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REVIEW

Methodological Considerations in Platelet Flow Cytometric Studies

János Kappelmayer | Lénárd Gonda | Bálint Krajcsir | Ildikó Beke Debreceni

Department of Laboratory Medicine, Faculty of Medicine, University of Debrecen, Debrecen, Hungary

Correspondence: János Kappelmayer (kappelmayer@med.unideb.hu)

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ABSTRACT

Platelets are anucleate cells that can be studied by benchtop flow cytometers and today there are several types of flow cytometric assays for platelets. Platelet structure studies in clinical flow cytometry mostly target rare congenital platelet glycoprotein deficiencies (e.g., Glanzmann-thrombasthenia, and Bernard-Soulier syndrome) that can be considered as the prototypes for platelet flow analysis, since the identification of complete or partial deficiency of these molecules provides the proper diagnosis. Another area of platelet flow cytometry is the detection of activated platelets. Mostly it means the gold standard platelet P-selectin assay or the investigation of PAC-1 binding—an assay where the activation dependent epitope of the GPIIb/IIIa receptor is investigated—but the measurement of platelet-leukocyte aggregates and microparticle analysis have gained increasing role. A unique platelet subtype is the subgroup designated as coated platelets that are formed by the simultaneous activation with collagen and thrombin. In clinical practice further functional assays are also informative tests: the monitoring of clopidogrel resistance by measuring the intracellular phosphorylated VASP and identifying heparin induced thrombocytopenia (HIT) by the use of patient plasma. The flow cytometric HIT test utilize the detection of phosphatidylserine expression on normal platelets or the formation of microparticles in the presence of the patient's plasma and therapeutic concentration of heparin, thus it seems reasonable to suggest it for the study of the functionality of the HIT antibodies. Nevertheless, there are also limitations to these assays as several of them require fresh samples, thus sample transport to specialized laboratories is not always feasible.

1 | Introduction

The analysis of platelets by flow cytometric methods was lagging behind leukocyte observations for several decades. There were a number of understandable reasons for this discrepancy, primarily that the first flow cytometers were barely capable for the identification of cells that were much smaller than a lymphocyte. In the early years of platelet flow cytometric studies, the dot-plots that were presented in those manuscripts from the 1980s would probably be unacceptable by recent journal editors. The other reason was that the attention of flow cytometric analysis in these years was mostly focused on major questions in immunology like lymphocyte subset analysis and on hematology like identifying leukemic cells by this novel immunofluorescent technique. Thirdly, many fluorescent probes and directly conjugated antibodies were developed later than for the investigation of leukocyte analysis.

The advent of technological developments in the past decades, however have considerably expanded and reshaped the field of clinical flow cytometry, thus recently platelet analysis is an everyday practice in several clinical laboratories. Today, the technologies have expanded far beyond the identification of congenital glycoprotein deficiencies by flow cytometry, as a part of these fluorescent methods is also utilized in routine hematology analyzers for accurate platelet enumeration. Most recently, the

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technological advancements also enable us to reliably analyze platelet-derived microparticles with dedicated cytometers that were developed particularly for this purpose.

This review describes some important methodological issues of structural and functional platelet analysis and highlights their role in the diagnostic algorithms.

2 | Preanalytical Aspects: A Cornerstone of Platelet Flow Cytometric Analysis

Garbage in, garbage out; this is the classical term used for any type of clinical laboratory analysis. More explicitly, if—for any reason—the sample is unsuitable for analysis, the laboratory can do its best but still no meaningful result will be obtained, and this is also true for all types of platelet studies.

Routinely, platelets are enumerated by an automated hematology analyser where a potential problem could be the presence of EDTA-induced thrombocytopenia that can be identified by the provided analyser flags and can usually be overcome by drawing the blood into citrate or alternatively into some other anticoagulant like magnesium-sulfate tube. The gold standard test for platelet function analysis is platelet lumi-aggregation, but this technique is out of the scope of the recent review.

