



Increased Platelet Adhesiveness in Patients with Venous Thromboembolic Disease

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Abstract

Background Association between global platelet function and the risk of venous thromboembolic disease (VTE) has been proposed, though the mechanisms do not involve increased platelet aggregation. However, platelet adhesiveness has not been systematically explored in VTE patients.

Objectives To evaluate platelet adhesive functions in VTE patients.

Methods Platelet adhesion was evaluated by using whole blood samples from VTE patients, selected based on short closure times on the PFA-100 ($n = 54$), and matched healthy individuals ($n = 57$) in: (i) the PFA-100, (ii) a cone plate analyzer (CPA), on a plastic surface, (iii) microfluidic devices, with two- and three-dimensional evaluation, and (iv) membrane glycoprotein analysis. Intraplatelet signaling was evaluated in isolated collagen type I (Col-I) activated platelets and platelets adhered on Col-I or von Willebrand factor (VWF) coated coverslips under flow. VWF antigen and ADAMTS-13 activity were measured in plasma samples.

Results PFA-100 closure times remained significantly shorter in patients. The CPA test showed a significant increase in the platelet aggregates size when using blood from VTE patients. Platelet adhesion on Col-I revealed a higher area covered by platelets and increased aggregate volume when exposed to samples from VTE patients. Protein P-

Keywords

- closure times
- intraplatelet protein
- platelet adhesiveness
- platelet aggregation
- venous thromboembolism

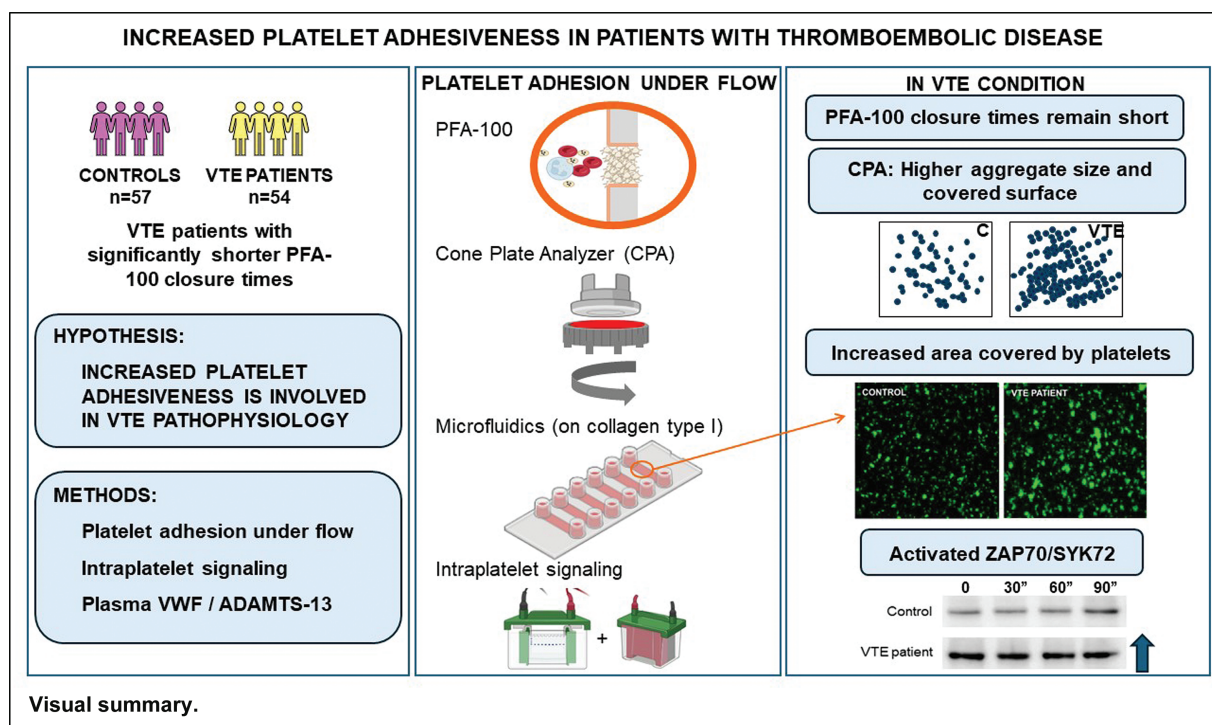
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ZAP70/SYK72 showed a phosphorylation level significantly increased in patients' platelets. Plasma VWF was significantly elevated in VTE patients.

Conclusions Platelets from VTE patients exhibit a proadhesive phenotype under flow conditions potentially related to the shortened occlusion times with the PFA-100. This enhanced adhesiveness may be explained by higher intraplatelet ZAP70/SYK72 phosphorylation and increased plasma VWF in patients. Therefore, primary hemostasis plays a significant role in the pathophysiology of VTE.

Introduction

The precise causes of venous thromboembolic disease (VTE) are still largely unknown. A complex and multifactorial nature resulting from the interaction of genetic and environmental factors is recognized. The genetic component is very considerable (around 60%, according to data from the Genetic Analysis of Idiopathic Thrombophilia 1 [GAIT 1] study¹), but the enormous efforts made in the recent years through global genome association studies (GWAS) have hardly added new information to what is already known (FVL, PT20210A, ABO).^{2,3} Both the GWAS and the more classical specific studies of candidate genes have detected some new genetic variants that carry an increased risk of venous thrombosis.³ Although some could be found in genes encoding platelet membrane glycoproteins,⁴ there is no incontestable evidence yet and none of the potential variants have been associated with global platelet function, and with platelet adhesiveness in particular. Therefore, although the genetic base of VTE is very large, to date only a relatively small part of the heritability is known. Epigenetics may play a role in increasing venous occlusive events⁵ and, also, further

attention should be paid to genes related to platelet over-reactivity.

