value was less than 0.05. The nonlinear regression curve fit of the data from coagulation assays was also carried out by using Prism; then, the inverse function of the five-parameter nonlinear regression model was obtained using Mathematica 12.0.0 (Wolfram Research, Champaign IL, USA).

3. Results

3.1. Features of the control and patient groups

Table 2 summarises the main clinical features of each study group and the antiphospholipid antibody profile of each resulting pooled IgG sample. Pregnancy loss, preeclampsia and intrauterine growth restriction were recorded in patients with pregnancy-related morbidity. Both venous and arterial thrombosis were identified in vascular APS groups. All pooled IgG samples from patients with APS were positive for the presence of at least one of the criteria aPL. Conversely, none of the control pools was positive for the presence of any tested antibody (neither criteria nor non-criteria).

Some preliminary verifications on the IgG samples are shown in the supplementary material (electrophoresis of proteins and assessment of endotoxin levels) (Fig. S3). Some coagulation tests, i.e., prothrombin time and dRVVT, were performed to assess the extrinsic pathway-related coagulation properties and the presence or absence of LA antibodies in the normal pooled plasma used as the substrate of the recalcified plasma-based assay. The results are also shown in the supplementary material (Fig. S4).

3.2. Coagulation activity of EDEV-rich supernatants

The capacity of aPL to modify clotting via inducing the release of

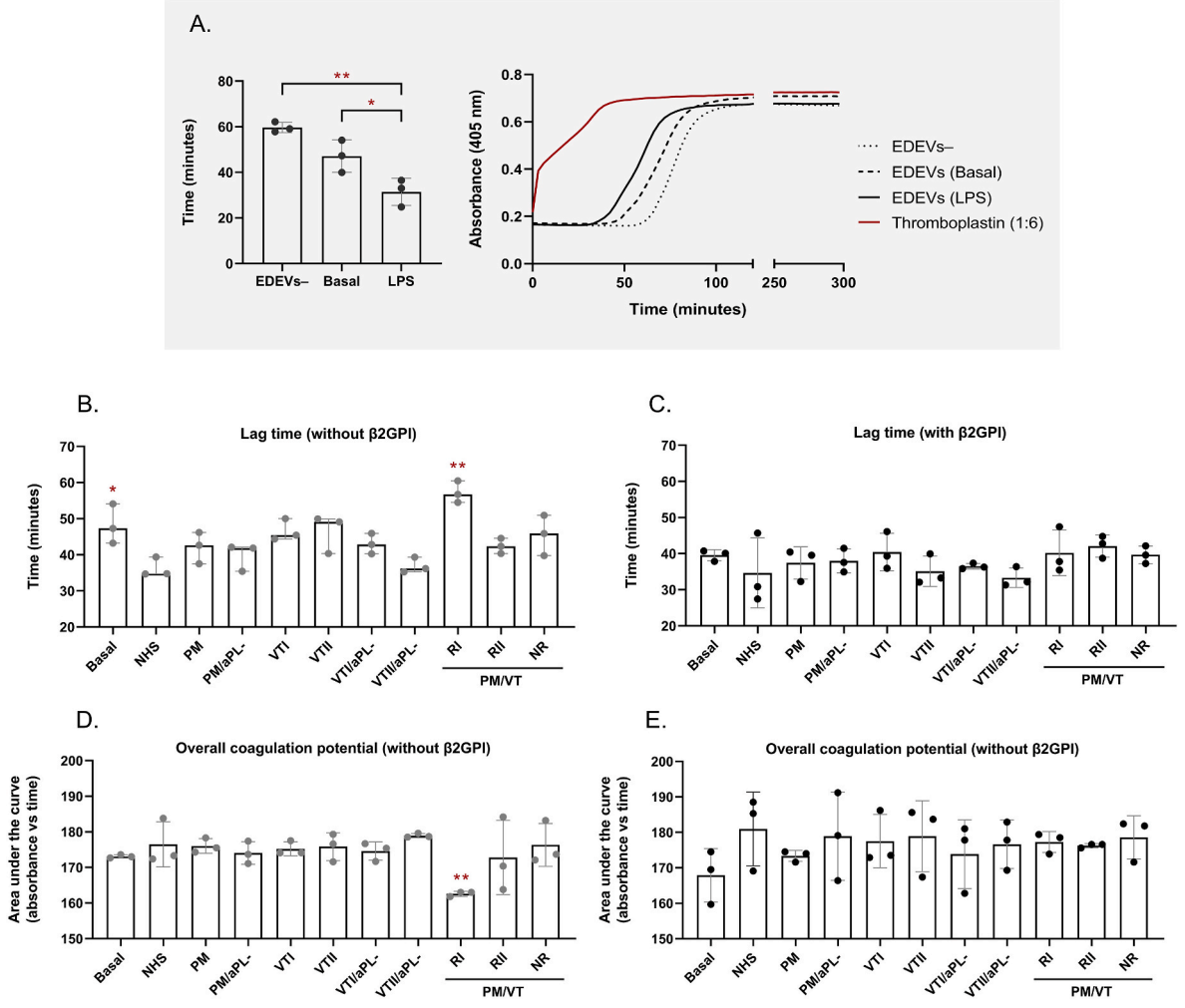

Fig. 1. EDEV-rich supernatant from endothelial cells stimulated with IgG from RI patients exhibits a dampened coagulation activity. This anticoagulant effect is abrogated by using β2GPI as a stimulus cofactor. A) Positive and negative controls of the recalcified plasma-based assay; Three independent replicates (left) and representative records of the coagulation curves (right). B) Lag time and D) overall coagulation potential of normal plasma, recalcified in the presence of EDEVs released by stimulation with IgG of different groups (Friedman test and repeated measures ANOVA test, respectively). C) Lag time and E) overall coagulation potential of normal plasma, recalcified in the presence of EDEVs released by stimulation with IgG of different groups and β2GPI as a cofactor (both, repeated measures ANOVA test). (For all cases differences are shown in comparison to the NHS control group, n = 3, *$p < 0.05$, **$p < 0.01$). EDEVs–, medium/large endothelial cell-derived extracellular vesicle-poor supernatant; LPS, lipopolysaccharide; EDEVs, endothelial cell-derived medium/large extracellular vesicles; β2GPI, β2 glycoprotein I. Refer to Table 1 for abbreviations of all patient and control groups.

