Cytometry Part B – 13-058_R1 (06.12.2013) Major changes underlined

# Decreased generation of procoagulant platelets detected by flow cytometric analysis in patients with bleeding diathesis

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**Running title:** Bleeding diathesis and COAT platelets

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**Words:** Abstract: 250
Main text: 3935

**Number of tables / figures:** 2 / 4

**Online supplementary materials:** 2

**Abstract**

**Background:** A clinically relevant bleeding diathesis is a frequent diagnostic challenge, which sometimes remains unexplained despite extensive investigations. The aim of our work was to evaluate the diagnostic utility of functional platelet testing by flow cytometry in this context.

**Methods:** In case of negative results after standard laboratory work-up, flow cytometric analysis (FCA) of platelet function was done. We performed analysis of surface glycoproteins (GP) Ibα, IIb, IIIa; P-selectin expression and PAC-1 binding after graded doses of ADP, collagen and thrombin; content/secretion of dense granules; ability to generate procoagulant platelets.

**Results:** Out of 437 patients investigated with standard tests between January 2007 and December 2011, we identified 67 (15.3%) with high bleeding scores and non-diagnostic standard laboratory work-up including platelet aggregation studies. Among these patients FCA revealed some potentially causative platelet defects: decreased dense-granule content/secretion (n=13); decreased alpha-granule secretion induced by ADP (n=10), convulxin (n=4) or thrombin (n=3); decreased fibrinogen-receptor activation induced by ADP (n=11), convulxin (n=11) or thrombin (n=8); decreased generation of COAT-platelets, i.e. highly procoagulant platelets induced by simultaneous activation with collagen and thrombin (n=16).

**Conclusion:** Our work confirms that storage pool defects are frequent in patients with a bleeding diathesis and normal coagulation and platelet aggregations studies. Additionally, flow cytometric analysis is able to identify discrete platelet activation defects. In particular, we show for the first time that a relevant proportion of these patients has an isolated impaired ability to generate COAT-platelets – a conceptually new defect in platelet procoagulant activity, that is missed by conventional laboratory work-up.

**Keywords**

platelet function, COAT platelets, platelet aggregation, flow cytometry, bleeding diathesis, bleeding scoring system

## Introduction

A clinically relevant bleeding diathesis is a frequent diagnostic challenge and identification of its etiology may be difficult (1). To assess the cause of bleeding a comprehensive clinical evaluation and a sequential laboratory work-up are necessary. Accurate acquisition of medical history is the first essential step. For this purpose, bleeding questionnaires have been developed and validated in clinical practice (2,3). In addition, standard laboratory tests are helpful to detect disorders of primary hemostasis or coagulation factor defects (4,5). However, identification of clinically relevant disorders of primary hemostasis may be difficult and work-up of a patient with a significant bleeding diathesis fails sometimes to detect an explanatory cause (1). The difficulty to determine the etiology is more pronounced if the bleeding is caused by a platelet dysfunction. A variety of different aspects of the platelet function can be impaired on a genetic basis (6) and multiple factors have an impact on the clinical phenotype – e.g., dietary and metabolic variables, smoking, inflammation, drugs, and other components of the hemostatic system (7). Because knowledge of bleeding's etiology is a prerequisite for optimal patient management, efforts to improve laboratory diagnostic procedures have been made. Presently various platelet function assays are available and the combination of multiple tests provides information about platelet function as a whole. Flow cytometric analysis is a very helpful tool in the diagnostic work-up of hematologic malignancies and several studies have shown its utility in diagnosing platelet defects as well (8-10).

At our institution a standardized procedure with step by step diagnostic is applied to out-patients referred for clarification of a bleeding diathesis (Figure 1). Acquisition of a detailed medical history and clinical examination are followed by analysis of plasmatic coagulation factors, including von Willebrand factor and whole blood

assessment of primary hemostasis. If no obvious cause of hemorrhagic diathesis is found, diagnostic procedure is widened to investigate platelet aggregation in vitro. As published by Quiroga et al. (1), also in our experience extended laboratory analyses are not always able to determine the underlying cause of the bleeding diathesis.

The aim of the present study was to evaluate the diagnostic utility of an extensive flow cytometric analysis of platelet function in patients with clinically relevant bleeding diathesis but unrevealing standard laboratory work-up.

## Materials and Methods

*Patients*

Patients referred to our hematology outpatient clinic for investigation of a possible hemorrhagic diathesis between January 2007 and December 2011 (n = 437). During their first visit, the patient's bleeding history was taken employing two bleeding score systems. One score is an in-house tool (University Clinic of Haematology Inselspital, "UCH-bleeding score"), to assess severity of bleeding at specific organs, including skin, nose, oral cavity, gastrointestinal, uro-genital, joints, and muscles, bleeding in association with minor injuries, dental procedures, and surgery, transfusion requirements, medications, and family history of a bleeding diathesis (maximal 26 points; see Online Supplemental Material for details). The second score used was the ISTH "Bleeding score and bleeding questionnaire for the diagnosis of type 1 von Willebrand disease" (maximal 30 points), which has already been published some years ago (2). Clinical examination and standard laboratory tests including full blood count, global coagulation tests, all relevant coagulation factors, von Willebrand factor (VWF) and platelet function analyzer (PFA) were also performed at first visit. Light transmission platelet aggregation testing (LTA) in platelet rich plasma (PRP) and flow cytometric analysis (FCA) of platelet function were stepwise added, if the bleeding diathesis was clinically relevant and the laboratory work-up failed to reveal an explanatory cause (Figure 1). The study was performed in accordance with local regulations for diagnostic-laboratory studies (Kantonale Ethikkommission Bern, www.kek-bern.ch). All patients gave written informed consent.

*Blood samples*

Blood was collected by standard venepuncture into EDTA for blood cell count (Monovette, Sarstedt, Nümbrecht, Germany), 0.106 M tri-sodium citrate (9:1 vol/vol) in plastic syringes for standard coagulation and platelet flow-cytometric assays (Monovette, Sarstedt, Nümbrecht, Germany), and buffered citrate for platelet aggregation studies (0.13 M tri-sodium citrate, pH 5.5).

*Analysis of coagulation parameters*

Details on coagulation assays performed in our laboratory have already been published (11). Clotting activities of factors VIII:C, IX:C, and XI:C were measured by a one stage clotting method, using factor deficient plasma (Siemens Healthcare Diagnostics, Marburg, Germany) and Pathromtin SL (Siemens Healthcare Diagnostics, Marburg), calibration curves were produced with lyophilized standard human plasma (SHP; Siemens Healthcare Diagnostics), the results were the average of 3 different sample dilutions (1:4, 1:8, 1:16). Clotting activity of factor XIII was measured by a chromogenic assay (Berichrom FXIII Kit, Siemens Healthcare Diagnostics) which was performed on a Behring Coagulation System (BCS) automated analyzer (Siemens Healthcare Diagnostics). Von Willebrand factor ristocetin cofactor activity (VWF:RCo) was measured with BC von Willebrand reagent (Siemens Healthcare Diagnostics) at 3 dilutions (1:4, 1:8, 1:16) using SHP for calibration. Von Willebrand antigen (VWF:Ag) was measured with the VIDAS VWF automated test system on a VIDAS analyzer (bioMérieux Suisse SA, Genève, Switzerland). Activity of $\alpha_2$-antiplasmin was measured by a chromogenic assay which was performed on a Behring Coagulation System (BCS) automated analyzer (Siemens Healthcare Diagnostics) after adding a defined amount of plasmin (Siemens Healthcare Diagnostics) to the patient's sample. As a standard, plasma

control samples of healthy donors with a defined $\alpha_2$-antiplasmin concentration were run in parallel.

*Platelet function analyzer (PFA)*

The PFA is a high shear-inducing test of platelet adhesion and aggregation (12). This test system is sensitive to a large number of variables like platelet count and function, von Willebrand factor defects, and hematocrit. Therefore testing was always performed with knowledge of full blood count and after a 10 days wash-out period of any compound known to affect platelet function. The test system consists of a microprocessor-controlled instrument (PFA-100® and INNOVANCE® PFA-100™ System, Siemens Healthcare Diagnostics, Germany) and a disposable test cartridge. We used two types of cartridges (Dade® PFA collagen/epinephrine, and Dade® PFA collagen/ADP, Siemens Healthcare Diagnostics, Germany) for all patients. In response to stimulation by collagen, together with either epinephrine or ADP, as well as by high shear rates, VWF and platelets become activated and adhere on the membrane surface in the test cartridge. Ultimately a platelet plug gets formed, occludes the aperture and the blood flow is stopped. The closure times (CT) for epinephrine and for ADP, which are defined as the time that is required to obtain occlusion of the aperture, were measured in two buffered citrated whole blood samples of each patient.

*Light transmission platelet aggregation tests (LTA) in platelet rich plasma (PRP)*

LTA testing was performed as already described in the literature (13,14). Specifically, venous blood samples were collected into buffered 0.13 M tri-sodium citrate in a ratio of 1:9 (1 part anticoagulant to 9 parts blood), transported to the laboratory at room temperature (RT) and processed immediately. PRP was prepared by centrifugation at

RT for 15 minutes at 150g. The PRP was carefully removed, placed into another plastic tube and stored at RT. In the PRP, the platelet count was adjusted to 250 $x10^9$/l with platelet poor plasma (PPP), prepared by further centrifugation of the remaining plasma at 1500g for 15 minutes at RT. In case of platelet counts between 100 and 250 $x10^9$/l, the count was not further corrected but documented on the protocol. In individuals with low platelet counts below 100 $x10^9$/l an additional centrifugation with 150g at RT for 5 minutes was performed (15).

The aggregometer (APACT 4004®, LABiTec GmbH, Ahrensburg, Germany) was then calibrated by using a cuvette containing PRP which equates to 0% light transmission and by using a second cuvette containing PPP which equates to 100% light transmission. Platelet aggregation was induced by increasing concentrations of four agonists. ADP (Sigma-Aldrich, St. Louis, MO, USA) was used at final concentrations of 4, 6, and 10 µM for male patients and concentrations of 3, 4, and 6 µM for female patients. Collagen (Horm®, Nycomed, Linz, A) was used at final concentrations of 1.5, 3, and 4 µg/ml. Arachidonic acid (Bio Data/Medonic Servotec AG, Interlaken, CH) was set at 2 mM. Ristocetin (Socochim SA, Lausanne, CH) concentrations were 1.5 mg/ml and 0.5 mg/ml. Two-hundred µL of PRP pre-warmed at 37°C for 1 min was added to the aggregometer cuvette and run for an additional minute in order to exclude spontaneous aggregation. Thereafter, 20 µL of the agonist was added and the response recorded. If the response to one agonist was out of normal range, the test was repeated once more either to confirm the result or to rule out technical failures. Normally, LTA started 1 hour after having drawn the venous blood samples from the patient and was completed within 2.5 hours. For interpretation of the platelet aggregation traces we used standardized in-house algorithms taking into account our validation (15) and published guidelines (13,14).

