Biomeditsinskaya Khimiya, 2025 vol. 71, issue 4, pp. 288–299.

CLINICAL-DIAGNOSTIC STUDIES

PLATELET FUNCTIONAL ACTIVITY IN PATIENTS WITH IMMUNE THROMBOCYTOPENIA

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Immune thrombocytopenia (ITP) is one of the most common causes of decreased platelet count. Bleeding is the main clinical symptom of ITP; although its severity correlates with the depth of thrombocytopenia, it may also depend on changes in the functional activity of platelets. In this study we have compared platelet functional activity in healthy volunteers (HV) and in ITP patients, as well as in groups of ITP patients with different levels of bleeding. The study included 65 HV and 84 ITP patients. Platelet activity was assessed by flow cytometry. Platelets were activated with thrombin receptor activating peptide (TRAP) or ADP, and the exposure of activation markers, activated form of glycoprotein (GP) IIb-IIIa and alpha-granule membrane protein P-selectin, was determined on their surface by measuring the binding of PAC-1 and CD62P antibodies, respectively. Platelet-associated IgG (PA-IgG, an indicator of the level of antiplatelet autoantibodies), the percentage of "young" reticular platelets (RP, %) and platelet light scatter (an indicator of their size) were also assessed using flow cytofluorimetry. Platelet binding of PAC-1 (and, to a lesser extent, CD62P binding) was lower in ITP patients than in HV. In ITP patients, PAC-1 binding inversely correlated with the PA-IgG content. In contrast to HV, in ITP patients, PAC-1 and CD62P binding did not directly correlate with the platelet size and RP, %. In ITP patients with severe bleeding, the platelet count was lower, PAC-1 and CD62P binding was reduced and PA-IgG and RP, % levels were increased. Thus, a decrease in the content of activation markers on the platelet surface was registered in ITP patients; it was more pronounced in patients with severe bleeding. It is suggested that the cause of this decrease may be due to the effect of autoantibodies (PA-IgG) on platelets, and in particular on GP IIb-IIIa.

Keywords: immune thrombocytopenia; platelet function; platelet antibodies; reticular platelets; platelet size; bleeding

DOI: 10.18097/PBMCR1596

INTRODUCTION

Immune thrombocytopenia (ITP) is a classic autoimmune disease and one of the most common causes of decreased platelet count in the circulation (2–4 cases per 100,000 population per year in the USA and European countries). Autoantibodies in ITP are usually directed against the main platelet antigens, glycoproteins (GP) IIb-IIIa and Ib. By binding to their targets, autoantibodies accelerate the destruction of platelets by macrophages in the spleen and liver and can suppress platelet production by bone marrow megakaryocytes (mainly in chronic ITP) [1–3].

Bleeding (or hemorrhagic syndrome) is the main clinical symptom of ITP. Although the frequency and severity of bleeding generally correlate with the depth of thrombocytopenia [4], bleeding may also depend on changes in platelet functional activity. Platelet function in patients with thrombocytopenia is usually assessed using flow cytometry. In contrast to the routine platelet aggregation test in platelet-rich plasma, platelet activity can be measured by flow cytometry at low platelet counts and in a small volume of whole blood. Using this approach, it has been demonstrated that ITP patients with hemorrhagic complications have a slight decrease in agonist-induced platelet surface exposure of specific markers of platelet activation (such as the activated form of the fibrinogen receptor, GP IIb-IIIa, and the alpha granule membrane protein P-selectin) [5–9].

Comparisons of platelet activity in ITP patients and healthy controls gave conflicting results; there were reports on both decreased [10, 11] and increased activity [12, 13] and also mixed results, which depended on the agonist used for platelet activation [14, 15]. The presence of antiplatelet autoantibodies is the main specific feature of ITP, which distinguishes it from other types of thrombocytopenia (e.g., hypoproductive forms due to impaired platelet production in the bone marrow). Possible effects of autoantibodies on platelet function have been investigated in only two studies. Panzer et al. found

Abbreviations used: FSC – forward scattering; GP – glycoprotein; HV – healthy volunteers; ITP – immune thrombocytopenia; MFI – mean fluorescence intensity, PA-IgG – platelet-associated IgG; RP – reticulated platelets; TO – thiazole orange; TPO – thrombopoietin; TRAP – thrombin receptor activating peptide.

no difference in platelet activity between ITP patients with and without anti-GP IIb-IIIa or anti-GP Ib autoantibodies [16], while Nishiura et al. found lower activity in ITP patients with anti-GP IIb-IIIa autoantibodies [12]. In ITP, platelets, due to their accelerated turnover, are characterized by an increased size [17–20] and an increased percentage of "young" reticulated platelets (RP) [21–24]. In healthy individuals, such changes directly correlate with an increase in platelet activity [25], but such relationships have not been described for ITP patients. Thus, it remains unclear, how platelet function changes in ITP and what factors influence these changes.

In this study we have compared platelet activity, which was measured by flow cytometry, in healthy volunteers (HV) and ITP patients, as well as in groups of ITP patients with different levels of bleeding. To identify factors that could affect platelet activity in patients with ITP, we analyzed the relationships between the parameters of their functional activity and (1) the level of antiplatelet autoantibodies (platelet-associated IgG, PA-IgG), (2) platelet size, (3) RP content.