EDEVs could be related to several factors, e.g., changes in the number of released vesicles, modifications in the anticoagulant or procoagulant cargos, or even a possible interaction between EDEVs and aPL. Therefore, we first considered evaluating the coagulation activity of the EDEV-rich fraction of supernatants (without any modifications) as an overall measure of EDEV-related coagulation properties. Using this fraction of the supernatant during standardisation tests, we found that it is possible to identify differences between the EDEV-related coagulation properties of IgG samples by employing the recalcified plasma-based assay protocol after 2-h stimuli with IgG (Fig. S5).

While performing the coagulation assay with IgG pools, we found a delay in the clot onset when plasma is recalcified in the presence of EDEVs isolated from HUVECs pretreated with IgG from patients with vascular and obstetric primary APS who manifest refractoriness to treatment (RI). According to our standardised protocol (Fig. 1A), this delay was statistically significant compared to the time exhibited by plasma recalcified with EDEVs released on the presence of IgG from normal human serum (NHS) (NHS = 34.77 min ± 4.73, RI = 56.74 min ± 5.92, $p < 0.01$). Furthermore, the late onset of clot activation was reflected in a lower overall coagulation potential (NHS = 176.5 ± 6.34, RI = 162.6 ± 0.6928, $p < 0.01$) (Fig. 1B and D).

In contrast to the previous results, assessing the coagulation activity of EDEVs released using β2GPI as a cofactor of the stimuli with IgG, no statistically significant difference was detected between groups (Fig. 1C and E). To further explore how co-stimulation of HUVECs with β2GPI modifies the coagulation activity of released EDEVs, we perform a multiple paired *t*-test analysis. This analysis compares the coagulation properties related to each study group in the presence and absence of β2GPI. We found that β2GPI has no significant effect over the EDEV-related coagulation activity of any IgG sample, except for the RI group. In this last group, β2GPI recovers the overall coagulation potential, returning this variable to baseline values, as the representative coagulation curves show (Fig. 2) (RI = 162.6 ± 0.6928, RI + β2GPI = 177.3 ± 2.950, $p < 0.05$).

3.3. Flow cytometry analysis of EDEVs

Results obtained in previous assays could be explained by differences in the quantities or contents of EDEVs. Therefore, it was feasible that the dampened EDEV-related coagulation activity of the IgG from RI patients would be due to a reduced capability of these antibodies to induce the release of EDEVs. To test this hypothesis, we analysed the samples of EDEV-rich supernatants by flow cytometry. We did not find any statistically significant difference between EDEV counts, neither in the presence nor absence of β2GPI ($p = 0.42$ and $p = 0.5$, respectively) (Fig. 3A to E). Another issue to be explored was the possible differences in the distribution of the estimated sizes of the extracellular vesicles in the enriched supernatants. In particular, we had to exclude an increase in extracellular vesicles with sizes between 1 and 2 μm (which have classically been understood as apoptotic bodies [41]) when using β2GPI. We found that in all cases the largest proportion of vesicles were approximately 0.5 μm in size (72% ± 3.3). This size has usually been associated with microparticles [35]. The relative frequency of 2 μm vesicles did not increase with any of the stimuli (Fig. S6). Despite the above, 2-way ANOVA analysis showed that β2GPI is an independent source of variation that increases in all cases the total count of EDEVs that are released from the endothelium ($p < 0.01$) (Fig. 3F). Strikingly, the increase in the number of EDEVs attributable to β2GPI did not result in an equivalent increase in the coagulation activity of the EDEV-rich supernatants, as shown by the correlation analysis ($R^2 = 0.002$, $p = 0.92$) (Fig. 3G).

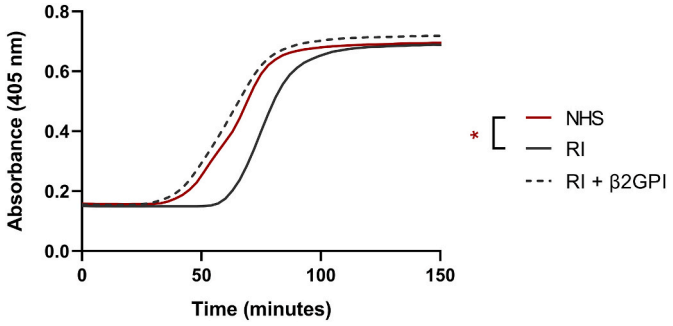

Fig. 2. Representative coagulation curves of the recalcified plasma-based assay exemplifying how β2GPI used as a cofactor during HUVEC stimulation with RI IgG restores de coagulation activity of EDEVs. Compared to the effect of IgG from NHS, the EDEV-rich supernatant from endothelial cells stimulated with RI IgG exhibits a lengthened lag time (rightward displacement of the coagulation curve). However, by using β2GPI as an IgG cofactor during the stimulation of endothelial cells, the coagulation activity related to EDEVs is reestablished. EDEVs, endothelial cell-derived medium/large extracellular vesicles; NHS, normal human serum; RI, Vascular and obstetric APS (primary and refractory).