*Flow cytometric analysis of platelet function*

PRP from the patient and a normal donor was diluted to 10 x$10^6$/ml with Tyrode's buffer. Analysis of surface glycoproteins (GP) Ibα (by a monoclonal anti-human CD42b antibody coupled with PE, Dako), GPIIb-IIIa (anti-hCD41-FITC and anti-hCD61-FITC, Becton Dickinson), baseline P-selectin expression (anti-CD62P-PE, Becton Dickinson) and PAC-1 binding (PAC1-FITC, Becton Dickinson) were performed in a 100 µl volume containing platelets at a final concentration of 5 x$10^6$/ml and combinations of relevant antibodies at saturating concentrations. After incubation in the dark for 15 min at room temperature (RT), 1'000 µl of Tyrode's buffer were added and platelets were immediately analyzed by flow cytometry (FACSCanto). Dose response of platelet reactivity was investigated with ADP (baseline and final ADP concentrations of 0.5, 5.0, and 50 µM), convulxin (5, 50, and 500 ng/ml), or thrombin (0.05, 0.5, and 5 nM) in a 100 µl volume containing platelets at a concentration of 5 x$10^6$/ml and anti-CD62P-PE and PAC1-FITC. After incubation in the dark for 10 min at 37°C, 1'000 µl of Tyrode's buffer were added to the platelets and the sample was analyzed immediately. Surface expression of negatively charged phopsholipids was investigated with Annexin V-FITC (Roche) at baseline and after activation for 10 min at 37°C in the dark with either 2 µM Ionophore A 23187 (Sigma) or the combination of 500 ng/ml convulxin and 5 nM thrombin (16). Immediately prior to analysis platelets were diluted with 500 µl calcium containing buffer. Finally, in order to evaluate content and secretion of dense granules (17), platelets were diluted to a final concentration of 5 x$10^6$/ml with Hank's buffer and loaded with mepacrine (final 0.17 and 1.7 µM) into a 100 µl volume for 20 min at 37°C in the dark. Secretion of dense granule was assessed after an additional 10 min incubation at 37°C with

buffer versus 5 nM thrombin. Immediately prior to analysis platelets were diluted with 1'000 µl Hank's buffer.

Flow cytometric analysis was repeated once at a later time point with different control platelets in order to confirm results. For P-selectin expression and GPIIb/IIIa-activation, the reported results are the mean value of the repeated measurements obtained with the two highest agonist concentrations (i.e., ADP 5.0 and 50 µM; convulxin 50 and 500 ng/ml; thrombin 0.5 and 5 nM). When comparing the median fluorescence intensity (MFI) of patient platelets surface GP, dense-granule content, or activation endpoints (surface expression of P-selectin, PAC-1 binding) to control platelets we defined, based on comparisons between 40 normal subjects, in-house cut-offs for the lower range (see results section and table 1). The ability to generate procoagulant COAT platelets was described by the absolute percentage of Annexin-V binding platelets after combined activation with convulxin 500 ng/ml and thrombin 5 nM (16). Based on 40 control subjects we defined the ability to generate <20% COAT platelets as "decreased" (Table 1).

*Statistical analysis*

Results are expressed as median ± interquartile range (IQR), minimum-maximum (min-max) or mean ± standard deviation (SD). Statistical analyses were performed with SigmaStat (version 3.5; Systat Software Inc., San Jose, CA, USA) using non parametric tests. $P < 0.05$ was considered statistically significant.

## Results

We developed an extensive flow-cytometric analysis (FCA) of platelet phenotypic and functional characteristics (Figures 2 and 3). Table 1 summarizes in-house cut-offs for phenotypic (platelet size and granularity, surface density of GPIbα, IIb, and IIIa, dense-granule content) and functional parameters (agonist-induced secretion of dense- and α-granules, activation of the fibrinogen-receptor, development of procoagulant COAT platelets). For most parameters we calculated relative MFI values compared to control platelets. As for COAT platelets, we employed the absolute percent values, finding results that are well in line with published data (16,18,19). For all parameters, in-house cut-offs for the lower range were defined based on the 2.5$^{th}$ and 5$^{th}$ percentile values.

Here we report our experience with FCA of platelet function in 67 patients with unexplained clinically significant bleeding diathesis. Between January 2007 and December 2011, 437 patients were referred to our hematology outpatient clinic in order to investigate a hemorrhagic diathesis. In 67 (15.3%) of them standard laboratory work-up failed to identify a clear cause. The median age was 47.9 years (range: 16.8 – 75.4 years) and the majority were female (50/67, 75%). Median bleeding score was 5 both for our in-house tool (“UCH-bleeding score”, see Online Supplemental Material) and for the ISTH “Bleeding score and bleeding questionnaire for the diagnosis of type 1 von Willebrand disease” (2). Standard coagulation parameters and VWF-analysis were normal (Online Supplementary Table), and CT by PFA was either normal or un-specifically prolonged (e.g., in presence of a low hematocrit). Examination of LTA-PRP with different platelet agonists (ADP, collagen,

arachidonic acid, ristocetin) revealed in 29 patients (43%) a normal test result, whereas in 38 patients (57%) was abnormal (Table 1). Thirty-three patients had a borderline result in platelet aggregation after stimulation with ADP. Less frequently borderline aggregation patterns were seen after stimulation with collagen (n=27), arachidonic acid (n=11), and ristocetin (n=8). In 28 out of 38 individuals (74%) with borderline LTA-PRP, more than one agonist showed an abnormal aggregation curve. However, these borderline results did not allow to clearly establish the presence of a platelet function defect.

Results of FCA are summarized in Table 2 and Figure 4. FCA did not reveal any defect in the surface density of the receptors for fibrinogen (GPIIb-IIIa) and VWF (GPIb-IX-V), and was completely normal in 18 out of the 67 (27%) patients (Table 2 and Fig. 4). FCA of platelet function was borderline in 2 (3%) and showed abnormal results in 47 (70%) patients.

Overall, the dense-granule content (detected by mepacrine stain) was <70% compared to control platelets in 9 out of 67 patients (13%): three of 29 (10%) patients with normal LTA-PRP and 6 of 38 (16%) patients with borderline LTA-PRP. Thrombin-induced secretion of dense-granules was reduced <90% in 3 patients (10%) with normal LTA-PRP and in 2 (5%) with borderline LTA-PRP.

Secretion of α-granules (detected by P-selectin surface expression) was evaluated after platelet activation by graded concentrations of ADP, convulxin and thrombin. Among the patients with normal LTA-PRP, 5 out of 29 patients (17%) had a decreased α-granule secretion <40% after exposure to ADP. In contrast, only one of these patients showed an impaired α-granule secretion after activation with thrombin

(<70% compared to control platelets) and 2 after convulxin (<65%). In the cohort with borderline LTA-PRP, 5 out of 38 patients (13%) showed an α-granule secretion <40% after ADP-induced activation. Again, only a few of the patients, had a decreased secretion of α-granules compared to control platelets after activation by thrombin (n=2) or convulxin (n=2).

Activation of GPIIb-IIIa receptor (detected by PAC1-binding) was evaluated after platelet activation by graded concentrations of ADP, convulxin and thrombin. Among patients with normal LTA-PRP, 4 out of 29 (14%) showed a decreased activation of the fibrinogen-receptor after ADP, 2/29 (7%) after thrombin, and 4/29 (14%) after convulxin. In the cohort with borderline LTA-PRP, 7 out of 38 (18%) patients showed a decreased GPIIb-IIIa activation after ADP, 6/38 (16%) after thrombin, and 7/38 (18%) after convulxin.

Procoagulant COAT platelets were generated by simultaneous platelet activation with convulxin and thrombin as already described in materials and methods. Overall, 16 out of 67 (24%) patients showed a decreased procoagulant platelet activity (Table 2 and Fig. 4). For comparison, the prevalence of COAT platelet levels <20% among normal controls employed for generating in-house cut-offs was 2/40 (p=0.024, Chi-square; see Table 1) and 9/100 in platelet donors serving as daily controls for diagnostic FCA (p=0.015, Chi-square; median 27%; range 15% – 57%; 2.5$^{th}$ percentile 17% and 5$^{th}$ percentile 18%).

For better visualization of the clustering of multiple defects in individual patients and in order to illustrate the relative frequency of potential platelet defects, we tabulated

the qualitative FCA results (white = normal; pale gray = borderline; dark gray = abnormal) together with the bleeding scores for each patient (Fig. 4).

## Discussion

Investigation of a bleeding diathesis includes several diagnostic steps and laboratory assays to identify its etiology (4,5). However, there is still a substantial percentage of patients having a positive bleeding diathesis in whom laboratory work-up is unable to detect a causative disorder (1).

In this study we evaluated the diagnostic utility of an extensive phenotypic and functional characterization of platelets by flow cytometry in patients with a clinically significant bleeding diathesis but non-diagnostic standard laboratory investigations.

We show that flow cytometric analysis (FCA) is able to identify several potentially explanatory platelet function defects (Table 2 and Fig. 4). However, we would also point out that among 18/67 patients (27%) FCA of platelet function was normal, indicating that either platelet defects not detectable by this technique or vessel wall defects could be responsible for the bleeding diathesis.

First, we have identified dense-granule defects in 13 out of 67 patients (19%): in 8 patients as an isolated anomaly and in 5 combined with other platelet function defects (Fig. 4). This observation confirms that symptomatic patients with normal platelet aggregation frequently have a storage pool disease (20,21) and is in line with the recommendation that platelet nucleotides content and release should specifically be investigated if aggregation studies are unrevealing (4,5).

Second, we have observed some peculiar functional anomalies, which may point to the underlying specific defect. For instance, a single patient with an isolated impairment of GPIIb/IIIa activation with all agonists (#59 Fig. 4A), suggesting a

dysfunctional fibrinogen receptor. A patient with a decreased ADP-induced platelet activation (#22 Fig. 4B) and another with a reduced convulxin-induced activation (#64 Fig. 4B), suggesting an involvement of the respective receptors. Other anomalies point to hypothetical defects in the pathways of specific agonist-induced activation end-points: for instance, secretion of α-granules by ADP (#15, #28, and #55 Figure 4A and #46 Figure 4B) or convulxin (#05 Fig. 4A and #44 Fig. 4B), and activation of the fibrinogen-receptor induced by ADP (#32 Fig. 4A and #45, #48, #50 Fig. 4B), convulxin (#20 Fig. 4A and #27 and #19 Fig. 4B) or thrombin (#37 Fig 4B). It is reasonable to speculate, that these distinct clusters point to the underlying defect and that elucidation of the involved pathway may lead to the identification of the pathogenic mechanism.

Third, we report that platelet α-granule secretion in response to the weak agonist ADP is more variable than that to thrombin or convulxin (Table 1) and that some patients show borderline results close to the cut-off values (Table 2). This is compatible with the hypothesis of minor platelet function defects, which are not detected by conventional platelet function studies but could lead to clinical relevant bleeding, especially if concomitant causes, such as surgical challenges, drugs or minor defects in other components of the hemostatic system are present (7).

The fourth and to us most fascinating observation is that a substantial proportion of the investigated patient (16/67, 24%) show a decreased ability to produce COAT platelets (Table 2). COAT platelets are a subpopulation of platelets, generated by combined activation with collagen and thrombin, which are very effective in sustaining thrombin generation (16). COAT platelets retain high levels of α-granule proteins on their surface, including factor V, fibrinogen and von Willebrand factor

(VWF) by a serotonin- and transglutaminase-dependent mechanism (22). Among healthy individuals, Dale and his colleagues described, similarly to our results (Table 1), an average of 33% COAT platelets and noted that the amount of an individual's COAT platelets seems to be stable over time (19).

