Flow cytometry-based platelet function testing is predictive of symptom burden in a cohort of bleeders

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Abstract

Platelet function disorders (PFDs) are common in patients with mild bleeding disorders (MBDs), yet the significance of laboratory findings suggestive of a PFD remain unclear due to the lack of evidence for a clinical correlation between the test results and the patient phenotype. Herein, we present the results from a study evaluating the potential utility of platelet function testing using whole-blood flow cytometry in a cohort of 105 patients undergoing investigation for MBD. Subjects were evaluated with a test panel comprising two different activation markers (fibrinogen binding and P-selectin exposure) and four physiologically relevant platelet agonists (ADP, PAR1-AP, PAR4-AP, and CRP-XL). Abnormal test results were identified by comparison with reference ranges constructed from 24 healthy controls or with the fifth percentile of the entire patient cohort. We found that the abnormal test results are predictive of bleeding symptom severity, and that the greatest predictive strength was achieved using a subset of the panel, comparing measurements of fibrinogen binding after activation with all four agonists with the fifth percentile of the patient cohort (p = 0.00008, hazard ratio 8.7; 95% CI 2.5–40). Our results suggest that whole-blood flow cytometry-based platelet function testing could become a feasible alternative for the investigation of MBDs. We also show that platelet function testing using whole-blood flow cytometry could provide a clinically relevant quantitative assessment of platelet-related hemostasis.

Introduction

Mild bleeding disorders (MBDs), characterized by disproportionate bleeding after trauma or surgery, menorrhagia, excessive and frequent mucocutaneous hemorrhage, easy bruising and often a positive family history, are common, yet constitute a genuine diagnostic challenge for the clinician [1]. Annually, at least 14 000 patients undergo investigation for MBDs globally [2], the diagnostic workup typically including tests of von Willebrand disease, platelet function defects (PFDs), and clotting factor deficiencies. Pathologic test results are only found in a minority of patients with MBDs [3–6], whereas most patients are unsatisfactorily categorized as bleeders of unidentified cause (BUC).

Since its introduction some 50 years ago, light transmission aggregometry (LTA) has been considered the “gold standard” for diagnosing PFDs [7]. Using this method, approximately 20% of patients with MBD are ultimately diagnosed with a PFD [5,8]. Despite ambitious attempts to harmonize LTA protocols worldwide [9], the technique remains poorly standardized [2]. More importantly, platelet function testing using LTA has not been shown to predict the risk of bleeding complications among patients with different PFDs [10–12], and pathologic test results indicative of PFD are not associated with more severe bleeding problems when compared to patients diagnosed with BUC [4,13], limiting the test’s clinical value [7].

Whole-blood flow cytometry for platelet function testing (FC-PFT) was introduced in 1987 by Shattil and colleagues [14]. As described previously [15,16], this method confers several advantages in comparison to LTA for platelet function analysis, e.g. that platelets are analyzed without the need for separation of blood components, that much smaller sample volumes are needed, that the results are not influenced by platelet count, enabling a direct comparison of platelet function between subjects irrespective of the presence of thrombocytopenia, and that the method gives quantitative results which are easy to interpret. Perhaps most importantly, FC-PFT allows for simultaneous measurement of a broad range of different markers for platelet function, allowing for a more comprehensive analysis of the extensive hemostatic repertoire of platelets. Despite being routinely used in clinical practice for this purpose by us and others [2], no studies have addressed the clinical utility of FC-PFT as a primary diagnostic method for the detection of PFDs.

In this study, we report the results from a three-year period of clinical use of FC-PFT in the investigation of patients with suspected platelet function disorders. Our findings reveal significantly lower test results in patients experiencing bleeding problems when compared to healthy individuals using median fluorescence
intensity (MFI) as a measure. Additionally, abnormal test results were significantly more common among patients experiencing moderate to pronounced bleeding problems when compared to patients with trivial to mild bleeding problems ($p < 0.0005$).

