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A design for an efficient functional panel that determines platelet exhaustion levels to differentiate responder and non-responder ITP patients

Nazanin Heidari[1,5], Ehteramolsadat Hosseini[1,5], Mohammad Faranoush[2], Seyed Mohammad Sadegh Pezeshki[1], Amir Teimourpour[3], Elizabeth E. Gardiner[4] & Mehran Ghasemzadeh[1]✉

Immune thrombocytopenia (ITP) is marked by decreased platelet counts and an increased bleeding risk, while functional platelet exhaustion may also underlie its clinical variability. This study investigates whether platelet functional competence can help differentiate "responder" (R) from "non-responder" (NR) ITP patients. Platelets obtained from 28 chronic ITP patients and 20 healthy controls, either in resting condition or in the presence of PMA, were subjected to flow cytometry, where the levels of platelet activation markers (P-selectin expression, PAC-1 binding) and reactive oxygen species (ROS) were assessed. In ITP, baseline levels of P-selectin and PAC-1 were higher compared to healthy controls, while their levels in response to PMA were significantly lower ($p<0.05$). Baseline ROS also showed a significant difference between ITP and controls, and ROS generation in response to PMA was reduced in ITP compared to controls ($p<0.05$). "NR" exhibited elevated basal P-selectin and diminished PMA-induced activation while bleeding scores showed no correlation with basal P-selectin. ROC curve analysis and multiple logistic regression models identified PMA-induced ROS and P-selectin responses as strong predictors of bleeding severity and treatment response in ITP. Panel analysis, including platelet activation markers and ROS generation in response to PMA, successfully distinguished the disease status with an adjusted AUC of 92 (95%CI:80.7–100), an optimum cut-off value of 0.228, sensitivity of 87.5%, and specificity of 85.0%. A novel composite index of platelet functional analysis was developed to overcome limitations of conventional assessment of ITP status. This multifactor panel accurately reflects platelet functional state while helping to distinguish between responder and non-responder patients. The panel could offer a promising tool for improved diagnosis, prognosis, and individualized patient management.

Keywords Immune thrombocytopenia (ITP), PAC-1 binding, Platelet activation, Platelet function tests, P-selectin, Reactive oxygen species, Thrombosis

Abbreviations

AUC	Area Under the Curve
BS	Bleeding Score
CCCP	Carbonyl Cyanide m-Chlorophenylhydrazone

[1]Blood Transfusion Research Center, High Institute for Research and Education in Transfusion Medicine, Iranian Blood Transfusion Organization Building, Hemmat Exp. Way, Next to the Milad Tower, Tehran, Iran. [2]Pediatric Growth and Development Research Center, Institute of Endocrinology and Metabolism, Iran University of Medical Sciences, Tehran, Iran. [3]Biological Products and Blood Safety Research Center, High Institute for Research and Education in Transfusion Medicine, Tehran, Iran. [4]Division of Genome Science and Cancer, John Curtin School of Medical Research, The Australian National University, Canberra, ACT, Australia. [5]These authors contributed equally to this work: Nazanin Heidari, Ehteramolsadat Hosseini, Elizabeth E. Gardiner and Mehran Ghasemzadeh. ✉email: mehran1476@yahoo.com

CRP	Collagen-Related Peptide
FITC	Fluorescein Isothiocyanate
FSC	Forward Scatter
GP	Glycoprotein
GSH	Glutathione
H2DCFDA	2',7'-Dichlorodihydrofluorescein Diacetate
ITP	Immune Thrombocytopenia
LZD	Linezolid
MAPK	Mitogen-activated protein kinase
MDA	Malondialdehyde
MFI	Mean Fluorescence Intensity
MPV	Mean Platelet Volume
NOX	NADPH oxidases
NPC	Normal Platelet Count
NR	Non-Responders
PE	Phycoerythrin
PDW	Platelet Distribution Width
PKC	Protein kinase C
PLT	Platelet
P-LCR	Platelet Larger Cell Ratio
PMA	Phorbol 12-Myristate 13-Acetate
PRP	Platelet-Rich Plasma
R	Responders
ROS	Reactive Oxygen Species
ROC	Receiver Operator Characteristic
TPO-RA	Thrombopoietin Receptor Agonists
TRAP	Thrombin Receptor Activating Peptide

Immune thrombocytopenia (ITP) is an autoimmune disorder characterized by a decrease in platelet (PLT) count, which can lead to an increased risk of bleeding[1]. The pathophysiology of ITP is complex and involves multiple contributing factors, including immunological disorders, bone marrow suppression, and platelet activation and clearance[2–4]. Platelet destruction and premature clearance are important factors that develop thrombocytopenia in these patients[5]. Whereas a definitive cure for this disease remains elusive, various supportive treatments are employed to manage bleeding risks, with corticosteroids and thrombopoietin receptor agonists (TPO-RAs) being the most frequent[6–9]. Given the responses to therapeutic approaches, ITP patients can be diagnosed as either "Responder, R" (with the PLT count $\geq 30 \times 10^3/\mu L$ or at least a twofold increase from the baseline count and no bleeding symptoms) and "Non-responder, NR" (with the PLT $< 30 \times 10^3/\mu L$, or less than a twofold increase from the baseline count, or presence of bleeding symptoms) states[10,11].

Based on their activation status, platelets exhibit distinct phenotypes, including pro-aggregatory, pro-inflammatory, and procoagulant profiles which are also observed in patients with ITP frequently demonstrated by PAC-1 binding (antibody against active $\alpha_{IIb}\beta_3$ integrin), P-selectin expression and phosphatidylserine exposure, respectively. However, despite extensive investigation, the relationship between platelet counts and its phenotypic alterations in the context of ITP has yielded inconclusive findings[4,12–15]. Similar efforts exploring the association between basal platelet activity and premature clearance with clinical manifestations of ITP have also reported inconsistent and often contradictory results[4,13,15,16]. These studies have yet to identify a definitive single platelet biomarker or panel-based diagnostic tool with sufficient reliability for prognostic or diagnostic utility.

A clinically evident, yet under-characterized, facet of ITP pathophysiology is the frequent presence of platelet functional incompetence[3,4]. This dysfunction may reflect a state of platelet exhaustion—a condition wherein platelets exhibit diminished responsiveness despite adequate circulating numbers[17–21]. Determining if this functional exhaustion is linked to disease activity or treatment response continues to be a significant gap in ITP research. To address this, our study explores platelet responsiveness to phorbol 12-myristate 13-acetate (PMA). This agonist is an analog of diacylglycerol (DAG) that has the ability to recruit and activate protein kinase C (PKC). Activated PKC induces platelet granule release, NOX activation, and the enhancement of signaling pathways (inside-out signaling as well as RhoA and mitogen-activated protein kinase (MAPK) signaling pathways) leading to activation and clustering of integrin αIIbβ3, all examined here by P-selectin expression, reactive oxygen species (ROS) generation and PAC-1 binding assays, respectively[22–24]. PMA is uniquely suited for such an investigation due to its ability to engage a broad spectrum of platelet activation pathways simultaneously[25]. This makes it a robust surrogate stimulus for assessing global platelet functional capacity and exhaustion[26,27]. The study assessed key PMA-induced platelet functional responses—including α-granule release, integrin αIIbβ3 activation, and intracellular ROS generation—using flow cytometric markers such as P-selectin expression, PAC-1 binding, and ROS-sensitive fluorescent probes, respectively. These parameters were analyzed as a composite panel to characterize the functional baseline and activation potential of platelets in ITP patients. Given PMA's comprehensive mechanism of action, surpassing the selective engagement of traditional physiological agonists, we propose it as a superior agent for evaluating platelet functionality in ITP. This study represents the first attempt to establish a cost-effective and practical panel analysis designed to quantify platelet functional exhaustion. We anticipate that this assay could not only delineate between "R" and "NR" disease states but also offer prognostic insight into patient trajectories.

