ORIGINAL ARTICLE

Validation of flow cytometric analysis of platelet function in patients with a suspected platelet function defect

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Essentials

- The diagnosis of mild platelet function disorders (PFDs) is challenging.
- Validation of flow cytometric testing in patients with suspected PFDs is required.
- Flow cytometry has added value to light transmission aggregometry (LTA) in diagnosis of PFDs.
- There is fair agreement in diagnosing PFDs between LTA and flow cytometry.

Summary. *Background:* Light transmission aggregometry (LTA) is the most commonly used test for the diagnosis of platelet function disorders (PFDs), but has moderate sensitivity for mild PFDs. Flow cytometry has been recommended for additional diagnostics of PFDs but is not yet standardized as a diagnostic test. We developed a standardized protocol for flow cytometric analysis of platelet function that measures fibrinogen binding and P-selectin expression as platelet activation markers in response to agonist stimulation. *Objectives:* To determine the additional value of flow cytometric platelet function testing to standard LTA screening in a cross-sectional cohort of patients with a suspected PFD. *Methods:* Platelet function was assessed with flow cytometry and LTA in 107 patients suspected of a PFD in whom von Willebrand disease and coagulation factor deficiencies were excluded. Both tests were compared in terms of agreement and discriminative ability for diagnosing patients with PFDs. *Results:* Out of 107 patients, 51 patients had an elevated bleeding score; 62.7% of the patients had abnormal platelet function measured with flow cytometry and 54.2% of the patients were abnormal based on LTA. There was fair agreement between LTA and flow cytometry ($\kappa = 0.32$). The discriminative ability of flow cytometric analysis in patients with an elevated bleeding score was good (AUC 0.82, 0.74–0.90), but moderate for LTA (AUC 0.70, 0.60–0.80). Both tests combined had a better discriminative ability (AUC 0.87, 0.80–0.94). *Conclusion:* Flow cytometric analysis of platelet function has added value in diagnostics of PFDs in patients with unexplained bleeding tendency.

Keywords: blood platelet disorders; blood platelets; flow cytometry; platelet activation; platelet function tests.

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Introduction

Platelets have an important role in the preservation of blood flow. Upon vascular damage, platelets prevent excessive blood loss by the formation of a stable platelet plug at the site of injury. Platelet function disorders (PFDs) are characterized by spontaneous mucocutaneous bleeding, easy bruising, menorrhagia, or an extended bleeding time [1]. PFDs are highly heterogeneous and may be inherited or acquired. Patients with PFDs may have an abnormal platelet count (thrombocytopenia) or impaired platelet function, but may also have both [2].

Mild PFDs are usually associated with mild bleeding symptoms that manifest after trauma or other hemostatic challenges. The incidence of mild bleeding symptoms in the general population is high, hampering identification of patients with mild PFDs. Standardized bleeding scores, such as the Bleeding Assessment Tool of the International Society on Thrombosis and Haemostasis (ISTH-BAT) [3],

have been developed to objectify bleeding symptoms and have shown high negative predictive value, but low specificity and positive predictive value [4]. One study showed that nearly 25% of healthy young women experienced two or more bleeding symptoms, indicating that these bleeding scores should be interpreted with caution [5].

Diagnosing severe inherited PFDs, such as Glanzmann thrombasthenia (GT) or Bernard Soulier syndrome (BSS), is relatively straightforward, because these disorders have a clear clinical presentation and the platelet function defects are readily detected with currently available diagnostic tools.

Laboratory diagnostics of mild PFDs are more challenging, as they often present with a heterogeneous phenotype. Phenotyping these disorders requires highly specialized laboratory techniques, which are not available at most diagnostic laboratories, precluding the diagnosis of a PFD [6,7]. If the diagnosis of a PFD can be made, the exact cause remains unclear in 34% to 60% of patients [6,8]. Nevertheless, a correct diagnosis in patients with a PFD is important for risk stratification, therapeutic intervention, and quality of life.

The most commonly used platelet function test for diagnosing PFDs is light transmission aggregometry (LTA) [9]. In combination with other functional tests, LTA is an important assay in the characterization of different (severe) PFDs, but lacks sensitivity for mild PFDs, including storage pool disease (SPD) [6,10,11]. Furthermore, LTA is time consuming, operator dependent, labor intensive and needs large volumes of blood [9]. Although the test itself is not very reproducible, the diagnosis of a PFD based on LTA can be confirmed in 90% of the cases [12]. There is poor consensus about which agonists and concentrations should be used, resulting in low agreement between laboratories [9,13]. Finally, LTA requires a minimum platelet count of 150×10^9 L^{-1} in platelet-rich plasma (PRP) [14]. Tests performed with samples with a platelet count $< 75 \times 10^9$ L^{-1} should be interpreted with caution, which is a problem when attempting to identify platelet function disorders in thrombocytopenic patients [15].

