Comprehensive platelet phenotyping supports the role of platelets in the pathogenesis of acute venous thromboembolism – results from clinical observation studies

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ABSTRACT

Background: The pathogenesis of arterial and venous thrombosis is in large part interlaced. How much platelet phenotype relates to acute venous thromboembolism (VTE) independent of the underlying cardiovascular profile is presently poorly investigated.

Methods: Platelet count and mean platelet volume (MPV), platelet aggregation in whole blood and platelet rich plasma (PRP), platelet-dependent thrombin generation (TG) and platelet surface activation markers were measured under standardized conditions. Machine learning was applied to identify the most relevant characteristics associated with VTE from a large array (N = 58) of clinical and platelet-related variables.

Findings: VTE cases (N = 159) presented with lower platelet count and MPV vs controls (N = 140). Whole blood aggregation showed shorter collagen/Epinephrine closure times in cases, particularly within aspirin (ASA) users. Within ASA users, higher PRP aggregation after adenosine diphosphate (ADP), epinephrine, collagen and arachidonic acid was observed in cases vs controls. Within non-ASA and/or subjects on anticoagulants, cases presented with lower aggregation after ADP and collagen vs controls. Lower platelet-dependent TG, higher CD63 on resting and lower PAC-1 expression after collagen/ADP in-vitro stimulated platelets further characterized VTE cases vs controls, independent of therapy. Lasso regression analysis identified 26 variables associated with VTE of which 69% were platelet-related.

Interpretation: Comprehensive phenotyping of platelet function identified a large proportion of low responders to ASA in VTE cases. Lower platelet-dependent TG and lower platelet reactivity after ex-vivo stimulation characterized the “platelet exhausted syndrome” in cases. Finally, from a large array of covariates including clinical risk factors, platelet biomarkers comprised 69% of all selected variables differentiating VTE cases vs controls.

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1. Introduction

Platelets are complex cells, without nucleus but rich in granules, which promptly respond to signals from circulating cells, vessel wall and other blood components. The role of platelets in atherogenesis, atherosclerosis and ultimately atherothrombosis is well
Research in context

Evidence before this study

The role of platelets in atherogenesis, atherosclerosis and ultimately atherothrombosis is well acknowledged. Recent evidence from animal models demonstrated an important role of platelets in development of venous thrombosis; however, the clinical evidence is rather limited. In humans, the pathogenesis of arterial and venous thrombosis is interlaced; individuals with venous thromboembolism (VTE) are shown to have an increased risk of atherosclerotic disease as well as individuals with atherosclerotic disease are at increased risk of subsequent VTE.

Added value of this study

This is the largest study comprehensively investigating platelet function in subjects with acute VTE to date. We demonstrated that platelet phenotype substantially differs between VTE cases and those with a VTE ruled out independent of the underlying cardiovascular risk profile. This study provides evidence for the occurrence of acetylsalicylic acid low-responsiveness in acute VTE which might explain the failure of acetylsalicylic acid to better protect subjects at risk, but also provides evidence for "exhausted" platelets in this disease. Using a machine learning technic including large array of the most relevant clinical and laboratory characteristics, platelet biomarkers comprised 69% of all selected variables differentiating VTE cases vs VTE controls.

Implications of all the available evidence

The available evidence substantiates the relevance of platelet function for the presence of an acute VTE event and gives insights into the role of platelets in the pathophysiology of this disease. Whether platelet phenotype characterization could improve identification of individuals at risk for recurrent VTE and/or worse outcome remains to be determined.

acknowledged [1]. Activated platelets are key players in arterial thrombosis, the principal cause of e.g. myocardial infarction and ischemic stroke. Antiplatelet agents interfere with platelet aggregation and represent a cornerstone for treatment and secondary prevention of atherothrombosis [2]. Conventional cardiovascular risk factors (CVRFs) are strongly related to development of cardiovascular adverse events and have been consistently associated with platelet activation [3,4]. Hence, there is solid evidence linking platelet activation and arterial prothrombotic conditions.

