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Full Length Article

Flow cytometric analysis of platelet function to improve the recognition of thrombocytopathy

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ABSTRACT

Introduction: Light transmission aggregometry (LTA) is the gold standard for diagnosing bleeding disorders. Although LTA is laborious, requires large volumes of blood and is relatively insensitive to small changes in platelet function, there is still no competing alternative approach to replace LTA for the diagnosis of platelet bleeding disorders.

Materials and methods: This study investigates the correlation between flow cytometry-based whole blood platelet activation test (WB-PACT) and LTA and whether WB-PACT is of additional value for the identification of bleeding disorders. In total, 161 patients with suspected bleeding diathesis were tested.

Results: A correlation of 0.41 between LTA and WB-PACT was found, and there was agreement between tests in 62% of cases ($\kappa = 0.23$). The WB-PACT is of additional value to LTA to detect platelet function disorders (PFD) as 10 patients with elevated bleeding score (BS) were detected with WB-PACT, 4 with LTA and 7 patients were positive with both tests. Interestingly, in contrast to LTA, WB-PACT has an additional option to detect VWF disfunctions.

Conclusion: WB-PACT may have added value for the routine diagnostic work-up in patients who need to have platelet function tested.

1. Introduction

The assessment of platelet (dys)function is required for the identification of mild bleeding disorders (MBDs) characterized by disproportional bleeding after trauma or surgery, menorrhagia, excessive and frequent mucocutaneous hemorrhage and easy bruising [1,2].

Currently, light transmission aggregometry (LTA) is the 'gold standard' platelet function test to define suspected bleeding disorders. Although LTA is used for more than four decades in diagnostic laboratories, it still lacks sensitivity for mild platelet function defects (PFDs) and it does not predict the risk of bleeding complications among patients with various platelet function defects [3–8]. It has been shown that only 60% of patients with excessive clinical bleeding and a suspected underlying inherited PFD, had a demonstrable abnormality in platelet aggregation [9], while in patients with mild bleeding, approximately 40% had a defect when assessed by LTA and serotonin and ATP release measurements [9,10]. Disadvantages of LTA are the poor standardization despite existing guidelines, the requirement of a considerable volume of a fresh blood sample and the fact that it is time and labor intensive and not applicable for samples with low platelet count [11–16]. Accordingly, alternative methods to measure platelet aggregation have been developed but despite potential advantages, none of these techniques competed with LTA with regard to bleeding diagnostics [16]. There is an urgent need for alternative methods to give complimentary information about mild platelet function defects.

The whole blood platelet activation test (WB-PACT), introduced in 1987 by Shattil et al. [17], is a flow cytometric approach that allows stimulation of platelets with multiple agonists, while platelet activation can be quantified with different types of activation markers of granule release, glycoprotein activation and phospholipid expression. Since many laboratories do not offer facilities for both aggregometry and flow cytometry, data regarding the agreement between both approaches are

