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ORIGINAL ARTICLE

Functional validation of a modified platelet desialylation test for immune and hereditary thrombocytopenias

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Abstract

Background: Thrombocytopenia arises from heterogeneous inherited and acquired disorders, and identifying the underlying platelet clearance mechanisms remains challenging. Platelet desialylation, characterised by loss of sialic acid and consequent exposure of terminal β-galactose residues recognised by the Ashwell–Morell receptor, represents an alternative pathway of hepatic platelet clearance. This study aimed to validate a modified platelet desialylation test (PDT) and assess its applicability in multitransfused, alloimmunized, and hereditary thrombocytopenia patients as a functional in vitro assay to detect plasma-induced platelet desialylation and explore its potential as a complementary biomarker of platelet clearance mechanisms.

Methods: PDT performance was evaluated using plasma from 20 healthy donor pools, 10 antibody-positive refractory patients, and 15 individuals with hereditary thrombocytopenias by FITC–RCA-I flow cytometry. Analytical validation included assessment of reproducibility, incubation time, lectin concentration, and ratio-based result interpretation.

Results: β-Lactose showed significantly lower expression mean fluorescence intensity (MFI) than both untreated reference control and neuraminidase-treated conditions ($p < 0.00001$), whereas patient plasma exhibited higher fluorescence than the reference control ($p < 0.0001$). A 1 h incubation using 0.3 µL per reaction RCA-I-FITC

Dante Langhi and Carla Luana Dinardo are co-last authors.

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stock solution (5 mg active conjugate/mL) provided stable and reproducible discrimination. In inherited thrombocytopenias, 66.7% of patients were PDT-positive despite HLA/HPA antibodies being detected in only 13.3%. PDT-positive samples showed increased desialylation-associated MFI ($p = 0.002$) with a strong correlation between MFI and ratio ($\rho = 0.877$). The assay required approximately 4 h and cost US$3.62 per test.

Conclusion: The PDT is a practical, reproducible, and cost-effective assay capable of detecting physiologic, enzymatic, and immune-mediated platelet desialylation, providing a complementary biomarker for investigating thrombocytopenic disorders.

KEYWORDS
alloimmunized patients, flow cytometry assay, immune platelet clearance, platelet desialylation, thrombocytopenia biomarkers

1 | INTRODUCTION

Thrombocytopenia is a haematological condition characterised by reduced platelet counts resulting from decreased production, increased peripheral destruction, sequestration, or immune-mediated mechanisms, including alloimmune platelet disorders.[1]

Its evaluation requires integration of clinical history, physical examination, and laboratory findings and encompasses both inherited and acquired etiologies.[2,3] Amongst inherited causes, Glanzmann Thrombasthenia and Bernard–Soulier Syndrome arise from defects in the GPIIb/IIIa and GPIb-IX-V complexes, respectively. These disorders typically present with significant mucocutaneous bleeding and, in the case of Bernard–Soulier Syndrome, giant platelets. Affected patients frequently require transfusional support, thereby increasing the risk of alloimmunization.[4,5]

Amongst acquired causes, immune thrombocytopenia (IT) results from the formation of allo- or autoantibodies directed against platelet glycoproteins, leading to Fcγ receptor-mediated destruction that occurs predominantly within the splenic reticuloendothelial system.[2,3,6–8]

The most prevalent clinical form is immune thrombocytopenic purpura (ITP), a chronic autoimmune disorder characterised by persistent thrombocytopenia and variable bleeding manifestations.[9–11]

Another clinically relevant acquired condition is platelet transfusion refractoriness (PTR), defined by inadequate platelet count increments following consecutive transfusions and attributable to immune, non-immune, or combined mechanisms.[12–14] PTR is associated with increased bleeding risk, prolonged hospitalisation, and higher healthcare costs, and is frequently linked to alloimmunization in chronically transfused patients, including those with hereditary disorders, malignancies, and benign hematologic diseases.[15,16]

Despite available therapies, approximately 15%–25% of patients with IT fail to respond adequately to conventional treatment, including splenectomy, suggesting that platelet destruction is not exclusively mediated by Fcγ receptor-dependent splenic clearance.[17,18] Platelets are continuously removed from circulation during senescence, activation, or disease states through signals such as phosphatidylserine exposure, P-selectin expression, or antibody binding, which promote clearance by splenic macrophages.[11,19,20] However, platelets also regulate their own through endogenous modification of surface glycans.[21]

Desialylation, characterised by the removal of terminal sialic acid residues, promotes the trafficking of desialylated platelets (dPLTs) to the liver for clearance, a process recognised as a major pathway during platelet aging and activation.[22–24] Premature desialylation may be induced by antiplatelet antibodies, CD8+ cytotoxic T lymphocytes, or microbial interactions.[25–27]

Exposure of β-galactose residues enables recognition by the hepatic Ashwell–Morell receptor (AMR), as well as potentially other cell surface receptors, redirecting platelet clearance from the spleen to Kupffer cells and hepatocytes and thereby establishing an Fc-independent clearance pathway.[21,22,28–31] This mechanism may help explain treatment refractoriness and can be partially modulated by sialidase inhibitors such as oseltamivir.[24,32–34]

Although desialylation occurs physiologically, it is markedly enhanced in autoimmune and alloimmune conditions in which antibodies targeting GPIIb/IIIa, GPIbα, GPIb/IX, or GPIa/IIa induce platelet activation and neuraminidase release.[2,35–38] Viral and bacterial infections may also trigger this process through immune activation and molecular mimicry.[26,35,39–44] Therefore, desialylation plays a significant role in multiple pathological processes associated with thrombocytopenia.[45]

Desialylation can be quantified by flow cytometry using fluorescent lectins such as RCA-I and ECL, which detect exposed galactose and β-GlcNAc residues.[22,28]

Emerging evidence suggests that platelet desialylation may serve as a prognostic biomarker of therapeutic failure in IT, particularly in patients receiving therapies targeting Fcγ receptor-mediated platelet clearance.[46] Therefore, sensitive laboratory tools capable of detecting this pathway may improve the mechanistic characterisation of thrombocytopenia.

Accordingly, this study aimed to validate a modified platelet desialylation test (PDT), developed as a functional in vitro assay using standardised donor platelets to determine whether circulating plasma factors can induce platelet desialylation, as indicated by the exposure

of terminal β-galactose residues. Additionally, its applicability was evaluated in multitransfused and alloimmunized patients, as well as in individuals with hereditary thrombocytopenias, to explore its potential as a complementary biomarker for investigating platelet clearance mechanisms across diverse thrombocytopenic conditions.