Most recent evidence from animal models demonstrated also an important role of platelets in development of venous thrombosis [5,6]; however, the clinical evidence is rather limited. In a small group of subjects with acute VTE, increased platelet activation with increased expression of platelet surface P-selectin and increased percentage of platelet-leukocyte conjugates, has been reported [7]. The most often investigated platelet parameter is mean platelet volume (MPV), a potential marker of platelet activation [8]. In a prospective population-based study, higher MPV was associated with higher risk of incident unprovoked VTE [9]. Differently, in patients presenting at the emergency department, lower MPV was associated with higher risk for objectively confirmed VTE diagnosis [10]. Likewise, in cancer patients, lower MPV was linked with higher VTE risk and worse survival [11]. The results from clinical trials assessing the effect of low-dose acetylsalicylic acid (ASA) against placebo showed a modest effect with around 40% reduction of recurrent VTE events, and at the same time reduction in major arterial events as well [12].

The pathogenesis of arterial and venous thrombosis is to some extent interlaced and individuals with VTE are shown to have an increased risk of atherosclerotic disease [13,14]. Similarly, it has been suggested that individuals with atherosclerotic disease are at increased risk of subsequent venous disease [15]. Whether arterial and venous thrombosis is the same disease at different vascular beds is still controversial considering the knowledge that traditional atherosclerotic risk factors are not entirely shared by both conditions [16].

To what extent platelet pathophysiology is involved in the acute VTE process, independent of the subjects’ underlying cardiovascular profile, is unknown. In the present work we undertook a comprehensive exploration of platelet function including platelet aggregation, platelet-dependent thrombin generation and platelet surface activation status in subjects presenting with signs and symptoms of deep vein thrombosis (DVT) and/or pulmonary embolism (PE) in an acute care setting. To better understand the role of platelets in the pathophysiology of VTE independent of underlying CVRFs and VTE risk factors, we used machine learning technic including large array of the most relevant clinical and laboratory characteristics predictive for VTE.

2. Methods

2.1. Study sample

Participants from the VTEval and FOCUS BioSeq studies at the Johannes Gutenberg University Medical Center Mainz in Germany, randomly selected at their baseline examination, were included in the platelet function study. The VTEval (NCT02156401) is a prospective observational single-center study including individuals with clinically suspicion for DVT and/or PE, recruited in the acute care setting such as emergency rooms, chest pain units and outpatient clinics, as described in detail before [17]. FOCUS BioSeq is a multi-center prospective study, conducted nationwide in Germany, enrolling individuals with objectively confirmed acute symptomatic PE [18]. In the platelet function study, confirmed subjects with DVT and/or PE were enrolled from both VTEval and FOCUS BioSeq study at the University Medical Center in Mainz. Controls were subjects with excluded VTE diagnosis and were selected from the VTEval study. Subjects with active cancer (n = 35) were excluded, leaving 294 individuals with platelet function measurements for the analysis. The VTEval and FOCUS BioSeq studies were implemented in accordance to the General Data Protection Regulation (EU 2016/679) and the Declaration of Helsinki (2013, 7th Revision). All participants provided written informed consent for participation.

Blood sampling and plasma preparation

Venous blood sampling was performed at inclusion of the subjects in the study when presenting at the University Medical Center in Mainz with signs and symptoms of VTE. Venous blood sampling was performed using tubes containing trisodium citrate (3.2%, 0.109 M, 1:9 vol:vol) and a PFA monovette (3.8%, 0.129 M) for the Innovance Platelet Function Analyzer (PFA)-200 (Siemens Healthcare, Marburg, Germany). Fresh citrated whole blood for platelet function analysis was hand delivered in the platelet epidemiology laboratory within 30 min after blood withdrawal at time of enrolment. Whole blood, platelet-rich plasma (PRP), platelet-poor plasma (PPP) and platelet-free plasma (PPP) were prepared as follows: PRP was isolated by centrifugation of whole blood at 200 × g for 10 min at room temperature (RT); after collecting the top 2/3rd of PRP, PPP was obtained by further centrifugation of the sample for 15 min at 2,000 × g at RT; PPP was obtained by centrifugation of whole blood for 5 min at 2,000 × g.
at RT. The collected PPP was further centrifuged for 10 min at 11,000 x g. The isolated PPP was stored at -80°C until further laboratory testing.

**Platelet function assays**

Platelet function testing was performed at the day of study subject's inclusion in a highly standardized manner with time tracking directly after blood withdrawal at the Platelet Epidemiology laboratory of the Johannes Gutenberg University Medical Center Mainz. In addition to standard platelet parameters available from blood count analysis, four platelet function assays were performed for assessing platelet aggregation, platelet-dependent thrombin generation and platelet surface activation. The flow of platelet function measurements has been described in detail before [19].