Materials and methods

Patients

Whole blood samples for this study were collected from patients diagnosed with primary ITP (ITP without an identifiable secondary cause)[28] who were aged between 10 and 40 years and were under the supervision of a subspecialist physician and referred to the oncology clinic between September 2024 and April 2025. All patients met the established diagnostic criteria for chronic ITP, which included patient's history, physical examination, persistent isolated thrombocytopenia lasting more than 12 months, normal or elevated megakaryocyte numbers in bone marrow biopsies, a normal spleen size, and the absence of other underlying causes for thrombocytopenia. In order to ensure consistency in evaluating treatment response, baseline platelet count was defined as the earliest available measurement before initiation of any ITP-specific treatment. The platelet count used to define treatment response and categorize patients as 'Responder' or 'Non-responder' was obtained at the same time as blood sampling for functional analysis, ensuring that platelet function markers reflected the patient's current clinical state. Control samples were obtained from age- and sex-matched healthy volunteers. To compute Informed consent was obtained from all participants. Informed consent was obtained from all participants, and if some of them were minors, these consents were obtained from their parents. The study was conducted in accordance with the Declaration of Helsinki, and this research received ethical approval from the Ethics Committee of the High Institute for Research and Education in Transfusion Medicine located in Tehran, Iran (Ethics approval number: IR.TMI.REC.1402.024).

Reagents and materials

Fluorescein isothiocyanate (FITC)- and phycoerythrin (PE)-conjugated mouse IgG1 isotype controls were sourced from (BD Biosciences, USA). The human monoclonal PE Anti-CD41 (ExBio, Czech), FITC Anti-CD41 (BD Biosciences, USA), PE Anti-CD42b (ExBio, Czech), FITC mouse Anti-Human CD62P (BD Biosciences, USA) and FITC mouse Anti- PAC-1, activation-specific integrin αIIbβ3 (BD Biosciences, USA) were also used. dichlorodihydrofluorescein-diacetate was purchased from Cayman Chemical (Cayman Chemical, AnnArbor, MI, USA). All other chemicals and reagents were procured from Sigma-Aldrich. Platelet counts, along with mean platelet volume (MPV), platelet distribution width (PDW) and platelet larger cell ratio (P-LCR) were quantified using a Sysmex XE-2100 instrument (Sysmex, Milton Keynes, UK).

Sample preparation

Peripheral blood samples were obtained using 3.2% sodium citrate Vacutainer tubes, with each participant providing 6 mL of blood and processed via centrifugation at 150 g for 10 min at room temperature to isolate platelet-rich plasma (PRP). The platelet concentration in each PRP sample was determined using the Sysmex XE-2100 instrument. All PRP preparations were incubated at 37 °C for a minimum of 30 min to allow platelet resting before flow cytometry analysis.

Flow cytometry to determine the levels of P-selectin expression and PAC-1 binding

Platelet counts for flow cytometry analysis were adjusted to 2×10^7/mL using Tyrode buffer (10 mM HEPES, 12 mM $NaHCO_3$, 137 mM NaCl, 2.7 mM KCl, 5 mM glucose, and 1 mM $CaCl_2$; pH 7.2–7.4). PRP (2×10^7/mL) were incubated with Anti-P-selectin (CD62P) or PAC-1 antibodies (with the designated amount recommended by manufacturer) for 45 min at 37 °C following the manufacturer's recommended dosage, without agonists and after in vitro exposure to 200 nM PMA. After incubation, the cells were fixed in 1% paraformaldehyde in PBS and subsequently analyzed using a flow cytometer (Partec GmbH, Germany) to get a total of 20,000 platelets. Data acquisition was optimized by adjusting the forward and side scatter channels, as well as fluorescence channels FL1 and FL2. Logarithmic amplification was applied to all four detectors. Platelet identification was confirmed by gating based on forward and side scatter properties, with CD41 and CD42b expression used as markers (supplementary Figure S1). The mean fluorescence of platelets expressing P-selectin above background levels (measured with isotype controls) was calculated, and also PAC-1 binding was assessed by mean fluorescence intensity (MFI). Data analysis was performed using FlowJo software (Tree Star Inc., OR, USA).

Analysis of intraplatelet reactive oxygen species (ROS) generation

To assess the production of reactive oxygen species (ROS), washed platelets (at a concentration of 10^7 cells/mL) were incubated with 10 µM dye 2',7'-dichlorodihydrofluorescein diacetate (H2DCFDA), a cell-permeable probe for detecting intracellular ROS. Platelets were exposed to H_2DCFDA with or without 200 nM PMA and 200 µM carbonyl cyanide m-chlorophenylhydrazone (CCCP) for ROS detection. Following incubation, samples were analyzed using the CyFlow® Space flow cytometer, acquiring 20,000 platelet events. Flow cytometric settings were optimized for platelet acquisition, using logarithmic amplification across all four detectors. Gating was performed to isolate intact platelet populations, defined by forward and side scatter properties and confirmed by CD41 and CD42b expression. The mean fluorescence of platelets exhibiting H_2DCFDA fluorescence, relative to the background (negative control), was recorded. Data were analyzed using FlowJo software.

Statistical analysis

To summarize quantitative variables, mean, standard deviation, median, and range were reported, and for qualitative variables, frequency along with percentage was reported. The normality assumption was evaluated by the Shapiro-Wilk test. Independent samples t-test or Mann-Whitney test was used to compare two independent samples based on the Shapiro-Wilk test. The Chi-square test was used to evaluate the association between two qualitative variables. In order to evaluate the performance of each parameter in identifying treatment response status, bleeding score status, and platelet count status, receiver operator characteristic (ROC) analysis was performed, and the AUC index was reported. The Youden index method was used to find the optimum cut-off

value, and its associated sensitivity and specificity were calculated. The adjusted AUC index was computed by the logistic regression model. For model building, we considered significant variables at 0.05 in univariate analysis. In addition, sensitivity analysis was performed by considering all potential factors for multiple logistic regression and the obtained results were reported in supplementary Table S1. All statistical analyses were performed in R software (version: 4.4.1) using the pROC package for conducting ROC analysis and the GraphPad Prism software (GraphPad Software, Inc., San Diego, CA).

Results

Patients and controls

This cross-sectional study involved 36 patients diagnosed with chronic ITP (persistent isolated thrombocytopenia lasting more than 12 months). Out of these, two patients declined to participate, and six were not within the specified age range, resulting in a final study group of 28 patients. The patients involved in the study were aged from 10 to 40 years, including 11 males (39.3%) and 17 females (60.7%). Additional demographic and clinical features of the ITP cohort were as follows: A history of bleeding manifestations was present in 64% of patients (n = 18), which included petechiae, ecchymoses, and mild mucosal bleeding; prior treatments showed that 8 patients (28.6%) were treatment-naïve, 14 (50%) were receiving TPO-RAs, and 6 (21.4%) had prior corticosteroid use; and ongoing treatment at the time of blood sampling included 8 patients (28.6%) receiving corticosteroids, 12 (42.8%) on thrombopoietin receptor agonists, and 8 (28.6%) under observation without active treatment (Table 1). Patients were stratified into two bleeding score (BS) groups based on the World Health Organization (WHO). Individuals with BS1 were asymptomatic for bleeding (corresponding to WHO bleeding score ≤ 1), while those with BS2 experienced minor symptoms such as bruising or occasional epistaxis, with little or no interference with daily living (WHO bleeding score 2)[30]. According to platelet count and BS, patients were divided into subgroups: "Responder, R" (with the PLT count ≥ 30 × 103/μL or at least a twofold increase from the baseline count and no bleeding symptoms) and "Non-responder, NR" (with the PLT ˂ 30 × 103/μL, or less than a twofold increase from the baseline count, or presence of bleeding symptoms) states[10,11].

Twenty healthy control volunteers with comparable demographics were included in the study. Analysis of platelet indices in both the ITP and control groups revealed that platelet counts in patients were markedly lower than those of healthy controls, whereas mean platelet volume (MPV), platelet distribution width (PDW), and platelet large cell ratio (PLCR) exhibited significant increases in patients compared to control (Table 1). The correlation between platelet indices was examined in patients with ITP, revealing a significant inverse relationship between MPV and platelet count (p =.048, r= -.37). Additionally, a direct relationship was observed between MPV with PDW (p˂ 0.001, r=.61) and PLCR (p˂ 0.001, r=.97).

