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ORIGINAL ARTICLE

Variation of platelet function in clinical phenotypes of acute venous thromboembolism – Results from the GMP-VTE project

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Abstract

Background: The role of platelets in the pathogenesis of venous thromboembolism (VTE) is receiving increasing attention; however, limited information is available on platelet function in the acute phase of the disease.

Objective: To characterize platelet function according to VTE phenotypes.

Patients/Methods: In total, 154 subjects (isolated pulmonary embolism [iPE], $n = 28$; isolated deep vein thrombosis [iDVT], $n = 35$; DVT+PE, $n = 91$) were included. In this study platelet function analyzer (PFA)-200, light transmission aggregometry (LTA), thrombin generation (TG) in presence (PRP) and absence (PFP) of platelets and platelet flow cytometry were investigated. LASSO regression was used to select clinical and platelet biomarkers that distinguish between VTE phenotypes.

Results: PFA-200 results did not differ between VTE phenotypes. LTA from DVT+PE subjects showed lowest maximum aggregation after epinephrine and adenosine diphosphate compared to iPE and iDVT. Lower % of PAC-1-positive platelets after in-vitro trigger were present in DVT+PE and iPE compared to iDVT. TG in PRP had lower peak height and velocity in DVT+PE and iPE against iDVT. The results of

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LASSO regression for the distinction between DVT+PE vs iDVT identified 18 variables (AUC =0.93) of which 72% were platelet biomarkers. For distinction between iPE and iDVT, 10 variables were selected (AUC = 0.96) of which 50% were platelet-related. Obesity was the only variable weakly discriminating between DVT+PE vs iPE (AUC = 0.66).

Conclusion: This explorative study suggests an important distinction between PE-related phenotypes and iDVT when considering clinical and platelet function data. Lower platelet-dependent TG along with reduced platelet reactivity suggest higher platelet degranulation in PE-dependent phenotypes compared to iDVT.

KEYWORDS
platelet function, venous thromboembolism, pulmonary embolism, deep vein thrombosis, thrombin generation

1 | INTRODUCTION

Venous thromboembolism (VTE) remains a major public health concern worldwide with substantial morbidity and mortality.[1] The mode of presentation with isolated deep vein thrombosis (iDVT) or isolated pulmonary embolism (iPE) has been associated with different clinical outcome and recurrence rates.[2] There is accumulating evidence linking iPE with chronic respiratory diseases and underlying atherosclerotic disease, suggesting a rather local trigger in disease conception.[3,4] Compared to iDVT, PE-related phenotype has been associated with worse clinical outcome.[5] In addition, increased risk of death in patients with acute symptomatic PE concomitant with DVT compared to iPE has been reported.[6]

Although platelets are traditionally thought to play a limited role in VTE, the observed partial protection against VTE by aspirin, as well as distinct mechanistic contributions of platelets to thromboinflammation, trigger new interest in the role of platelets in VTE.[7,8] Mean platelet volume (MPV) is one of the most extensively studied platelet-related biomarkers in the setting of VTE.[9,10] Conversely to the positive association between higher MPV and occurrence of cardiovascular disease, acute venous thrombosis has been related to lower MPV.[10-12] Lower MPV and decreased platelet reactivity have been particularly linked to high risks of VTE and worse prognosis in active cancer patients.[13,14] In addition to lower MPV, higher platelet count is an established risk factor for venous thrombosis in active cancer patients on chemotherapy or hospitalization.[15] Data from a large population-based study suggests an association between increased platelet aggregation and risk for incident VTE.[16] Whereas the available clinical and epidemiological studies are not sufficiently robust to establish a role of platelets in venous thrombosis, animal studies clearly support the contribution of platelets to DVT. Platelet-von Willebrand factor interaction, platelet cross talk with leukocytes and C-type lectin-like receptor 2 specific platelet recruitment have been recognized critical to initiate and propagate venous thrombosis in mouse models.[17-20]

ESSENTIALS

- Platelets are traditionally thought to play a limited role in VTE; however, the observed partial protection by aspirin, as well as distinct mechanistic contributions of platelets to thromboinflammation, trigger new interest in the role of platelets in VTE.
- Using a large panel of clinical covariates and for the first time platelet function characteristics, we phenotypically profiled a large cohort of patients with different acute manifestations of VTE.
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- PE-related phenotypes differ from isolated DVT in respect to both, clinical and platelet function characteristics.
- This explorative study further indicates the concept of higher platelet degranulation in PE-dependent phenotypes compared to isolated DVT.

We recently demonstrated that platelet phenotype substantially differs between VTE cases and those with a VTE ruled out independent of the underlying clinical and cardiovascular risk profile. Including a large range of the most relevant clinical and laboratory characteristics and by using a machine learning approach, we showed that platelet biomarkers comprised 69% of all selected variables differentiating VTE cases vs VTE controls.[21] Although several associations between platelets and VTE have been established, there is a lack of studies of the possible platelet related mechanisms that link to different VTE phenotypes. To fill this gap, we undertook a comprehensive investigation of platelet function in relation to distinct VTE phenotypes in the setting of an acute event. To characterize platelet function according to the manifestation of VTE, i.e. in iDVT, iPE and concomitant DVT+PE, we studied platelet aggregation,

platelet surface activation markers and platelet-dependent thrombin generation (TG). By applying machine learning on comprehensive data sets with information on clinical status and platelet function, we explored the most prominent characteristics differentiating between VTE phenotypes.