2.1 | Collection of Anticoagulated Samples

The most commonly used anticoagulant for flow cytometry analysis is buffered sodium citrate [1]. Expert opinions differed on the acceptability of other anticoagulants. Ethylenediaminetetraacetic acid (EDTA) should be avoided since it dissociates the fibrinogen receptor αIIbβ3 integrin irreversibly, thus preventing fibrinogen binding. Strong calcium-chelating agents, like EDTA, result in loss of binding of complex specific antibodies apparently due to disassembly of the heterodimer [2]. In addition, EDTA also results in swelling of platelets [3]. However, in a clinical study, there was a positive correlation between the percentage of reticulated platelets (RP) and the mean platelet volume (MPV) in both EDTA- and citrate-anticoagulated blood [4]. For P-selectin analysis, blood anticoagulated with sodium citrate should be used instead of K_3-EDTA, as it had a statistically significant increasing effect on P-selectin expression [5].

Elevated levels of platelet-derived microparticles (PMP) were detected in heparin and D-Phe-Pro-Arg-chloromethyl ketone (PPACK)-anticoagulated samples as compared to samples anticoagulated with acid-citrate-dextrose (ACD) or sodium citrate [6]. Similarly, PMP count determination was carried out from citrated and heparinized plasma, and a more accurate PMP quantification was observed in citrated plasma [7]. Furthermore, heparin enhances the activation of platelets by adenosine diphosphate (ADP); therefore, flow cytometric results of platelet activation in patients receiving heparin should be interpreted carefully [8]. In addition, anticoagulation with heparin, PPACK, sodium citrate, and EDTA resulted in significant differences in platelet-monocyte aggregation, and a direct thrombin inhibitor as an anticoagulant was suggested [9].

2.2 | Collection of Samples in Fixative

Sample fixation is an important issue in flow cytometric platelet assays. Paraformaldehyde (PFA) is considered highly beneficial to prevent spontaneous in vitro platelet activation. However, PFA induces a systematic stimulation of platelets [10]. In contrast, P-selectin expression was not significantly altered by fixation with a low concentration of formaldehyde solution (0.2% or 0.5%), but was slightly decreased by 1.0% formaldehyde solution or PFA fixation [11].

Annexin V has a high affinity to negatively charged phospholipids and can be used to identify procoagulant platelets. For annexin V staining the fixation is not commonly recommended, because formaldehyde can cause factitious annexin V binding. However, a low concentration of calcium-free formaldehyde solution (1.1%) did not alter the proportion of annexin V positive platelets [12]. Nevertheless, it should be noted that formaldehyde-fixed platelet samples should be analyzed within 12 h of the fixation to avoid artifactual increases in fluorescence [13].

ThromboFix is a platelet stabilizer (from Beckman Coulter) and may be used in conjunction with platelet monoclonal antibody-dye conjugates for flow cytometric analysis. Fixation of samples by ThromboFix is helpful in flow cytometric analysis of the platelet activation marker, CD62P, but it should not be used for the investigation of leukocyte-platelet aggregate formation [14].

For flow cytometric analysis of the activated conformation of αIIbβ3 integrin the PAC-1 antibody (pentameric IgM κ-immunoglobulin) can be used, but the activation-dependent antibody binding can be affected by the fixation and is often decreased compared to unfixed platelets [15].

2.3 | Analysis of Platelets in Different Sample Types

Platelet function can be comprehensively evaluated by flow cytometry in whole blood. It can be used to measure platelet reactivity, circulating activated platelets, platelet–platelet aggregates, leukocyte-platelet aggregates, procoagulant platelet-derived microparticles, reticulated platelets, and calcium flux [15]. Furthermore, whole blood is recommended for the assessment of platelet function and activation by flow cytometry, especially in patients with low platelet count [16].

However, in specific cases, it might be necessary to evaluate platelet function in the absence of other blood cells. Platelet rich plasma (PRP) can be used in flow cytometric assays for setting up the diagnosis [17] or the investigation of the effect of drugs on platelet function [18]. The advantage of using PRP in flow cytometry testing is that no significant platelet activation was observed after the separation of PRP (baseline), which was characterized by very low P-selectin expression.