There is an unmet need for methods that improve the diagnostic accuracy of (mild) PFDs. An alternative approach to test platelet function is by means of flow cytometry. Here, platelet activation can be determined by quantifying fibrinogen binding to integrin αIIbβ3, or expression of P-selectin or CD63 on the platelet surface upon stimulation [16,17], and also ristocetin-induced VWF-platelet binding can be determined [18,19]. Flow cytometry-based approaches for the assessment of platelet function have already been used for many years in research settings [20,21]. The major advantage of a flow cytometry-based approach is that it requires a small amount of blood and allows the analysis of platelet function in thrombocytopenia [22,23]. The ISTH SSC recommends the use of flow cytometry in the diagnostic work-up of PFDs [9], but lack of standardization prevents the use of flow cytometry as a diagnostic test. Therefore, we optimized a flow cytometry-based platelet activation test (PACT) for diagnostic use, which measures P-selectin expression and αIIbβ3 activation after stimulation with a variety of different agonists.

In this study, we validated our assay in patients with a well-defined hereditary platelet function disorder. We subsequently determined the added value of flow cytometric analysis of platelet function in a cross-sectional cohort of patients with unidentified bleeding tendency in whom VWD or coagulation disorders were excluded and who were referred for platelet function testing to a tertiary referral hospital.

Methods

Participants

Healthy volunteers. Blood from healthy participants was obtained through the Mini Donor Service, a blood donation facility for research purposes that is approved by the medical ethics committee of the University Medical Center Utrecht and for which all donors have provided written informed consent, in accordance with the declaration of Helsinki. All participants reported being healthy and free from antiplatelet drugs or non-steroid anti-inflammatory drugs for at least 10 days prior to blood donation.

Patients. Two patient cohorts were used in this study. In cohort 1, patients with a previously diagnosed PFD were included. These patients were diagnosed with SPD, GT or BSS. Cohort 1 was used for the proof of principle of the flow cytometric PACT assay. In cohort 2, 143 consecutive patients with a mucocutaneous bleed pattern who were referred to a hemophilia treatment center for LTA analysis were included. After visiting the hematologist, a bleeding score was calculated using the ISTH-BAT [3]. Exclusion criteria were von Willebrand disease, coagulation factor deficiencies, pregnancy and age < 18 years. Approval for this study was obtained from the medical ethics review board of the UMC Utrecht. Written informed consent was obtained from patients with hereditary platelet function disorders (cohort 1) in accordance with the declaration of Helsinki. Informed consent requirement was waived by the Institutional Review Board for patients included in cohort 2.

Blood collection

Peripheral venous blood from patients and controls was drawn by venipuncture into 109-mM trisodium citrate Sarstedt tubes in a 9:1 (v:v) blood to anticoagulant ratio. All blood samples were processed within 1–6 h after blood collection.

Reagents

PE-conjugated anti-P-selectin (AK4) and APC-conjugated anti-GP1b (HIP1) antibodies were purchased from BD Biosciences (Franklin Lakes, NJ, USA), FITC-conjugated rabbit anti-human fibrinogen was obtained from DAKO (Glostrup, Denmark) and FITC-conjugated goat anti-human VWF was obtained from Bio-Rad laboratories (Veenendaal, the Netherlands). Adenosine diphosphate (ADP) and indomethacin were from Sigma-Aldrich (Zwijndrecht, the Netherlands), fibrillar equine collagen I (Horm collagen) was from Takeda (Linz, Austria), cross-linked collagen-related peptide (CRP-xl) was a generous gift from Professor Richard Farndale (University of Cambridge, Cambridge, UK), ristocetin was purchased from American Biochemical and Pharmaceuticals Ltd (Marlton, NJ, USA), arachidonic acid was from the Bio/data corporation (Horsham, PA, USA), protease activating receptor (PAR)-1-activating peptide SFFLRN (PAR1-AP) was obtained from Bachem (Weil am Rhein, Germany) and PAR-4-activating peptide AYPGKF (PAR4-AP) was from the Netherlands Cancer Institute (Amsterdam, the Netherlands).

Light transmission aggregometry

LTA was performed at 37 °C with the PAP-8E platelet aggregometer (Sysmex, Etten-Leur, Netherlands) within 3 hours after blood collection. PRP was obtained by centrifugation of whole blood at 160 g for 15 min at 20 °C. Platelet counts were adjusted to 250×10^9 platelets L^{-1} with platelet-poor plasma, obtained by centrifugation of the remaining blood (2000 g, 15 min, 20 °C). LTA is dependent on platelet count in PRP and can be inaccurate at lower platelet counts [24]. LTA data were not obtained when platelet count was $< 75 \times 10^9$ L^{-1}. Aggregation was initiated with ADP (2.5 and 5.0 μM), Horm collagen (1.0 and 4.0 μg mL^{-1}), arachidonic acid (1.5 mM) or ristocetin (1.0 mg mL^{-1}). Samples were stirred at 900 rpm and aggregation traces were recorded for 15 min and the final amplitude (FA) of the aggregation curve was evaluated. Cut-off levels for differentiation between normal and abnormal responses were based on the 2.5th percentile of the FA of 58 healthy controls for each agonist.