MATERIALS AND METHODS

HV and ITP Patients

The study included 65 HV and 84 ITP patients, which were observed in hematology clinics in Moscow, mainly in the National Medical Research Center of Hematology. ITP was diagnosed according to international recommendations [2] using the following criteria: (1) isolated thrombocytopenia (platelet count $<100\times10^9$/l), (2) the absence of diseases causing secondary thrombocytopenia (antiphospholipid syndrome, systemic lupus erythematosus, lymphoproliferative diseases, etc.). Platelet-associated IgG (PA-IgG) and plasma glycocalicin were measured in all patients with thrombocytopenia. Patients without increased PA-IgG (<200% of control values in HV), an indicator of the presence of autoantibodies on the platelet surface, and with a significant decrease in plasma glycocalicin (<50% of control values in HV), an indicator of impaired platelet production in the bone marrow, were excluded from the study. Most patients had newly diagnosed (<3 months, n=24) or persistent ITP (3–12 months, n=47), and a minority had chronic ITP (>12 months, n=13). At the time of the examination, none of the patients received specific ITP therapy: intravenous immunoglobulin, corticosteroids, thrombopoietin receptor agonists (TPO), or other treatment. Patients with newly diagnosed ITP were examined before starting any therapy, and patients with persistent and chronic ITP were examined while they had not received therapy for at least one week (in most cases after an ineffective course of corticosteroids). The degree of bleeding was assessed using the scale recommended by the International ITP Working Group (grades 1–4) [26]. The assessment was performed by a qualified hematologist based on the patient's history and complaints (recent episodes of internal bleeding, nosebleeds, gingival bleeding, post-traumatic bleeding, menorrhagia in women, etc.), physical examination (petychia, ecchymoses, hematomas, etc.) and blood tests (red blood cell count and hemoglobin level). ITP was initially diagnosed in hematology clinics, and the diagnosis was then confirmed after additional laboratory tests during the patient's visit to Chazov National Medical Research Center of Cardiology. The platelet count of the patients included in the study was $\geq150\times10^9$/l and the patients were not taking drugs that affect platelet function.

Blood Sampling

Blood from HV and ITP patients was collected from the cubital vein in 5% EDTA or 3.8% sodium citrate in a blood:anticoagulant ratio of 9:1 using needles of at least 18 G (thinner needles and vacutainers were not used to prevent possible platelet activation). EDTA-anticoagulated blood was used to count platelets, measure RP, platelet forward scattering (FSC), and PA-IgG, and also to prepare plasma samples for glycocalicin and TPO assays. Sodium citrate-anticoagulated blood was used to assess platelet activity.

Platelet Counting

Platelet counting was performed in an Abacus Junior B hematology analyzer (Diatron Ltd., Austria). The device was regularly calibrated using standard blood samples.

Reticulated Platelets

Reticulated platelets (RP) were determined by their residual nucleic acid content, assayed using thiazole orange (TO) as a dye as described previously [25]. For samples without TO, 5 μl of whole blood was mixed with 990 μl of the BD FACS Flow reagent (BD Bioscience, USA), and 5 μl of CD42b-APC (BD Biosciences). For samples with TO, 5 μl of whole blood was mixed with 940 μl of the BD FACS Flow reagent, 5 μl of CD42b-APC and 50 μl of TO solution (10 μg/ml). Samples were incubated for 30 min at room temperature, centrifuged at 2500 *g* for 3 min, and the pellet was resuspended in 350 μl of the BD FACS Flow reagent. The analysis was performed in a BD FACSCanto II flow cytometer (BD Biosciences) using BD FACS Diva software (BD Biosciences). Platelets were detected (gated) according to their size and staining with the specific marker CD42b-APC. Platelets were considered positive for TO staining when their fluorescence exceeded the fluorescence of >99% of platelets in the sample without TO, and the percentage of TO positive platelets was calculated, i.e. RP in the total population (RP, %).

Platelet Light Scattering

The average value of forward scattering of platelets (FSC), characterizing the size of platelets, was determined by flow cytometry in the same sample as RP (see above) and expressed in arbitrary units (a.u.).

Platelet-Associated IgG

Platelet-associated IgG (PA-IgG) was determined using flow cytofluorimetry by binding of FITC-labeled affinity-purified goat polyclonal antibodies against human IgG (anti-IgGh-FITC) (IMTEK, Russia) to platelets. Because of the low platelet count in ITP patients, EDTA-anticoagulated blood was left to stand for 30 min at room temperature for partial sedimentation of erythrocytes, and the platelet-rich supernatant was collected. Samples were diluted 1:5 with phosphate-buffered saline (150 mM NaCl, 10 mM Na_2HPO_4/NaH_2PO_4, pH 7.4) containing 5 mM EDTA and 2% BSA, and after addition of 10 μg/ml CD42b-Alexa Fluor 647 (IMTEK) and 30 μg/ml anti-IgGh-FITC were incubated at 37°C for 30 min in the dark. Samples (60 μl) were diluted with 250 μl of the BD FACS Flow reagent and analyzed on a BD FACSCanto II flow cytometer using BD FACS Diva software. Platelets were detected (gated) according to their size and CD42b-Alexa Fluor 647 staining. The mean fluorescence intensity (MFI) for anti-IgGh-FITC binding was estimated in arbitrary units (a.u.) in each ITP patient and compared with that in the control group of HV. PA-IgG in patients was expressed as a percent of HV control samples defined as 100%. An example of PA-IgG determination is given in the Supplementary Materials (Fig. S1).