For the study of the intrinsic properties or specific signaling pathways of platelets, washed platelets (WPs) or gel-filtered platelets (GFPs) can be used [19, 20]. The advantage of these types of samples is that platelets are separated from the anticoagulant and their plasma environment [21]. For preparation

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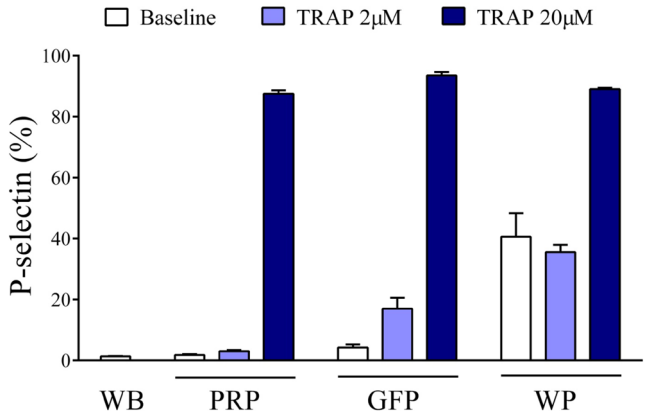

FIGURE 1 | Platelet activation in different sample types. Platelet function can be investigated in whole blood and different platelet isolates. However, platelets may be activated to varying degrees during the separation. P-selectin expression is negligible on platelets in whole blood (WB) and platelet-rich plasma (PRP) and relatively low on gel-filtered platelets (GFP); however, considerably more on washed platelets (WP). In addition, WPs are insensitive to a low concentration of thrombin receptor activating peptide (TRAP); as a result, surface P-selectin expression did not increase significantly after the activation with 2 μM of TRAP, but all sample types showed very high P-selectin expression in the presence of 20 μM of TRAP. Bars represent the mean and SD values of $n = 3$ experiments.

of GFP whole blood was drawn into ACD containing tube. After a two-fold dilution with buffered saline-glucose-citrate (BSGC; pH 7.3) at room temperature (RT), PRP was immediately prepared during centrifugation at 170 x g for 8 min at RT. Gel filtration was performed on a column of Sepharose CL-2B (Sigma-Aldrich, Saint Louis, MO, USA), and purified platelets were adjusted to the cell concentration of 40 G/L in BSGC. However, the isolation-induced platelet activation is considerably more in WPs than in PRPs or GFPs. During this procedure PRP was prepared by centrifugation from ACD-anticoagulated whole blood (150 g, 15 min, 37°C). Subsequently, platelets were washed in citrate buffer (128 mM NaCl, 4,3 mM Na_2HPO_4, 7,5 mM Na_2HPO4, 4,8 mM Na-citrate, 2,4 mM citric acid, 11 mM glucose, 0,35 m/V % bovine serum albumin (BSA), pH: 6,5) with PGE1 (300 ng/mL) and apyrase (1,2 U/mL). Finally, platelets were resuspended in HEPES buffer (10 mM Hepes, 137 mM NaCl, 2.8 mM KCl, 1 mM $MgCl_2$, 12 mM Na_2CO_3, 5,5 mM glucose, 0,4 mM Na_2HPO_4, 0,35 m/V % BSA, pH: 7,4). WPs were insensitive to activation (15 min at RT) with low concentration of thrombin receptor activating peptide (TRAP; Sigma-Aldrich, Saint Louis, MO, USA), as a result surface P-selectin expression did not increase significantly after activation with a low concentration (2 μM) of TRAP (Figure 1). Nevertheless, all sample types showed similar P-selectin expression upon activation with a high concentration (20 μM) of TRAP.

