Extracellular vesicles released upon antiphospholipid antibody stimulus: an actual direct procoagulant mechanism or a new factor in the lupus anticoagulant paradox?

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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 endothelial cell-derived extracellular vesicles released upon stimulation with aPL (aPL-EDEVs) are related or not 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. Although 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.

Keywords

Antiphospholipid antibodies, cell-derived extracellular vesicles, lupus coagulation inhibitor, thrombosis, mitogen-activated protein kinase kinases.

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 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[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.

Methods

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 tested 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.

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

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 test (Aeskulisa Phospholipid-8Pro-GM, Aesku diagnostics, Wendelsheim, Germany) in order to confirm or reject the presence of aPL in pooled IgG samples.

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 the 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 5mg/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 1-3 culture passages, $7x10^4$ HUVECs per well were seeded in 24-well microplates (Corning, Corning NY, USA) to perform the corresponding stimuli. In this regard, 24 hours after seeding, once a 100% confluence was reached, 250 µg/mL IgG 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), 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 purified human β2GPI (Louisville APL Diagnostics, Texas city TX, USA).

In some final verifications, along with IgG stimulus, we perform a 1-hour 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 p38 MAPK, Sigma-Aldrich, St. Louis MO, USA), 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).

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 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 x g for 5 min). Subsequently, EDEVs were spun down in a 25 µL pellet (15000 x g for 60 min). In selected experiments, EDEV-poor supernatants were used as a negative control.

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 hour 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 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 (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 to 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 seconds. 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 to 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 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 to 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.

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/EDEVs 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 two hours of sample collection, platelet-poor plasma was extracted by double centrifugation (2000 x g for 15 min, and then 2500 x 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 (15000 x g for 60 min).

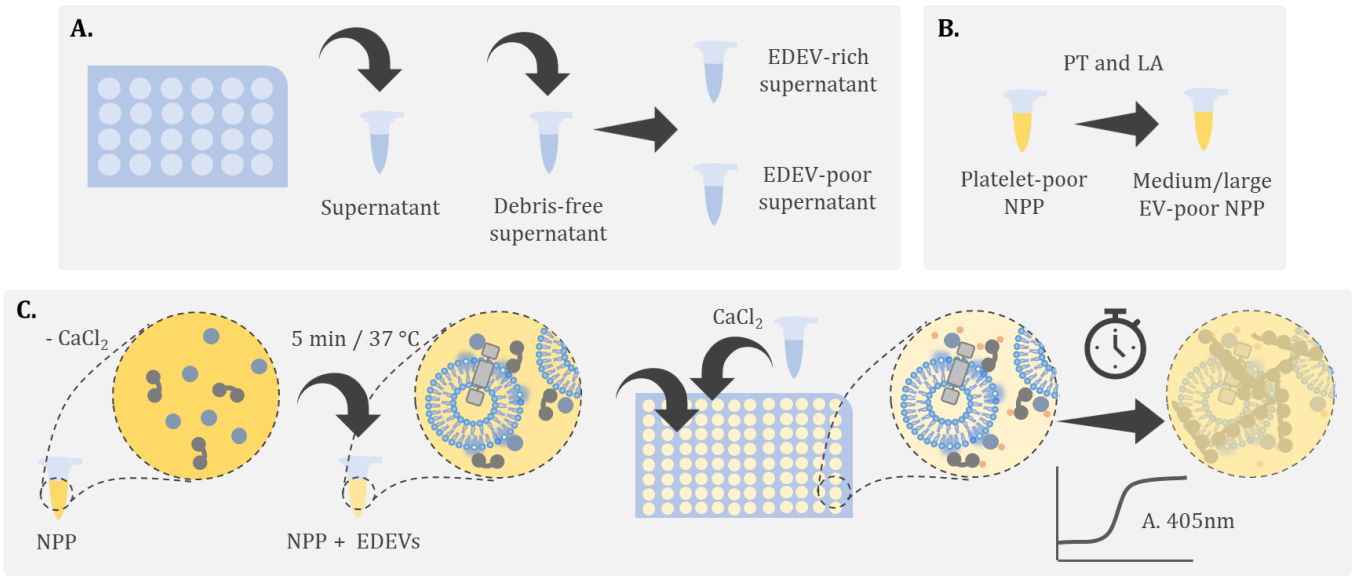

Fig. S1. Graphical overview of the recalcified plasma-based assay. A) Supernatants were collected after 2-hour stimuli to enrich EDEVs through washing with fresh EV-depleted medium and centrifugation. B) Previously, platelet-poor plasma samples were obtained from 20 healthy volunteers to make a pool of normal human plasma (NPP). C) Each sample of EDEVs was then incubated with NPP and ulteriorly recalcified. Plasma absorbance was kinetically recorded to obtain the coagulation curves. EVs, extracellular vesicles; PT, prothrombin time; LA, lupus anticoagulant; NPP, normal pooled plasma; EDEVs, endothelial cell-derived medium/large extracellular vesicles.

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 λ = 405 nm were taken every 3 minutes during 5 hours 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, $2.1x10^5$ HUVECs were seeded, equally divided into three wells of a 24-well microplate, and stimulated with 8 µg/mL LPS for 2 hours. 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 two hours, EDEVs were washed, spun down and tested in the recalcified plasma-based assay.

Analysis of coagulation curves

Kinetic records of absorbance from the recalcified plasma-based assay constitute coagulation curves with a sigmoidal shape. Briefly, since 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 (5PL) 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].