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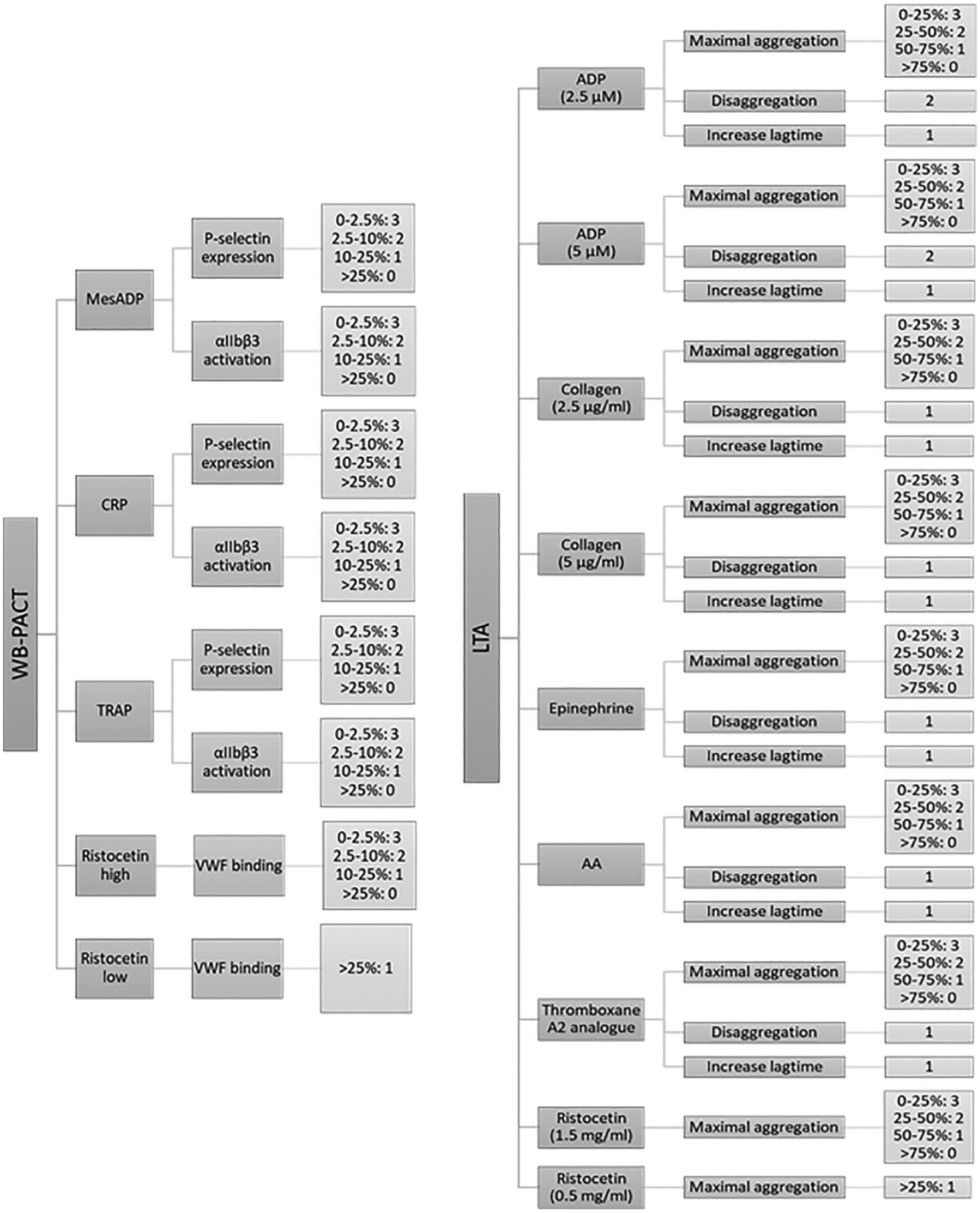

Fig. 1. The WB-PACT was scored by first determining the 2.5th, 10th and 25th percentiles for WB-PACT parameters in 56 healthy donors. In patients, if MFI was lower than the 2.5th percentile (0–2.5%), between the 2.5th and 10th percentiles (2.5–10%), between the 10th and 25th percentiles (10–25%) or higher than the 25th percentile (> 25%) a score of 3, 2, 1 or 0 was given, respectively (panel A). For LTA, maximal aggregation, disaggregation and prolongation of the lagtime were scored. In patients, if maximum aggregation was between 0% and 25%, between 25% and 50%, between 50% and 75% or more than 75% as score of 3, 2, 1, 0 was given, respectively. If disaggregation was present, a score of 2 or 1 was given depending on the agonist and if an increase in lagtime was measured a score of 1 was given (panel B). Patients with at least one parameter lower than the 10th percentile measured with WB-PACT and at least one value deviating from the normal range measured with LTA were categorized as abnormal for PFD.

Table 1
Baseline characteristics.

	All	Men	Women	Children
1. Patients with bleeding diathesis				
N	161	31	100	30
Age, years; median (IQR)	32 (20–51)	54 (27–61)	35.5 (24–50)	9 (4–13)
VWF:Ag (%); median (IQR)	93 (68–122)			
VWF:Rco; median (IQR)	87 (64–120)			
BS				
N of BS reported	117	22	72	23
median BS	3	2	3	2
% of elevated BS	25	32	18	39

N, number of patients; IQR, interquartile ranges; BS, bleeding score; N of BS reported, number of patients interviewed to determine a bleeding score.

scarce and these data would be of great value to correctly interpret the results achieved with either of these two approaches. For the diagnosis of MBDs, recently, Boknäs et al. [18] suggested that flow cytometric analysis of platelet function could become a feasible alternative for LTA, while van Asten et al. [19] concluded that this approach has added value to LTA.

In this study, we compared LTA and WB-PACT for the detection of PFDs in patients with bleeding diathesis.

