Pediatric immune thrombocytopenia: Catching platelets

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Citation for published version (APA):
Functional platelet defects in children with severe chronic ITP as tested with two novel assays applicable for low platelet counts

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Abstract

Immune thrombocytopenia (ITP) is an autoimmune disease with a complex heterogeneous pathogenesis and a bleeding phenotype that is not necessarily correlated to platelet count. In this study, the platelet function was assessed in a well-defined cohort of 33 pediatric chronic ITP patients. Since regular platelet function test cannot be performed in patients with low platelet counts, two new assays were developed to determine platelet function. First, the microaggregation test measuring in platelets isolated from 10 ml whole blood, the platelet potential to form micro-aggregates in response to an agonist. Second, the platelet reactivity assay, measuring platelet reactivity to ADP, convulxin (CVX) and thrombin receptor activator peptide (TRAP) in only 150 μL unprocessed whole blood. Patients with a severe bleeding phenotype, demonstrated a decreased aggregation potential upon phorbol myristate acetate (PMA) stimulation, decreased platelet degranulation following ADP stimulation and a higher concentration of ADP and convulxin needed to activate the glycoprotein IIbIIIa complex compared to patients with a mild bleeding phenotype. In conclusion, here we have established two functional tests that allows for evaluation of platelet function in patients with extremely low platelet counts (<10⁹). These tests show that platelet function is related to bleeding phenotype in chronic ITP.
Introduction

Platelets play a critical role in hemostasis. When the vascular endothelium is disrupted, platelets adhere to subendothelium and initiate primary hemostasis. Excessive bleeding can occur if primary hemostasis is abnormal, either because of deficient platelet number or function. In vivo, primary hemostasis can be tested via bleeding time. However, this test does not distinguish between the varieties of causes of disturbed primary hemostasis.\(^1\)\(^-\)\(^3\) This can be tested more specifically in vitro, but current methods require relative high numbers of platelets and are consequently unsuitable for patients with low platelet counts.\(^1\)\(^-\)\(^4\)

Immune thrombocytopenia (ITP) is the most common cause of primary thrombocytopenia in children, with an incidence of about 1 in 20,000 children.\(^5\)\(^-\)\(^6\) Although the pathophysiology of ITP is not fully understood, two major forms are recognized: acute ITP and chronic ITP. Acute ITP is characterized by a sudden onset of bruising and bleeding in an otherwise healthy child. Often there is a history of viral illness in the weeks preceding the onset of bruising.\(^5\) Full blood counts show low platelet numbers (frequently < 20*10\(^9\)/L) as the only abnormality. In acute ITP, auto antibodies, recognizing glycoproteins on the surface of platelets and megakaryocytes, are considered the underlying cause.\(^7\)\(^-\)\(^8\) These antibodies are thought to result in accelerated clearance of platelets and megakaryocytes, and thereby, may also lead to decreased production of platelets.\(^7\)\(^-\)\(^9\) In chronic ITP, the attribution of auto antibodies to the pathogenesis of thrombocytopenia is less clear.