2 | METHODS

2.1 | Study sample

Platelet function characteristics were assessed in a subgroup of 180 confirmed VTE patients randomly selected from the Genotyping and Molecular Phenotyping in Venous ThromboEmbolism (GMP-VTE) project, as described before in detail.[22] In brief, GMP-VTE comprises two prospective, observational studies enrolling individuals with DVT and PE at the Johannes Gutenberg University Medical Center Mainz in Mainz, Germany. DVT was confirmed after a whole-leg compression color Doppler ultrasonography, whereas PE after a computed tomographic pulmonary angiography or ventilation/perfusion (V/Q) scintigraphy. Patients in whom PE was confirmed were additionally examined with whole-leg ultrasonography. All diagnoses were independently confirmed by board-certified angiologists or radiologists connected to the study site. All subjects gave written informed consent before entering the study. Study protocol and documents were approved by the local Ethics Committee and the studies have been conducted in accordance with the Declaration from Helsinki. Subjects with known cancer diagnosis ($n = 21$) were excluded from analysis for the following reasons: (1) low number and heterogeneity of cancer types in the present sample; (2) indications that malignancy had a profound effect on platelet and coagulation biomarkers. Subjects with unknown VTE phenotype ($n = 5$) were also excluded leaving 154 individuals for the present analysis.

2.2 | Categorization of medications

Medications that study participants were taking including the therapy given before the blood draw were registered during patient inclusion at the study site. The medications were classified according to the Anatomical Therapeutic Chemical (ATC) classification system. The following medication groups were selected for analysis: acetylsalicylic acid (ASA, B01AC56), clopidogrel (B01AC04), Vitamin K antagonists (B01AA), heparin (B01AB) and direct factor Xa inhibitors (B01AF).

2.3 | Blood sampling and plasma preparation

Venous blood sampling was performed using tubes containing trisodium citrate (3.2%, 0.109 M, 1:9 vol:vol). Fresh citrated whole blood for platelet function analysis was hand delivered in the platelet epidemiology laboratory within 30 min after blood withdrawal from the baseline examination. Depending on the type of test, whole blood, platelet-rich plasma (PRP), platelet-poor plasma (PPP) or platelet-free plasma (PFP) was used. PRP was isolated by centrifugation of whole blood at 200 *g* for 10 min at room temperature (RT); after collecting PRP, the remaining blood sample is further centrifuged for 15 min at 2,000 *g* at RT to obtain PPP used for adjusting the concentration of PRP to 150,000 platelets/μL. PFP was obtained by double centrifugation as follows: whole blood was centrifuged for 5 min at 2000 *g* at RT and the collected PPP was further centrifuged for 10 min at 11,000 *g*. The isolated PFP was stored at −80°C until further laboratory testing.

2.4 | Platelet function assays

Three different assays of platelet function were carried out, enabling investigations into platelet aggregation, activation and TG. Platelet function measurements were performed according to standardized operating protocols established in the platelet epidemiology laboratory of the Center for Thrombosis and Hemostasis, University Medical Center Mainz in Mainz, Germany.

2.4.1 | Platelet Function analyser-200

Platelet aggregation of citrated whole blood at high shear rate was studied by using the Platelet Function analyzer (PFA)-200 (Siemens Healthcare Diagnostics, Marburg, Germany). For each sample, two ready cartridges were used with collagen-adenosine diphosphate (collagen-ADP) and collagen-epinephrine (collagen-EPI) according to the manufacturer`s instructions. Results were expressed as closure time in seconds.

2.4.2 | Light transmission aggregometry

Platelet aggregation after adding different platelet agonists, obtained from DiaSys Diagnostic Systems GmbH, Flacht, Germany, was measured in undiluted PRP using light transmission aggregometry (LABiTec, Ahrensburg, Germany). The following platelet agonists were used: adenosine diphosphate (ADP) at 0.5 and 2 μM, epinephrine (EPI) at 0.5 and 5 μM, collagen at 2 mg/mL, arachidonic acid at 1 mM and thrombin receptor activating peptide-6 (TRAP-6) at 10 μM, final concentrations. Furthermore, a sample without addition of an agonist under stirring conditions was investigated and noted as aggregation without trigger. The aggregation measurement time was 600 seconds and results were expressed as maximum aggregation (%) and aggregation velocity (%/min).

2.4.3 | Thromboxane B2 measurements

Thromboxane B2 (TxB_2) levels were assessed in citrated plasma by in vitro competitive ELISA assay (ab133022; Abcam, Cambridge, UK),

according to manufacturer instructions. The results, assessed by the Tecan Infinite M200 Pro microplate reader (Tecan Group Ltd., Männedorf, Switzerland), were expressed as nanograms per milliliter (ng/ml).

2.4.4 | Flow cytometry

Activation-dependent platelet surface antigens (i.e. P-selectin, CD63, fibrinogen binding and tissue factor) at resting whole blood and PAC-1 after triggering whole blood with collagen-ADP by the PFA-200, were assessed by flow cytometry using a BD Accuri C6 (BD Biosciences, San Jose, CA). Briefly, 5 μL of resting whole blood was double stained with CD42a-PerCP (Becton Dickinson, Heidelberg, Germany) for platelet gating and one of the following monoclonal antibodies: CD41-PE (Beckman Coultier, Krefeld, Germany), CD62p-FITC (Becton Dickinson, Heidelberg, Germany), CD63-FITC (Beckman Coultier, Krefeld, Germany), anti-human Fibrinogen-FITC (DAKO, Glostrup, Denmark), anti-human Tissue Factor-FITC (Sekusui Diagnostics, Stamford, Connecticut, USA). For the platelet agonist (collagen/ADP) stimulated whole blood, samples were double stained with CD42a-PerCP and PAC-1-FITC (Becton Dickinson, Heidelberg, Germany). All samples were incubated for 20 min at room temperature in dark after which the reaction was stopped by adding 500 μL phosphate-buffered saline. The samples were then immediately analyzed and percentages (%) of platelets as well as mean fluorescence intensity (MFI) expressing the specific antigens were recorded.