There are previous studies that support the role of platelets in the development of thrombosis, especially arterial. The presence of platelets in vascular thrombi and emboli and the verification of the effects due to their activation, such as local secretion of vasoconstrictor, procoagulant or activating substances, and inflammation mediators^{6,7} explain their involvement in atherothrombotic disease. Increased platelet reactivity has been reported by multiple research groups in association with arterial thromboembolic pathology.^{8–16}

The platelet mechanisms that determine the risk of venous thromboembolism have been much less studied. The results of the study by von Brühl et al¹⁷ revealed that the cellular cooperation between platelets, neutrophils, and monocytes is essential for the initiation and propagation of the venous thrombus. This study demonstrated that venous thrombi formation does not occur in severely thrombocytopenic mice, supporting previous observations.¹⁸ Von Willebrand factor (VWF), essential for platelet adhesion, also seems to be crucial in the formation of venous thrombus, as demonstrated in animal models,¹⁹ and some relate VWF to

extracellular neutrophil traps (NETs).^{20,21} Even more, a recent study in a murine model of spontaneous deep vein thrombosis (DVT)²² confirms the fundamental role of platelets in the genesis of the venous thrombus. A recent review on the topic⁴ reports the scarcity of literature on the different processes that could explain the involvement of platelets in venous thrombosis, highlighting those related to the presence of platelet microparticles, increased platelet aggregability and activation, and the possible relationship with both circulating platelet count and size. Recent results from our group corroborate the contribution of elevated platelet counts²³ and lower ADAMTS13 levels²⁴ in the risk of venous thrombosis, especially in women. However, no reference to publications on platelet adhesion mechanisms in humans is made.

We have previously investigated the contribution of the global platelet function and aggregation in the development of VTE in a cohort of patients ($n = 400$) included in the RETROVE (Riesgo de Enfermedad TROMboembólica VENosa) study.²⁵ In our results, the whole blood aggregation parameters did not show any association with the VTE risk, as previously suggested in some studies using classical platelet aggregometry with platelet-rich plasma.^{26,27} Nevertheless, we demonstrated an association between short closure times on the Platelet Function Analyzer (PFA-100®) and VTE risk.²⁵ These divergent results suggest that there are other platelet function mechanisms, such as adhesion, which may be responsible for VTE risk.

In the present study, we aimed to explore the adhesive function of platelets in a specific subtype of VTE patients who present with shortened closure times on the PFA, by using adhesion devices under flow conditions. Differences in platelet membrane glycoprotein expression, signaling proteins,

and in plasma levels of VWF and ADAMTS-13 activity, crucial in platelet function, were also analyzed.

Material and Methods

Patients and Controls

All the individuals were recruited from the RETROVE study at the Hospital de la Santa Creu i Sant Pau, Barcelona (Spain). Their initial inclusion took place between 2012 and 2016. RETROVE was a prospective case-control study that included 400 consecutive adult (≥ 18 years) patients with VTE (according to specific clinical inclusion criteria) and 400 healthy volunteers who served as controls.²⁵

The diagnosis of venous thrombosis was based on Doppler ultrasonography, tomography, magnetic resonance, arteriography, phlebography, and pulmonary gammagraphy. Any type of venous thrombosis was included except those related to cancer. For the control group, 400 unpaid healthy volunteers (not blood bank donors and unrelated with cases and neither among them) were included. They were recruited to match the age and sex distribution of the Spanish population (2001 census).

From these initial cohorts, we performed a second re-sampling with all the available subjects in 2019. The main inclusion criterion for the patients was to have a PFA occlusion time value below the 10th percentile (obtained from the control population) in at least one of the PFA tests (Col-ADP or Col-EPI), to include patients with the shortest times. Controls were selected from the 400 controls of the RETROVE cohort, matching 1:1 for sex and age with the VTE patients included. ►Table 1 summarizes the clinical characteristics of the included subjects.

Table 1 Clinical characteristics of VTE patients and controls

		Controls		Patients		P-value (*)
Baseline characteristics		$n = 57$		$n = 54$		
Sex (men)	n (%)	24	(42.1%)	23	(42.6%)	0.959
Age (years)	Mean (SD)	66.7	(16.4)	66.6	(17.3)	0.901
BMI (kg/m^2)	Mean (SD)	26.8	(4.0)	27.6	(4.55)	0.367
Smoking	n (%)	10	(17.5%)	10	(18.5%)	0.894
Alcohol consumption	n (%)	28	(49.1%)	26	(48.1%)	0.918
Hypertension	n (%)	23	(40.4%)	26	(48.1%)	0.408
Dyslipidemia	n (%)	19	(33.3%)	17	(31.5%)	0.835
Statins	n (%)	14	(24.6%)	14	(25.9%)	0.869
Diabetes mellitus	n (%)	7	(12.3%)	2	(3.7%)	0.163
Autoimmune disease	n (%)	7	(12.3%)	5	(9.3%)	0.608
Ictus hemorrhagic	n (%)	0	(0.0%)	0	(0.0%)	1
Arterial thrombosis background	n (%)	0	(0.0%)	2	(3.7%)	0.234
Nonsteroidal anti-inflammatory drugs	n (%)	9	(15.8%)	15	(27.8%)	0.125
Antiplatelet drugs	n (%)	2	(3.5%)	8	(14.8%)	0.049

Abbreviations: BMI, body mass index; VTE, venous thromboembolism.

(*) Mann-Whitney U test for continuous variables and Chi-Square/Fisher test for categorical variables.

Our study was performed following the ethical guidelines of the Declaration of Helsinki. Written informed consent was obtained from all the participants and all of the procedures were approved by the Institutional Review Board at the Hospital de la Santa Creu i Sant Pau.

Blood was collected and anticoagulated with citrate-phosphate-dextrose (100 mmol/L sodium citrate, 16 mmol/L citric acid, 18 mmol/L sodium hydrogen phosphate, and 130 mmol/L dextrose) (at a final citrate concentration of 19 mmol/L).

Experimental Design

Differences in platelet adhesion characteristics between VTE patients with shortened PFA values ($n = 54$) and matched healthy individuals by age and sex ($n = 57$) were evaluated, by using citrated whole blood samples, in PFA-100 test, to measure global platelet function as closure times with collagen-ADP (Col-ADP) and collagen-epinephrine (Col-EPI) as inductors; cone plate(let) analyzer (CPA) (IMPACT) test, to evaluate platelet adhesiveness on a plastic surface (shear rate of 1800 s^{-1} for 2 min); and microfluidic devices, to explore platelet adhesion on collagen type I (Col-I, shear rate of 800 s^{-1} for 5 min) with two- and three-dimensional evaluation by confocal microscopy. Platelet membrane glycoprotein evaluation was performed by flow cytometry. Intraplatelet signaling was evaluated in both isolated Col-I activated platelets (30, 60, and 90 s) and platelets adhered on Col-I or VWF coated coverslips after perfusion with citrated whole blood (800 s^{-1} , for 5 min). Plasma levels of VWF and ADAMTS-13 activity were measured.