**Standard platelet parameters**

Platelet indices, namely platelet count and mean platelet volume (MPV) were automatically assessed on an ADVIA 120 Hematology System (Siemens, Erlangen, Germany) between 30 and 90 min after blood sampling as described in detail before [20].

**Platelet aggregation**

Platelet aggregation was investigated by two methods, in whole blood by PFA-200 and in PRP by Light Transmission Aggregometry (LTA).

PFA-200 (Siemens Healthcare, Marburg, Germany) is an automated assay using provided cartridges (Siemens Healthcare, Marburg, Germany) coated with platelet agonists (collagen/adenosine-diphosphate [ADP] and collagen/epinephrine [EPI]) to assess primary hemostasis at high shear rates. In the aperture of the cartridges 800 mL of whole blood was pipetted. The occlusion time until complete central aperture sealing is automatically reported as closure time (CT), expressed in seconds (sec). The maximum measurable CT was 300 s.

LTA was performed using APACT 4S Plus aggregometer (LABItec, Ahrensburg, Germany). Parameters for response were adjusted to 100% using PPP and 0% using PRP from each participant. Agonists were added to the PRP (unadjusted for platelet count), and platelet aggregation was monitored for 10 min by aggregometer tracings. Final test concentrations of agonists were 0.5 μmol/L and 2 μmol/L for ADP, 0.5 μmol/L and 5 μmol/L for EPI, 2 μg/mL for collagen, 1 mmol/L for arachidonic acid and 10 μmol/L for thrombin receptor activated peptide-6 (TRAP-6). In addition, no-trigger aggregation without addition of platelet agonists was assessed. The maximum percent aggregation (%) and velocity aggregation (%/min) were the recorded parameters of interest.

**Thrombin generation**

Thrombin generation (TG) was measured by the Calibrated automated thrombogram (CAT) assay (Thrombinscope BV, Maastricht, The Netherlands) according to standardized protocols as previously reported [21]. TG in PRP was assessed in fresh material, whereas TG in PPP was assessed in frozen material. For TG measurements in PRP, 20 μl of the exogenous PRP reagent (1pM TF) were added to 80 μl PRP (with adjusted platelet concentration of 150,000 platelets/μl using autologous PPP), whereas for the measurements in PPP, 20 μl of the exogenous low PPP reagent (1pM TF together with 4 μM phospholipids) were added to 80 μl plasma. Following 10 min incubation at 37°C in the fluorimeter, 20 μl of low-affinity fluorogenic substrate for thrombin (Z-Gly-Gly-Arg-AMC) and calcium chloride mixture (FluCa), were automatically dispensed into each well. To correct for inner filter effects and substrate consumption, TG measurements were calibrated against a signal from the calibration well obtained in a sample from the same plasma (80 μL PRP or PPP, respectively), supplemented with a fixed amount of thrombin–o2-macroglobulin complex (20 μL of Thrombin Calibrator) and 20 μL of FluCa by means of Thrombinscope software (Thrombinscope BV). All CAT reagents were obtained from Stago Deutschland GmbH (Düsseldorf, Germany). After 120 min, the resulting TG curve was analysed for the following parameters: lag time, as the time for minimum thrombin formed (min), peak height, as the maximum concentration of thrombin formed (nM thrombin), endogenous thrombin potential (ETP), as the total amount of thrombin formed over time (nM*min), time to peak, as the time until the peak height (min) and velocity index defined with the formula: peak height/(time to peak – lag time), as a measure of the speed of TG (nM/min).

Flow cytometric analysis of platelets

Platelet surface activation antigens were assessed in citrated whole blood on Accuri C6 (BD, Heidelberg, Germany). Briefly, 5 μl of whole blood at resting condition was double stained with a platelet identifying monoclonal antibody CD42a-PerCP (Becton Dickinson, Heidelberg, Germany) for gating plus one of the following antibodies: CD41-PE (Beckman Coulter, Krefeld, Germany), CD62p-FITC (Becton Dickinson, Heidelberg, Germany), CD63-FITC (Beckman Coulter, Krefeld, Germany), anti-human Fibrinogen-FITC (DAKO, Glostrup, Denmark), anti-human Tissue Factor–FITC (Sekusui Diagnostics, Stamford, Connecticut, US). In addition, 5/μl of whole blood after Collagen/ADP test with the PFA-200 assay was double stained with CD42a-PerCP and PAC-1-FITC (Becton Dickinson, Heidelberg, Germany). The samples were incubated for 20 min at room temperature in dark after which the reaction was stopped with addition of 500 μl phosphate-buffered saline. The samples were then immediately analyzed and percentages (%) of platelets as well as the mean fluorescence intensity (MFI) expressing the specific antigens were recorded.