### Methods

#### Patients and healthy controls

This retrospective study is based on journal records and test results for 105 patients referred to Linköping University Hospital from the County of Östergötland (with approximately 410 000 inhabitants) between September 2007 and September 2010 for evaluation of platelet function. During this period, all MDs could refer patients for FC-PFT and all referrals were accepted, provided that von Willebrand disease (VWD) had been excluded. The study was approved by the local Ethics Committee and was conducted in accordance with the Declaration of Helsinki on human subject research. The patient cohort was defined according to the following inclusion criteria: (A) a complete diagnostic workup at our local laboratory including a full blood cell count, Activated Partial Thrombin Time (APTT), Prothrombin Time (PT), Factor VIII (FVIII), and von Willebrand factor (antigen and ristocetin cofactor activity; < 0.50 kIU/L considered abnormal) without pathological findings; and (B) FC-PFT and patient interviews performed during the same visit to the specialized outpatient clinic performing the investigation. All referred patients fulfilled the above criteria, and were thus included in the study. Twenty-four healthy age and sex-matched volunteers were also included as a reference cohort. During the inclusion period, light transmission aggregometry was not available for use in any patients. All patients were instructed by mail not to take drugs known to affect platelet function for 10 days preceding the visit. At the time of the inclusion period, no bleeding assessment tool (BAT) designed specifically for the assessment of platelet dysfunction was available. Bleeding symptoms were, therefore, assessed and scored by an experienced clinician blinded to the laboratory results of the study, using an interview-based quantitative BAT developed for the diagnosis of type 1 VWD [17], which has been shown to have a high negative predictive value (NPV) and moderate positive predictive value (PPV) for the investigation of MBDS [6]. To allow for a more general assessment of symptom burden, i.e. the clinical severity of the experienced bleeding problems, BAT scores were complemented with a bleeding symptom assessment (BS) based on the professional judgment of the clinician, as has been recommended previously [1,18]. Using the BS, patient symptom burden was assessed according to a five-grade scale, with 0 indicating trivial bleeding symptoms, 1 indicating mild bleeding symptoms, 2 indicating moderate bleeding symptoms, 3 indicating pronounced bleeding symptoms, and 4 indicating severe bleeding symptoms (no patients in the study were found to have severe bleeding symptoms). Bleeding time (BT) was evaluated in most patients (96/105) using a Surgicutt® instrument (International Technidyne Corporation, Edison, NJ, USA) by trained and experienced staff at the central laboratory. A summary of characteristics for patients and healthy controls is provided in Table I.

#### Materials

Fluorescein isothiocyanate (FITC)-conjugated chicken antibodies directed toward human fibrinogen, P-selectin and insulin (used as a negative control) were purchased from Diapensia HB (Linköping, Sweden; http://www.diapensia.se). These antibodies have extensive scientific documentation regarding use in flow cytometry applications [19–21] and, in contrast to mammalian-derived antibodies, do not provoke artifactual Fc-receptor mediated platelet activation by immune complexes [22,23]. Antibodies were diluted in HEPES buffer (composed of 137 mM NaCl, 2.7 mM KCl, 1 mM MgCl2, 20 mM HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid), 1 g/l bovine serum albumin and 5.6 mM glucose, pH 7.4) and used in a dilution where strong stimulation could render all platelets positive with minimal unspecific fluorescence. The thrombin receptor agonist peptides PAR1-activating peptide (PAR1-AP) (amino acid sequence SFLLRN, also known as Trap-6) and PAR4-AP (AYPGKF) were synthesized by the Biotechnology Centre of Oslo, Oslo University (Oslo, Norway). Cross-linked collagen related peptide (CRP-XL) was synthesized at the Department of Biochemistry, University of Cambridge (Cambridge, UK). ADP, PAR1-AP, and PAR4-AP were diluted in 0.9% saline and CRP-XL was diluted in 0.05% acetic acid. Apyrase (grade VII, from potato), chemicals for the HEPES buffer, as well as all other reagents used, were obtained from Sigma-Aldrich (St. Louis, MO, USA).