In the majority of pediatric ITP patients, thrombocytopenia resolves spontaneously within weeks or months. In about 25% of the patients, thrombocytopenia persists and becomes chronic.\(^5\)\(^-\)\(^10\)\(^-\)\(^11\) During chronic ITP platelet counts can vary in time from very low (<10*10\(^9\)/L) to almost normal. However, the observed bleeding tendency does not correlate strictly with platelet count. Cases with either low platelet counts without bleeding, or relatively high platelet counts with severe bleeding do occur. Causes for this variation in bleeding tendency are unknown. We hypothesize that variation in platelet function can account for the differences observed in bleeding phenotypes. Until now it has not been possible to predict if an individual child with chronic ITP is at risk for severe bleeding due to platelet malfunction, because of the lack of reliable tests for platelet function in patients with low platelet counts.\(^2\)\(^-\)\(^4\)
We here describe two functional platelet tests that can be used on patient material with very low (≥10^9/L) platelet numbers. The first test, the platelet micro aggregation test, based on a recently developed test\textsuperscript{12}, was adjusted to test platelet function directly by determining the potential of patients’ platelets to form micro-aggregates together with platelets from a healthy control in response to an agonist. Ristocetin and PMA were used to activate platelets through the von Willebrand Factor (vWF) and the fibrinogen binding site on glycoproteinIbIX (GPIbIX) and GPIIbIIIa respectively.\textsuperscript{12-14} In the second assay, the platelet reactivity assay, the reactivity in three major physiological platelet activation pathways was determined in unprocessed whole blood by flow cytometry on the level of individual platelets.\textsuperscript{15} ADP, convulxin (CVX) and thrombin receptor activator peptide (TRAP) were used to activate platelets via P2Y receptors (P2Y\textsubscript{1} and P2Y\textsubscript{12}), glycoprotein VI receptor and Proteinase Activated Receptor-1 (PAR-1), respectively. Furthermore the platelet activation test quantifies both degranulation (P-selectin expression on the platelet surface) and activation of glycoprotein IIbIIIa (binding of fibrinogen to platelets). Both assays require a minimum of blood compared to classical functional platelet assays, i.e. 10 ml of whole blood for the platelet micro aggregation test and 150 μl whole blood for the platelet reactivity assay. After validating both tests for low platelet numbers with platelets from healthy controls, the tests were used within a well-defined cohort of children with chronic ITP and results were correlated with reported bleeding scores. Our results show that patients suffering from serious bleedings have impaired functional platelet capacities compared to patients with no or mild bleeding and healthy controls. Both tests yield valuable information that may be used to predict future bleeding tendencies in chronic ITP patients.

Methods

Patients

Children aged 6 to 13 years with chronic ITP were included in this multicenter observational study. Parents and patients aged 12 years and older gave written informed consent. The study was approved by the Institutional Review Board of the University Medical Center Utrecht and performed in accordance with the Declaration of Helsinki.
Chronic ITP was defined as isolated thrombocytopenia with a platelet count of less than 100*10^9/L for more than 12 months. Patients were classified as having either a mild grade 0-3, or a severe grade 4-5 bleeding phenotype, according to the Buchanan bleeding score. For scoring, all bleeding problems during the course of ITP were taken into account, irrespective of platelet count. These chronic patients were scored severe if they received grade 4/5 on the Buchanan scale based on both their worst bleeding event, and recurrence of at least one more episode of grade 4/5 bleeding needing medical intervention. All except one of the severe patients also experienced a grade 4/5 in the two months prior to inclusion.

Data on duration of the ITP, bleeding tendency and medication were collected by questionnaire and from the patient’s medical files. All patients were tested for the presence of autoantibodies with indirect platelet immunofluorescence tests (PIFT) and indirect monoclonal antibody immobilization of platelet antigens (MAIPA).

Blood obtained from adult healthy individuals served as positive controls, and two Glanzmann Thrombasthenia patients were included as negative controls for the platelet micro-aggregation tests. Acquired Glanzmann patients have α-GPIIbIIIa auto antibodies which inhibit platelet aggregation via the GPIIbIIIa route, whereas other pathways are normal. Primary Glanzmann patients lack GPIIbIIIa almost completely, but other pathways may also be hampered.12,17