**Determination of levels of thromboxane B2 (TXB2) by ELISA**

Levels of TXB2, a stable metabolite of thromboxane A2 (TXA2), were determined colorimetrically in citrated plasma by competitive ELISA (ab133022; Abcam, Cambridge, UK), according to the instructions of the manufacturer. The generated color intensity was determined at 405 nm with correction at 580 nm using a Teco Infinite M200 Pro microplate reader (Tecan Group Ltd., Männedorf, Switzerland). The results were expressed as nanogram per milliliter (ng/ml).

**Data management and statistical analysis**

All data underwent quality control ensuring completeness and plausibility by using predefined algorithms and criteria by a central data management team. Definitions of tradition cardiovascular risk factors (CVRFs), VTE risk factors and categorization of medications are presented in supplemental material (part A).

Data are reported as mean (standard deviation) when normally distributed or median (interquartile range) when skewed distribution. Categorical variables are presented as absolute numbers and percentages (%). Differences between groups have been calculated using the t test and/or Mann-Whitney U test, if needed. Because of the explorative character of the analysis, a threshold of significance was not defined for P values. P values should rather be interpreted as a continuous measure of statistical evidence.

Least absolute shrinkage and selection operator (LASSO) regularized logistic regression, a common technique for supervised machine learning, was selected for the ability to deal with p > n situations, when number of variables (p) exceeds the number of observations (n) [22]. This method was used to select variables from a comprehensive panel of clinical markers and platelet function markers that most
Data Statement

Data are not made available for the scientific community outside the established and controlled workflows and algorithms. To meet the general idea of verification and reproducibility of scientific findings, we offer access to data at the local database in accordance with the ethics vote upon request at any time. Interested researchers make their requests to the coordinating principal investigator of the VTE studies (Dr. Philipp S. Wild; philipp.wild@unimedizin-mainz.de).”

Results

Characteristics of the study subjects

Descriptive data are presented in Table 1 for 159 individuals with confirmed acute VTE (case group) and 140 individuals with objectively excluded VTE (control group). Females were 20% less frequent in the VTE group compared to the control group. Age and body mass index did not differ between groups whereas, as expected, D-Dimer and PE frequency was notably different between VTE cases and controls. The optimal \( \lambda \) selected by cross-validation.

Table 1: Study subject characteristics

| Characteristic                  | VTE case | VTE ruled out |
|--------------------------------|----------|---------------|
| Number                         | 159      | 140           |
| Females, % (N)                 | 41% (65) | 61% (86)      |
| Age (years)                    | 60 (50/73) | 59 (45/72)  |
| Body Mass Index (kg/m²²)       | 28 (25/32) | 28 (24/32)  |
| D-Dimer (mg/l)                 | 5 (2/11) | 0.8 (0.5/1)  |
| VTE risk factors               |          |               |
| VTE (history)                  | 35% (51/148) | 19% (27/139) |
| DVT (history)                  | 31% (46/148) | 18% (25/139) |
| PE (history)                   | 15% (22/148) | 7% (9/138)   |
| Immobilization (last 30 days)  | 16% (24/149) | 5% (7/138)   |
| Long-distance flight/travel     | 14% (21/148) | 10% (14/137) |
| Pregnancy (current)            | 0% (158) | 1% (2/140)   |
| Surgery (last 30 days)         | 3% (5/148) | 2% (3/136)   |
| Thrombophilia                  | 4% (5/126) | 3% (7/138)   |
| Trauma (last 30 days)          | 8% (11/147) | 1% (2/138)   |
| Cardiovascular risk factors    |          |               |
| Arterial Hypertension          | 57% (81/143) | 48% (67/139) |
| Diabetes                       | 17% (25/144) | 12% (16/139) |
| Smoking                        | 19% (27/142) | 16% (22/138) |
| Obesity                        | 37% (58/158) | 37% (51/137) |
| Cardiovascular diseases        |          |               |
| Atrial fibrillation            | 6% (8/144) | 11% (15/137) |
| Congestive heart failure       | 6% (8/143) | 8% (11/137)  |
| Coronary artery disease        | 9% (13/145) | 16% (22/136) |
| Stroke                         | 6% (8/145) | 7% (9/138)   |
| Peripheral artery disease      | 5% (6/114) | 4% (5/138)   |
| Therapy                        |          |               |
| Antithrombotic agents (B01A)   | 93% (148/159) | 63% (88/140) |
| Acetylsalicylic acid (B01AC06) | 35% (55/159) | 29% (41/140) |
| Clopidogrel (B01AC04)          | 3% (4/159) | 4% (6/140)   |
| Anticoagulant agents*          | 81% (128/159) | 44% (61/140) |
| Vitamin K antagonists (B01AA)  | 5% (8/159) | 4% (6/140)   |
| Heparin group (B01AB)          | 67% (107/159) | 36% (50/140) |
| Direct FXa inhibitors (B01AF)  | 25% (39/159) | 4% (6/140)   |
| Cardiovascular agents**        | 60% (95/159) | 42% (59/140) |

