FLOW CYTOMETRIC INVESTIGATION OF CLASSICAL AND ALTERNATIVE PLATELET ACTIVATION MARKERS

Béla Nagy Jr, Ildikó Beke Debreceni, János Kappelmayer
Department of Laboratory Medicine, Medical and Health Science Center, University of Debrecen, Debrecen, Hungary

Corresponding Author:

Béla Nagy Jr, MD, PhD
Department of Laboratory Medicine, Medical and Health Science Center, University of
Debrecen, Debrecen, Hungary
Tel: +36 52 340 006
Fax: +36 52 417 631
e-mail: nagyb80@gmail.com

ABSTRACT

Platelets show a substantial role in the maintenance of vascular integrity when these cells after a rapid activation adhere to the vessel wall lesion, aggregate with other platelets and leukocytes resulting in an arterial thrombosis. Analysis of in vivo platelet activation at an early time point is crucial in the detection of developing thrombotic events. In addition, the forecast of future complications as well as the evaluation of the efficacy of anti- platelet medication are also essential in a large group of patients. Changes in the levels of platelet receptors or alteration in other surface properties due to intra- and extracellular responses to a stimulus can be measurable primarily by flow cytometry with specific antibodies via the assessment of classical and alternative platelet activation markers. Some of these biomarkers have been already used in routine laboratory settings in many cases, while others still stand in the phase of research applications. Deficiency in platelet receptors is also accessible with this technique for the diagnosis of certain bleeding disorders. We here describe the most important types of platelet activation markers, and give an overview how the levels of these markers are altered in different diseases.

INTRODUCTION

Platelets are involved in the regulation of hemostasis, as activated platelets normally adhere to the injured vessel wall. Thrombocytes form aggregates with each other, but also interact with leukocytes to avoid a substantial blood loss from the circulation. These cellular complexes also contribute to the development of local inflammatory events. In contrast, abnormal platelet function may result in thrombotic or bleeding complications.

In arterial thrombosis, the level of platelet reactivity increases, and the expression of several platelet activation proteins (markers) can be measured on the cell surface. In addition, distinct platelet subpopulations (e.g. coated-platelets) may be also investigated using such experiments. Discovery of novel biomarkers is still of interest to predict emergency thrombotic states, and to monitor the effects of anti-platelet therapy. The deficiency or lack of platelet receptors may generate a dysfunction in platelet aggregation and cause hemorrhage. Early detection of all these anomalies is demanding, and flow cytometry is a reliable laboratory method to analyze platelet function in ex vivo clinical samples. This tool is now getting available in more and more laboratories, and a combination of two or three antibodies against platelet receptors allows a sensitive and specific analysis of platelets. However, there are several preanalytical and methodological pitfalls, which may influence the measurement and the interpretation of these results. In this review, the classic and alternative platelet activation markers on flow cytometry are summarized, which have been assessed in a large number of studies to evaluate altered platelet function.

I. CLASSICAL PLATELET ACTIVATION MARKERS

I/1. CD62P

P-selectin (CD62P) is one of the most abundant proteins in the α-granules of platelets, which is exposed on the cell surface within seconds after platelet activation [1]. Flow cytometric analysis of surface-bound CD62P alone or in a combination with other markers (see below) has been used as the 'gold standard' marker for the assessment of platelet activation in ex vivo patient samples in the last 3 decades reviewed in [2].

