Mechanisms sensitive to different drugs mediate a pro-adhesive and pro-thrombotic platelet phenotype in anti-phospholipid syndrome

Marina Camera,[1,2] Marta Brambilla,[1] Paola Adele Lonati,[3] Alessia Becchetti,[2] Claudia Grossi,[3] Kevin Nallio,[2] Arianna Da Via,[3] Laura Trespidi,[4] Maria Orietta Borghi,[3,5] Francesco Tedesco[3] and Pier Luigi Meroni[3]

[1]Department of Pharmaceutical Sciences, University of Milan; [2]Unit of Cell and Molecular Biology in Cardiovascular Diseases, IRCCS Centro Cardiologico Monzino; [3]Laboratory of Immunorheumatologic Researches, IRCCS Istituto Auxologico Italiano; [4]Dipartimento Area Materno-Infantile, IRCCS Fondazione Ospedale Maggiore Policlinico and [5]Dipartimento di Scienze Cliniche e di Comunità, Dipartimento di Eccellenza 2023-2027, University of Milan, Milan, Italy

Correspondence: M. Camera
marina.camera@unimi.it

P. L. Meroni
p.meroni@auxologico.it

Received: December 10, 2024.
Accepted: June 25, 2025.
Early view: July 3, 2025.

https://doi.org/10.3324/haematol.2024.287133

Abstract

Anti-phospholipid antibodies (aPL) mediate platelet- and leukocyte-interaction with damaged endothelium, contributing to anti-phospholipid syndrome (APS) vasculopathy. This study aimed to understand the mechanisms sustaining the pro-adhesive/-thrombotic platelet phenotype and the *in vitro* effects of different drugs. We included 34 primary APS (PAPS) patients and 12 healthy subjects (HS). All patients had medium/high aPL levels with vascular/obstetric symptoms according to the 2023 ACR/EULAR classification. *In vivo*, we evaluated by flowcytometry platelet activation markers (P-selectin, activated GPIIbIIIa [aGPIIbIII], tissue factor [TF], ApoER2 and β2GPI expression and platelet-monocyte and -granulocyte aggregates [PMA and PGA]). *In vitro*, the impact of antiplatelet and anti-inflammatory drugs on platelet activation induced by different aPL subpopulations was investigated. PAPS patients exhibited greater percentages of circulating ApoER2^{pos}-, P-selectin^{pos}-, aGPIIbIIIa$_{pos}$-, TF^{pos}-platelets, and TF^{pos}-platelet-leukocyte aggregates. *In vitro*, HS blood incubation with PAPS plasma fully reproduced the activation found *in vivo*. While anti-β2GPI-Domain(D)1, but not anti-D4,5, immunoglobulin (Ig)G upregulated platelet TF expression only, the addition of interleukin (IL)-6 also induced P-selectin and aGPIIbIIIa upregulation. An IL-6 receptor-blocking monoclonal antibody prevented the pro-adhesive/-coagulant platelet phenotype and the formation of platelet-leukocyte aggregates mediated by PAPS plasma or by total IgG plus exogenous IL-6. While aspirin and P2Y$_{12}$ inhibitor fully inhibited platelet activation, hydroxychloroquine (HCQ) did not blunt TF expression. PAPS patients exhibit circulating pro-adhesive/-coagulant (TF-positive) platelets and platelet-leukocyte aggregates mediated by β2GPI-D1-dependent IgG and an inflammatory trigger. While aspirin and P2Y$_{12}$ inhibitor significantly inhibited the aPL-mediated P-Selectin and TF upregulation, HCQ affected the adhesion phenotype only, and might not be adequate to prevent platelet-mediated thrombosis.

Introduction

Anti-phospholipid syndrome (APS) is an autoimmune thrombotic vasculopathy mainly mediated by β2glycoprotein I (β2GPI)-dependent anti-phospholipid antibodies (aPL).[1] Vitamin K antagonists (VKA) are the mainstream treatment for secondary prophylaxis in vascular APS, while low-dose aspirin (LDA) is suggested for asymptomatic aPL carriers.[2-4] Additional therapeutic approaches are included in the EULAR recommendations for APS, such as the combination of antiplatelet and VKA.[3] Moreover, preliminary evidence supports the usefulness of hydroxychloroquine (HCQ) and statins as additional therapeutic tools in some patient subpopulations.[3,5] However, the adequate strategy for primary and secondary thrombosis prophylaxis in aPL patients remains controversial, and new vascular events are reported despite therapy in some cases.[2,6,7]

While endothelium perturbation is generally accepted as the upstream step in the pathogenesis of vascular APS, the involvement of other cell types is crucial for thrombus formation. In particular, much attention has been recently paid to the pro-thrombotic and pro-inflammatory role of platelets and neutrophils in addition to monocytes.[1] The reported increased urinary arachidonic acid metabolites

support the presence of an activated platelet phenotype in APS patients.[8] The platelets' role in clot formation has been then shown by platelet-dependent thrombi in one APS animal model and by the greater number of tissue factor (TF)-positive platelets in APS patients.[9-11] Platelets may form complexes with white blood cells during activation, providing a novel link between inflammation and thrombosis.[12,13] Platelet-leukocyte aggregates are present in healthy people but significantly increase in patients with cardiovascular or infectious disorders. They predict severe outcomes after surgery or percutaneous treatment in patients with cardiovascular disease.[14,15] Platelet-leukocyte aggregates have also been reported in patients with primary APS, but the mechanisms underlying their formation are still scanty.[16,17] As mentioned above, therapy with antiplatelet drugs and anticoagulation is adequate in most APS patients, but recurrences are reported.[2] A deeper understanding of the molecular player supporting the adhesive and thrombophilic platelet phenotype may also drive toward a more specific therapeutic approach (pharmacological interventions). Thus, the study aimed to gain insights into the mechanisms responsible for the presence and characteristics of platelet-leukocyte aggregates in APS and the effect of antiplatelet and anti-inflammatory drugs on platelets' adhesive and pro-coagulant properties.

Methods

Patient selection and anti-phospholipid antibody detection

Patients with primary APS (PAPS; N=34) displaying medium/high double or triple positivity for aPL were classified according to the 2023 ACR/EULAR APS classification criteria.[18] Eight aPL-negative chronic coronary artery disease (CAD) patients were enrolled for comparison. Twelve age- and sex-matched healthy subjects (HS) were included as normal controls. Table 1 reports the clinical and laboratory characteristics of the enrolled patients and normal controls. The anticoagulant and/or antiplatelet drugs reported in the table are those taken by patients at the moment of blood collection, far from the initial diagnosis.

The aPL profiles were carried out as previously described.[19-23] There is growing evidence that anti-β2GPI immunoglobulin (Ig)G antibodies specifically reacting with the domain-1(D1) display the strongest association with LAC and in particular with the APS clinical manifestations. In order to make our cohort as homogeneous as possible, we also characterized the domain specificity of the included samples. All the included patients were positive for anti-β2GPI D1 detected as described.[19-23]

The study was approved by the Istituto Auxologico Italiano Ethics Committee (22.07.2010), and informed consent was obtained from all participants according to the principles of the Declaration of Helsinki.

Table 1. Clinical and laboratory characteristics of the investigated patients and controls.

Characteristic, N	PAPS N=34	CAD N=8	HS N=12
Age, years, mean+SD	37.2±4.1	72.5±5.6	39±11
Female/Male	31/3	5/3	10/2
Arterial thrombosis	11	8	0
Venous thrombosis	17	0	0
Arterial & venous thrombosis	6	0	0
Obstetric & vascular	5	0	0
aCL IgG	21	0	0
aCL IgM	3	0	0
Anti-β_2GPI IgG	34	0	0
Anti- β_2GPI IgM	3	0	0
Anti- β_2GPI-D1 IgG	34	0	0
LAC	21	0	0
Double positive	14	0	0
Triple positive	20	0	0
Anticoagulant therapy (VKA)	27	0	0
Low-dose aspirin	4	8	0
$P2Y_{12}$ inhibitor	0	0	0
No therapy	3	0	0

PAPS: primary anti-phospholipid syndrome; CAD: anti-phospholipid antibodies-negative coronary artery disease; HS: healthy subjects; LAC: lupus anticoagulant; aCL: anti-cardiolipin antibodies; anti-β2GPI: anti-β_2 glycoprotein I antibodies; SD: standard deviation; VKA: vitamin K antagonist; Ig: immunoglobulin.