Platelet Function

Platelet functional activity was assessed by flow cytometry assay for platelet surface expression of activation markers, activated GP IIb-IIIa, and the alpha granule membrane protein P-selectin, by binding of PAC-1 and CD62P antibodies, respectively, as described in detail previously [25]. Because of low platelet counts in ITP patients, sodium citrate-anticoagulated blood was left to stand at room temperature for 30 min for partial sedimentation of erythrocytes, and platelet-rich supernatant was collected. Samples were diluted with $CaCl_2$-free Tyrode/HEPES solution (137 mM NaCl, 2.7 mM KCl, 0.36 mM NaH_2PO_4, 0.1% dextrose, 5 mM HEPES, pH 7.35, 1 mM $MgCl_2$), containing 0.35% BSA. For HV and ITP patients with platelet counts $>50\times10^9$/l, a 1:5 dilution was used, and for ITP patients with lower platelet counts, lower dilutions (up to 1:2) were used. To 60 μl of the diluted blood samples, 3 μl of CD42b-APC and either 10 μl of PAC-1-FITC (BD Biosciences), 5 μl of CD62P-FITC (IMTEK), or 5 μl of mouse IgG-FITC (IMTEK) were added. The mouse IgG-FITC control was used to assess nonspecific binding of the IgG anti-CD62P antibody to platelets. However, this control was not used for PAC-1 binding (an IgM antibody). Platelets were either unactivated or activated with 10 μM TRAP (thrombin receptor activating peptide, the SFLLRN sequence), kindly provided by M.V. Ovchinnikov, Chazov National Medical Research Center of Cardiology) or 20 μM or 2.5 μM ADP (AppliChem, Germany). Samples with antibodies and agonists were incubated for 15 min at 37°C in the dark, fixed with an equal volume of 2% paraformaldehyde for 40 min in the dark, diluted with 250 μl of the BD FACS Flow reagent and analyzed in a BD FACSCanto II flow cytometer using BD FACS Diva software. Platelets were detected (gated) according to their size and by CD42b-APC staining. MFI for PAC-1-FITC and CD62P-FITC binding (in a.u.) and the percentage of platelets positive for PAC-1 and CD62P (PAC-1+ and CD62P+) were determined. Platelets were considered PAC-1+ and CD62P+ if their fluorescence exceeded that of 95% of platelets in control samples (non-activated platelets for PAC-1 and non-activated platelets with mouse control IgG-FITC for CD62P). Examples of PAC-1 and CD62P binding in HV and ITP patients are shown in Figure 1.

Glycocalicin

Plasma glycocalicin levels were measured using a home-developed ELISA protocol as described previously [20, 27]. Plasma samples from several HV were mixed, frozen in aliquots at -70°C, and used as a standard plasma pool for the calibration curve. Glycocalicin levels in patients and HV were expressed as % of the glycocalicin content in the standard plasma pool, which was defined as 100%.

Thrombopoietin

Plasma thrombopoietin (TPO) levels were measured using the Quantikine® ELISA for human TPO (R&D Systems, USA) according to the manufacturer's recommendations.

Statistics

Statistical analysis was performed using Statistica 12 software (Stat. Soft, USA). Most of the variables did not follow normal distribution (Shapiro-Wilk test) and, therefore, nonparametric statistics were used to analyze the results. Variables were presented as medians and 25–75 percentiles. For comparison of groups, the following tests were used: the Mann-Whitney test (for quantitative variables), the Chi-square criterion (for qualitative variables). For correlation analysis, the Spearman test was used. Construction and analysis of ROC curves were performed using the Med Calc 15.8 program (MedCalc Software, USA).