The potential clinical relevance of COAT platelets in hemostasis is revealed by the work of Dale and Prodan. Their investigations have shown a link between COAT platelet level and the appearance of spontaneous intracerebral hemorrhages (23) and bleed volume (24). A low level of COAT platelets is also correlated with lacunar stroke morphology (18) and early hemorrhagic transformation in non-lacunar strokes (25). On the other hand, a high amount of COAT platelets seems to correlate with transient ischemic attack and ischemic stroke (26,27). Recently, the same group found a higher level of COAT platelets in patients with subarachnoid hemorrhage and described an increased 30-day mortality in association with the amount of COAT platelets in these patient population (28). Finally, COAT platelets appear to have an impact on the bleeding phenotype in severe haemophilia (29). However, to draw robust conclusions about how individual differences in the level of COAT platelets contributes to a hemorrhagic or thrombotic phenotype, more clinical data are still required.

In the present work we identified 16 out of 67 (24%) patients with a clinically significant bleeding diathesis but unrevealing laboratory work-up, who generated <20% COAT platelets. Of note, in the majority of them this was the only anomaly identified (n=10) and in the remainders it was usually associated with just a single additional platelet activation defect (Fig. 4). Since platelets of all 16 patients showed normal surface expression of negatively charged phospholipids upon activation with

ionophore A23187 this is not a primary defect in platelet procoagulant activity, such as in Scott Syndrome (30,31). We hypothesize that the impaired ability to generate procoagulant COAT platelets is likely to be secondary to an insufficient intracellular calcium rise upon combined activation with convulxin and thrombin. We are currently searching for causative defects (32).

This work, showing a significantly higher prevalence of an impaired ability to generate COAT platelets in patients with a bleeding diathesis (16/67) than in normal controls (2/38 and 9/100; p=0.024 and 0.015, respectively) underscores the role of platelet procoagulant activity for the formation of a stable hemostatic plug. Of note, platelets ability to differentially regulate thrombin generation is an increasingly recognized aspect of hemostasis and thrombosis (33), beside their ability to adhere and aggregate.

In perspective, COAT platelets could represent an interesting therapeutic target. If elevated COAT platelets will be shown to be independently associated with arterial thrombotic disease, drugs that specifically inhibit the mechanisms of platelet procoagulant transformation would result in potential new antithrombotic therapies. Conversely, up-regulating COAT platelet generation might be expected to represent a therapeutic option for patients suffering from bleeding diathesis (Colucci et al. Manuscript submitted). Finally, in vitro studies have shown that recombinant FVIIa preferentially binds to procoagulant platelets (34,35), and similar preferential binding to COAT platelets has been described for vatreptacog alfa, a novel recombinant FVIIa variant (36). Taking these data into account, the individual level of COAT platelets might be an important component for explaining the difference in hemostatic response to these drugs.

In summary, we show that phenotypic and functional platelet analysis by flow cytometry among patients with a significant bleeding diathesis and non-diagnostic standard laboratory work-up is able to identify potentially explanatory defect. We confirm that storage pool defects are frequent and should be searched in this population (in this report 19%). In addition, we show for the first time that a relevant proportion of these patients present with an isolated impairment of their ability to generate COAT platelets (in this report 24%). This is a conceptually novel (33), clinically potentially relevant defect in platelet procoagulant activity, that is missed by conventional laboratory work-up focusing on platelet adhesive and aggregating properties.

**Acknowledgements**

We thank Tiziana Conte, Evelyne Giabbani, Marco Pavicic, Anh Huynh, Marianne Reusser, and Katja Weller for their excellence in performing flow cytometric platelet studies, the technicians of the hemostasis laboratory and the physicians of our clinic. We also thank Therese Jost for her outstanding administrative assistance.

**Authorship section**

Michael Daskalakis and Giuseppe Colucci collected, analyzed and interpreted the data and wrote the manuscript.

Peter Keller developed the flow cytometry analysis and did critical reading and writing upon the manuscript.

Sophie Rochat performed flow cytometric analysis analyzed and interpreted the data.

Tobias Silzle collected patient's data and did critical reading and writing upon the manuscript.

Franziska Demarmels-Biasiutti did clinical follow up of the patients in our outpatient clinic and did critical reading and writing of the manuscript.

Gabriela Barizzi supervised/coordinated the laboratory diagnostic work up and did critical reading and writing of the manuscript.

Lorenzo Alberio developed the flow cytometry analysis, did clinical follow up of the patients in our outpatient clinic, analyzed and interpreted the data and wrote the manuscript.

**Disclosure of Conflicts of Interest**

All authors of the manuscript hereby confirm that they do not have any direct and indirect conflicts of interest, especially no financial relationships with industry (through investments, employment, consultancies, stock ownership, honoraria).

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**Table 1. In house reference ranges for flow cytometric platelet function analysis (n=40)**

| Parameter | Indicator | Unit | Median | 2.5th-percentile | 5th-percentile | In-house cut-off |
|---|---|---|---|---|---|---|
| **Size** | FSC | MFI (*) | 104 | 68 | 69 | **70** |
| **Granularity** | SSC | MFI (*) | 102 | 82 | 82 | **80** |
| **GPIIb** | Anti-CD41 mAb | MFI (*) | 102 | 84 | 87 | **80** |
| **GPIIIa** | Anti-CD61 mAb | MFI (*) | 98 | 85 | 86 | **80** |
| **Glb-alpha** | Anti-CD42b mAb | MFI (*) | 101 | 83 | 84 | **80** |
| | | | | | | |
| **Dense granules** | Mepacrine | | | | | |
| **- content** | | MFI (*) | 103 | 70 | 72 | **70** |
| **- secretion (Thr 5 nM)** | | MFI (*) | 100 | 96 | 97 | **90** |
| | | | | | | |
| **α-granule secretion** | Anti-CD62 mAb | | | | | |
| **- Baseline** | | Absolute % | 0.9 | | | **4** |
| **- ADP 5 µM** | | MFI (*) | 102 | 35 | 44 | **40** |
| **- ADP 50 µM** | | MFI (*) | 99 | 39 | 40 | |
| **- Thrombin 0.5 nM** | | MFI (*) | 100 | 65 | 70 | **70** |
| **- Thrombin 5 nM** | | MFI (*) | 107 | 70 | 75 | |
| **- CVX 50 ng/ml** | | MFI (*) | 99 | 67 | 68 | **65** |
| **- CVX 500 ng/ml** | | MFI (*) | 106 | 67 | 67 | |
| | | | | | | |
| **GPIIb/IIIa-activation** | PAC-1 | | | | | |
| **- Baseline** | | Absolute % | 0.5 | | | **3** |
| **- ADP 5 µM** | | MFI (*) | 95 | 63 | 69 | **70** |
| **- ADP 50 µM** | | MFI (*) | 94 | 65 | 72 | |
| **- Thrombin 0.5 nM** | | MFI (*) | 111 | 58 | 60 | **70** |
| **- Thrombin 5 nM** | | MFI (*) | 107 | 71 | 72 | |
| **- CVX 50 ng/ml** | | MFI (*) | 98 | 57 | 58 | **60** |
| **- CVX 500 ng/ml** | | MFI (*) | 98 | 58 | 61 | |
| | | | | | | |
| **Procoagulant platelets** | Annexin-V | | | | | |
| **- Baseline** | | Absolute % | 1.9 | | | **5** |
| **- COAT platelets** | | Absolute % | 31 | 19 | 20 | **20** |

Legend: ADP, adenosine diphosphate; CVX, convulxin; FSC, forward scatter; mAb, monoclonal antibody; MFI = median fluorescence intensity; SSC, sideward scatter; Thr, thrombin; (*) = relative to control platelets

**Table 2. Patient characteristics and type of platelet dysfunction revealed by flow-cytometric analysis (FCA) in patients with significant bleeding diathesis and non-diagnostic standard laboratory workup (n = 67).**

*(see next page)*

†, borderline values are defined as lying within 3% above the cut-off level

*, individual results

Legend: ADP, adenosine diphosphate; FCA, flow-cytometric analysis; GPIIb/IIa, Glycoprotein IIb/IIIa-receptor; ISTH, International Society on Thrombosis and Hemostasis; LTA-PRP, light transmission aggregometry in platelet-rich plasma; UCH, University Clinic for Haematology, Inselspital, Bern

| | Patients with normal LTA-PRP | Patients with borderline LTA-PRP |
|---|---|---|
| **Total number of patients** (n, %) | 29 (43%) | 38 (57%) |
| | | |
| **Sex** (women/men) | 24/5 | 26/12 |
| | | |
| **Age** (years, median, min/max) | 52.8 (23.5/75.4) | 43.2 (16.8/72.9) |
| | | |
| **Bleeding score UCH** (median, min/max) | 6 (3/17) | 4 (1/19) |
| **Bleeding score ISTH** (median, min/max) | 6 (1/14) | 4 (0/16) |
| | | |
| **Platelet FCA findings** (n, %) | | |
| Normal | 10 (35%) | 8 (21%) |
| Borderline (†) | 1 (3%) | 1 (<3%) |
| Abnormal | 18 (62%) | 29 (76%) |
| | | |
| **Delta storage pool disease** | | |
| Dense-granule content **(<70%)** | **3** (54%, 57%, 67%)* | **6** (57%, 60%, 63%, 66%, 66%, 68%)* |
| (borderline†) | 0 | 3 (70%, 71%, 71%) |
| Dense-granule secretion, thrombin **(<90%)** | **3** (51%, 82%, 89%)* | **2** (76%, 88%)* |
| Dense-gra (borderline†) | 1 (92%)* | 1 (92%)* |
| | | |
| **Decreased α-granules secretion** | | |
| ADP **(<40%)** | **5** (24%, 26%, 28%, 37%, 39%)* | **5** (31%, 32%, 36%, 36%, 37%)* |
| (borderline†) | 2 (42,%, 42%)* | 0 |
| Thrombin **(<70%)** | **1** (66%)* | **2** (65%, 69%)* |
| Throm (borderline†) | 0 | 0 |
| Convulxin **(<65%)** | **2** (53%, 63%)* | **2** (46%, 49%)* |
| Convulxin (borderline†) | 0 | 1 (65%)* |
| | | |
| **Decreased GPIIb/IIIa-receptor activation** | | |
| ADP **(<70%)** | **4** (62%, 64%, 64%, 66%)* | **7** (47%, 52%, 56%, 63%, 64%, 65%, 67%)* |
| (borderline†) | 1 (70%)* | 3 (70%, 70%, 72%)* |
| Thrombin **(<70%)** | **2** (49%, 53%)* | **6** (50%, 53%, 63%, 65%, 66%, 66%)* |
| Thrombi (borderline†) | 0 | 2 (71%, 71%)* |
| Convulxin **(<60%)** | **4** (45%, 51%, 52%, 57%)* | **7** (33%, 45%, 49%, 56%, 57%, 57%, 58%)* |
| Convul (borderline†) | 0 | 0 |
| | | |
| **Procoagulant COAT platelets** | | |
| Thrombin + Convulxin **(<20%)** | **7** (13%, 13%, 15%, 16%, 16%, 17%, 19%)* | **9** (9%, 11%, 13%, 15%, 16%, 17%, 18%, 19%, 19%)* |
| (borderline†) | 1 (21%)* | 2 (21%, 22%)* |
| | | |

## Figure legends

**Figure 1. Diagnostic flow-chart for patients with bleeding diathesis.**

The flow chart describes the standardized procedure with step by step diagnostic work-up of out-patients referred to our institution for clarification of a bleeding diathesis.