2.4.5 | Calibrated automated thrombogram

The calibrated automated thrombogram (CAT, Thrombinoscope BV, Maastricht, the Netherlands) was used to measure TG in PRP (with an adjusted platelet concentration to 150,000 platelets/μL using autologous PPP) after exposure to a trigger (e.g., low tissue factor ~1pM TF). To understand the contribution of platelets, TG in PFP after exposure to PPP low (low TF and 4μM phospholipids) was also investigated. TG measurements were performed following a standard operating procedure with a total measurement time of 90 min, as described in detail previously.[23] TG curves were calculated by means of Thrombin scope software (Thrombinoscope BV). All CAT reagents were purchased from Stago Deutschland GmbH (Düsseldorf, Germany). The i vestigated parameters of interest were lag time, expressed in minutes, representing the time until minimum thrombin n is formed, (arbitrarily defined as the moment when 10 nM thrombin is formed), peak height representing the maximum concentration of thrombin formed (expressed in nM thrombin), endogenous thrombin potential (ETP) representing the net effect of procoagulant and anticoagulant actions in the plasma (expressed in nM*min) and velocity representing the speed of TG (expressed in nM/min).

2.5 | Statistical methods

Distribution of demographic characteristics, VTE risk factors and cardiovascular risk factors (CVRFs) were presented as mean (±standard deviation) for continuous variables with normal distribution, median with interquartile range for variables with skewed distribution and as percentages (absolute numbers) for categorical variables. Differences in biomarkers of platelet function between iPE, iDVT, and DVT+PE were calculated by t-test for normally distributed variables, Wilcoxon test for variables with skewed distribution and proportional test for variables expressed as proportions. Least absolute shrinkage and selection operator regularized logistic regression (LASSO regression), a common supervised machine learning technique, was used to select clinical variables and platelet function biomarkers that most relevantly distinguished between VTE clinical phenotypes. Fractional polynomial transformations were applied to all variables included in the regression models to account for non-linear relationships. The characteristics were ordered by lambda ratio (LR), a scale-invariant measure of predictive robustness, which was calculated by computing the ratio of λ at which a variable was omitted from the model to the optimal λ selected by cross-validation. Missing values were imputed using random forest imputation, implemented in the R package 'missForest'.[24] No threshold for significance was defined because of the explorative character of the analysis. Therefore, *P*-values should be interpreted as continuous measure of statistical evidence. Statistical analysis was performed with software program R, version 3.6.1 (http://www.R-project.org).

3 | RESULTS

3.1 | Clinical characteristics of the study sample

The study sample consisted of 154 subjects with average age of 60 ± 16 years that was similar between VTE subgroups (**Table 1**). Females were 42% of the total sample with lower proportion in the PE subgroup (36%). Subjects with iDVT and iPE had a comparable body mass index (BMI), whereas subjects in the DVT+PE subgroup presented with higher BMI. History of VTE including history of DVT was more prevalent in the iDVT subgroup, whereas history of PE was highest in the DVT+PE subgroup. Presence of hypertension was more prevalent in PE-related phenotypes (iPE and DVT+PE), whereas obesity was more prevalent in DVT-related phenotypes (iDVT, 31.4% and DVT+PE, 46.7%). Intake of anticoagulant medication at enrollment was 83.5% in DVT+PE, 82.1% in iPE and 71.4% in iDVT subgroup. Intake of antiplatelet therapy was highest in participants with iPE (46.4%), followed by iDVT (44.0%), and 14.3% in DVT+PE subgroup. The majority of the participants reported taking ASA ($n = 55$) and four participants only (iDVT, $n = 1$; iPE, $n = 2$ and DVT+PE, $n = 1$) were taking clopidogrel as an antiplatelet medication. The participants stopped taking the antiplatelet therapy with a median of 0 days (IQR: 0–2 days) after a VTE diagnosis.

TABLE 1 Clinical characteristics of the study sample ($N = 154$) according to disease phenotype

Variable	DVT+PE	iDVT	iPE	*P*-value
Number	91	35	28	
Female sex (number)	42.9% (39)	42.9% (15)	35.7% (10)	0.79
Age (years)	61.7 (±14.9)	57.9 (±16)	60.4 (±20.1)	0.50
BMI (kg/m²)	30.6 (±6.0)	27.1 (±5.4)	27.1 (±6.1)	0.0017
VTE risk factors				
Hx of VTE	35.7% (30/84)	42.9% (15/35)	16.0% (4/25)	0.085
Hx of DVT	31.0% (26/84)	42.9% (15/35)	12.0% (3/25)	0.038
Hx of PE	18.1% (15/83)	8.6% (3/34)	8.0% (2/25)	0.25
Immobilization	14.3% (12/84)	11.4% (4/35)	24.0% (6/25)	0.38
Long-distance flight/ travel	14.5% (12/83)	17.1% (6/35)	12.0% (3/25)	0.85
Surgery	2.4% (2/83)	0% (0/35)	8.0% (2/25)	0.17
Trauma	7.2% (6/83)	14.3% (5/35)	0% (0/25)	0.12
Thrombophilia	1.5% (1/67)	8.8% (3/34)	4.3% (1/23)	0.21
Pregnancy (current)	1.1% (1/91)	0% (0/35)	0% (0/27)	0.71
Cardiovascular risk factors				
Arterial hypertension	67.1% (53/79)	35.3% (12/34)	52.0% (13/25)	0.0066
Diabetes mellitus	20.3% (16/79)	11.4% (4/35)	20.0% (5/25)	0.51
Smoking	15.2% (12/79)	22.9% (8/35)	21.7% (5/23)	0.55
Obesity	46.7% (42/90)	31.4% (11/35)	14.3% (4/28)	0.0060
Therapy				
Antithrombotics[a]	97.8% (89/91)	80.0% (28/35)	96.4% (27/28)	0.0011
Antiplatelets	44.0% (40/91)	14.3% (5/35)	46.4% (13/28)	0.0050
ASA	42.9% (39/91)	11.4% (4/35)	42.9% (12/28)	0.0030
Clopidogrel	1.1% (1/91)	2.9% (1/35)	7.1% (2/28)	0.21
Anticoagulants[b]	83.5% (76/91)	71.4% (25/35)	82.1% (23/28)	0.30

Percentages are based on non-missing data. Plus-minus signs denote standard deviations.