**Sample preparation**

Before initiating the study, experiments were conducted to optimize protocol parameters such as agonist concentrations, handling of reagents, time of day for sampling etc. in the flow cytometry protocol (detailed in supplemental methods). Venous blood was collected via a 21-gauge needle (0.8x40 mm) without stasis into 2.7 ml plastic vacuum tubes (BD Diagnostics, Vacutainer, Plymouth UK), containing 0.3 ml of 0.129 M sodium citrate. The experimental protocol is illustrated in Figure 1. Samples were turned 10 times to enable mixing and left at room temperature for 1 h. 10 µl of blood was then added to pre-prepared plastic tubes containing 100 µl HEPES buffer and 10 µl diluted FITC-conjugated antibody and the mixture was incubated for 10 minutes. In experiments where apyrase was used to assess dense granule release, apyrase in a final concentration of 0.25 U/ml was added to the HEPES buffer before incubation. Then 10 µl of agonist solution was added, bringing the final concentrations of agonists to 15 µM for PAR1-AP, 10 µM for ADP, 60 or 400 µM for PAR4-AP (60 µM for measurements of P-selectin.

### Table I. Patient and control characteristics.

The bleeding symptoms were assessed by clinical assessment and a quantitative bleeding assessment tool (BAT) developed for the diagnosis of type 1 VWD. The bleeding time was performed in 96/105 patients.

| Age (years) | Patients ($n = 105$) | Healthy controls ($n = 24$) |
|------------|----------------------|---------------------------|
| Mean       | 35.4                 | 36.8                      |
| Min        | 1                    | 3                         |
| 1st Quartile | 25                  | 23.5                      |
| Median     | 32                   | 35                        |
| 3rd Quartile | 45                  | 47                        |
| Max        | 86                   | 61                        |

| Bleeding symptoms |
|-------------------|
| Trivial           | 17                  |
| Mild              | 46                  |
| Moderate          | 24                  |
| Pronounced        | 18                  |

| Bleeding time |
|---------------|
| Mean (sec)    | 460                 |
| > 1000 sec    | 8                   |

| BAT |
|-----|
| Median | 5        |
|       | 5        |
|       | na       |

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exposure and 400 µM for measurements of fibrinogen binding) and 0.85 µg/ml for CRP-XL. The reaction mixture was incubated for 10 minutes. Samples were then diluted in 1 ml of HEPES buffer and gently vortexed to quench further activation. After quenching, samples were subdivided into a set of flow cytometer tubes with an additional 1:3 dilution in HEPES to obtain optimal concentrations for subsequent analysis. Samples were left standing in the dark for 30 minutes before measurements were initiated.

Flow cytometry

Flow cytometry was performed on a Coulter Epics XL MCL flow cytometer with Expo 32 ADC software (Beckman Coulter, Miami, FL, USA). Platelets were identified by their specific forward and side scatter characteristics and not with a labeled antibody in order to avoid possible problems with over-lapping emission spectra. For every measurement, Hepes-EDTA (10 mM) or FITC-conjugated anti-insulin-Ab (matched with regard to both concentration and fluorophore per protein (F/P) ratio) were used as negative (background fluorescence) controls for measurements of fibrinogen binding and P-selectin exposure, respectively. The threshold for distinguishing negative and positive platelets was set to get 1–2% positive platelets in the control sample, as previously recommended [16,24]. Additionally, one tube was always incubated with buffer instead of agonist to identify pre-activated samples. Each sample was prepared in duplicate for subsequent analysis of coefficient of variance (CV). The mean value of duplicate measurements was used in subsequent analysis. Two alternative methods were used to provide a quantitative measure of the degree of binding to the fluorescent activation marker; the percentage of positive platelets (% pos) and median fluorescence intensity (MFI), which report the median signal from bound antibody for the total platelet population, and would be...
advantageous in situations where most platelets express the antigen, but where the level of expression could vary between individuals [15,16,25].

Data from experiments involving addition of apyrase were interpreted as follows. The relative signal reduction in the presence of apyrase was compared to a reference range constructed from healthy volunteers. A signal reduction less than the reference range in conjunction with a below average signal intensity without apyrase was interpreted as indicative of a defect in dense granule storage and/or release, in analogy with a previously published protocol [26]. As a control experiment, tests involving pre-incubation with apyrase were also performed using ADP as agonist to ensure complete signal reduction as proof of efficient ADP-depletion. Tests involving pre-incubation with apyrase were only performed using fibrinogen binding as activation marker.