**Figure 2. Flow-cytometric analysis of platelet phenotype and function**

Flow-cytometric analysis of platelet function was performed as described in the Methods section. **Panel A**: Resting platelets stained for GPIIb (CD41) and P-Selectin (CD62). **Panel B**: Surface expression of GPIIIa (CD61) and GPIbα (CD42b) on resting platelets. **Panel C**: Dense granule content and secretion (Dotted line: unstained platelets; interrupted line, platelets loaded with mepacrine 1.7 μM; continuous line: mepacrine-loaded platelets after activation with 5nM thrombin). **Panel D**: Platelet procoagulant activity (Dotted line: resting platelets; interrupted line: platelets activated with ionophore A 23187; continuous line: platelets simultaneously activated with convulxin 500 ng/ml and thrombin 5 nM, the bar highlights COAT platelets).

**Figure 3. Flow-cytometric analysis of agonist-induced platelet activation**

Flow-cytometric analysis of platelet function was performed as described in the Methods section. **Panel A**: Resting platelets stained with PAC-1 (recognizing an activation-dependent conformational change of the fibrinogen-receptor, GPIIb/IIIa) and anti-CD62 mAb (detecting surface-expression of P-selectin, a marker of α-granule secretion). Platelets after activation with graded concentrations of thrombin

(**Panel B**: 0.5 nM; **Panel C**: 5 nM) or convulxin (**Panel D**: 5 ng/ml, **Panel E**: 50 ng/ml, **Panel F**: 500 ng/ml).

**Figure 4. Synoptic overview of FCA results of platelet function in 67 patients with clinically significant bleeding diathesis and non-diagnostic standard laboratory work-up.**

**Panel A:** Patients with normal LTA-PRP. **Panel B:** Patients with borderline LTA-PRP. Each row represents one single patient with its study number, hypothetical defect and bleeding scores. Endpoint/agonist combinations are shown in different lanes and individual FCA results (%) are displayed. Agonist-response below in-house cut-offs (Table 1) is marked as a dark-gray box; borderline agonist response (Table 2) is labeled as a pale-gray box; normal response is left in white. “Defect?” indicates the hypothetical underlying platelet function defect: e.g., GPIIb/IIIa (CVX): pathway involved in convulxin-induced activation of the fibrinogen-receptor; Alpha (ADP): pathway involved in ADP-induced secretion of α-granules; Dense: storage-pool disease.

Abbreviations: ADP, adenosine diphosphate; BS, bleeding score; CVX, convulxin; FCA, flow cytometric analysis; GPVI, collagen receptor; ISTH, International Society on Thrombosis and Hemostasis; mepa, mepacrine; $P2Y_1$ and $P2Y_{12}$, ADP receptors; PAC-1, antibody that recognizes an activation-dependent epitope on GPIIb-IIIa (integrin $\alpha_{IIb}\beta_3$); UCH, University Clinic of Hematology, Inselspital, Bern.

**Figure 1**

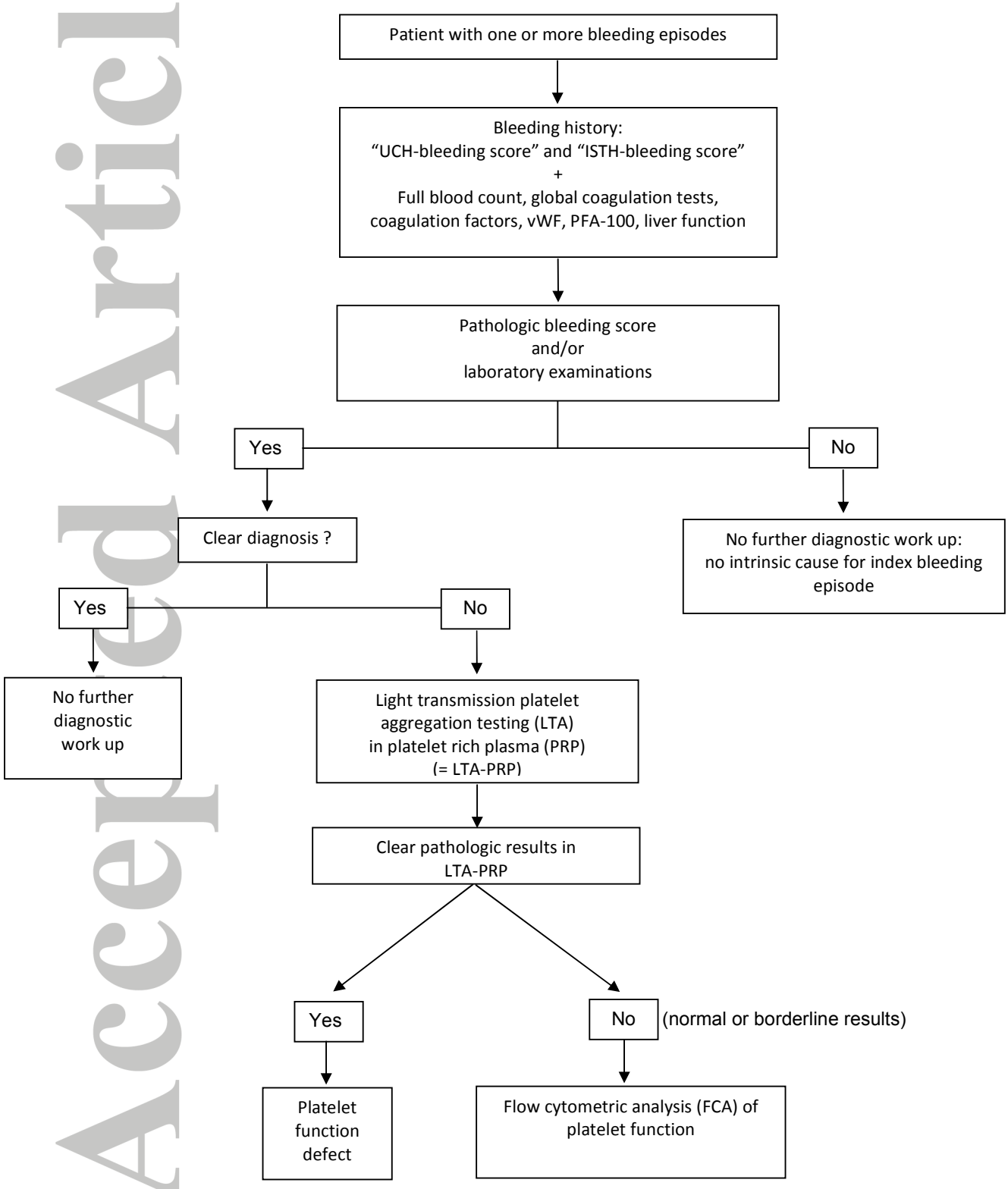
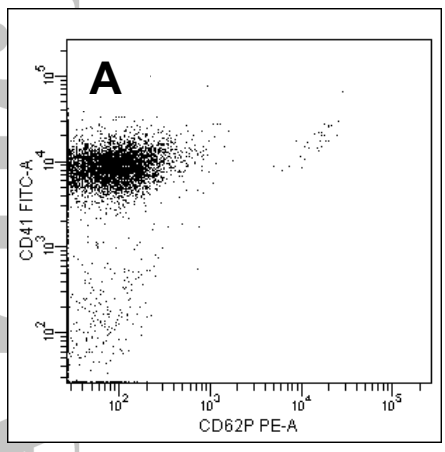
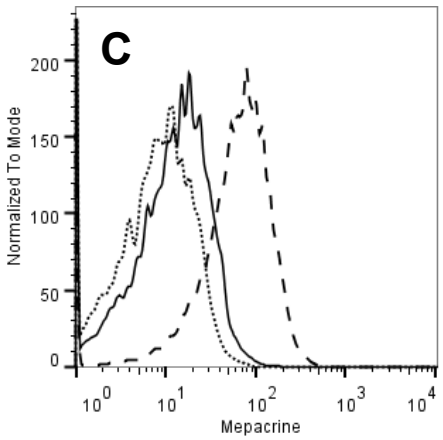
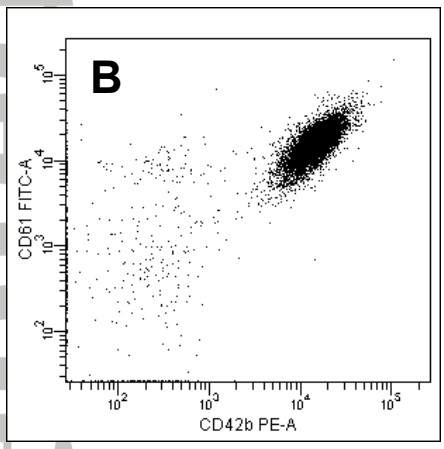
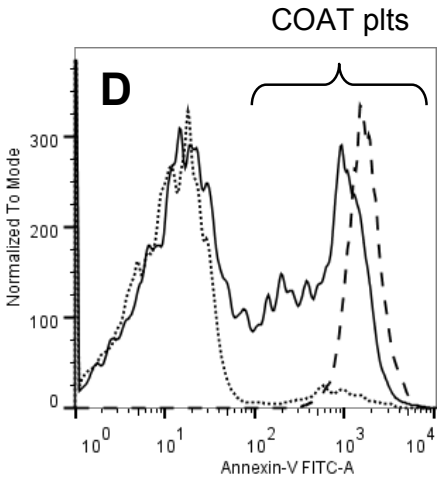


**Figure 2**



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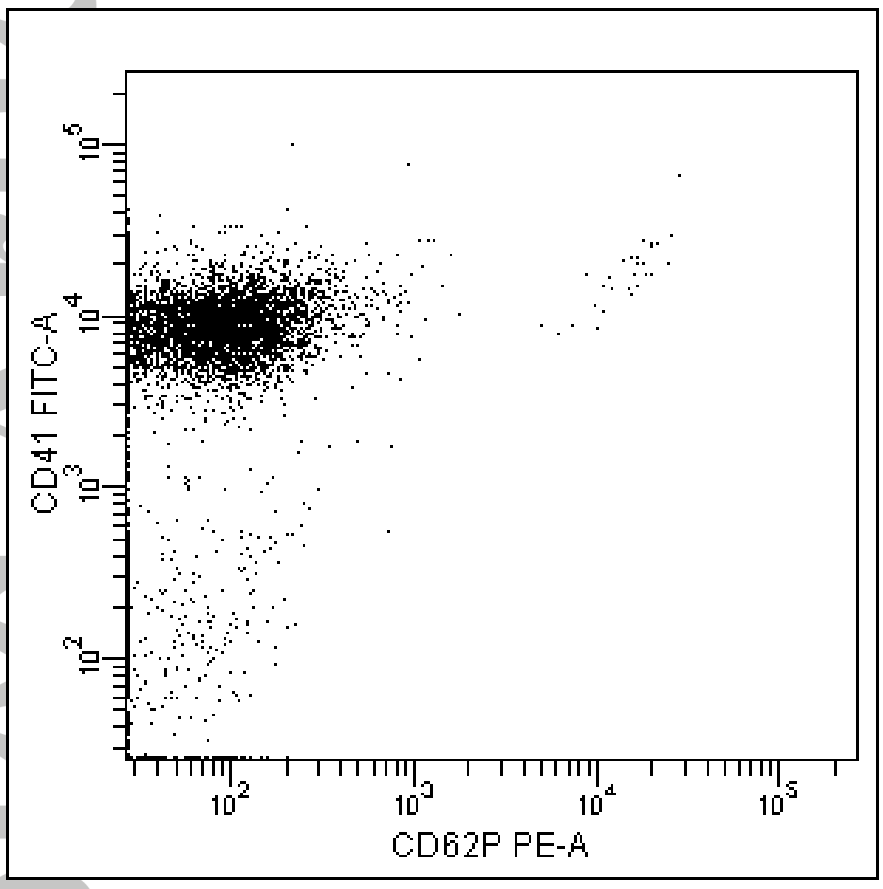