**Platelet micro-aggregation test**

For the platelet micro-aggregation assays peripheral whole blood was collected into 10 mL BD Vacutainer with 17 IU sodium heparin (Becton Dickinson and Company, Plymouth, United Kingdom), and centrifuged for 15 minutes at 218 g to obtain platelet rich plasma (PRP). PRP was washed 1:1 in sequestrin buffer (17.5 mM Na2HPO4, 8.9 mM Na2EDTA, 154 mM NaCl, pH 6.9, 0.1% (w/v) BSA) and centrifuged for 6 minutes at 2374 G. Platelets were washed again by adding 10 ml sequestrin buffer and finally dissolved in 20mM HEPES medium enriched with 132 mM NaCl, 6 mM KCl, 1 mM MgSO4, 1.2 mM KH2PO4 and 5 mM glucose. To measure aggregation function, patients’ platelets were suspended in HEPES medium to a final concentration of 10^6/ml, a minimum of 400 µl was required. Control platelets, from a healthy donor, were suspended in HEPES medium to a final concentration of 90*10^6/ml, a minimum of 800 µl was required. The platelets from the patients were stained with 0.8 µM PKH-26 (PKH26GL,
Sigma-Aldrich), whereas the control platelets were stained with 0.2 μM carboxyfluorescein diacetate succinimidyl ester (CFSE) (C1157, Invitrogen). Both were incubated in the dark for 15 minutes at room temperature. Staining was stopped by adding citrate-phosphate-dextrose (CPD) plasma from pooled AB+ blood from healthy donors (20% final concentration). Subsequently, 500 μl 10*10^6/ml patient platelets were mixed with 500 μl 90*10^6/ml control platelets and incubated with 20 μM PPACK (Mercks Biochemicals, Cat.520222) for 5 minutes at 37°C while shaking at 300 rpm. Platelet mixes were incubated for 5 minutes with 3 mM CaCl₂ to enable platelet activation. A healthy control was always tested next to a patient (positive control). Platelets were activated with 100 ng/ml PMA (SC-3576, Santa Cruz), or with 1.5 mg/ml ristocetin (Biopool, Trinity Biotech, Cat.50705), to activate GPIIbIIIa and GPIbIX respectively. Samples without an agonist served as a negative control. Samples were taken and fixed in 0.5% formaldehyde (Buffered Formaldehyde 4%, Klinipath, Duiven, The Netherlands) in a V-bottom 96 wells plate (Nunc, Thermo Fisher Scientific), at 0, 5 and 10 minutes. Fixed samples were measured on a FACS CantoII+HTS (BD Biosciences, San Jose, CA), analyzed with BD Facs DIVA 6.1 software (BD Biosciences, San Jose, CA), and aggregation calculated as: Aggregation(%) = (#aggregates (CFSE·PKH⁺ events)/(#patients' platelets (PKH⁺ events) x 100%).

**Platelet reactivity assay**

Blood was collected into a 4.5 mL BD Vacutainer with 0.5 mL sodium citrate 3.2% (Becton, Dickinson and Company, Plymouth, United Kingdom). Serial dilutions of adenosine diphosphate (ADP; Roche, Almere, The Netherlands) to stimulate the P2Y receptors (starting from 125 μM), Convulxin (CVX; Pentapharm, Basel, Switzerland) to stimulate the GPVI receptor (starting from 39 ng/mL), and thrombin receptor activator peptide (TRAP; Bachem AG, Bubendorf, Switzerland) to stimulate the PAR-1 receptor (starting from 625 μM), all in eight 4-fold dilutions, were prepared in a mixture of 47.5 μL HEPES buffered saline (HBS; consisting of 10 mM HEPES, 150 mM NaCl, 1 mM MgSO₄, and 5 mM KCl, pH 7.4, filtered through a 0.22 μm filter), 2 μL R-phycoerythrin (RPE) labeled mouse anti-human P-selectin antibodies (#555524; BD biosciences, Franklin Lakes, NJ), and 0.5 μL Alexa Fluor 488-labeled fibrinogen (Invitrogen, Eugene, OR).
A control sample, only containing 47.5 μL HBS, 2 μL RPE-labeled mouse anti-human P-selectin antibodies, and 0.5 μL Alexa Fluor 488-labeled fibrinogen, was prepared to determine platelet basal activation levels.

To measure patient and healthy control platelet reactivity, 5 μL fresh, citrate anti-coagulated, whole blood was added to all samples. After 20 minutes of incubation, 50 μL Optilyse B (Beckman Coulter Inc., Fullerton, CA) was added to fix the samples. After 10 minutes, 395 μL distilled water was added to lyse the erythrocytes. After half an hour of incubation at room temperature the samples were kept at 4°C until analysis on the FACS Canto II flow cytometer from BD Biosciences, which was at all times performed within 24 hours. Single platelets were gated based on forward and side scatter properties, 10000 single platelets were measured in each sample. The median fluorescence intensity (MFI) of RPE-labeled mouse anti-human P-selectin antibodies and Alexa Fluor 488-labeled fibrinogen on platelets was measured with FACS analysis, representing the quantity of P-selectin and open GPIIbIIIa receptor per platelet.