Presented are demographic and clinical characteristics as relative frequencies in percentage (%), rounded to one informative digit, for the venous thromboembolism (VTE) cases and VTE ruled out. Age and body mass index are presented as median (25% quartile and 75% quartile). Anatomical Therapeutic Chemical Classification System code:

* B01AA, B01AB, B01 AF.
** C01-C10.

Platelet aggregation

The results of platelet aggregometry on collagen/adenosinediphosphate (collagen/ADP) and collagen/epinephrine (collagen/EPI) assessed by PFA-200 are presented in Fig. 1. VTE cases showed shorter closure time for collagen/ADP (86.5 [70.0/104.2] sec.) and collagen/EPI (125.0 [102.3/251.7] s.) compared to VTE controls (collagen/ADP: 94.5 [79.0/115.0] s.; collagen/EPI: 155.0 [115.3/300.0] s.). Within the subgroup of subjects taking ASA, the differences between cases and controls remained with diminished statistical power, for both collagen/ADP and collagen/EPI. In subjects without ASA, VTE cases had shorter CTs than the control group for both triggers, collagen/ADP and collagen/EPI.

The results from LTA stratified for presence of VTE is presented in the whole sample and in subgroups according to intake of antithrombotic therapy in Supplemental Table S2. The strength of evidence for the difference (reflected by the p-value for testing) in maximum aggregation and/or velocity aggregation between cases and controls according to therapy is reported in Fig. 2. Aggregation velocity after low EPI (0.5 µM) was higher and after ADP (2 µM) and collagen
lower in VTE cases not only in the whole sample, but also in the sub-
groups of ASA non-users and in subjects on anticoagulation. Further-
more, within subjects taking ASA, VTE cases had higher maximum
aggregation particularly after EPI (5 μM), collagen and arachidonic
acid compared to controls.

**Thromboxane B2**

To confirm the low response on ASA, observed as higher platelets
aggregation in cases vs controls, levels of Thromboxane B in citrated
plasma were assessed. As shown in Figure S1, VTE cases presented
with higher Thromboxane B2 levels compared to VTE controls in the
whole sample (cases: 3.4 [2.2/6.1]; controls: 1.7 [1.0/3.9]), within
subgroup of ASA users (cases: 3.0 [1.4/4.3]; controls: 1.0 [0.7/1.5] and
in the subgroup of subjects not on ASA (cases: 3.6 [2.5/6.3]; controls:
2.6 [1.4/4.9]).

**Thrombin generation in presence and absence of platelets**

In presence of platelets, TG from VTE cases presented with longer
lag time and time to peak, lower ETP and peak height and lower
velocity compared to the control group (Table 2). As anticoagulation
therapy has a substantial impact on TG parameters, the analysis was
stratified according to intake of anticoagulant agents. Within subjects
on anticoagulants, differences between groups were not observed for
most of TG parameters, except for lower velocity index and a ten-
dency to lower peak height in VTE cases. In subjects not on anticoa-
gulation, peak height and velocity were also lower in VTE cases
(though with a clearer difference between groups), but additionally
lag time and time to peak were longer in the cases. Interestingly, ETP
did not differ with presence or absence of VTE in individuals not tak-
ing anticoagulants.

In absence of platelets, similar findings as in presence of platelets
were observed with strongly diminished TG parameters in VTE cases
compared to controls. Within those on anticoagulant agents, in more
than 50% of VTE cases TG presented as flat line without thrombin for-
mation (e.g. for lag time: 25th and 75th percentile were non applicable
and 14.23, respectively). In subjects without anticoagulants TG
parameters were comparable between cases and controls.