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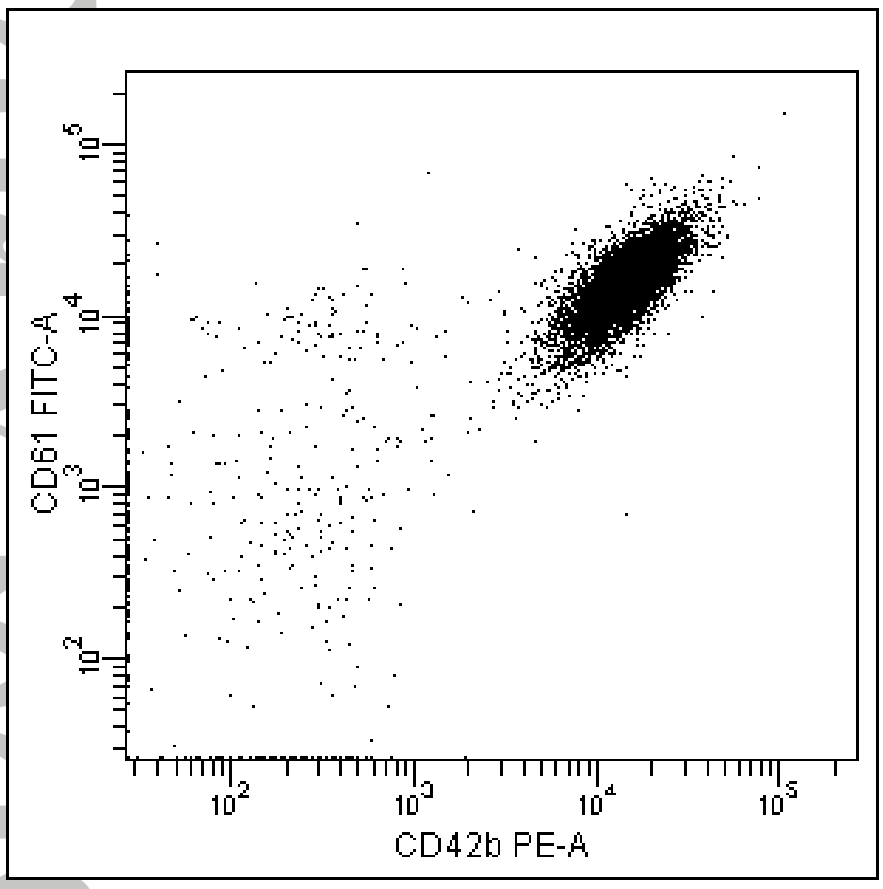
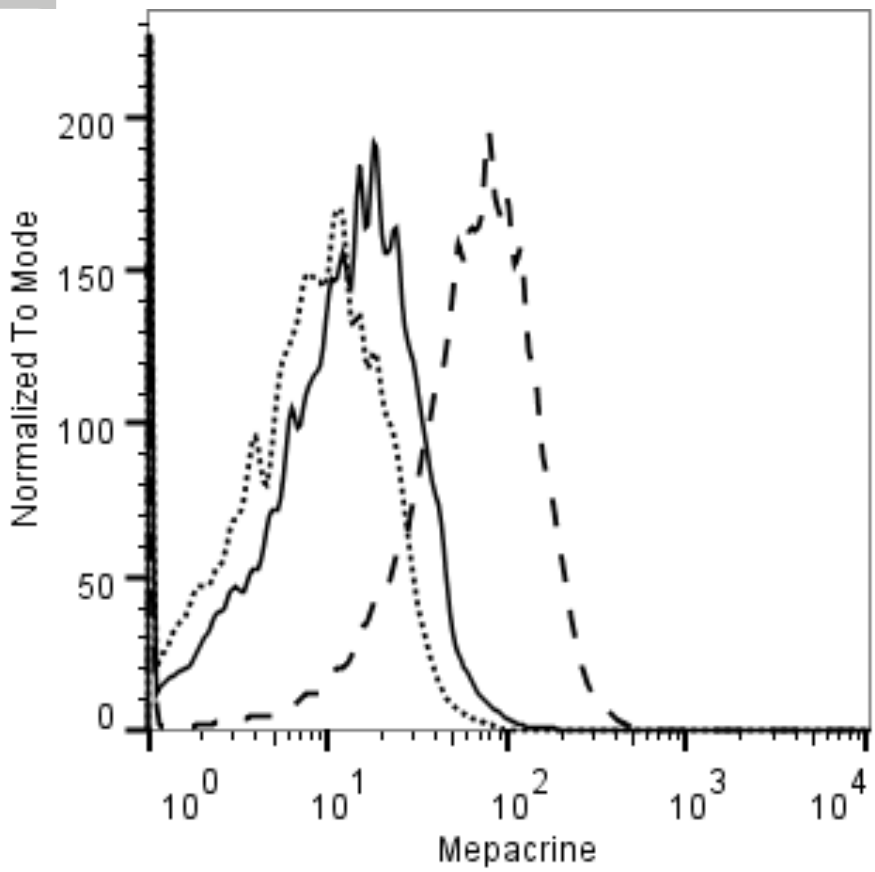


**Figure 2C**



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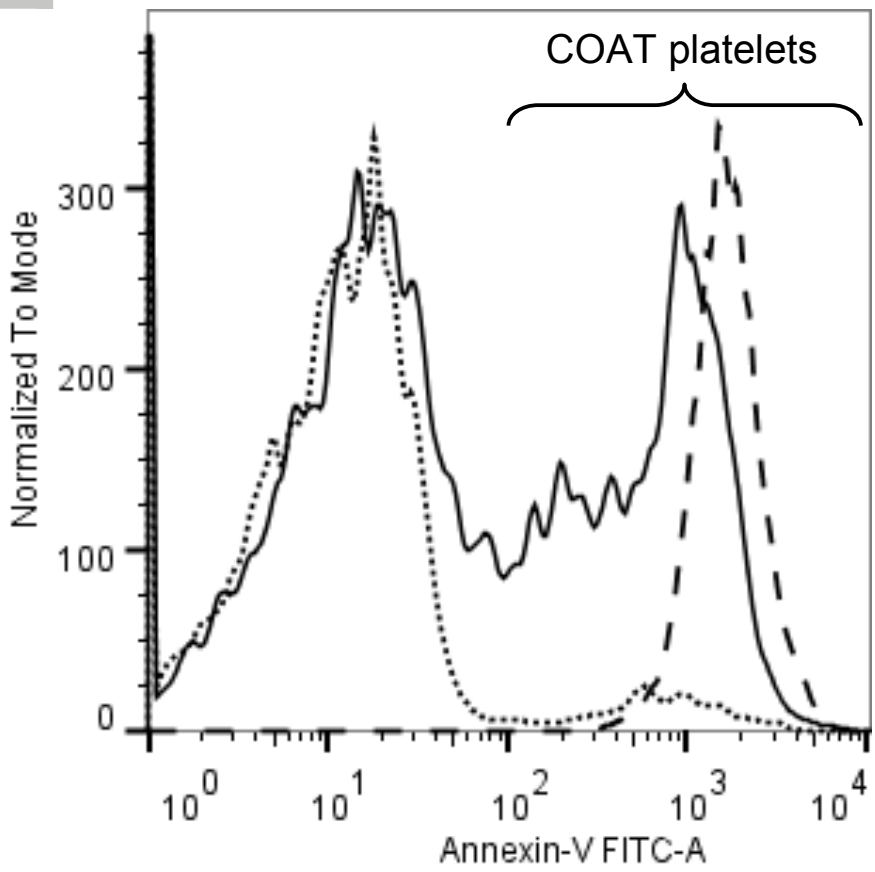


**Figure 3**

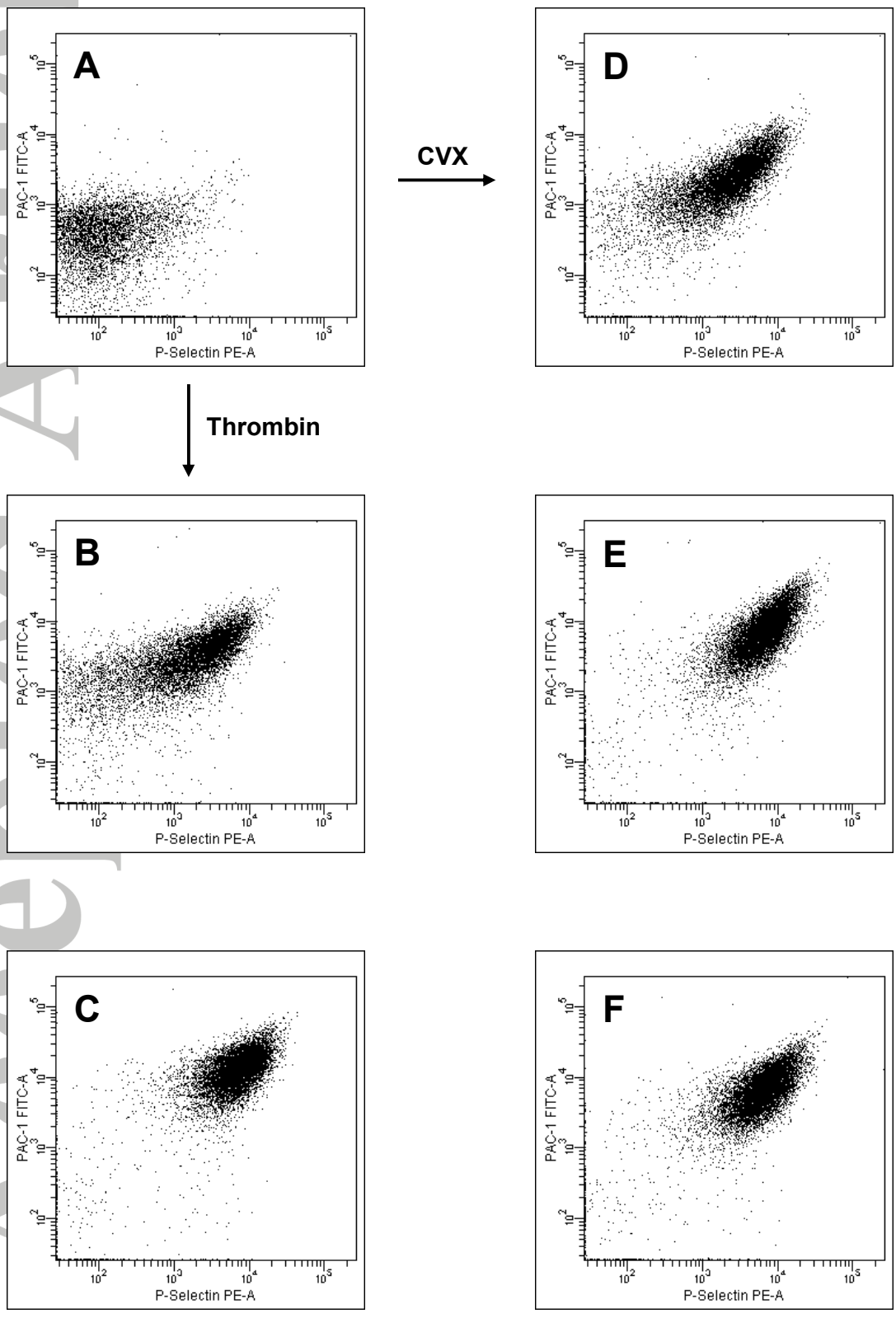
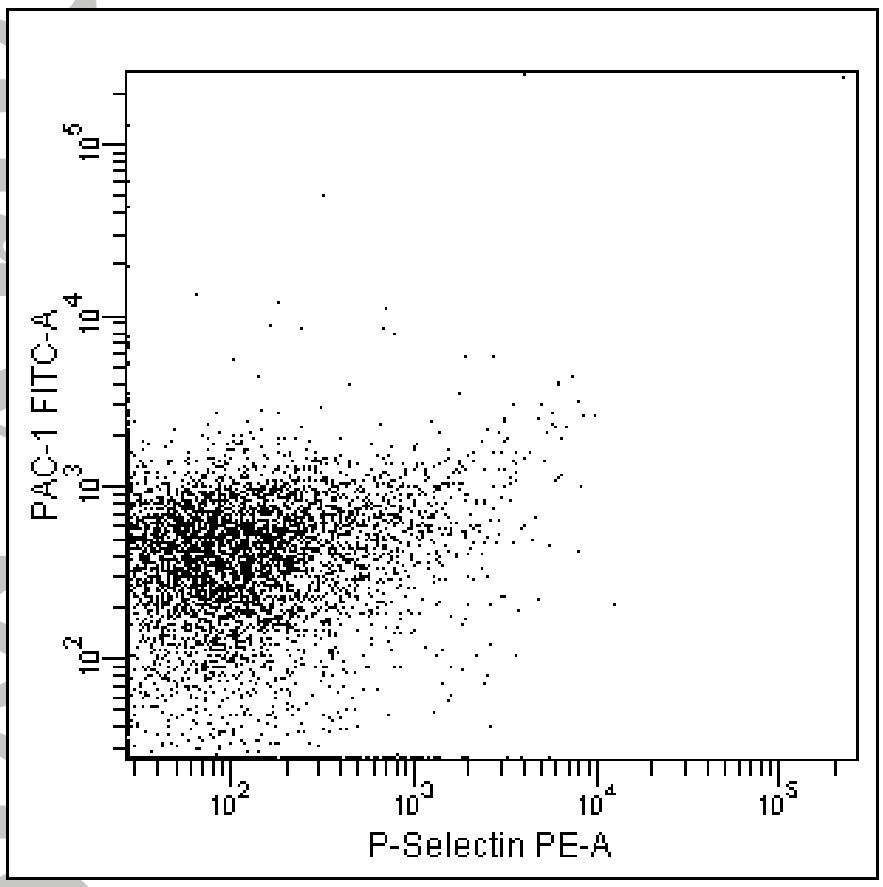