2 | METHODS

2.1 | Study design

This methodological, experimental, and cross-sectional study was designed to standardise and analytically validate the modified PDT, developed as a functional in vitro plasma-based assay using standardised donor platelets as the target substrate. The study encompassed assay development, optimisation, and comprehensive performance evaluation, including assessments of precision, reproducibility, linearity, and analytical stability. Controlled experiments were conducted to evaluate intra- and inter-assay consistency and to establish reference ranges using samples from healthy individuals and patients with thrombocytopenia.[47]

The PDT implemented in this study was developed through strategic adaptations of the platelet immunofluorescence test (PIFT) originally described by von dem Borne[48] and of desialylation assays previously reported by Li et al.,[24] Tao et al.,[22] and Lasne et al.[49]

2.2 | Population and ethical aspects

The study was conducted at a quaternary care centre and included blood donors considered healthy individuals, who voluntarily participated after providing written informed consent during the donor screening process.

Hospitalised patients enrolled in the study provided written informed consent prior to inclusion, authorising transfusion procedures and laboratory investigations. Additionally, patients with hereditary thrombocytopenias, including Glanzmann thrombasthenia and Bernard–Soulier syndrome, provided written informed consent before participation, authorising the investigation of platelet antibodies and related laboratory analyses, in accordance with approval by the local Research Ethics Committee.

2.3 | Selection and preparation

A total of 260 blood donors with blood group O were recruited from two independent collection sites between May and June 2024. In parallel, 10 patients with PTR referred for platelet antibody investigation were included. Plasma samples from these patients were initially screened by platelet crossmatch using the PIFT.[48] Samples demonstrating positive reactivity were subsequently analysed using the Luminex PakLx platform to identify human platelet antibodies (HPA) and/or human leukocyte antibodies (HLA).[48]

Additionally, patients with hereditary thrombocytopenias who were admitted for investigation of platelet antibody status were also tested using both PIFT and Luminex PakLx assays to confirm the absence of immune reactivity. Following completion of immunohaematological testing, all collected samples, including those obtained from both donors and patients, were subsequently evaluated using the PDT to assess platelet surface desialylation and to establish baseline and pathological desialylation profiles.

2.4 | Platelet desialylation test protocol

The platelet desialylation assay was performed according to the following standardised protocol (Figure 1):

I. Platelet pool preparation: Three apheresis platelet units collected within 24 h of donation were selected. A 10-cm tubing segment from each unit was aseptically separated, and platelets were transferred to a conical tube, washed twice with phosphate-buffered saline (PBS) containing 0.1% EDTA (1.0×10^{-3}; Sigma-Aldrich, St. Louis, MO, USA), and centrifuged at 250 × g for 2 min. The final platelet concentration was adjusted to $1.5–2.5 \times 10^{11}$/L using a Sysmex XN-550 haematology analyser.
II. Platelet adherence: Fifty microliters of the platelet suspension were dispensed into each well of a microplate and centrifuged at 250 × g for 2 min to promote platelet adherence to the well surface.
III. Addition of plasma and controls: For reagent validation, β-lactose (200 mmol/L; Sigma-Aldrich) was used as a negative control, whereas neuraminidase from *Clostridium perfringens* (NeuC; 0.5 U/mL; Sigma-Aldrich) served as the positive control. Patient plasma samples and pooled plasma from healthy donors (five donors per pool) were added and gently mixed to minimise platelet activation.
IV. Incubation: Plates were incubated at 37°C for 30 min to allow antigen–antibody interactions.
V. Washing: Samples were washed three times with 0.1% PBS/EDTA, with centrifugation performed after each wash to remove unbound components.
VI. Lectin staining: Ricinus communis agglutinin I conjugated to fluorescein (RCA-I–FITC; Vector Laboratories, Burlingame, CA, USA), supplied at 5 mg active conjugate/mL in 10 mM HEPES, 0.15 M NaCl (pH 7.5), was used at a standardised volume of 0.3 μL of stock solution per reaction, diluted in 0.1% PBS/EDTA. A final volume of 50 μL per reaction was added to each well, followed by incubation for 1 h at room temperature, protected from light.
VII. Final wash: After a final wash with 0.1% PBS/EDTA, the supernatant was carefully discarded.
VIII. Flow cytometry preparation: Platelets were resuspended in 100 μL of 0.1% PBS/EDTA at a platelet concentration ranging from 1.5 to 2.5×10^{11}/L and were gently transferred to acrylic tubes for flow cytometric analysis.