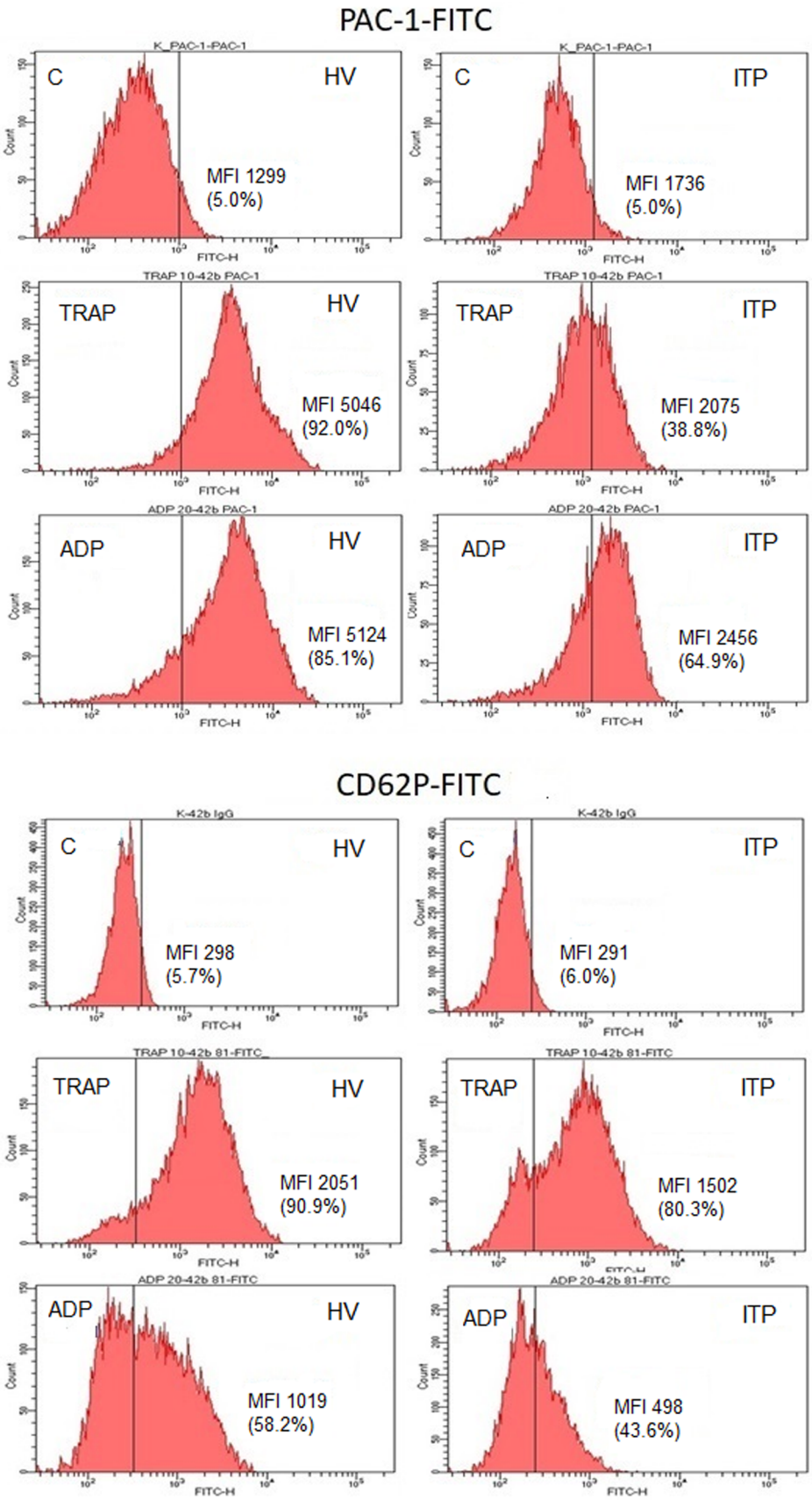

Figure 1. Platelet functional activity in HV and ITP patients (as indicated). Platelet binding of PAC-1-FITC antibody against activated GP IIb-IIIa (upper panel) and CD62P-FITC antibody against P-selectin (lower panel). Flow cytometry, examples of the fluorescence histogram. Platelets were not activated (control without agonists (C)) or activated by 10 μM TRAP (TRAP) or 20 μM ADP (ADP). MFI values and % of PAC-1+ or CD62P+ platelets (in brackets) are shown.

RESULTS

Characteristics of HV and ITP Patients

The main characteristics of HV and ITP patients, given in Table 1, indicate no significant differences between the groups in age and a men to women ratio. The platelet count was significantly lower in the ITP group compared to HV (medians of 29×10^9/l and 199×10^9/l, respectively). The platelet size (assessed using the FSC index) and the percentage of RP (RP, %) were significantly higher in the ITP group. The PA-IgG level in ITP patients was 500% (median) of the control level in the HV group (defined as 100%). Plasma glycocalicin and TPO were approximately the same in both groups.

A strong inverse correlation was found between the platelet count and PA-IgG in ITP patients ($R = -0.605$, $p<0.001$). The platelet size (FSC index) and RP, % directly correlated with each other, both in ITP patients and in HV ($R = 0.578$, $p<0.001$ and $R = 0.605$, $p<0.001$, respectively).

Functional Activity of Platelets in ITP Patients

Comparative analysis of functional activity of platelets in ITP patients and HV was assessed using flow cytometry by determining PAC-1-FITC antibody binding to platelets (recognizing activated GP IIb-IIIa), and CD62P-FITC antibody binding (recognizing the membrane protein α-granules P-selectin). MFI and the percentage of PAC-1+ and CD62P+ were assessed before and after platelet activation. In HV and ITP patients, the low baseline levels of PAC-1 and CD62P binding by unactivated platelets ("without agonists") did not differ. However, activation of platelets from ITP patients with TRAP and ADP resulted a significant decrease of most PAC-1 and CD62P binding parameters (Fig. 2). When platelets were activated with 10 μM TRAP, the reduction was more pronounced for PAC-1 than for CD62P: both PAC-1 MFI and PAC-1+, % reduced by about 30–40%, while CD62P MFI remained unchanged and CD62P+, % was lower by only 8%. When platelets were activated with ADP (used at both concentrations) the reduction was almost the same (about 20%) for all parameters, except for PAC-1+, % for 2.5 μM ADP (39%).

In ITP patients all the parameters of agonist-induced PAC-1 binding (MFI and PAC-1+, % for all agonists) were inversely correlated with PA-IgG levels (R from -0.302 to -0.446) (Table 2). However, for CD62P binding, a significant correlation was found only for CD62P+, % at platelet activation with 10 μM TRAP ($R = -0.389$) (Table 2).

In accordance with our earlier results [25], in HV, most MFI values for PAC-1 and CD62 binding directly correlated with the platelet size (FSC index) and RP, % (Table 3). However, in ITP patients, significant correlations were found only for CD62P MFI during platelet activation with 10 μM TRAP.

ITP Patients with Different Bleeding Levels

We have compared ITP patients with no bleeding and low bleeding levels corresponding to grades 0/1 of the bleeding assessment scale (group 0/1) and patients with more severe bleeding corresponding to grades 2/3 (group 2/3) (our group did not include patients with the most dangerous bleeding corresponding to grade 4). These groups did not differ in age and the men to women ratio. There were no differences in the levels of glycocalicin and TPO and the platelet size (FSC index) (TPO and FSC were slightly higher in the group 2/3, but the differences were not statistically significant). In the group 2/3, the platelet count was lower (by 34%), and PA-IgG and RP, % were higher (by 55% and 59%, respectively) compared with the group 0/1 (Table 4).

Several indices of the platelet functional activity were reduced in the group 2/3 (Fig. 3). These differences were more pronounced for PAC-1 binding. Significant differences were found for PAC-1 MFI after platelet activation with 10 μM TRAP and 20 μM ADP (20% decrease in both cases) and for the initial level without agonists (21% decrease) and in the case of PAC-1+, % after platelet activation with 10 μM TRAP (24% decrease). However, no differences

Table 1. The main characteristics of HV and ITP patients

	HV (n=65)	ITP patients (n=84)
Age	57 (48–66)	52 (37–61)
Gender, m/f	27/38	32/52
Platelets, $\times10^9$/l	199 (168–243)	29 (19–46)***
FSC, a.u.	19696 (16747–23406)	30760 (25585–39673)***
RP, %	11,4 (8.5–15.4)	16.5 (10.2–21.1)**
PA-IgG, % of control[1]	100 (88–115)	500 (370–820)***
Glycocalicin, % of control[2]	100 (68–120)	75 (60–100)
TPO, ng/ml	0.86 (0.36–2.34)	1.16 (0.56–3.36)

1 – Patients with PA-IgG < 200% of control values in HV were not included in the study. 2 – Patients with glycocalicin <50% of control values in HV were not included in the study. Data represent medians (and 25–75 percentiles). **$p<0.01$, ***$p<0.001$ – significance of differences from HV.