The obtained FACS data were quantified using BD FACSDiva software 6.1.2. The platelet responsiveness to agonists was qualified by calculation of the EC50 and the maximal response using GraphPad Prism 5.03 (GraphPad Software, San Diego, CA, U.S.A.). The EC50 represents the concentration agonist generating a response halfway between baseline and maximum response. The response of the platelets to the highest agonist concentration in the dilution series represents the maximal effect of stimulation.

To validate the assay for use at low platelet count, platelet rich plasma (PRP) form healthy donors was isolated by centrifugation for 15 minutes at 160 g, and platelet poor plasma (PPP) was obtained by centrifugation twice for 10 minutes at 2000 g. Platelet number was set at 250, 50, 25 and 10*10^9/L by diluting PRP by addition of PPP. 5 μL of these mixtures was added to ADP, CVX and TRAP samples to determine platelet reactivity at different platelet numbers.

**Statistical analysis**

Statistical analyses were performed using IBM SPSS Statistics 20.0.0 for Windows (International Business Machines Corporation, New York, U.S.A.). Data are shown as median with interquartile range (IQR) unless otherwise indicated. Wilcoxon signed ranks tests were performed to analyze data from related samples used in the validation experiments. For analysis of patient data, comparison between two groups was performed by Mann-Whitney U test for
numerical data and by Fisher’s exact test for categorical data. Correlation of two numerical variables was performed by Spearman’s rank correlation. P-values lower than .05 were considered to be statistically significant.

Results

Baseline characteristics

A total of 33 patients, all outpatients at Dutch university medical hospitals, were included in this study. As such, these patients represent a sub-group of ITP patients with increased bleeding phenotype. Ten patients were classified as having a severe bleeding phenotype (30.3%) and 23 patients as having a mild bleeding phenotype (69.7%) (Table 1). Platelet reactive auto antibodies were found in a minority of patients: 3 were found positive by indirect PIFT, and 6 by indirect MAIPA. These patients were equally distributed between groups.

<table>
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<th>Mild bleeding phenotype (n=23)</th>
<th>Severe bleeding phenotype (n=10)</th>
<th>P-value</th>
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<tr>
<td>Age at inclusion, years</td>
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<td>12 (8-15)</td>
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<tr>
<td>Age at diagnosis, years</td>
<td>3 (2-9)</td>
<td>4(2-12)</td>
<td>0.773</td>
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<td>Sex, male</td>
<td>6 (26)</td>
<td>3 (30)</td>
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<td>Platelet count at day of inclusion, *10⁹/L</td>
<td>74 (53-117)</td>
<td>58 (14-156)</td>
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<tr>
<td>MPV at day of inclusion, fL</td>
<td>9.4 (8.6-11.6)</td>
<td>10.8 (8.4-14.8)</td>
<td>0.216</td>
</tr>
<tr>
<td>Positive indirect PIFT</td>
<td>2 (9)</td>
<td>1 (10)</td>
<td>1.000</td>
</tr>
<tr>
<td>Positive indirect MAIPA</td>
<td>4 (17)</td>
<td>2 (20)</td>
<td>1.000</td>
</tr>
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</table>

Table 1 Baseline characteristics

† Data represent median (interquartile range), P was calculated using Mann Whitney U test.

‡ Data represent number (percentage), P was calculated using Fisher’s exact test.