Hemostatic Capacity in the PFA-100

The PFA-100 is considered a substitute for the classic bleeding time test. It comprises a microprocessor-controlled instrument and a disposable test cartridge containing a biologically active membrane.²⁸ The system monitors platelet interaction on membranes coated with Col-ADP and Col-EPI. The device aspirates a citrated blood sample, under constant vacuum, from a sample reservoir through a capillary and a microscopic aperture cut into the membrane. The presence of collagen together with either ADP or EPI, as well as the high shear rates generated under the standardized flow conditions, results in platelet attachment, activation, and aggregation, building a stable platelet plug at the aperture within seconds. The time required to obtain full occlusion of the aperture is reported as the “closure time.”

Cone Plate(let) Analyzer (CPA) Testing

The CPA was used as previously described.²⁹ The device tests platelet adhesion and aggregation in citrated whole blood under arterial flow conditions (1,800/second for 2 min). Blood is in contact with the polystyrene surface of a well, where plasma proteins adsorb, by using laminar flow. Three parameters were evaluated: the average size of the polystyrene-bound platelet clusters or platelet aggregates (AS, μm^2), the number of platelet groups (OB, n), and the surface covered by platelets (expressed in percentage, %SC).

Adhesion Studies Under Flow Conditions in Microfluidic Chambers

Microfluidic studies were carried out using commercially available slide-based chambers (μ -SlideVI 0.4, IBIDI) coated with fibrillar Col-I. Aliquots of citrated whole blood were perfused through the slide micro-channels at a shear rate of 800 s^{-1} , for 5 min. Perfused channels were rinsed with 0.15 M phosphate-buffered saline (PBS), fixed with paraformaldehyde 1% (15 min, 4°C), and further incubated with glycine 1% for 10 min to reduce high background staining due to free unreactive aldehyde groups. Thereafter, perfused channels were exposed to 1% bovine serum albumin (BSA) for 15 min before incubation with a specific mouse antibody to human platelet GPIV (CD36, C88456, Life Span BioSciences, Seattle, WA, USA). Then, a secondary anti-mouse antibody conjugated with Alexa fluor 488 (Molecular probes, New York, NY, USA) was added. Incubations with antibodies were performed for 1 hour, at room temperature (RT), in a humidified chamber.

Images of platelet interactions were captured using a microscope (DM4000 B) equipped with fluorescence filters (Leica, Barcelona, Spain), and analyzed (ImageJ Fiji, Bethesda, Rockville, MD, USA) to calculate the surface covered by platelets, expressed as a percentage of the total area explored (%SC).

Confocal microscopy to evaluate platelet aggregate volumes, count, and height was used in a limited number of experiments (controls $n = 6$ and VTE patients $n = 6$). Perfused channels were observed on a Leica TCS-SP5 Laser Scanning Confocal Microscope. The confocal pinhole was set at 1 Airy unit. Transmitted light bright field images were acquired simultaneously through a transmitted light detector. A total of 10 different fields/chamber channels were evaluated, and the aggregate volumes, count, and height of these platelet interactions were calculated using Image-J software, and expressed as μm^3 , n , and μm , respectively.

Flow Cytometry Analysis of Platelet Membrane Glycoproteins

Platelets were analyzed by dual flow cytometry using combinations of antibodies or markers conjugated with fluorescein isothiocyanate (FITC) or phycoerythrin (PE). The platelet population was gated using an antibody to GPIIb/IIIa (CD41a PerCP-Cy5.5, HIP8, BD Biosciences, San Jose, CA, USA). Presence of GPIb (CD42b-PE, BD Biosciences), GPIV (CD36-FITC, Sanquin Plesmanlaan, Amsterdam, Netherlands), GPIX (CD42a-FITC, Beb1, BD Biosciences), P-selectin (CD62P-PE, Beckman Coulter, Barcelona, Spain), and lysosomal antigen (CD63-FITC, Beckman Coulter) was analyzed. Nonspecific membrane immunofluorescence was determined by using an IgG1 (mouse)-FITC (Beckman Coulter), and an IgG1 (mouse)-PE (Beckman Coulter) as a negative control. Immunolabeling of platelets with monoclonal antibodies (MoAbs) was performed using dual-color analysis as previously described.³⁰ Briefly, after collection 2.5 mL aliquots of blood were added to polypropylene tubes preloaded with 50 mL of PBS. Samples were first incubated with saturating

concentrations of anti-CD41a-PE in the dark, without stirring, for 15 min at RT, followed by the addition of labeled MoAbs and an additional incubation for 15 min. Samples were then diluted with 1 mL of PBS and analyzed immediately with a flow cytometer (Navios EX, Beckman Coulter) as previously described.³¹

Evaluation of Intraplatelet Signaling Proteins in Platelets

To perform studies on signaling proteins in platelets activated by adhesion, a parallel-plate perfusion chamber was used to expose coverslips coated with Col-I or VWF to flowing citrated whole blood (800 s^{-1} , for 5 min) from VTE patients or healthy controls. Coverslips were incubated with Laemmli's buffer (125 mmol/L Tris-HCl, 2% SDS, 5% glycerol, and 0.003% bromophenol blue), containing 2 mmol/L orthovanadate and 5 mmol/L N-ethylmaleimide, at 4°C for 15 min. Adherent platelets were then recovered by scraping and the total lysate was collected in an Eppendorf tube and heated at 90°C for 5 min. Samples were kept at -20°C until electrophoretic analysis.