3.4. The direct effect of aPL on the coagulation activity of EDEVs

Since it is unlikely that EDEV count could explain the dampened coagulation activity of the EDEV-rich supernatant from HUVECs pretreated with RI IgG, we tested the hypothesis that aPL from this group could exert an anticoagulant influence over EDEVs by direct interaction. Then, we replicated the recalcified plasma-based assay with some modifications. Briefly, EDEVs resulting from the stimulation of HUVECs with LPS (LPS-EDEVs) were incubated with IgG from NHS, IgG from RI patients, or filtered medium without any serum supplementation as control.

If the anticoagulant effect of RI IgG were exerted directly over EDEVs, results obtained in initial assays should be reproducible with this modified protocol. As it was approached, LPS-EDEVs incubated with RI IgG exhibited a dampened coagulation activity compared with LPS-EDEVs incubated with NHS IgG (NHS = 34.73 ± 17.66, RI = 47.18 ± 19.42, $p < 0.05$) (Fig. 4A and B). To assess whether the direct anticoagulant effect of RI IgG on EDEVs could be influenced by the transfer from supernatants to plasma of non-EDEV-associated antibodies, we performed a second modified version of the coagulation assay while maintaining the same proportions of plasma, medium and clot activator volumes. Then, we used purified IgG, EDEV-poor supernatants and Russell's viper venom as a clot activator. Results show that LA antibodies are often detectable in plasma diluted in a medium with an initial IgG concentration equal to that of the stimuli (see methods, section 2.3) (NR = 1.25, 1.16, 0.80). However, when plasma is diluted in EDEV-poor supernatant from previous assays (after washing carried out during the isolation of EDEVs), LA antibodies are undetectable (NR = 0.85, 1.10, 1.10) (Fig. 4C and D).

3.5. Relation between LA, the attenuated coagulation activity of EDEVs, and the β2GPI-dependent procoagulant effect

Our results indicate that aPL can exhaust the coagulation activity of EDEVs by direct means. Therefore, we attempted to explore whether our results in the absence of β2GPI could be related to the LA-like activity of autoantibodies. We found a positive and statistically significant correlation between the lag time values of EDEVs and the mean normalised ratio values of dRVVT of patients ($R^2 = 0.76$, $p < 0.01$) (Fig. 5A). In contrast, when we compared the lag time of EDEVs released upon the stimulation with aPL in the presence of β2GPI and the mean normalised ratio values of dRVVT, there was no statistically significant correlation, thus suggesting other factors along with LA must be conditioning the clot activation ($R^2 = 0.2$, $p = 0.2$) (Fig. 5B).

We then calculated the difference in the lag time of EDEVs released in the absence and presence of β2GPI as a measure of the capacity of this cofactor to enhance clotting by shortening the lag time (Fig. 5C). We found that this measure increases proportionally along with the LA-like

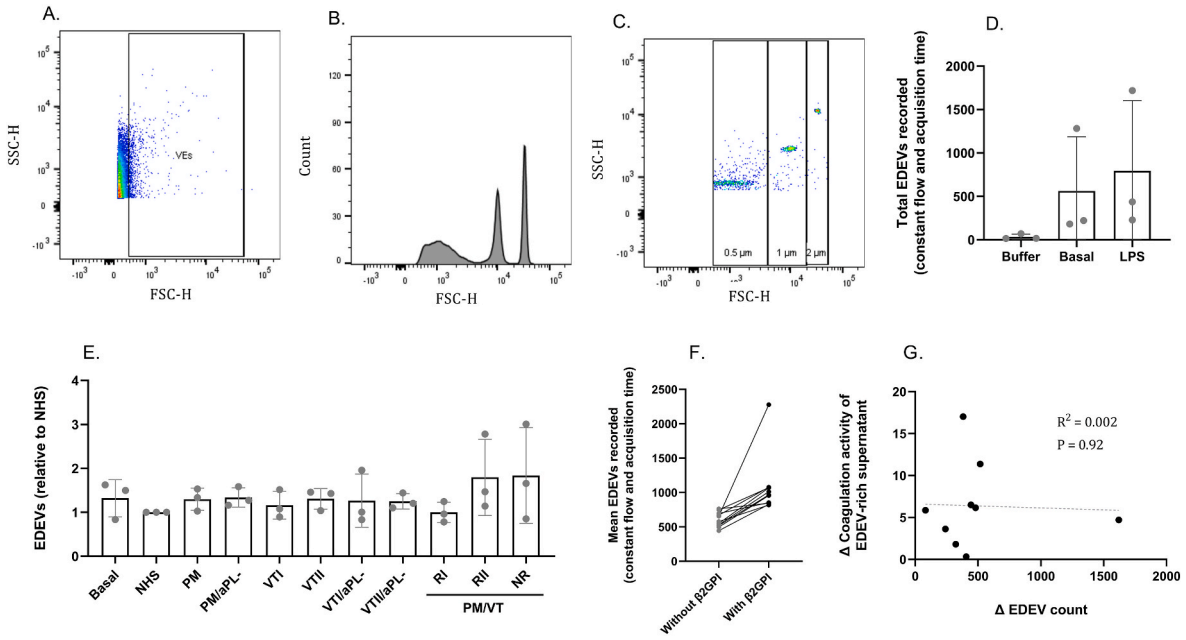

Fig. 3. The dampened coagulation activity exhibited by the EDEV-rich supernatant from RI IgG-stimulated HUVECs cannot be explained by a decreased amount of EDEVs. Instead, the restoring action of β2GPI may be associated with an increase in the amount of EDEVs that are released. A) Delimitation of the electronic noise in the flow cytometer. B) Testing the resolution to differentiate 0.5, 1, and 2 μm-sized particles using FSC. C) Setting the gates for sizing using commercial beads. D) Controls of flow cytometry assay. Double negative events (for PS and CD31) were excluded from the analysis, as described in the methods (section 2.5). E) Relative number of EDEVs released after stimulation with IgG in the absence of β2GPI (n = 3, repeated measures ANOVA test, no statistically significant differences were found). F) For each IgG sample, the count of EDEVs released on account of *in vitro* stimulation with and without β2GPI as a cofactor. G) Correlation between the change in EDEV count and the change in coagulation activity (lag time) of the EDEV-rich supernatants, both attributable to the use of β2GPI as a cofactor during HUVEC stimulation (simple linear regression and Pearson's test). SSC, side scatter; FSC, forward scatter; LPS, lipopolysaccharide; EDEVs, endothelial cell-derived medium/large extracellular vesicles; PS, phosphatidylserine; β2GPI, β2 glycoprotein I. Refer to Table 1 for abbreviations of all patient and control groups.