Validation of two new functional platelet assays at low platelet count

For the micro aggregation test, platelets were gated based on their forward / sideward scatter characteristics (Figure 1A). After mixing PKH26 labeled patients samples with CFSE-labeled control platelets, a time dependent increase in aggregation was observed, with a maximal aggregation after 10 minutes. Aggregation was defined as events double positive in PKH and CFSE, and
quantified as shown in Figure 1B. We first determined the optimal and the lowest platelet number required for reliable results, by testing a range of proportions in healthy controls (Supplementary Figure 1). This was accomplished by changing the concentration of PKH-labeled platelets, whereas CFSE-labeled platelets were kept constant at 90*10⁶/ml using healthy control platelets. A ratio of approximately 1 PKH26 (test platelet) to 10 CFSE-labeled control platelets was considered reliable and feasible, indicating that 10 ml whole blood with only 10*10⁹/platelet/L was sufficient. We also crossed platelets from different healthy controls, to assess whether well functioning platelets from one donor will reach a similar aggregation level when aggregated with platelets from different healthy controls. Similar aggregation levels were reached (Supplementary Figure 2).

For the platelet reactivity assay, single platelets were gated (Figure 1A), and both P-selectin expression and open GPIIb/IIa were determined based on MFI levels (Figure 1C). To validate the use of the platelet reactivity assay for the determination of platelet function in samples with low platelet numbers, platelet reactivity was measured in platelet rich plasma samples of healthy controls diluted to a platelet number of 250, 50, 25 and 10*10⁹/L. Dilution was accomplished by addition of plasma, so that only platelet count was diluted without dilution of plasma proteins. Platelet reactivity within donors, quantified by agonist concentration needed to obtain half maximal activation (EC50) and by response to maximal agonist concentration, did not change when platelet number was reduced to 10*10⁹/L (Supplementary Figure 3). Although validation experiments already showed that parameters of platelet reactivity were not dependent on platelet number, we investigated if platelet count possible affected platelet reactivity in our study population. In the 33 patients, no correlation was observed between platelet number at day of inclusion and platelet reactivity (rho=0.165; P=.367; Supplementary Figure 4).

Micro aggregation of platelets

To assess whether the test correctly identified patients with known functional platelet defects, an acquired and a primary Glanzmann patient were tested. Baseline levels were completely normal (Figure 2A). Aggregation upon stimulation with Ristocetin via GPIbIX was normal in the acquired Glanzmann patient, whereas the primary Glanzmann patient reached approximately half that of the healthy control (Figure 2B). As expected, almost complete inhibition
of platelet aggregation was observed in both patients when stimulated with PMA via GPIIbIIIa (Figure 2C).