Variable	ITP	Control	*P*-value
Gender, n (%)			0.629
Male	11 (39.3%)	9 (45%)	
Female	17 (60.7%)	11 (55%)	
Age (y), Mean ± SD	20.68 ± 10.67	25.20 ± 10.20	0.108
Platelet indices, Mean ± SD			
PLT count (*10^3/ml)	73.61 ± 59.42	196 ± 36	< 0.001
MPV[13]	11.48 ± 1.17	9.3 ± 0.96	< 0.001
PDW[13]	14.77 ± 2.21	11 ± 1.5	< 0.001
PLCR (%)	35.08 ± 7.33	21 ± 6.5	< 0.001
Clinical findings of patients, n (%)			
Bleeding Score (BS)			
BS 1	10 (35.7%)		
BS 2	18 (64.3%)		
Prior treatment			
Treatment-naïve	8 (28.6%)		
TPORA	14 (50%)		
Corticosteroids	6 (21.4%)		
Splenectomy	1 (4%)		
Ongoing treatment at the time of sampling:			
Corticosteroids (prednisone/dexamethasone)	8 (28.6%)		
TPORA	12 (42.8%)		
No active treatment (watch and wait)	8 (28.6%)		

Table 1. The comparison of variables between ITP and control. BS, bleeding Score; MPV, mean platelet volume; PDW, platelet distribution width; P-LCR, platelet larger cell ratio; PLT, platelet; TPO-RA, thrombopoietin receptor agonists.

Comparison of platelet basal activation state and its functional competence in ITP patients versus controls

In patients with ITP, basal P-selectin levels were significantly higher (p =.002), whereas the P-selectin response to PMA was significantly lower compared to healthy controls (p =.048) (Fig. 1A). In addition, while baseline PAC-1 binding in ITP patients significantly raised compared to healthy controls (p =.003), integrin αIIbβ3 activation in response to PMA treatment (p˂ 0.001) was significantly diminished in ITP patients (Fig. 1B).

The baseline levels of ROS showed significant difference between individuals with ITP and healthy controls (p =.006), also, the response to PMA was notably reduced in ITP patients compared to controls (p˂ 0.001), whereas there was no significant different in ROS generation in response carbonyl cyanide m-chlorophenyl hydrazone (CCCP) (p =.057) between controls and patients (Fig. 1C).

Figure 2 demonstrates the representative dot plots and histograms for flowcytometry profiles of abovementioned platelet activation markers in a healthy control vs. an ITP patient. Figures 2A and C presents platelets scattergrams under the resting and PMA stimulated conditions of platelets, where PMA scattergrams show higher level of platelet activation in control vs. patient in addition a comparison between Fig. 2B and D indicates the superior platelet response to PMA in control vs. patient, where basal levels of platelet activation was higher in patient than control.

Comparison of platelet basal activation state and its functional competence in two groups of ITP patients with respect to platelet count

An analysis of the correlation between platelet counts and platelet activation markers, revealed an inverse relationship with basal P-selectin (r= -.4; P =.03) a direct correlation with basal ROS generation (r =.44; P = 002) with no relevance to baseline PAC-1 levels (Fig. 3A). On the other hand, platelet count was showed a significant direct correlation with P-selectin (r =.56; P =.006), PAC-1 binding (r =.62; P <.001) and ROS generation (r =.75; P <.001), all in response to PMA (Fig. 3B).

Considering a 30 × 10^3/μL count as an important cutoff point in patient management[10], further analysis was performed where patients were categorized into two groups based on platelet count: ≥30 and ˂30 × 10^3/μL (because a 30 × 10^3/μL count is important in patient management). The analysis of platelet function in these groups indicated that the baseline expression of P-selectin decreased as platelet count increased to some extent, with patients with platelet counts ≥ 30 × 10^3/μL showing a decreasing trend in P-selectin expression compared to those with platelet counts ˂ 30 × 10^3/μL. Notably, the expression of P-selectin significantly increased in response to PMA in the group with platelet counts ≥ 30 × 10^3/μL compared with patients with platelet counts ˂ 30 × 10^3/μL (Fig. 3C). The basal level of PAC-1 binding showed significantly increased levels in patients with platelet counts ˂ 30 × 10^3/μL compared to the group with platelet counts ≥ 30 × 10^3/μL. It is noteworthy that, patients with platelet counts ˂ 30 × 10^3/μL showed a declining trend in agonist-induced PAC-1 binding (Fig. 3D).

The examination of ROS expression indicated that with an increase in platelet counts, the baseline levels of ROS were also significantly elevated. Furthermore, ITP with platelet counts ˂ 30 × 103/μL showed a significantly diminished ROS response to PMA and CCCP agonists when compared to the group with platelet counts ≥ 30 × 10^3/μL (Fig. 3E).

Table 2 presents a platelet count-based categorization of ITP patients in relation to their platelet activation status. This categorization helps illustrate how platelet functionality, not just count, may influence bleeding risk. As shown, there were significant differences in platelet activation parameters [PAC-1 (Base); 2.1 ± 0.4 vs. 1.81 ± 0.24; P-value = 0.044; AUC, 76.19; cut-off value of 1.915, sensitivity of 85.7% and specificity of 85.7%], [ROS (Base); 1.4 ± 0.21 vs. 1.8 ± 0.54; P-value = 0.009; AUC, 70.7; cut-off value of 1.705, sensitivity of 47.6% and specificity of 100%], [P-sel (PMA); 3.11 ± 1.18 vs. 4.27 ± 1.52; P-value = 0.038; AUC, 76.9; cut-off value of 2.725/3.785, sensitivity of 90.4/57.1% and specificity of 61.9/85.7%], and [ROS (PMA); 4.96 ± 0.66 vs. 6.82 ± 1.67;

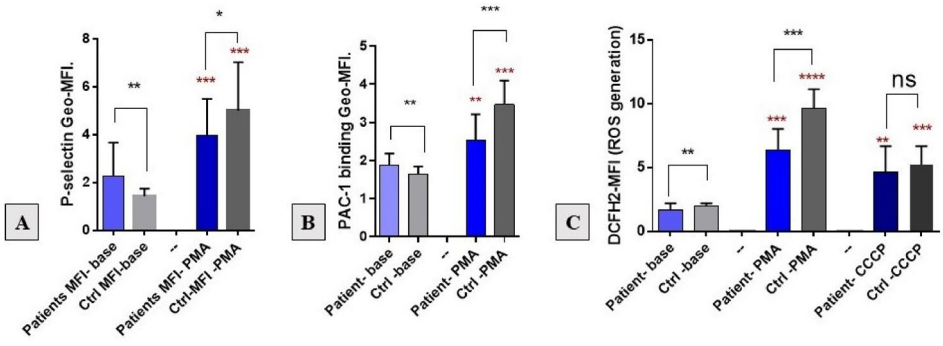

Fig. 1. Assessment of platelet basal activation state and its functional competence in ITP patients and controls. **(A)** P-selectin expression, **(B)** PAC-1 binding, **(C)** and ROS generation in individuals with ITP (blue) compared to healthy controls (gray) at baseline and after agonist treatment. Red asterisks show statistical differences of each agonist group vs., the related base group, while black asterisks illustrate statistical differences between ITP (n = 28) and control (n = 20). *P <.05, **P <.01, and ***P <.001. Data presented as mean ± SD. CCCP, carbonyl cyanide m-chlorophenylhydrazone; PMA, Phorbol 12-Myristate 13-Acetate; ROS, Reactive Oxygen Species.