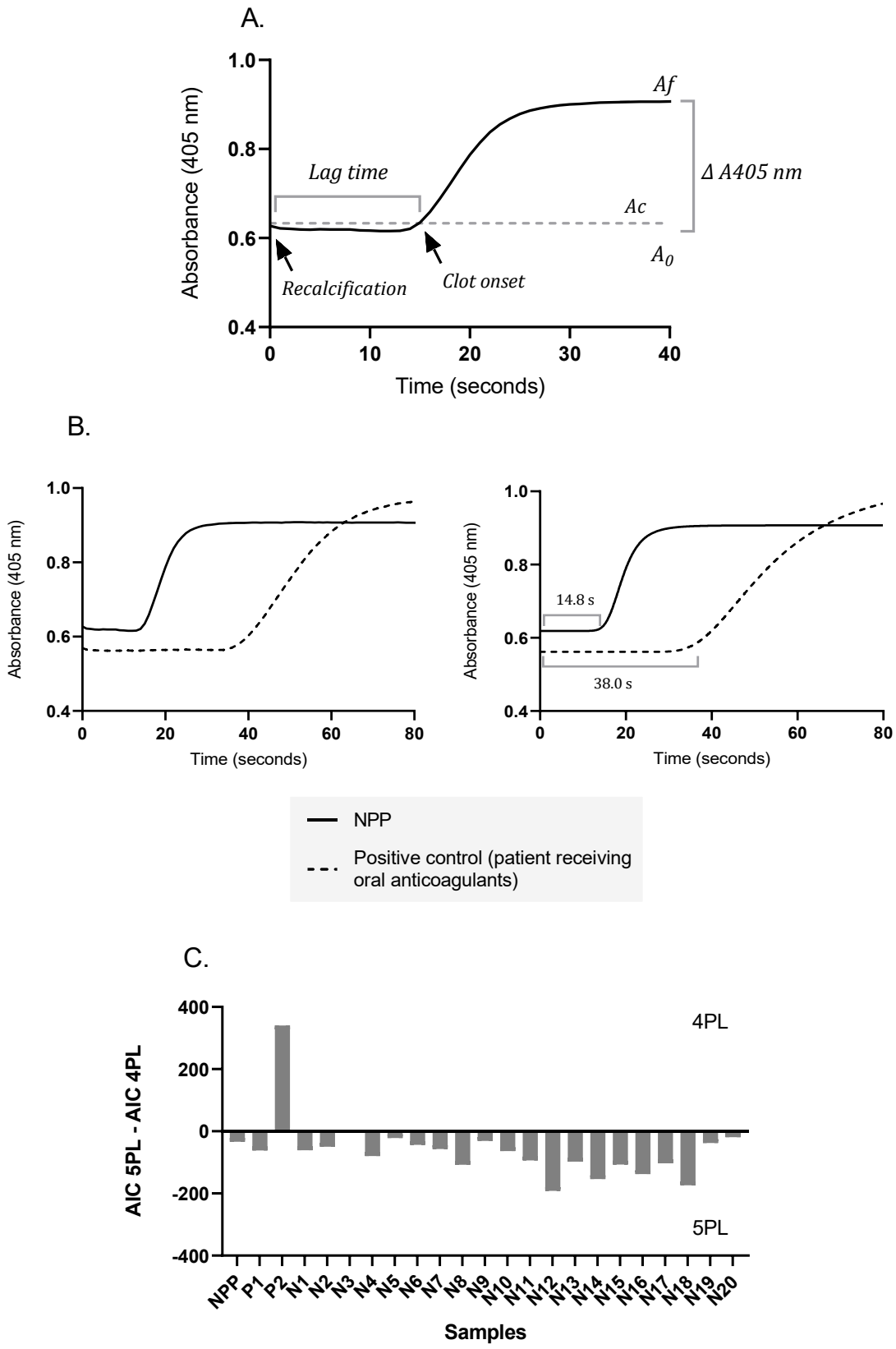

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Fig. S2. Strategy for the analysis of coagulation curves. A) Graphical explanation of the lag time according to the mathematical definition published by Shustova et al. B) An example of the nonlinear regression curve fit using the five-parameter logistic model (based on Richard's curve). NPP and anticoagulated plasma were recalcified with commercial total-thromboplastin. The records of absorbance every second (left) were used to calculate the model of absorbance as a function of time (right). Then we used this function to interpolate the time point at which 5% of the total change in the absorbance is reached (lag time). C) Comparison of the relative quality with which 4PL and 5PL models represent the coagulation curves of the 20 healthy volunteers. In all healthy volunteers, the Akaike information criterion value of the 5PL model was equal to or lower than the AIC value of the 4PL model, thus suggesting the 5PL model best represents the data sets. NPP, normal pooled plasma; AIC, Akaike information criterion; P, anticoagulated patient; N, healthy volunteer; 4PL, four-parameter nonlinear regression model; 5PL, five-parameter nonlinear regression model.

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). In all cases, differences were considered statistically significant when the p-value was less than 0.05. 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).

Results

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 (either criteria or non-criteria).

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
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
Venous/arterial thrombosis (n)	0	6	4	3	2	6	0	5	5	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

(n) Number of patients in each group with the corresponding clinical feature. † Values shown in the antiphospholipid antibody profile, other than for the normalised ratio of dRVVT, correspond to semi-quantitative results obtained by testing the purified IgG samples at a final concentration of 250 μg/mL and using a commercial ELISA kit. 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. 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.

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. Results are also shown in the supplementary material (Fig. S4).

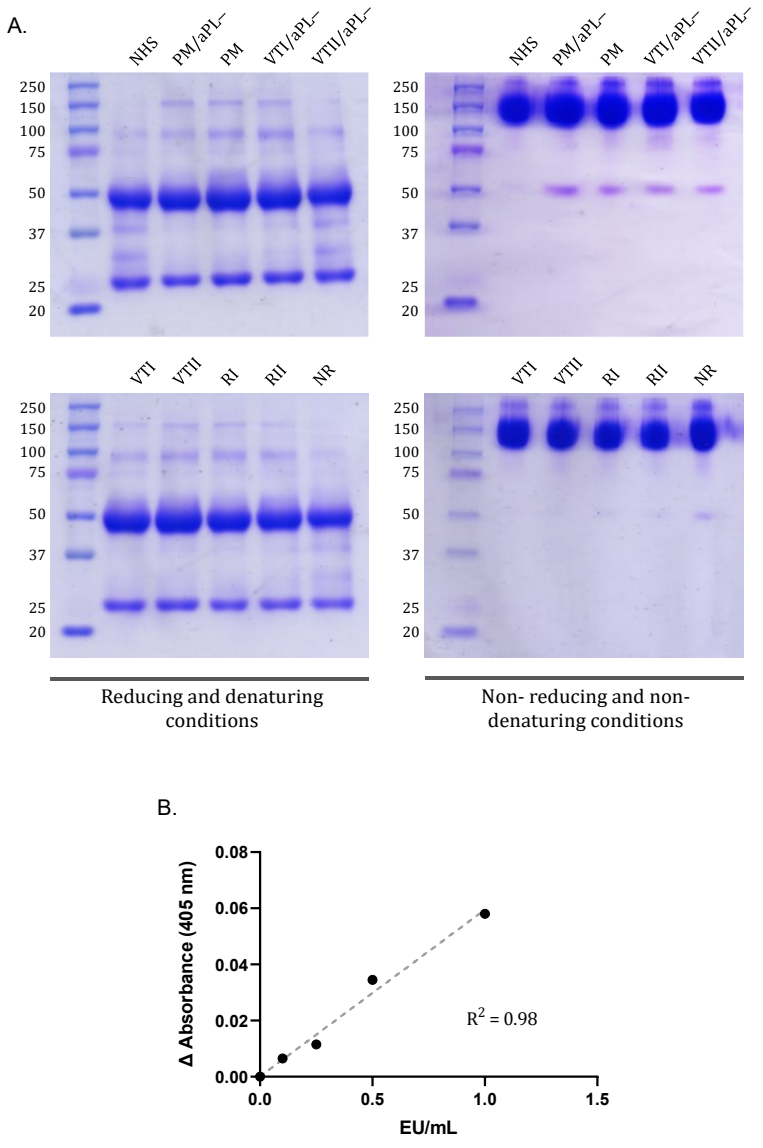

Fig. S3. Characterisation of IgG samples. A) Gel electrophoresis of IgG samples. Gels were prepared with 10% polyacrylamide, and then each lane was loaded with protein standard or 20 μg of IgG sample. B) Dot plot of endotoxin standards in the LAL test performed after sample detoxification. The interpolation of absorbance values shows endotoxin concentration was below 0.06 EU/mL in all samples. LAL, Limulus amebocyte lysate. Refer to table 1 for abbreviations of all patient and control groups.