Abbreviations: ASA, acetylsalicylic acid; BMI, body mass index; DVT, deep vein thrombosis; Hx, history; PE, pulmonary embolism; VTE, venous thromboembolism.

[a]Antiplatelet and anticoagulant agents.

[b]Heparin, vitamin K antagonists, direct factor Xa inhibitors.

3.2 | Platelet aggregation and TxB_2 levels

Results of aggregation in whole blood with PFA-200 and in PRP with LTA after different platelet agonists including aggregation without trigger are presented in **Table 2**. DVT+PE subjects presented with lowest values on maximum aggregation, particularly after low dose EPI (0.5μM) and high dose ADP (2μM) agonism. Results on velocity aggregation did not differ between VTE phenotypes. TxB2 levels (ng/ml) were also no different between the VTE phenotypes (DVT+PE = 3.06 [1.99/6.39], iDVT = 3.29 [2.47/4.83] and iPE = 3.61 [2.68/5.33]). **Figure S1** shows the results from PFA and Figure S2 maximum platelet aggregation after different agonists and according to use of ASA. Subjects using clopidogrel ($n = 4$) were excluded from the analysis. ASA users showed longer closure time after collagen-epinephrine compared to non-ASA users independent of the VTE phenotype (**Figure S1A**). No differences between the subgroups were observed for collagen-ADP agonist (**Figure S1B**). Similarly, maximum platelet aggregation after arachidonic acid showed lower maximum aggregation for ASA users vs non users independent of the VTE phenotype (**Figure S2E**). The differences between ASA users and non-users were not statistically important for the remaining platelet agonists-induced aggregation (**Figure S2**).

3.3 | Platelet surface activation markers

The flow cytometric analysis for platelet surface activation markers in resting platelets showed no difference in % of platelets expressing platelet surface P-selectin, CD-63 and fibrinogen binding. Platelet surface tissue factor (TF) expression was higher in iDVT compared to DVT+PE subgroup (supplemental **Table S1**). Expression of PAC-1, measured in whole blood after collagen-ADP agonism with PFA-200 system, presented with lower % of platelets in both PE-related phenotypes, iPE (16.09% ± 13.03) and DVT+PE (21.90 ± 15.59), compared to iDVT (37.51 ± 18.26).

TABLE 2 Platelet aggregation according to disease phenotype

Platelet aggregation	DVT+PE (*N* = 91)	iDVT (*N* = 35)	iPE (*N* = 28)	*P*-value (iDVT vs DVT+PE)	*P*-value (iPE vs DVT+PE)	*P*-value (iDVT vs iPE)
PFA–200 (seconds)						
Collagen-ADP	84.0 (69.0–106.0)	87.0 (77.3–98.0)	88.5 (64.7–104.2)	0.62	0.89	0.93
Collagen-epinephrine	173.9 (±86.6)	144.3 (±68.3)	175.9 (±90.8)	0.050	0.92	0.14
LTA - Maximum aggregation (%)						
Without trigger	4.66 (2.14–7.87)	3.10 (1.50–7.98)	2.86 (2.12–5.55)	0.41	0.12	0.56
ADP (0.5 μM)	21.21 (14.69–27.71)	23.36 (15.54–33.43)	16.51 (11.55–29.38)	0.15	0.52	0.13
ADP (2 μM)	49.04 (±20.70)	59.24 (±22.44)	53.47 (±23.71)	0.025	0.39	0.34
Epinephrine (0.5 μM)	38.71 (±22.81)	53.56 (±28.31)	46.12 (±26.00)	0.0085	0.19	0.29
Epinephrine (5 μM)	49.86 (±26.22)	60.62 (±26.85)	60.62 (±24.20)	0.050	0.053	1.00
Collagen (2 mg/mL)	55.73 (±25.02)	60.71 (±26.70)	59.19 (±24.05)	0.35	0.52	0.82
Arachidonic acid (1 mM)	44.73 (±34.08)	48.36 (±36.52)	43.63 (±34.74)	0.62	0.89	0.61
TRAP–6 (10 μM)	52.06 (±31.90)	56.03 (±29.69)	54.89 (±29.07)	0.52	0.67	0.88
LTA - Velocity aggregation (%/min)						
Without trigger	5.52 (3.56–6.82)	4.20 (3.13–5.52)	4.17 (3.53–5.45)	0.083	0.080	0.65
ADP (0.5 μM)	35.54 (±15.91)	38.83 (±17.20)	34.78 (±17.65)	0.34	0.84	0.17
ADP (2 μM)	69.7 (±25.0)	73.0 (±23.0)	72.3 (±24.3)	0.48	0.64	0.90
Epinephrine (0.5 μM)	46.89 (43.83–50.96)	45.7 (43.6–54.7)	47.40 (40.73–50.68)	0.92	0.38	0.32
Epinephrine (5 μM)	37.2 (±8.7)	36.5 (31.1–46.1)	38.8 (±12.1)	0.69	0.52	0.95
Collagen (2 mg/mL)	50.91 (43.96–69.73)	59.12 (48.19–76.13)	51.93 (43.32–78.22)	0.21	0.71	0.63
Arachidonic acid (1 mM)	39.38 (29.92–90.91)	58.82 (29.29–108.84)	36.74 (30.43–82.12)	0.63	0.72	0.40
TRAP–6 (10 μM)	73.76 (±39.42)	82.68 (±40.69)	83.66 (±35.97)	0.28	0.23	0.92

Note: Data presented are mean (±standard deviation), tested with a *t*-test and/or median (interquartile range), tested with a Wilcoxon test.
Abbreviations: ADP, adenosine diphosphate; iDVT, isolated deep vein thrombosis; iPE, isolated pulmonary embolism; LTA, light transmission aggregometry; LTA, light transmission aggregometry; N, Number; TRAP-6, thrombin receptor activating peptide-6.