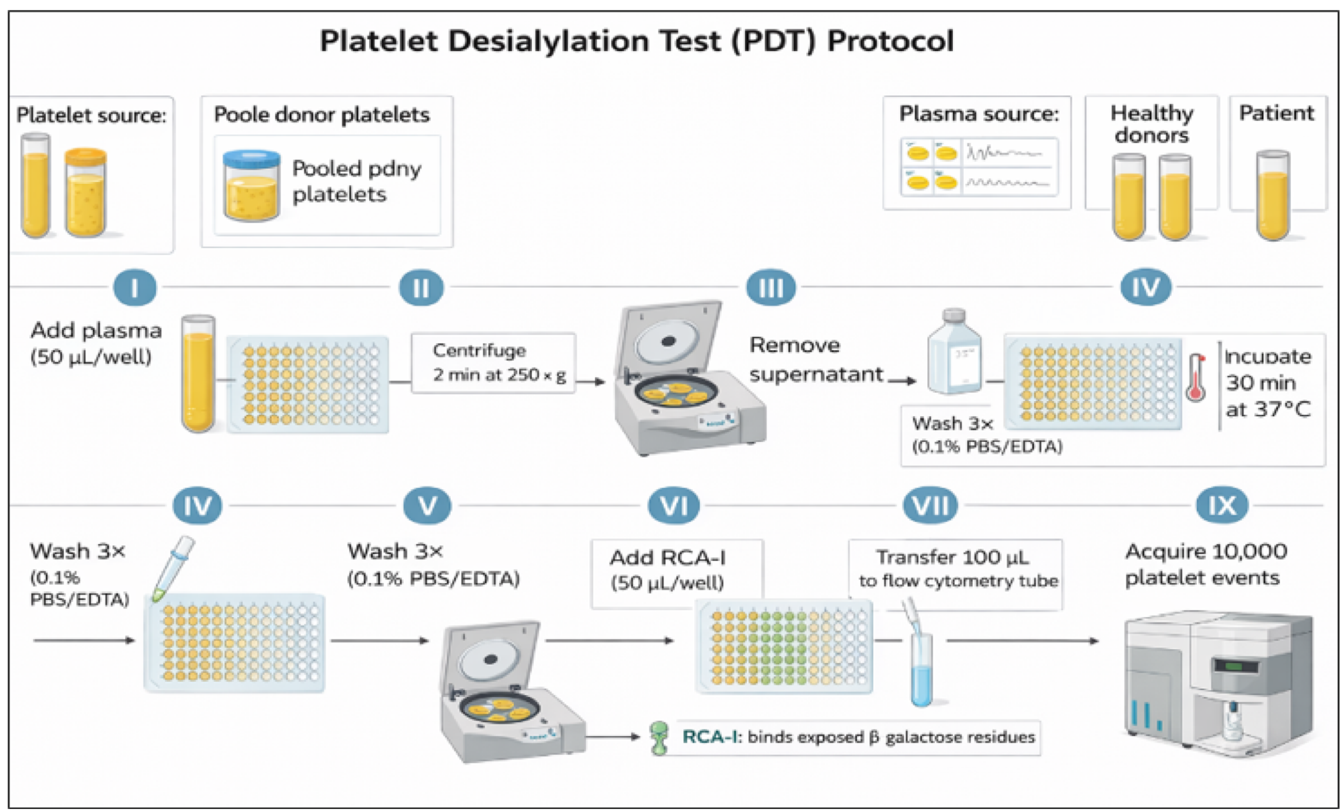


FIGURE 1 Workflow of the modified platelet desialylation test (PDT). Platelets obtained from pooled donor platelet concentrates were distributed into 96-well plates and incubated with plasma samples. Following centrifugation (250 × g for 2 min) and supernatant removal, platelets were washed with 0.1% PBS/EDTA and incubated for 30 min at 37°C. After additional washing steps, FITC-conjugated Ricinus communis agglutinin I (RCA-I) lectin was added and incubated for 1 h at room temperature in the dark to detect exposed terminal β-galactose residues resulting from platelet desialylation. Samples were subsequently transferred to flow cytometry tubes and analysed by flow cytometry, with acquisition of 10 000 platelet events per sample. Platelet desialylation was quantified by measuring mean fluorescence intensity (MFI).

IX. Flow cytometry analysis: Samples were analysed on a DxFLEX flow cytometer (Beckman Coulter, Brea, CA, USA), with acquisition of 1.0×10^4 events (10 000 platelets) per sample. Mean fluorescence intensity (MFI) was recorded to quantify RCA-I binding. Data were analysed using FlowJo Software version 10.10.0.

2.5 | Analytical standardisation and diagnostic interpretation of the PDT

To standardise result interpretation, a statistical ratio (*R*) was calculated for each sample using an approach analogous to that applied in platelet immunofluorescence assays (PIFT):

$$R = \text{median fluorescence of the sample} / \text{median fluorescence of the normal reference control (healthy plasma pool)}$$

The healthy plasma pool served as the physiological baseline for normalisation, whilst β-lactose confirmed minimal background binding and neuraminidase verified assay responsiveness. Plasma from previously characterised anti-platelet antibody-positive patients was used as a clinical positive control. Diagnostic thresholds were determined from the distribution of *R* values in the reference population, incorporating analytical variability. The upper limit of normality was defined as mean + 2SD (95th percentile), and intra- and inter-assay coefficients of variation were considered to establish a borderline interval. Samples were classified as:

- PDT-negative: $R < 1.30$
- PDT-inconclusive: $1.30 \le R \le 1.45$
- PDT-positive: $R > 1.45$

Thresholds were internally validated for the flow cytometer used in this study. As absolute fluorescence values depend on instrument configuration and detector settings, laboratory-specific validation is recommended when implementing the PDT on other cytometry platforms.

2.6 | Statistical analysis

Non-parametric statistical tests were applied due to the small sample size and non-normal distribution of PDT variables. Group comparisons

were performed using the Friedman test followed by Dunn's post hoc correction, whilst paired analyses were conducted using the Wilcoxon signed-rank test. The effect of RCA-I concentration was evaluated using the Kruskal–Wallis test, whereas differences between PDT-positive and PDT-negative patients were analysed using the Mann–Whitney *U* test. Correlations between MFI and the ratio were assessed using Spearman's rank correlation coefficient. Wilson's method was used to calculate 95% confidence intervals, and statistical significance was defined as $p < 0.05$. All analyses were performed using GraphPad Prism version 10.0 (GraphPad Software, San Diego, CA, USA) and R version 4.3.1.

3 | RESULTS

3.1 | General findings

Plasma samples from 20 healthy donors, used as the normal reference control (healthy plasma pool), and from 10 antibody-positive refractory patients were evaluated under untreated conditions as well as in the presence of β-lactose (technical negative control) and neuraminidase (NeuC, positive control).

In the antibody-positive validation subset ($n = 10$), PDT measurements showed broad analytical dispersion, with MFI values ranging from 1032.3 to 5080.7 a.u. and ratio values from 1.49 to 3.54, indicating strong discriminatory capacity across the assay's dynamic range.

The clinical heterogeneity of this cohort, including aplastic anaemia (30%), myelodysplastic syndrome (30%), acute leukaemia (10%), solid tumours (10%), and hereditary platelet disorders (20%) did not impair assay performance. Antibody profiles were predominantly anti-HLA (60%), followed by mixed anti-HLA/anti-HPA reactivity (30%) and anti-GPIIb (10%), with higher PDT values observed in samples exhibiting broader or dual antibody specificities (Tables 1 and S1).

TABLE 1 PDT quantitative values and associated clinical and antibody profiles in the study cohort.

A. Continuous variables

Parameter	MFI (a.u.)	Ratio
Mean	2131.94	2.35
Median	1647.55	2.04
Standard deviation	1325.13	0.69
IQR	1432.23	1.06
Min–Max	1032.3–5080.7	1.49–3.54
Coefficient of variation (%)	62.1	29.4

B. Distribution of clinical diagnoses

Diagnosis	*n*	Proportion (%)
Aplastic anaemia	3	30%
Myelodysplastic syndrome	3	30%
Acute myeloid leukaemia	1	10%
Breast cancer	1	10%
Glanzmann thrombasthenia	1	10%
Bernard–Soulier syndrome	1	10%
Total	10	100%

C. Antibody profiles

Specificity	*n*	Proportion (%)	MFI (a.u.)	Ratio
Anti-HLA	6	60%	2031.85	2.00
Anti-HLA + Anti-HPA	3	30%	2623.63	2.65
Anti-GPIIb	1	10%	1257.40	3.54
Total	10	100%	–	–

3.2 | Reference-based detection of platelet desialylation

Statistical comparisons across experimental conditions demonstrated a clear distinction between baseline and clinically relevant desialylation profiles using untreated donor plasma as the reference control. Incubation with β-lactose yielded significantly lower MFI values than both neuraminidase-treated samples and untreated donor plasma (Wilcoxon signed-rank test, $p < 0.00001$ for both comparisons), confirming effective inhibition of RCA-I binding and validating β-lactose as the technical negative control.