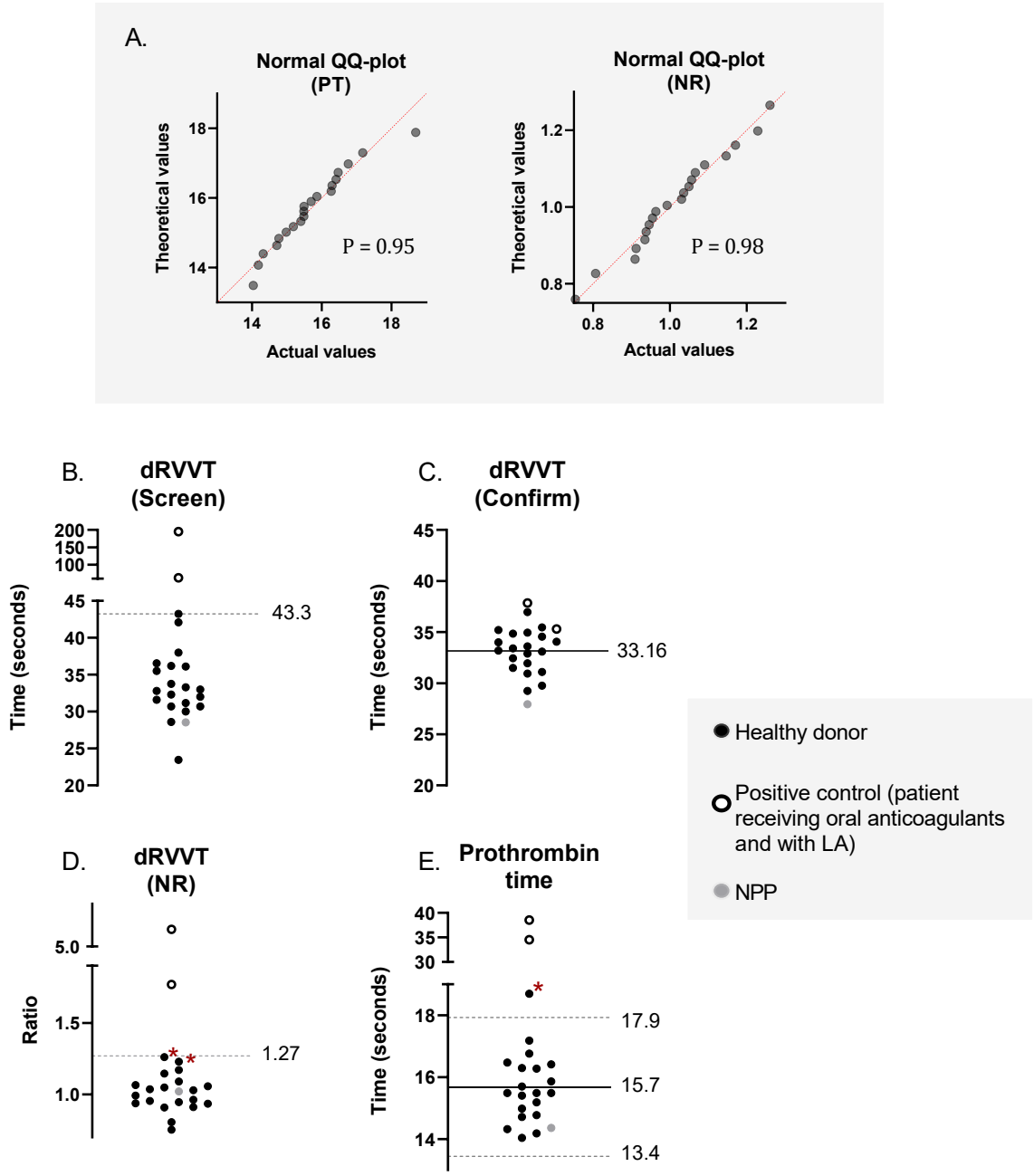

Fig. S4. Prothrombin time and dilute Russell's viper venom time of the normal pooled plasma and the healthy volunteers. A) Quantile-quantile plots showing normal distribution of prothrombin time values (left) and the normalised ratio of dRVVT values (right) from 20 healthy volunteers (Shapiro-Wilk test). B) Screen and C) confirm test of dRVVT. The dashed line represents the 97.5 percentile; the continuous line represents the mean. D) Normalised ratio of dRVVT and E) prothrombin time. The continuous line represents the mean; dashed lines represent the mean + 2SD (D), or mean ± 2SD (E). In all cases, red asterisks highlight individuals excluded from normal pooled plasma. For positive controls, dRVVT was measured using a mixing protocol. PT, prothrombin time; NR, normalised ratio; dRVVT, dilute Russell's viper venom time; NPP, normal pooled plasma.

Coagulation activity of EDEV-rich supernatants

The capacity of aPL to modify clotting via inducing the release of 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. For that reason, 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, employing the recalcified plasma-based assay protocol after 2-hour stimuli with IgG samples (Fig. S5).