2. Materials and methods

2.1. Study population

Platelet function by LTA and WB-PACT was measured in parallel in 161 patients investigated for bleeding diathesis and in 56 healthy controls. We expected a 20% lower expression in patients with bleeding diathesis. Based on an alpha of 0.05 and a 1-beta of 0.8, we calculated that 46 subjects per group are sufficient to find significant differences between patients and controls. We used the following website for the power calculations: https://www.stat.ubc.ca/~rollin/stats/ssize/n2.html. For the patients investigated for bleeding diathesis, a bleeding score was calculated using the ISTH-BAT (elevated BS: > 5 in women, > 3 in men and > 2 in children) [27]. Our study protocol was approved by the ethical committee of the Ghent University Hospital. Citrated whole blood (BD Vacutainer, Becton Dickinson, Plymouth, UK) was collected and processed according to the recommendations for the standardization of LTA [16].

2.2. Reagents

Platelet agonists used for flow cytometric analysis of platelet function include the $P2Y_{12}$ agonists 2MeS-ADP (1624, Tocris), the protease activated receptor (PAR)-1 agonist thrombin receptor activator peptide (TRAP-6 (SFLLRN), H-2936; Bachem, Germany), the glycoprotein VI (GPVI) agonist collagen-related peptide (CRP, purchased from Professor Farndale, University of Cambridge, UK) [28]. Ristocetin was purchased from ABP Ltd. (Germany). The monoclonal antibodies used were FITC-conjugated PAC1, directed against the activated αIIbβ3 receptor, PE-conjugated anti-P-selectin (CD62P, clone AK4), APC-conjugated anti-GPIb (CD42b, clone HIP1) and APC-conjugated anti-αIIbβ3 (CD41a), all purchased from BD Pharmingen (NJ, USA) and FITC-conjugated anti-VWF (CL7616F, Cedarlane, Burlington, USA).

Platelet agonists, used for LTA were ADP, epinephrine, arachidonic acid, ristocetin (Avant- Medical, The Netherlands), U46619 (Sigma Aldrich, Belgium) and collagen (Takeda Pharma, Austria).

2.3. Whole blood platelet activation test (WB-PACT)

Platelet activation tests for flow cytometric analysis were prepared as published earlier [29,30]. In short, test strips consisting of no agonist, 30 μmol/l TRAP, 5 μg/ml CRP and 2 μmol/l MeSADP were prepared in advance and stored at −20 °C. Each reaction mixture with a total volume of 20 μl consists of 2 μl FITC-conjugated PAC-1, 1.5 μl PE-conjugated anti-P-selectin and 0.5 μl APC-conjugated anti-CD42b with or without agonist in HEPES-buffered saline (HBS, 10 mmol/l HEPES, 150 mmol/l NaCl, 1 mmol/l MgSO4, 5 mmol/l KCL, pH 7.4). In addition, test strips were prepared with no agonist, 1.2 mg/ml or 0.2 mg/ml ristocetin in a reaction mixture of 2 μl FITC-conjugated anti-VWF and 0.5 μl APC-conjugated anti-CD41a in HEPES buffered saline, and stored at −20 °C.

Whole blood was kept at RT for at least 30 min and maximum 4 h. Test strips were thawed to 37 °C and shortly centrifuged. Subsequently, whole blood was incubated at 37 °C for 10 min and diluted 1:4 in pre-heated HEPES-buffered saline to minimize the formation of platelet aggregates. From this diluted blood, 5 μl was added to each reaction mixture (20 μl, final dilution 1:20) and the tests were incubated for exactly 20 min at 37 °C. Reactions were stopped by adding 250 μl fixation solution (137 mmol/l NaCl, 2.7 mmol/l KCl, 1.12 mmol/l NaH_2PO_4, 1.15 mmol/l KH_2PO_4, 10.2 mmol/l Na_2HPO_4, 4 mmol/l EDTA, 0.5% formaldehyde).

A FACS Canto™ II (BD Biosciences, Erembodegem, Belgium) was used to analyze the samples (Supplemental Fig. 1). First, using the forward and sideward scatter pattern and gating on the CD42b or CD41a positive cells, platelets were discriminated from other cells. Fluorescent intensity in the FITC gate and PE gate was selected to determine activated αIIbβ3 or VWF binding and P-selectin density, respectively and results are expressed as median fluorescent intensity (MFI) minus the MFI of the unstimulated cells. Single measurements were performed as intra-assay precision for this assay is below 5% with the exception of P-selectin expression in response to MesADP (below 10%) [29].