Statistical analyses

All statistical calculations were performed using R software (R Foundation for Statistical Computing, Vienna, Austria; www.Rproject.org). The Anderson-Darling normality test was performed on samples (n = 19–26) obtained from healthy volunteers for each of the 16 tests included in the test panel using robust estimates, and outliers were removed according to Horn’s method for outlier detection, which has been shown to improve the estimation of reference intervals from a small number of observations [27]. When normality was indicated, reference intervals were calculated using the robust method, which has previously been shown to provide accurate estimates when performed on small samples drawn from populations with symmetric distributions [28]. If non-normality was implied (p < 0.05), Cox–Box transformation was applied to adjust for possible skewness. Receiver operator curves (ROC) where constructed using the R software packages pROC [29] for calculating confidence intervals for the area under the curve (AUC) using bootstrapping and ROCR [30] for graphic presentation. Proportions were compared using the two-sided Fisher’s exact t-test. Differences between samples consisting of continuous data were evaluated with the two-sided unpaired Mann–Whitney U-test. Pearson’s correlation coefficient was used to calculate correlations between continuous variables. Common odds ratios (OR) were calculated using Mantel–Haenszel Common Odds Ratio Estimate. Linear regression was performed using least squares fitting of data.

Results

Validation of the experimental protocol

Agonist concentrations for use in FC-PFT were selected after evaluating their respective dose-response curves when tested on blood from healthy volunteers (Figure S1). Due to high inter-individual variability among healthy volunteers when using the calculated EC50 (Tables S1 and S2), agonist concentrations were selected as the lowest one giving near maximal percentage of positive platelets for all tests except for when testing P-selectin exposure after activation with PAR4-AP. Experiments were also conducted to assess the impact of variations in pre-analytical handling, food intake, physical exercise and time of day for sampling on the flow cytometry results (Figures S2–S4). The apyrase assay used for indirect assessment of dense granule signaling defects was validated experimentally (Figure S5).

Patient characteristics and bleeding assessments

Twenty-two male (21%) and 83 female (79%) patients were included, with ages ranging from 1 to 86 years (Table I). The age distribution of males and females stratified according to bleeding symptoms was evenly matched between sexes (Figure S6A). Bleeding symptoms and BAT exhibited a strong correlation (p < 0.001, r = 0.79, Figure S6B). Due to the previously reported association between a prolonged bleeding time and defective primary hemostasis [31–33], bleeding time was used to evaluate the two methods employed for bleeding symptom assessment (Figure S6C). Linear regression revealed a significant association between bleeding time and bleeding symptom severity (p = 0.004), but not between bleeding time and BAT (p = 0.106, data not shown). There was a significantly higher frequency of abnormal BT (> 1000 sec) in the group with moderate to pronounced bleeding symptoms compared to those with trivial to mild bleeding symptoms (p = 0.027, common odds ratio (OR) 6.0; 95% CI 1.14–31.6, Figure S6C). This difference was not observed when comparing groups with a cut-off of 4 points using BAT (p = 0.21, data not shown).

Flow cytometry-based platelet function testing; significantly lower test results among bleeders

The coefficient of variation between duplicate samples for the flow cytometry test panel ranged between 2.3% (positive platelets for P-selectin with CRP-XL) and 20% (MFI for fibrinogen binding with ADP) (Figure S7). Overall, the coefficient of variation was higher using antibodies directed toward fibrinogen as activation marker and when using MFI as detection method. Correlation coefficients for the different analytical variables (i.e., fibrinogen binding versus P-selectin and MFI versus percent positive platelets) were generally higher when analyzing MFI for fibrinogen binding across the whole panel of agonists (0.693–0.830, Figure S9). Comparing the panel of agonists, correlations were higher across the different analytical modalities for CRP-XL. The observed wide range of test results among healthy controls is consistent with previous findings using both flow cytometry and LTA [8,34–36].