Blood collection and plasma preparation

Whole blood (WB) from PAPS patients, CAD and HS was drawn with a 19-gauge needle without venous stasis into κ_2-EDTA or sodium citrate (0.129 M, 1/10 volume/volume) containing tubes (Vacutainer, Becton Dickinson) for blood cell counting or flow cytometry analysis, and plasma preparation, respectively, and processed within 15 minutes (min). For platelet poor plasma preparation, whole blood (WB) was centrifuged at 1,700g for 10 min, 4°C. Plasma was collected into a fresh tube and stored at -80°C until analysis.

For the *ex vivo* studies, five PAPS patients with double (N=2) or triple (N=3) high aPL positivity were selected for total IgG and plasma pool preparation. IgG were obtained by affinity purification from serum samples of PAPS or age and sex-matched HS, as described, and their reactivity against whole β2GPI and D1 was confirmed.[19-23] For the plasma pool preparation, 1.5 mL of plasma from each of the five PAPS patients was pooled in order to obtain the amount necessary (~7 mL) to carry out the large number

of experiments, which would not have been feasible with the single-patient plasma.

Flow cytometry

The phenotypic characterization of PAPS platelets was performed on 27 of 34 enrolled PAPS patients. The assessment of aGPIIbIIIa, intracellular TF, TFPI, ApoER2 and β2GPI was performed as proof-of-concept on ten patients. Flow cytometry analysis was performed as previously described[24,25] and modified as reported in the *Online Supplementary Appendix.* The gating strategy is shown in *Online Supplementary Figure S1.*

Ex vivo studies

To evaluate the effect of PAPS patients' plasma and IgG on platelet activation, blood from HS with ABO group O (N=7) was centrifuged at 1,700g for 10 min at room temperature (RT), plasma-depleted, and replaced with plasma pool from PAPS patients, plasma from CAD patients or with autologous plasma as control. The stimulating activity of PAPS IgG was tested by spiking blood from HS with affinity-purified total PAPS IgG (200 μg/mL). To evaluate the impact of IL-6 on platelet activation and platelet-leukocyte formation, HS blood was stimulated with IL-6 (100 pg/mL; Merk Millipore) or IL-6 plus PAPS IgG at RT for 30 min. The effect of ApoER2 inhibitor (RAP, 13.7 μg/mL; Euroclone), IL-6R inhibitor (tocilizumab, 300 μg/mL; Roche Pharma AG), HCQ (100 μg/mL; Sigma Aldrich) and antiplatelet drugs (aspirin, 8 μM; AR-C69931MX, 100 nM; Tocris Bioscience) on platelet activation was investigated incubating each of these compounds with HS WB for 30 min at RT before the addition of PAPS patients' plasma or PAPS IgG. Affinity-purified total IgG from healthy sera pools (N=5 age-/sex-matched HS) were used as controls. After 30 min of incubation, the blood was labeled for P-selectin, aGPIIbIIIa, TF, and CD41/CD14 or CD41/CD66 and analyzed by flow cytometry.

Statistical analysis

A sample of 46 patients (34 PAPS patients and 12 HS) was estimated to provide 80% statistical power to detect as significant (P<0.05) a difference between groups of at least 0.962 standard deviations (for example, in the case of TF^{pos} platelets this would correspond to a mean absolute variation of about 2.9%).

Quantitative variables are presented as mean ± standard deviation (SD); within-group comparisons were made with the Wilcoxon signed-rank test, and between-group comparisons were made with the Wilcoxon rank-sum test. The normality of the variable distributions was assessed using either the D'Agostino-Pearson omnibus K2 test or Shapiro-Wilk test, as appropriate, along with the visualization through normal probability plots. Categorical variables are presented as N (%) and were compared by χ^2 or Fisher's exact test when appropriate. Spearman's correlation coefficient assessed associations between variables. A P value <0.050 was considered statistically significant. All analyses were performed using Prism Graphpad version 9.0 and SAS v. 9.4 (SAS Institute, Cary, NC, USA).

Results

Patients

Table 1 reports the laboratory and clinical characteristics of the 34 patients with PAPS included in the study. All the patients were classified as macrovascular, and five as vascular/obstetric PAPS according to the new ACR/EULAR classification criteria.[18] The patients displayed a high-risk aPL profile being 20/34 triple positive and all positive for anti-β2GPI-Domain-1(D1) IgG.[18]

While three patients were investigated before starting any therapy, 27 of 34 were on VKA, four of 34 on antiplatelet therapy only (Table 1). All the patients were investigated between 6 and 12 months after the clinical event and/or APS diagnosis. CAD patients were included for comparison while age-/sex-matched HS were enrolled as healthy controls.

Platelet-granulocyte and -monocyte aggregates in primary anti-phospholipid syndrome patients

Whole blood flow cytometry analysis showed that PAPS patients had 2-fold greater percentages of platelet-granulocyte and platelet-monocyte aggregates than HS (Figure 1A, B). This finding is consistent with the significant trend toward an increased number of platelets expressing adhesion markers P-selectin (2-fold), and activated GPIIbIIIa (aGPIIbIIIa; 1.5-fold; N=10) (Figure 1C, D).

As the primary antigenic target of aPL is β2GPI, we investigated the presence of β2GPI and ApoER2, one of the β2GPI receptors on the cell membrane, in a subgroup of ten PAPS patients. A 3-fold greater number of platelets expressing ApoER2 under resting conditions was observed in PAPS than in HS, with a trend towards a higher number of $\beta2GPI^{pos}$-platelets (Figure 1E, F). This finding highlights the overall increased potential of β2GPI-dependent aPL to recognize β2GPI on platelet membranes, as previously reported.[21] Interestingly, a trend toward a positive association between ApoER2 expression and an increased low-grade platelet activation in terms of platelet-granulocyte and platelet-monocyte aggregates, TF and aGPIIbIIIa expression was observed (*data not shown*).

Prothrombotic platelet phenotype in primary anti-phospholipid syndrome patients and controls

Similar to P-selectin and aGPIIbIIIa a significantly greater number of TF^{pos}-platelets was present in PAPS patients under resting conditions *versus* HS (5.6±4.2% *vs*. 2.4±1.4%; P=0.02; Figure 2A). This increase was not paralleled by

an increase in the percentage of TFPI^{pos}-platelets in a subgroup of ten PAPS patients, thus shifting the balance towards a more prothrombotic situation (Figure 2B). The percentages of platelets carrying intracellular-stored TF (ICTF) were comparable between PAPS patients and HS in the same subgroup (Figure 2C).

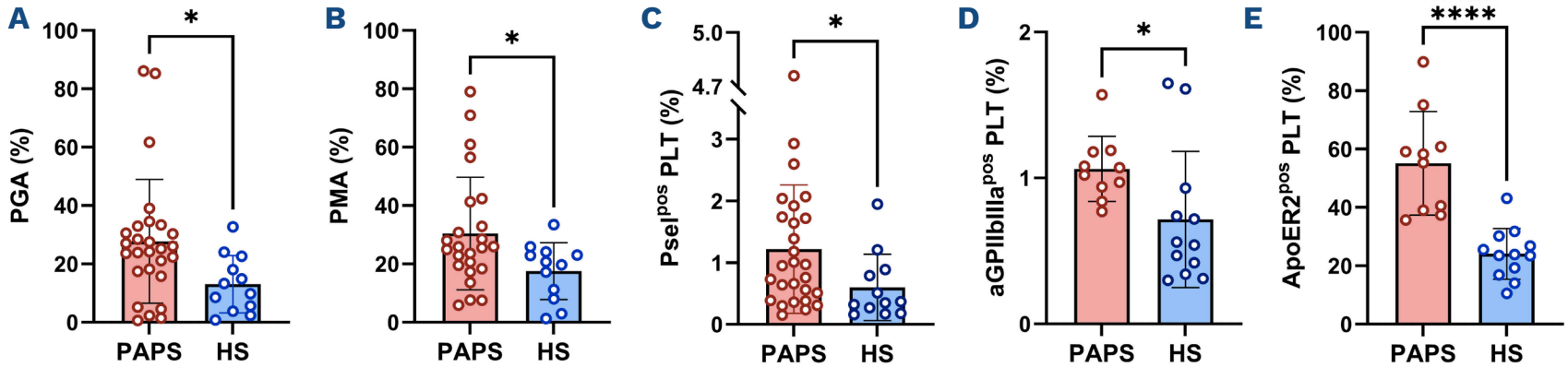
A 4-fold higher percentage of TF^{pos} platelet-leukocyte aggregates was observed in PAPS patients compared to HS (Figure 2D, E), which is consistent with the greater percentage of TF^{pos}-platelets. Indeed, imaging flow cytometry analysis of platelet-leukocyte aggregates showed that TF^{pos} signal (green) overlapped with that of the platelet (red) in ~88% of platelet-leukocyte aggregates and only with ~20% of the leukocyte population marker (white), corroborating the localization of TF mainly on platelets (yellow signal; Figure 3A, B). Furthermore, it is worth mentioning that a comparable percentage of TF^{pos}-monocytes and -granulocytes was found in PAPS patients and HS (*Online Supplementary Figure S2*).