3.4 | Thrombin generation

The results on TG in PRP showed that subjects with iDVT presented with higher peak height and higher velocity in comparison to those with DVT+PE and iPE. Subjects with iPE showed no different TG results compared to those with DVT+PE (**Table 3**). Differently, in the absence of platelets, no TG was generated for DVT+PE and iDVT. In subjects with iPE, TG was significantly reduced, however still present in more than 75% of subjects. **Figure 1** is showing TG results for all three VTE phenotypes within subjects taking anticoagulants. Higher peak height and velocity were confirmed in PRP for iDVT subjects compared to DVT+PE. TG in PRP of subjects with iPE did not differ compared to the subgroup with DVT+PE. Results in absence of platelets showed no generation of thrombin for the DVT+PE and iDVT subgroups. Differently, subjects with iPE demonstrated generation of low thrombin, similarly as in presence of platelets (supplemental **Table S2**). In subjects not on anticoagulant therapy, as shown in **Table S3**, iDVT presented with the highest platelet-dependent TG peak height and velocity compared to PE-related phenotypes. In absence of platelets, generation of thrombin was registered in all subjects, without important statistical differences between the groups.

3.5 | Machine learning with clinical and platelet-related biomarkers for discrimination between VTE phenotypes

Variables selected by the machine learning LASSO-regularized logistic regression with fractional polynomials adjusted for age, sex, traditional CVRFs, comorbidities and VTE risk factors are presented in **Figure 2** (full list of variables is available in the supplemental material, part B). For the distinction between DVT+PE and iDVT (reference), 18 variables were identified with high discriminatory power (AUC = 0.93). Platelet biomarkers comprised 72% of all variables selected. The analysis for distinction between DVT+PE and iPE (reference) resulted in obesity as only variable with modest discriminatory ability (AUC = 0.66). Differently, distinction between iPE and iDVT (reference) resulted in 10 variables, again with a high discriminatory ability (AUC = 0.96). In the latter distinction between the two isolated phenotypes, platelet biomarkers comprised 50% of all selected variables.

The percentage of platelets expressing PAC-1 after collagen-ADP agonism showed the highest LR (supplemental **Table S4**) for DVT+PE (LR = 5.2) and iPE (LR = 4.2) in relation to iDVT phenotype. The direction of the association was negative for PE-related phenotypes

TABLE 3 Thrombin generation in platelet rich and platelet free plasma according to disease phenotype

	Variable	DVT+PE	iDVT	iPE	*P*-value (iDVT vs DVT+PE)	*P*-value (iPE vs DVT+PE)	*P*-value (iDVT vs iPE)
	Number	91	35	28	-	-	-
PRP	Lag time (min)	10.49 (±9.13)	13.34 (±8.46)	14.80 (±10.28)	0.13	0.073	0.58
	ETP (nM*min)	1059.14 (0–1632.94)	1279.74 (797.8–1666.68)	1149.75 (329.16–1551.5)	0.15	0.80	0.27
	Peak height (nM)	35.02 (0–85.77)	67.71 (45.30–108.51)	48.53 (9.01–78.29)	0.0052	0.62	0.020
	Velocity (nM/min)	1.94 (0–8.52)	6.11 (3.47–13.31)	3.15 (0.58–7.21)	0.0010	0.38	0.040
PFP	Lag time (min)	n.a. (n.a.–13.94)	n.a. (n.a.–8.94)	8.66 (n.a.–12.34)	0.41	0.39	0.10
	ETP (nM*min)	0 (0–568.20)	0 (0–886.74)	339.96 (0–953.41)	0.86	0.17	0.65
	Peak height (nM)	0 (0–36.65)	0 (0–108.03)	19.93 (0–134.69)	0.82	0.14	0.61
	Velocity (nM/min)	n.a. (n.a.–7.92)	n.a. (n.a.–17.42)	2.68 (n.a.–30.87)	1.00	0.12	0.16

Note: Data presented are mean (±standard deviation), tested with a t-test and/or median (interquartile range), tested with a Wilcoxon test.

Abbreviations: ETP, endogenous thrombin potential; iDVT, isolated deep vein thrombosis; iPE, isolated pulmonary embolism; n.a., non-available (the thrombin generation curve was a flat line).

in comparison to iDVT. Lower maximum aggregation with low EPI (0.5 μM, LR = 1.7) and lower velocity of TG in PRP (LR = 1.7) were further identified for DVT+PE compared to iDVT phenotype. The maximum aggregation after low ADP (0.5 μM) was positively and collagen-ADP aggregation time was negatively linked with DVT+PE in respect to iDVT phenotype. Lower peak height in PRP (LR = 2.7) and higher maximum aggregation after TRAP-6 further differentiated iPE vs iDVT phenotype.

Concerning the clinical characteristics, presence of arterial hypertension (LR = 3.5), absence of (diagnosed) thrombophilia (LR = 2.6) and a negative history of DVT (LR = 2.2) characterized the DVT+PE phenotype in respect to iDVT. When comparing the isolated phenotypes, negative history of DVT (LR = 2.6), absence of obesity (LR = 1.6) and trauma (LR = 1.4) and positive history of diabetes mellitus (LR = 1.1) and atrial fibrillation (LR = 1.1) were identified as predictors for the iPE phenotype.