No statistically significant difference was observed between neuraminidase-treated and untreated donor plasma samples ($p = 0.097$), indicating overlapping levels of platelet desialylation. This supports the role of untreated donor plasma as a physiological baseline for lectin binding, likely reflecting low-grade plasma-mediated activity and inter-pool biological variability rather than enzymatic saturation alone. Accordingly, untreated donor plasma was retained as the reference control because its purpose was normalisation rather than discrimination. The donor pool defines the physiological baseline, whereas neuraminidase represents a maximal enzymatic response used exclusively to confirm assay reactivity. Diagnostic thresholds require a stable biological baseline rather than the lowest possible signal; therefore, untreated donor plasma was used for ratio calculation, whilst neuraminidase served exclusively as the positive control.

In contrast, incubation with patient plasma produced a marked increase in MFI compared with the untreated donor reference (Mann–Whitney *U* test, $p < 0.0001$), supporting the presence of circulating factors associated with enhanced platelet desialylation (Figure 2; Table S2).

Collectively, these findings demonstrate that the PDT effectively discriminates physiological baseline desialylation from antibody-mediated desialylation whilst remaining distinct from the inhibited background signal, confirming the assay's robustness and sensitivity in clinically relevant antibody-positive samples.

3.3 | Optimization of RCA-I incubation time

To assess the influence of RCA-I incubation time on the PDT, 47 platelet samples from healthy donors were analysed after 1 and 3 h of

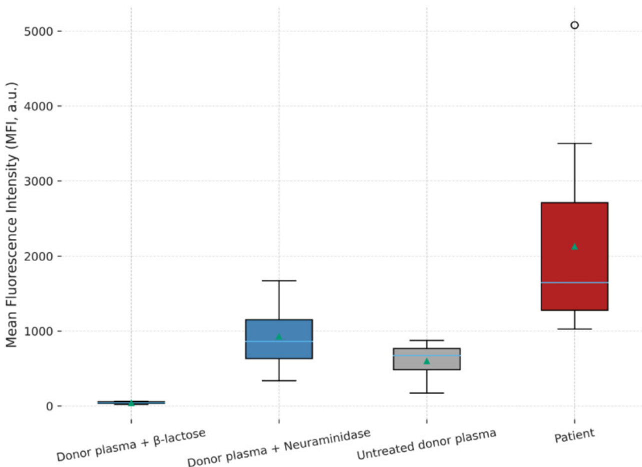


FIGURE 2 Mean fluorescence intensity (MFI) measured by the modified platelet desialylation test (PDT) under four experimental conditions. β-Lactose-treated donor plasma was used as the technical negative control, neuraminidase (NeuC)-treated samples as the enzymatic positive control, and untreated donor plasma as the physiological reference control. Patient plasma samples exhibited significantly higher MFI values than the untreated control, indicating increased exposure of terminal β-galactose residues and enhanced platelet desialylation. Statistical comparisons demonstrated significant differences between β-lactose and untreated controls ($p < 0.00001$), β-lactose and neuraminidase-treated samples ($p < 0.00001$), and patient plasma and untreated controls ($p < 0.0001$), whereas no significant difference was observed between neuraminidase-treated and untreated samples ($p = 0.097$). Data are presented as median and interquartile range (IQR); whiskers represent minimum and maximum values, and triangles (▲) indicate the mean.

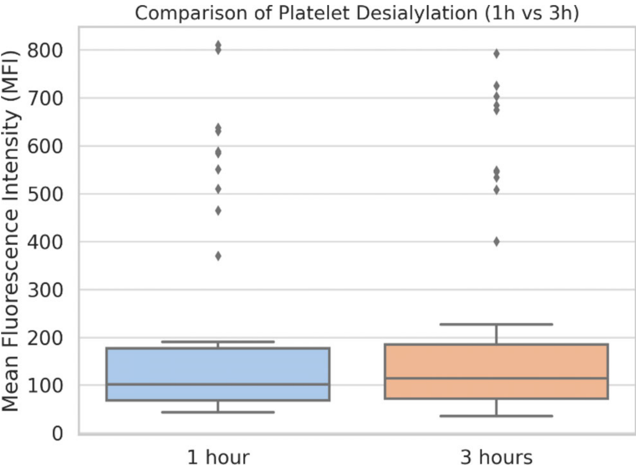


FIGURE 3 Comparison of platelet desialylation following 1- and 3-h incubation periods. Boxplots illustrate the distribution of mean fluorescence intensity (MFI) values obtained with the modified platelet desialylation test (PDT) after 1 and 3 h of incubation. Each dot represents an individual measurement. Although a slight increase in MFI was observed after 3 h of incubation, the overall distributions and variability patterns remained comparable between conditions. These findings indicate that extending the incubation period from 1 to 3 h does not substantially affect assay performance, supporting the reproducibility and stability of the PDT under both conditions.

incubation. MFI values were comparable between the two time points (202.2 ± 218.0 vs. 214.1 ± 219.7).

The paired Wilcoxon signed-rank test showed no statistically significant difference ($W = 439.0$, $p = 0.19$), indicating that prolonged incubation does not significantly enhance lectin binding. A strong positive correlation was observed between the 1 and 3 h measurements (Spearman's $\rho = 0.68$, $p < 0.0001$), supporting high intra-assay consistency.

These findings demonstrate that 1 h of RCA-I incubation is sufficient to achieve stable and reproducible platelet–lectin interactions without compromising assay performance (Figure 3; Table S3). Table S3 summarises the comparison of RCA-I fluorescence after 1- and 3-h incubation across three independent assay runs performed on separate days using freshly collected donor platelet samples.

3.4 | Optimization of RCA-I volume per reaction

The effect of RCA-I volume per reaction well on platelet desialylation detection was evaluated under three conditions: untreated plasma (pooled healthy donors, reference control), neuraminidase-treated (NeuC), and β-lactose controls. Increasing volumes of RCA-I stock solution 0.1, 0.2, 0.3, 0.5, and 0.6 μL per reaction (tube) were tested to determine the concentration that provided optimal signal discrimination whilst maintaining analytical stability. Lower volumes yielded limited fluorescence separation, whereas higher volumes increased signal variability without improving group differentiation.