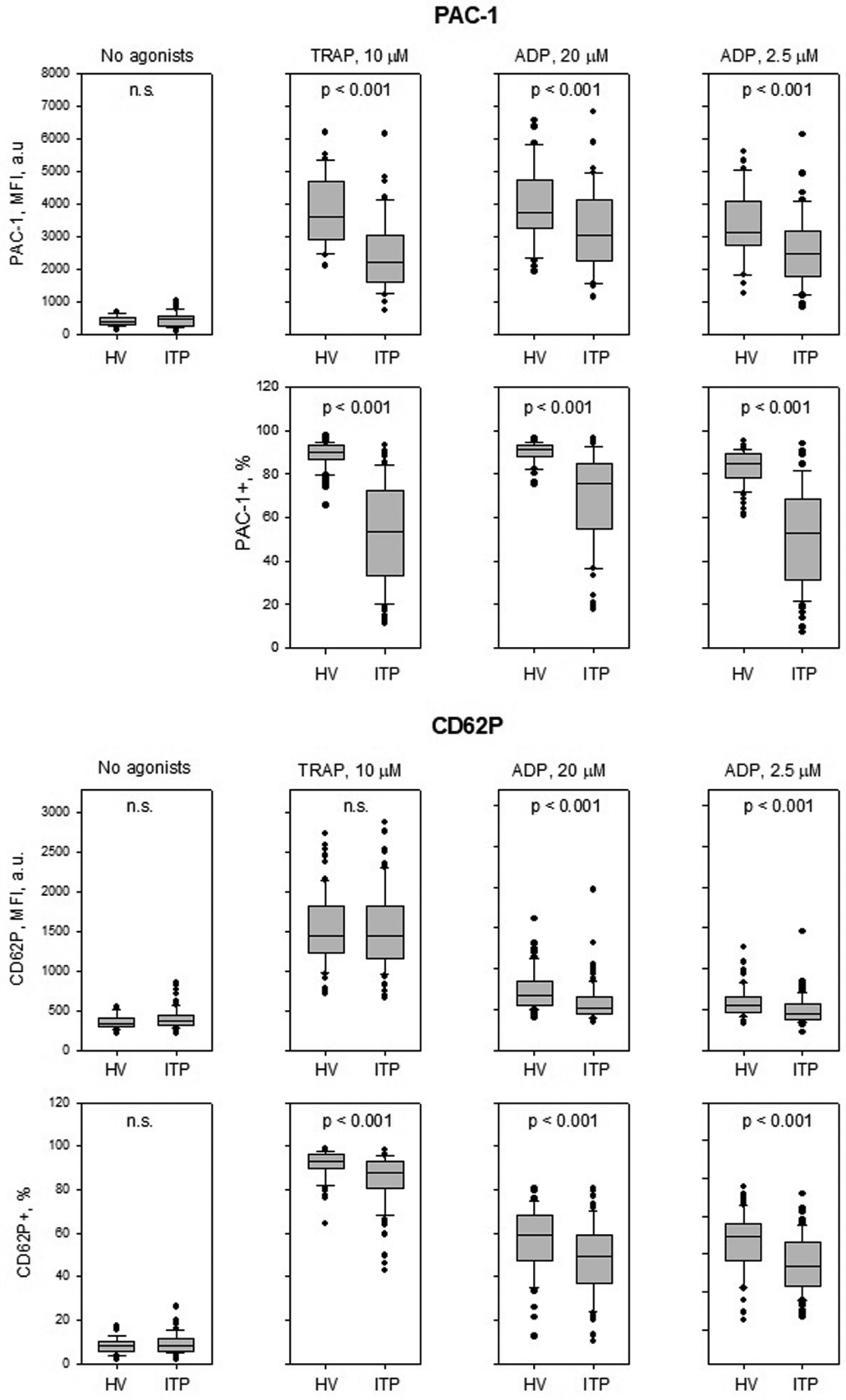

Figure 2. Platelet functional activity in HV and ITP patients (as indicated). Display of activated GP IIb-IIIa (PAC-1 antibody binding, upper panel) and P-selectin (CD62P antibody binding, lower panel). Platelets were not activated ("no agonist") or activated by 10 μM TRAP, 20 μM ADP, and 2.5 μM ADP. Medians, 25–75%, 5–95%, and individual values above and below 5–95% are shown for PAC-1 MFI (a.u.), PAC+, %, and CD62 MFI (a.u.), and CD62P+, %. *p* – significance of differences between HV and ITP patients, n.s. – not significant.