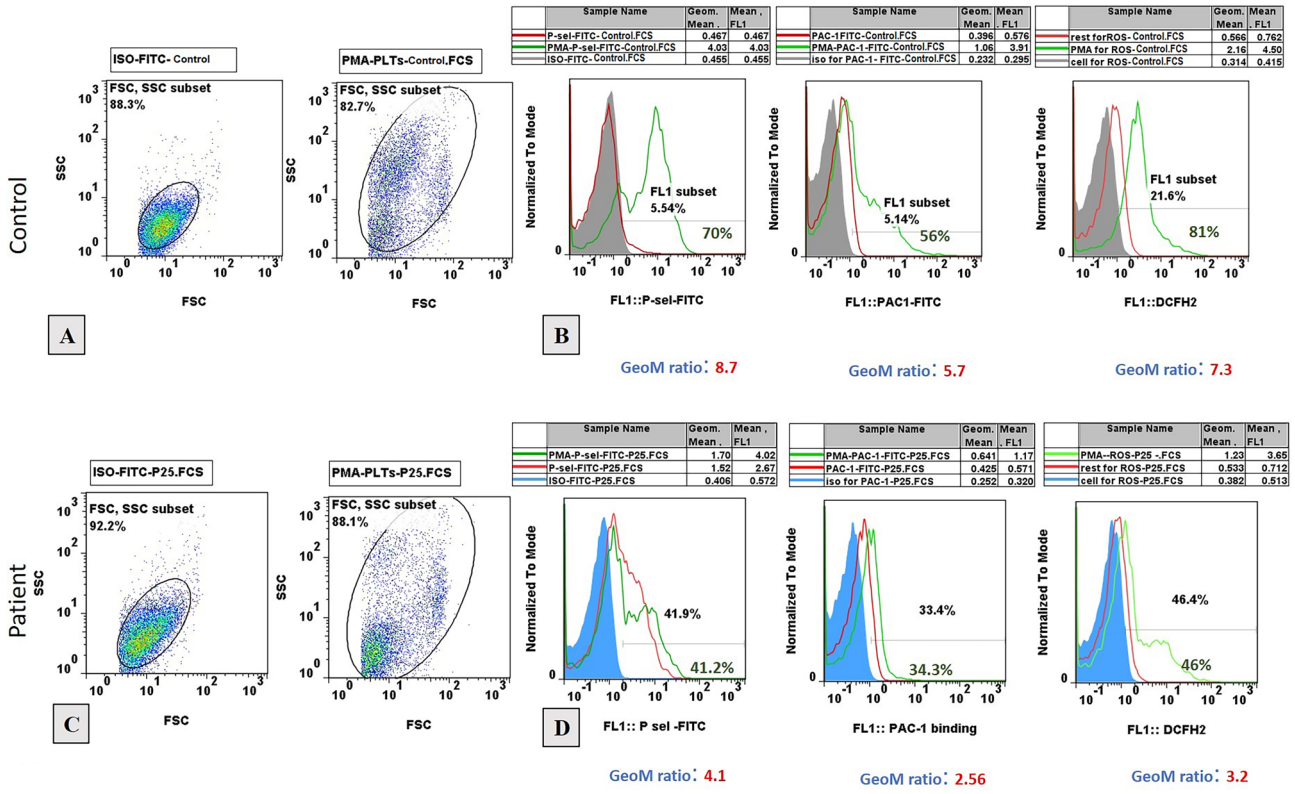

Fig. 2. Demonstrative dot plots and histograms of flow cytometry profiles for platelet activation markers in an ITP patient vs. a control. **(A)** shows dot plot scattergrams of resting and PMA activated platelets of control sample gated for flow cytometry analysis. **(B)** shows corresponding histograms of P-selectin expression, PAC-1 binding and DCFH2 expression (indicating ROS generation) with gray, red and green histograms are representative for isotype/cell alone, resting status of platelet and PMA induced platelet activation, respectively. **(C** and **D)** demonstrate similar abovementioned flowcytometric profiles for an ITP patient, where here the blue histograms are representatives for isotype/cell alone, resting status of platelet and PMA induced platelet activation. *Note: the green percentages show the positive gating for stimulated platelets, while the black ones identify the percentages positive gating events for resting platelets.*

P-value < 0.001; AUC, 87.4; cut-off value of 5.85, sensitivity of 71.4% and specificity of 100%] among ITP patients, based on platelet count less than $30 \times 10^3/\mu L$ and equal to greater than $30 \times 10^3/\mu L$. Furthermore, considering the parameters that were significant at the 0.05 level in univariate analysis, PAC-1 (Base), ROS (Base), P-sel (PMA), and ROS (PMA) (as the independent variables) and platelet count (as the dependent variable) were also subjected to a multiple logistic regression model, in which the calculated adjusted AUC was 97.3 (95%CI: 92.3–100, P-value < 0.001) with an optimum cut-off value of 0.715, sensitivity of 90.5 and specificity of 100 (Fig. 4A).

Comparison of platelet basal activation state and its functional competence in two groups of ITP patients categorized by BSs

Analysis of platelet counts in bleeding scores (BS) 1 and 2 indicated no significant differences between the two groups (Fig. 5A). Furthermore, there was no significant difference in the MPV, PLCR and PDW indices between the two groups of BS1 and BS2 (Fig. 5B).

An examination of baseline P-selectin levels revealed a not significantly different result in BS2 vs. BS1. However, BS2 exhibited a notable decrease in P-selectin response to the PMA when compared to BS1. In this regard, a direct comparison between both groups of BSs with their corresponding controls showed a significant increase in basal P-selectin and a notable decrease in P-selectin response to the agonist in BS2 (Fig. 5C). An assessment of baseline PAC-1 binding revealed no significant difference between the two BSs; however, after exposure to the agonist, participants in BS2 exhibited a trend reduction compared to the BS1 (Fig. 5D).

Basal ROS examinations also showed no significant different between BS1 and BS2; however, the response rates to the PMA in BS2 were notably lower than BS1. Research examining the response of ROS to CCCP did not yield significant different between BS groups (Fig. 5E).

Table 3 describes the BS in relation to platelet activation status among ITP patients. It highlights how variations in platelet activation may correlate with bleeding severity, regardless of platelet count alone. There were significant differences in platelet activation parameters [P-selectin (PMA); 3.38 ± 1.07 vs. 5.05 ± 1.65; P-value = 0.013; AUC, 81.4; cut-off value of 3.36, sensitivity of 61.1% and specificity of 100%], [ROS (PMA); 5.76 ± 1.32 vs.7.41 ± 1.8; P-value = 0.015; AUC, 78.3; cut-off value of 8.020/5.415, sensitivity of 100/50% and specificity of 50/100%]

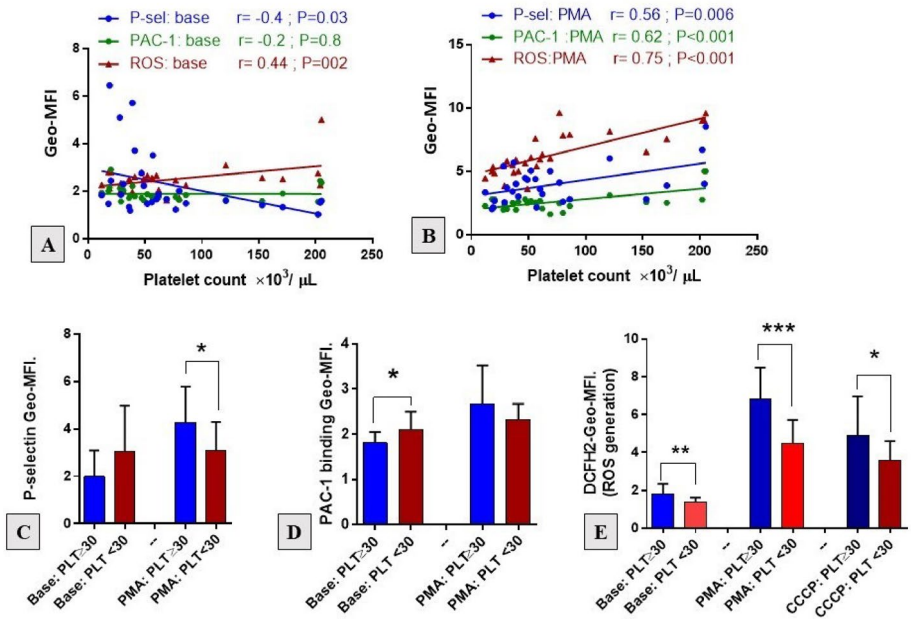

Fig. 3. Assessment of platelet basal activation state and its functional competence in ITP patients with respect to platelet count. **(A)** The correlation of platelet counts with P-selectin expression, (blue) PAC-1 binding, and (green) ROS generation (red) at baseline and **(B)** response to PMA states were analyzed in ITP patients ($n = 28$). Further, patients were divided into two PLT count groups (with a cutoff point of $30 \times 10^3/\mu L$). Then, **(C)** P-selectin expression, **(D)** PAC-1 binding and **(E)** ROS generation at baseline (blue) and in response to PMA (red) were evaluated. Black asterisks illustrate statistical differences between BS groups of ITP at baseline and after agonist treatment. $^*P < .05$, $^{**}P < .01$, and $^{***}P < .001$. Data presented as mean ± SD. CCCP, carbonyl cyanide m-chlorophenylhydrazone; PMA, Phorbol 12-Myristate 13-Acetate; ROS, Reactive Oxygen Species.