Flow cytometric analysis of platelet activation markers in whole blood

Whole blood was diluted 1 : 10 in HEPES buffered saline (HBS; 10 mM HEPES, 150 mM NaCl, 1 mM $MgSO_4 \times 6H_2O$, 5 mM KCl pH 7.4), which contained a platelet agonist and either FITC-conjugated anti-fibrinogen (1 : 100) antibodies and PE-conjugated anti-P-selectin antibodies (1 : 25) or FITC-conjugated anti-VWF antibodies (1 : 1000). We used a streamlined agonist panel similar to that described for LTA [25]. To confirm BSS diagnosis, APC-conjugated anti-GP1b (1 : 25) was diluted in HBS. Whole blood was stimulated for 20 min with either a single concentration of agonist (30 μM ADP, 100 μM PAR1-AP, 1500 μM PAR4-AP or 1 μg mL^{-1} CRP-xl) or serial dilutions (0.008–125 μM ADP, 0.153–2500 ng mL^{-1} CRP-xl, 0.038–625 μM PAR1-AP, 0.2–4000 μM PAR4-AP or 0.00–0.75 mg mL^{-1} ristocetin) at room temperature as indicated. Platelet count does not influence platelet responses to agonists [26]. Samples were fixed (0.148% formaldehyde, 137 mM NaCl, 2.7 mM KCl, 1.12 mM NaH_2HPO_4, 10.2 mM Na_2HPO_4, 1.15 mM KH_2PO_4, 4 mM EDTA, pH 6.8) for 20 min and analyzed on a BD Accuri flow cytometer (BD Biosciences). Prior to analysis, the flow cytometer was calibrated using fluorescent beads. Platelets were identified with forward and sideward scatter and median fluorescent intensity (MFI) data were obtained. MFI was normalized to correct for lot-to-lot variations. Area under the curve (AUC) and EC50 were calculated in samples where a concentration range of agonists was used to stimulate platelets.

Statistical analysis

Measurements were screened for normality with the Shapiro–Wilk normality test, and test reference values of the LTA ($n = 58$) and the standardized flow cytometry-based platelet reactivity test ($n = 202$) were determined according to CSLI guideline EP09A3 [27]. Percentage final amplitude (FA) in LTA and the MFI of P-selectin expression and fibrinogen binding to αIIbβ3 were considered abnormal if they were below the 2.5th percentile of the control population.

A receiver operator curve (ROC) was based on a probability score created with multiple logistic regression analysis of the nine variables for the flow cytometric platelet reactivity test (MFI of P-selectin expression and fibrinogen binding after stimulation by PAR1-AP, PAR4-AP, ADP and CRP-xl, and ristocetin-induced VWF binding) and four variables for LTA (FA after ADP, arachidonic acid, collagen and ristocetin incubation). Sensitivity was plotted against the false-positive rate to show the discriminating ability of both tests in diagnosis.

All statistical analyses were performed using Graphpad Prism software version 6.0 (San Diego, CA, USA) and IBM SPSS statistics version 21 (Armonk, NY, USA).

Results

Severe inherited platelet function disorders can be detected with a standardized flow cytometry-based platelet activation assay

Dose–response curves after platelet stimulation with ADP, CRP-xl, PAR1-AP, PAR4-AP or ristocetin were obtained from 17 healthy controls. P-selectin expression

was used as a marker for granule release (Fig. 1A), fibrinogen binding was used as a marker for αIIbβ3 activation (Fig. 1B) and VWF binding was assessed as a marker for GPIb-IX-V functionality (Fig. 1C). All platelet activation markers increased with increasing concentrations of agonist and were maximal at the highest agonist concentrations used. To determine whether this flow cytometry-based assay was able to discriminate between normal and abnormal platelet responses, dose–response curves were obtained in patients with severe inherited PFDs, including five patients with δ-storage pool disease (SPD), four patients with Glanzmann thrombasthenia (GT) and one Bernard Soulier syndrome (BSS) patient. The AUC was calculated for patients and controls as a measure of total platelet reactivity. Cut-off values to discriminate normal from abnormal responses were based on the 5th–95th percentile of the response in 17 healthy controls (Fig 1D–F). All patients with SPD had an abnormal response to one or more platelet agonists, but VWF binding was normal in all SPD patients. As expected, fibrinogen binding was absent in platelets from patients with GT in response to all agonists. In contrast, P-selectin expression in GT patients was normal for all agonists, with the exception of ADP. VWF binding was normal in all GT patients. In the patient with BSS, all platelet responses were normal, except for VWF binding, which was reduced. Taken together, these data indicate that the flow cytometric platelet reactivity assay PACT can discriminate between patients with severe PFDs and healthy controls.