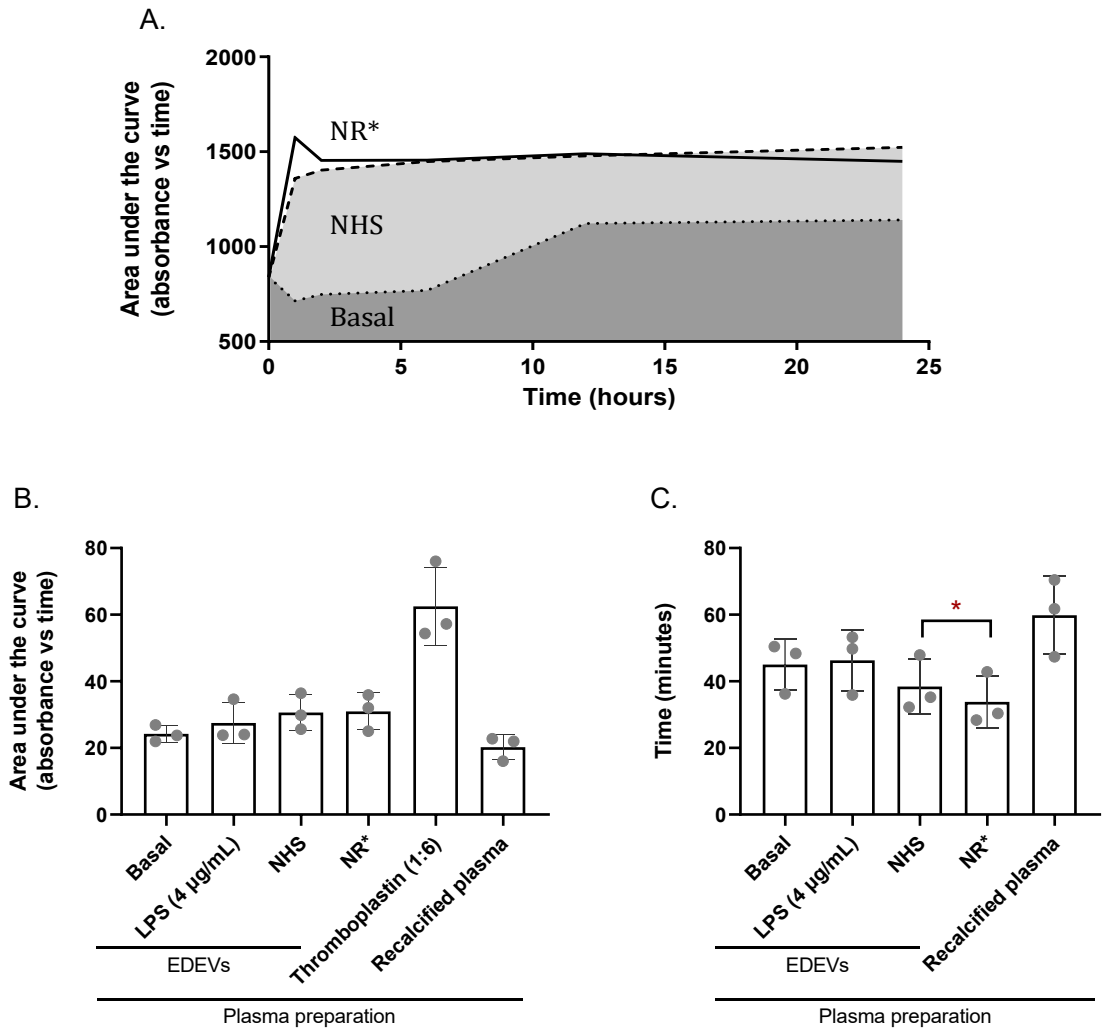

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Fig. S5. Coagulation activity of EDEVs over time. A) 24-hour follow-up of the overall coagulation potential in the presence of β2GPI. NR* represents EDEVs released upon stimulation of HUVECs with the IgG from one patient of the NR group. B) Overall coagulation potential of EDEVs collected after a 2-hour stimulus in the presence of β2GPI. No statistically significant differences were found. C) Time to reach 50% clot activation in the presence of EDEVs released upon a 2-hour stimulus with IgG and β2GPI. (Paired t-test, n = 3, * $p < 0.05$). NR, vascular and obstetric APS (non-refractory); NHS, normal human serum; LPS, lipopolysaccharide; EDEVs, endothelial cell-derived medium/large extracellular vesicles; β2GPI, β2 glycoprotein I.

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) (Fig. 1). According to our standardised protocol (Fig. 2A), 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. 2B and 2D).

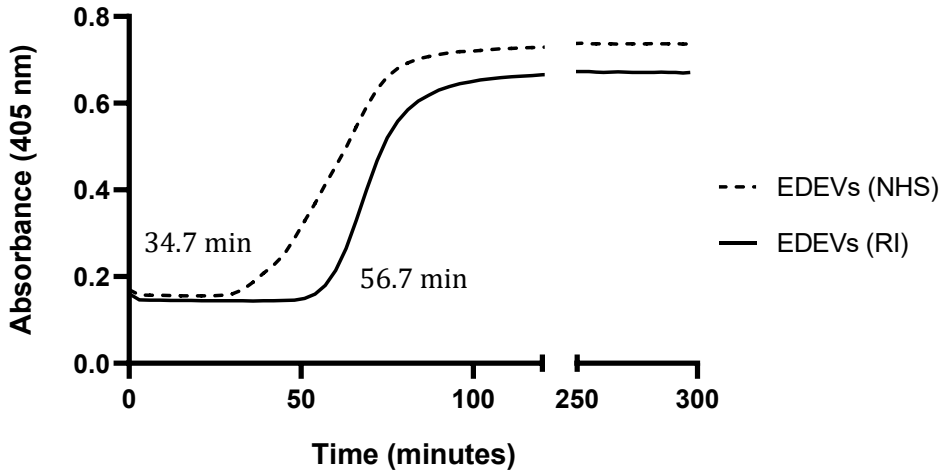

Fig. 1. Representative coagulation curves of the recalcified plasma-based assay. The time course of plasma absorbance after recalcification in the presence of EDEVs released upon stimulation with NHS IgG (dashed line) or RI IgG (continuous line). Lag time is indicated in minutes for both coagulation curves. EDEVs, endothelial cell-derived medium/large extracellular vesicles; NHS, normal human serum; RI, Vascular and obstetric APS (primary and refractory).

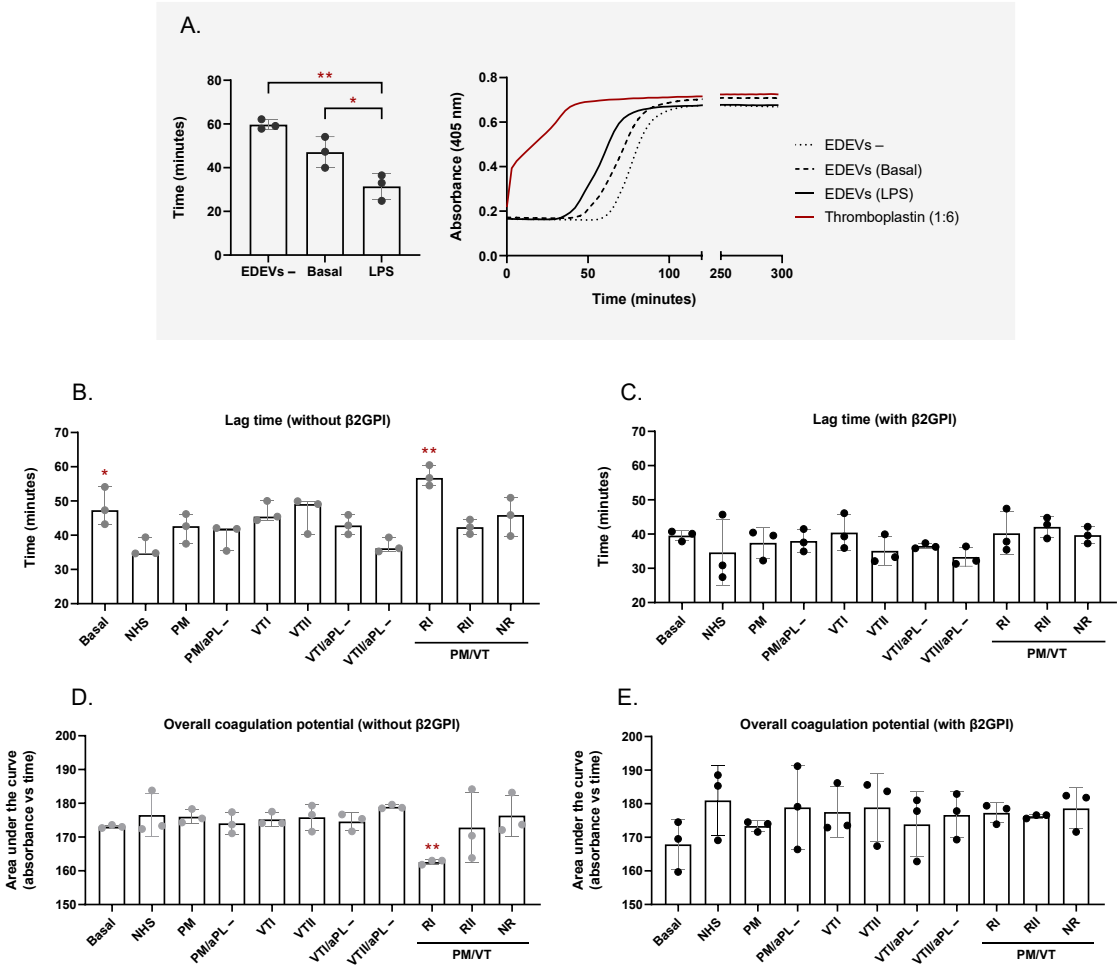

Fig. 2. EDEV-rich supernatant from endothelial cells stimulated with IgG from RI patients exhibits a dampened coagulation activity. This anticoagulant effect is abrogated by using β2GPI as a stimulus cofactor. A) Positive and negative controls of the recalcified plasma-based assay. Three independent 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.