Characteristics	PLT count ($*10^3/\mu L$)	*N*	Mean ± SD	Median (Min-Max)	*P*-value*	AUC 95%CI	Cut-off	Sensitivity	Specificity
Age	< 30	7	15.14 ± 7.27	11.00 (10–30)	0.156	68.4 47.8–88.9	16.5/18.5	66.7/52.4	71.4/85.7
	≥ 30	21	22.52 ± 11.12	19.00 (10–40)					
Psel (Base)	< 30	7	3.06 ± 1.92	2.30 (1.46–6.45)	0.059	74.5 53.3–95.7	1.745	66.7	85.7
	≥ 30	21	1.99 ± 1.11	1.59 (1.02–5.71)					
PAC1 (Base)	< 30	7	2.1 ± 0.4	2.03 (1.56–2.9)	**0.044**	**76.19 50.03–100.0**	**1.915**	**85.7**	**85.7**
	≥ 30	21	1.81 ± 0.24	1.75 (1.55–2.43)					
ROS (Base)	< 30	7	1.4 ± 0.21	1.40 (1.07–1.68)	**0.009**	**70.7 50.3–91.2**	**1.705**	**47.6**	**100**
	≥ 30	21	1.8 ± 0.54	1.60 (1.15–2.88)					
Psel (PMA)	< 30	7	3.11 ± 1.18	2.66 (2–5.4.4)	**0.038**	**76.9 53.5–1000.0**	**2.725/3.785**	**90.4/57.1**	**61.9/85.7**
	≥ 30	21	4.27 ± 1.52	4.00 (2.14–8.52)					
PAC1 (PMA)	< 30	7	2.32 ± 0.35	2.25 (1.95–2.8)	0.300	63.6 36.5–90.7	2.38	76.2	71.4
	≥ 30	21	2.62 ± 0.73	2.50 (1.61–5.61)					
ROS (PMA)	< 30	7	4.96 ± 0.66	5.00 (3.86–5.8)	**< 0.001**	**87.4 74.4–100.0**	**5.85**	**71.4**	**100**
	≥ 30	21	6.82 ± 1.67	6.33 (3.62–9.6)					

Table 2. Platelet count-based category with respect to platelet activation status in ITP patients. *Comparing two groups of PLT count. AUC, Area Under the Curve; ITP, Immune Thrombocytopenic; PLT, platelet.

among ITP patients, based on bleeding score. Furthermore, considering the parameters that were significant at the 0.05 level in univariate analysis, age, P-selectin (PMA), and ROS (PMA) (as the independent variables) and bleeding score (as the dependent variable) were also subjected to a multiple logistic regression model, in which the calculated adjusted AUC of bleeding score was 85 (95%CI: 70.1–99.9, P-value < 0.001) with an optimum cut-off value of 0.539, sensitivity of 88.9% and specificity of 70% (Fig. 4B).

Given the significant difference observed between the BS groups in terms of patients' mean age, further analyses were conducted to examine the relationship between platelet functional analysis and age. The results showed a significant association between patients' age and their ROS response to PMA ($r = .390$, P-value = 0.038), while no significant association was found with the other platelet phenotypic or functional analysis

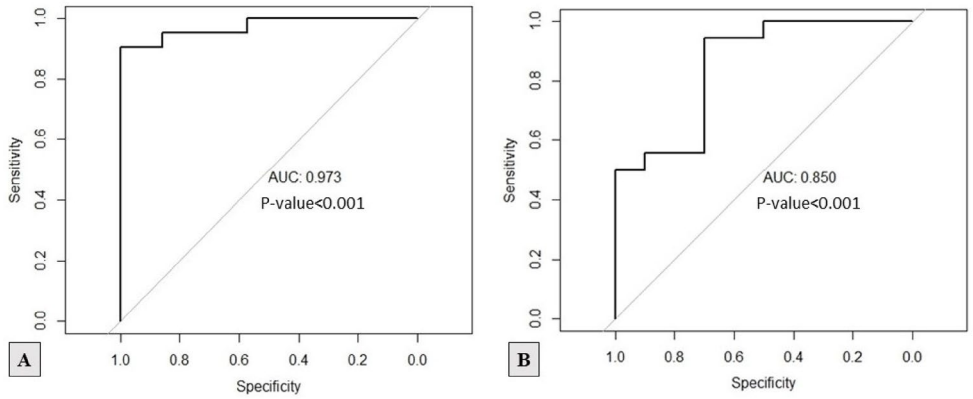

Fig. 4. The performance of multiple logistic regression model of predicting platelet count status of ITP patients using ROC curve. **(A)** ROS (Base), P-selectin (PMA), and ROS (PMA), the parameters that were significant at the 0.05 level in univariate analysis and platelet count were subjected to a multiple logistic regression model. **(B)** P-selectin (PMA), and ROS (PMA), the parameters that were significant at the 0.05 level in univariate analysis and bleeding score were subjected to a multiple logistic regression model.

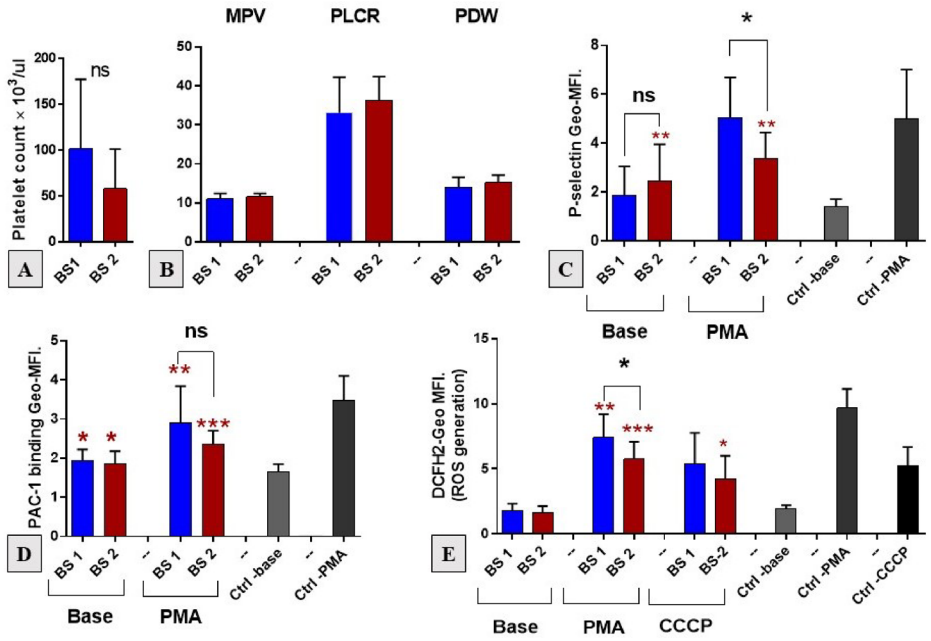

Fig. 5. Assessment of platelet basal activation state and its functional competence in ITP patients with respect to bleeding score. **(A)** Analysis of platelet counts conducted between BS1 (blue) and BS2 (red). Furthermore, **(B)** the MPV, PLCR, RP, and PDW indices were evaluated between these groups. The BS groups were also analyzed according to **(C)** P-selectin expression, **(D)** PAC-1 binding, and **(E)** ROS generation at baseline and in response to an agonist in comparison with healthy controls (gray). Black asterisks illustrate statistical differences between BS1 and 2 groups. Red asterisks show statistical differences of ITP ($n = 28$) vs., the related control ($n = 20$). $^{*}P < .05$, $^{**}P < .01$, and $^{***}P < .001$. Data presented as mean ± SD. CCCP, carbonyl cyanide m-chlorophenylhydrazone; MPV, mean platelet volume; PDW, platelet distribution width; P-LCR, platelet larger cell ratio; PMA, Phorbol 12-Myristate 13-Acetate; ROS, Reactive Oxygen Species.

(P-value > 0.05). In addition, age status also showed a trend toward lower platelet counts (P-value = 0.062) (please see the supplementary Table S2). In this context, further statistical analysis (supplementary Table S3) was also conducted where the cohort was divided into two groups of patients above/equal and less than 15 years old. This analysis also showed significantly lower ROS response to PMA (P-value = 0.019) as well as a trend toward lower platelet counts in patients less than 15 years old (P-value = 0.059).