Performance characteristics of the standardized flow cytometry-based platelet reactivity test PACT

Analysis of the area under the dose–response curve yielded results that were similar to those obtained with analysis of the response to a high concentration of agonist (Supplementary Table 1). We therefore simplified the test by including a single concentration for each agonist derived from the shoulder region of the dose–response curve: 30 μM ADP, 100 μM PAR1-AP, 1500 μM PAR4-AP, 1 μg mL^{-1} CRP-xl or 0.4 mg mL^{-1} ristocetin. Reproducibility was assessed with six repeated measurements from two healthy controls on 3 days, at least 1 week apart. Blood from one of these donors was treated with 100 μM indomethacin to mimic reduced platelet reactivity. The mean coefficient of variation of the response to each agonist was 7.4% in healthy platelets and 5.8% in platelets with decreased reactivity, indicating good reproducibility. All coefficients of variation for single agonists were < 10%.

Standardized flow cytometry-based platelet reactivity testing has added value on top of LTA in identification of PFDs

In cohort 2, 143 consecutive patients with a suspected PFD were enrolled (Fig. 2). Twenty out of the 143 patients were below 18 years of age, six patients were pregnant, seven patients had von Willebrand disease and three patients had a coagulation factor deficiency. In the remaining 107 patients with a suspected PFD (Table S2), we determined platelet reactivity with both LTA and the PACT to determine whether flow cytometry can be used to identify patients with a PFD. The mean platelet count was 224×10^9 L^{-1} (range, 7–640). LTA data were obtained from 100 patients (93%); LTA data were missing in the remaining seven patients due to platelet counts $< 75 \times 10^9$ L^{-1}. Flow cytometric analysis of platelet function was performed in all patients. The reference values for the LTA were based on the 2.5th percentile in 58 healthy controls, but at 2.5 μM ADP and 4.0 μg mL^{-1} collagen, the cut-off value was at 0% aggregation. Therefore, only 5.0 μM ADP, 1.5 mM arachidonic acid, 4.0 μg mL^{-1} collagen and 1.0 mg mL^{-1} ristocetin were used for analysis. Reference values for the PACT were established, based on the 2.5th percentile of P-selectin expression, fibrinogen binding and VWF binding in 202 healthy volunteers (Table S3). As expected, platelet reactivity towards agonists varied substantially in the general population (Fig. 3A–D).

There was large variation between individuals in platelet reactivity measured with flow cytometry, especially when αIIbβ3 activation was measured in our patients (Fig. 3E-H). Out of 107 patients, 57 patients (53.3%) had at least one reduced response to a platelet agonist, of whom 26 patients (45.6%) showed both reduced fibrinogen binding and decreased P-selectin expression, 22 patients (38.6%) had reduced fibrinogen binding but normal P-selectin expression, and nine patients (16%) had reduced P-selectin expression but normal fibrinogen binding.

Out of 107 patients with a suspected PFD, 51 patients had an elevated bleeding score [28] (> 3 in men; > 5 in women). Thirty-two out of these 51 patients showed decreased platelet reactivity with the PACT, whereas 26 out of 48 patients showed decreased platelet reactivity

Fig. 1. Platelet responses in healthy controls and patients with δ-storage pool disease (SPD), Glanzmann thrombasthenia (GT) or Bernard Soulier syndrome (BSS). Whole blood obtained from 17 healthy controls was stimulated with ADP, CRP-xl, PAR1-AP, PAR4-AP and ristocetin at the indicated concentrations for 20 min, fixed and subjected to flow cytometric analysis. P-selectin expression (A) was assessed as a measure of granule release, fibrinogen binding (B) as a measure of αIIbβ3 activation, and VWF binding (C) to determine functionality of the GPIb-V-IX complex. Data were normalized on the median maximal result and expressed as median fluorescent intensity with 95% confidence interval. The area under the curve (AUC) of normalized P-selectin expression (D), fibrinogen binding (E) and VWF binding (F) of five patients with SPD, four patients with GT and one BSS patient was compared with the AUC in healthy controls (box and whiskers). The error bars indicate the 95% confidence interval of the control population.