**Platelet surface activation**

Flow cytometric results of platelet surface activation markers
between groups according to presence of VTE are displayed in supple-
mental Table S3. At resting platelet conditions, a higher percent-
age of platelets expressing P-selectin, CD63 and fibrinogen were
found in VTE cases compared to controls. No relevant differences
were observed for MFI of these activation markers. Differently, the
percentage of platelets expressing PAC-1, after collagen/ADP trigger
with the PFA-200 system, showed trend for lower expression in cases
(20.8 [9.7/38.0] vs. 27.6 [11.0/46.0]). Within ASA users the differences
remained unchanged for most of the activation markers. The differ-
ence for percentage of platelets expressing P-selectin was not present
anymore, however an important difference for P-selectin MFI was
observed between VTE cases (2580 [2092/4486]) and controls (3612
[2327/9272]). Within subjects on anticoagulants, CD63 expression
remained higher and PAC-1 remained lower in cases vs controls.
Within the combined anticoagulant and ASA therapy, cases presented
with lower percentage of platelets expressing PAC-1 compared to
controls.

**Variables predicting VTE by applying machine learning**

Least absolute shrinkage and selection operator (LASSO)-regular-
ized logistic regression including a large variable set of 58 covariates
including age, sex, clinical information on traditional CVRFs and VTE
risk factors, and platelet biomarkers as predictive variable was
applied to distinguish VTE cases from individuals without VTE. The
variables selected with this approach are presented in supplemental
Table S4 with 3 fold cross-validated AUC= 0.88. Fig. 3 summarizes the
selected variables in the whole sample and in the subsample taking
anticoagulants (without presenting the same variable — if selected —
in several transformations). When analyzing the whole sample, 26
variables were identified as predictors or presence of VTE. Of these
26 variables, 69% (18 variables) were platelet-related biomarkers. The largest effect for presence of a VTE event was the velocity index in presence of platelets with a lambda ratio of 3.97 and a negative association (being lower in VTE cases) followed by a positively associated expression of CD63 (% of platelets), as a platelet dense granule protein, with lambda ratio of 3.65. Within the subgroup of subjects taking anticoagulant agents, in total 13 variables predictive of VTE were selected of which even greater percentage, 84.6% (11 variables), were platelet-related biomarkers. The highest lambda ratio of 3.18, negatively associated with VTE, was attributed to expression of PAC-1 (% of platelets) followed by the positively associated velocity of aggregation after low EPI with lambda ratio of 3.02. The selection of variables predictive of VTE in the smaller subgroups of ASA users is presented in supplemental Figure S1.

Discussion

This comprehensive investigation including platelet aggregation, platelet-dependent and -independent thrombin generation and platelet-surface activation reports important evidence for distinct platelet characteristics in individuals with acute VTE independent of the underlying cardiovascular profile.

Individuals with VTE presented with different platelet aggregation, compared to subjects without VTE, also under intake of ASA: platelet aggregation in whole blood showed shorter CT after Collagen/ADP and Collagen/EPI in cases and shorter Collagen/EPI CT was also observed in the subgroup taking low dose ASA. The findings were confirmed by LTA using the agonists EPI and arachidonic acid, both agents sensitive for assessing inhibition of the acetylic acid