Interestingly, the percentage of P-selectin^{pos}-, aGPIIbIIIa^{pos}- and TF^{pos}-platelets in PAPS and HS blood samples stimulated with ADP was similar, as were the total and TF^{pos}-platelet-leucocyte aggregates (*Online Supplementary Figure S3A-G*).

There was no relationship between platelet/platelet-leukocyte aggregates' activation phenotypes and clinical APS variants, aPL profile, and therapy (*data not shown*).

Primary anti-phospholipid syndrome IgG and plasma differently affect platelet phenotype *in vitro*

To investigate the role of aPL in modulating platelet phenotype, HS blood was spiked with affinity-purified total



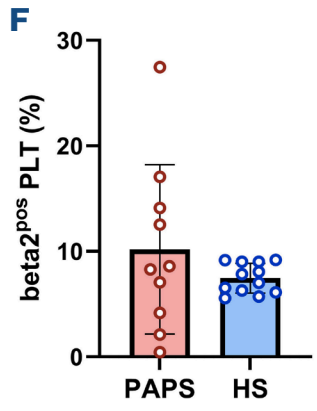


Figure 1. Markers of platelet activation in primary anti-phospholipid syndrome patients and healthy subjects. Platelet-granulocyte (PGA) (A) and platelet-monocyte (PMA) (B) aggregate formation as well as platelet-associated P-selectin (Psel) (C), activated GPIIbIIIa (aGPIIbIIIa) (D), ApoER2 (E) and β_2 glycoprotein I (β_2GPI) (F) expression were evaluated by whole blood flow cytometry in primary anti-phospholipid syndrome (PAPS) patients and healthy subjects (HS). Data are reported as individual values (PAPS N=24-27 for A-C and N=10 for D-F; HS N=12 for A-F; please see Methods for details) and mean ± standard deviation of antigen-positive cells. $^{*}P<0.05$; $^{****}P<0.001$.

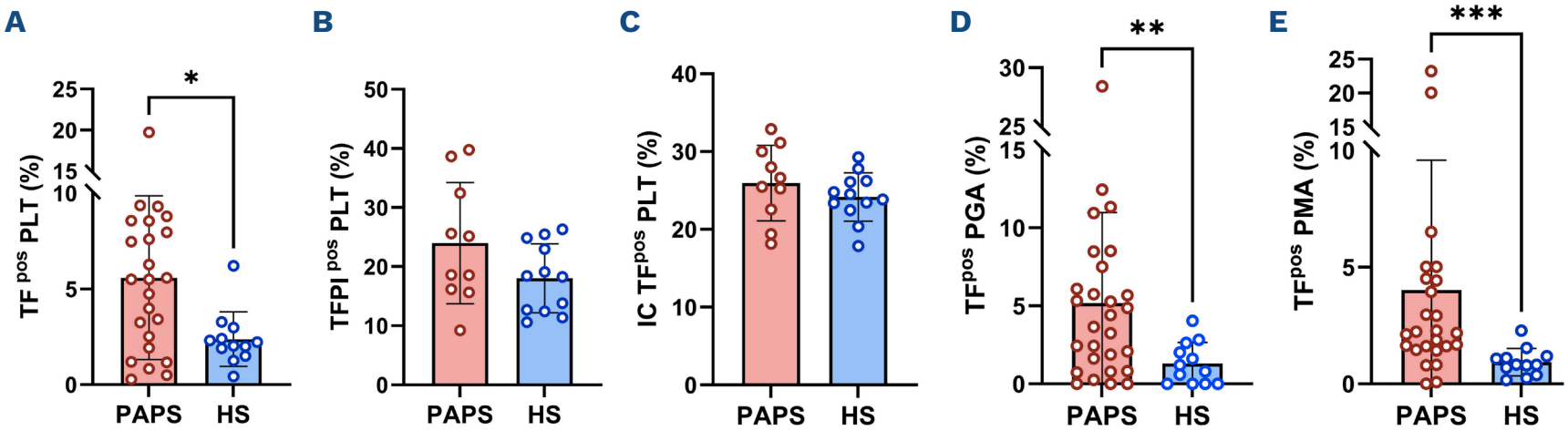


Figure 2. Procoagulant platelet phenotype in primary anti-phospholipid syndrome patients and healthy subjects. Flow cytometry analysis of platelets expressing surface-associated tissue factor (TF) (A) and tissue factor pathway inhibitor (TFPI) (B), as well as that of intracellular (IC) TF^{pos}-platelets (C) and of TF^{pos}-platelet-granulocyte (PGA) (D) and -platelet monocyte (PMA) (E) aggregates. Data are reported as individual values (primary anti-phospholipid syndrome [PAPS] N=24-28 for A, D, E and N=10 for B, C; healthy subjects [HS] N=12 for A-E; please see Methods for details) and mean ± standard deviation of antigen-positive cells. $^{*}P<0.05$; $^{**}P<0.01$; $^{***}P<0.001$.

PAPS IgG. The results show that PAPS IgG, unlike HS IgG used as control, significantly increased TF (9.9±1.2% *vs*. 2.5±0.9%; *P*=0.01) but not P-selectin and aGPIIbIIIa platelet expression (Figure 4A-C). The lack of effect of PAPS IgG on P-selectin expression is consistent with their inability to influence the percentages of platelet-leukocyte-aggregates (Figure 4D, E). To compare the effect of PAPS plasma *versus* IgG on platelet phenotype, mixing experiments were carried out reconstituting plasma-depleted blood from HS with PAPS plasma pool or HS autologous plasma. As a further pathological control, plasmas from CAD patients were also tested in a comparable assay. Only pooled plasma from PAPS patients significantly increased P-Selectin, aGPIIbIIIa, and platelet-leukocyte aggregates as well as TF expression on platelets and aggregates, fully reproducing the activated phenotype observed in the patients (Figure 5A-E).

In vitro effect of IL-6R-inhibitor, ApoER2, hydroxychloroquine, and anti-platelet drugs on healthy platelet phenotypes after incubation with anti-phospholipid syndrome IgG and plasma

Animal models of APS require a second inflammatory hit to display the aPL thrombotic effect in agreement with the clinical observation that infectious/inflammatory conditions are frequently associated with vascular events.[1] Thus, to understand the different activated phenotypes induced by affinity-purified IgG and plasma from PAPS patients, we also investigated the impact of a prototype inflammatory cytokine (i.e., IL-6) on affinity-purified IgG-treated blood from HS in *in vitro* experiments. The results showed that