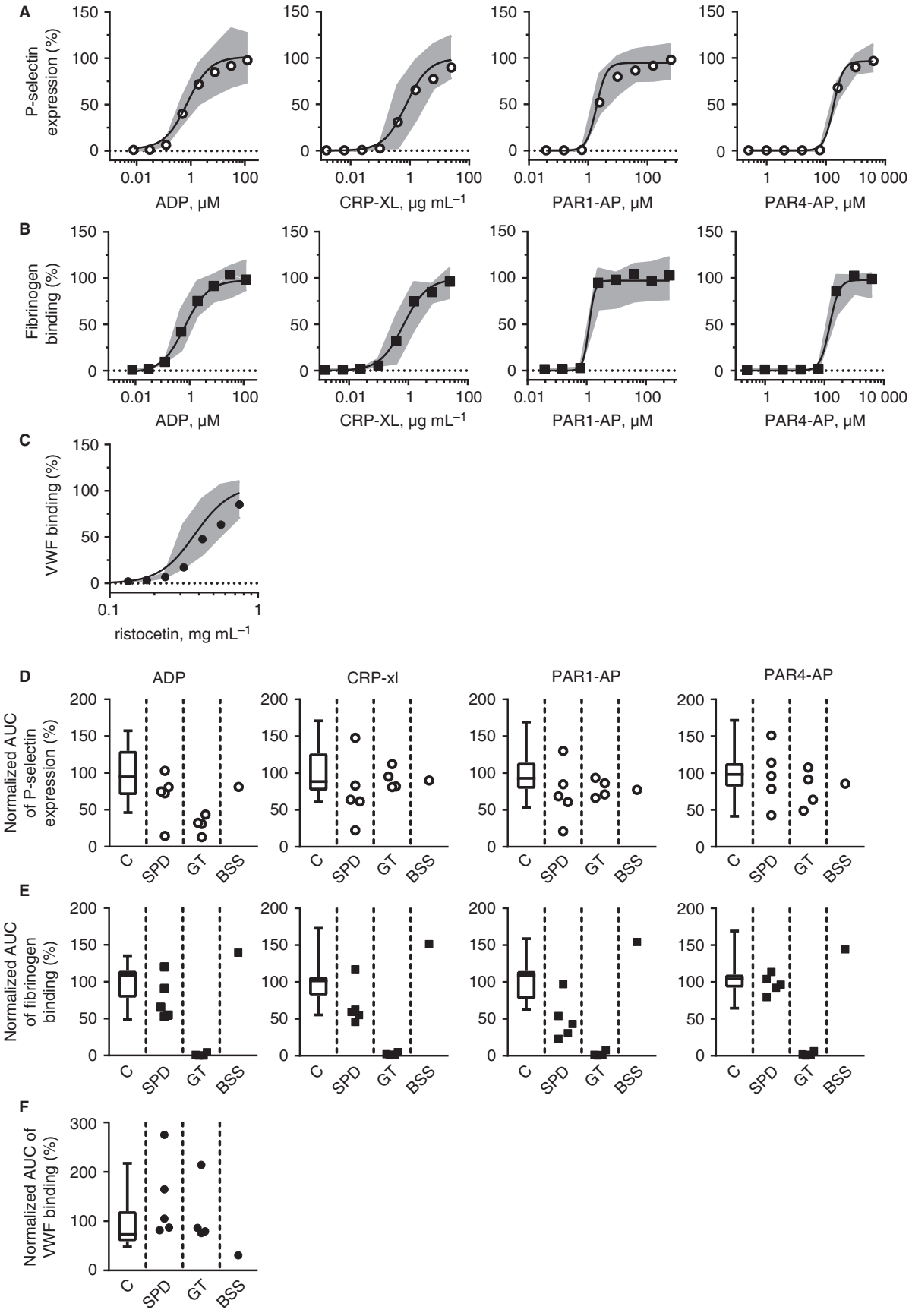

Table 1 Flow cytometry-based analysis of patients with an elevated bleeding score and a suspected platelet function disorder compared with light transmission aggregometry (LTA)

Flow cytometric platelet reactivity (n = 51)	
Normal reactivity	19 (37.3%)
Abnormal	32 (62.7%)
Reduced, 1 agonist	5 (9.8%)
Reduced, 2 agonists	6 (11.8%)
Reduced, 3 agonists	3 (5.9%)
Reduced, 4 agonists	13 (25.5%)
Reduced, 5 agonists	5 (9.8%)
Platelet aggregation with LTA (n = 48)*	
Normal aggregation	22 (45.8%)
Abnormal	26 (54.2%)
Reduced, 1 agonist	14 (29.2%)
Reduced, 2 agonists	5 (10.4%)
Reduced, 3 agonists	7 (14.6%)
Reduced, 4 agonists	0

*No data available in three patients due to thrombocytopenia.

with LTA (Table 1), and therefore had a PFD. LTA data were unavailable in three patients due to thrombocytopenia. Twenty of 26 patients with an abnormal LTA also had abnormal PACT results. Nine out of 32 patients with abnormal PACT results showed normal platelet aggregation. Agreement between LTA and the PACT was fair ($\kappa = 0.32$; $P < 0.05$) (Table 2).

Discriminative ability between patients and healthy controls

Next, diagnostic accuracy was estimated with ROC analysis. PACT and LTA data from all 107 patients, the 51 patients with a high bleeding score or the 56 patients with a low bleeding score were compared with PACT and LTA results obtained in a cohort of 58 healthy controls (Fig 4). LTA data from seven patients were missing due to a platelet concentration $< 75 \times 10^9$ L^{-1}, of whom three had a high bleeding score and four had a low bleeding score. Therefore, LTA data were available in 48 patients with a high bleeding score and in 52 patients with a low bleeding score. The flow cytometry-based test data were obtained from all patients.

Performance of the PACT (AUC 0.74, 0.66–0.82) was similar to performance of LTA (AUC 0.65, 0.57–0.74; $P = 0.14$) (Table 3). Combined, LTA and flow cytometry (AUC 0.80, 0.74–0.87) performed better than LTA alone ($P < 0.01$), but performance of the combination of LTA and flow cytometry was similar to the PACT alone ($P = 0.23$). When analysis was limited to patients with an elevated bleeding score, the performance of the PACT improved (AUC 0.82, 0.74–0.90) compared with LTA (AUC 0.70, 0.60–0.80; $P = 0.07$). The combination of both tests performed better than LTA alone (AUC 0.87, 0.80–0.94; $P < 0.01$). The better performance of the PACT than LTA could be due to the use of an extra agonist in the PACT compared with LTA. A comparison of the performance of single agonists in the PACT and LTA showed similar results in both patients with a high and those with a low bleeding score (Table 4).