Detection of elevated surface P-selectin was the subject of numerous clinical studies in acute coronary syndrome (ACS) [3-5], in type 1 and 2 diabetes mellitus (DM) [6-8], untreated hypertension [9], obesity with or without DM [10-11], peripheral artery disease (PAD) [12], acute ischemic stroke [13-16], essential thrombocythemia (ET) [17], and in those clinical conditions where platelet activation is one part of the disease pathomechanism such as in primary Raynaud's disease [18]. Platelet-bound P-selectin values are typically determined in percent positivity (%), however, even a small change in mean fluorescence intensity (MFI) values may demonstrate a larger alteration in surface P-selectin measured on a logarithmic scale. P-selectin analysis in non-activated samples is for 'baseline' CD62P positivity in the current in vivo platelet activation status, while platelet reactivity can be evaluated with CD62P levels on stimulated platelets by using submaximal concentrations of classic agonists adenosine-diphosphate (ADP) (0.5-5 µM), collagen (1-2 µg/mL), or thrombin-receptor activating peptide (TRAP) (1-8 µM) [7,10,19,20]. Significantly higher P-selectin levels were independently correlated with the body mass index (BMI) [10], the atherosclerosis indicator carotid intima-media thickness (IMT), and the inflammation marker C-reactive protein (CRP) [11]. Stellos et al. further investigated surface-bound P-selectin as a prognostic marker in myocardial infarction (MI), and they found a positive association between the extent of myocardial injury measured with the levels of troponin-I plus creatine kinase-MB and CD62P positivity independently of age, gender, and baseline medication [5]. CD62P values were significantly increased in ST-segment elevation MI (STEMI) patients that reflected a greater degree of occlusive thrombus formation in these patients versus others with non- ST-segment elevation MI (NSTEMI) or Troponin-I-positive unstable angina (UA). On the other hand, P-selectin positivity showed a limited sensitivity (57.5%) and specificity (69%) for detection of ACS and discrimination of chest pain of different origins [5]. In monitoring of anti-platelet medication, CD62P had a minor sensitivity to the effects of ADP-receptor blocker clopidogrel and acetylsalicylic acid (ASA) therapy in stroke [21]. Surprisingly, opposite findings were also reported when less CD62P positive platelets were measured in MI [20,22] and (convalescent) cerebral infarction as baseline values and in response to agonist stimulation compared to clinical control cohorts [19,23,24]. Likewise, CD62P expression rapidly declined after the onset of acute ischemic stroke [25]. These phenomena were explained to be due to the rapid shedding of P-selectin from circulating platelets, and the sequestration of these activated cells into heterotypic aggregates [26,27]. In fact, the plasma concentration of released/shed receptors (i.e. soluble P-selectin) was measured in parallel with immunoassays as an additional platelet marker in these studies [11,19,20,24]. It was also suggested that platelets were exhausted and failed to respond to thrombin *in vitro* after a substantial cellular activation during stroke [28]. Therefore, detection of CD62P by flow cytometry seems to be a more reliable tool for monitoring platelet function at acute but not chronic stimulus of platelets.

I/2. CD40L

CD40L expression was first described on activated T-cells [29], and was later shown to be liberated to the platelet surface from α-granules, similarly to P-selectin [30]. It is now considered as an emerging platelet activation marker, and its level (CD154) was also increased when platelet activation was associated with endothelial dysfunction and inflammation in MI and UA [31]. Patients with UA who needed coronary angioplasty or had recurrent angina showed even higher CD40L expression on platelets compared with those without such complications [32]. Moreover, significant increase in CD40L on platelets was already detected in transient ischemic attack (TIA), not only complete stroke [33]. Especially in atherosclerotic ischemic stroke, CD40L positivity was enlarged compared to that in asymptomatic carotid stenosis [14]. Consequently, upregulated CD40L level was thought to initiate ischemic stroke from large artery atherosclerosis, and the concentration of this marker was correlated with worse clinical outcome after cerebral infarction [16,34].

I/3. CD63

CD63 (granulophysin, LAMP-3) is translocated from dense-granules and lysosomes to the plasma membrane after platelet activation [35]. CD63 expression was higher on day 1 in the stroke group versus control group, which remained significantly elevated until day 90 [25]. Similarly, Cha et al. found significantly higher CD63 platelet positivity in patients with atherosclerotic ischemic stroke than in normal subjects; however, no significant differences were seen between atherosclerotic ischemic stroke and asymptomatic carotid stenosis [14]. Additionally, increased CD63 level was predominantly detected in the acute stage of ischemic stroke compared with its convalescent stage and the control group [16,36]. In contrast, others found no elevation in CD63 positivity in either acute or convalescent stroke patients versus subjects without vascular disease [15]. Similarly to P-selectin, CD63 had an inferior role to detect the effects of clopidogrel and ASA in stroke patients [21].
Immunofluorescence analysis of CD63 by flow cytometry was a suitable method for the diagnosis of Hermansky-Pudlak syndrome

accompanied with bruise and bleeding complications, where the significantly lower number of dense-granules and lysosomes in platelets was recognized by using anti-CD63 antibody versus a normal sample [35].