activity directly exerted by autoantibodies over EDEVs in the absence of β2GPI ($R^2 = 0.77$, $P < 0.01$) (Fig. 5D), thus explaining that the anticoagulant effect is eclipsed, but not surpassed, in the presence of this cofactor.

It has been described that LA phenomenon could be driven by aβ2GPI antibodies. For this reason, we also explored the relationship between the amount of aβ2GPI antibodies in IgG samples and the ability of these antibodies to dampen the direct coagulation activity of EDEVs. These two variables showed a positive and statistically significant relationship ($R^2 = 0.62$, $P < 0.05$) (Fig. S7).

3.6. MAPK pathways and the β2GPI-dependent procoagulant effect

Given a possible role of β2GPI in mediating the aPL-dependent activation of proinflammatory and procoagulant signalling pathways, we explored the participation of mitogen-activated protein kinase kinases 1 and 2 (MEK1/2) and p38 mitogen-activated protein kinase (p38MAPK) in the β2GPI-dependent procoagulant activity of aPL-EDEVs. These protein kinases have previously been described as downstream mediators of effects of aPL on endothelial cells and monocytes, leading to the expression of adhesion molecules, cytokines, and procoagulant factors such as tissue factor [16,42].

Interestingly, using a selective inhibitor of MEK1/2, the β2GPI-dependent procoagulant effect, which hitherto constrained the direct anticoagulant effect of autoantibodies from RI patients, was suspended. Thus, a prolongation on the lag time, similar to that observed in the absence of β2GPI, was again apparent, suggesting that the MEK1/2 pathway mediates the β2GPI-dependent procoagulant effect (NHS = 24.03 ± 23.16, RI + β2GPI + U0126 = 43.51 ± 20.07, $p < 0.05$) (Fig. 6). In contrast, SB203580 (a selective inhibitor of p38MAPK) at a final concentration able to abrogate the enhancement of EDEV production upon LPS stimulation (Fig. S8) showed no effect over the procoagulant action of β2GPI.

Previously (Fig. 3F) we described that β2GPI increases the number of released EDEVs. Here, we found that although the MEK1/2 inhibitor attenuates the procoagulant influence of β2GPI on aPL-EDEVs, it does not affect the number of vesicles detected by flow cytometry (Fig. 7). We also independently compared the number of EDEVs of each size and positive for CD106 or CD62E/P without finding statistically significant differences (data not shown).

Finally, we wanted to assess the effect of some pharmacological agents usually employed in the management of obstetric APS that could be useful in vascular APS given their capability to inhibit endothelial dysfunction/activation [14,43,44]. We found no significant effect using acetylsalicylic acid or hydroxychloroquine. The effect of low-molecular-weight heparin (enoxaparin) was also assessed, but no clot activation was detected after 5 h (data not shown). Acetylsalicylic acid increased the amount of released EDEVs in the presence of β2GPI and RI IgG (Fig. 7). However, this phenomenon was not mirrored in the coagulation activity of the corresponding EDEV-rich supernatant.

4. Discussion

We could summarise our findings in four core concepts: First, in the complex interaction between plasma, aPL-EDEVs, and the same autoantibodies that condition the release of these EDEVs, an anticoagulant effect prevails. Second, this anticoagulant effect can be described by some features; namely, it is not explained by a lower amount of EDEVs, it is exerted by direct interaction between aPL and EDEVs, and it has a positive and statistically significant correlation with the mean values of dRVVT. Third, β2GPI, a plasma protein that performs as the primary cofactor of aPL, restores the coagulation properties of EDEV-rich supernatants. It is noteworthy that the greater the anticoagulant effect initially exerted on the EDEVs, the greater the procoagulant effect of β2GPI that reestablishes their coagulation activity. Finally, the procoagulant effect of β2GPI is suspended by inhibiting the MEK1/2 pathway