An intermediate volume of 0.3 μL per reaction provided the best balance between signal intensity and background separation, enabling reliable detection of platelet desialylation across all experimental conditions. No significant overall differences were observed between immediate and 24 h measurements when each condition was analysed over time ($p = 0.0625$), indicating short-term stability of the fluorescence signal. In contrast, the Kruskal–Wallis test demonstrated significant differences amongst the three conditions both immediately after preparation and after 24 h ($H = 9.98$, $p = 0.0068$). Post hoc analysis revealed significantly lower fluorescence in the β-lactose group compared with both the reference control and NeuC ($p < 0.05$), whereas no significant difference was detected between the reference control and NeuC groups. These findings confirm the assay's specificity, reproducibility, and sustained discriminatory capacity over time. An RCA-I volume of 0.3 μL per reaction was identified as the optimal condition, providing the best analytical stability and signal-to-noise discrimination (Figure 4; Table S4). Table S4 specifically addresses the short-term stability of the fluorescence signal after staining, comparing measurements obtained immediately after preparation with those acquired after 24 h under different RCA-I volumes and control conditions. This analysis evaluates post-staining signal stability rather than lectin incubation kinetics.

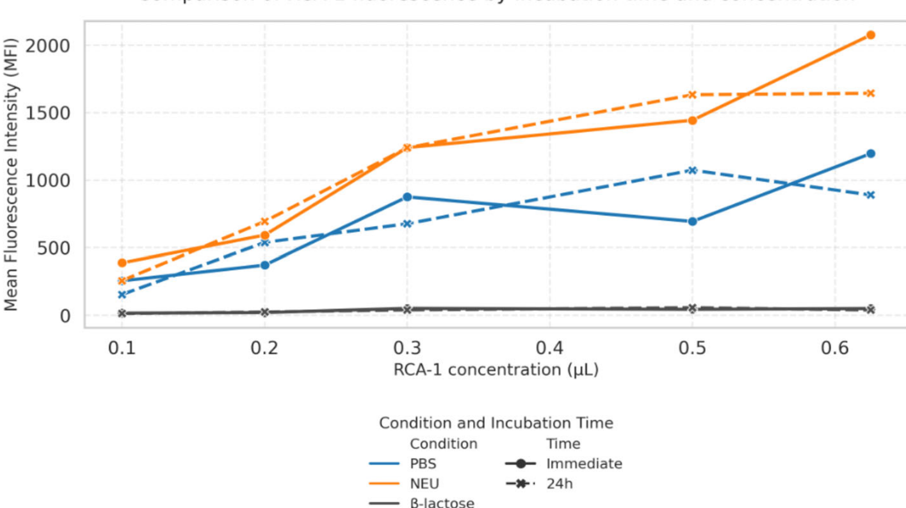


FIGURE 4 RCA-I fluorescence according to lectin concentration, incubation time, and experimental condition. Mean fluorescence intensity (MFI) increased in a concentration-dependent manner in both PBS and neuraminidase (NEU)-treated samples, with NEU consistently exhibiting the highest fluorescence signals, reflecting enhanced exposure of terminal β-galactose residues following enzymatic desialylation. In contrast, β-lactose-treated samples remained near baseline across all RCA-I concentrations, confirming effective inhibition of lectin binding. Comparison of immediate acquisition (solid lines) and 24-h post-staining acquisition (dashed lines) demonstrated higher MFI values after prolonged incubation, particularly in NEU-treated samples, whilst preserving the overall fluorescence pattern across experimental conditions.

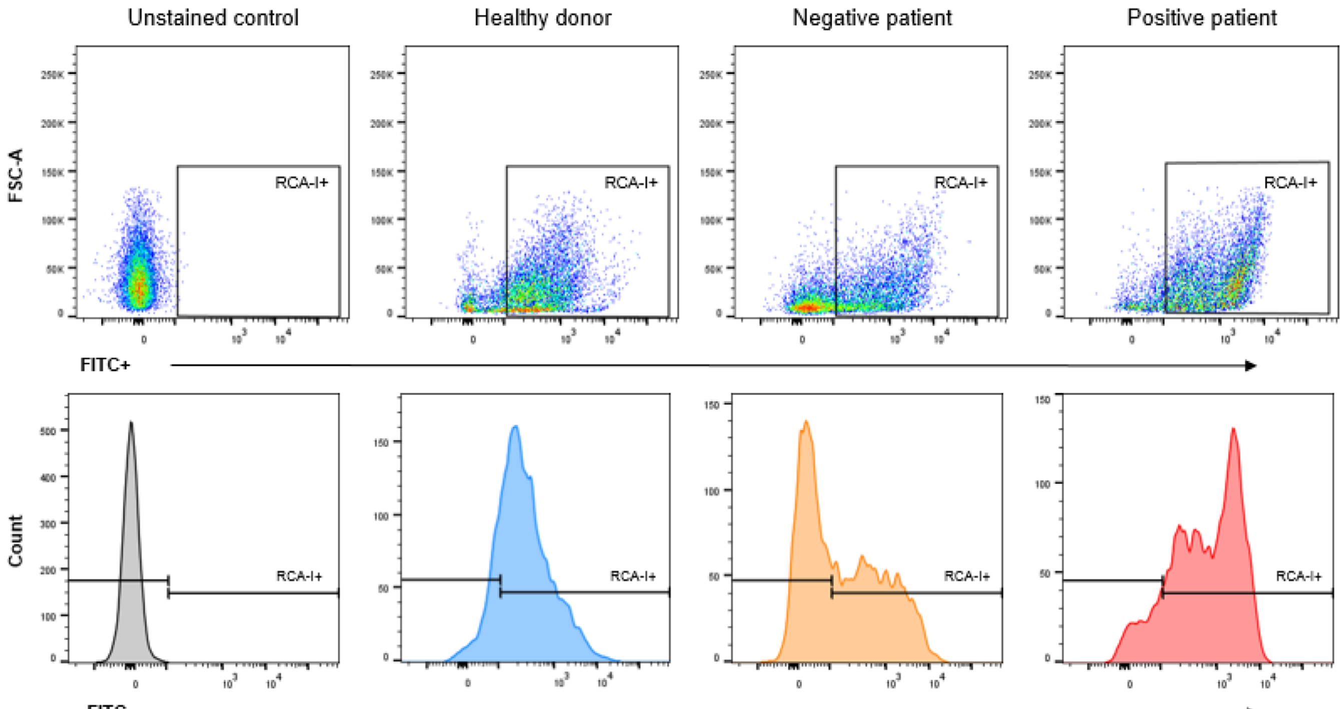
3.5 | Flow cytometric signature of platelet desialylation

Flow cytometric analysis of RCA-I binding, shown in Figure 5A, demonstrated distinct fluorescence patterns amongst the study groups. Unstained platelets (grey) were used to define the negative region. Healthy donors (blue) and PDT-negative patients (orange) exhibited comparable profiles, with a detectable basal proportion of RCA-I$^+$ platelets but without a pronounced rightward shift, indicating low to intermediate RCA-I binding within the expected assay background. In contrast, PDT-positive patients (red), as illustrated by the dot plots and histograms, showed a clear rightward shift, accompanied by a higher proportion of RCA-I$^+$ platelets and increased FITC fluorescence intensity (Figure 5B). These findings indicate greater RCA-I binding in the PDT-positive group, reflecting greater exposure of terminal β-galactose residues compatible with higher relative platelet desialylation under the standardised assay conditions.