I/4. GPIIb/IIIa receptor (PAC-1 binding)

Fibrinogen receptors undergo a conformational change during platelet activation [37]. PAC-1 antibody was formerly developed by Shattil and his coworkers [37], and nowadays it is a commercially available monoclonal antibody, which specifically binds to the activated form of GPIIb/IIIa receptor complex induced by shear stress upon platelet aggregation. Increasing level of activated GPIIb/IIIa receptors was studied from clinically stable to unstable coronary artery diseases [38]. Also, constantly elevated PAC-1 binding at 3-month follow-up was associated with an increased incidence of recurrent stroke [36]. On the contrary, McCabe et al. did not find any difference in PAC-1 percent positivity between those with acute or convalescent cerebrovascular disease [15]. In manifest metabolic syndrome, higher expression of PAC-1 with augmented fibrinogen binding was observed compared to subjects with vascular disease [39]. PAC-1 was also found as a sensitive parameter in following clopidogrel effect along with decreased level of the intracellular vasodilator-stimulated phosphoprotein (VASP) [40].

II. ALTERNATIVE BIOMARKERS OF PLATELET ACTIVATION

II/1. PMPs

Platelet-derived microparticles (PMPs) has been employed as an alternative evaluation of platelet activation in recent years. These vesicles are formed during platelet 'budding', and thus contain several components from platelet cytoplasm and outer membrane. Consequently, PMPs were positive for CD62P and CD63 [41]. Moreover, those PMPs shed from phosphatidylserine (PS)-positive platelets were also positive for PS, and had 50- to 100-fold higher procoagulant activity than activated platelets [42]. Definition and analysis of PMPs are still a debated area of clinical flow cytometry. Due to the variable storage and preparation of samples, isolation of PMPs as well as differences in the settings of measurement, PMP numbers fairly varied even in the same disease causing potential inappropriate interpretations [43]. Yet, the need for standardized protocols is still demanding. The analysis of PMPs was first set by using fluorescent beads with standard size and amount for enumerating PMPs below 1 μm [44]. Beads were initially processed, and then clinical samples were measured within a standard collection time of 30 seconds. The numbers of PMPs were calculated based on the event count from the bead tube collected for the same time period. PMPs were gated into a restricted area by FSC and SSC parameters, and then identified by the presence of PS with Annexin V- FITC and their CD41 positivity. In addition, CD62P expression was also measured on these vesicles [44] (Figure 1). Previous studies described elevated number of PMPs in MI, atrial fibrillation, and ischemic stroke with severe carotid atherosclerosis compared to healthy controls [23,41,45,46]. Others recently claimed that PMPs act as an independent marker of cardiovascular events in high-risk ACS patients, because atherosclerotic burden did not affect PMP number in stable angina subjects [47]. Furthermore, in ACS patients who underwent coronary stenting had even higher PMP numbers at 15 minutes after the intervention induced by the procedure-mediated trauma compared to those with diagnostic catheterization alone [44] (Figure 1). In terms of abnormal metabolic and inflammatory conditions, Csongrádi and her colleagues demonstrated
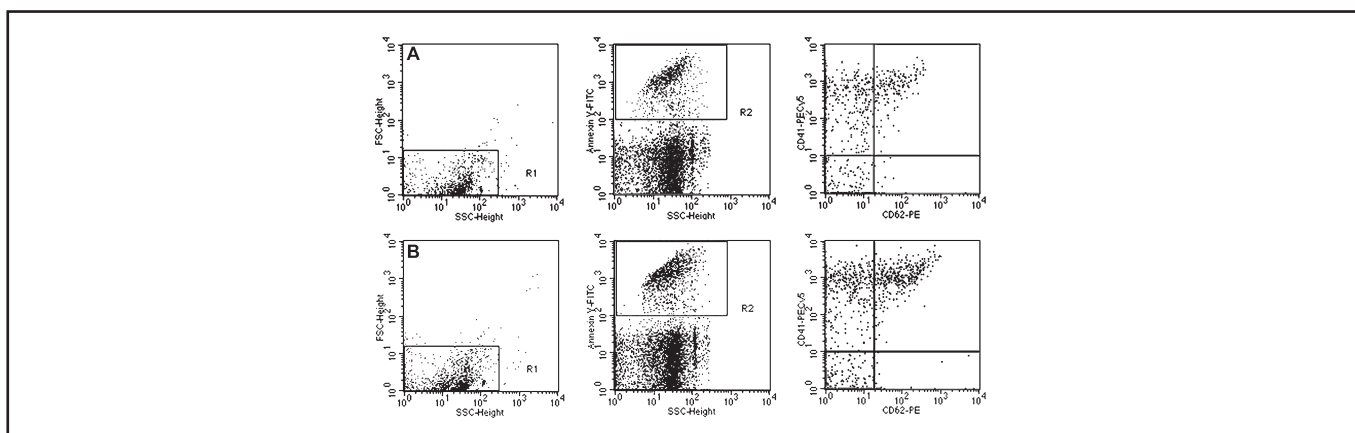
Figure 1