2.3.1. Light transmission aggregometry

Light transmission aggregometry (LTA) was performed on a chrono-log model 700 (Chrono-log corporation, Havertown, US) according to the manufacturer's instructions and to the North American Consensus Guideline on platelet function testing [15]. Platelet-rich citrate plasma was obtained after centrifugation (10 min, 180g/min) (Hettich Universal 32; DJB Labcare, Buckinghamshire, UK). LTA curves were assessed after addition of 25 μl of the agonists ADP (2.5 and 5 μmol/l), collagen (2.5 and 5 μg/ml), ristocetin (0.5 and 1.5 mg/ml), epinephrine (10 μmol/l), arachidonic acid (AA) (1 mmol/l), and thromboxane A2 analogue (1 μmol/l) to 225 μl platelet rich citrate plasma. Light transmission was recorded for 6 min and compared to platelet poor plasma (PPP). Aggregation was expressed as % maximum aggregation compared to PPP, lagtime was expressed in seconds. Disaggregation was defined as the difference between maximal and final platelet aggregation after 6 min expressed as percentage and > 20% of disaggregation was considered significant. For LTA imprecision is below 8% for all parameters, ristocetin low concentration not considered because of the low maximal aggregation as expected in normal individuals. In routine setting, single measurements are performed, and abnormal results are repeated as confirmation for decreased maximal aggregation or disaggregation.

2.4. VWF antigen and VWF activity

The HemosIL AcuStar VWF:Ag assay (VWF:Ag Acustar) and HemosIL AcuStar VWF:RCo assay (Instrumentation Laboratory) was performed on an ACL AcuStar™ (Instrumentation Laboratory) using magnetic particles coated with anti-VWF polyclonal antibodies (antigen detection) or Recombinant GPIbα (rGPIbα) (activity measurement) [31]. The assay was executed according to the instructions of the manufacturer.