3.6 | Clinical utility of PDT in inherited thrombocytopenias

A total of 15 patients with inherited thrombocytopenias were included, comprising 11 with Glanzmann thrombasthenia and four with Bernard–Soulier syndrome. The cohort was predominantly female (86.7%), with ages ranging from 18 to 56 years. Transfusion exposure was highly heterogeneous: platelet transfusions ranged from 0 to 521 units and RBC transfusions from 0 to 73 units. Patients with Glanzmann thrombasthenia exhibited greater transfusion requirements and a higher frequency of alloimmunization (63.6%) than those with Bernard–Soulier syndrome (25%) (Tables 2 and S5). Platelet alloimmunization was documented in 13% of patients, whereas RBC alloimmunization occurred in 40%, predominantly directed against Rh antigens, followed by anti-K, anti-Dia, anti-Cw, and cold autoantibodies. All RBC alloantibodies were identified in female patients.

PDT analysis revealed marked interindividual variability, with a mean MFI of 1105.8 a.u. (median 933.5) and a mean ratio of 2.624 (median 2.28). Two-thirds of patients were classified as PDT-positive (10/15; 66.7%; 95% CI: 41.7%–84.8%), whereas 26.7% (4/15) were negative PDT-negative and 6.7% (1/15) had inconclusive results (Tables 3 and S6). PIFT detected antiplatelet reactivity in 53.3% of cases, whereas the Luminex PAkLx assay identified specific HLA/HPA antibodies in 13.3%. As these assays assess different aspects of platelet immunoreactivity, their results were interpreted as complementary rather than directly comparable. Most PDT-positive patients were PIFT-positive but Luminex-negative, which may reflect antibody reactivities not detected by the HLA/HPA panel or alternative immune-mediated mechanisms beyond those identified by conventional alloantibody screening assays which may reflect antibody reactivity not detected by the specific HLA/HPA panel or other immune mechanisms beyond those identified by conventional alloantibody screening assays. Statistical analysis demonstrated significantly higher desialylation markers in PDT-positive samples; the patient with an inconclusive result was excluded from the statistical analysis. Both MFI and ratio values were significantly higher in PDT-positive (10/15) than in PDT-negative patients (4/15) (Mann–Whitney $U = 40.0$; $p = 0.002$ for both). A strong positive correlation was observed between MFI and ratio values (Spearman $\rho = 0.877$; $p < 0.0001$) (Table 4). For interpretation of results in this patient group, the PDT was designed to assess



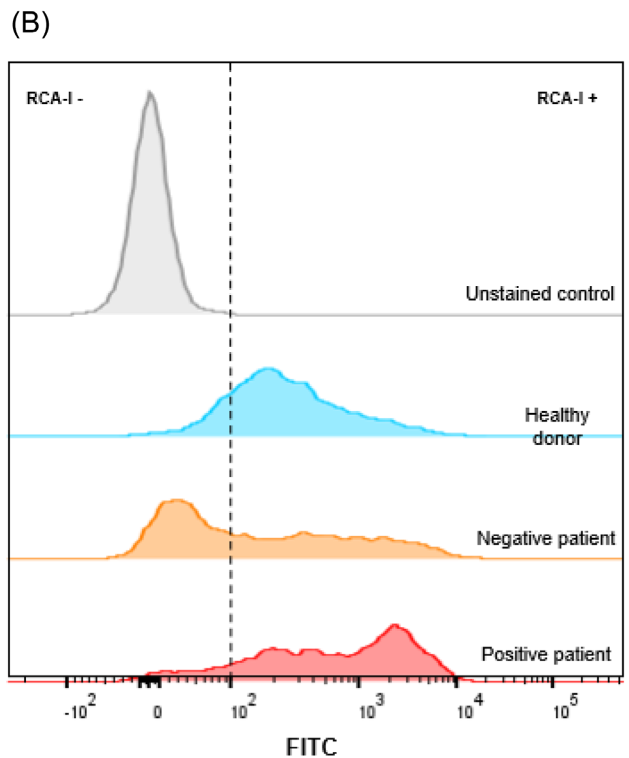


FIGURE 5 Legend on next page.

whether circulating plasma factors are capable of inducing platelet desialylation.

PDT-positive: results indicate increased exposure of terminal β-galactose residues, suggesting the presence of an additional plasma-mediated platelet clearance mechanism.

PDT-negative: results indicate the absence of relevant desialylation under the experimental conditions.

PDT-inconclusive: the inconclusive sample was excluded from comparative statistical analyses to avoid potential.

Despite the limited cohort size, reflecting the rarity of these disorders, the high frequency of PDT-positive results and their association with transfusion exposure support the biological relevance of the assay. Collectively, these findings indicate that platelet desialylation is common in inherited thrombocytopenias and that the PDT can detect clinically relevant platelet alterations that may not be identified by conventional antibody detection assays.

3.7 | Practical performance and cost of the PDT

The standardised PDT protocol required an overall assay time of approximately 4 h, with fluorescence measurements obtained from the acquisition of 10 000 platelet events. Using the predefined ratio thresholds (negative <1.30; inconclusive 1.30–1.45; positive >1.45), the assay reliably distinguished desialylated from non-desialylated platelet profiles. Furthermore, the method demonstrated a low operational cost, with an estimated expense of US$3.62 per test (Tables S7 and S8).

4 | DISCUSSION

In this study, we standardised and analytically validated a modified PDT, developed as a functional in vitro assay to investigate platelet desialylation as a potential mechanism of platelet clearance in thrombocytopenic disorders. The assay was designed to reproduce, under standardised experimental conditions, a biologically relevant interaction between circulating plasma factors and platelet targets. Using donor platelets as a controlled substrate, the modified PDT assesses whether patient plasma contains soluble factors capable of inducing exposure of terminal β-galactose residues, detected through increased RCA-I binding and consistent with platelet desialylation. These circulating factors may include platelet-reactive antibodies, immune complexes, inflammatory mediators, transfusion-related immune stimuli, or other plasma components associated with platelet activation and glycan remodelling. Furthermore, the assay was designed to distinguish physiological baseline lectin binding from increased desialylation resulting from enzymatic activity or immune-mediated mechanisms.