Representative dot plots of PMP analysis. PMPs were collected in patients after diagnostic catheterization without stenting (A) and clinical subjects after coronary stenting (B). PMPs were identified in R1 gate according to FSC and SSC parameters, and then by their Annexin V (PS) positivity (PMP number: 881 events [A] vs. 1117 events [B] in R2). During further analysis, PMPs were stained by anti-CD41-PECy5 and anti-CD62-PE antibodies to measure the activation status of PMPs (20.5% vs. 33.7%) (adapted after some minor modifications with permission from [44]).

significantly higher PMP concentrations in obese subjects and type 2 DM patients versus healthy individuals, where PMP levels were strongly associated with carotid IMT, BMI values, and CRP concentrations [11]. In agreement with this study, PMP levels were positively and independently correlated with carotid IMT in the convalescent phase of ischemic stroke as well [19]. Finally, PMPs could be also measured in the flow cytometric detection of heparin-induced thrombocytopenia (HIT) (see below). In summary, although increased PMP levels were documented even in the early phase of vascular diseases, it is still questionable whether flow cytometric analysis of PMPs is ready to be used as a biomarker for routine laboratory purposes due to relatively lower sensitivity and specificity, and the rather variable conditions of measurement [47].

II/2. Heterotypic aggregates

Platelet-leukocyte aggregates are generated in the blood stream when activated leukocytes and platelets rapidly produce cellular complexes with each other via exposed receptors, notably with CD62P through an interaction with P-selectin Glycoprotein Ligand-1 (PSGL-1) [48]. These heterotypic aggregates accumulate in the site of thrombus formation facilitating the development of variable vascular infarctions. Thus, therapeutic interference of these interactions may be a potential target of anti-platelet medication reviewed in [49]. The half-life of circulating interactions (platelet-monocytes) is much longer (about 30 minutes) compared to P-selectin expression [26]. Thus, the analysis of platelet-leukocyte aggregates may be a more consistent indicator of platelet activation than measuring the amount of P-selectin positive single platelets.