**Figure 3A**



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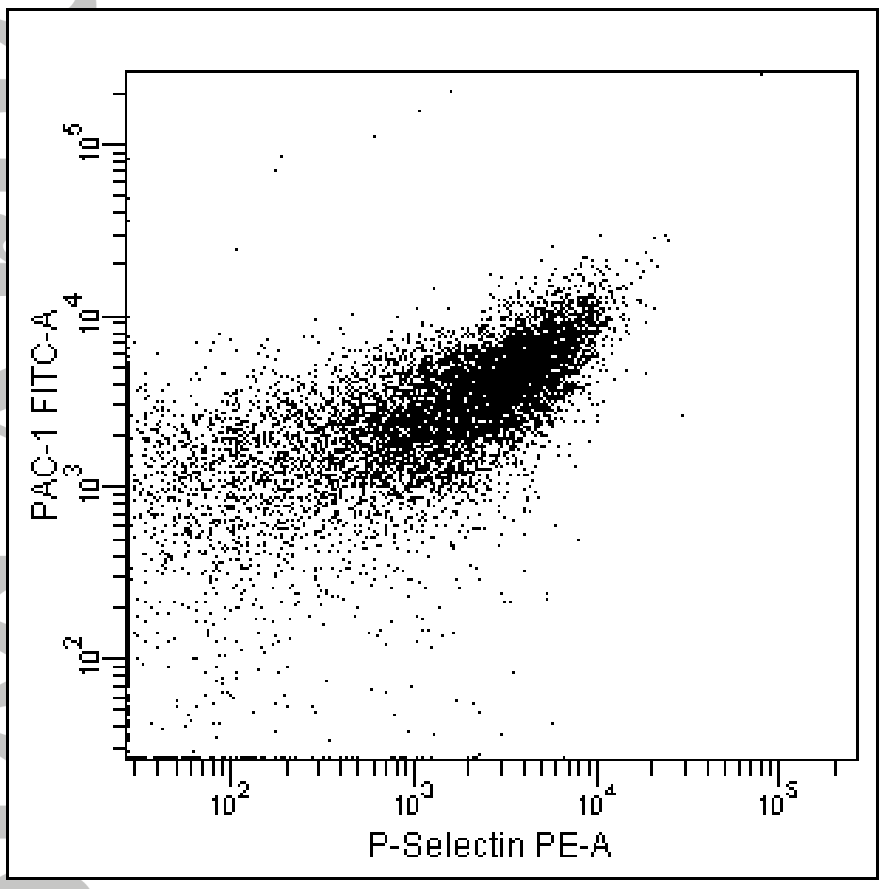

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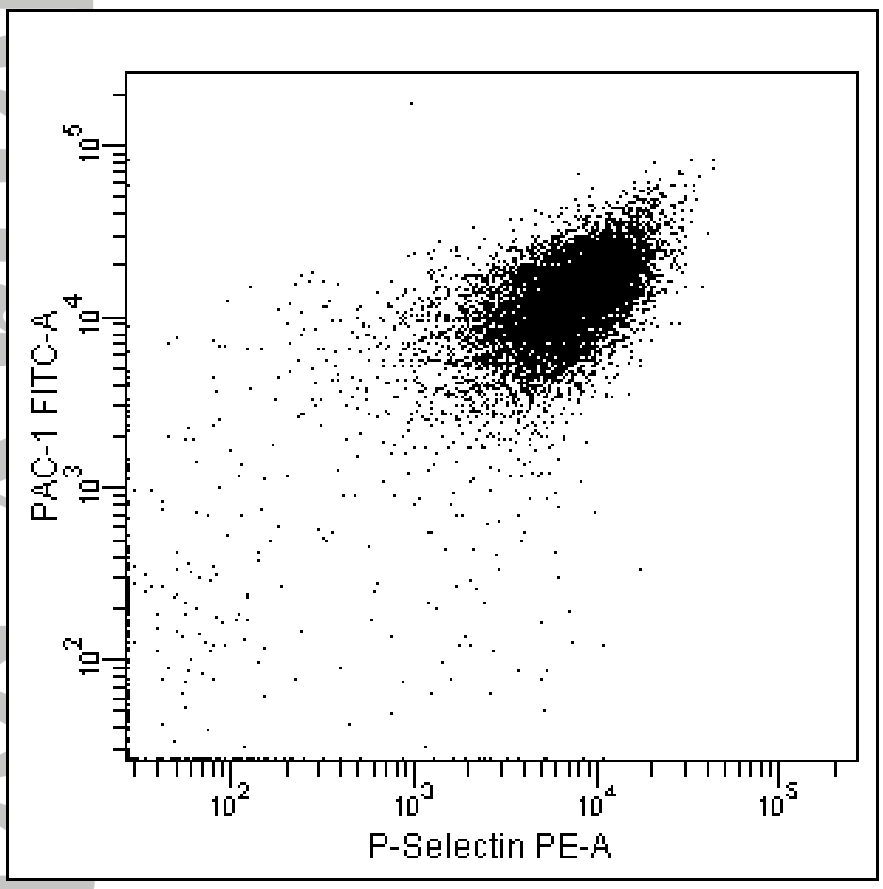

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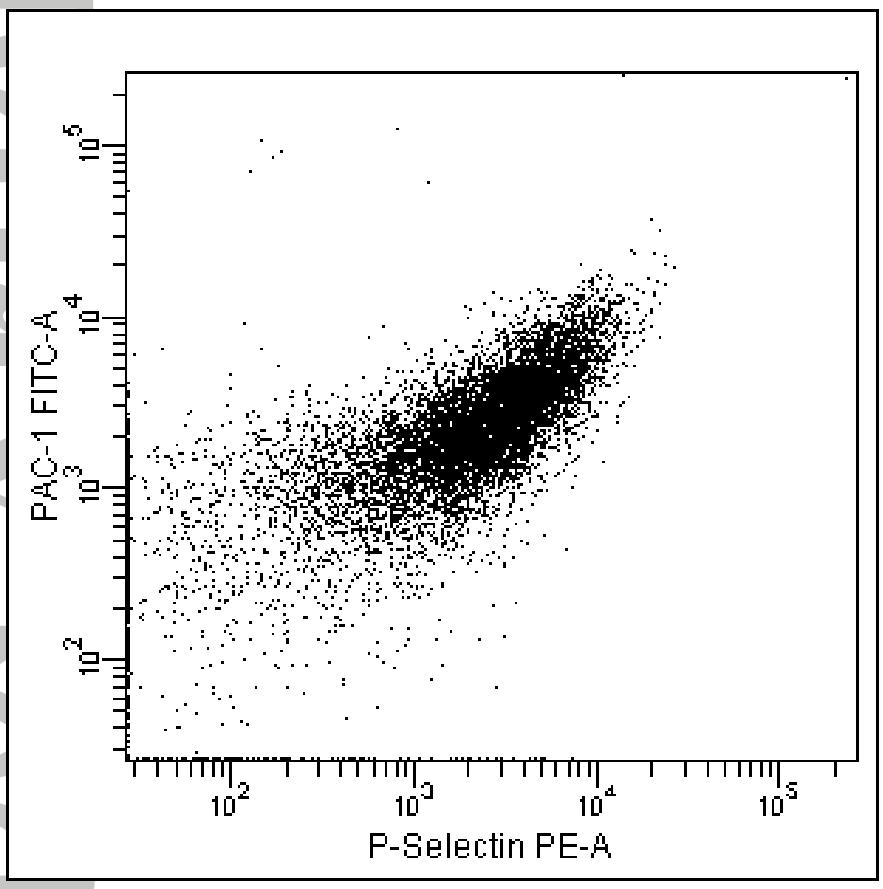