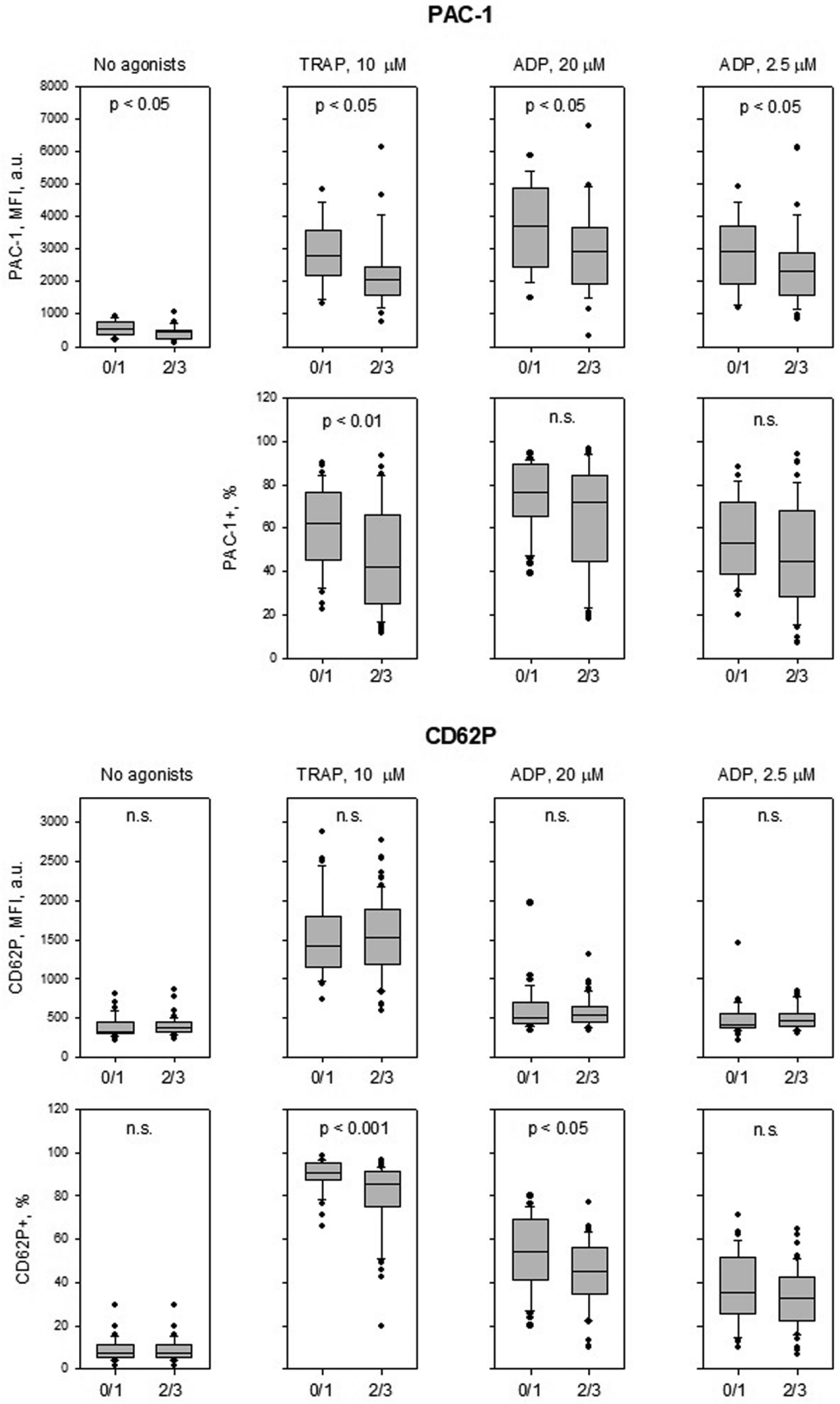

Figure 3. Platelet functional activity in ITP patients with different bleeding grades – groups 0/1 and 2/3. Exposure to activated GP IIb-IIIa (PAC-1 antibody binding, upper panel) and P-selectin (CD62P antibody binding, lower panel). Platelets were not activated ("no agonist") or activated by 10 μM TRAP, 20 μM ADP, and 2.5 μM ADP (as indicated). Medians, 25–75%, 5–95%, and individual values above and below 5-95% are shown for PAC-1 MFI (a.u.), PAC+, %, and CD62 MFI (a.u.), and CD62P+, %. *p* – significance of differences between groups 0/1 and 2/3, n.s. – not significant.

Table 2. Correlations of platelet functional activity parameters and PA-IgG in ITP patients

	PAC-1/PA-IgG binding	
	PAC-1 MFI, a.u..	PAC-1+, %
Without agonists	-0.127	—
10 μM TRAP	-0.445***	-0.373***
20 μM ADP	-0.426***	-0.302**
2.5 μM ADP	-0.442***	-0.380***
	CD62P/PA-IgG binding	
	CD62P MFI, a.u..	CD62P+, %
Without agonists	0.181	0.080
10 μM TRAP	0.018	-0.389***
20 μM ADP	-0.020	-0.202
2.5 μM ADP	0.020	-0.191

Data represent correlation coefficients. **p<0.01, ***p<0.001 – significance of correlations.

Table 3. Correlations of platelet functional activity parameters, FSC, and RP, % in HV and ITP patients

	HV	**ITP**	**HV**	**ITP**
	PAC-1, MFI/FSC		PAC-1, MFI/RP	
Without agonists	0.194	0.288*	0.431***	-0.016
10 μM TRAP	0.311*	0.185	0.386**	0.069
20 μM ADP	0.376**	0.012	0.465***	-0.085
2.5 μM ADP	0.291*	0.052	0.376**	-0.062
	CD62P, MFI/FSC		CD62P, MFI/RP	
Without agonists	0.151	0.284*	0.012	0.239
10 μM TRAP	0.464***	0.599***	0.495***	0.446***
20 μM ADP	0.450***	0.141	0.507***	0.029
2.5 μM ADP	0.452***	0.193	0.536***	0.099

Data represent correlation coefficients. **p<0.01, ***p<0.001 – significance of correlations.