Patients with UA showed a significant increase in the level of neutrophil-platelet aggregates compared with patients with stable angina [50]. In ACS, not only the total level of platelet-monocyte complexes was augmented, but such tissue factor (TF)-positive population as well in contrast to stable angina or controls [51]. Significantly elevated levels of platelet-monocyte aggregates were published in the acute stage of cerebral infarction compared to control groups [15,16,33] that showed a good predictive value in early outcome and long-term prognosis after stroke in a recent study [34]. Yet, there were some contradictory data on the presence of platelet-leukocyte interactions in atrial fibrillation showing decreased levels versus control healthy individuals [20]. In terms of metabolic diseases, neutrophil-platelet aggregates were higher in type 1 DM patients with nephropathy compared to DM patients with normal renal function as well as non-diabetic persons [7]. There was a significant difference in the percentage of monocyte-platelet aggregates but not platelet-neutrophil or platelet-lymphocyte interactions between the diabetic especially with proliferative retinopathy and nephropathy and control groups [52]. Similarly to these data, enhanced leukocyte-platelet adhesion was correlated to platelet hyperreactivity among DM patients especially those with microangiopathy [53]. In chronic myeloproliferative diseases, the increased level of platelet-monocyte aggregates may also contribute to the vascular complications [54].

II/3. FXIII

Coagulation factor XIII (FXIII) is a protransglutaminase that is essential for maintaining hemostasis as a key regulator of fibrinolysis, and accelerates the fibrin cross-linking process [55]. FXIII is targeted and concentrated at the site where platelet-rich thrombi are formed. FXIII binds to activated platelets (Figure 2), and this interaction occurs via GPIIb/IIIa and αVβ3 receptors [56,57], and the surface-bound form was suggested to cross-link secreted α-granule proteins when coated-platelets are generated [58]. In a clinical study [59] in patients with PAD, platelet-associated FXIII was found significantly higher than in healthy controls, and the detection of FXIII on platelets was proposed as an alternative marker of platelet activation [59,60].

II/4. Phosphatidylserine

Phosphatidylserine (PS), a negatively charged lipid in the inner-leaflet of phospholipid membranes, is exposed to cell surface upon platelet activation to propagate coagulation events. Via cleaving FX and prothrombin into their active form, PS facilitates the assembly and activation of tenase and prothrombinase complexes. As a result, fibrin fibers are formed in the early phase of clot formation reviewed in [61]. PS exposure can be detected by the binding of Annexin V to platelets, which requires extracellular Ca^{2+}, so it should be supported during such experiments [62]. Interestingly, this marker did not become a conventional platelet activation marker for ex vivo clinical samples, but was an available tool for studying *in vitro* procoagulant platelet responses, and identifying PMPs by flow cytometry.

Apart from these processes, PS expression also occurs during platelet apoptosis via caspase and calpain activation, when platelets undergo a cellular death pathway resulting in their clearance from the circulation by scavenger cells [63,64]. These events could be also induced *in vitro* by the classic platelet agonist, thrombin [65]. Aging, and stored platelets after several days were also positive for PS [66,67]. Overall, analysis of other biomarkers is necessary with PS to distinguish platelet activation and apoptosis-mediated changes from each other.