Discussion

This study shows that a standardized flow cytometric approach can be used to identify patients with a PFD. Patients with a severe inherited platelet disorder showed decreased platelet reactivity compared with healthy

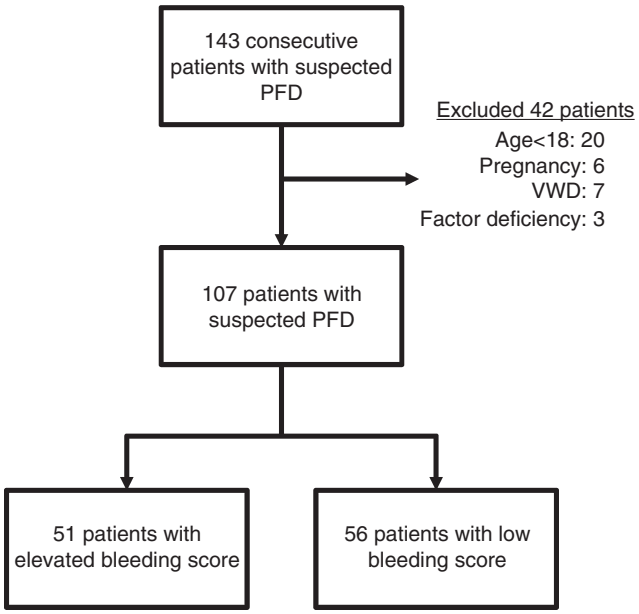

Fig. 2. Inclusion of patients with suspected platelet function disorders (PFDs) in cohort 2.

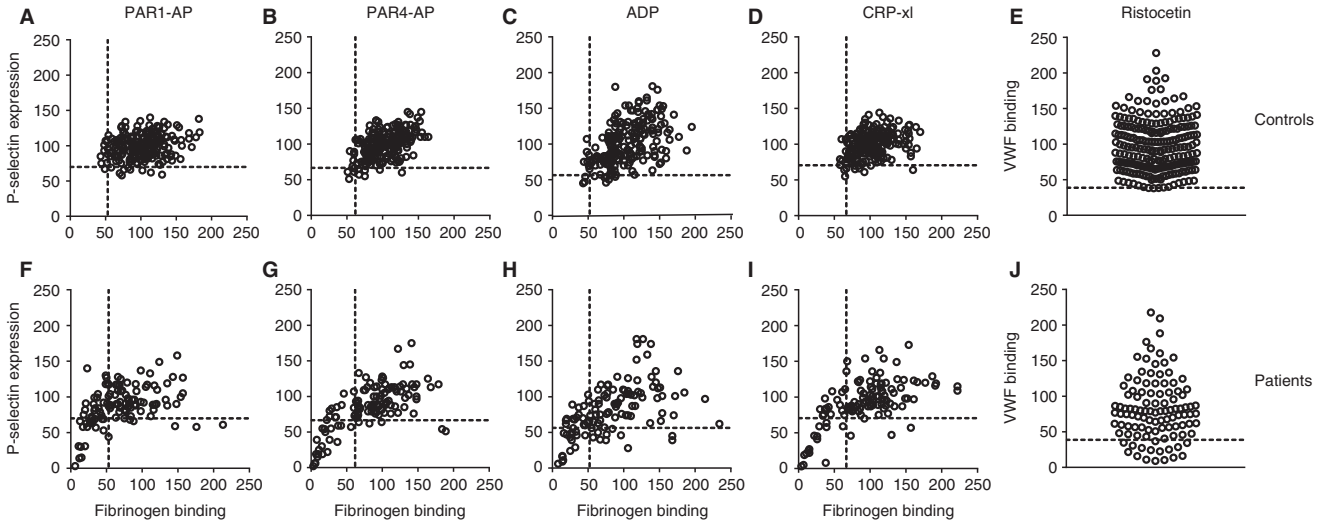

Fig. 3. Platelet reactivity after stimulation of different agonists in healthy controls (n = 202) and patients with suspected platelet function disorders (PFDs) (n = 107). Platelet reactivity was measured after stimulation with PAR1-AP (A&F), PAR4-AP (B&G), ADP (C&H), CRP-xl (D&I) and ristocetin-induced von Willebrand factor (VWF) binding (E&J). Every dot represents the P-selectin expression (y-axis) and fibrinogen binding (x-axis) of one individual per agonist. The dotted line per agonist is based on the 2.5th percentile of the healthy control population. Healthy controls are plotted in A–E and the patient population is plotted in F–J.