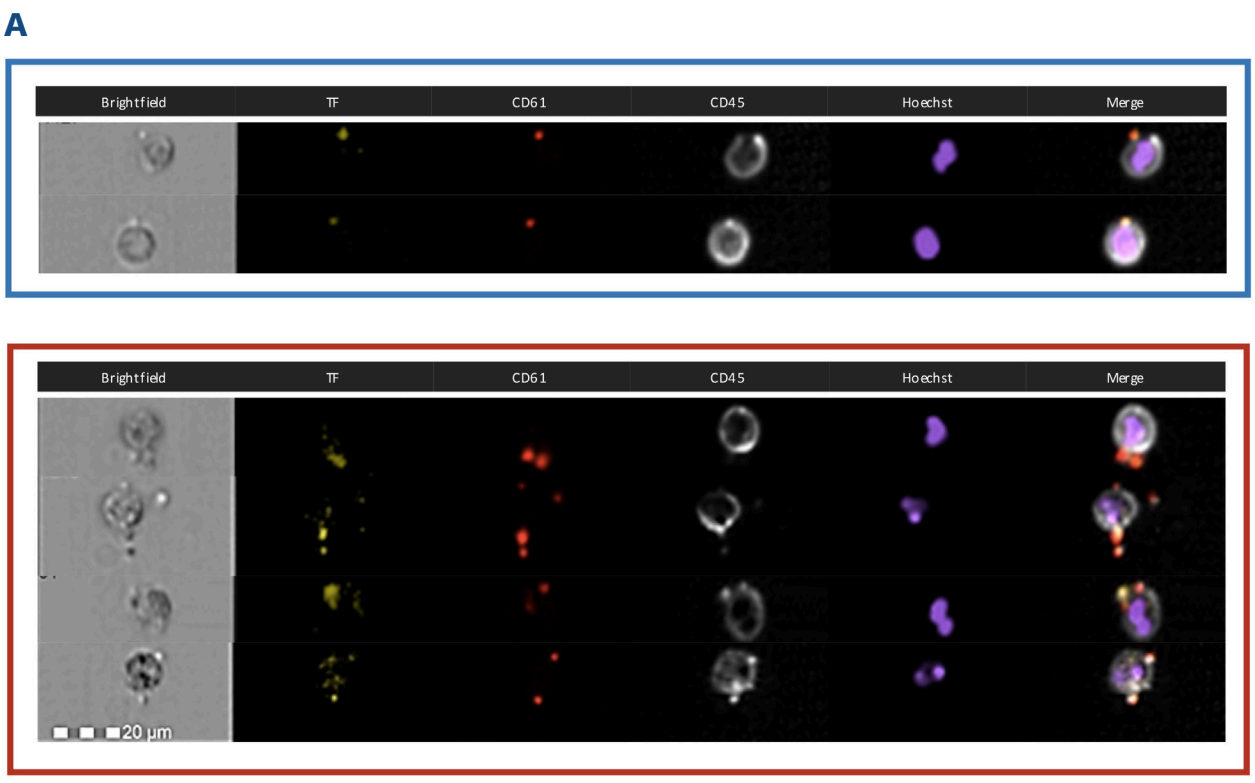


Figure 3. Cellular localization of tissue factor expressed on platelet-leukocyte aggregates. Analysis of tissue factor (TF)^{pos}-platelet-granulocyte (PGA) (A) and TF-^{pos}-platelet-monocyte (PMA) (B) aggregates in whole blood from healthy subjects (HS) (blue frame) and primary anti-phospholipid syndrome (PAPS) patients (red frame) by imaging flow cytometry. Representative channel images of brightfield (gray), TF (green), platelet population marker CD61 (red), leukocyte population marker CD45 (white) expression, and nuclear staining (Hoechst, magenta) acquired by ImageStream^{X} Mk II, at 60X magnification, are shown. In the merged images, the yellow color represents the co-localization of TF (green) with CD61 (red) corroborating the platelet localization of TF.

incubation of blood from HS with IL-6 alone increased the percentage of TF^{pos}-platelets while not affecting P-selectin as well as aGPIIbIIIa expression. The whole platelet thrombo-inflammatory phenotype was fully reproduced when blood from HS was treated with IL-6 and PAPS IgG (Figure 6) but not with HS IgG (*Online Supplementary Figure S4*).

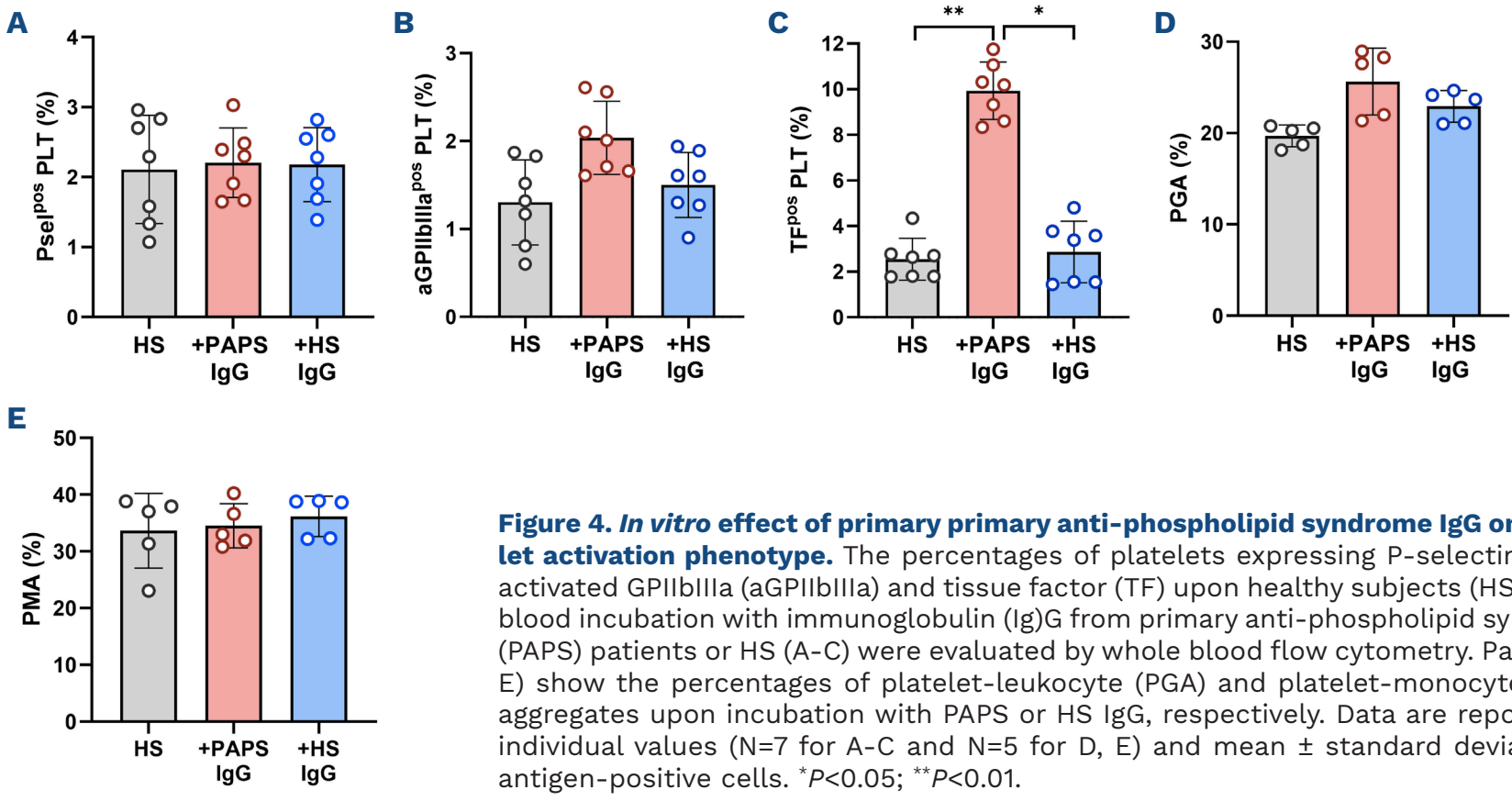


Figure 4. *In vitro* effect of primary primary anti-phospholipid syndrome IgG on platelet activation phenotype. The percentages of platelets expressing P-selectin (Psel), activated GPIIbIIIa (aGPIIbIIIa) and tissue factor (TF) upon healthy subjects (HS) whole blood incubation with immunoglobulin (Ig)G from primary anti-phospholipid syndrome (PAPS) patients or HS (A-C) were evaluated by whole blood flow cytometry. Panels (D, E) show the percentages of platelet-leukocyte (PGA) and platelet-monocyte (PMA) aggregates upon incubation with PAPS or HS IgG, respectively. Data are reported as individual values (N=7 for A-C and N=5 for D, E) and mean ± standard deviation of antigen-positive cells. $^{*}P<0.05$; $^{**}P<0.01$.