In contrast to the previous results, assessing the coagulation activity of EDEVs released using β2GPI as a cofactor of the stimuli with aPL, no statistically significant difference was detected between groups (Fig. 2C and 2E). 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 (RI = 162.6 ± 0.6928, RI + β2GPI = 177.3 ± 2.950, $p < 0.05$).

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, either in the presence or absence of β2GPI ($p = 0.42$ and $p = 0.5$, respectively) (Fig. 3). We further independently analysed the number of EDEVs corresponding to 0.5, 1, and 2 μm. No statistically significant difference was found (data not shown).

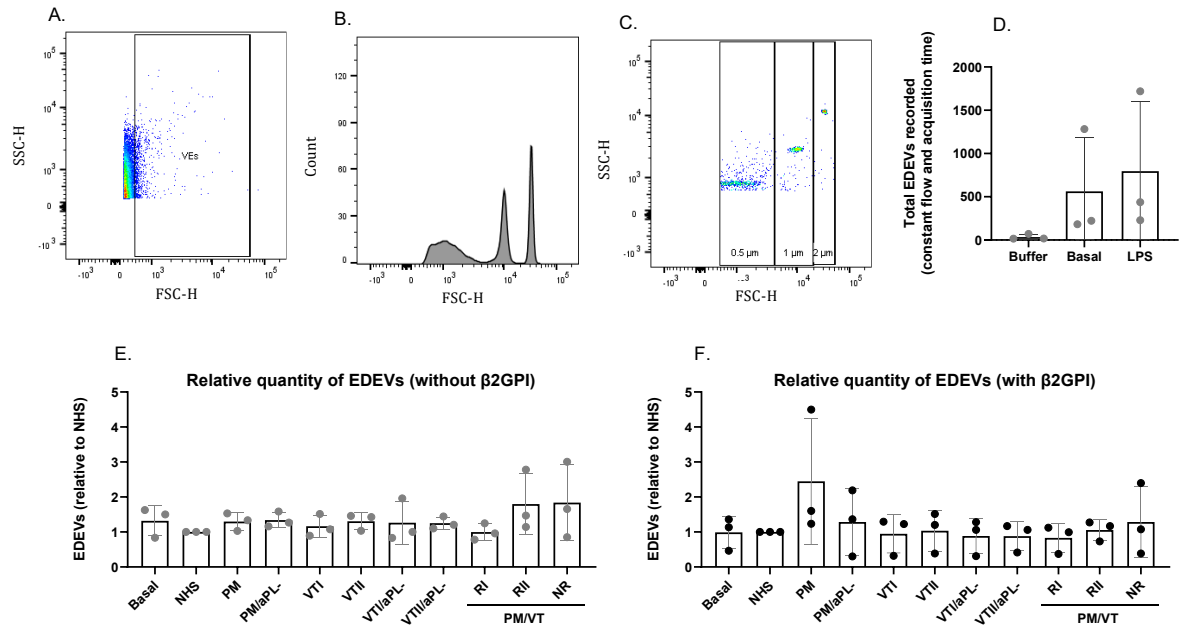

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Fig. 3. The dampened coagulation activity exhibited by the EDEV-rich supernatant from RI IgG-stimulated HUVECs cannot be explained by a decreased count of EDEVs. A) Delimitation of the electronic noise in the flow cytometer. B) Testing the resolution 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. E, F) Relative number of EDEVs released after stimulation with IgG in the presence or absence of β2GPI (n = 3, repeated measures ANOVA test, no statistically significant differences were found). 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.

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 4B). 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 stimulus (see methods) (NR = 1.3 and 1.2). 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 (Fig. 4C and 4D).

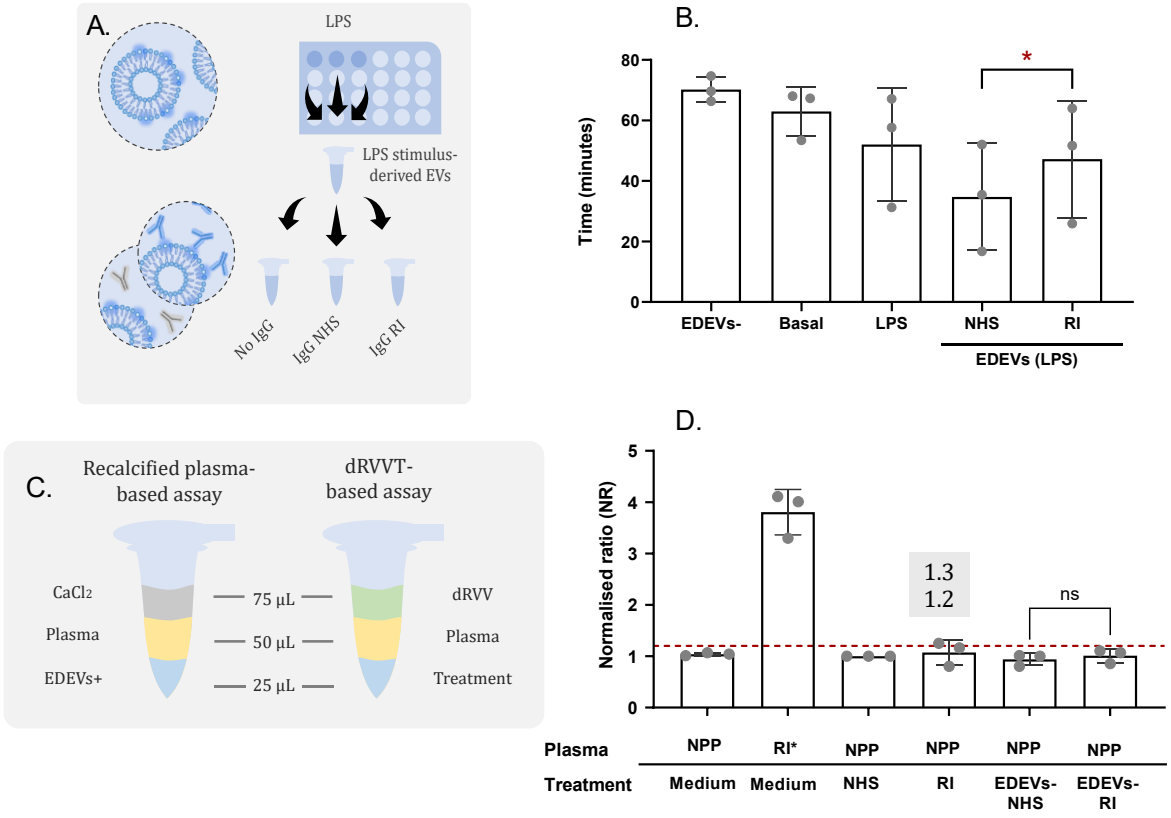

Fig. 4. IgG from patients of the RI group exerts a direct anticoagulant effect on EDEVs released on account of a proinflammatory stimulus. A) Graphical overview of the modified protocol employed to reproduce the recalcified plasma-based assay. Briefly, a pool of EDEVs released upon LPS stimulus was collected 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.