3 | Platelet Structural Phenotyping by Flow Cytometry

Platelet biology has moved beyond simple counts and light-transmission aggregation to high-content single-cell phenotyping, with flow cytometry (FC) becoming a standard research and clinical tool for assessing platelet structure, receptor expression, and activation state. By using FC we have the ability to (i) analyze thousands of individual platelets in small sample volumes, (ii) combine lineage gating (CD41/CD61) with multiple fluorescent labels, and (iii) detect both surface-exposed and intracellular antigens. These has driven its adoption for diagnostic workflows (inherited platelet disorders, immune/autoimmune thrombocytopenias, drug effect monitoring) as well as in mechanistic research. Recently, consensus and methodological papers emphasize standard panels (integrin activation, P-selectin expression, fibrinogen binding) and provide best-practice guidance for preanalytics, gating, and controls—a clear sign that platelet FC moved from niche to mainstream in both labs and translational studies [16].

3.1 | Key Surface Structural Platelet Markers

Surface glycoproteins define the platelet's adhesive/aggregation machinery and are essential readouts in both diagnosis and functional studies. CD41 (GPIIb/integrin αIIb) and CD61 (GPIIIa/integrin β3) form a core integrin heterodimer (αIIbβ3) that is widely used as platelet identity/gating markers and as functional readouts (bound ligand, PAC-1 reactivity). It can be used representatively for broad platelet immunophenotyping and for functional panels [22]. CD42a (GPIX), CD42b (GPIbα), CD42c (GPIbβ), and CD42d (GPV) of the GPIb–IX–V complex mediates VWF binding and initial tethering under shear; FC is used to quantify expression and to detect defects (e.g., Bernard–Soulier syndrome) [23]. Further platelet markers are used less frequently, like CD31 (PECAM-1), an adhesion/signaling molecule that is mainly used in platelet–leukocyte aggregate studies and to probe shear/adhesion biology [24]. Morphological gating of platelets alone is insufficient. Over the last decade, a consensus has emerged that integrin αIIbβ3 (GPIIb/IIIa) and the GPIb-IX-V complex are the most robust and specific antigenic determinants for identifying platelets [25].

3.2 | Activation Markers

Activation markers are the standard readouts for platelet functional assays by flow cytometry. CD62P (P-selectin) is a classical α-granule membrane protein that translocates to surface upon activation and is measured routinely by FC as a sensitive activation marker [26]. CD63 (LAMP-3) is another secretion induced dense-granule/lysosomal membrane protein that appears on the surface after degranulation [27]. CD107a/CD107b (LAMP-1/LAMP-2) are degranulation markers, in which CD107a is commonly used in degranulation assays. Platelet CD40L (CD154) expression (surface-associated or platelet-bound) is measured by FC in studies linking platelets to inflammation/immune modulation [28]. PAC-1 binding, PAC-1 is a conformation-specific monoclonal antibody that binds the activation-dependent epitope of αIIbβ3 or fluorescent fibrinogen binding, are central to nearly all FC platelet activation protocols and is in consensus panel recommendations [29].

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3.3 | Conformational Probes—LIBS/RIBS/Conformation-Sensitive mAbs

Ligand-induced binding sites (LIBS) antigens are epitopes that become exposed on integrins when they adopt ligand-competent conformations. LIBS-reactive mAbs were among the first probes of integrin activation; they allow detection of conformational changes independently of ligand occupancy. Early and illustrative studies used LIBS-reactive antibodies to follow αIIbβ3 activation and to probe how antagonists/ligands change receptor shape [30]. PMI-1 (a LIBS-sensitive mAb) and related LIBS reagents have been used in mechanistic work to show affinity modulation and to map glycosylation/conformational effects on αIIbβ3 [31].

As a receptor-induced binding site (RIBS-type) reagent 9F9 was historically described as a binding site on fibrinogen when the ligand is bound to platelet receptors. 9F9 has been used to detect fibrinogen bound to activated platelets (i.e., ligand-occupied complexes)—an approach that gives information about ligand conformation and receptor–ligand complexes rather than only receptor exposure [32].