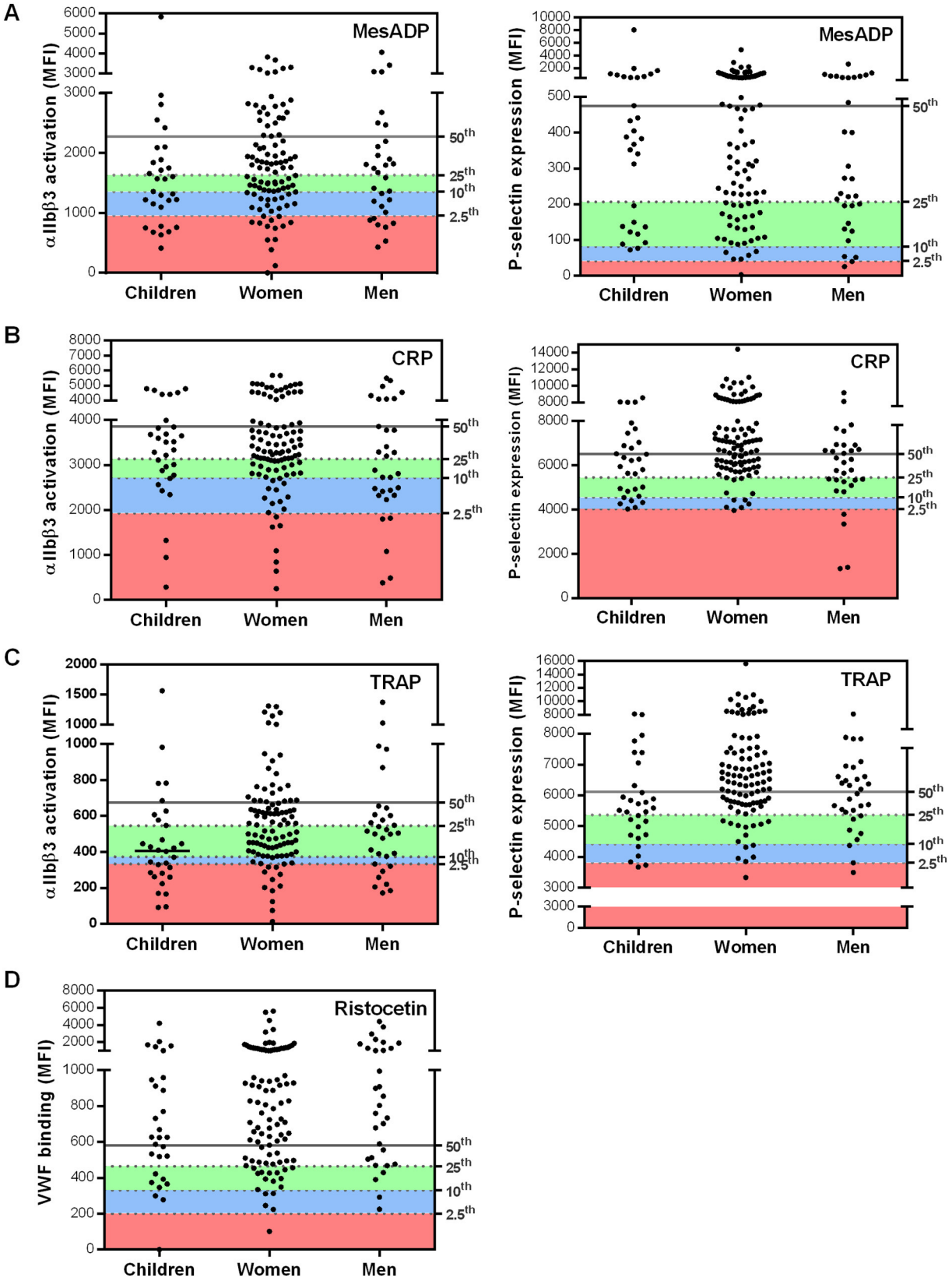

Fig. 2. Both αIIbβ3 receptor activation (left panels) and P-selectin expression (right panels) in response to MeSADP (A), CRP (B) and TRAP (C) was determined in blood of patients with bleeding diathesis. In addition, VWF binding was determined in response to 1.5 mg/ml ristocetin (D) in the same population. The 50th (median), 25th, 10th and 2.5th percentiles, of the healthy population are indicated. If patients are between the 25th and 10th percentile (green), they received a score of one point for each agonist. If patients are between the 10th and 2.5th percentile (blue), they received a score of two points for each agonist. If patients are below the 2.5th percentile (red), they received a score of three points for each agonist.

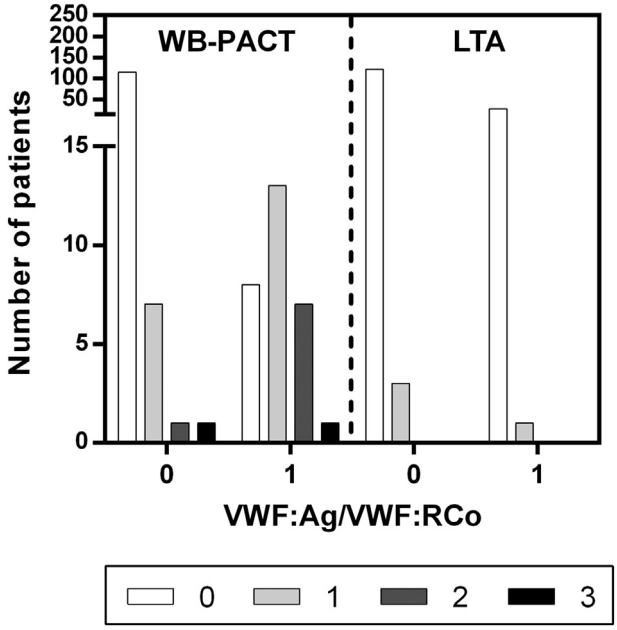

Fig. 3. The scoring of the conventional methods to investigate VWF concentration and function was based on low VWF:Ag and/or low VWF:RCo (score 1) and normal VWF:Ag and VWF:RCo (score 0). The binding of VWF to platelets (WB-PACT) and platelet agglutination (LTA) in response to low and high concentrations of ristocetin is scored according to Fig. 1.

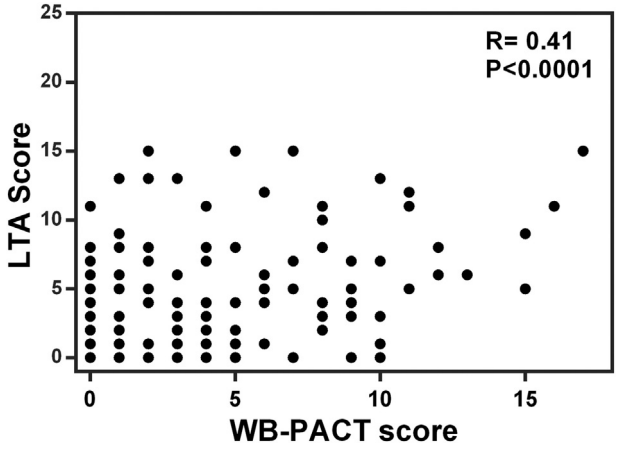

Fig. 4. Correlation of WB-PACT with LTA for determining platelet function in patients suspected for bleeding diathesis. The Spearman correlation coefficient and *P* value are depicted.