A group-wise comparison of the test results between patients and healthy controls revealed significant differences for all tests using MFI as analytical method, while only two out of eight tests differed significantly when measuring percent positive platelets (Figure 2). In general, the test results did not appear normally distributed, with a high frequency of apparently bimodal and asymmetric distributions. For most tests, the patient test interval included an asymptotic tail in the lower end of the range, consistent with the presence of pathologically unreactive platelets among a subset of patients (Figure 2). The mean test values and standard deviations for patients stratified according to bleeding symptoms and healthy controls are provided in Table S3.

When each test was analyzed as an independent continuous variable, linear regression revealed no significant association between patient test results and bleeding symptom severity (data not shown). The test results were then converted to binary variables (normal versus abnormal test results (ATR)) by two different methods: (i) by comparing each test result to the reference range constructed from data obtained with healthy controls, and (ii) by defining values below the fifth percentile of the results from the entire patient cohort as abnormal (Figure 3). Out of 272 tests performed on 17 patients with trivial bleeding problems, only three test results pertained to the fifth percentile of the entire patient cohort (Figure 3B), as compared to 29 out of 288 tests performed on 18 patients with pronounced bleeding problems (p < 0.0001 for difference between groups). Among patients with at least one ATR, the proportion of patients with more than one ATR was higher in patients with moderate or severe bleeding (horizontal bars in Figure 3A–B).

The bleeding symptom severity was then dichotomized by categorizing trivial to mild bleeding symptoms as low symptom burden and moderate to pronounced symptoms as high symptom
Figure 2. Distribution of test results among patients and healthy controls. Platelets were activated according to the protocol described in Figure 1. The distribution of test results among patients (black) and healthy controls (grey) are shown in a split bean plot, each graph showing the distribution of results for a specific combination of activation marker (P-selectin exposure or fibrinogen binding) and analytical modality (median fluorescence intensity (MFI) or percent positive platelets (% pos)) across the panel of agonists (ADP, CRP-XL, PAR1-AP, and PAR4-AP). Thin white horizontal lines represent individual values, the length of the lines being proportional to the number of observations. Black horizontal bars represent median values for each individual agonist. Dotted horizontal lines represent the median value for all measurements performed across the panel of agonists. Annotations: n.s. $p > .05$, * $p \leq .05$, ** $p \leq .01$, *** $p \leq .001$.

Figure 3. Occurrence of abnormal test results among individual patients stratified according to bleeding symptoms. Platelets from patient samples were activated with PAR1-AP, ADP, PAR4-AP or CRP-XL and analyzed for P-selectin exposure and fibrinogen binding according to Figure 1. Results were compared to the reference range calculated from healthy controls (A) or the fifth percentile of the entire patient cohort (B). Test results within the normal range are colored grey, test results below the defined range are colored red with a continuous color gradient using the deviation from the mean divided by the standard deviation as a measure of degree of abnormality. Grey horizontal bars illustrate the number of abnormal test results for each patient.
burden. Analyzing each test individually using method (i), only MFI for fibrinogen binding after activation with CRP-XL was significantly associated with high symptom burden ($p = 0.03$). Using method (ii), all patients with test results in the lowest fifth percentile had high symptom burden when measuring MFI for either fibrinogen binding or P-selectin exposure after activation with PAR1-AP and MFI for fibrinogen binding after activation with PAR4-AP ($p = 0.009$ for all three tests. Figure 3B).

Aggregate measures of platelet function based on FC-PFT can predict bleeding symptom severity

To construct an aggregate measure of results for the entire test panel, ROC curve analysis was performed to calculate the optimal cut-off for the number of ATRs used as predictor of bleeding symptom severity (Figure S8). A comparison of method (i) and (ii) for the identification of ATRs yielded highly significant models with similar area under the curve (AUC, Table II), with a tendency for higher specificity using the method (ii) and improved sensitivity using the method (i). Optimizing specificity and sensitivity with equal weights yielded an ideal cut-off of $> 2$ ATR for both methods. As shown in Table II and Figure 4, patients with $> 2$ ATRs were 4.8 or 7.8 times more likely to have a high symptom burden as compared to patients with $\leq 2$ ATRs, indicating that $> 2$ ATR is a significant predictor of a more severe bleeding diathesis. The predictive strength was superior when using the fifth percentile as predictor, yielding a positive predictive value of 80% and a specificity of 95%. When comparing the different detection methods (% positive platelets and MFI), the specificity and overall significance of the predictive model was superior for MFI (Table II).