As presented in the supplemental **Figure S3**, further considering the antiplatelet and anticoagulant agents in the LASSO model, resulted in 16 variables differentiating between iDVT and DVT+PE phenotype with AUC = 0.91. The percentage of platelets expressing PAC-1 after collagen-ADP agonism remained the top variable with the highest predictive power (LR = 4.3) followed by the intake of antiplatelet agents with LR = 2.9. Platelet biomarkers comprised 56% of all variables differentiating between iDVT and DVT+PE phenotype. The distinction between iDVT and iPE resulted in 10 variables of which 60% were platelet biomarkers with high discriminatory power (AUC = 0.92). Lower platelet surface PAC-1 expression remained the top biomarker followed by intake of antiplatelet agents more prevalent for iPE vs iDVT subgroup. The distinction between DVT+PE and iPE did not change and obesity remained the unique differentiating variable.

4 | DISCUSSION

Although the risk factors for VTE are quite comparable for DVT and PE, hence the term VTE is used for a condition that was deemed to comprise one entity, recent insights suggest that isolated PE has distinctly different features from DVT. This led to the postulation of different endophenotypes of VTE, which may be clinically relevant in terms of the management. While PE remains the third most common cause of death after myocardial infarction and stroke,[25] specific components in the pathophysiology, like the contribution of platelets, may differ from DVT, a condition that is generally managed with oral anticoagulant therapy only.

Using a large panel of clinical covariates and for the first time platelet function characteristics, we phenotypically profiled a large cohort of patients with different acute manifestations of VTE. A substantial number of platelet biomarkers covering various aspects of platelet function were measured in the acute event in addition to clinical characteristics to deeper characterize VTE phenotypes. At presentation and after confirmation of VTE, patients are asked to stop with the antiplatelet agents and start with an anticoagulant

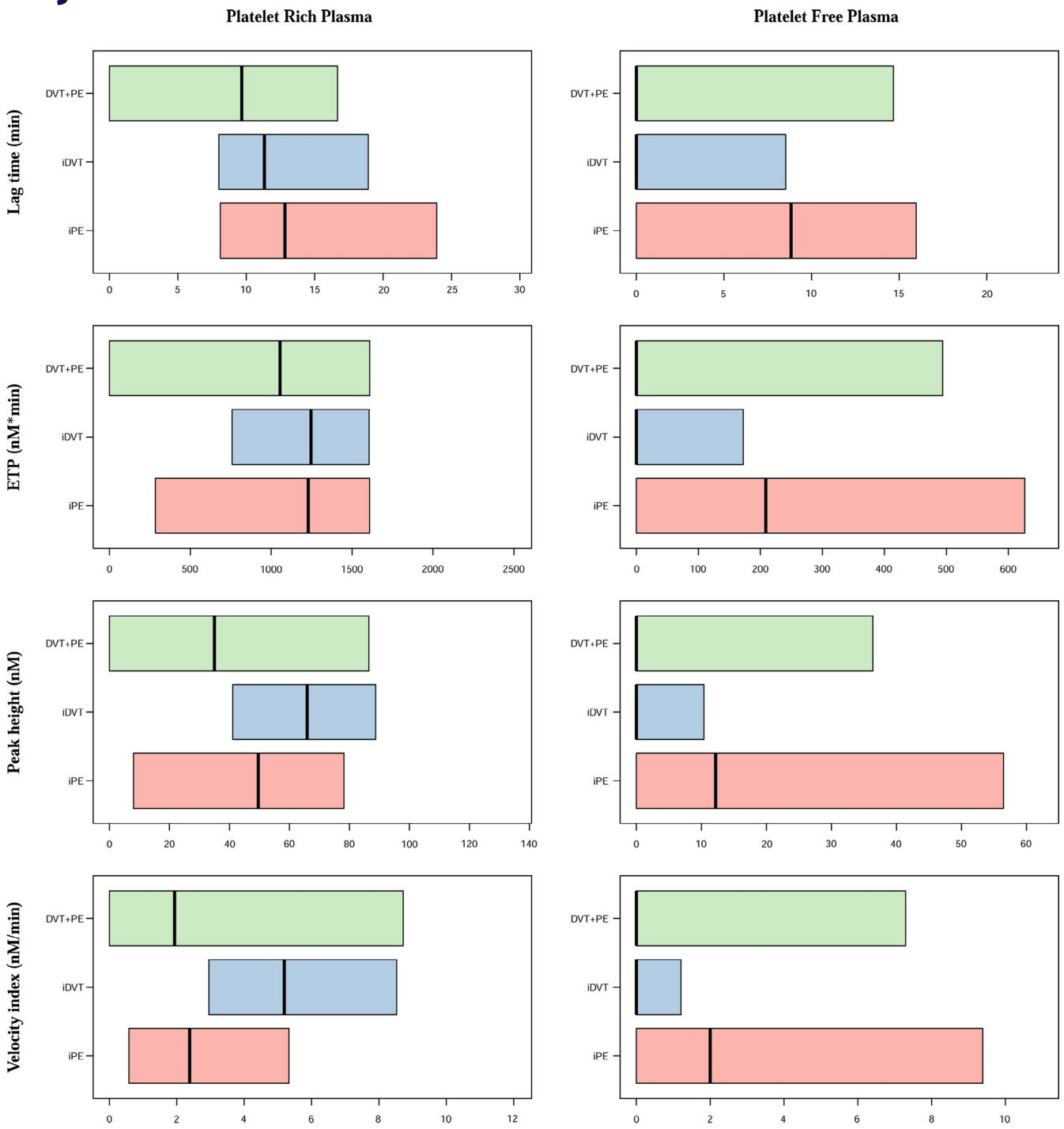

FIGURE 1 Thrombin generation in subjects taking anticoagulants in plasma with and without platelets. Presented are lag time (min), endogenous thrombin potential (ETP, nM*min), peak height (nM) and velocity index (nM/min) measured in platelet rich plasma (plots on left) and platelet free plasma (plots on right). Calculated p-values by t-test for normally distributed variables and Wilcoxon test for variables with skewed distribution between all three groups are available in supplemental Table S2. Abbreviations: iDVT, isolated deep vein thrombosis ($n = 25$); iPE, isolated pulmonary embolism ($n = 23$); DVT+PE ($n = 76$)."