III. Deficiency in platelet glycoproteins

Inherited platelet disorders are characterized by abnormalities of platelet function and production causing mucocutaneous bleeding symptoms with distinct intensity reviewed in [68]. When platelets show defects with an absence or malfunction of
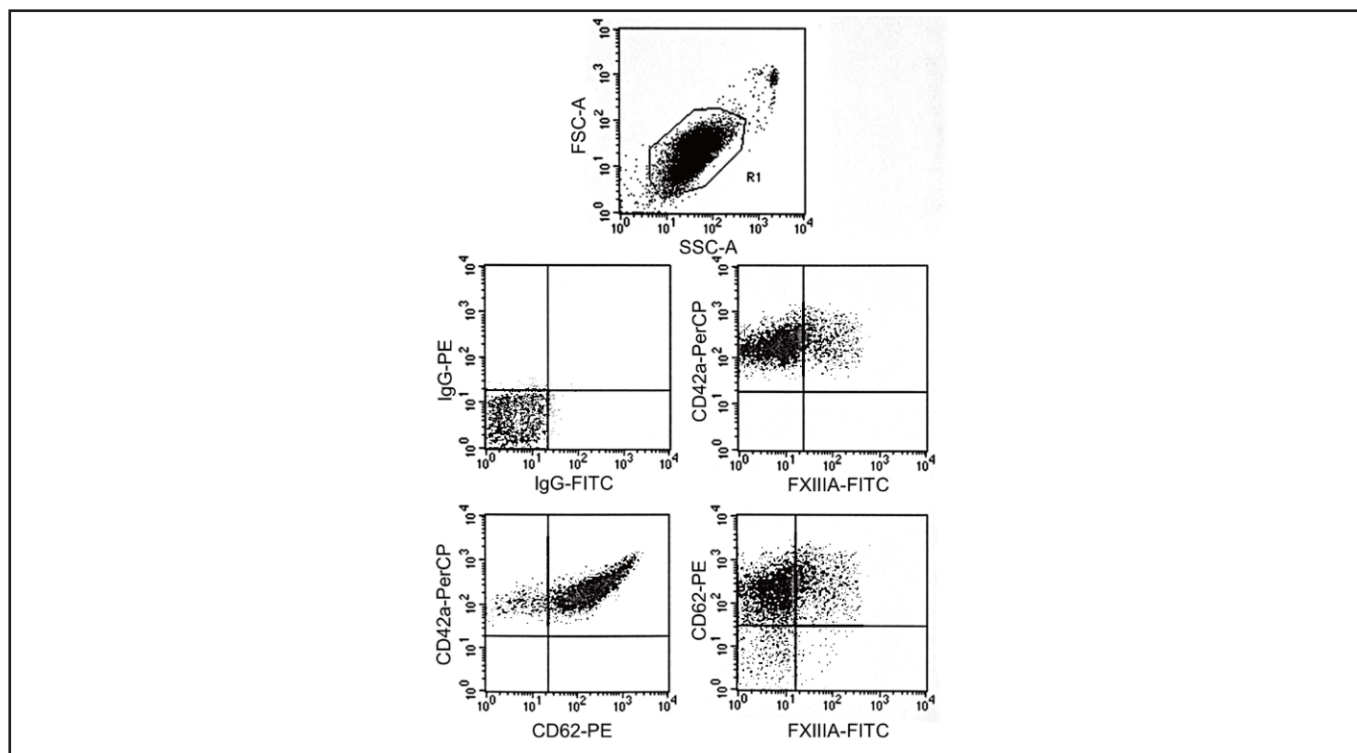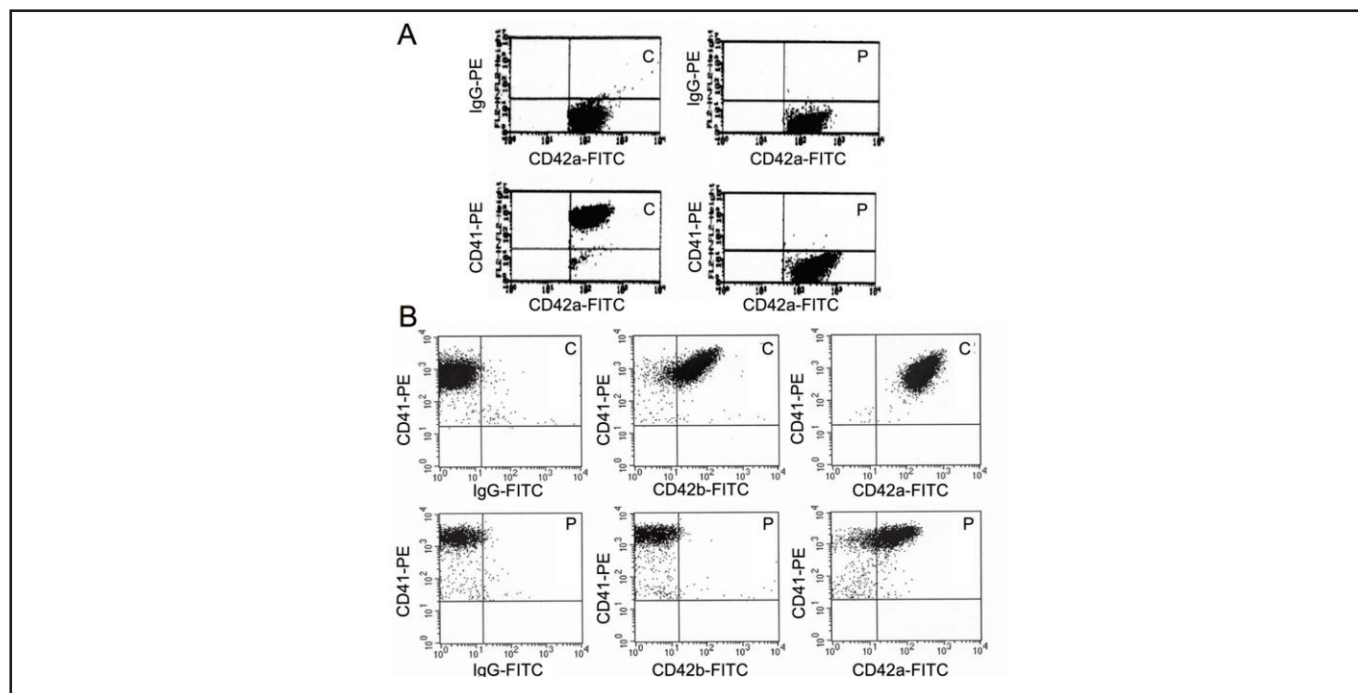
Figure 2
Representative dots plot series on TRAP- activated (20 μM) platelets (R1) in a normal whole blood sample analyzed by flow cytometry in three-color labeling experiments with anti-FXIIIA-FITC, anti- CD62-PE and anti-CD42a-PerCP antibodies. FXIII-A showed a co-expression with CD62P (23%). CD62% was 94% due to full platelet activation.