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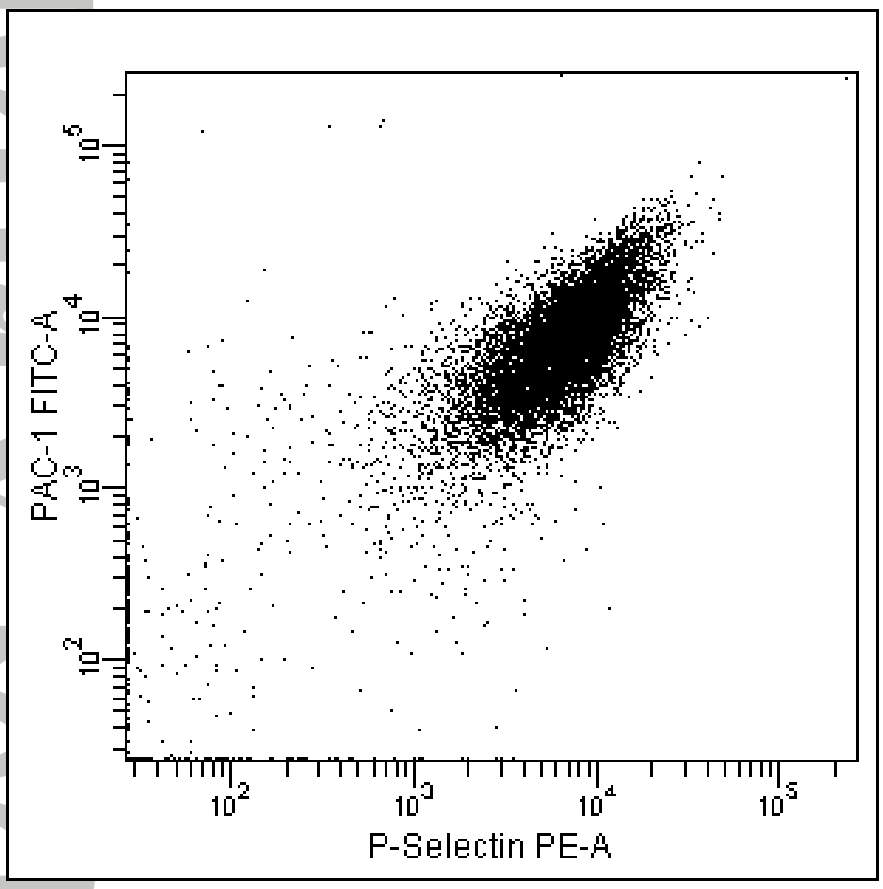

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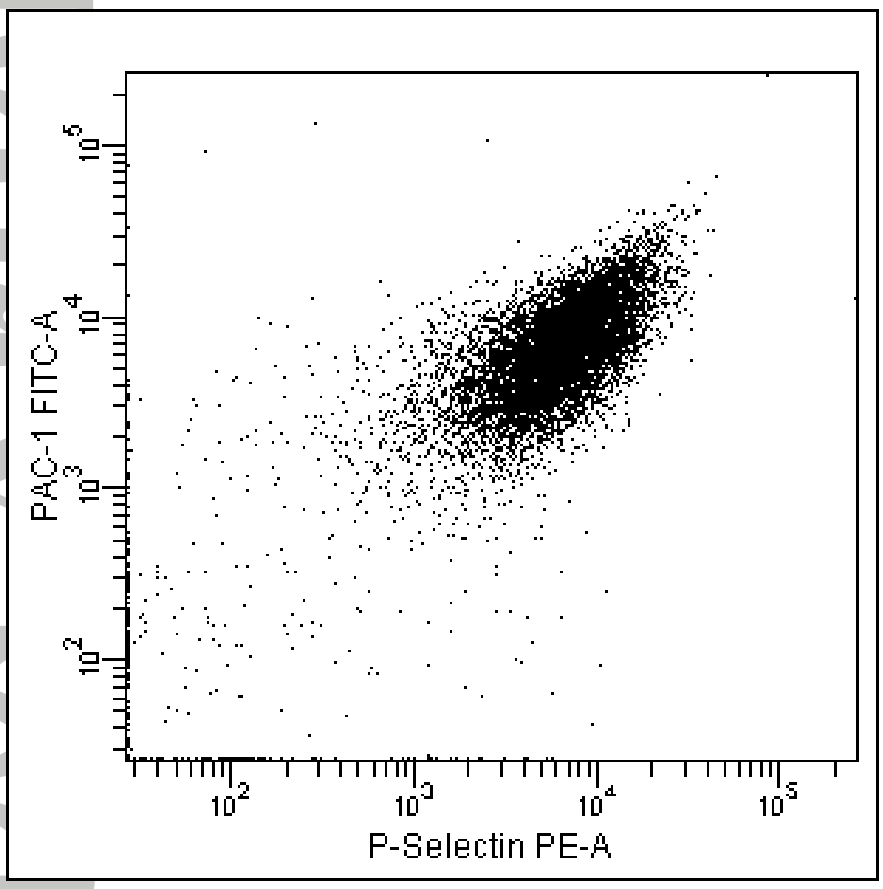

## Figure 4A

| Parameter / Pat.-No | P-selectin ADP | PAC-1 ADP | P-selectin CVX | PAC-1 CVX | P-selectin thrombin | PAC-1 thrombin | δ granula content | δ granula secretion | COAT plts | Defect ? | BS UCH/ISTH |
|---|---|---|---|---|---|---|---|---|---|---|---|
| 04 | 28 | 84 | 83 | 67 | 88 | 53 | 93 | 89 | 13 | complex | 9/6 |
| 01 | 39 | 82 | 79 | 46 | 81 | 78 | 90 | 51 | 52 | complex | 8/8 |
| 59 | 61 | 86 | 92 | 51 | 90 | 49 | 83 | 98 | 27 | GPIb/IIa | 8/9 |
| 20 | 82 | *17 | 71 | 57 | *22 | *04 | 96 | 99 | 29 | GPIIb/IIa (CVX) | 6/5 |
| 05 | 43 | 87 | 53 | 70 | 77 | 73 | 96 | 82 | 23 | Alpha (CVX) | 6/6 |
| 32 | 132 | 84 | 94 | 68 | 112 | 95 | 116 | 110 | 29 | GPIIb/IIa (ADP) | 3/3 |
| 16 | 37 | 123 | 75 | 90 | 82 | 107 | 91 | 99 | 48 | Alpha (ADP) | 17/14 |
| 28 | 24 | 79 | 114 | 100 | 108 | 100 | 92 | 93 | 32 | Alpha (ADP) | 7/7 |
| 55 | 26 | 89 | 84 | 75 | 88 | 91 | 91 | 97 | 34 | Alpha (ADP) | 3/4 |
| 14 | 98 | 69 | 129 | 64 | 122 | 73 | 54 | 92 | 42 | Dense | 7/5 |
| 26 | 157 | 92 | 88 | 90 | 88 | 93 | 67 | 99 | 28 | Dense | 7/1 |
| 58 | 123 | 105 | 89 | 72 | 102 | 114 | 57 | 99 | 27 | Dense | 8/9 |
| 02 | 55 | 79 | 76 | 52 | 97 | 79 | 77 | 100 | 15 | COAT | 4/5 |
| 18 | 42 | 86 | 109 | 78 | 107 | 73 | 100 | 96 | 19 | COAT | 13/10 |
| 06 | 62 | 105 | 114 | 93 | 122 | 102 | 103 | 98 | 16 | COAT | 8/9 |
| 07 | 58 | 82 | 97 | 81 | 120 | 107 | 133 | 127 | 16 | COAT | 11/10 |
| 09 | 79 | 113 | 102 | 124 | 107 | 130 | 114 | 104 | 13 | COAT | 4/6 |
| 35 | 57 | 80 | 138 | 91 | 122 | 101 | 93 | 103 | 17 | COAT | 5/5 |
| 12 | 42 | 127 | 87 | 107 | 97 | 113 | 83 | 93 | 21 | ---- | 3/2 |
| 13 | 47 | 93 | 103 | 85 | 110 | 131 | 155 | 102 | 24 | ---- | 4/5 |
| 30 | 57 | 115 | 86 | 95 | 96 | 142 | 99 | 93 | 40 | ---- | 6/6 |
| 31 | 67 | 74 | 116 | 91 | 112 | 80 | 93 | 94 | 35 | ---- | 5/3 |
| 51 | 59 | 92 | 92 | 109 | 96 | 100 | 77 | 98 | 60 | ---- | 3/1 |
| 53 | 91 | 116 | 104 | 104 | 110 | 111 | 112 | 94 | 32 | ---- | 6/6 |
| 54 | 94 | 97 | 99 | 147 | 75 | 88 | 77 | 98 | 39 | | 3/2 |
| 56 | 96 | 161 | 125 | 129 | 127 | 141 | 94 | 100 | 37 | ---- | 5/5 |
| 60 | 45 | 95 | 109 | 125 | 90 | 125 | 99 | 100 | 57 | ---- | 8/7 |
| 62 | 60 | 74 | 131 | 82 | 128 | 85 | 88 | 94 | 26 | ---- | 4/5 |
| 65 | 77 | 107 | 112 | 88 | 128 | 125 | 78 | 96 | 26 | ---- | 12/7 |
| Number | 5/2 | 4/1 | 2/0 | 4/0 | 1/0 | 2/0 | 3/0 | 3/1 | 7/1 | | |