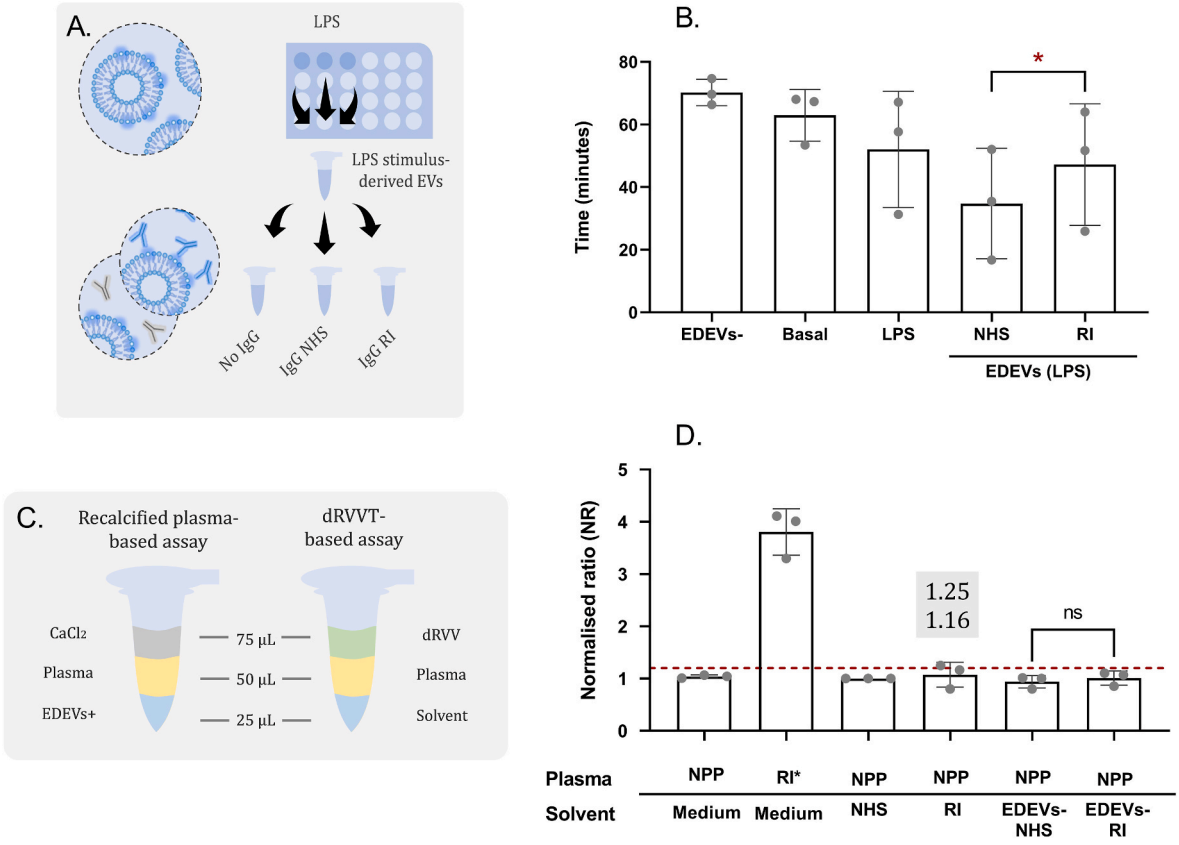

Fig. 4. IgG from patients of the RI group exerts a direct anticoagulant effect on EDEVs released on account of a proinflammatory stimulus. A) Graphical overview of the modified protocol employed to reproduce the recalcified plasma-based assay. Briefly, a pool of EDEVs released upon LPS stimulus was collected and then used in normalised quantities to assess the effect of IgG samples on their coagulation activity. B) Lag time of plasma recalcified in the presence of EDEVs released upon LPS stimulus previously incubated with IgG from NHS or IgG from RI patients (n = 3, paired *t*-test, *p < 0.05). Also shown is the lag time of vesicles released after stimulation with LPS that were not incubated with IgG (LPS) as well as vesicles released from endothelium in the absence of LPS (basal). C) Graphical overview of the modified protocol employed to assess the presence of LA antibodies in EDEV-poor supernatants. Briefly, plasma was diluted with fresh medium, medium with 250 µg/mL IgG (RI and NHS), or EDEV-poor supernatants (as a measure of the transfer from supernatant to plasma of EDEV-non-associated LA antibodies), then, dilute Russell's viper venom in the presence and absence of an excess of phospholipids was added to each plasma mixture. Clotting times were recorded. D) Normalised ratios of dRVVT for each plasma mixture. Plasma from one patient with vascular and obstetric APS who manifested refractoriness to treatment (RI*) was used as a positive control. The dashed line represents the cut-off value (1.2) (n = 3, paired *t*-test). LPS, lipopolysaccharide; NHS, normal human serum; RI, vascular and obstetric APS (primary and refractory); EDEVs–, medium/large endothelial cell-derived extracellular vesicle-poor supernatant; EDEVs, endothelial cell-derived medium/large extracellular vesicles; dRVVT, dilute Russell's viper venom time; dRVV, dilute Russell's viper venom; NPP, normal pooled plasma.

before and during stimulation of endothelial cells (which does not occur when inhibiting p38MAPK). However, this phenomenon does not occur by decreasing the amount of released EDEVs (see graphical abstract).

From these findings, two issues stand out as being relevant to discuss, i.e., do LA autoantibodies actually exert a direct anticoagulant effect on aPL-EDEVs? and how does β2GPI restore the coagulation activity of EDEV-rich supernatants?

Regarding the first question, previously, some authors have measured the coagulation activity of medium/large EVs from patients with aPL. In this context, it is contradictory that, even if the presence of aPL has been widely related to a higher quantity of plasma EVs [23,24], neither the tissue factor-like activity [28] nor the total quantity of PS available for the formation of prothrombinase complexes [23,27] exhibit an increase in comparison with seronegative healthy volunteers. That is to say, contrary to other non-aPL-related contexts [26], an increased quantity of medium/large EVs in the presence of aPL is not reflected in a proportional increase of the clotting capacities related to these structures. According to our findings, this apparent contradiction is due to the ability of aPL themselves to exhaust the procoagulant activity of medium/large EVs.

Remarkably, the features we attribute to this anticoagulant action of aPL over EDEVs led us to suggest that LA-like autoantibodies are responsible for this phenomenon. Although the exact mechanism by which some aPL perform as anticoagulant agents *in vitro* is unknown, two basic hypotheses have been well described. First, it was suggested that anti-β2 glycoprotein I (aβ2GPI) antibodies could be the main causatives of the LA phenomenon [2]. This approach arises from the observation that domain V of β2GPI exposes a PS-binding site that presumptively competes for the anionic surfaces with coagulation factors. Some aβ2GPI antibodies enhance this binding activity [45]. However, the aβ2GPI-based model of LA has been challenged by evidence showing that, in physiologically relevant calcium concentrations, the affinity of β2GPI to PS is negligible; so competition with other proteins such as prothrombin on PS-sites is not likely of interest [46]. aβ2GPI antibodies may still be involved in the LA phenomenon through other more recently postulated mechanisms such as direct interaction with the coagulation factor V (FV) [9]. In fact, correlation analysis of our data showed that the higher the amount of aβ2GPI antibodies in a purified IgG sample, the higher the direct anticoagulant activity exerted by these antibodies on EDEVs. The second type of aPL to which LA activity is attributed is the anti-prothrombin (aPT) antibodies. These autoantibodies form immunocomplexes with prothrombin, which compete with the coagulation factor X (FX) on PS-sites [9].