To perform studies on signaling proteins in platelets activated in suspension, platelet-rich plasma (PRP) was obtained by centrifugation of citrated blood samples ($200 \times g$, 10 min, 22°C). Platelets were isolated from PRP ($800 \times g$, 10 min, 22°C) by washing twice with equal volumes of citrate-citric acid-dextrose (93 mmol/L sodium citrate, 7 mmol/L citric acid, and 140 mmol/L dextrose), pH 6.5, containing 5 mmol/L adenosine and 3 mmol/L theophylline.³² The final pellet was resuspended in Hanks' balanced salt solution (136.8 mmol/L NaCl, 5.4 mmol/L KCl, 0.2 mmol/L $\text{MgSO}_4 \times 7\text{H}_2\text{O}$, 0.6 mmol/L Na_2HPO_4 , 0.4 mmol/L KH_2PO_4 , 0.5 mmol/L $\text{MgCl}_2 \times 6\text{H}_2\text{O}$, 1.3 mmol/L CaCl_2) and platelet counts adjusted to 1.2×10^6 platelets/ μL . Suspensions were kept at 37°C for 30 min before activation. Aliquots of platelet suspensions were kept undisturbed or were activated with Col-I (20 $\mu\text{g/mL}$) for 30, 60, and 90 seconds. To obtain platelet lysates, activation was stopped by the addition of Laemmli's buffer, and heated for 5 min at 90°C. Samples were kept at -20°C until electrophoretic analysis.

Then, all platelet lysates were resolved by SDS-PAGE (8% gels).³³ Proteins present in the gels were transferred to nitrocellulose membranes.³⁴ After blocking for nonspecific binding, Western blots were incubated with a phosphotyrosine antibody (PY20) HRP conjugated (sc-508 HRP, Santa Cruz Biotechnology, Dallas, TX, USA) for 1 hour at RT or with a Phospho-Zap-70/Syk primary antibody (2701S, Cell Signaling, Danvers, MA, USA) overnight at 4°C, followed by a polyclonal goat anti-rabbit secondary antibody HRP conjugated (P0448, Dako, Glostrup, Denmark) for 1 hour at RT. Excess antibody was removed by extensive washing, and blots were developed by Enhanced chemiluminescence (ECL) Western Blotting Substrate.³⁵

Changes in phosphorylation levels of intraplatelet signaling proteins were visually inspected and densitometrically quantified in the gel. This intraplatelet signaling protein evaluation was performed in a limited number of experiments (controls $n = 7$ and VTE patients $n = 7$).

Plasma Levels of VWF and ADAMTS-13 Activity

Plasma levels of circulating VWF antigen (VWF:Ag) were measured by immunoturbidimetry in the Atellica 360 COAG coagulometer (Siemens Healthineers, Germany).

For ADAMTS-13 activity, fluorescence resonance energy transfer (FRET) methodology was applied using a synthetic 73-amino acid VWF peptide as a fluorescence-quenching substrate (FRET-VWF73).³⁶ Plasma samples were diluted in buffer containing 5 mM Tris HCl, 25 mM calcium chloride (pH 6), 0.005% Tween-20, 100 mM 4-(2-aminoethyl)-benzene sulfonyl fluoride, and hydrochloride Pefabloc SC (Roche, Mannheim, Germany). After adding 100 μL of calibration test and control samples into a 96-well white plate (Sterilin Ltd., Newport, UK), it was incubated at 37°C for 10 min. Then, 100 μL of the FRET-VWF73 substrate solution was added to each well and fluorescence was measured by a fluorescence microplate reader (Fluoroskan Ascent FL; Thermolab Systems, MA, USA) ($\lambda_{\text{ex}} = 340\text{ nm}$, $\lambda_{\text{em}} = 450\text{ nm}$ at 37°C) every 5 min up to 1 hour.

Analysis of Plasma Proteins Related to Endothelial Function and Immunoinflammation

Plasma concentration of 80 proteins, including cytokines and chemokines related to the immunoinflammatory response, markers of endothelial dysfunction and cardiovascular disease, were measured using Luminex xMAP® technology with a MILLIPLEX® MAP multiplexed assay kit (Merck Millipore, Burlington, Massachusetts, United States), following the protocol provided by the manufacturer. The xPONENT version 3.1 (Luminexcorp) and MILLIPLEX® Analyst Version 3.5 (Merck Millipore) were used for acquisition and data analysis, respectively. Proteins in which the plasma levels were undetectable in more than 60% of the individuals were not considered.

Statistics

Results of continuous variables such as age, platelet adhesion analyses, and membrane glycoproteins are expressed as mean $\pm$ standard deviation (SD) while categorical variables (health background variables) are described with frequencies and percentages. Comparisons between the VTE and non-VTE groups (healthy individuals, controls) were performed with raw data, using parametric or non-parametric tests, as needed: unpaired Student's t-test or the Mann-Whitney U test for continuous variables and the chi-square test or Fisher exact test for categorical variables. Dunn non-parametric test was used for comparing multiple values between both groups in three-dimensional evaluation of microfluidic studies. Correlations between continuous variables were assessed with Spearman's Rho correlation coefficient. Statistical analysis was processed with SPSS statistical software (V.25; SPSS, Chicago, Illinois, USA). $P < 0.05$ was considered as statistically significant.

Results

PFA-100

Closure times in both Col-ADP and Col-EPI cartridges were significantly shorter in VTE patients with respect to controls

Table 2 Platelet function and laboratory parameters of VTE patients and controls

Laboratory parameters		Controls		Patients		P-value (*)
		n = 57		n = 54		
PFA-ADP (2012–2016)	Mean (SD)	87	(22.5)	59	(6.4)	<0.001
PFA-ADP (2019)	Mean (SD)	94	(26.7)	71	(11.4)	<0.001
PFA-EPI (2012–2016)	Mean (SD)	124	(33.9)	83	(9.9)	<0.001
PFA-EPI (2019)	Mean (SD)	104	(26.2)	80	(13.3)	<0.001
Platelets (x10E9/L)	Mean (SD)	239	(57.60)	240	(57.7)	0.969
Mean platelet volume (fL)	Mean (SD)	8.60	(.94)	8.37	(0.80)	0.218
Plateletcrit (%)	Mean (SD)	0.26	(.06)	0.27	(0.06)	0.749
Platelet distribution wide (%)	Mean (SD)	13.8	(2.3)	13.2	(1.92)	0.264
FVIIIc (%)	Mean (SD)	172.8	(63.8)	267.7	(78.1)	<0.001
VWF (antigen levels in %)	Mean (SD)	153.9	(125.0)	238.3	(74.2)	<0.001
ADAMTS-13 activity (%)	Mean (SD)	101.3	(23.74)	96.2	(28.06)	0.194

Abbreviations: PFA, Platelet Function Analyzer; VTE, venous thromboembolism.
Notes: PFA values at the time of recruitment into the study (2012–2016) are given for comparison with the PFA values determined again in 2019.
*Comparison performed with Mann-Whitney U test.