Characteristics	Bleeding Score	*N*	Mean ± SD	Median (Min-Max)	*P*-value*	AUC (95%CI)	Cut-off value	Sensitivity	Specificity
Age	1	10	25.8 ± 9.23	24.00 (16–40)	**0.023**	**76.4 (58.5–94.3)**	**15**	**61.1**	**100**
	2	18	17.83 ± 10.57	11.00 (10–39)					
P-selectin (Base)	1	10	1.88 ± 1.18	1.55 (1.02–5.1)	0.137	67.5 (45.9–89.1)	1.745	61.1	80.0
	2	18	2.46 ± 1.5	1.85 (1.18–6.45)					
PAC-1 (Base)	1	10	1.92 ± 0.3	1.83 (1.55–2.43)	0.533	57.5 (33.6–81.4)	2.015	83.3	40.0
	2	18	1.86 ± 0.32	1.77 (1.56–2.9)					
ROS (Base)	1	10	1.82 ± 0.51	1.61 (1.17–2.7)	0.280	62.8 (40.6–84.9)	1.475	55.6	80.0
	2	18	1.64 ± 0.51	1.47 (1.07–2.88)					
P-sel (PMA)	1	10	**5.05 ± 1.65**	4.58 (3.39–8.52)	**0.013**	**81.4 (65.4–97.4)**	**3.36**	**61.1**	**100.0**
	2	18	**3.38 ± 1.07**	3.10 (2–5.68.68)					
PAC-1 (PMA)	1	10	3.01 ± 1.1	2.65 (1.95–5.95)	0.113	68.6 (45.6–91.6)	2.625	83.3	60.0
	2	18	2.35 ± 0.35	2.46 (1.61–2.8)					
ROS (PMA)	1	10	**7.41 ± 1.8**	7.12 (5.43–9.6)	**0.015**	**78.3 (60.2–96.4)**	**8.020/5.415****	**100/50****	**50/100****
	2	18	**5.76 ± 1.32**	5.53 (3.62–7.89)					
PLT count	1	10	101.8 ± 75.68	68.50 (28–207)	0.160	66.7 (44.1–89.3)	186.5	100	30.0
	2	18	58.06 ± 43.4	48.00 (12–171)					

Table 3. Bleeding score with respect to platelet activation status in ITP patients. *Comparing two groups bleeding score. AUC, Area Under the Curve; ITP, Immune Thrombocytopenic; PLT, platelet.

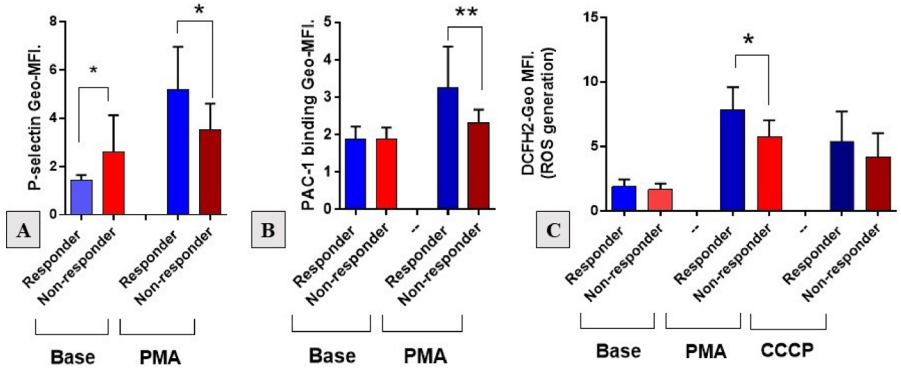

Fig. 6. Assessment of platelet basal activation state and its functional competence with respect to treatment response status. "NR" and "R" groups of ITP patients were studied according to **(A)** P-selectin expression, **(B)** PAC-1 binding, and **(C)** ROS generation at baseline (blue) and in response to agonist (red). Black asterisks illustrate statistical differences between BS groups of ITP ($n = 28$) and control ($n = 20$) at baseline and after agonist treatment. *$P < .05$, and **$P < .01$. Data presented as mean ± SD. CCCP, carbonyl cyanide m-chlorophenylhydrazone; PMA, Phorbol 12-Myristate 13-Acetate; ROS, Reactive Oxygen Species.

Comparison of platelet basal activation state and its functional competence in "NR" ITP patients compared to those under "R" status

The baseline levels of P-selectin were notably elevated in "NR" patients compared to those in "R". Additionally, the P-selectin response to PMA was significantly diminished in "NR" patients (Fig. 6A). Regarding PAC-1, which identifies the active form of αIIbβ3, there was no significant difference noted between the two disease statuses at baseline; however, in "NR" patients, the PAC-1 response to PMA was significantly lower compared to the "R" group (Fig. 6B). Similarly, ROS also exhibited no significant difference in basal levels between both groups. However, in "NR" patients, the ROS response to PMA, but not CCCP, was notably diminished when compared to the "R" group (Fig. 6C).

Table 4 shows the treatment response in relation to platelet activation status in ITP patients. It illustrates how the level of platelet activation may influence or predict the effectiveness of therapy. As revealed, among the platelet activation parameters at baseline, only the P-selectin (Base) parameter showed a significant relevance with treatment response in ITP patients, with a significantly higher level in non-responder than in responder group (2.59 ± 1.53 vs. 1.43 ± 0.22, respectively; P-value = 0.013). It is predicted that with an AUC of 80.9%, individuals with P-selectin (Base) higher than the cut-off value of 1.745 could be considered as non-responder to treatment with a sensitivity of 100% and specificity of 65%. In addition, considering the parameters that were significant at the 0.05 level in univariate analysis, P-selectin (Base), P-selectin (PMA), PAC-1 (PMA), ROS (PMA), platelet count (as the independent variables) and treatment response status (as the dependent variable)

Characteristics	Group	*N*	Mean ± SD	Median (Min-Mix)	*P*-value*	AUC (95%CI)	Cut-off value	Sensitivity	Specificity
Age	Non-responder	20	17.75 ± 10.01	12.50 (10–39)	**0.013**	**80.6 (64.5–96.8)**	**16.5**	**100**	**60.0**
	Responder	8	28 ± 9.04	28.50 (17–40)					
P-selectin (Base)	Non-responder	20	2.59 ± 1.53	1.93 (1.18–6.45)	**0.013**	**80.9 (65.0–96.9.0.9)**	**1.745**	**100**	**65.0**
	Responder	8	1.43 ± 0.22	1.50 (1.02–1.66)					
PAC-1 (Base)	Non-responder	20	1.88 ± 0.31	1.79 (1.56–2.9)	0.919	51.6 (25.0–78.1.0.1)	1.88	75.0	40.0
	Responder	8	1.88 ± 0.33	1.77 (1.55–2.43)					
ROS (Base)	Non-responder	20	1.64 ± 0.48	1.51 (1.07–2.88)	0.334	62.2 (37.0–87.4.0.4)	2.05	50.0	85.0
	Responder	8	1.87 ± 0.56	1.82 (1.17–2.7)					
P-sel (PMA)	Non-responder	20	3.5 ± 1.1	3.27 (2–5.68.68)	**0.036**	**79.7 (62.0–97.3.0.3)**	**3.36**	**100.0**	**55.0**
	Responder	8	5.17 ± 1.79	4.58 (3.39–8.52)					
PAC-1 (PMA)	Non-responder	20	2.31 ± 0.35	2.38 (1.61–2.8)	**0.006**	**84.1 (68.6–99.5)**	**2.625**	**75.0**	**85.0**
	Responder	8	3.26 ± 1.09	2.70 (2.45–5.45)					
ROS (PMA)	Non-responder	20	5.75 ± 1.25	5.54 (3.62–7.89)	**0.011**	**85.6 (69.5–100.0)**	**8.02**	**62.5**	**100.0**
	Responder	8	7.86 ± 1.74	8.57 (5.5–9.6)					
PLT count	non-responder	20	55.2 ± 41.98	44.00 (12–171)	**0.016**	**79.4 (60.5–98.2)**	**50.5**	**87.5**	**60.0**
	Responder	8	119.88 ± 74.14	99.00 (36–207)					

Table 4. Treatment response with respect to platelet activation status in ITP patients. *Comparing two groups treatment response. AUC, Area Under the Curve; ITP, Immune Thrombocytopenic; PLT, platelet.