Regardless of the case, it is expected that the LA phenomenon can be

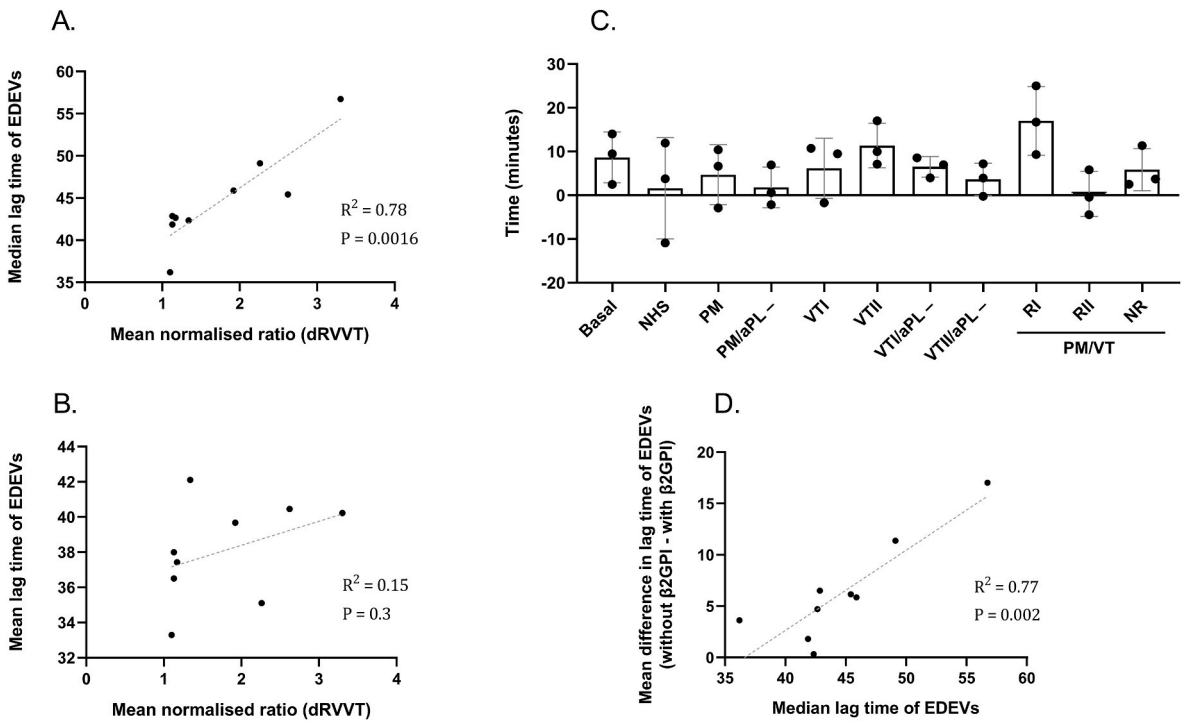

Fig. 5. The direct anticoagulant effect of aPL seems to correspond to the LA phenomenon and is proportional to the β2GPI-dependent procoagulant effect. For each control and patient group, the mean normalised ratio value of the dRVVT test was compared with A) the median lag time obtained by assessing the coagulation activity of EDEVs released in the absence of β2GPI and B) the mean lag time obtained by assessing the coagulation activity of EDEVs released in the presence of β2GPI. C) Capacity of β2GPI to shorten the lag time of EDEVs. Difference between the lag time values in the absence and presence of β2GPI. (n = 3, repeated measures ANOVA test). D) Correlation between the direct anticoagulant effect exerted by aPL (expressed as the median lag time obtained by assessing the coagulation activity of EDEVs released in the absence of β2GPI) and the procoagulant effect of β2GPI (expressed as the mean difference in the lag time with and without this cofactor) (A, B and D, simple linear regression and Pearson's test). LA, lupus anticoagulant; EDEVs, endothelial cell-derived medium/large extracellular vesicles; dRVVT, dilute Russell's viper venom time; β2GPI, β2 glycoprotein I.