(►Table 2 and ►Fig. 1). In Col-ADP cartridges, closure times were 71 ± 11.4 s in patients ($n = 54$) versus 94 ± 26.7 s in controls ($n = 57$) ($p < 0.01$). In Col-EPI cartridges, results were 80 ± 13.3 s in patients versus 104 ± 26.2 s in controls ($p < 0.01$). CPA Testing with Blood Samples from VTE Patients and Healthy Controls

Parameters AS (μm^2), OB (n), and SC (%) were measured after performing CPA testing in VTE patient ($n = 54$) and healthy control ($n = 57$) blood samples (►Fig. 2). The AS was significantly ($p < 0.05$) higher in VTE patients ($118.9 \pm 38.4 \mu\text{m}^2$) when compared with controls

($101.2 \pm 40.6 \mu\text{m}^2$). Regarding OB, there was a decrease, although not statistically significant, in VTE patients (760.8 ± 321.2) in comparison to controls (835.6 ± 324.7). Concerning the %SC, a slight increase was observed in VTE patients ($13.5 \pm 3.3\%$) versus controls ($12.7 \pm 4.0\%$), though differences did not reach statistical significance.

Platelet Adhesion on Collagen Under Flow Conditions Using Microfluidic Chambers

Collagen-coated chambers were perfused with blood from VTE patients ($n = 54$). The area covered by platelets in VTE

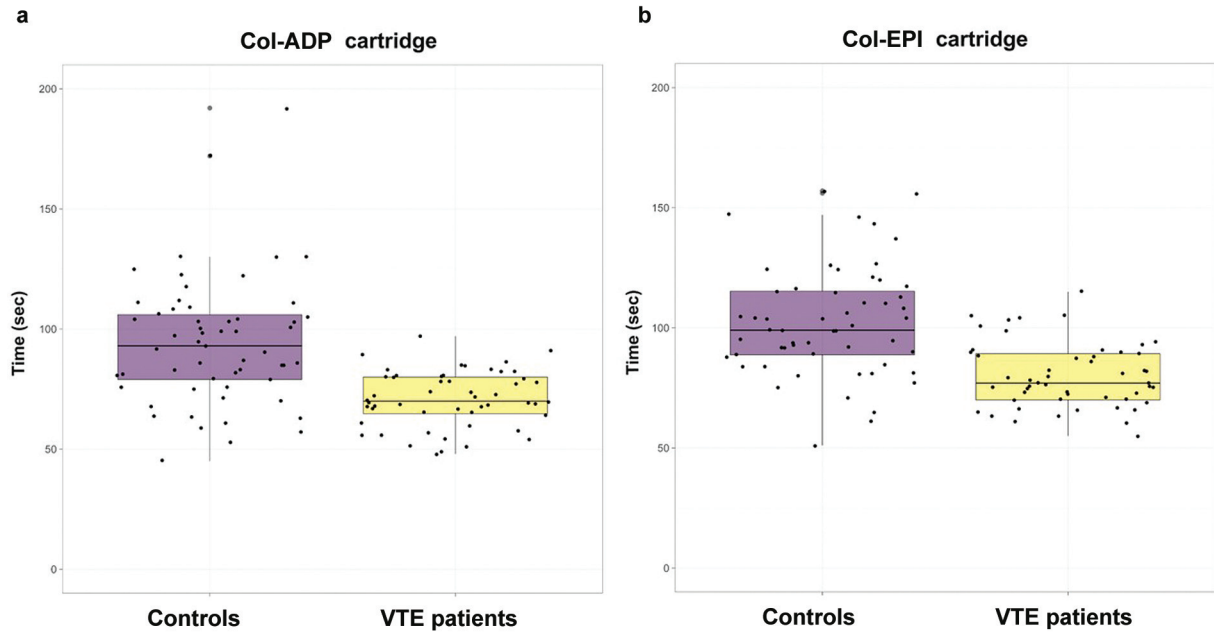


Fig. 1 PFA-100 test showing closure times with both (a) Col-ADP and (b) Col-EPI cartridges when using blood samples from venous thromboembolism (VTE) patients with previous results of short closure times on the PFA-100 (yellow box, $n = 54$) with respect to controls (purple box, $n = 57$).

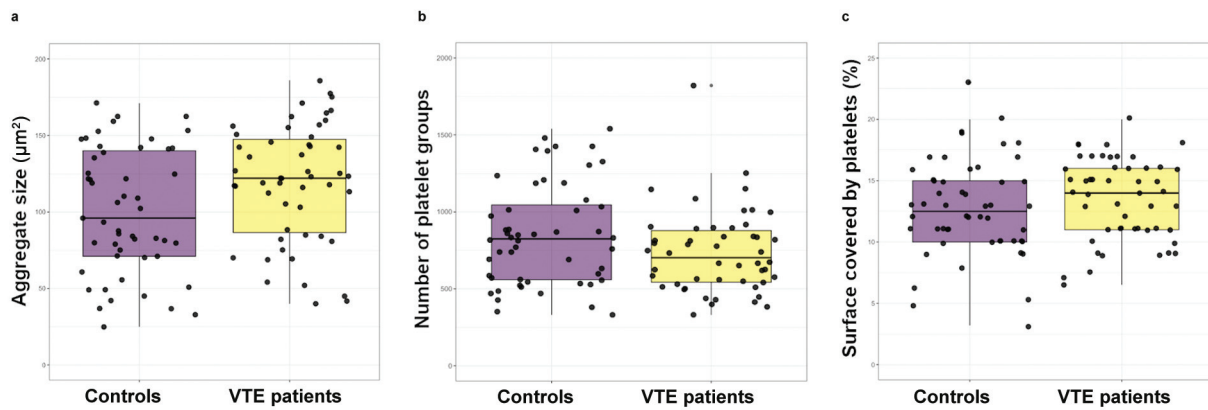


Fig. 2 Cone plate analyzer (CPA) evaluating three parameters: (a) The average size of the polystyrene-bound platelet clusters or platelet aggregates (AS, μm^2), (b) the number of platelet groups (OB, n), and (c) the surface covered by platelets (expressed in percentage, %SC) when using blood samples from venous thromboembolism (VTE) patients selected based on short closure times on the PFA-100 (yellow box, $n = 54$) with respect to controls (purple box, $n = 57$).