Table 2 Fair agreement between light transmission aggregometry (LTA) and the platelet activation test (PACT)

		Abnormal PACT		
		No	Yes	
Abnormal LTA	No	13	9	22
	Yes	6	20	26
	Missing	0	3	3
		19	32	51

controls and flow cytometry could be used to discriminate between patients with a suspected PFD and healthy controls in a real-life setting.

The ISTH/SSC guidelines recommend the use of flow cytometry in the diagnostic work-up of patients with PFDs [9]. Previous studies have shown that a flow cytometry-based platelet function test corresponds well with the bleeding severity in patients with immune thrombocytopenia [22] and that it is a promising screening tool for patients with mild bleeding disorders. An additional advantage of flow cytometry compared with conventional platelet diagnostics is the small sample volume. Whereas conventional diagnostics require a large blood volume, flow cytometry can be performed with as little as 100 μL of whole blood, thus enabling platelet diagnostics in infants and small children [29]. Nevertheless, the technique needs standardization and validation before it can be implemented in a diagnostic laboratory. It has been shown that flow cytometry allows the sub-classification of PFDs using CD63 expression and P-selectin expression in a patient population with previously categorized platelet abnormalities [16]. In our study, we demonstrated that flow cytometry has additional value to LTA in the diagnosis of patients with a suspected PFD by using fibrinogen binding instead of CD63 expression. As our population reflects the real-life patient population seen by the hematologist, our study shows that a standardized flow cytometry approach has diagnostic potential in patients with a suspected bleeding disorder.

Light transmission aggregometry is still considered the primary diagnostic tool during work-up of PFDs, despite known limitations such as large variability in response in individual donors and low sensitivity for mild PFDs. Our study indicates that there is fair agreement between LTA and platelet function measured with flow cytometry. The differences between LTA and flow cytometry in our study can be explained in part by the agonist panels that were used in both tests. LTA was not performed with PAR1-AP and PAR4-AP, whereas the flow cytometric test did not contain arachidonic acid-induced platelet activation, which is frequently impaired in patients with unidentified bleeding problems [30].

Interestingly, the diagnostic accuracy of flow cytometry for detection of patients with a suspected PFD was similar to that of LTA, with poor accuracy for LTA and moderate accuracy for flow cytometry. Accuracy was good when data obtained with LTA and flow cytometry were combined, suggesting both tests detect different patient populations and that a flow cytometric approach provides added diagnostic value. In patients with a high bleeding score, the diagnostic accuracy of the PACT appeared to improve more than LTA, but with only 51 patients with an increased bleeding score our study lacked sufficient power to attain statistical significance. However, in further support of the better performance of the

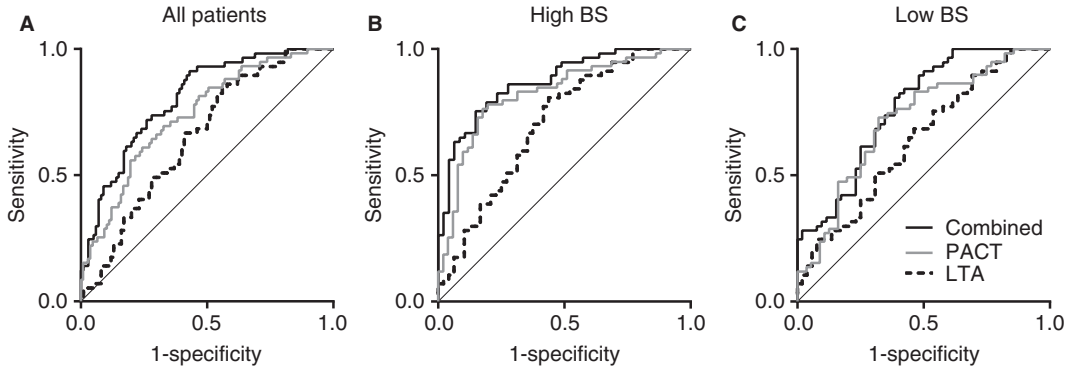

Fig. 4. The discriminative ability of light transmission aggregometry (LTA) and flow cytometry between healthy controls and patients with a suspected platelet function disorder (PFD). Probability scores for patients with a suspected PFD and 58 healthy controls were calculated with multiple logistic regression and plotted in a receiver operator curve (ROC) curve to determine the discriminative ability of flow cytometric platelet function testing and LTA. The discriminative ability of the platelet activation test (PACT) and LTA was determined in all 107 patients (A), in 51 patients with a high bleeding score (BS) (B) and in 56 patients with a low bleeding score (C). LTA data were unavailable in seven patients due to thrombocytopenia. LTA data were available in 48 patients with a high bleeding score and in 52 patients with a low bleeding score.