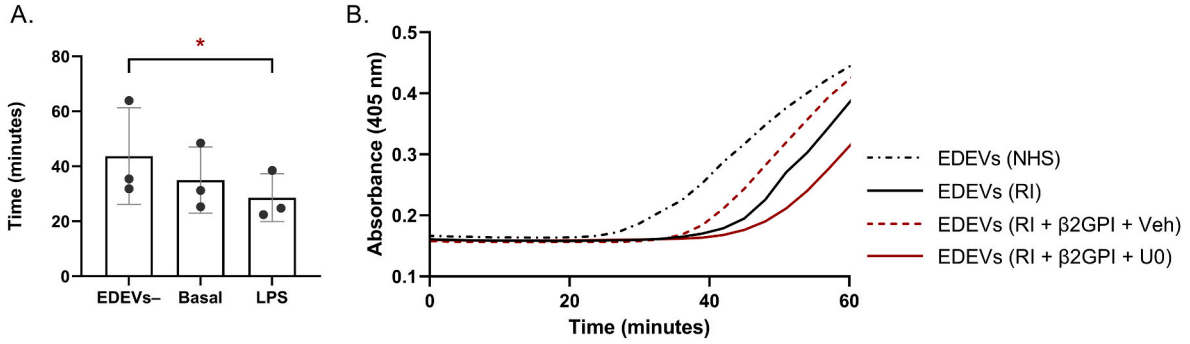

Fig. 6. Inhibiting the MEK1/2 pathway hinders the procoagulant action that β2GPI as an IgG cofactor exerts on the EDEV-rich supernatant. A) Positive and negative controls of the recalcified plasma-based assay. B) Representative coagulation curves (first hour zoom-in following recalcification). The selective inhibitor of MEK1/2 pathway U0126 induces a rightward displacement of the coagulation curve (increased lag time) in the presence of β2GPI. EDEVs, endothelial cell-derived medium/large extracellular vesicles; LPS, lipopolysaccharide; NHS, normal human serum; RI, vascular and obstetric APS (primary and refractory); EDEVs–, medium/large endothelial cell-derived extracellular vesicle-poor supernatant; β2GPI, β2 glycoprotein I; Veh, the vehicle of U0126 (dimethyl sulphoxide); U0, U0126. The final concentrations of each reagent are listed in the methods (section 2.3).

attributed to more than one kind of aPL with different anticoagulant mechanisms [47]. That is why, as the clinical data shows, there is no single absolutely sensitive method to detect LA [48], and it has been recommended to use more than one test to diagnose APS [8,49]. Hence, our findings would reflect just some LA-like autoantibodies [47]. In fact, it is even possible that the LA-like activity exerted over EDEVs in the absence of β2GPI and prothrombin could be explained by other types of aPL, or the transfer of non-detected non-vesicle-associated autoantibodies, which once in plasma would exert their anticoagulant influence.

Another critical issue to be discussed concerns the procoagulant action of β2GPI by which it reestablishes the coagulation activity of EDEV-rich supernatants. As the correlation analysis showed, the greater the capability of antibodies to dampen the coagulation activity of EDEVs by direct means in the absence of β2GPI, the greater the capability of β2GPI to restore the coagulation properties of vesicles by stimulating endothelial cells. This balance between procoagulant and anticoagulant effects explains why neither phenomenon prevails, and ultimately, there are no differences in the coagulation activity of the EDEVs. Both mechanisms, procoagulant and anticoagulant, were maximal for IgG of RI patients, the group with higher levels of LA as shown by the mean