2.5. Scoring system platelet function tests

The WB-PACT by flow cytometry quantifies αIIbβ3 activation and P-selectin expression in response to MeSADP, TRAP and CRP. Similarly, this test can be used to quantify VWF binding to platelets in response to ristocetin. The 2.5th, 10th and 25th percentiles for WB-PACT parameters were determined in 56 healthy donors to score the patients samples and the scoring system is shown in Fig. 1A. For LTA, maximal aggregation, disaggregation and prolongation of the lagtime in response to ADP, collagen, epinephrine, AA, a thromboxane A2 analogue and ristocetin was scored (Fig. 1B).

To study the agreement between both tests in patients investigated for bleeding diathesis, patients with at least one parameter lower than the 10th percentile measured with WB-PACT or at least one parameter deviating from the normal range measured with LTA (< 50% maximal aggregation, prolonged lagtime and/or disaggregation > 20%) were categorized as abnormal for PFD.

2.6. Statistics

Analyses were performed using the software packages SPSS version 23.0 and GraphPad Prism 6. Spearman rank correlations were used to test for correlations between the different methods. Agreement between the methods was assessed using two-by-two analysis and Cohen's kappa statistic. *P*-values < 0.05 were considered statistically significant.

3. Results

Patient characteristics, bleeding assessment and platelet function.

In total, 161 patients with a bleeding diathesis (median age 32 years) were included in the study (Table 1). A bleeding score was reported for 117 of 161 patients and an elevated BS was reported for 32%, 18% and 39% of men, women and children, respectively.

Platelet function was measured in response to MeSADP, CRP, TRAP and ristocetin and the extent of αIIbβ3 activation, P-selectin expression and VWF binding to platelets is shown in Fig. 2. In addition, platelet aggregation with LTA was measured according to general clinical practice [15]. For each agonist, a score was calculated for both WB-PACT and LTA (Fig. 1). For WB-PACT the median score (minimum-maximum) in patients with expected bleeding diathesis was 1 (0–6), 0 (0–6), 1 (0–6) and 0 (0–3) for MeSADP, CRP, TRAP and ristocetin, respectively. For LTA the median score (minimum-maximum) was 1 (0 − 10), 0 (0–5), 0 (0–4), 0 (0–5), 0 (0–4) and 0 (0–1) in response to ADP, collagen, epinephrine, TXA2 analogue, arachidonic acid and ristocetin, respectively. The distribution of the scores in the patients with bleeding diathesis is shown in Supplemental Fig. 2. The inter-individual variation was between 23% and 53% when platelets were activated with TRAP or CRP and 52% and 140% when stimulated with MesADP for P-selectin expression and αIIbβ3 activation, respectively.

More in detail, the binding of VWF to platelets detected by flow cytometry was more sensitive for diagnosing deviations in VWF concentration or function than ristocetin induced platelet agglutination measured with LTA (Fig. 3). In total, 29 patients were detected with abnormal VWF:Ag and/or VWF:RCo, and interestingly, 21/29 patients had an increased score for ristocetin (high concentration) induced binding of VWF to platelets (WB-PACT). More in detail, 8/29 patients were abnormal for VWF-binding to platelets (score > 1 i.e. < 10th percentile). In contrast, for LTA, only 1 out of 29 patients had a score different from zero (score 1 i.e. < 75% agglutination) for ristocetin induced agglutination of platelets.

3.1. Correlation and agreement between LTA and WB-PACT

Furthermore, a Spearman correlation coefficient of 0.41 between LTA platelet activation score versus WB-PACT platelet activation score was observed (Fig. 4). Furthermore, for 99 of 161 patients (62%) there was agreement between the tests (κ = 0.23, *P* = .003) with 46 patients (29%) diagnosed with PFDs and 53 patients (33%) without PFD. In total, 25 (15%) and 37 (23%) patients were diagnosed with PFD according to LTA and WB-PACT, respectively.