patients was significantly higher than in control experiments ($n = 57$) ($16.8 \pm 5.9\%$ vs. $13.3 \pm 5.7\%$, $p < 0.01$) (**►Fig. 3a**). Confocal analysis ($n = 6$ each group) revealed that the volume of the aggregates on the perfused surface was superior using VTE patients' blood samples than controls ($335 \pm 802 \mu\text{m}^3$ vs. $257 \pm 522 \mu\text{m}^3$, $p < 0.01$, $n = 6$) (**►Fig. 3b**). The number of platelet aggregates attached to the surface was significantly lower in perfusion studies using

blood from VTE patients compared to controls' blood (226 ± 102 vs. 251 ± 112 , $p < 0.01$, $n = 6$) (**►Fig. 3c**). The platelet aggregates height was slightly, though significantly, reduced in the experiments carried out with VTE patients' blood versus controls' blood ($26.8 \pm 12.9 \mu\text{m}$ vs. $32.0 \pm 10.7 \mu\text{m}$, $p < 0.01$, $n = 6$) (**►Fig. 3d**).

►Supplementary Fig. S1 shows three-dimensional images of the perfused surfaces.

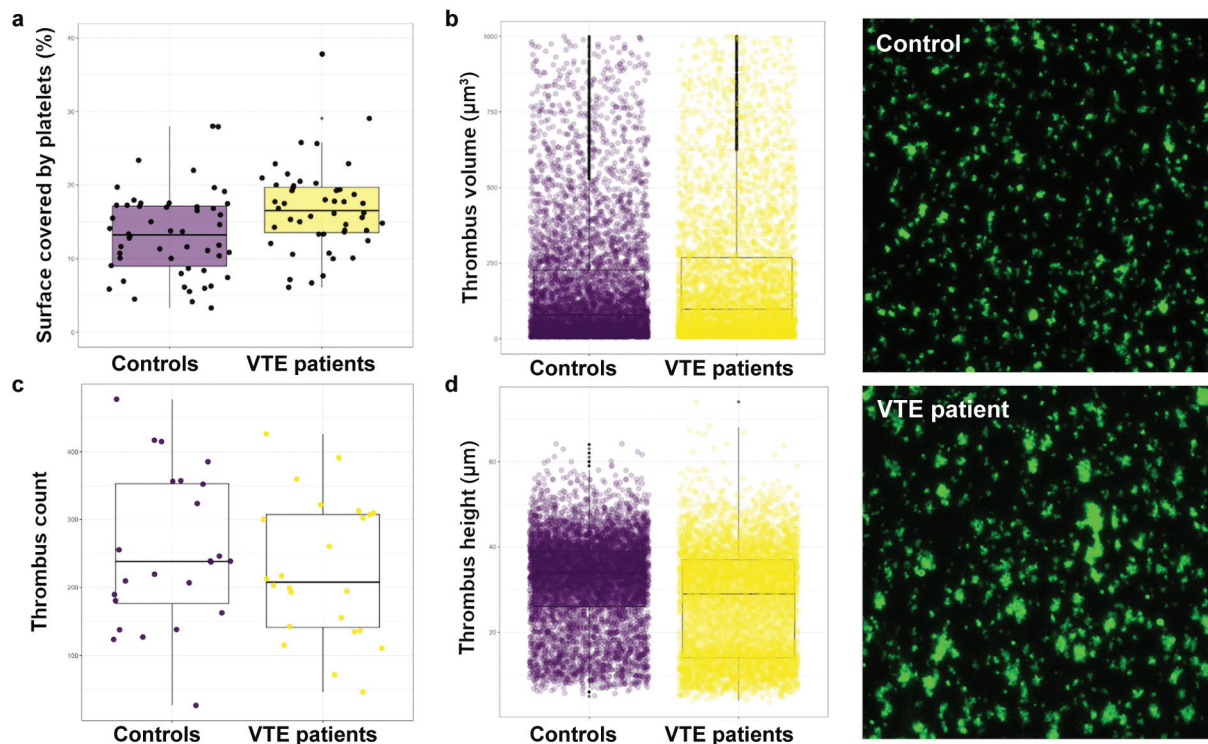


Fig. 3 (a) Microfluidic studies two-dimensional evaluation analyzing the surface covered by platelets (%) when chambers were perfused with blood from venous thromboembolism (VTE) patients selected based on short closure times on the PFA-100 (yellow box, $n = 54$) and controls (purple box, $n = 57$). Micrographs (on the right) showing platelet adhesion (stained in green) on the surface covered by collagen type I. Three-dimensional evaluation analyses: (b) platelet aggregate volumes (μm^3), (c) platelet aggregate counts (n) and (d) platelet aggregate heights (μm) of platelets attached to the surface covered by collagen type I, when chambers were perfused with blood from VTE patients selected based on short closure times on the PFA-100 (yellow box, $n = 6$) and controls (purple box, $n = 6$).