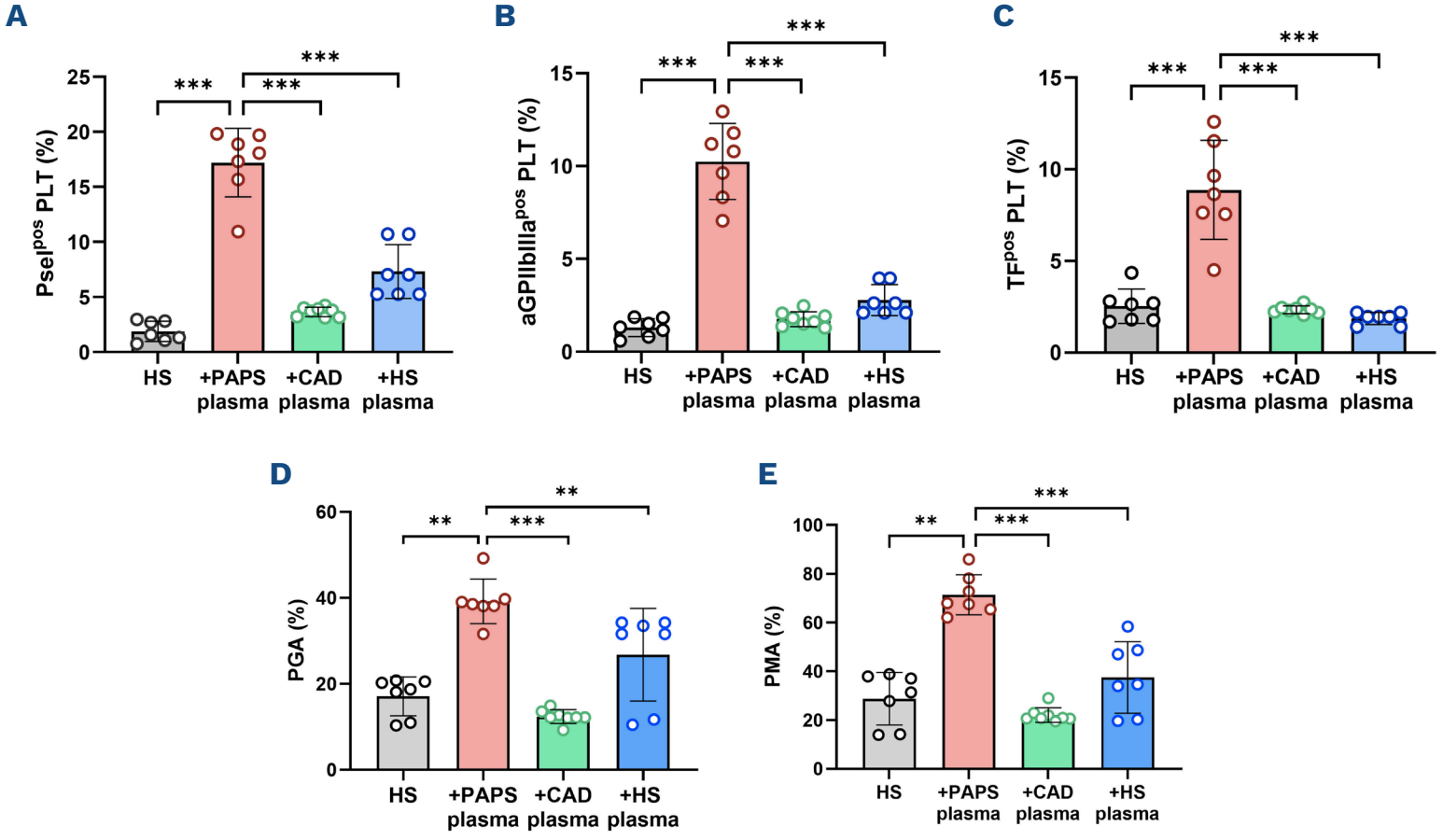


Figure 5. *In vitro* effect of primary anti-phospholipid syndrome plasmas on adhesive/prothrombotic platelet phenotype and heteroaggregate formation. The figure shows the percentages of platelets expressing P-selectin (Psel; A), activated GPIIbIIIa (aGPIIbIIIa; B) and tissue factor (TF; C) upon whole blood incubation with pooled primary anti-phospholipid syndrome plasma (PAPS; red bars), plasma from chronic coronary artery diseas (CAD) patients (green bars) or autologous healthy subjects' plasmas (HS; blue bars). Panels (D, E) show the percentages of platelet-granulocyte (PGA) and platelet-monocyte (PMA) aggregates upon incubation with PAPS, CAD or autologous HS plasmas, respectively. The expression of platelet activation markers in whole blood of HS was reported as reference (grey bars). Data are reported as individual values (N=7) and mean ± standard deviation of antigen-positive cells. $^{**}P<0.01$; $^{***}P<0.001$.

This effect was inhibited by a monoclonal IgG blocking IL-6R (tocilizumab; Figure 6), which was also effective in mixing experiments with PAPS plasma (Figure 7).

The platelet activation and platelet-leukocyte formation induced by PAPS plasma and PAPS IgG plus IL-6 were reduced by the ApoER2 inhibitor (RAP), further supporting the involvement of β2GPI engaged by this receptor (Figures 6 and 7).

Interestingly, the activated platelet phenotype was also reduced by aspirin and Clopidogrel/AR-C69931MX (a $P2Y_{12}$ antagonist), drugs frequently used in primary and secondary vascular prophylaxis.[2] Of note, HCQ, another molecule effective on platelet activation,[26-28] prevented P-selectin and aGPIIbIIIa upregulation without significantly affecting TF expression either induced by PAPS IgG (14.3±2.8% and 14.2±3.7% TF^{pos} platelets, without and upon HCQ preincubation, respectively; *P*=0.963) (Figure 8) or PAPS plasma (8.8±2.7% and 5.1±1.7% TF^{pos} platelets, without and upon HCQ preincubation, respectively; *P*=0.123; Figure 7).

Control experiments with HS plasmas did not affect P-selectin, aGPIIbIIIa, TF, and platelet-leukocyte aggregate formation (*data not shown*).

β2GPI-dependent anti-phospholipid antibodies epitope specificity is pivotal in affecting platelet phenotype modulation *in vitro*

Sera from APS patients may contain β2GPI-dependent aPL reacting with epitopes located in different molecule domains and with different pathogenic/predictive values. In particular, aPL reacting with β2GPI-D1 are widely accepted

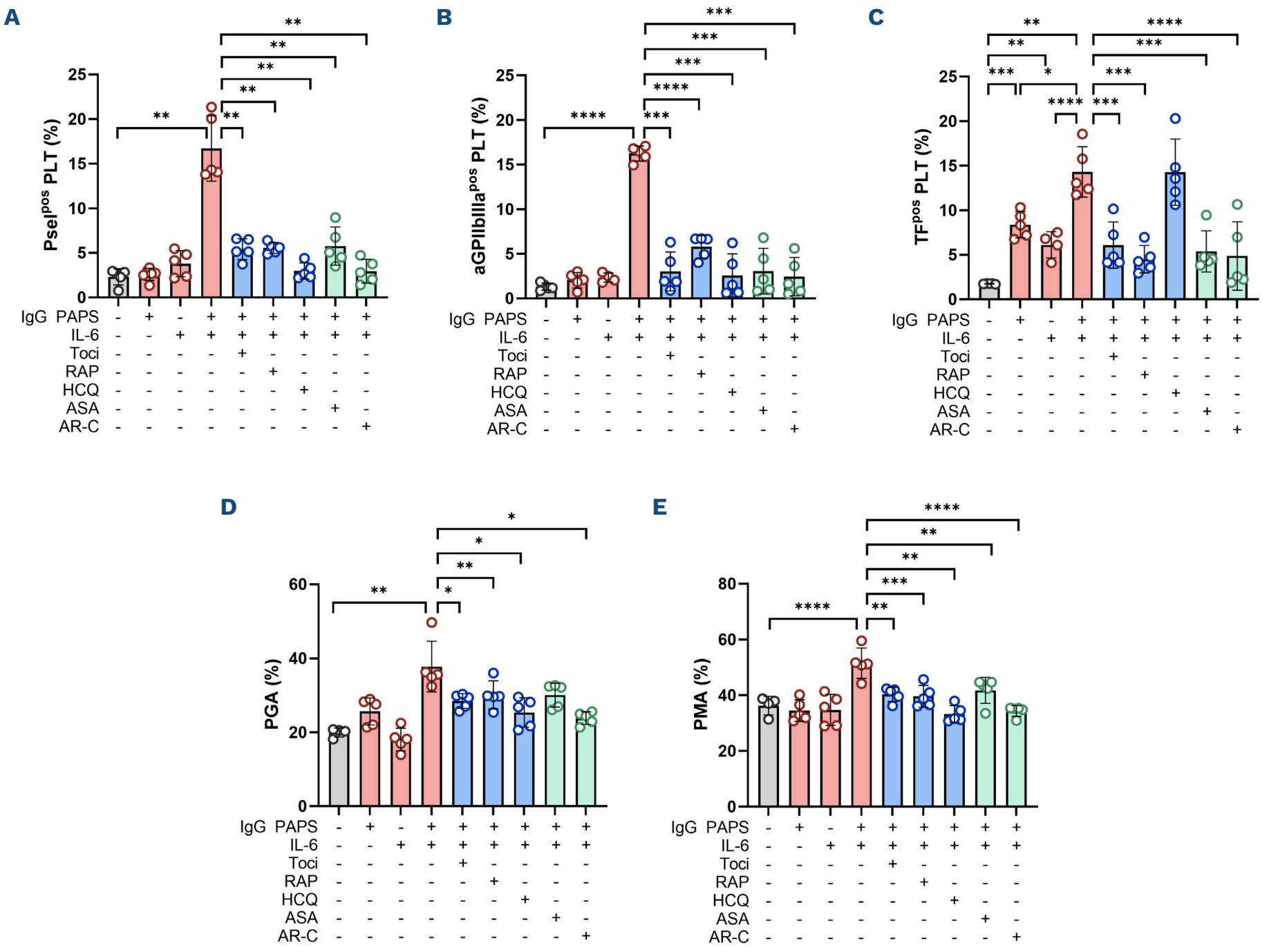


Figure 6. Pharmacological inhibition of IgG-mediated P-selectin and tissue factor platelet expression. Percentages of platelets expressing P-selectin (Psel), activated GPIIbIIIa (aGPIIbIIIa) and tissue factor (TF) (A-C) were analyzed upon healthy subjects (HS) whole blood incubation with primary anti-phospholipid syndrome (PAPS) immunoglobulin (Ig)G in the presence or absence of interleukin (IL)-6 (100 pg/mL), IL-6R inhibitors (tocilizumab [toci] 300 μg/mL), ApoER2 inhibitor (RAP, 13.7 μg/mL), hydroxychloroquine (HCQ) (10 μg/mL), aspirin (ASA) (8 μM) or the selective platelet $P2Y_{12}$ antagonist AR-C69931MX (AR-C) (100 nM). Comparable experiments measuring platelet-granulocyte (PGA) (D) and -monocyte (PMA) (E) aggregates were performed. Data are reported as individual value (N=5) and mean ± standard deviation of antigen-positive cells. $^{*}P<0.05$; $^{**}P<0.01$; $^{***}P<0.001$ and $^{****}P<0.0001$.