Depending on which of the alternative data subsets and thresholds used (Table II), between 29% and 68% of patients with high symptom burden were categorized as having a clinically relevant platelet function disorder. When maintaining a specificity of $> 90\%$, this interval narrowed down to 29–38%, a figure in line with previous estimations obtained using LTA when excluding patients with vWD or clotting factor deficiencies [4,5,37]. There was a significant association between abnormal BT ($> 1000$ sec) and the occurrence of $> 2$ ATR ($p = 0.027$, OR 5.9, 95% CI 1.22–31.1).

The results from experiments with apyrase identified 9 patients with tests results indicative of defective dense granule storage and/or release. Although the test results by themselves were not associated with more pronounced bleeding problems in this patient cohort, inclusion of the results in the aggregate model comprising the entire test panel compared with the reference range resulted in an improvement in the overall significance and predictive strength of the test ($p = 0.0003$, OR 5.4; 95% CI 2.0–15.6).

**Table II.** Comparison of the predictive strength of different discriminatory thresholds for predicting bleeding symptom severity using FC-PFT. The statistical measures area under the curve (AUC), $p$-value, Odds Ratio, positive predictive value (PPV), negative predictive value (NPV), specificity (Spec), and sensitivity (Sens) were calculated from a contingency table showing the frequency distributions of low versus high symptom burden among bleeding patients dichotomized by comparing the number of abnormal test results as defined by the predictor variable with the cut-off established using ROC curve analysis.

| Predictor                  | AUC        | Cut-off | $p$-value | Odds Ratio | PPV | NPV | Spec | Sens |
|----------------------------|------------|---------|-----------|------------|-----|-----|------|------|
| Below reference range      | 0.68; 95% CI 0.58–0.79 | $> 2$   | 0.0005    | 4.9; 95% CI 1.8–14.2 | 68% | 70% | 86% | 45% |
| Ref. range – MFI           | 0.68; 95% CI 0.59–0.78 | $> 0$   | 0.003     | 3.7; 95% CI 1.5–9.3  | 56% | 65% | 65% | 67% |
| Ref. range – % pos         | 0.60; 95% CI 0.30–0.70 | $> 0$   | 0.07      | 2.1; 95% CI 0.9–5.2  | 49% | 67% | 68% | 50% |
| Fifth percentile           | 0.68; 95% CI 0.59–0.77 | $> 2$   | 0.001     | 7.8; 95% CI 1.9–46  | 80% | 67% | 95% | 29% |
| Fifth percentile – MFI     | 0.66; 95% CI 0.58–0.74 | $> 0$   | 0.00008   | 8.7; 95% CI 2.5–40   | 80% | 69% | 94% | 38% |
| Fifth percentile – % pos   | 0.61; 95% CI 0.52–0.69 | $> 0$   | 0.007     | 3.9; 95% CI 1.3–12.9 | 67% | 67% | 89% | 33% |

**Discussion**

Despite major theoretical advances in our understanding of how platelets contribute to hemostasis, the last decades have provided the clinician with remarkably few new tests improving our understanding of how platelet dysfunction cause symptoms in real-life patients with bleeding problems. Translating laboratory results from platelet function testing into clinically useful information requires a clear clinical correlate to pathological findings, something currently lacking except from the rare cases of well-defined platelet disorders such as Glanzmann thrombastenia and Bernard–Soulier syndrome [1,4]. The establishment of tests that provide such a link between the laboratory and the clinic could help to bring day-to-day decisions such as whether to investigate family members, whether to give bleeding prophylaxis in the event of frequent bleeding or surgery and assessing risks when deciding on the use of thromboprophylaxis after thrombosis and surgery, into the realms of evidence-based medicine. Today, the so-called “global” tests of primary hemostasis are often used as primary screening tools before the use of more specific tests such as LTA, but this approach requires intricate investigation algorithms with sequential testing and does not serve to establish a clear link between a detected defect and the phenotype of the patient [38].