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 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.

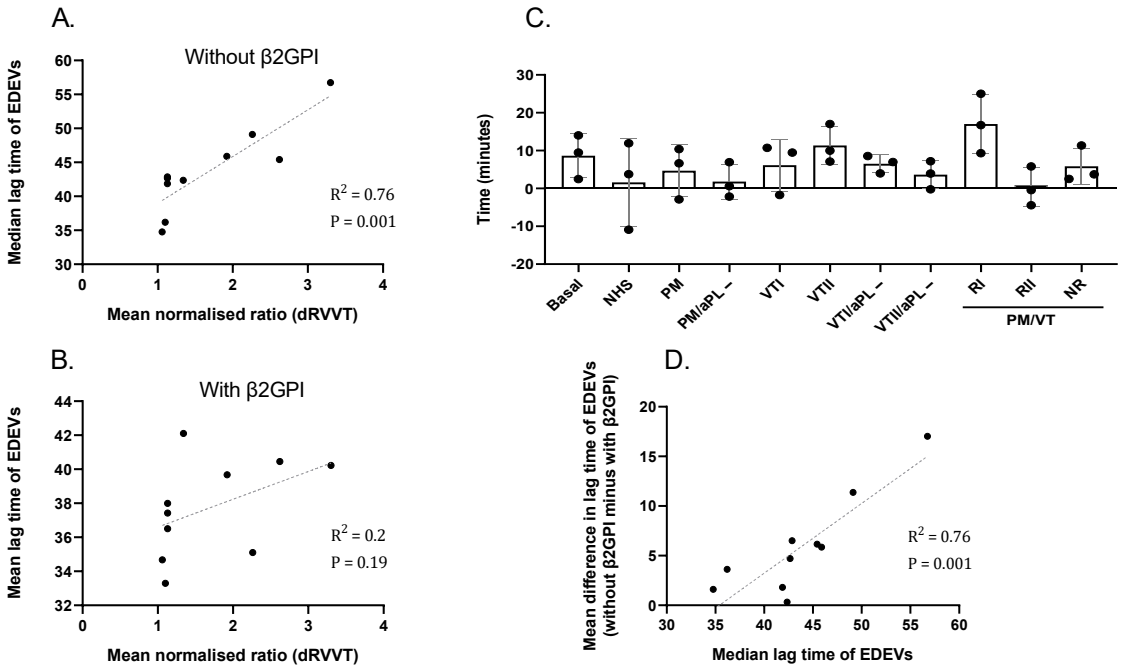

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Fig. 5. The direct anticoagulant effect of aPL seems to correspond to the LA phenomenon and is proportional to the β2GPI-dependent procoagulant effect. For each control and patient group, the mean normalised ratio value of the dRVVT test was compared 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.

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 coagulation 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[16,41].

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, proving 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. S6) showed no effect over the procoagulant action of β2GPI.

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, without finding statistically significant differences (data not shown).

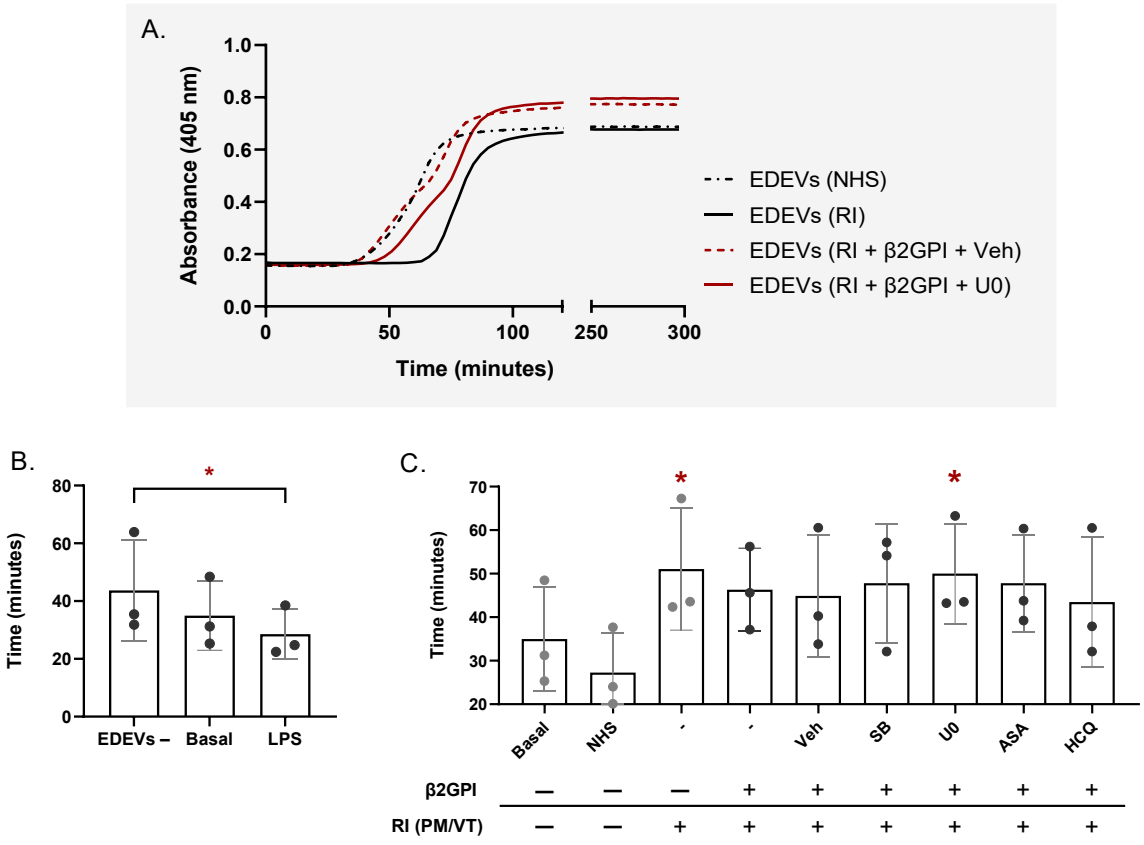

Fig. 6. The β2GPI-dependent procoagulant effect relies on the function of the MEK1/2 pathway. A) Representative coagulation curves. The selective inhibitor of MEK1/2 pathway U0126 induces a rightward displacement of the coagulation curve (increased lag time) in the presence of β2GPI. B) Positive and negative controls of the recalcified plasma-based assay. C) Lag time of normal plasma recalcified in the presence of EDEVs released by HUVECs stimulated with RI IgG in the presence or absence of β2GPI and with different pretreatments. (Differences between groups are shown compared to the NHS control group, n = 3, Friedman test, * $p < 0.05$). 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.

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[14,42,43]. We found no significant effect using acetylsalicylic acid or hydroxychloroquine (Fig. 6). The effect of low-molecular-weight heparin (enoxaparin) was also assessed, but no clot activation was

detected after 5 hours (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 (Fig. 6).