as the antibodies with the strongest predictive/diagnostic value. At variance, antibodies directed against epitopes located in D4-5 are not associated with the APS clinical manifestations and are not thrombogenic in an animal model.[22,23,29,30] We also tested IgG fractions from two APS patients specifically reacting only with a synthetic D4-5 of β2GPI.[22] Unlike anti-D1, which induced a 4-fold increase in the percentage of TF^{pos}-platelets, anti-D4-5 β2GPI IgG did not affect platelet phenotype in the *in vitro* experiments (*Online Supplementary Figure S5*). Interestingly, adding IL-6 did not modify the effect of anti-D4-5 IgG on platelet phenotype (*data not shown*).

Discussion

This study shows for the first time that (i) in PAPS patients levels of circulating platelets expressing ApoER2, one of the β2GPI receptors on the cell membrane, are three times greater than in HS; (ii) PAPS IgG, reacting with β2GPI-D1, added to HS blood induce platelet-associated TF expression, favoring the onset of a prothrombotic phenotype; (iii) PAPS IgG added to HS blood induce the activated platelet phenotype observed in PAPS patients - with activated GPIIbIIIa expression and increased P-selectin presence which leads to platelet-leukocyte formation - only in the presence of low-grade inflammation; (iv) plasma of PAPS patients added to plasma-depleted blood of HS reproduces the platelet activated phenotype observed in PAPS patients and this effect is blunted by tocilizumab; (v) antiplatelet drugs, such as aspirin or $P2Y_{12}$ inhibitors, inhibit platelet activation induced by PAPS IgG or PAPS plasma while HCQ does not affect TF expression in a statistical significant manner.

Our data extend the findings of activated platelets in

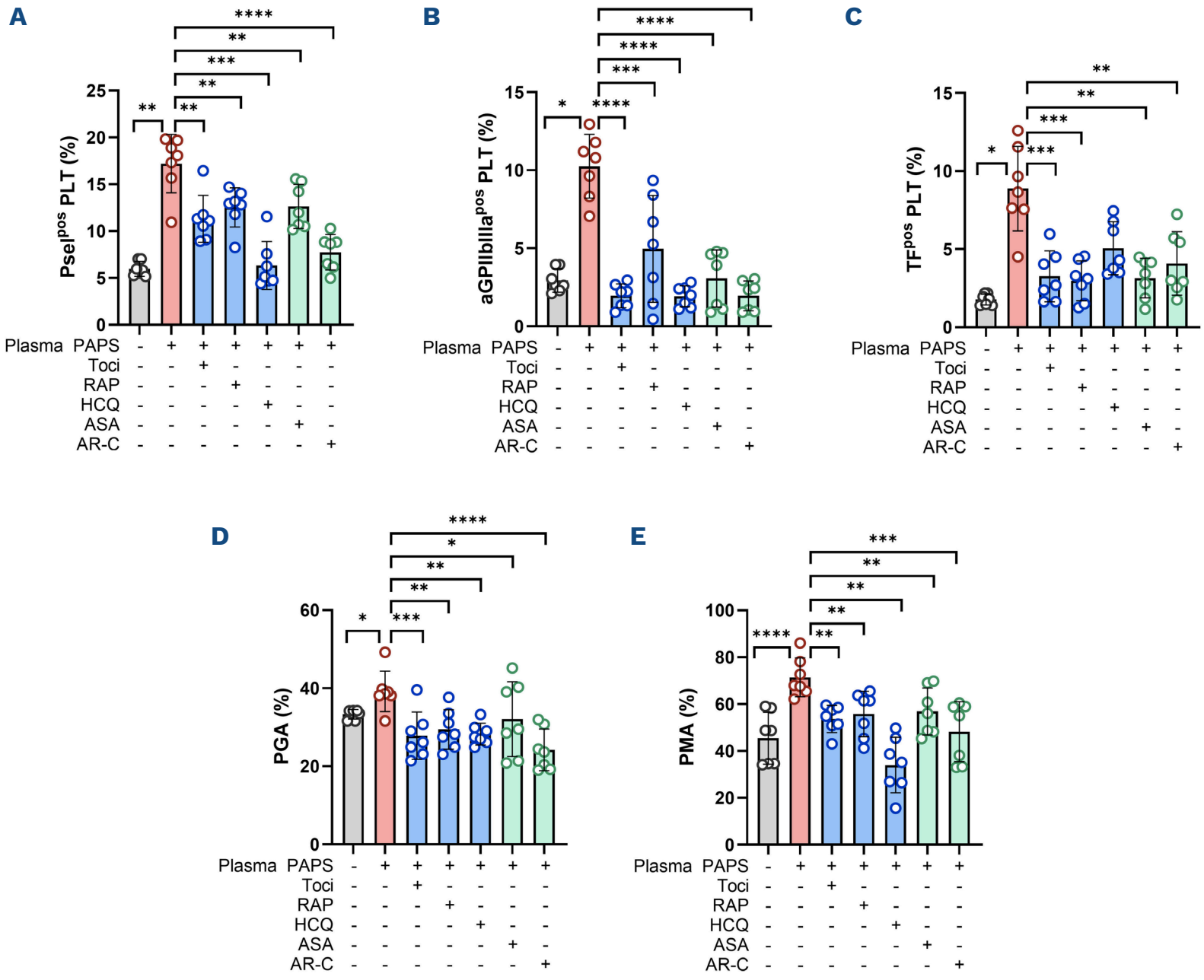


Figure 7. Pharmacological inhibition of plasma-mediated adhesive and thrombophilic platelet phenotype. Percentages of platelets expressing P-selectin (Psel), activated GPIIbIIIa (aGPIIBIII) and tissue factor (TF) (A-C) were analyzed upon healthy subjects (HS) whole blood incubation with primary anti-phospholipid syndrome (PAPS) plasma in the presence or absence of interleukin (IL)-6R inhibitors (tocilizumab [toci] 300 μg/mL), ApoER2 inhibitor (RAP, 13.7 μg/mL), hydroxychloroquine (HCQ) (100 μg/mL), aspirin (ASA) (8 μM) or the selective platelet $P2Y_{12}$ antagonist AR-C69931MX (AR-C) (100 nM). Comparable experiments measuring platelet-granulocyte (PGA) (D) and -monocyte (PMA) (E) aggregates were performed. Data are reported as individual value (N=7) and mean ± standard deviation of antigen-positive cells. $^{*}P<0.05$; $^{**}P<0.01$; $^{***}P<0.001$ and $^{****}P<0.0001$.

PAPS patients, characterized by a pro-inflammatory and pro-thrombotic phenotype in a homogeneous cohort selected according to the new classification criteria and displaying an anti-β2GPI-D1 specificity.[8,10,21] The increased expression of P-selectin and activated GPIIbIIIa is indeed consistent with the pro-adhesive phenotype and levels of platelet-leukocyte aggregates greater than in HS.[16,17] The classical vascular syndrome is mainly a localized thrombotic vasculopathy at variance with the systemic catastrophic APS variant. The interaction between platelets and leukocytes at the site of endothelial injury contributes to the APS thrombo-inflammatory process.[1] Within this process, we found an increased percentage of circulating TF_{pos}-platelets and hetero-aggregates, further supporting the idea that platelets and leukocytes, neutrophils in particular, play a critical role downstream of the initial endothelial perturbation mediated by aPL. Interestingly, as far as the prothrombotic phenotype is concerned, it is worth mentioning that TF was not up-regulated in monocytes and granulocytes of PAPS patients - among the circulating blood cells - but only in platelets, that were the source of the protein also in the hetero-aggregates, as clearly evidenced by image flow cytometry. These data point to a selective effect of APS β2GPI-dependent IgG on platelets, which is also consistent with the upregulation of ApoER2, one of the cell membrane receptors for β2GPI.[1] The involvement of this receptor in the observed platelet activation is supported by *in vitro* experiments in which a specific inhibitor prevents the IgG effect.