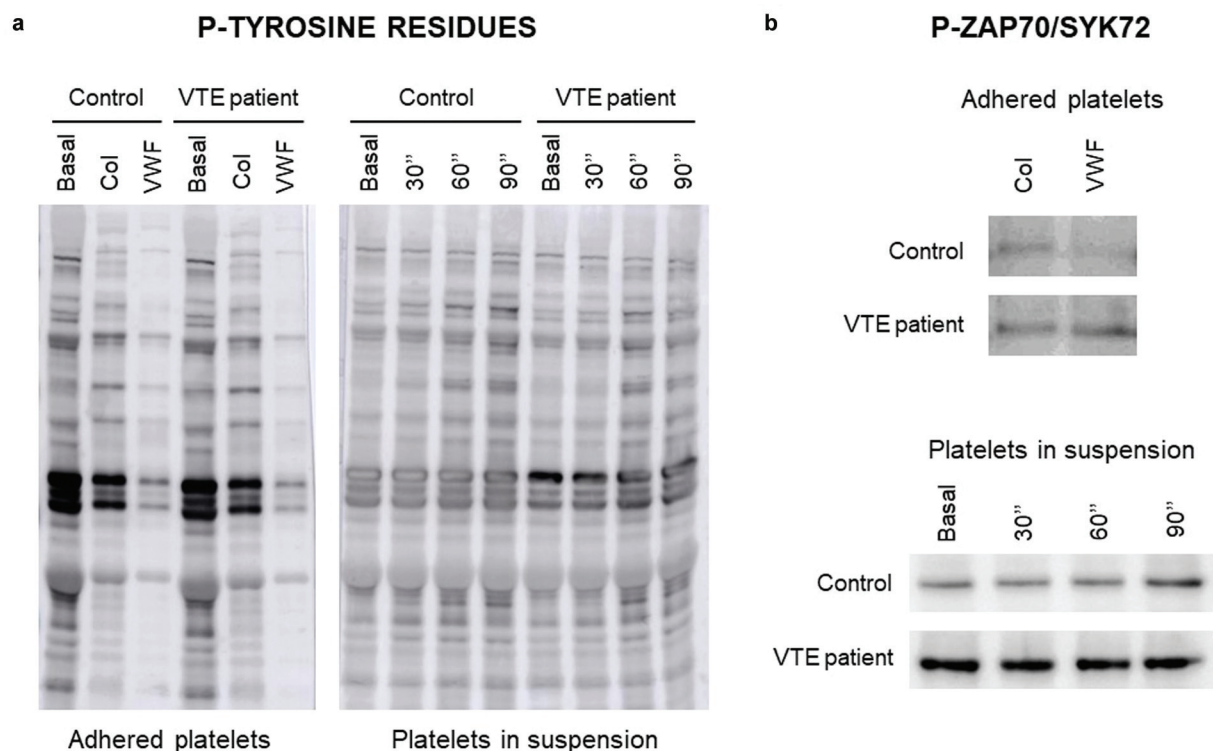


Fig. 4 Changes in phosphorylation levels of intraplatelet signaling proteins evaluated in platelets activated in suspension by collagen type I (Col-I), and by adhesion under flow conditions on Col-I and von Willebrand factor (VWF)-coated surfaces. Activation of (a) tyrosine residues and (b) ZAP70/SYK72 corresponding to blood samples from venous thromboembolism (VTE) patients selected based on short closure times on the PFA-100 ($n = 7$) and controls ($n = 7$).

Flow Cytometry Analysis of Platelet Membrane Glycoproteins

No differences in the expression of the platelet surface glycoproteins (GP) IIb–IIIa, Ib, IV, and IX, P-selectin, and lysosomal antigen were observed between VTE patients ($n = 54$) and control subjects ($n = 57$).

Intraplatelet Signaling

Changes in phosphorylation levels of intraplatelet signaling proteins were evaluated in platelets activated in suspension by Col-I, and by adhesion under flow conditions on Col-I and VWF-coated surfaces. Tyrosine phosphorylation of proteins in VTE patients' platelets did not differ significantly from healthy controls' platelets, either activated in suspension or by adhesion (►Fig. 4a). When activated in suspension, a progressive time-dependent phosphorylation was visually observed in both VTE and control platelets. After adhesion on Col-I or VWF, no differences either in phosphorylated proteins or in the phosphorylation intensity were detected.

P-ZAP70/SYK72 evaluation was performed in blood samples from VTE patients ($n = 7$) and controls ($n = 7$). When exploring P-ZAP70/SYK72 protein in platelets activated in suspension (►Fig. 4b, bottom), an earlier and significant increased activation was observed in VTE patients' platelets compared to control (fold increases $\pm$ standard error of the mean [SEM] of 1.7 ± 1.0 [$p < 0.05$], 1.5 ± 0.4 [$p < 0.05$], 1.5 ± 0.3 [$p < 0.05$], and 1.2 ± 0.2 at basal state, 30, 60, and 90 seconds, respectively). After adhesion on Col-I and VWF,

an increase in the phosphorylation of ZAP70/SYK72 was detected in patients' platelets, though moderate (fold increase of 1.2 ± 0.3 and 1.4 ± 0.3 , respectively) (►Fig. 4b, top).

Plasma Levels of VWF and ADAMTS-13 Activity

Plasma levels of circulating VWF antigen (VWF:Ag) significantly increased in VTE patients ($n = 54$) in comparison with controls ($n = 57$) ($238.3 \pm 74.2\%$ vs. $153.9 \pm 125.0\%$, $p < 0.01$). No significant differences in ADAMTS-13 activity were observed between plasma of VTE patients ($n = 54$) and that of controls ($n = 57$) ($96.2 \pm 28.1\%$ vs. $101.3 \pm 23.7\%$).

Plasma Proteins Related to Endothelial Function and Immunoinflammation

Of the 80 plasma proteins analyzed, 58 exhibited detectable levels. Statistical differences between cases and controls were only observed in three proteins: CTAK, eotaxin, and IL-27. Three additional proteins, IL-10, IL-1 RA, and MDC, showed a trend for statistical significance ($p < 0.08$). Results are shown in ►Supplementary Table S1.

Correlations between some of these six proteins and the PFA results or platelet adhesion measurements were slight and none of them appeared relevant from a biological point of view ($\rho < 0.3$). See ►Supplementary Table S2.

Discussion

Previous studies indicate an association between global platelet function and VTE risk, though hyperaggregability

has not been found in patients suffering from this condition. The present study aimed at investigating the association between the adhesive functions of platelets and VTE risk, in a cohort with previous history of VTE and short PFA-100 closure times. Using different strategies to evaluate platelet adhesion, platelets from VTE patients exhibited a proadhesive phenotype under flow conditions, potentially explaining the shortened PFA-100 occlusion times. The enhanced platelet adhesiveness may be explained by the significantly increased levels of VWF in the plasma of these individuals, as well as by the activation of intraplatelet signaling proteins, such as ZAP70/SYK72. Therefore, a platelet proadhesive behavior, rather than a hyperaggregability state, may constitute a crucial step in the pathophysiology of VTE.