3.4 | Intracellular/Cytoplasmic Platelet Markers Beyond FXIII

Factor XIII (FXIII) is an intracellular transglutaminase abundantly expressed in the platelet cytoplasm, where it contributes to fibrin cross-linking and thrombus stabilization. Intracellular detection of FXIII subunit A (FXIII-A) by flow cytometry has been used to characterize activated and procoagulant platelet subpopulations. Co-expression of FXIII-A with classical activation markers such as P-selectin further supports its association with functionally activated platelet states [27]. Historically, FXIII-A was the canonical intracellular platelet marker, but multiple intracellular granule and signaling proteins are now routinely or experimentally measured by flow cytometry in permeabilized cells and have diagnostic or mechanistic value.

CD63 and CD107a are used both as surface activation markers and (when measured intracellularly) as granule content indicators. Flow cytometry papers use CD63/CD107 stains to readout degranulation and granule content [27]. Platelet serotonin (5-HT) content and release can be quantified by intracellular flow cytometric staining, and this has been used in studies of platelet biology, psychiatric drug effects, and immune interactions [33]. Platelet factor 4 (PF4, CXCL4) is measurable in platelets by intracellular FC. Von Willebrand factor (vWF), although predominantly derived from endothelial cells, is stored in platelet α-granules, and platelet vWF binding has also been measured by FC in neonatal and adult studies. vWF binding to platelets is also quantifiable as a functional readout [34]. Flow cytometry and microscopy studies show fibrinogen internalization and intracellular pools, which can be assessed after permeabilization [35]. Thrombospondin-1 (TSP-1/THBS1) is an α-granule protein, and flow cytometry has been used to detect platelet TSP-1 content and surface exposure after stimulation [36]. The major platelet structural markers are summarized in Table 1.

TABLE 1 | Some important platelet markers used in flow cytometric studies.

Cluster of differentiation (if available)	Target molecule
Constitutive markers	
CD31	PECAM-1
CD41	GPIIb, αIIb integrin
CD42a	GPIX (GPIb-V-IX complex)
CD42b	GPIbα (VWF receptor)
CD42d	GPV (GPIb-V-IX complex)
CD61	GPIIIa, ß3 integrin
Activation markers	
CD40L	CD154, CD40 ligand
CD62P	P-selectin
CD63	Tetraspanin, LAMP-3
CD107a	LAMP-1
CD107b	LAMP-2
Intracellular markers	
FGA	Fibrinogen
FXIIIa	Factor XIII subunit A
PF4	Platelet factor 4
TSP-1	Thrombospondin
vWF	von Willebrand Factor
5-HT	Serotonin

3.5 | Platelet Subtypes

Understanding platelet heterogeneity has become crucial in both basic and translational hemostasis research. Beyond single-value assays (platelet count, aggregation), modern approaches emphasize functionally distinct platelet subpopulations that are generated during thrombus formation. These subpopulations differ in surface phenotype, intracellular cargo exposure, procoagulant activity, mechanical behavior, and interactions with other blood cells. Flow cytometry (with both surface and intracellular stains, plus functional probes like annexin V, PAC-1 and fluorescent fibrinogen/vWF binding) is a central method to enumerate and phenotype these subpopulations.

4 | Aggregatory/Contractile (Aggregating) Platelets

These are the platelets that mediate adhesion, spreading, and contraction within a developing thrombus: they possess activated integrin αIIbβ3 (bind fibrinogen and vWF), generate traction forces on fibrin, and drive clot contraction (retraction). On a single-cell level, they display strong PAC-1 reactivity and high fibrinogen binding, and mechanically they generate substantial

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contractile force. These can be identified by PAC-1 binding or fluorescent-fibrinogen binding for integrin activation [37].

5 | Procoagulant/Phosphatidylserine-Exposing Platelets

A specialized subpopulation formed in response to strong stimulation (e.g., collagen) that exposes phosphatidylserine (PS) on the outer leaflet, provides a negatively charged surface for assembly of coagulation enzyme complexes (tenase/prothrombinase), and supports explosive thrombin generation. These are also called "procoagulant platelets" and are distinct from aggregatory platelets. A common characteristic for investigating this subtype is high annexin V or lactadherin binding (PS marker) and CD62P/CD63 may be coexpressed [38].