## Figure 4B

| Parameter / Pat..No | P-selectin ADP | PAC-1 ADP | P-selectin CVX | PAC-1 CVX | P-selectin thrombin | PAC-1 thrombin | δ granula content | δ granula secretion | COAT plts | Defect? | BS UCH/ISTH |
|---|---|---|---|---|---|---|---|---|---|---|---|
| 52 | 32 | 52 | 69 | 45 | 83 | 66 | 57 | 97 | 36 | Complex | 4/5 |
| 34 | 36 | 76 | 93 | 63 | 69 | 53 | 90 | 92 | 25 | Complex | 7/7 |
| 38 | 164 | 81 | 131 | 49 | 83 | 50 | 81 | 97 | 36 | GPIIb/IIIa | 5/4 |
| 67 | 125 | 88 | 104 | 58 | 109 | 66 | 101 | 90 | 40 | GPIIb/IIIa | 10/9 |
| 63 | 58 | 47 | 81 | 57 | 93 | 86 | 71 | 100 | 31 | GPIIb/IIIa | 3/3 |
| 37 | 130 | 77 | 179 | 86 | 198 | 65 | 70 | 97 | 43 | GPIIb/IIIa (Thr) | 3/3 |
| 64 | 46 | 70 | 46 | 33 | 82 | 76 | 89 | 98 | 29 | GPVI | 3/4 |
| 27 | 58 | 95 | 89 | 57 | 81 | 76 | 137 | 101 | 22 | GPIIb/IIIa (CVX) | 2/2 |
| 19 | 98 | 137 | 100 | 57 | 105 | 74 | 111 | 100 | 28 | GPIIb/IIIa (CVX) | 3/2 |
| 44 | 95 | 72 | 48 | 75 | 97 | 84 | 89 | 98 | 50 | Alpha (CVX) | 7/6 |
| 22 | 31 | 56 | 85 | 62 | 81 | 71 | 111 | 100 | 34 | P2Y1/12 | 2/2 |
| 45 | 46 | 64 | 91 | 77 | 94 | 70 | 98 | 101 | 43 | GPIIb/IIIa (ADP) | 4/4 |
| 48 | 64 | 63 | 87 | 104 | 85 | 87 | 81 | 101 | 34 | GPIIb/IIIa (ADP) | 2/0 |
| 50 | 146 | 65 | 147 | 131 | 131 | 102 | 147 | 103 | 41 | GPIIb/IIIa (ADP) | 3/2 |
| 46 | 37 | 101 | 81 | 81 | 93 | 94 | 155 | 105 | 36 | Alpha (ADP) | 2/2 |
| 47 | 44 | 86 | 70 | 71 | 65 | 79 | 66 | 98 | 34 | Dense | 4/1 |
| 41 | 57 | 106 | 93 | 96 | 93 | 90 | 68 | 70 | 42 | Dense | 4/3 |
| 39 | 103 | 95 | 72 | 90 | 77 | 83 | 60 | 90 | 43 | Dense | 4/4 |
| 43 | 98 | 89 | 105 | 97 | 111 | 91 | 66 | 99 | 45 | Dense | 9/8 |
| 61 | 101 | 93 | 69 | 68 | 83 | 101 | 63 | 100 | 28 | Dense | 4/4 |
| 10 | 177 | 216 | 121 | 170 | 101 | 118 | 71 | 88 | 12 | COAT | 3/1 |
| 3 | 67 | 112 | 153 | 87 | 117 | 63 | 115 | 104 | 13 | COAT | 14/14 |
| 16 | 55 | 67 | 97 | 88 | 83 | 78 | 91 | 100 | 19 | COAT | 19/16 |
| 24 | 36 | 76 | 92 | 85 | 107 | 93 | 78 | 96 | 19 | COAT | 2/2 |
| 8 | 124 | 106 | 124 | 97 | 115 | 89 | 126 | 99 | 18 | COAT | 3/3 |
| 23 | 93 | 98 | 94 | 85 | 121 | 111 | 104 | 100 | 15 | COAT | 3/3 |
| 25 | 61 | 107 | 118 | 103 | 120 | 109 | 77 | 95 | 17 | COAT | 7/7 |
| 29 | 53 | 81 | 126 | 112 | 112 | 84 | 104 | 94 | 9 | COAT | 1/1 |
| 66 | 145 | 149 | 155 | 166 | 134 | 142 | 129 | 99 | 17 | COAT | 11/10 |
| 17 | 60 | 69 | 92 | 132 | 86 | 92 | 75 | 97 | 21 | | 12/10 |
| 11 | 71 | 96 | 100 | 80 | 107 | 97 | 83 | 98 | 25 | | 6/5 |
| 21 | 180 | 133 | 117 | 109 | 81 | 95 | 85 | 97 | 36 | ---- | 5/5 |
| 33 | 102 | 108 | 142 | 106 | 151 | 115 | 147 | 102 | 23 | ---- | 4/5 |
| 30 | 125 | 110 | 136 | 117 | 123 | 106 | 100 | 100 | 25 | ---- | 3/2 |
| 40 | 80 | 109 | 89 | 68 | 79 | 78 | 92 | 96 | 24 | ---- | 7/10 |
| 42 | 189 | 184 | 106 | 97 | 102 | 110 | 81 | 106 | 43 | ---- | 6/7 |
| 49 | 103 | 124 | 93 | 162 | 88 | 138 | 131 | 102 | 34 | ---- | 6/5 |
| 57 | 47 | 79 | 104 | 86 | 82 | 92 | 93 | 99 | 50 | ---- | 6/3 |
| Number | 5/0 | 7/3 | 2/1 | 7/1 | 2/0 | 6/2 | 6/3 | 2/1 | 9/2 | | |

**Supplemental Table 1.**

**Characteristics of patients with clinically significant bleeding diathesis and non-diagnostic standard laboratory work-up (n = 67).**

| | | | Normal LTA-PRP | | Borderline LTA-PRP | | p |
|---|---|---|---|---|---|---|---|
| | | | | | | | |
| Total number of patients | | | 29 | | 38 | | --- |
| | | | | | | | |
| Sex (women/men) | | | 24/5 | | 26/12 | | --- |
| | | | | | | | |
| Age (years, median, min/max) | | | 52.8 (23.5/75.4) | | 43.2 (16.8/72.9) | | |
| | | | | | | | |
| Bleeding score UCH (median, min/max) | | | 6 (3/17) | | 4 (1/19) | | ns |
| Bleeding score ISTH (median, min/max) | | | 6 (1/14) | | 4 (0/16) | | ns |
| | | | | | | | |
| **Coagulation parameters (normal range)** | | | **Median (min/max)** | | **Median (min/max)** | | **p** |
| **item** | **unit** | **normal range** | | | | | |
| VWF:RCo | % | 42-168 | 89.0 | (47.0/260.0) | 82.5 | (55.0/165.0) | ns |
| VWF:Ag | % | 42-136 | 89.0 | (50.0/235.0) | 82.5 | (55.0/150.0) | ns |
| VWF:ratio | | $\geq$ 0.7 | 1.0 | (0.7/1.3) | 1.0 | (0.7/1.2) | ns |
| FVIII:C | % | 55-164 | 96 | (63/152) | 87 | (65/145) | ns |
| FIX:C | % | 69-134 | 98 | (70/130) | 100 | (74/212) | ns |
| FXI:C | % | 70-139 | 100 | (71/135) | 103 | (74/134) | ns |
| FXIII qualitative | | | normal | | normal | | ns |
| FXIII activity | % | 70-140 | 104 | (62/135)* | 106 | (73/179)† | ns |
| α-2-Antiplasmin | % | 73-126 | 107 | (80/129) | 112 | (89/130) | ns |
| PFA ADP/collagen | sec | 65-121 | 104 | (65/211) | 105 | (70/185) | ns |
| PFA Epi/collagen | sec | 91-157 | 145 | (83/300) | 151 | (74/246) | ns |

Abbreviations: pts, patients; LTA-PRP, light transmission aggregometry in platelet-rich plasma; SD, standard deviation; UCH, University Clinic Haematology, Bern; ISTH, International Society of Thrombosis and Haemostasis; VWF:RCo, von-Willebrand-Ristocetin-Cofactor; VWF:Ag, von-Willebrand-Antigen; VWF:ratio, von-Willebrand-Factor-ratio; PFA, platelet function analyzer-100 closure time; Epi; epinephrine; sec, seconds; ns, not significant. *, FXIII activity was measured in 7/29 patients. †, FXIII activity was measured in 12/38 patients.

# Questionnaire for bleeding diathesis

"UCH-bleeding score"

**Department of Hematology and Central Hematology Laboratory**

**University Hospital, Inselspital Bern Switzerland**

| **1. Cutaneous symptoms** | **Point(s):** |
|---|---|
| • No bruising/no hematoma, except after adequate trauma; no petechiae. Occasional minor bruising after a mild insult, especially in women, is still normal. | 0 |
| • Occasional minor bruising/hematoma without memory of any trauma or one time or occasional appearance of larger hematoma without any appropriate trauma. | 1 |
| • Often multiple bruising without memory of any trauma or frequent petechiae/hematoma along with solitary bruising events or repeated large hematoma without any trauma or with only very mild trauma. (petechiae alone, without other signs of hemorrhagia, should be described in 11.) | 2 |

| **2. Epistaxis** | |
|---|---|
| • No or rare epistaxis events (≤ 2x per year), duration < 15 minutes, hemostatic treatment effective with commonly used household methods. | 0 |
| • Occasional epistaxis (≥ 3x per year), duration > 15 but < 30 minutes, hemostatic treatment effective with commonly used household methods. No underlying local anatomic problem known. | 1 |
| • Frequent epistaxis (≥ 1x every 2 months), duration often > 30 minutes, specific medical treatment for hemostasis necessary in some cases, including transfusion of blood products. No underlying local anatomic problem known. | 2 |

| **3. Oral cavity (without dental treatment)** | **Point(s):** |
|---|---|
| • No bleeding signs of the gingiva or elsewhere in the oral cavity except in case of using a new/hard toothbrush. | 0 |
| • Occasional gingival bleeding independent of tooth cleaning. | 1 |
| • Often and/or repeated strong bleeding of the gingiva or elsewhere in the oral cavity. Medical treatment for hemostasis necessary.<br>or<br>Many petechiae of the oral mucosa without a known underlying medical or anatomic cause (e.g. Morbus Rendu-Weber-Osler). | 2 |
| **4. Bleeding signs/complications during/after dental treatment/maxillary surgery** | |
| • No bleeding signs during or after dental treatment/maxillary surgery. | 0 |
| • One bleeding complication over several hours during/after dental treatment/ maxillary surgery. | 1 |
| • Repeated (> 2x) bleeding complications over several hours, with necessity of dental re-treatment/maxillary re-surgery. | 2 |
| **5. Bleeding after minor injury** | |
| • No prolonged bleeding after a cut-wound or abrasion; bleeding stops in less than 10 minutes either spontaneously or by using a small bandage. | 0 |
| • Occasional prolonged bleeding following minor wounds; treatment with small bandage results in bleeding cessation. | 1 |
| • Recurrence of prolonged and greater bleeding complications after minor wounds, regular bandage not sufficient for bleeding cessation. Additional specific hemostatic treatment required. | 2 |
| **6. Muscle hematomas or hemarthroses** | |
| • No muscle hematomas or hemarthroses except after severe injury. | 0 |
| • One episode of muscle hematoma or hemarthroses either spontaneously or after inadequate trauma. | 1 |
| • Several (> 2x) episodes of muscle hematomas or hemarthroses, spontaneously or after inadequate trauma, and without underlying medical or anatomic cause. Degenerative arthropathy as long-term consequence. | 2 |

| **7. Bleeding of the gastrointestinal tract or genitourinary tract** | **Point(s):** |
|---|---|
| • No bleeding events or only bleeding events due to an underlying local cause. | 0 |
| • Gastrointestinal or genitourinary bleeding of unknown etiology. Prolonged and/or strong bleeding, e.g. in the case of hemorrhoids. | 1 |
| • Recurrent gastrointestinal or genitourinary bleeding episodes without pathological findings and not drug-induced. | 2 |

| **8. Menorrhagia** | |
|---|---|
| • Regular menstrual bleeding (duration of 3-5 days, 2-5 pads/tampons per day). | 0 |
| • Possible or distinct symptoms of menorrhagia/hypermenorrhea without definite exclusion of an underlying gynecological cause. | 1 |
| • Severe menorrhagia (duration > 7 days) or hypermenorrhea (replacement of pads/tampons even during night time) without underlying gynecological cause. | 2 |

| **9. Bleeding complications during/after surgery** | |
|---|---|
| • No bleeding complications during/after surgery. No need for transfusions. | 0 |
| • One single episode of bleeding complication during/after surgery. | 1 |
| • Several (> 2x) bleeding episodes during/after surgeries with excessive bleeding, requiring transfusions, suture or re-surgery. | 2 |

| **10. Transfusions** | |
|---|---|
| • No need for transfusions, except for large surgical interventions or injuries. | 0 |
| • One single episode of transfusion during/after minor surgical intervention or due to minor injury. | 1 |
| • Several (> 2) episodes of transfusions during/after minor surgical interventions or after minor injuries. | 2 |

| **11. Bleeding complications of unknown origin/reason** | **Point(s):** |
|---|---|
| • No episode. | 0 |
| • Major bleeding complication/organ bleeding without clarification of underlying cause. | 1 |
| • Major bleeding complication/organ bleeding of unknown reason after adequate medical examination. | 2 |

| **12. Bleeding diathesis linked to medication intake (e.g. NSAIDs, aspirin)** | |
|---|---|
| • No bleeding diathesis after medication intake. | 0 |
| • Doubtful correlation of bleeding diathesis and medication intake. | 1 |
| • Definite correlation of bleeding diathesis and medication intake. | 2 |

| **13. Hereditary reasons for bleeding diathesis** | |
|---|---|
| • No bleeding diathesis in the patient's family. | 0 |
| • Suspicious positive bleeding diathesis for one or more members in the patient's family. | 1 |
| • Definite positive bleeding diathesis for one or more members in the patient's family. | 2 |