Platelet-associated TF expression is a feature of a subset of platelets released from bone marrow megakaryocytes through a finely regulated mechanism.[31,32] Under physiological conditions, TF is stored in the open canalicular system (OCS) of platelets, making it inaccessible to antibody binding and detection by flow cytometry.[25] As a result, only a small number of TF^{pos}-platelets can be detected under resting condition. Upon activation with the most common platelet agonists such as ADP, thrombin, thromboxane, rapid events such as shape change, cytoskeleton reorganization and OCS externalization occur, exposing this protein to the blood where it can bind and activate the coagulation factor VII.[25,33-35] TF expression on the platelet surface is also induced following activation with PAPS IgG, as shown in this study and also previously reported,[10] resulting in an increase in the number of detectable TF^{pos}-platelets. PAPS IgG induce a signaling pathway mediated by the TLR-4 receptor through IRAK phosphorylation and NF-κB activation, and modulated by heparanase inhibitors.[36] Interestingly, platelet-associated TF expression induced by PAPS IgG is also modulated by $P2Y_{12}$ inhibitors, as demonstrated in this study. This finding highlights a shared signaling pathway between PAPS IgG and ADP and suggests a cross-talk between these two signaling molecules. Pathological conditions may affect the number of $ICTF^{pos}$ and TF^{pos}-platelets released into the bloodstream.[37] In this regard, the fact that the total number of platelets with intracellularly stored TF was similar in patients with PAPS and HS suggests that the mechanism supporting the more activated platelet phenotype found in patients does not act on megakaryocytes in the bone marrow, but rather acts directly on platelets in the bloodstream. This finding is supported by the evidence that *ex vivo* stimulation of platelets from PAPS patients and HS with ADP resulted in a similar expression of TF, further highlighting the presence of an altered basal state rather than an increased overall responsiveness. The clinical relevance of the greater number of TF^{pos}-platelets found in PAPS patients lies in the significant correlation between platelet-associated TF expression and its functional activity, measured as thrombin generation capacity,[38,39] thus providing the rationale for the prothrombotic phenotype of PAPS patients.

Data from *in vitro* mixing experiments further brought knowledge on the etiopathogenesis of thrombotic complications in PAPS patients. Indeed, they showed that incubation of blood from HS with affinity-purified PAPS IgG fractions increased TF but not P-selectin and activated GPIIbIIIa expression and hetero-aggregate formation at variance with HS IgG. It is important to emphasize that IgG from patients with positivity only for anti-β2GPI-D4,5 did not affect platelet phenotype in contrast to PAPS IgG, all positive for anti-β2GPI D1. Conversely, PAPS plasmas upregulated both HS platelets' P-selectin, activated GPIIbIIIa and TF expression, and increased the percentages of TF^{pos} hetero-aggregates. The fact that aPL-negative plasmas from CAD patients did not affect platelet phenotype and did not increase hetero-aggregate formation further supports the crucial role of aPL. However, the finding that PAPS IgG alone did not affect P-selectin and activated GPIIbIIIa expression suggests that additional plasma components play a role in mediating the pro-adhesive platelet phenotype. The increased expression of P-selectin and activated GPIIbIIIa is a characteristic of activated platelets in inflammatory conditions.[12,13] Even though PAPS is an autoimmune thrombotic vasculopathy not associated with a full-blown systemic inflammation, an "inflammatory second hit" has been demonstrated in animal models and suggested in the clinical setting.[1,40] The demonstration that adding a prototype inflammatory mediator, such as IL-6, to PAPS IgG restores P-selectin and activated GPIIbIIIa upregulation is consistent with the hypothesis that thrombo-inflammation is needed to form platelet-leukocyte aggregates with a thrombophilic profile.[1] The role of IL-6 in supporting platelet activation is further demonstrated by the inhibitory effect of the blocking monoclonal anti-IL-6R IgG (Tocilizumab) added to the plasma of PAPS patients.

Both ASA and $P2Y_{12}$ antagonist inhibit P-selectin, activated GPIIbIIIa and TF upregulation and the formation of hetero-aggregates when HS blood is incubated with PAPS plasmas or with PAPS IgG together with exogenous IL-6. These data are consistent with meta-analysis studies showing a strong decrease in the risk of first thrombosis in aPL carriers treated with low-dose ASA.[2,4] Moreover, the inhibition of platelet pro-adhesive and pro-thrombotic phenotype by

ASA and $P2Y_{12}$ antagonists speaks in favor of their single or dual use in the secondary prevention of recurrent thrombosis in people with APS, despite the very low-certainty evidence reported in a recent meta-analysis study.[2]

There is evidence of HCQ's beneficial adjunctive role in preventing thrombosis in APS, mainly in patients suffering from systemic lupus erythematosus (SLE).[5] HCQ inhibition of platelet adhesive phenotype upregulation and the consequent formation of hetero-aggregates is consistent with its suggested clinical efficacy. However, the lack of a statistically significant effect on TF expression raises concerns about its use as a single therapy and suggests its efficacy, at least in SLE-APS, may be due to other pharmacological mechanisms.[4,41]

All the investigated PAPS plasmas were positive for anti-β2GPI antibodies reacting with D1 and could induce the platelet adhesive phenotype and TF upregulation. This finding is consistent with the widely accepted idea that this type of antibody represents the leading pathogenic group of aPL.[1] On the contrary, antibodies reacting only with D4,5 are not associated with the clinical manifestations and are not thrombogenic in animals.[29,30] Interestingly, sera monospecific for D4,5 did not mediate the platelet pro-adhesive and pro-thrombotic phenotype at variance with the sera positive for anti-β2GPI-D1. These findings further support the specificity of the reported data and the critical pathogenic role of anti-β2GPI-D1 aPL.

Limitations of this study are that most of our patients displayed a high-risk aPL profile (i.e., double/triple positivity, medium/high aPL titers, and reactivity against β2GPI D1) and were on anticoagulant or antiplatelet prophylactic therapy. The high aPL risk profile and the concomitant therapy may be confounding variables potentially responsible for the lack of association between the levels of platelet-leukocyte aggregates and aPL profile or treatment. Increased platelet-leuko-aggregates have been found in patients treated with VKA as well as in a few patients on LDASA only. This finding is consistent with the risk of recurrences even in treated patients as reported in large registry studies.[2,4] Despite that, we did not find significant differences in the platelet phenotype in the three patients investigated before therapy.

In conclusion, analyzing a well-defined and very homogeneous cohort of PAPS, we have investigated the ability of anti-β2GPI D1 IgG to induce a pro-adhesive and pro-thrombotic platelet phenotype in PAPS pathogenesis and evaluated the effect of molecules with different pharmacological mechanisms. Further studies are needed to determine whether the experimental models can predict the clinical response to the therapy.

Disclosures

No conflicts of interest to disclose.

Contribution

PLM, MC, FT, and MOB planned the study and wrote the manuscript. PLM and LT enrolled patients. MB, PAL, AB, CG, KN and ADV carried out the experiments. MB and PAL analyzed results and made the figures. All authors agreed on the content of the manuscript, reviewed drafts, and approved the final version.

Funding

This work was supported by the Italian Ministry of Health - Ricerca Corrente.

Data-sharing statement

Data are available on request from the corresponding authors.