Under the current guidelines, the use of flow cytometry is already recommended as an integral component of the investigation of MBDs [2,39], and recently, measurements of fibrinogen binding upon stimulation with agonists utilizing FITC-conjugated chicken antibodies have been studied in Glanzmann’s thrombasthenia and other well defined platelet disorders [35]. Additionally, the utility of flow cytometry as a primary screening tool in the investigation of MBD has been evaluated, demonstrating a high degree of correlation with findings using LTA [40]. In this study, we wanted to know whether flow cytometry could be used to identify subjects with a clinically relevant platelet function defect among patients referred for investigation of a suspected MBD. We demonstrate that abnormal test results using FC-PFT are clearly correlated with a more pronounced bleeding diathesis in this setting. Interestingly, a recent study investigating the use of FC-PFT in pediatric patients with immune thrombocytopenia showed similar results, with a clear association between platelet reactivity as measured by FC-PFT and bleeding symptoms [41], illustrating the potential broad applicability of FC-PFT for assessing bleeding risk in a wide range of different disease states.

When using the entire test panel of 16 different tests, we found that more than two abnormal test results are needed to give a strong association between test results and symptom burden for the individual patient, possibly due to the redundancy of pathways eliciting platelet activation [42]. Even though this finding was highly significant, the relatively small patient sample is reflected in large uncertainties regarding the effect size when using our test panel as a predictor of symptoms. Nevertheless, our findings strongly suggest that the
presence of more than two abnormal test results in a patient would indicate the presence of a clinically relevant platelet function disorder which would be expected to cause moderate to pronounced bleeding problems. Measuring fibrinogen binding after activation with a panel of physiologically relevant agonists was found to be of superior predictive value as compared to P-selectin exposure. We also conclude that MFI is a more informative measure than percent positive platelets in this material. Further, we demonstrate that it is necessary to account for the non-normal distribution of analytical variables when constructing reference intervals for the different tests. Of note, we used a different and smaller set of platelet agonists than those conventionally used for the investigation of PFDs with LTA, choosing to focus on the major platelet activating receptors. This approach could arguably reduce the sensitivity of the protocol for subtle defects related to adrenoceptor signaling.

When evaluating methods for identifying platelet function defects among patients with MBD, it is important to bear in mind that MBD is a poorly defined disease entity comprising a heterogeneous group of disorders with different etiologies that, apart from platelet function defects, also probably include structural and anatomical defects in connective and mucosal tissue and possibly also hitherto undefined defects in the coagulation system. Thus, even an “ideal” platelet function test would most probably only have limited sensitivity when evaluated for its capacity to identify patients with more serious hemostatic defects among patients with MBD. On the other hand, an optimal platelet function test would have high specificity, since an identified platelet function defect would invariably cause bleeding problems of a certain magnitude. One strength of our study in this regard is that our sample consisted of an unselected patient cohort defined by a clinical suspicion of a PFD. Thus, our sample is likely to reflect the patient composition at a specialist unit in real-life.

Being a pilot study, the present study has a number of weaknesses including the low number of healthy donors and that it did not include a direct comparison with LTA. However, the promising results in this and other studies illustrate the potential of PC-PFT as a method to identify clinically relevant platelet function defects among patients with MBD and warrant further validating studies in this setting.

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Conflict of Interest Disclosures
Tomas Lindahl is the owner of Diapensia HB, the manufacturer of the chicken antibodies used in the study. There are other, competing companies selling similar FITC-conjugated chicken antibodies. The other authors state that they have no actual or potential conflicting interests.

Supplemental data
Supplemental data for this article can be accessed www.tandfonline.com/iplt

Notes on contributor
N.B. performed the research, analyzed and interpreted the data and wrote the manuscript. L.F. analyzed and interpreted the data and provided critical revisions of the manuscript. S.R. and T.L.L. conceived the study, performed the research and provided critical revisions of the manuscript.

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