therapy, according to the guidelines of antithrombotic therapy for VTE.[26] Considering that the effects of ASA last for the duration of the life of the platelet (≈10 days), and the time between VTE diagnosis and stopping the antiplatelet medication was with a median of 0 days (IQR: 0–2 days) we considered in our analysis also the intake of both antiplatelet and anticoagulant agents.

We demonstrated that PE-related phenotypes differ from isolated DVT in respect to both, clinical and platelet function characteristics. Interestingly, 72% and 50% of the relevant variables for the differentiation of DVT+PE and iPE from the iDVT phenotype were platelet-related biomarkers and these had the highest lambda ratios as predictive measure. However, DVT+PE did not relevantly differ

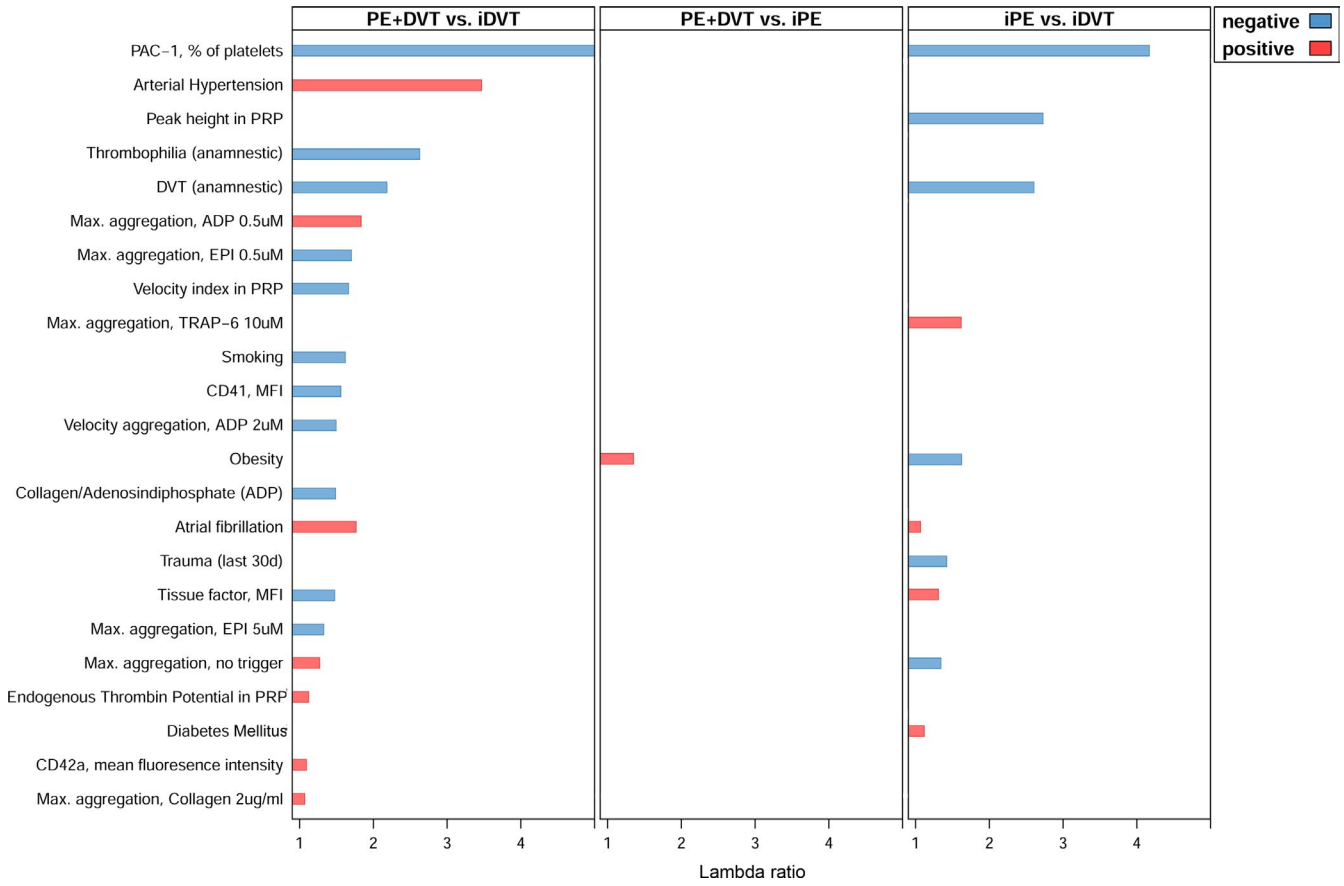

FIGURE 2 Variables differentiating between VTE phenotypes. Variables in blue have a negative association, e.g. the variable is lower in the investigated group when compared to the reference group. Variables in red show a positive association, e.g. the variable is higher in the investigated group when compared to the reference group. LASSO regression with fractional polynomial transformations, was used to select clinical variables and platelet function biomarkers that most relevantly distinguished between VTE clinical phenotypes. Number of total predictors, $N = 60$ (platelet biomarker predictors, $N = 38$). iDVT, isolated deep vein thrombosis; iPE, isolated pulmonary embolism; PRP, platelet rich plasma; EPI, epinephrine; MFI, mean fluorescence intensity; TRAP-6, thrombin activated peptide-6.

from iPE, considering the clinical and platelet function variables, and obesity was the only differing characteristic.