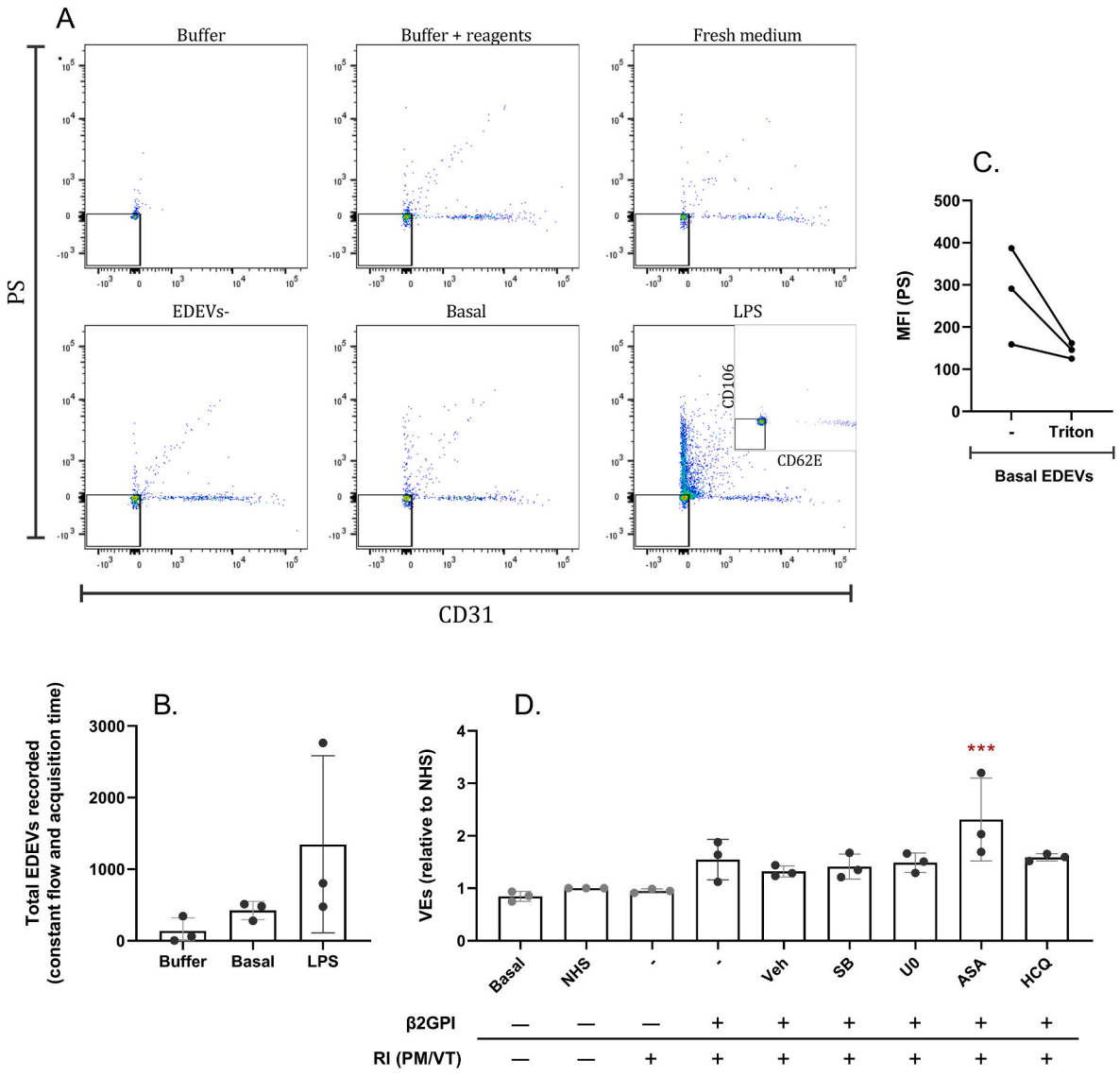

Fig. 7. Inhibiting the MEK1/2 pathway does not exert an anticoagulant effect over the coagulation activity of EDEV-rich fraction of supernatant by reducing the count of EDEVs. A, B) Representative dot plots and EDEV counts of controls in the flow cytometry assay. EDEV detection was optimised by using two additional markers (CD106 and CD62E/P). C) Effect of Triton x-100 at a final concentration of 0.075% in the mean fluorescence intensity related to phosphatidylserine expression. D) Relative number of EDEVs released after stimulation of HUVECs (repeated measures ANOVA test, n = 3, ***p < 0.01, differences are shown in comparison to the NHS control group). PS, phosphatidylserine; EDEVs, endothelial cell-derived medium/large extracellular vesicles; LPS, lipopolysaccharide; NHS, normal human serum; RI, vascular and obstetric APS (primary and refractory); EDEVs–, medium/large endothelial cell-derived extracellular vesicle-poor supernatant; β2GPI, β2 glycoprotein I; Veh, the vehicle of SB203580 and U0126 (dimethyl sulphoxide); SB, SB203580; U0, U0126, ASA, acetylsalicylic acid; HCQ, hydroxychloroquine. The final concentrations of each reagent are listed in the methods (section 2.3).

normalised ratio of dRVVT.

Indeed, the capability of β2GPI to proportionally restore the coagulation activity of EDEVs is highly suggestive of the presence of LA-like aPL, whose *in vitro* anticoagulant properties would be neutralised in the presence of an excess of anionic phospholipids. For this reason, β2GPI may actually increase the production of EDEVs to a point at which LA antibodies are undetectable. Our analysis suggests that β2GPI is an independent source of variation that increased the number of released EDEVs in all cases.

Despite the above, there are two findings suggesting that other mechanisms along with an increase in the number of EDEVs could be involved in the restorative effect of β2GPI over de EDEV-rich supernatants. First, we were able to prevent the β2GPI-dependent procoagulant action by inhibiting MEK1/2 pathway, which again dampened the coagulation activity of the EDEV-rich supernatants without any statistically significant change in the number of vesicles. Hence, it is also possible that β2GPI (which is a common cofactor of aPL [13,50]) could act via enabling an aPL-driven procoagulant and proinflammatory phenotype on endothelial cells that would well be reflected on their daughter EVs. In fact, it was previously shown that aPL down-regulate the expression of anticoagulant proteins and up-regulate the expression of procoagulant proteins in endothelial cells [14]. Our findings strongly support this hypothesis inasmuch as MEK1/2 pathway is a well-described pathway activated by aPL that in monocytes leads to the expression of tissue factor [42]. Furthermore, the sparse evidence addressing aPL-induced biogenesis of medium/large EVs has suggested that MEK1/2 may be involved in this process by activating the Rho-associated protein kinase (ROCK), leading to cytoskeletal remodelling and contraction [51]. Second, it should be noted that LPS stimulus, which successfully increases the number of released EDEVs as the β2GPI did, was not equally sufficient to exhaust the LA-like effect of IgG at the same final concentration. Therefore, β2GPI stimulus must be

increasing not only the number of released EDEVs, but also de coagulation activity of these structures. Evaluating tissue factor expression in aPL-EDEVs is a reasonable step to follow in order to explore the above possible explanation.

Although our approach is novel and allowed us to assess the overall effect resulting from the interaction between different factors related to EDEVs, aPL, β2GPI, and plasma, we found it critical to address each of these factors separately to consider their potential influence on coagulation *in vivo*. For example, it should not be ruled out that the coagulation activity of EDEVs could be increased by the action of other types of aPL with no direct anticoagulant influence over the vesicles. In this regard, during our standardisation assays, we could notice that, in the presence of β2GPI, the IgG from one patient with non-refractory vascular and obstetric APS induces a slight but consistent shortening in the time to reach the 50% clot activation (Fig. S5). Results with IgG pools show that this is not a common feature of a certain group of patients, yet we consider this a limitation in our work.

5. Conclusions

In conclusion, our results show that stimulation of endothelial cells with aPL in the presence of β2GPI (as would occur *in vivo*) is not sufficient to exceed, although it does eclipse, the LA-like effect that the same autoantibodies exert on EDEVs. This finding constitutes a tentative explanation not only for why the LA phenomenon has not been shown to be significant *in vivo* (with some exceptions) [52,53], but also for why EDEVs have not been shown to represent a direct procoagulant mechanism in APS [23,28].

Instead, EDEVs in this experimental setting constitute a new factor that brings additional complexity in understanding the LA paradox. The biological meaning of this new factor should be elucidated in further studies. As suggested by other authors [34], the direct interaction between LA-like aPL and EDEVs is likely to lead to the formation of immunocomplexes. For this reason, we encourage new investigations to analyse the formation of these complexes and how they could lead to thrombosis *in vivo* by indirect means.

Author contributions

Álvarez Daniel: Conceptualization, methodology, formal analysis, investigation, writing – original draft, visualization; Rúa Carolina: Writing – review and editing, supervision; Velásquez-Berrío Manuela: Writing – review and editing, funding acquisition, conceptualization; Cataño John Ubeimar; Writing – review and editing; Escudero Carlos: Writing – review and editing, visualization; Cadavid J. Ángela P.; Conceptualization, methodology, validation, resources, writing – review and editing, visualization, supervision, project administration, funding acquisition.

Declaration of competing interest

None.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jaut.2022.102905.

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