The role of platelets in atherogenesis, atherosclerosis, and atherothrombosis is well recognized nowadays.³⁷ Platelet activation plays a key role in arterial thrombosis, and antiplatelet agents are essential for both the treatment and the secondary prevention of atherothrombosis.³⁸ However, the contribution of platelet pathophysiology to acute VTE has been poorly investigated. Increased P-selectin expression,^{39,40} higher VWF¹⁹ and tissue factor (TF),⁴¹ and the release of endothelial microparticles (EMP) seem to play an active role in the thrombotic process. Therefore, the activation of the platelets, leukocytes, and endothelium is key in the pathophysiology of VTE.³⁹ Moreover, the formation of EMP–leukocyte and platelet–leukocyte conjugates has been proposed to be implicated in VTE and its inflammatory process.³⁹ Changes in the mean platelet volume (MPV), a surrogate marker of platelet activation, have been also related to the presence of VTE.^{42–44}

In a previous study, applying machine learning strategies, combining clinical and several platelet function related variables,⁴⁵ authors found that 69% of the selected variables that were capable of differentiating VTE cases (159 patients) from controls (140) corresponded to platelet biomarkers. VTE cases showed lower platelet count and MPV versus controls, with shorter closure times of Col-EPI, particularly in those patients treated with acetylsalicylic acid (ASA). Furthermore, the lower platelet-dependent thrombin generation and the lower reactivity found after ex-vivo stimulation could indicate the presence of a “platelet exhausted syndrome” in individuals with VTE. The contribution of platelets to two subtypes of pulmonary embolism (PE), isolated PE (iPE) and DVT-associated PE (DVT-PE), has also been evaluated.⁴⁶ The main conclusion was that the platelet protein release contributes to the differential regulation of plasma proteins in both the entities, which may be associated with different platelet activation patterns.

Earlier studies by our group indicate that platelet phenotypes are involved in the pathophysiology of venous thrombosis.²⁵ Also, in further studies we demonstrated that elevated platelet counts and high levels of the serotonin transporter (SERT) and the protein VAMP8 (human vesicle-associated membrane protein 8) were related to a double risk

of VTE in females.^{23,47} More recently, we showed that platelet aggregability and, mainly, hyperaggregability are not associated with the risk of venous thrombosis, especially when results are corrected by age.⁴⁸ The results of the present study demonstrate that platelets from VTE patients exhibit enhanced adhesiveness, evaluated through different strategies, rather than increased aggregability. Adhesion on both a plastic surface and a collagen-coated surface, under flow conditions, resulted in an increased surface covered by platelets when exposed to blood samples from VTE patients. These results paralleled the augmented plasma levels of VWF detected in these patients.

Whether elevated plasma levels of VWF represent a risk factor or a consequence of VTE is still unclear. VWF could be useful as a biomarker to predict first-lifetime VTE. Some studies hypothesize that the relation between VWF and VTE probably is biologically mediated by the elevated levels of FVIII.⁴⁹ Those patients with elevated plasma VWF levels are likely to have a higher endogenous thrombin potential due to the simultaneous elevation of FVIII levels.⁵⁰ In this regard, there is evidence demonstrating the role of VWF and GPIb α in platelet adhesion and of FVIII in subsequent thrombus growth in experimental thrombosis under venous flow conditions.⁵¹ In addition, in a case–control study, the combination of elevated plasma VWF levels and high MPV, or a high platelet count, had an additive effect on the VTE risk.⁵² In our cohort of patients, platelet counts did not differ among both the groups.

Intraplatelet signaling events after platelet contact may be also of interest in explaining the VTE risk. Genetic analyses in VTE patients performed (data not shown) by our group uncovered the potential contribution of platelet signaling genes in the proadhesive behavior of the VTE patients' platelets. Among the genes identified, the LAT gene was of interest since the protein encoded is phosphorylated by ZAP70/SYK72 protein tyrosine kinases. Activation of platelets in suspension resulted in a significant increase of platelet ZAP70/SYK72 phosphorylation in our VTE patients' cohort versus control platelets. This protein has been previously associated with platelet activation by collagen through a GPIIb-IIIa dependent mechanism.⁵³ Moreover, Arderiu et al⁵⁴ found that the initial arrest of platelets on subendothelial components under flow conditions causes SYK phosphorylation, in a GPIIb-dependent manner, which is followed by activation and phosphorylation of intraplatelet cortactin and focal adhesion kinase (FAK). Therefore, these results could justify the proadhesive behavior of VTE patients' platelets.

In summary, platelets from VTE patients with short closure times on the PFA exhibit a proadhesive phenotype under flow conditions, expressed by an enhanced adhesiveness on type I collagen-coated surfaces. The platelet proadhesive performance in VTE patients could be explained by increased plasma levels of VWF. In addition, the adhesion-related signaling protein ZAP70/SYK72, activated significantly more in platelets from VTE patients, reinforces this proadhesive phenotype of platelets. Results from the present study

substantiate the key role of platelet adhesion in the pathophysiology of VTE.

What is Known About this Topic?

- Previous studies support the role of platelets in the development of thrombosis, especially arterial.
- Increased platelet reactivity in association with arterial thromboembolic pathology has been reported by multiple research groups.
- Whole blood aggregation parameters do not show an association with the VTE risk, as previously suggested in some studies using classical platelet aggregometry with platelet-rich plasma.

What Does this Paper Add?

- Platelets from VTE patients are more proadhesive under flow conditions potentially related to the shortened occlusion times on the PFA-100.
- Area covered by platelets and aggregate volumes are higher when exposed to samples from VTE patients using cone plate analyzer (CPA) and microfluidic devices with two- and three-dimensional evaluation.
- Phosphorylation of ZAP70/SYK72 protein is significantly increased in platelets from VTE patients.
- Plasma levels of VWF is significantly elevated in VTE patients.

Author Contributions

JMS and MDR wrote the manuscript; MDR and JCS designed the study; JCS recruited the patients included in the study and described the patients' characteristics; JMS, DJD, MC, and LM were responsible for the experimental work; JMS, STM, PS, and SM performed the statistical analysis of results; JMS, STM, ABMC, MP, GE, and MDR designed the figures; SM, MP, FV, GE, MDR, and JCS contributed to discussion of the results; all the authors contributed to the review and editing of the final manuscript.

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Conflict of Interest

None of the authors have conflicts of interest directly related to this work. JMS and MDR have received honoraria from Jazz Pharmaceuticals.

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