We subsequently performed the platelet micro-aggregation test on 18 chronic ITP patients, 11 with mild, and 7 with severe bleeding phenotype. Unstimulated platelets of mild and severe phenotype patients did not aggregate over time (Figure 2D,G). After stimulation with ristocetin mild phenotype patients equaled their healthy control after both five (47.6% [25.2-58.1], 49.9% [33.0-56.9]; P=.971) and ten minutes (53.8% [37.9-65.7], 54.3% [41.4-71.2]; P=.684) (Figure 2E). Whereas severe phenotype patients tended to have lower aggregation levels after five (27.1% [24.0-41.7]) and ten minutes (35.3% [29.2-52.7]) compared to healthy controls (49.2% [31.5-55.7]; P=.073, 55.8% [40.9-66.2]; P=.125), but the differences were not significant. (Figure 2H). Upon stimulation with PMA mild phenotype patients equaled their healthy control after five (45.2% [34.7-70.0], 63.0% [47.3-70.9]; P=.481) and ten minutes (71.9% [50.4-84.9], 80.6% [62.2-85.83]; P=.631). (Figure 2F). In contrast, severe phenotype patients had significantly lower aggregation levels after five (30.9% [16.0-42.8]) and ten minutes (52.4% [28.7-66.45]) of PMA stimulation compared to healthy control platelets (64.2% [51.4-65.2]; P=.021, 80.8% [67.3-80.9]; P=.017) after 5 and 10 minutes, respectively (Figure 2I).
Figure 1 Methods platelet micro aggregation test and platelet reactivity assay. A) Platelets were gated based on forward and sideward scatter properties (FSC/SSC) B) Platelet micro-aggregation test. Test platelets, derived either from a patient or healthy control were stained with PKH and mixed in 1:9 ratio with healthy control platelets, stained with CFSE. Platelets were analyzed in the PE (PKH) and FITC (CFSE) channel, platelets positive in both channels were identified as aggregates. The left panel shows unstimulated platelets after 0 minutes, the middle panel unstimulated platelets after 10 minutes and the right panel PMA stimulated platelets after 10 minutes. C) Platelet reactivity assay. The median fluorescence intensity (MFI) of RPE-labeled mouse anti-human P-selectin antibodies and Alexa Fluor 488-labeled fibrinogen on platelets was measured by FACS analysis, representing the quantity of P-selectin and open GPIIbIIIa receptor per platelet. The left panel shows unstimulated platelets, the right panel shows platelets stimulated with 625 μM TRAP.
Figure 2 Severe chronic ITP patients show functional defects in platelet microaggregation upon PMA stimulation. An acquired Glanzmann patient, with functional GPIIbIIIa and an anti-GPIIbIIIa antibody, and a primary Glanzmann patient, without functional GPIIbIIIa, served as negative controls for the assay (A, B, C). Eleven mild chronic ITP patients (D, E, F) and 7 severe chronic ITP patients (G, H, I) were tested in the platelet micro-aggregation test. Results are given as percentage of aggregation reached after 0, 5 and 10 minutes without (A, D, G), after Ristocetin (B, E, H) or after PMA stimulus (C, F, I). Outcomes are depicted pair-wise, the patient compared to a healthy control tested in parallel. Statistical analysis was performed by Wilcoxon signed ranks testing.
Figure 3 Severe chronic ITP patients show functional defects platelet reactivity upon ADP and convulxin stimulation. To determine baseline platelet reactivity, mean fluorescence intensity (MFI) in arbitrary units (AU) of fluorescent labeled anti-P-selectin antibody (A) and of fluorescent labeled fibrinogen (B) bound to platelets was determined in the absence of agonists. Maximal MFI in AU in response to stimulation with ADP, convulxin and TRAP, and concentration of these agonists needed to obtain half maximal MFI were determined for both platelet P-selectin expression (C,D,G,H,K,L) and opening of GPIIbIIIa receptor (E,F,I,J,M,N). The dots represent individual results for the 10 severe patients, 23 mild patients and 8 healthy controls, with a line at the median. Statistical analysis was performed by Mann-Whitney U testing.
CHAPTER 5

Platelet reactivity assay

Basal level of platelet activation and platelet reactivity to the agonists ADP, CVX and TRAP was determined for all included patients by measuring platelet P-selectin expression and opening of the GPIIbIIIa receptor in whole blood (Figure 3). There were no significant differences when comparing patients with chronic ITP with a mild phenotype to healthy controls. Basal levels of platelet activation did not differ between chronic ITP patients with a mild (n=23) and with a severe bleeding phenotype (n=10), nor when compared to healthy controls (n=8) (Figure 3A-B). Platelet degranulation, determined via measurement of P-selectin expression, in response to maximal ADP stimulation was less in patients with a severe bleeding phenotype (9.6*10^2 AU [3.7-11.8]), compared to both patients with a mild phenotype (13.0*10^2 AU [11.2-15.3]; P=.020) and to controls (22.4*10^2 AU [10.7-25.5]; P=.016) (Figure 3C). When stimulated with CVX or TRAP, no differences in P-selectin expression were observed between mild and severe bleeders (Figure 3G, K-L). Patients with a severe phenotype did need more CVX (11.0 ng/ml [5.6-393.8]) to stimulate for half maximal P-selectin expression compared to healthy controls (4.7 ng/ml [2.6-6.4]; P=.021) (Figure 3H).