To further dissect the platelet involvement, we showed that platelet surface expression of PAC-1, after in-vitro collagen-ADP agonism, was the best discriminator for DVT+PE and/or iPE, compared to iDVT. Reduced platelet reactivity in PE-related phenotypes, both concomitant DVT+PE and iPE, is in line with lower platelet-dependent TG peak height and TG velocity in iPE and/or DVT+PE compared to iDVT, respectively. In vivo, platelets are crucial for TG amplification by exposing negatively charged phospholipids, thus providing a procoagulant surface for the assembly of tenase and prothrombinase complexes.[27] Furthermore, as observed by the in-vitro CAT assay, phosphatidylserine exposure on activated platelets regulates both the onset and the rate of TG, when assessed in TF triggered PRP.[28] The expression of phosphatidylserine on the platelet surface was not determined in the present sample, however the low expression of the available platelet-surface biomarkers (e.g. P-selectin and CD63) suggests a prior platelet release reaction in this clinical context. Platelet surface TF expression and fibrinogen binding were also lower in PE-related phenotypes compared to iDVT. These results, along with reduced platelet reactivity after in-vitro platelet agonism, further support the hypothesis of degranulated platelets in the PE-dependent phenotype ("exhausted platelet" syndrome), presumably as consequence of a previous activation.[29] Reduced platelet responsiveness to platelet agonists in-vitro and lower percentage of platelets expressing P-selectin have been reported in cancer patients at risk for VTE and increased mortality.[14]

The results of the present study are important to grasp the profile of platelet function in an acute VTE event and to highlight potential different roles of platelets in PE associated conditions. There may be several reasons for a more prominent role of platelets in PE, including the different vascular bed-related flow conditions, the etiology of PE which in part may have a local origin, e.g. in the setting of isolated PE, and the clustering of platelets within the pulmonary circulation. Moreover, there is a marked overlap in risk factors for venous and arterial thromboembolic manifestations, as well as an increased risk of major adverse cardiovascular events in subjects with VTE, which may indirectly support an overlap in pathophysiology, including platelets in VTE and coagulation factors in arterial thrombosis.[30,31] Increased expression of platelet surface activation

markers, shorter PFA-100 closure times and higher TG have been reported in individuals with CVRFs, acute coronary syndrome or stable cardiovascular disease.[23,32-34] On the other hand, CVRFs have also been associated with VTE risk.[35] In a recent, large population-based study including more than 700,000 individuals, older age, smoking and obesity were consistently associated with higher VTE risk.[36] Obesity as a complex chronic relapsing disease has been characterized with prothrombotic properties and could differentially contribute to coagulation and platelet activation in different acute VTE phenotypes.[37] Interestingly, the current study revealed that all platelet-related biomarkers were linked to distinct VTE phenotypes independent of the individual's cardiovascular profile. A therapeutic implication could be to reconsider the use of platelet inhibitors in selected patients with acute VTE, in analogy to the setting of acute coronary syndrome where the combination of low dose rivaroxaban and aspirin provided a gain in efficacy, albeit at a price of bleeding.[38] Beyond antithrombotic protection, aspirin administration can further significantly alleviate the pulmonary vasoconstriction and impaired oxygenation associated with acute PE.[39]

The present study has also potential limitations which need to be addressed: The still relatively small sample size of individuals with platelet function testing available, limited the possibility to address the relevance of the "exhausted platelet" syndrome for clinical outcome. The majority of subjects enrolled were taking anticoagulant therapy, antiplatelet agents or both drugs at the time of presentation in the hospital. Antithrombotic therapy is well acknowledged to affect platelet function testing. In specific, antiplatelet agents have profound effect on platelet aggregation particularly after epinephrine and arachidonic acid trigger, whereas anticoagulant agents have important effect on TG. The differences between VTE phenotypes we are reporting here were unlikely to be drug dependent, as the same differences were observed after stratification for the therapy regimen. Because of the acuteness of the disease and the clinical need to start with anticoagulant therapy as soon as possible, the number of subjects without anticoagulant therapy at baseline was very low. This did not allow for evaluating the status in absence of the respective therapy. This study has an important and unique strength: It is the first study to demonstrate a different role of platelets in phenotypes of VTE in humans. Until now, platelet function testing was predominantly applied in the diagnostic work-up of conditions with bleeding diathesis, but had no potential impact in acute venous thrombosis. Finally, the small sample size of patients with available platelet function testing prevented us from assessing the association with clinical outcome. Larger longitudinal studies with available platelet phenotype data are needed to ascertain which platelet biomarkers are linked with worse clinical outcome and to identify subpopulations of VTE patients at different response to therapy and/or risk for VTE complications.

This study provides an important insight on platelet function varying with the phenotype of acute VTE. A set of clinical and platelet markers could clearly discriminate PE-related phenotypes from iDVT. It remains to be further investigated whether platelet function is of relevance to improve clinical outcome after an acute VTE event.

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CONFLICT OF INTEREST

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AUTHOR CONTRIBUTIONS

MP-N. and PSW. conceived the study and MP-N., HtC., and PSW drafted the manuscript. BW performed platelet function assays. MP-N, TK, VtC, LE, JHP, IM, CG, HMS, KJL, HtC, KL, SH, SK and PSW were involved in the analysis strategy. MN performed the statistical analyses. MP-N, BW, MN, TK, VtC, LE, JHP, IM, CG, HMS, KJL, HtC, KL, SH, SK and PSW participated in the interpretation of the findings, reviewed the manuscript and revised it critically before submission. All authors have seen and approved the final version of the manuscript.

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SUPPORTING INFORMATION

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