6 | Coated-Platelets (COAT-Platelets)

"Coated" or COAT-platelets are a distinct subpopulation that forms after dual-agonist stimulation (e.g., collagen + thrombin). They differ from the previously described PS exposing subgroup as these platelets retain high levels of α-granule proteins on their surface (a "protein coat"), but also expose PS, and bind coagulation factors—making them strongly procoagulant and important for localized fibrin formation. COAT-platelets have been linked to pathologies and to inter-individual differences in clotting. Different assays that measure retention/display of α-granule proteins (e.g., thrombospondin, fibrinogen), combined with annexin V or other procoagulant markers show a great potential to measure COAT-platelets. Flow cytometric methods allow the quantification of the proportion of platelets that form a COAT-phenotype after a specific stimulation [39].

Characteristics flow cytometric signals of the aggregatory and procoagulant platelet subtypes are shown in Figure 2. During this experiment, platelets were activated by convulxin and identified by their CD41 labeling (panel A). The active conformation of the integrin alphaIIb/beta3 was determined by binding of FITC-conjugated PAC-1 and PECy5-conjugated CD41a antibodies (panel B). Platelet PS expression was determined by annexin V binding to platelet surface using FITC-conjugated annexin V and for platelet identification (panel C). Five microliters of PRP was stained with 5 μL of annexin V-FITC and 5 μL of CD41-PE in 35 μL annexin V binding buffer (it provides calcium for binding of annexin V), and the mixture was incubated for 15 min at RT in the dark.

6.1 | Reticulated/Immature Platelets (Young Platelets)

Reticulated platelets (RP) or immature platelet fraction (IPF) are newly released platelets from megakaryocytes; they are larger, more RNA- and ribosome-rich, metabolically active and often hyper-reactive. Clinically, IPF is used as an indicator of thrombopoietic activity and to help distinguish causes of thrombocytopenia. A common method to identify IPF is RNA-binding dyes (e.g., thiazole orange, oxazine) and size (FSC) and CD41 gating [40]. Most modern automated hematology analyzers capable of fluorescent platelet RNA detection can measure reticulated or immature platelets. This includes Sysmex, Abbott, Beckman Coulter, Mindray, and Horiba instruments, with the results expressed as IPF% and/or absolute immature platelet counts. These measurements are useful for assessing thrombopoiesis, platelet turnover, and bone marrow recovery.

7 | Platelet Functional Studies by Flow Cytometry

Current platelet functional assays routinely performed by light transmission aggregometry have methodological limitations in samples with lower platelet count. Platelet aggregation as measured by flow cytometry provides an opportunity to observe platelet aggregates in small blood volumes and with low platelet counts. Flow cytometry assays have been developed previously [41] and demonstrated assessment of aggregation in thrombocytopenic patients [42]. However, this assay may not represent the whole blood platelet function as centrifugation may remove