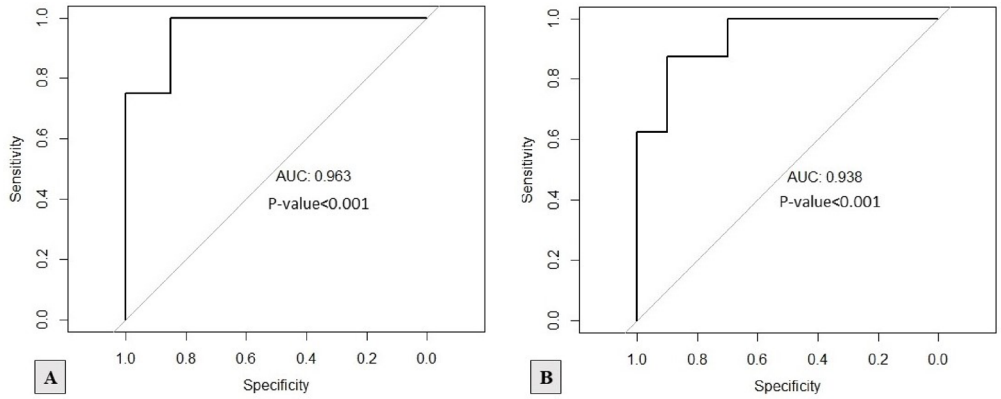

Fig. 7. The performance of multiple logistic regression model of predicting treatment response status of ITP patients using ROC curve. **(A)** P-selectin (Base), P-selectin (PMA), PAC-1 (PMA), ROS (PMA), the parameters that were significant at the 0.05 level in univariate analysis and treatment response were subjected to a multiple logistic regression model. (**B**) The functional assays of the platelet in response to PMA agonist including P-selectin (PMA), PAC-1 (PMA), ROS (PMA), Logit (probability of response) = −20.33 + PAC-1 (PMA)*4.83 + Psel (PMA)*0.673 + ROS (PMA)*0.683; Logit(P) = Log(P/1-P).

were also subjected to a multiple logistic regression model, in which the calculated adjusted AUC of treatment response was 96.3 (95%CI: 90.0–100.0, P-value < 0.001) with an optimum cut-off value of 0.215, sensitivity of 100 and specificity of 85 (Fig. 7A). On the other hand, considering the functional assays of the platelet in response to PMA agonist, the adjusted AUC would be 93.8 (95%CI: 84.8–100, P-valued < 0.001), with an optimum cut-off value of 0.229, sensitivity of 87.5% and specificity of 90.0% (Fig. 7B). Representative raw data relevant to the results presented here were included in supplementary Figure S2.

Discussion

This study first investigated the relationship between platelet indices in ITP patients, as well as the levels of basal platelet activation state, oxidative stress and platelet functional competence in these patients compared to healthy individuals. Then, as a more novel part of this research, the status of platelet activation and its functional competence was examined according to the clinical performance of the patients, in whom chronic ITP was divided into different categories based on platelet count, bleeding score, and response to treatment.

The findings confirm and expand upon previous evidence that ITP patients exhibit significantly elevated levels of platelet indices including MPV, PDW, and PLCR[4,13,31–33]. These changed indices may reflect increased bone marrow activity in response to peripheral platelet destruction. This response is often insufficient and thrombopoiesis may not fully compensate for platelet loss. However, our data underscore that these size-based

indices, while indicative of immature platelet release, are insufficient alone to describe platelet functional competence or predict clinical outcomes.

Functional assays demonstrated impaired agonist-stimulated platelet reactivity in ITP patients. Specifically, patients showed elevated basal expression of P-selectin and diminished responsiveness to PMA stimulation, suggesting a phenotype of pre-activation coupled with defective signaling capacity[16,29,34]. This finding is in line with previous studies reporting enhanced basal platelet activation yet reduced functional responsiveness in ITP platelets[16,29,34] (Table 5). In these patients, the initial binding of PAC-1 was significantly increased compared to the healthy control group, which may indicate a pre-aggregatory state of platelets that in certain clinical cases may be predictive of the risk of thrombosis. However, upon stimulation with PMA, a notable attenuation in integrin αIIbβ3 activation was observed. The presence of increased basal platelet activation coupled with decreased responsiveness to agonists suggests disruption of downstream signaling pathways, which is attributed to a chronic exaggerated activation state leading to platelet exhaustion, an observation that has also been demonstrated in ITP patients[4,13,35–38]. (Table 5). Despite the changes in platelet activation markers, the insufficient platelet activity is also extended to the changes in redox regulation, where the generated ROS should play a critical role in the enhancement of signaling pathways that support platelet functional competence. Although basal ROS levels were notably lower in patients than in controls, ITP platelets demonstrated a markedly reduced ROS response to PMA, suggesting compromised function of exhausted platelets that are not able to present a competent oxidative response to the designated agonist. Given that ROS levels did not significantly change upon CCCP stimulation in these patients compared to controls, this impairment may stem from altered NADPH oxidase activity rather than mitochondrial dysfunction, the evidence suggesting inadequate potential of platelet activating signals[39–42]. While these findings are consistent with previous work suggesting that oxidative stress plays a pivotal role in ITP[43–46], they also, along with other parameters, highlight the complexities of platelet activation dynamics in ITP and emphasize the need for further research into multifactorial targeted tests to determine patients' clinical status or predict outcomes.

Amongst the clinical parameters, platelet count and variable manifestations of bleeding (defined by scores) are the two key indicators commonly used to evaluate the status of ITP patients[10]. Platelet count and bleeding score are common tools in ITP assessment, but they may not reflect the full clinical picture. Both quantitative and qualitative platelet abnormalities contribute to disease manifestations. The present study showed no statistically significant difference in platelet counts between patients with BS1 and BS2. Other research has also indicated that there is no correlation between platelet count and the severity of bleeding[3,4,34]. It was shown that platelet

Author, Year	Studied population	Tests for platelet function or oxidative status	Results
Platelet functional tests			
Frelinger et al., 2013[3]	Pediatric ITP patients	– P-selectin – GPIIb/IIIa – GPIb, phosphatidylserine expression	– Higher unstimulated P-selectin, TRAP-stimulated GPIb, and FSC associated with more bleeding. – TRAP-stimulated P-selectin and GPIIb/IIIa were associated with reduced bleeding risk – Platelet function markers help predict bleeding severity beyond platelet count
Frelinger et al., 2015[4]	Pediatric ITP patients	– P-selectin – GPIIb/IIIa activation – CD42b – platelet FSC	– Higher immature platelet fraction, unstimulated P-selectin, and FSC associated with increased bleeding. – TRAP-stimulated P-selectin and GPIIb/IIIa linked to reduced bleeding – Platelet function markers, independent of platelet count, correlate with bleeding severity
Bhoria et al., 2015[16]	Chronic ITP: complete or good responders, R, and NR	– P-selectin – PAC-1 at baseline and after treatment with ADP	– P-selectin expression and PAC-1 binding showed no significant difference in the activation status of R group, when compared to the NR group. – Expression of PAC-1 is significantly more in the CR group after activation with ADP. – The fold activation of platelets upon treatment with ADP is more in healthy controls than in ITP patients. – Treatment with steroids causes platelets in R group become more responsive to ADP-activation, similar to healthy controls.
Middelburg et al., 2016[34]	Pediatric ITP patients: Active and Remission	– Baseline P-selectin expression and – after stimulation with TRAP and CRP	– Remission patients never showed deceased platelet activity. – Some ITP patients in remission did show increased platelet activity. – In ITP patients in the lowest count category, the risk of bleeding was reduced. – Platelet activity can be a predictor of bleeding risk in ITP patients with low platelet counts.
Frelinger et al., 2018[12]	Pediatric ITP patients at two visits over 10 months	– P-selectin – GPIIb/IIIa activation – immature platelet fraction	– Platelet function tests correlated with both concurrent and future bleeding severity. – Platelet function, independent of count, is stable over time and predicts bleeding risk
Mehic et al., 2023[13]	ITP patients, thrombocytopenic and healthy controls	– P-selectin – GPIIb/IIIa activation	– No evidence that hyper-reactive platelets reduce bleeding tendency – Impaired platelet function, not activation, plays a role in bleeding in non-immune thrombocytopenia
– **Oxidative status**			
Zhang et al., 2011[48]	Pediatric ITP patients (acute vs. chronic)	– GSH – Oxidative stress pathways – Vanin-1 expression	– Oxidative stress pathways, reduced GSH levels in chronic ITP – Oxidative stress may drive chronic ITP pathogenesis
Kamhieh-Milz et al., 2012[44]	ITP patients (active/remission), healthy controls	– Intracellular antioxidant capacity – glutathione peroxidase	– Reduced antioxidant capacity in active ITP, higher oxidative stress. – Oxidative stress may contribute to platelet dysfunction in ITP.

Table 5. Conducted studies on platelet functional and oxidative States in ITP patients. CRP, collagen-related peptide; FSC, forward scatter; GP, glycoprotein; GSH, Glutathione; ITP, Immune thrombocytopenia; LZD, linezolid; MDA, malondialdehyde; NPC, normal platelet count; NR, non-responders; R, responders; ROS, Reactive oxygen species; TRAP, thrombin receptor activating peptide.