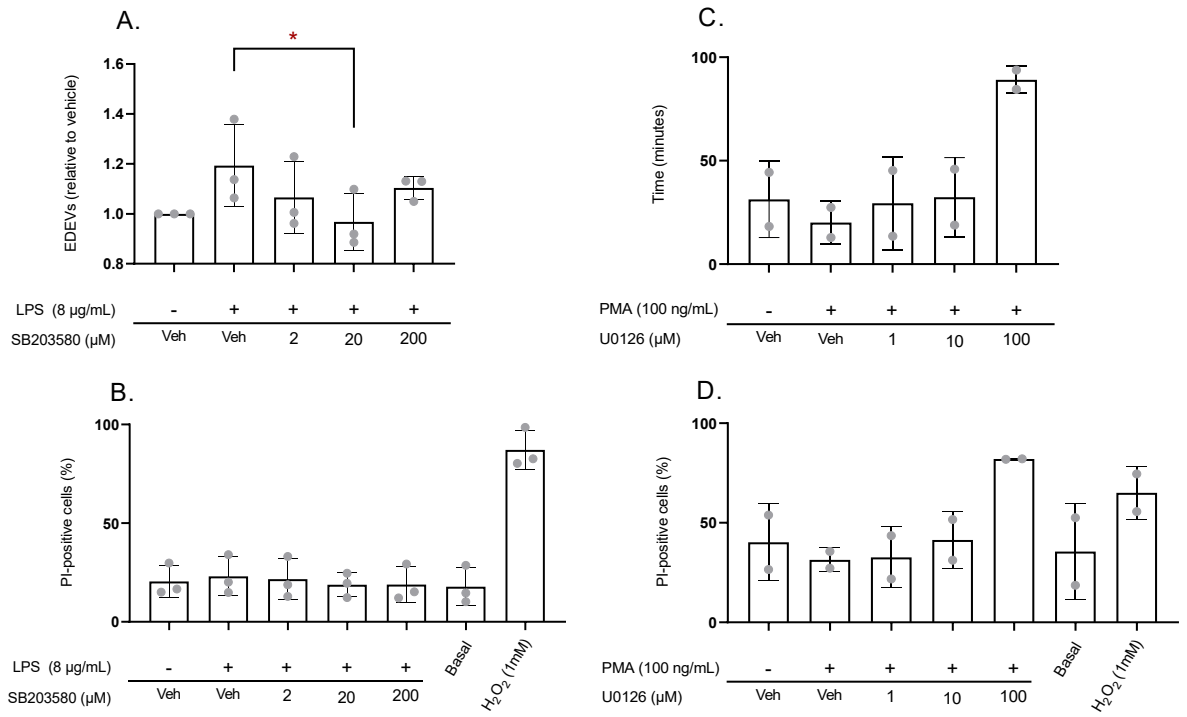

Fig. S6. Standardisation of SB203580 and U0126 concentrations. A, B) Evaluation of different concentrations of the p38MAPK inhibitor, SB203580. HUVECs were pre-treated for 1 hour with vehicle (DMSO) or inhibitor. Then, cells were stimulated with 8 µg/mL LPS for two hours. Subsequently, released EDEVs were isolated and counted by flow cytometry (A) (Friedman test, n = 3, * $p < 0.05$), while cells were detached and stained with propidium iodide to assess the integrity of their membranes by flow cytometry (B). C, D) Evaluation of different concentrations of the MEK 1/2 inhibitor, U0126. HUVECs were pre-treated for 1 hour with vehicle (DMSO) or inhibitor. Then, cells were stimulated with 100 ng/mL PMA for two hours. Subsequently, released EDEVs were isolated and used in the recalcified plasma-based coagulation assay (C), while cells were detached and stained with propidium iodide to assess the integrity of their membranes by flow cytometry (D). EDEVs, endothelial cell-derived medium/large extracellular vesicles; LPS, lipopolysaccharide; PMA, phorbol 12-myristate 13-acetate; PI, propidium iodide.

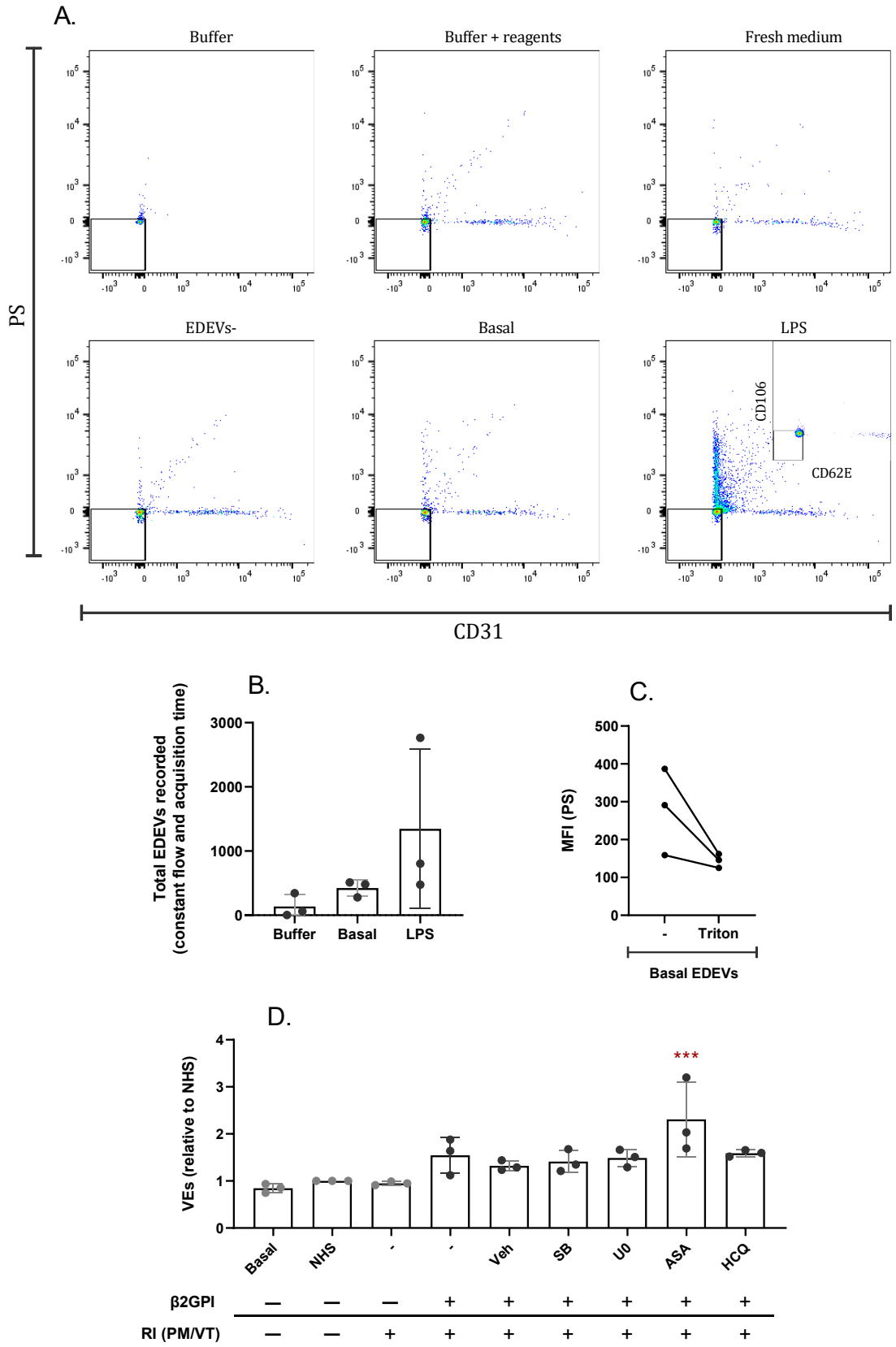

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Fig. 7. Inhibition of the MEK1/2 pathway does not exert an anticoagulant effect over the coagulation activity of EDEV-rich fraction of supernatant by reducing the count of EDEVs. A, B) Representative dot plots and EDEV counts of controls in the flow cytometry assay. EDEV detection was optimised by using two additional markers (CD106 and CD62E). C) Effect of triton x-100 at a final concentration of 0.075% in mean fluorescence intensity associated with 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.