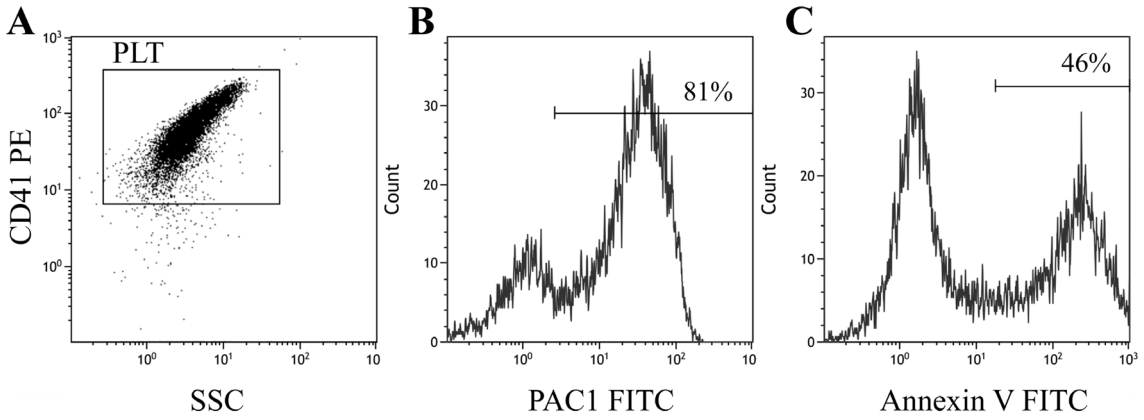

FIGURE 2 | Characteristics of aggregating and procoagulant platelets. Subpopulations of platelets (PLT) differ in the surface phenotype. Aggregating and procoagulatory platelets show a positivity to CD41 (A). In addition, aggregating platelets possess high expression of activated αIIbβ3 integrin, which can be identified by PAC-1 binding (B), while procoagulant platelets show high phosphatidylserine (PS) exposure. In this experiment, platelets were activated by convulxin, PAC-1 was detected by a conformation-dependent antibody, and PS expression was detected by annexin V binding (C).

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large platelets. Moreover, the lack of standardization limits the use of this assay.

Platelet P2Y12 receptor inhibitor drugs are widely utilized in several indications, but interindividual response has also been shown to vary in prevalence based on the assay used to assess [43]. Flow cytometric assessment of phosphorylation of vasodilator stimulated phosphoprotein (VASP), an intracellular protein which is dependent on P2Y12 receptor activation and correlates well with the degree of functional platelet inhibition, has been early described [44]. The result of flow cytometric phosphorylated-VASP assay showed good correlation with the results of light transmission aggregometry [45] and an ELISA based phosphorylated-VASP assessment assay [46]. VASP phosphorylation has been shown to index efficacy of P2Y12 inhibitors [47] and several studies showed clinical implications of this flow cytometric test [48, 49].

Diagnostics of heparin induced thrombocytopenia (HIT) begins with pre-test evaluation of 4T score, followed by immunological detection assays of antibodies against PF4-heparin complexes. However, definitive diagnosis and thrombotic potential assessment can be achieved by functional assays. The serotonin release assay is considered gold standard in HIT diagnosis, but its availability is restricted by its complexity and specialized equipment. Functional flow cytometric assays using annexin V-binding for the diagnosis of HIT have been described decades ago [50, 51]. It has also been demonstrated that positive assay results correlate with higher antibody titer [52]. Functional flow cytometric assays used for HIT diagnosis, as shown by a large cohort study, demonstrate high sensitivity and specificity compared with clinical diagnosis [53]. The efficacy of the functional flow cytometric assay can be enhanced by combining it with a positive immunoassay [54]. A typical flow cytometric pattern from a HIT positive patient is demonstrated in Figure 3. Here, PRP was obtained from trisodium citrate anticoagulated blood of healthy donors having O blood type. To avoid donor platelet variation, during our tests, PRP was obtained from the same selected high responder donors as described previously [55]. Donor PRP was separated by centrifugation (150 g, 5 min, 22°C). PRP (10 μL) was added to 10 μL patient plasma. The mixture was incubated in the presence of therapeutic (0.3 IU/mL) dose of unfractionated heparin in a final volume of 50 μL adjusted by phosphate buffered saline. Platelets were gated based on their CD41