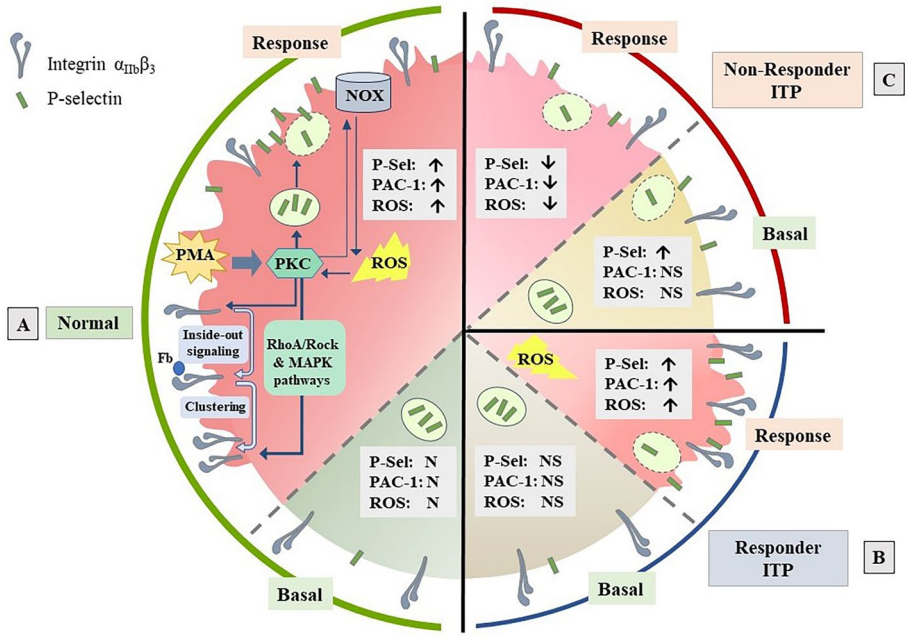

Fig. 8. A comparison of the platelet functional panel in ITP patients, distinguishing between responders and non-responders, in relation to healthy individuals. (**A**) At baseline, platelets of healthy controls exhibit normal levels (N) of P-selectin expression, PAC-1 binding, and ROS generation. Following treatment with PMA, there is an increase in the levels of all three parameters in response. PMA has the ability to recruit and activate PKC. This enzyme induces platelet granule release, NOX activation, and the enhancement of inside-out signaling as well as RhoA and MAPK signaling pathways, leading to activation and clustering of integrin αIIbβ3. (**B**) In the platelets of "Responder" ITP, baseline levels of P-selectin expression, PAC-1 binding, and ROS generation were not significantly (NS) different from those in normal platelets. Following PMA treatment, all three factors showed an increase (slightly less than normal platelets). (**C**) In the platelets of "Non-Responder" ITP, the baseline levels of PAC-1 binding and ROS generation were not significantly (NS) different from those observed in normal platelets. Upon exposure to PMA, all three factors exhibited a decrease when compared to both normal platelets and the platelets of ITP responders. Fb, Fibrinogen; ITP, immune thrombocytopenia; MAPK, Mitogen-activated protein kinase; N, Normal; NS, Not Significant, NOX, NADPH oxidases; PKC, protein kinase C; PMA, Phorbol 12-Myristate 13-Acetate; ROS, Reactive Oxygen Species.

function, rather than count alone, plays a significant role in bleeding risk in ITP. This highlights the importance of including functional platelet assessments when evaluating bleeding severity. Therefore, this study examined platelet functional markers with respect to platelet count and bleeding state to improve prediction of bleeding risk and treatment response[13,15]. Given this, combined approaches supported by empirical and clinical evidence can be useful for correctly diagnosing disease status while also predicting patient outcomes[10,47]. To achieve this goal, a combination of a bleeding score with a platelet threshold defined by a platelet count less than or greater than $30 \times 10^3/\mu L$ is considered a practical approach that clinically distinguishes between "NR" and "R" patients[10,47]. However, such an approach may not easily lead to a definitive and reliable result in every situation, as it is completely influenced by clinical experience and the accuracy of the medical staff in determining the necessary clinical findings. In this regard, studying the relationship between platelet hemostatic function and patient conditions and outcomes is a valuable quantitative and qualitative measure, because if this correlation is confirmed, it can be used as an indicator to aid diagnosis or predict patients' responses. In light of this, the study presented here first examined the association between platelet activation status and functional competence with a defined platelet threshold or BS separately. Overall, the findings revealed a limited and inconsistent correlation between platelet count or BS alone and platelet activation state and functionality. To address this limitation, since the issue could be due to the lack of comprehensiveness of each of the mentioned clinical indicators alone, platelet activation markers were then compared separately in patients with definitive "response" or "non-response" conditions. Clinical definitions were obtained through verification by an experienced physician using a combination of bleeding score and platelet threshold. In this regard, our data established that basal levels of P-selectin and responses of P-selectin, PAC-1 binding, and ROS generation to PMA are robust functional biomarkers capable of differentiating between "NR" and "R" states in ITP (Fig. 8).

The integration of the abovementioned markers into a functional assay panel offers an even more powerful tool for clinical evaluation. Through this combined approach, we identified a novel composite index, "Logit(probability of response)= −21.97 + PAC-1 (PMA)*8.16 + P-selectin (PMA)*0.61 + ROS (PMA)*0.437-P-selectin (Base)*3.21; Logit(P) = Log(P/1-P), derived from a combination of those multiple meaningful laboratory assays, while the calculated adjusted AUC of treatment response was 94.4 (95%CI: 85.8–100) with an optimum cut-off value of 0.129/0.569, sensitivity of 100/75 and specificity of 75/100. This newly proposed

index may serve as a laboratory panel to more accurately reflect the functional status and therapeutic response in ITP patients, potentially overcoming the limitations associated with relying on single parameters alone while providing further confirmation of the determined clinical status of patients by medical staff. Taken together, the promising results obtained here recommend that future studies be designed to longitudinally follow up with newly diagnosed ITP patients using this "Logit(P) = platelet functional index (PFI)" index, so that they can provide further validation for the predictive value of this panel analysis with respect to treatment response and long-term clinical outcomes. Such follow-up investigations could provide critical insights into the utility of our obtained platelet functional index as a prognostic tool and its potential indication in guiding therapeutic strategies.

Regardless of the findings presented here, since our study cohort is somewhat small, further studies with a larger cohort would be appreciated to confirm our conclusion. Another limitation of this study was the lower number of responder cases compared to Non-responders. This was mainly due to the referral nature of our center, which often manages more complex ITP cases. Therefore, future studies involving a wider ITP patient population could be much appreciated to provide a better balance between different categories of patients.

Conclusion

The main focus of the study presented here was to determine whether platelet functional competence can be relevant to the disease status in chronic ITP patients. For this purpose, patients were divided into different groups with respect to their platelet count and BS as the major markers that dictate disease status. Given this, the present study, for the first time, achieved to introduce a valuable panel analysis of platelet activation state to discriminate between "NR" r "R" patients. Taken together, this multifactorial diagnostic panel—including functional assays of P-selectin, PAC-1 binding and ROS generation—is recommended to enhance diagnostic accuracy and guide individualized follow-up strategies for ITP patients.

Data availability

The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

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Author contributions

M.G. provided conceptual input and the idea of research, supervised and designed the study, performed the experiments, analyzed the data, depicted the figures and wrote the final version of the paper; N.H. provided conceptual input, performed the experiments, helped with paper preparation, depicted the figures and wrote the first draft of paper; E.H. provided conceptual input, co-supervised the study, performed the experiments and statistical analysis and helped with manuscript writing; M.F provided conceptual input, managed patient access, provided clinical results as a consultant physician; M.S.P. Provided conceptual input managed sample collection and preparation; A.T. performed ROC analysis and logistic regression model while acting as statistics consultant. E.G. constructively reviewed the paper, provided conceptual input and suggestions for proper presentation results and figures, and suggested advanced statistical analyses.

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Declarations

Ethics approval and consent to participate

This study approved by the Ethics Committee of the High Institute for Research and Education in Transfusion Medicine, Tehran, Iran (Ethics approval number: IR.TMI.REC.1402.024).

Competing interests
The authors declare no competing interests.

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Correspondence and requests for materials should be addressed to M.G.

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