The concentration of ADP and CVX needed to obtain opening of the half maximal amount of GPIIbIIIa receptors, was higher in severe bleeders (1.1 μM ADP [0.9-1.5] and 9.0 ng/ml CVX [5.2-12.9]), compared to both mild bleeders (0.7 μM ADP [0.4-1.0] and 5.8 ng/ml CVX [2.8-9.0]; P=.031 and P=.031) and healthy controls (0.5 μM ADP [0.4-0.9] and 3.0 ng/ml CVX [1.4-4.0]; P=.027 and P=.006) (Figure 3F, J). Reactivity to TRAP was equal between groups (Figure 3M-N). Maximal GPIIbIIIa response to all agonists did not differ between groups (Figure 3E, I, M).

Platelet micro aggregation versus platelet reactivity

In both the micro aggregation and platelet reactivity assay, the platelets from severe ITP patients showed diminished activity compared to healthy control. Indeed, PMA-induced aggregation and maximal platelet P-selectin response to ADP, the parameters giving the most discrimination between healthy controls and patients within the assays, correlated significantly for the 18 patients in which both assays were performed (rho=.519; P=.027) (Figure 4). In Supplementary Table 1, the characteristics of both assays are listed.
Figure 4 Correlation of platelet micro-aggregation and platelet reactivity. Response to PMA induced platelet micro-aggregation and ADP induced maximal platelet reactivity correlated with a Spearman correlation coefficient of .519 (P=.027) for all 18 patients in which both assays were performed.

Discussion

A variation in bleeding severity exists between patients with chronic ITP that cannot be explained by platelet counts alone. We therefore hypothesized that platelet function might be affected diversely between patients with chronic ITP. In the current study we observed a functional platelet defect in chronic ITP patients with a severe bleeding phenotype. These platelets displayed a decreased potential to form micro-aggregates following PMA stimulation, decreased platelet degranulation following ADP stimulation and higher ADP and convulxin concentrations needed for half maximal activation of the GPIIbIIIa complex.

To our knowledge, this is the first study to establish platelet function in individuals with low platelet counts. Classical platelet function tests are not reliable with platelet counts below 50*10^9/L.\textsuperscript{24} In this study, we present the micro aggregation test and the platelet reactivity assay for their use in samples containing low platelet numbers down to 10*10^9/L. We show that the micro aggregation test can be performed in 10 ml blood with this minimal platelet number to assess platelet function directly as well as pathway specific. In the platelet reactivity assay, only 150 μL of whole blood was needed for all performed platelet reactivity measurements. The assay determines the reactivity of single platelets independently of platelet number. Only 3 quick handling steps, incubation, fixation and red cell lysis, are needed to perform the assay, since platelet isolation is not required. The serial dilutions used for platelet activation can be stored at -20 degrees Celsius for several weeks, and can be
used instantly when needed. The assay has a broad and quantitative detection range, measuring both platelet degranulation and GPIIbIIIa opening, and it distinguishes between multiple specific activation pathways. In the scope of this research we have decided to test pathways of specific interest in ITP, but naturally, each agonist of choice can be used in both assays. Both tests might be of great diagnostic value in a broad range of patients suffering from thrombocytopenia. In this study in children with chronic ITP we proved that we can determine platelet function and correlate this with bleeding phenotype.

We classified patients as having a mild or a severe bleeding phenotype using the overall Buchanan bleeding score.\textsuperscript{20} We compared patients with a mild bleeding phenotype scoring a grade 0-3, to patients with a severe phenotype scoring a grade 4-5 in our search for a relation between platelet function and bleeding phenotype. This established score for ITP patients, which grades bleeding severity based on skin and mucosal bleeds showed to be associated with platelet function.