Table 3 Area under the curve of discriminative ability in patients with suspected platelet function disorder (PFD)

	LTA		PACT			Combined		
	Area	95% CI*	Area	95% CI	*P*-value†	Area	95% CI	*P*-value†
All	0.65	0.57–0.74	0.74	0.66–0.82	0.14	0.80	0.74–0.87	0.006
High BS	0.70	0.60–0.80	0.82	0.74–0.90	0.07	0.87	0.80–0.94	0.009
Low BS	0.64	0.53–0.74	0.72	0.62–0.81	0.27	0.77	0.68–0.86	0.06

LTA, light transmission aggregometry; PACT, platelet activation test; BS, bleeding score. *Indicates the 95% confidence interval. †*P*-value in comparison with LTA.

Table 4 Area under the curve of single variables in light transmission aggregometry (LTA) and the platelet activation test (PACT)

	LTA			PACT		
		Area	95% CI*		Area	95% CI
High BS	ADP	0.68	0.57–0.78	ADP	0.67	0.57–0.78
	Collagen	0.64	0.43–0.74	CRP-xl	0.58	0.47–0.69
	Ristocetin	0.62	0.53–0.75	Ristocetin	0.64	0.51–0.73
	AA	0.59	0.48–0.70	PAR1-AP	0.7	0.57–0.79
				PAR4-AP	0.62	0.51–0.73
Low BS	ADP	0.6	0.49–0.71	ADP	0.6	0.50–0.71
	Collagen	0.57	0.47–0.68	CRP-xl	0.57	0.47–0.68
	Ristocetin	0.56	0.44–0.67	Ristocetin	0.55	0.44–0.66
	AA	0.65	0.55–0.75	PAR1-AP	0.57	0.46–0.67
				PAR4-AP	0.57	0.46–0.67

AA, arachidonic acid; ADP, adenosine 5′-diphosphate; BS, bleeding score. *Indicates the 95% confidence interval.

PACT, the combination of the PACT and LTA performed similarly to the PACT alone. We cannot exclude that the better performance of the PACT is caused by the number of agonists that were analyzed in each test. Whereas the PACT investigated five agonists, LTA investigated only four. The equal performance of single agonists in both tests, combined with the added value of the PACT on top of LTA in our regression model, indicates that the PACT is non-inferior to LTA in the identification of patients with decreased platelet reactivity.

Bleeding symptoms are rare in patients with a platelet count above 50×10^9 L^{-1} but normal platelet function. Therefore, measuring platelet function in patients with thrombocytopenia is important [31]. Flow cytometry allows the measurement of platelet function in thrombocytopenia [32]. In our study, all seven patients with a platelet count lower than 75×10^9 L^{-1} had decreased platelet reactivity, whereas platelet function could not be assessed in this population with LTA [15].

Validation of new diagnostic tests for PFDs is difficult because of the absence of a reference standard test. The increasing knowledge of the genetic background in PFDs might help in the evaluation of new approaches for platelet function testing. However, prediction of bleeding severity based on genetics is difficult [33] and is not helpful in acquired PFDs. Ideally, diagnostic tests can be compared with future bleeding episodes with a follow-up study design, but the low frequency of bleeding episodes in mild PFDs and prophylactic treatment of moderate to severe bleeding disorders preclude the possibility of such a strategy [34]. For this reason, we validated the flow

cytometry-based test in a population of patients with a positive bleeding history in whom a PFD is suspected, which corresponds to the real-life patient population seen by the hematologist. A drawback of this population is that there is no demonstrable PFD in some patients with a clear bleeding diathesis, making it impossible to determine whether a patient is true- or false-negative. Furthermore, we did not perform repeated measurements to verify the number of false-positive measurements.

The strength of this validation study is that a standardized assay with reference values was used for comparison with LTA. The investigators were not blinded to case or control status in the determination of diagnostic accuracy, but data analysis was unbiased due to the use of a multiple logistic regression model.

With our study, we confirmed the utility of flow cytometric platelet function testing in severe PFDs and thrombocytopenia, but also showed that it provides added value to the routine diagnostic work-up in patients with a positive bleeding history and a suspected PFD. Therefore, flow cytometry-based platelet function testing should be considered as a promising tool in the diagnostic approach to PFDs and effort should be made to further validate and standardize flow cytometric tests for platelet function.

Addendum

R. E. G. Schutgens, M. Roest, G. Pasterkamp, and R. T. Urbanus designed the study. I. van Asten, M. Baaij, and J. Zandstra performed the experiments. I. van Asten, R. E. G. Schutgens, M. Baaij, J. Zandstra, A. Huisman, S. J. A. Korporaal, and R. T. Urbanus analyzed the data. I. van Asten, R. E. G. Schutgens, M. Baaij, J. Zandstra, M. Roest, G. Pasterkamp, A. Huisman, S. J. A. Korporaal, and R. T. Urbanus wrote the manuscript.

Disclosure of Conflict of Interests

S. J. A. Korporaal and R. T. Urbanus are stockholders in U-PACT BV, a spin-off company from UMC Utrecht. The other authors state that they have no conflict of interest.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1. Discriminative ability of dose–response curve (DRC) vs. single agonist concentrations PACT.
Table S2. Clinical characteristics of patients with a suspected PFD.
Table S3. Reference intervals for fibrinogen binding, P-selectin expression and VWF binding for PACT per platelet agonist.

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