|                | WS       | ASA     | w/o ASA  | AC      | w/o AC   | ASA+AC  |
|----------------|----------|---------|----------|---------|----------|---------|
| Max. agg., no trigger | 0.77     | 0.68    | 0.86     | 0.82    | 0.26     | 0.74    |
| Velocity agg., no trigger | 0.30     | 0.67    | 0.44     | 0.57    | 0.85     | 0.52    |
| Max. agg., ADP 0.5uM | 0.50     | 0.47    | 0.87     | 0.85    | 0.80     | 0.38    |
| Velocity agg., ADP 0.5uM | 0.63     | 0.22    | 0.56     | 0.73    | 0.68     | 0.48    |
| Max. agg., ADP 2uM | 0.49     | 0.16    | 0.14     | 0.13    | 0.82     | 0.69    |
| Velocity agg., ADP 2uM | 0.022    | 0.91    | 0.0028   | 0.16    | 0.42     | 0.66    |
| Max. agg., EPI 0.5uM | 0.34     | 0.38    | 0.16     | 0.19    | 0.51     | 0.64    |
| Velocity agg., EPI 0.5uM | 0.00010  | 0.70    | 0.00010  | 0.0049  | 0.091    | 0.35    |
| Max. agg., EPI 5uM | 0.49     | 0.063   | 0.12     | 0.31    | 0.60     | 0.15    |
| Velocity agg., EPI 5uM | 0.18     | 0.87    | 0.20     | 0.36    | 0.51     | 0.82    |
| Max. agg., Collagen 2ug/ml | 0.10     | 0.024   | 0.0026   | 0.35    | 0.044    | 0.25    |
| Velocity agg., Collagen 2ug/ml | 0.0029    | 0.45    | 0.00017  | 0.079   | 0.018    | 0.88    |
| Max. agg., AA 1mM | 0.95     | 0.011   | 0.48     | 0.32    | 0.87     | 0.43    |
| Velocity agg., AA AA 1mM | 0.40     | 0.066   | 0.87     | 0.86    | 0.90     | 0.87    |
| Max. agg., TRAP-6 10uM | 0.68     | 0.70    | 0.85     | 0.56    | 0.26     | 0.87    |
| Velocity agg., TRAP-6 10uM | 0.10     | 0.19    | 0.28     | 0.83    | 0.075    | 0.98    |

Fig. 2. Platelet aggregation in platelet rich plasma according to intake of antithrombotic medication in individuals with suspected venous thromboembolism. Presented data indicate the evidence for a difference of LTA aggregation in VTE cases vs individuals with a rule out of VTE by p-values. The color indicates the direction of the difference with red if aggregation is higher and with blue if aggregation is lower in VTE cases. The intensity of the color indicates the strength of evidence for the difference between groups, ranging from $p < 0.001$ to $p = 0.2$. Abbreviations: WS, whole sample; ASA, acetylsalicylic acid; AC, anticoagulant; ADP, adenosine diphosphate; EPI, epinephrine; TRAP-6, thrombin receptor activating peptide-6.
Table 2

| Platelet Rich | Platelet Free |
|---------------|--------------|
| Lag time (min) | 10.17 (5.21/18.67) | 2.03 (0/11.97) |
| ETP (nM*min)   | 1140.26 (336.36/1608.99) | 25.79 (0/774.62) |
| Peak height (nM) | 48.31 (9.46/86.20) | 1.52 (0/76.34) |
| Time to peak (min) | 20.11 (11.46/39.44) | 12.20 (6.67/16.84) |
| Velocity index (nM/min) | 2.84 (0.37/8.64) | 0.95 (0.37/2.71) |

Presented data are median (25th and 75th percentile); Subjects on anticoagulants were taking one of the following agents: low molecular weight heparin, factor Xa inhibitor or Vitamin K antagonist; VTE, venous thromboembolism; w/o, without; N.A., non applicable (within 120 min thrombin generation curve was not generated).
reported in acute medical conditions such as subjects with cancer at risk for VTE as well as subjects with trauma-induced coagulopathy \[36\]. In addition, coagulation factor consumption at the time of the acute thrombus formation could further contribute to the observed reduction of TG in cases compared to controls. Decreased plasma Factor VII levels, lower fibrinogen concentration and Factor XIII levels have been frequently reported in patients with acute thrombosis, such as DVT and PE \[37,38\].

Our study has limitations. VTE is an acute disease that encompasses two different clinical manifestations of PE and DVT, frequently presenting with different severity and duration of the clinical signs and symptoms. These aspects including the different timing and duration of intake for both anticoagulant and antiplatelet agents could affect the results on platelet function.

This study has several strengths: It is the largest study comprehensively investigating platelet function in subjects with acute VTE to date. The highly standardized assessment of platelet function minimized pre-analytical variability that might have affected results. LTA measurements without trigger support the absence of in-vitro platelet activation. On the other side, certain limitations merit consideration: data on ASA compliance were not available, as ASA effectiveness was not the aim of this explorative study. However, in conclusion from the present analysis, low response to ASA will be subject of future investigations. The sample size was unique for the comprehensive assessment, however was still comparably small for analyzing the relation of platelet phenotype to clinical outcome in an acute VTE. It has been previously reported that higher TG is associated with higher risk for incident or recurrent VTE. This is the first study that reports the TG profile in presence and absence of platelets at the time of the acute venous thrombus formation \[39,40\].