With the micro aggregation assay, we found patients classified as severe bleeders to have significantly lower aggregation levels compared to healthy controls upon 5 and 10 minutes of PMA stimulation. However, when stimulating with Ristocetin no differences between patients and healthy controls were observed. A possible explanation for the decreased PMA response might be an interference of auto antibodies present on patient platelets not detectable by indirect MAIPA or indirect PIFT as both assays have very low sensitivities (25-39% and 30%, respectively).\textsuperscript{21-23} The presence of anti-GPIIbIIIa antibodies in these patients might explain why we predominantly see an effect with PMA stimulation and not with Ristocetin, as these auto antibodies are most frequently found in ITP patients.\textsuperscript{7,24} With the platelet reactivity assay, we found the severe patients to have significant lower P-selectin expression after activation with ADP, but not after activation with CVX or TRAP. Next to platelet P-selectin expression, opening of the platelet glycoprotein IIbIIIa receptor was measured. We observed that for both stimulation with ADP and CVX, higher agonist concentrations were needed in patients with a severe phenotype to obtain opening of the half maximal amount of GPIIbIIIa receptors, when compared both with mild patients and controls. Interference of auto antibodies in binding of FITC labeled fibrinogen to open GPIIbIIIa in the platelet reactivity assay seems unlikely, since differences in GPIIbIIIa opening are seen only for ADP and CVX stimulation, and not following TRAP stimulation. Theoretically, the decrease in platelet reactivity observed in severe patients might be caused by an
interaction of auto antibodies with platelets or megakaryocytes, leading to outside-in signalling influencing platelet reactivity to natural stimuli or influencing megakaryocyte maturation and differentiation.

A limitation to our study is that we cannot confirm if the decreased platelet activity, we observed in the more severe ITP patients, was due to antiplatelet antibodies. Although direct variants of PIFT and MAIPA are more sensitive, these still require high number of platelets and are therefore unsuitable for severe cases with low number of platelets. Therefore, direct confirmation whether or not autoantibodies are to blame, will have to wait until more reliable methods are developed to detect low levels of antiplatelet antibodies using low number of platelets. Also, a larger prospective study, determining platelet function, estimating bleeding tendency with the new ITP bleeding assessment Tool (ITP BAT) after several months, will be required to determine if these assays reported here can predict severity.

Within healthy adult individuals, platelet parameters are known to be stable when measured at several time points. A single measurement represents a person’s innate platelet reactivity, making repeated measurements unnecessary. However, we cannot exclude antibodies responsible for ITP to influence this innate platelet reactivity via outside in signalling. Platelet reactivity in chronic ITP patients could therefore vary along with disease activity. Future longitudinal studies will be necessary to determine whether platelet reactivity is stable in chronic ITP.

In this study we have focused on in vitro platelet function in the context of bleeding severity in chronic ITP. Nevertheless in vivo other factors could influence bleeding severity as well, such as endothelial function, and plasma factors. These factors might, together with platelet function, influence bleeding phenotype.

In summary, here we have established two functional tests that allows for evaluation of platelet function in children with chronic ITP, and to associate the results with the bleeding phenotype. Patients with a severe bleeding phenotype were found to have a decreased platelet function, shown by decreased platelet aggregation following PMA stimulation in the platelet micro-aggregation test, decreased platelet degranulation following ADP stimulation in the platelet reactivity assay and higher ADP and convulxin concentrations needed for half maximal activation in the platelet reactivity assay. Longitudinal studies will have to confirm if the micro aggregation test and the platelet reactivity assay can be used to predict bleeding phenotype in chronic ITP.
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Acknowledgments

The authors would like to thank M. Peters, J. Verhage, W.A. Kors, A.M.J. van Meurs, N. Dors, F.J. Smiers, R.Y.J. Tamminga and A. Beishuizen for enrolling patients in this study. This work was partly funded by the Landsteiner Foundation for Bloodtransfusion Research (Landsteiner Stichting voor Bloedtransfusie Research, LSBR 0842) to AL.

Authorship Contributions

EB, AL, KH, LH, PG, GV, MB designed the study. EB, LH, MR, PG were responsible for the experimental design of the platelet reactivity assay. AL, LP, ES, MH, GV were responsible for the experimental design of the platelet micro-aggregation assay. EB and LH performed all platelet reactivity assays, AL performed all platelet micro-aggregations tests. EB and AL carried out the data analysis. All authors contributed to data interpretation. EB, AL, LH, PG, GV, MB wrote the manuscript. All authors contributed to revising the manuscript for intellectual content and style and all approved the final version.

Conflict of Interest Disclosures

The authors declare no competing financial interests.
Reference List