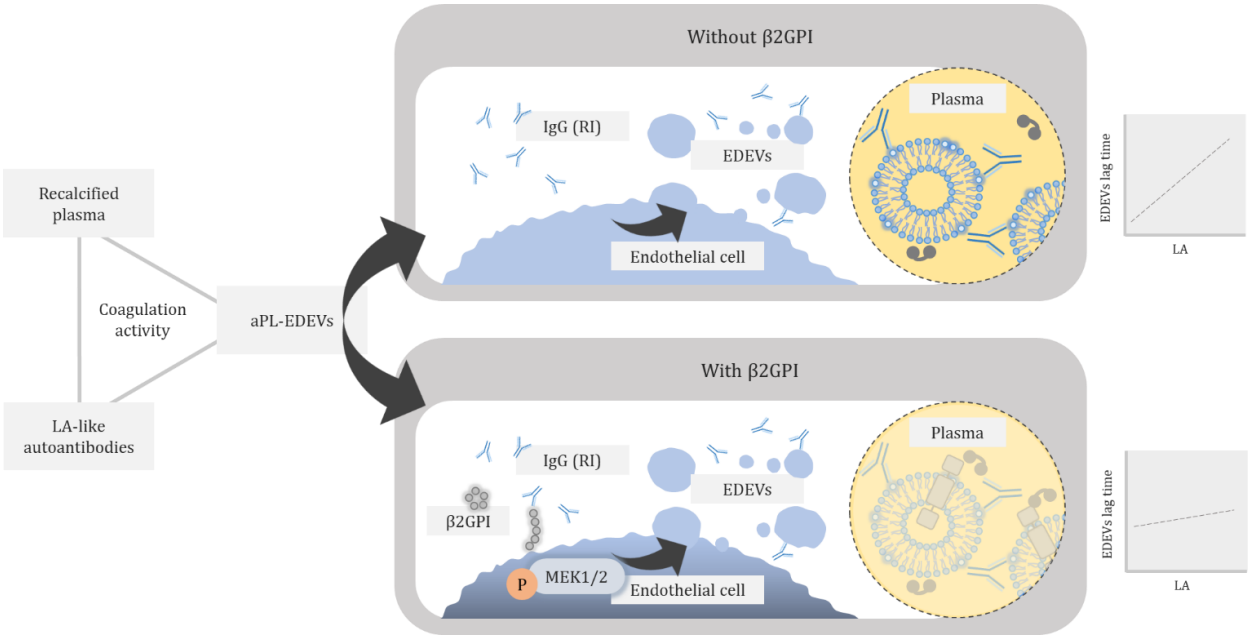
Discussion

We could summarise our findings in four core concepts: 1) in the complex interaction between plasma, aPL-EDEVs, and the same autoantibodies that condition the release of these EDEVs, an anticoagulant effect prevails. 2) 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. 3) In addition, β2GPI, a plasma protein that performs as the primary cofactor of aPLs, restores the coagulation properties of EDEVs. 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. 4) Finally, the procoagulant effect of β2GPI is suspended by inhibiting the MEK1/2 pathway 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 exert a direct anticoagulant effect on aPL-EDEVs? and how does β2GPI restore the coagulation activity of EDEVs?

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.



Graphical abstract. The coagulation activity resulting from the interaction between recalcified plasma, antiphospholipid antibodies, and endothelial cell-derived extracellular vesicles released upon stimulation with antiphospholipid antibodies is strongly conditioned by autoantibodies with lupus anticoagulant-like activity. Nevertheless, β2 glycoprotein I can overcome this anticoagulant effect in a MEK1/2-dependent manner. The lupus anticoagulant-like activity directly exerted over extracellular vesicles and the β2 glycoprotein I-dependent procoagulant activity are directly proportional and have shown to be higher in antibodies from patients with vascular and obstetric primary antiphospholipid syndrome who manifest refractoriness to treatment. aPL, antiphospholipid antibodies; LA, lupus anticoagulant; EDEVs, endothelial cell-derived medium/large extracellular vesicles; IgG, immunoglobulin G; RI, Vascular and obstetric APS (primary and refractory); β2GPI, β2 glycoprotein I; MEK1/2, mitogen-activated protein kinase kinases 1 and 2.

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[44]. 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[45]. aβ2GPI antibodies may still be linked to the LA phenomenon through other more recently postulated mechanisms such as direct interaction with the coagulation factor V (FV)[9]. 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 attributed to more than one kind of aPL with different anticoagulant mechanisms[46]. That is why, as the clinical data shows, there is no single absolutely sensitive method to detect LA[47], and it has been recommended to use more than one test to diagnose APS[48]. Hence, our findings would reflect just some LA-like autoantibodies[46]. In fact, it is even possible that the LA-like activity exerted over EDEVs in the absence of β2GPI could be explained by the presence of non-vesicle-associated autoantibodies undetectable for the dRVVT test, 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 EDEVs. 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 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. Nevertheless, our final flow cytometry assay suggests that changes in the number of EDEVs do not explain, at least in their entirety, neither the β2GPI-dependent procoagulant effect nor the anticoagulant effect of inhibiting MEK1/2.

Since we did not find significant differences in the number of vesicles released in the presence or absence of β2GPI, it is possible that β2GPI (which is a common cofactor of aPL[13,49]) 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, in as much as inhibition of the MEK1/2 pathway, a well-described pathway activated by aPL that in monocytes leads to the expression of tissue factor[41], abrogates the β2GPI-dependent procoagulant effect. 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[50]. 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 associated with EDEVs, aPL 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 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.

In summary, our results indicate 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 for why the LA phenomena have not been shown to be significant *in vivo* with some exceptions[51,52], but also 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.

Acknowledgements

Minciencias, Colombia, supported this research (Grant no. 111580762949). We acknowledge the kind assistance of Dr Mauricio Rojas and the flow cytometry unit of Universidad de Antioquia. The authors wish to thank Hospital San Vicente Fundación; Clínica Universitaria Bolivariana; Unidad Hospitalaria Manrique, E.S.E. Metrosalud, Medellín, Colombia; and especially, all women who selflessly collaborated in this study. The author MVB is a recipient of a fellowship awarded by Minciencias, Colombia (Grant no. 757 from 2016). Fondecyt, Chile (1200250) financially supports CE.

Conflict of Interest

None declared.

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