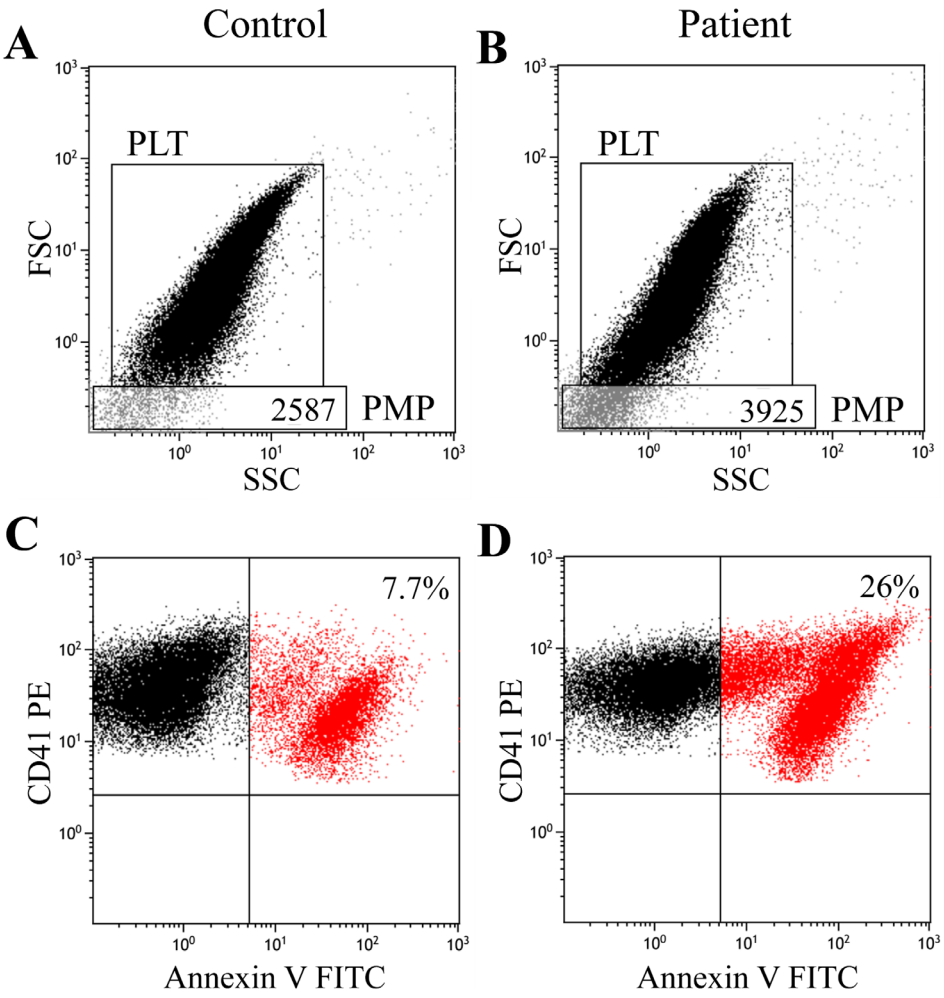

FIGURE 3 | Flow cytometric functional assay for heparin-induced thrombocytopenia (HIT). Donor platelet-rich plasma was incubated with control plasma or plasma from HIT patients in the presence of pharmacological concentration (0.3 IU/mL) of heparin. Platelet-derived microparticles (PMP) were identified based on their light scattering properties (A, B), and platelet activation was investigated by annexin V binding (C, D). Patient plasma-induced platelet activation was shown by increased counts of PMP and percentage of annexin V binding during a 1 min of the acquisition period.

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expression. PMPs produced by activated platelets were identified by size-selection based on their forward (FSC) and side scatter (SSC) properties. Size-calibrated latex spheres (diameter with 0.5, 1 and 3 μm) were used to test the correctness of PMP gating.

Vaccine-induced immune thrombotic thrombocytopenia (VITT), a prothrombotic state induced by vaccination, could be assessed by the detection of procoagulant platelets. A study describing a combination of a cell death marker and P-selectin indicated that flow cytometric test could be used for diagnosis of VITT [56].

In conclusion, platelet flow cytometric studies are getting more and more utilized by clinical laboratories, but even in developed regions like Northern Europe only 36% of the laboratories perform routinely flow cytometry for evaluating inherited platelet function defects [57] thus there is still plenty of space for development.

Author Contributions

János Kappelmayer conceptualized the work and wrote a large part of the manuscript, Lénárd Gonda and Bálint Krajcsir did the literature search and participated in the measurements and did the formatting of the manuscript, Ildikó Beke Debreceni carried out most measurements, prepared figures and wrote a part of the manuscript.

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Disclosure

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Ethics Statement

Ethical approval was obtained from the Ethics Committee of the University of Debrecen (identification code: RKEB/IKEB 4875–2017).

Consent

From controls and patients, the required consent documents were obtained.

Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

The data that support the findings of this study are available on request from the corresponding author. The data are not publicly available due to privacy or ethical restrictions.

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