In the group that was diagnosed with PFDs by both LTA and WB-PACT, 25% of the patients had an elevated bleeding score (Fig. 5A). For patients with PFDs according to WB-PACT only and LTA only, an elevated BS was recorded for 33% and 21% of the patients, respectively (Fig. 5B and C). In total, 8 (6 women and 2 men) out of 29 patients (28%) with an elevated BS were not diagnosed with PACT nor LTA (Supplemental Fig. 3). In the group of children with elevated BS, 7 out of 9 children were detected with WB-PACT (78%), while only 4 out of 9 children were detected with LTA (44%). However, when combining both tests, all children with elevated BS were detected. Remarkably, in the group patients with a normal BS, 24% were diagnosed with PFD with both tests, 17% with LTA, 23% with WB-PACT, and 36% of

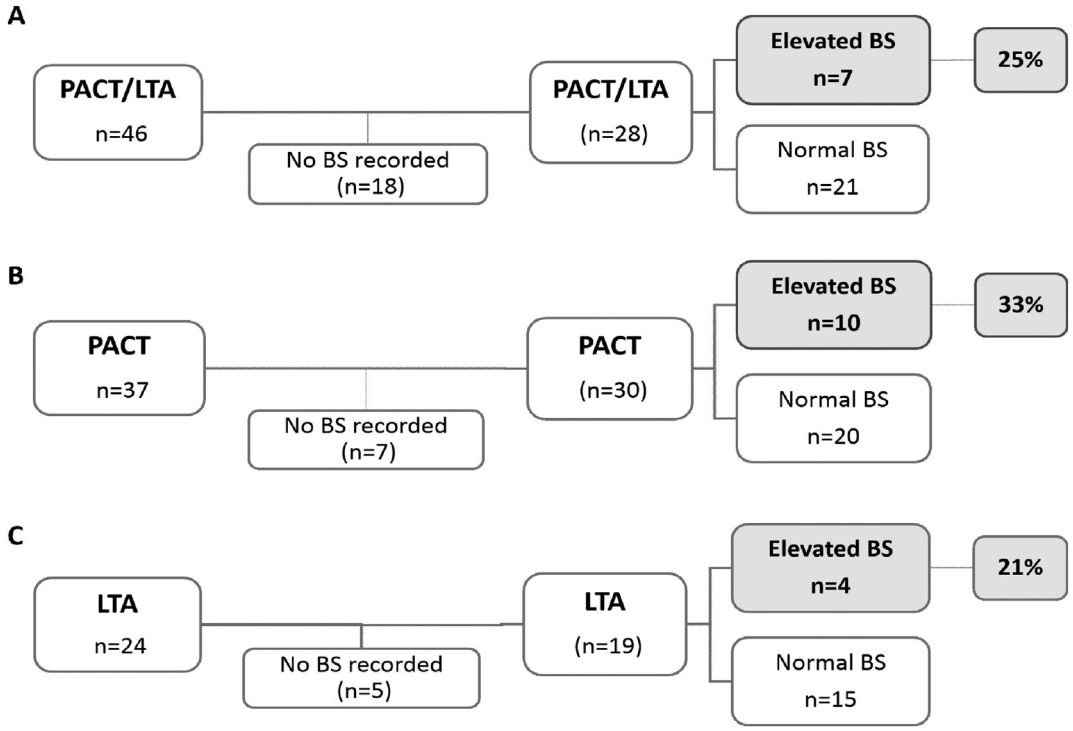

Fig. 5. Agreement of bleeding score (BS) with PFD according to both LTA and WB-PACT (panel A), to only WB-PACT (panel B) and to only LTA (panel C).

patients were found negative for PFDs (Supplemental Fig. 3B).

4. Discussion

This study investigates the (additional) value of flow cytometric analysis of platelet function in patients with suspected thrombocytopathy.

For the WB-PACT, the inter-individual variation in patients with suspected thrombocytopathy was high with %CV up to 55% (apart from P-selectin expression in response to MeSADP). This is higher compared to a healthy population ($N = 129$) where the inter-individual variation was around 30% (apart from P-selectin expression in response to ADP) [29]. The higher variation can be explained by the presence of platelet defects in some patients while others have a normal platelet response.

As expected, we observed a weak correlation between the total scores of all parameters/agonists of LTA and WB-PACT in the study population. It was in line with our expectations that there was no complete correlation, because there are major differences between LTA (a semi-quantitative method) and whole blood PACT (a quantitative analysis) [33]. Our findings and findings of others [19] show that LTA and WB-PACT are complimentary tests, which give supplemental insights in the bleeding risk, indicating that the tests improve each other's prediction.