References

1. Raschi E, Borghi MO, Tedesco F, Meroni PL. Antiphospholipid syndrome pathogenesis in 2023: an update of new mechanisms or just a reconsideration of the old ones? Rheumatology (Oxford). 2024;63(SI):SI4-SI13.
2. Bala MM, Celinska-Lowenho M, Szot W, et al. Antiplatelet and anticoagulant agents for secondary prevention of stroke and other thromboembolic events in people with antiphospholipid syndrome. Cochrane Database Syst Rev. 2020;10(10):CD012169.
3. Tektonidou MG, Andreoli L, Limper M, et al. EULAR recommendations for the management of antiphospholipid syndrome in adults. Ann Rheum Dis. 2019;78(10):1296-1304.
4. Arnaud L, Mathian A, Devilliers H, et al. Patient-level analysis of five international cohorts further confirms the efficacy of aspirin for the primary prevention of thrombosis in patients with antiphospholipid antibodies. Autoimmun Rev. 2015;14(3):192-200.
5. Ruiz-Irastorza G, Tektonidou MG, Khamashta M. Anticoagulant and non-anticoagulant therapy in thrombotic antiphospholipid syndrome: old drugs and new treatment targets. Rheumatology (Oxford). 2024;63(SI):SI96-SI106.
6. Cervera R, Serrano R, Pons-Estel GJ, et al. Morbidity and mortality in the antiphospholipid syndrome during a 10-year period: a multicentre prospective study of 1000 patients. Ann Rheum Dis. 2015;74(6):1011-1018.
7. De Pascali F, Filippova YA, Donadini MP, Pengo V, Squizzato A. Efficacy and safety of low-dose acetylsalicylic acid for the prevention of thromboembolic events in individuals positive for antiphospholipid antibodies: a systematic review and meta-analysis. Thromb Res. 2025;245:109225.
8. Huang S, Ninivaggi M, Chayoua W, de Laat B. VWF, Platelets and the antiphospholipid syndrome. Int J Mol Sci. 2021;22(8):4200.
9. Proulle V, Furie RA, Merrill-Skoloff G, Furie BC, Furie B. Platelets are required for enhanced activation of the endothelium and fibrinogen in a mouse thrombosis model of APS. Blood. 2014;124(4):611-622.
10. Capozzi A, Manganelli V, Riitano G, et al. Tissue factor overexpression in platelets of patients with anti-phospholipid syndrome: induction role of anti-β2-GPI antibodies. Clin Exp

Immunol. 2019;196(1):59-66.
11. Griem K, Falter T, Hollerbach A, et al. Association of specific antiphospholipid antibodies to platelet count and thrombocytopenia. Thromb Haemost. 2025 Jan 27. doi: 10.1055/a-2510-6147. [Epub ahead of print]
12. Nicolai L, Pekayvaz K, Massberg S. Platelets: orchestrators of immunity in host defense and beyond. Immunity. 2024;57(5):957-972.
13. Mack A, Vanden Hoek T, Du X. Thromboinflammation and the role of platelets. Arterioscler Thromb Vasc Biol. 2024;44(6):1175-1180.
14. Pluta K, Porębska K, Urbanowicz T, et al. Platelet-leucocyte aggregates as novel biomarkers in cardiovascular diseases. Biology (Basel). 2022;11(2):224. Erratum in: Biology (Basel). 2022;11(11):1554.
15. Brambilla M, Camera M, Colnago D, et al. Tissue factor in patients with acute coronary syndromes: expression in platelets, leukocytes, and platelet-leukocyte aggregates. Arterioscler Thromb Vasc Biol. 2008;28(5):947-953.
16. Joseph JE, Harrison P, Mackie IJ, Isenberg DA, Machin SJ. Increased circulating platelet-leucocyte complexes and platelet activation in patients with antiphospholipid syndrome, systemic lupus erythematosus and rheumatoid arthritis. Br J Haematol. 2001;115(2):451-459.
17. NaveenKumar SK, Tambralli A, Fonseca BM, et al. Low ectonucleotidase activity and increased neutrophil-platelet aggregates in patients with antiphospholipid syndrome. Blood. 2024;143(12):1193-1197.
18. Barbhaiya M, Zuily S, Naden R, et al. 2023 ACR/EULAR antiphospholipid syndrome classification criteria. Ann Rheum Dis. 2023;82(10):1258-1270.
19. Andreoli L, Chighizola CB, Nalli C, et al. Clinical characterization of antiphospholipid syndrome by detection of IgG antibodies against β2 -glycoprotein i domain 1 and domain 4/5: ratio of anti-domain 1 to anti-domain 4/5 as a useful new biomarker for antiphospholipid syndrome. Arthritis Rheumatol. 2015;67(8):2196-2204.
20. Chighizola CB, Pregnolato F, Andreoli L, et al. Beyond thrombosis: anti-β2GPI domain 1 antibodies identify late pregnancy morbidity in anti-phospholipid syndrome. J Autoimmun. 2018;90:76-83.
21. Lonati PA, Scavone M, Gerosa M, et al. Blood cell-bound C4d as a marker of complement activation in patients with the antiphospholipid syndrome. Front Immunol. 2019;10:773.
22. Durigutto P, Grossi C, Borghi MO, et al. New insight into antiphospholipid syndrome: antibodies to β2glycoprotein I-domain 5 fail to induce thrombi in rats. Haematologica. 2019;104(4):819-826.
23. Agostinis C, Durigutto P, Sblattero D, et al. A non-complement-fixing antibody to β2 glycoprotein I as a novel therapy for antiphospholipid syndrome. Blood. 2014;123(22):3478-3487.
24. Brambilla M, Canzano P, Valle PD, et al. Head-to-head comparison of four COVID-19 vaccines on platelet activation, coagulation and inflammation. The TREASURE study. Thromb Res. 2023;223:24-33.
25. Brambilla M, Becchetti A, Rovati GE, et al. Cell surface platelet tissue factor expression: regulation by P2Y12 and link to residual platelet reactivity. Arterioscler Thromb Vasc Biol. 2023;43(10):2042-2057.
26. Meroni PL. Prevention and treatment of obstetrical complications in APS: is hydroxychloroquine the Holy Grail we are looking for? J Autoimmun. 2016;75:1-5.
27. Espinola RG, Pierangeli SS, Gharavi AE, Harris EN. Hydroxychloroquine reverses platelet activation induced by human IgG antiphospholipid antibodies. Thromb Haemost. 2002;87(3):518-522.
28. Rand JH, Wu XX, Quinn AS, Chen PP, Hathcock JJ, Taatjes DJ. Hydroxychloroquine directly reduces the binding of antiphospholipid antibody-beta2-glycoprotein I complexes to phospholipid bilayers. Blood. 2008;112(5):1687-1695.
29. Meroni PL, Borghi MO. Antiphospholipid antibody assays in 2021: looking for a predictive value in addition to a diagnostic one. Front Immunol. 2021;12:726820.
30. Radin M, Cecchi I, Roccatello D, Meroni PL, Sciascia S. Prevalence and thrombotic risk assessment of anti-β2 glycoprotein I domain I antibodies: a systematic review. Semin Thromb Hemost. 2018;44(5):466-474.
31. Brambilla M, Facchinetti L, Canzano P, et al. Human megakaryocytes confer tissue factor to a subset of shed platelets to stimulate thrombin generation. Thromb Haemost. 2015;114(3):579-92.
32. Brambilla M, Rossetti L, Zara C, et al. Do methodological differences account for the current controversy on tissue factor expression in platelets? Platelets. 2018;29(4):406-414.
33. Camera M, Frigerio M, Toschi V, et al. Platelet activation induces cell-surface immunoreactive tissue factor expression, which is modulated differently by antiplatelet drugs. Arterioscler Thromb Vasc Biol. 2003;23(9):1690-1696.
34. Camera M, Brambilla M, Toschi V, Tremoli E. Tissue factor expression on platelets is a dynamic event. Blood. 2010;116(23):5076-5077.
35. Camera M, Brambilla M, Facchinetti L, et al. Tissue factor and atherosclerosis: not only vessel wall-derived TF, but also platelet-associated TF. Thromb Res. 2012;129(3):279-284.
36. Capozzi A, Riitano G, Recalchi S, et al. Effect of heparanase inhibitor on tissue factor overexpression in platelets and endothelial cells induced by anti-β2-GPI antibodies. J Thromb Haemost. 2021;19(9):2302-2313.
37. Brambilla M, Gelosa P, Rossetti L, et al. Impact of angiotensin-converting enzyme inhibition on platelet tissue factor expression in stroke-prone rats. J Hypertens. 2018;36(6):1360-1371.
38. Canzano P, Brambilla M, Porro B, et al. Platelet and endothelial activation as potential mechanisms behind the thrombotic complications of COVID-19 patients. JACC Basic Transl Sci 2021;6(3):202-218.
39. Trabattoni D, Brambilla M, Canzano P, et al. Migraine in patients undergoing PFO closure: characterization of a platelet-associated pathophysiological mechanism: the LEARNER study. JACC Basic Transl Sci 2022;7(6):525-540.
40. Erkan D, Willis R, Murthy VL, et al. A prospective open-label pilot study of fluvastatin on proinflammatory and prothrombotic biomarkers in antiphospholipid antibody positive patients. Ann Rheum Dis. 2014;73(6):1176-1180.
41. Tektonidou MG, Andreoli L, Limper M, et al. EULAR recommendations for the management of antiphospholipid syndrome in adults. Ann Rheum Dis. 2019;78(10):1296-1304.