Table 4. The main characteristics of ITP patients with different levels (grades) of bleeding

	Bleeding grade 0/1 (n=34)	**Bleeding grade 2/3 (n=50)**
Age	51 (36–62)	53 (39–60)
Gender, m/f	14/20	18/32
Platelets, $\times 10^9$/l	38 (23–48)	25 (14–38)**
FSC, a.u.	29970 (25219–35543)	32883 (26524–44038)
RP, %	11.3 (7.9–18.8)	18 (13.1–23.0)**
PA-IgG, % of control	400 (340–570)	620 (410–850)**
Glycocalicin, % of control	75 (60–110)	73 (60–95)
TPO, ng/ml	0.95 (0.51–2.93)	1.21 (0.62–3.41)

Data represent medians (and 25–75 percentiles). **p<0.01, ***p<0.001 – significance of differences from group 0/1.

were found for CD62P MFI after platelet activation with either agonist or for the basal level without agonists, and only a small, albeit statistically significant, decrease was found for CD62P+, % when platelets were activated with 10 μM TRAP and 20 μM ADP (11% and 16%, respectively).

We have performed ROC analysis for potential markers of severe bleeding by using the parameters with the highest difference between groups 0/1 and 2/3: platelet count, PA-IgG, RP, % and PAC-1+, % after platelet activation with 10 μM TRAP (other parameters of platelet activity were reduced to a lesser extent in the group 2/3). Optimal cut-off values were calculated for all markers and all models were statistically significant with areas under the curve from 0.668 to 0.697. Specificity of all markers was quite high: more than 80% (low false-positive rate), but sensitivity was insufficient, from 42% to 56% (high false-negative rate) (Table 5; ROC curves, Figure S2 in Supplementary Materials).

Table 5. ROC-analysis of bleeding markets. Comparisons of groups of ITP patients with bleeding grades 0/1 and 2/3

Marker	Threshold value	AUC	Sensitivity	Specificity
Platelets, $\times 10^9$/l	$\leq 20 \times 10^9$/l	0.673**	42%	85%
PA-IgG, % of control	>610%	0.697***	50%	82%
RP, %	>19%	0.668**	45%	85%
PAC-1+, % (10 μM TRAP)	≤44%	0.681**	56%	81%

AUC – areas under the curve. **p<0.01, ***p<0.001 – significance of the model.

DISCUSSION

In this study, we have assessed the functional activity of platelets in ITP patients in comparison with HV. Using flow cytometry, we determined the exposure of two activation markers on the platelet surface, the activated form of the fibrinogen receptor, GP IIb-IIIa, and the alpha granule membrane protein, P-selectin, by measuring binding of fluorescently labeled antibodies against PAC-1 and CD62P, respectively. Binding of both antibodies, slightly reduced in ITP patients, is consistent with some of the results obtained in separate studies, also indicating a decrease in the functional activity of platelets in ITP patients, determined using flow cytometry [10, 11]. However, in other studies, an increase in activation parameters was registered [12, 13] or differently directed results were obtained: a simultaneous decrease and increase in activation parameters depending on the platelet agonist used [14, 15]. These discrepancies can be partly explained by differences in the inclusion criteria for patients with ITP. Unlike other authors, we did not include in the study patients without an increase in PA-Ig (i.e. without a proven autoimmune component in the development of thrombocytopenia) and with a significant decrease in plasma glycocalicin (i.e. with signs of hypoproductive thrombocytopenia). Another reason for the inconsistency of the results obtained may be the differences in the analyzed groups: in some studies, patients were examined exclusively with a chronic course of the disease [12] or pediatric patients [13]. Some authors have found an increase in the level of basal platelet activation (without addition of exogenous agonists) in ITP patients [11, 13, 15, 16]. However, like some other authors [6, 12], we did not observe such an increase, possibly due to precautions taken to reduce platelet activation during blood sampling (e.g. not using thin needles and vacutainers).

PAC-1 antibody binding to platelets in ITP-patients negatively correlated with the content of PA-IgG, characterizing the total level of antiplatelet autoantibodies on the platelet surface. An inverse correlation was observed for both parameters of PAC-1 binding (MFI and PAC-1+, %) during platelet activation by all agonists used. Since antiplatelet autoantibodies are most often directed against GP IIb-IIIa [1–3], it can be assumed that they can interfere with PAC-1 binding to its activation-dependent epitope in GP IIb-IIIa. It is known that the PAC-1 antibody recognizes an epitope within or close to the fibrinogen binding site exposed during platelet activation. This suggests that some autoantibodies that inhibit its binding can also suppress the activity of GP IIb-IIIa in relation to the binding of fibrinogen, its physiological ligand. However, PAC-1 is an IgM antibody and, due to its large size, PAC-1 binding may be impaired not only by autoantibodies to its specific epitope but also by epitopes located at some distance that do not necessarily inhibit fibrinogen binding and subsequent platelet aggregation. This issue requires clarification, perhaps by testing fibrinogen interactions directly with ITP patient platelets. The reduction in CD62P antibody binding cannot be directly explained by autoantibodies, which are rarely directed against P-selectin [28]. In contrast to PAC-1 a negative correlation between CD62P and PA-IgG binding was found only for CD62+, % after platelet activation with 10 μM TRAP.

We have previously demonstrated that in healthy individuals, MFI values for PAC-1 and CD62 antibody binding correlated with platelet size and PT, % [25] and confirmed the results previously obtained in the present study. In accordance with numerous previously obtained data [17–24], we have also observed a significant increase in the platelet size (FSC index) and RP, % in ITP patients. However, these changes were not accompanied by an increase in PAC-1 MFI or CD62P MFI. Moreover, these parameters were slightly reduced in ITP patients and did not correlate with FSC and RP, %. These results indicate that the exposure of platelet activation markers in ITP is influenced not by the platelet size and RP content, but also by other factors, one of which may be autoantibodies interacting with platelet antigens and, in particular, with GP IIb-IIIa.