Our data seem to demonstrate that LTA is insensitive to detect deviations in VWF concentration or function by measuring platelet agglutination induced by high concentrations of ristocetin as none of the patients had an abnormal response (score > 1). Indeed, LTA is mostly used to detect gain-of-function defects VWD (type2B, platelet type) by low ristocetin induced agglutination [8]. Interestingly, WB-PACT was able to detect a reduced binding of VWF in response to ristocetin in a population of patients with suspected thrombocytopathy, and data corresponded well with decreased VWF:Ag and/or VWF:RCo. Other differences between the LTA and the WB-PACT include different agonists that were used. Unlike LTA, the WB-PACT does not contain a thromboxane analogue, AA or epinephrine, however, it does include a PAR-1 activating peptide. In the past we validated U46619 and epinephrine with WB-PACT, however both agonists gave too much variation between measurements. The triple helical cross-linked CRP is a powerful GPVI agonist, however, CRP is sensitive to GPVI density on the platelet surface. Furthermore, despite an essential role of GPVI in the formation of a stable platelet thrombus, no overt bleeding phenotype was observed either in GPVI deficient mice or in patients with a congenital GPVI deficiency [34,35]. Although GPVI plays a crucial role in collagen-dependent thrombus formation, it shows a low affinity for collagen and the adhesion of platelets on collagen is considerably enhanced by interaction with integrin α2β1 [36,37]. Together, in response to collagen, α2β1 and GPVI synergistically stimulate Ca^{2+} signaling, and platelet aggregation [36,38]. Furthermore, a limitation of this study is the determination of the cut-off values for WB-PACT. The reference population only consisted of 56 healthy individuals instead of the 120 recommended by the CSLI guidelines and the 10th percentile was used as the cut-off value. Also, the score model was not previously validated in a group of severe defined PFD. However, in a previous study, we showed that the PACT assay discriminates Bernard-Soulier from non-Bernard-Soulier by the absence of binding of the anti-GPIb antibody, while Glanzmann thrombasthenia is discriminated from non-Glanzmann thrombasthenia by the absence of αIIbβ3 antibody binding to platelets [19].

In patients presenting with suspicion of bleeding diathesis, we show a weak agreement between the two tests (concordant in 62% of the cases, $\kappa = 0.23$). In other studies, this agreement was higher (concordant in 65% of the cases, $\kappa = 0.32$), which may be explained by the different agonist panels and the different patient populations (exclusion of children, pregnancy, von Willebrand disease and factor deficiency) [19]. In patient populations with a history of excessive bleeding (median BS = 13), the agreement between the tests is much higher (concordant in 84% of cases, $\kappa = 0.67$) [39] then in populations with milder bleeding phenotypes.

The unified ISTH-BAT, has gained general acceptance, and the uniform reporting of reference ranges according to age and gender allows standardization of the results and comparison between studies [27,40]. We observed that 28% of patients with elevated BS were not diagnosed with WB-PACT nor LTA and that 64% of patients with normal BS were detected with PFD according to LTA and/or WB-PACT.

This is in contrast with the high negative predictive value of the ISTH-BAT reported earlier [41,42]. However, a systemic review does not support this finding and report that, as disease prevalence in the studies was low (because most patients do not have a MBD) there are many true-negative test results, but the lower sensitivity in these studies indicates that the few patients with a MBD still could be missed by the ISTH-BAT [43].

In conclusion, in this population consisting of patients suspected for thrombocytopathy, a weak correlation was found between WB-PACT test results and the results from the widely used LTA test. Although 28% of patients with high BS were not detected with WB-PACT nor LTA, all children with elevated BS had either a positive LTA or a positive WB PACT test. A major advantage of WB-PACT is that the ristocetin induced VWF-binding to platelets can be measured in exactly the same setting as platelet activation. Ristocetin induced VWF-binding to platelets is a representative measurement for VWF:Ag and VWF:RCo positivity. Therefore, based on the present results in combination with the advantages compared to LTA (little amount of blood needed, less preprocessing steps and quantitative results), WB-PACT may have added value for the routine diagnostic work-up in patients who need to have platelet function tested.

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Declaration of competing interest

The authors report no declarations of interest. Some of the authors are employees of the research company Synapse BV (member of the Diagnostica Stago group).

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.thromres.2020.06.037.

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