The application of machine learning technic identified the most relevant differentiating factors between confirmed and objectively excluded VTE. Our finding that 69% of the selected variables were platelet-related puts special emphasis on the relevance of platelets and platelet function testing in venous thrombosis. It has been reported that hemodynamics affects the proportion of activated platelets, e.g at low shear rate, such as in the venous system, larger proportion of platelets get activated and release their granule content, unlike in the arterial system at high shear hemodynamics \[41\]. This study supports the value of platelet function testing to explore the degree of platelet activation in the thrombotic process.

In conclusion, this study characterizes the platelet phenotype in individuals with acute VTE and proves platelet function to substantially differ between subjects with VTE and those with a VTE ruled out independent of the present clinical profile and risk factors. The data further provide evidence for the occurrence of ASA low-responsiveness in acute VTE which might explain the failure of ASA to better protect subjects at risk, but also provides evidence for “exhausted” platelets in this disease. Whether the platelet phenotype could improve identification of individuals at risk for recurrent VTE and/or worse outcome remains to be determined.

**Authorship Contributions**

MP-N, SH, VL, SK, KL and PSW designed the study. MP-N, SH, IM, CG, VL, KL and PSW designed this analysis. MN performed the statistical analysis. MP-N, BW, VtC, JHP, HMS, TK, TM and KJL performed research and contributed to the interpretation of results. MP-N, HtC and PSW drafted the manuscript. All authors contributed to critical review and the composition of the final manuscript.
Disclosures of Conflict of Interest

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/ebiomed.2020.102978.

References

[1] Davi G, Patrono C. Platelet activation and atherothrombosis. N Engl J Med 2007;357(24):2482–94.
[2] Collins R, Petro R, Hennekens C, et al. Aspirin in the primary and secondary prevention of vascular disease: a collaborative meta-analysis of individual participant data from randomised trials. Lancet 2009;373(9678):1849–60.
[3] Santilli F, Vazzana N, Laini R, Guagnano MT, Davi G. Platelet activation in obesity and metabolic syndrome. Obes Rev: J Int Assoc Study of Obes 2012;13(1):27–42.
[4] Ferroni P, Basili S, Falco A, Davi G. Platelet activation in type 2 diabetes mellitus. J Thromb Haemost 2004;2(8):98–101.
[5] Brill A, Fuchs TA, Chauhan AK, et al. von Willebrand factor-mediated platelet adhesion is critical for deep vein thrombosis in mouse models. Blood 2011;117(4):1400–7.
[6] Heestermans M, Salloum-Asfar S, Street T, et al. Mouse venous thrombosis upon silencing of anticoagulants depends on tissue factor and platelets, not FXII or neutrophils. Blood 2019;133(20):3595–603.
[7] Chirinoi JL, Heeris GA, Velasquez H, et al. Elevation of endothelial microparticles, platelets, and leukocyte activation in patients with venous thromboembolism. J Am Coll Cardiol 2005;45(9):1467–71.
[8] Gasparayan AV, Ayazyan L, Mikhailidou DP, Kitas GD. Mean platelet volume: a link between thrombosis and inflammation? Curr Pharm Des 2011;17(1):47–58.
[9] Braekkan SK, Mathiesen EB, Bjølsen T, Wilsgaard T, Stormer J, Hansen JB. Mean platelet volume is a risk factor for venous thromboembolism: the Tromso Study. Thromb Haemost 2010;103(1):157–61.
[10] Lippi G, Buonocore R, Cervellin G. The mean platelet volume is decreased in patients diagnosed with Venous Thromboembolism in the emergency department. Semin. Thromb. Haemost. 2016;42(6):632–5.
[11] Riedl J, Kader A, Reitter EM, et al. Association of mean platelet volume with risk of venous thromboembolism and mortality in patients with cancer, results from the Vienna Cancer and Thrombosis Study (CATS). Thromb Haemost. 2014;111(4):570–8.
[12] Mines J, Becattini C, Agnelli G, et al. Aspirin for the prevention of recurrent venous thromboembolism: the INSPIRE collaboration. Circulation 2014;130(13):1062–71.
[13] Madridano O, del Toro J, Lorenzo A, et al. Subsequent arterial ischemic events in patients receiving anticoagulant therapy for venous thromboembolism. J Vasc Surg. Venous Lymphat. 2015;3(2):135–41.