The observed decrease in the levels of activated GP IIb-IIIa and P-selectin expression on the platelet surface in ITP patients cannot be explained by a decrease in the total content of these proteins. Platelets in ITP patients are “younger” (increased proportion of RP) and larger than in HV. It is known that “young” and large platelets are characterized by an increased content of platelet surface receptors and intracellular granules (the source of P-selectin) [29].

In the present study, using CD42b antibodies against GP Ib as a marker for "gating" platelets in whole blood, we observed an increase, rather than a decrease, in the content of this receptor in ITP patients (data not shown). We also did not observe a decrease in maximum P-selectin expression levels (CD62P, MFI) in ITP patients when platelets were activated with 10 μM TRAP, a potent agonist, stimulating almost total (100%) granule release and P-selectin translocation to the platelet surface.

ITP patients with more severe hemorrhagic complications (group 2/3 vs. group 0/1 according to the bleeding assessment scale score) were characterized by lower platelet counts, higher PA-IgG and RP, % values, and a decrease in some parameters of platelet activation. In ROC analysis, these markers demonstrated high specificity but insufficient sensitivity. In other words, we observed a large number of false negative results when, contrary to the proposed models, the platelet count and PAC-1+, % (the activation index that changes the most) were higher, and PA-IgG and PT, % were below the threshold values in patients with severe bleeding (group 2/3). The fact that some patients develop hemorrhagic syndrome with a relatively high platelet count (in some cases $>50\times10^9$/l) and unchanged platelet activity indicates that we still do not know all the factors that determine the severity of bleeding in ITP.

Exclusion of ITP patients with the most dangerous bleeding (grade 4 on the bleeding scale), who required emergency hospitalization and immediate intensive treatment was a limitation of our study. This was due to the fact that all types of ITP-specific therapy (intravenous immunoglobulin, corticosteroids, TPO receptor agonists, etc.) could affect the studied platelet characteristics, including their size, RP content, and functional activity.

CONCLUSIONS

The data obtained indicate a decrease in the functional activity of platelets in ITP patients (decreased exposure of activation markers on their surface: activated GP IIb-IIIa and alpha-granule membrane protein P-selectin), and this decrease was more pronounced in patients with severe bleeding.

ACKNOWLEDGEMENTS

The authors are very grateful to M.A. Ovchinnikov (Chazov National Medical Research Center of Cardiology) for providing TRAP.

FUNDING

This study was supported by the grant from the Russian Ministry of Health (project No. 124020200125-3).

COMPLIANCE WITH ETHICAL STANDARDS

All volunteers and patients gave voluntary informed consent for the use of their blood samples for research purposes. The study was approved by the independent ethics committee of the Chazov National Medical Research Center of Cardiology (protocol No. 298 dated January 29, 2024).

CONFLICT OF INTEREST

The authors declare no conflicts of interest.

Supplementary materials are available in the electronic version at the journal site (pbmc.ibmc.msk.ru).

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Received: 25.06.2025.
Revised: 04.07.2025.
Accepted: 07.07.2025.

ФУНКЦИОНАЛЬНАЯ АКТИВНОСТЬ ТРОМБОЦИТОВ У ПАЦИЕНТОВ С ИММУННОЙ ТРОМБОЦИТОПЕНИЕЙ

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Иммунная тромбоцитопения (ИТП) — одна из частых причин снижения числа тромбоцитов. Кровотечения являются основным клиническим симптомом ИТП, их тяжесть коррелирует с глубиной тромбоцитопении, но может зависеть и от изменений функциональной активности тромбоцитов. Мы сравнили активность тромбоцитов у здоровых добровольцев (ЗД) и пациентов с ИТП, а также в группах пациентов с ИТП с разным уровнем кровотечений. В исследование были включены 65 ЗД и 84 пациента с ИТП. Активность тромбоцитов оценивали с помощью проточной цитофлуориметрии. Тромбоциты активировали пептидом, активирующим рецептор тромбина (TRAP), или ADP и определяли экспонирование на их поверхности маркеров активации, активированной формы гликопротеина (ГП) IIb-IIIa и белка мембран альфа-гранул P-селектина, измеряя связывание антител PAC-1 и CD62P соответственно. Тромбоцит-ассоциированные IgG (TA-IgG, показатель уровня антитромбоцитарных аутоантител), процент “молодых” ретикулярных тромбоцитов (РТ, %) и светорассеяние тромбоцитов (показатель их размера) также оценивали с помощью проточной цитофлуориметрии. Связывание с тромбоцитами PAC-1 и в меньшей степени CD62P было ниже у пациентов с ИТП, чем у ЗД. У пациентов с ИТП связывание PAC-1 обратно коррелировало с содержанием TA-IgG. В отличие от ЗД, у пациентов с ИТП связывание PAC-1 и CD62P не коррелировало напрямую с размером тромбоцитов и РТ, %. У пациентов с ИТП с тяжёлыми кровотечениями количество тромбоцитов было ниже, было снижено связывание PAC-1 и CD62P и повышены уровни TA-IgG и РТ, %. Таким образом, у пациентов с ИТП было зарегистрировано снижение содержания на поверхности тромбоцитов маркеров активации, которое было в большей степени выражено у пациентов с тяжёлыми кровотечениями. Предполагается, что причиной этого снижения может быть воздействие аутоантител (TA-IgG) на тромбоциты и, в частности, на ГП IIb-IIIa.

Полный текст статьи на русском языке доступен на сайте журнала (http://pbmc.ibmc.msk.ru).

Ключевые слова: иммунная тромбоцитопения; функция тромбоцитов; антитела к тромбоцитам; ретикулярные тромбоциты; размер тромбоцитов; кровотечения

Финансирование. Работа выполнена при поддержке гранта Министерства здравоохранения № 124020200125-3.

Поступила в редакцию: 25.06.2025; после доработки: 04.07.2025; принята к печати: 07.07.2025.