TABLE 2 Clinical characteristics of patients with hereditary thrombocytopenia.

Characteristics	Total	Female ($n = 13$)	Male ($n = 2$)
Age, years, median [IQR]	36 [24–45]	36 [25–47]	23–43
Ethnicity			
Caucasian	11 (73%)	10	1
African descent	4 (27%)	3	1
Platelet transfusions (*n*)	1153	591	526
Median [IQR]	14 [4–120]	NA	NA
RBC transfusion (*n*)	189	116	73
Platelet alloimmunization (*n*)	2 (13%)	2	0
Anti-HLA	1	1	0
Anti-HPA (Anti-GPIIb)	1	1	0
RBC alloimmunization	6 (40%)	5	1
Rh system (D, C, E, c)	4	–	–
Kell system (K)	1	–	–
Others (Dia, Cw)	2	–	–
Cold autoantibodies	2	–	0

FIGURE 5 Flow cytometric assessment of platelet desialylation by RCA-I binding. (A) Representative flow cytometry dot plots (FSC-A vs. FITC fluorescence) illustrating the binding of FITC-conjugated Ricinus communis agglutinin I (RCA-I) to platelets. Unstained platelets (grey) were used to define the negative fluorescence region. Healthy donors (blue) and PDT-negative patients (orange) exhibited comparable fluorescence profiles, with detectable RCA-I-positive platelets but no substantial increase in fluorescence intensity. In contrast, PDT-positive patients (red) displayed an expanded RCA-I-positive platelet population, consistent with increased exposure of terminal β-galactose residues. (B) Representative FITC fluorescence histograms corresponding to the samples shown in panel A. PDT-positive patients demonstrated a marked rightward shift in fluorescence intensity compared with healthy donors and PDT-negative patients, indicating increased RCA-I binding and enhanced platelet desialylation.

As such, it provides a complementary tool for investigating non-classical pathways of platelet clearance.

Platelet clearance has traditionally been viewed as a splenic Fc-receptor-mediated process. However, accumulating evidence indicates that desialylated platelets are preferentially cleared in the liver through Ashwell–Morell receptor and likely other hepatic surface receptors, with contributions from Kupffer cells and hepatocytes.[23–26,30,31,45,50,51]

This alternative mechanism of platelet clearance has been increasingly recognised in immune thrombocytopenia and may contribute to suboptimal responses to conventional first-line therapies, including corticosteroids, intravenous immunoglobulin (IVIG), splenectomy, and even HLA/HPA-compatible platelet transfusions. Collectively, these findings support the hypothesis that antibody-mediated platelet desialylation constitutes a clinically relevant pathway underlying therapeutic refractoriness, particularly in patients harbouring anti-GPIbα antibodies.[12,14,22,24,30,51–55]

The modified PDT was conceptually inspired from the PIFT, originally described by Von dem Borne,[48] as well as by platelet desialylation assays developed by Li et al.,[24] Tao et al.,[22] and Lasne et al.[49] Whilst PIFT primarily detects Fc-mediated IgG binding to platelet surface antigens, the modified PDT assesses a downstream functional consequence of platelet injury by measuring glycan remodelling through FITC-conjugated Ricinus communis agglutinin I (RCA-I) binding. This distinction approach enables the detection of Platelet alterations that may not be identified by conventional antibody-based assays, including Fc-independent, antibody-independent, or mixed immune mechanisms involved in platelet clearance.

During assay development, several methodological parameters were systematically optimised to ensure analytical robustness, reproducibility and feasibility for routine laboratory implementation. Platelet age emerged as a critical variable, as desialylation progressively increases during storage, supporting the use of freshly collected platelets (<24 h) to minimise background glycan alterations and preserve assay sensitivity.[19,45,56–61]

Furthermore, variations in incubation time did not significantly affect fluorescence measurements, indicating the stability of the assay within the evaluated conditions. Optimization experiments also demonstrated that an RCA-I stock solution volume of 0.3 μL per reaction provided the most favourable signal-to-noise ratio whilst maintaining reagent economy. Although baseline fluorescence values varied amongst platelet donors, this variability appeared to reflect biological heterogeneity rather than analytical instability, supporting the use of pooled donor plasma as a normalisation reference to improve assay standardisation and inter-assay comparability.

Analytical validation demonstrated the expected responses to both enzymatic desialylation and inhibition controls, confirming the functional performance and specificity of the assay. Notably, antibody-positive refractory samples exhibited higher desialylation-associated signals than untreated controls, suggesting that circulating plasma factors may promote platelet surface glycan remodelling beyond the direct effects of antibody binding alone. These observations support the concept that platelet desialylation is not driven by a single pathogenic mechanism but rather reflects a complex interplay amongst immune-mediated processes, platelet activation, and glycan remodelling pathways. Collectively, these findings reinforce the emerging view that platelet clearance in thrombocytopenic disorders is a multifactorial process involving both Fc-dependent and Fc-independent mechanisms.

A particularly important finding of this study was the application of the modified PDT in inherited thrombocytopenias, specifically Glanzmann thrombasthenia and Bernard–Soulier syndrome. These rare disorders are often characterised by lifelong transfusion exposure and an increased risk of alloimmunization.[4] Notably, a substantial proportion of patients demonstrated positive modified PDT results despite negative HLA/HPA antibody testing.

The observed discordance amongst modified PDT, PIFT, and Luminex findings suggests that platelet alterations in inherited thrombocytopenias may not be fully explained by conventionally detectable

TABLE 3 PDT, PIFT, and Luminex PAKLx results in hereditary thrombocytopenias.

A. PDT—Continuous variables (MFI and ratio)			
Parameter		**MFI (a.u.)**	**Ratio**
Mean		1105.8	2.624
Median		933.5	2.28
Std. deviation		831.2	1.51
B. Frequency distribution and 95% CI			
PDT result	***n***	**Proportion (%)**	**95% CI (Wilson)**
Positive	10	66.7	41.7%–84.8%
Negative	4	26.7	10.9%–51.9%
Inconclusive	1	6.7	1.2%–29.8%
C. PIFT result			
Positive	8	53.3	30.1%–75.2%
Negative	7	46.7	24.8%–69.9%
D. Luminex PAKLx result			
Positive	2	13.3%	3.7%–37.9%
Negative	13	86.7%	62.1%–96.3%

TABLE 4 Statistical analysis of PDT performance.

Analysis	Groups/variables compared	Test	Statistic	*p*-Value	Interpretation
MFI: PDT+ versus PDT−	PDT+ ($n = 10$) versus PDT− ($n = 4$)	Mann–Whitney *U*	$U = 40.0$	0.002	Significant; MFI higher in PDT+
Ratio: PDT+ versus PDT−	PDT+ ($n = 10$) versus PDT− ($n = 4$)	Mann–Whitney *U*	$U = 40.0$	0.002	Significant; Ratio higher in PDT+
MFI × Ratio	Continuous variables	Spearman correlation	$\rho = 0.877$	0.0001	Strong positive correlation

alloantibody responses. Alternative mechanisms may include low-affinity or low-titre antibodies below the detection threshold of current assays, antibodies targeting non-canonical epitopes, immune complexes, or chronic platelet activation resulting from cumulative transfusion exposure. These processes may converge to promote platelet glycan remodelling and desialylation, thereby facilitating platelet clearance through both Fc-dependent and Fc-independent pathways.

Accordingly, the modified PDT provides complementary mechanistic information that is not captured by conventional antibody-based assays. Whilst traditional immunohaematological methods primarily detect antigen-specific immune responses, the modified PDT evaluates the functional susceptibility of platelets to desialylation-associated clearance. Because desialylated platelets are preferentially removed through hepatic receptor-mediated pathways, including the Ashwell–Morell receptor system, rather than through classical splenic Fcγ receptor-dependent mechanisms, identification of this pathway may improve the interpretation of transfusion failure. More broadly, assessment of platelet desialylation may facilitate a mechanism-oriented understanding of refractoriness and help inform individualised therapeutic strategies.

The classification thresholds were defined to capture the continuous nature of platelet desialylation whilst accommodating the analytical variability inherent to biological assays. Results below the lower cutoff were interpreted as falling within the range of physiological variability, whereas values exceeding the upper threshold were considered suggestive of clinically meaningful desialylation activity. Intermediate values constituted an indeterminate zone, reflecting the overlap between biological heterogeneity and measurement uncertainty. Therefore, results within this range should be interpreted cautiously and in conjunction with the broader clinical and laboratory context.

Several technical limitations should be acknowledged. The modified PDT relies on freshly collected platelets (<24 h), as storage-associated glycan remodelling and spontaneous desialylation may alter lectin binding patterns and influence assay results. Moreover, fluorescence intensity measurements are inherently dependent on flow cytometer characteristics, acquisition settings, and analytical platforms, necessitating local assay validation and laboratory-specific cutoff determination. Finally, the biological heterogeneity observed amongst platelet donors underscores the importance of using pooled reference plasma for normalisation, which may enhance reproducibility and reduce variability attributable to individual donor characteristics.

Autologous platelet testing was not feasible because of the limited availability of platelets in thrombocytopenic patients and the inherent variability associated with severely reduced platelet counts. To ensure assay standardisation and reproducibility, healthy donor platelets were employed as a uniform biological substrate to simulate, under controlled in vitro conditions, the interaction between circulating plasma factors and platelet targets. As a result, the modified PDT evaluates the capacity of patient plasma to induce platelet desialylation rather than directly measuring desialylation on autologous platelets. Consequently, the assay should be interpreted as a functional assessment of plasma-mediated pro-desialylation activity. Whilst this approach does not directly demonstrate desialylation occurring in vivo, it may reveal biologically relevant mechanisms capable of promoting platelet glycan remodelling and subsequent clearance.

An additional limitation concerns the interpretation of negative platelet antibody results. The lack of detectable antibodies should not be considered evidence of their absence, as clinically relevant antibodies may target antigenic specificities not assessed by the available assays or may be present at low titers below the analytical sensitivity of the methods used. Moreover, immunoglobulin depletion experiments were not performed on refractory plasma samples, precluding a direct assessment of the specific contribution of antibody-mediated mechanisms to the induction of platelet desialylation. As a result, the relative roles of antibodies and other circulating plasma factors in promoting glycan remodelling could not be fully delineated. Accordingly, the results should be interpreted in light of the complex and multifactorial nature of platelet refractoriness, which likely involves the interaction of immune-mediated, inflammatory, and non-immune pathways.

Finally, the modified PDT demonstrated practical feasibility, characterised a short turnaround time, compatibility with standard flow cytometry platforms, and low operational cost, supporting its potential applicability in specialised laboratories. Nevertheless, multicenter studies and external validation will be required before its incorporation into routine clinical practice.

In summary, the modified PDT represents a reproducible functional assay capable of detecting platelet desialylation across a broad spectrum of thrombocytopenic conditions. Rather than replacing conventional antibody-based assays, it provides complementary mechanistic information by offering biological insight into platelet clearance pathways and contributing to a more comprehensive understanding of thrombocytopenia. Moreover, the assay demonstrated excellent cost-effectiveness, with an estimated operational cost of only US $3.62 (United States dollars) per test, further supporting its feasibility for routine laboratory implementation. Collectively, these findings suggest that the modified PDT may serve as a valuable adjunctive tool for investigating desialylation-associated platelet clearance and improving the mechanistic characterisation of thrombocytopenic disorders.

5 | CONCLUSIONS

The modified PDT represents a practical, reproducible, and cost-effective functional assay for the assessment of platelet desialylation. By providing insight into platelet clearance pathways that are not readily detected by conventional immunohaematological methods, the PDT complements existing diagnostic approaches and expands the biological understanding of thrombocytopenic disorders. Its ability to identify desialylation-associated platelet clearance may support a more comprehensive evaluation of patients with immune and non-immune thrombocytopenia and contribute to mechanism-informed clinical decision-making.

AUTHOR CONTRIBUTIONS

K.N.C.Z. was responsible for the experimental procedures, statistical analysis, and manuscript drafting. T.C.S.S. contributed to the statistical analysis. M.D., M.C.A.V., J.V.B.O., and A.C. provided support during the experimental phase of the study. F.A.R. prepared the flow cytometry figures. C.G., P.V., V.R., and A.M.J. contributed to the clinical aspects of the study. E.M. and J.B. assisted with manuscript preparation and approved the final version for submission. C.L.D. and L.D. contributed to the study conception and design, experimental execution, statistical analysis, manuscript preparation, and critical revision of the manuscript. All authors reviewed and approved the final version of the manuscript.

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CONFLICT OF INTEREST STATEMENT

The authors have no competing interests.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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