receptor(s) in adhesion receptors (GPIb/V/IX complex; Bernard-Soulier syndrome [BS]), or aggregation receptors (GPIIb/GPIIIa complex; Glanzmann-thrombasthenia [GT]), platelets fail to bind to the main ligands von Willebrand factor (vWF) and fibrinogen, respectively. BS was characterized with lacking ristocetin-induced aggregation, prolonged bleeding time, large platelets and thrombocytopenia resulting in epistaxis, gingival and cutaneous bleeding in an adult female patient [69]. GT platelets showed impaired aggregation to natural agonists (ADP, collagen, arachidonic acid) causing mucosal bleeding or epistaxis in a young male subject [70]. In both diseases, flow cytometric analysis of surface glycoprotein expression was essential for the final diagnosis when surface properties of patient platelets were compared to those from a healthy age-matched sample. In the type II GT patient, platelets were identified by anti- CD42a (GPIX) with no difference between patient and control in this term, while GPIIb receptors (CD41) were hardly detectable (Figure 3A). In addition, GPIIIa receptors were also absent by using anti-CD61 antibody (data not shown) [70]. In case of the BS person, GPIX by anti-CD42a showed a significantly lower expression, and GPIb receptors with anti-CD42b antibodies demonstrated a null level versus those of a healthy individual. Platelets were identified by their CD41 positivity [69] (Figure 3B).

IV. Reticulated platelets

Percent of reticulated/immature platelets was suggested as a useful marker of augmented production or turnover of platelets in subjects with increased platelet activation long ago [71]. These platelets have large size with higher density compared to normal platelets. They also demonstrate an enhanced reactivity as they secrete more granule contents upon activation than smaller platelets [72]. Elevated level of reticulated platelets was measured in increased thrombopoiesis such as in ET, or when a compensatory mechanism occurs due to a large platelet loss (e.g. immune thrombocytopenic purpura) [73]. Flow cytometric analysis of reticulated platelets was formerly set using thiazole orange staining to detect their mRNA content and a platelet-specific (e.g. anti-GPIb) antibody for platelet gating [71]. ACS patients had significantly higher level of reticulated platelets versus healthy individuals [74]. Moreover, reticulated platelet percent was increased in both early and late phase of ischemic stroke/TIA after adjustment for age [75]. In terms of monitoring of anti-platelet drugs, larger immature platelet fraction was observed in aspirin treatment in those after stent thrombosis showing an increased platelet turnover [76]; however, it was not confirmed in subjects with stroke [75].
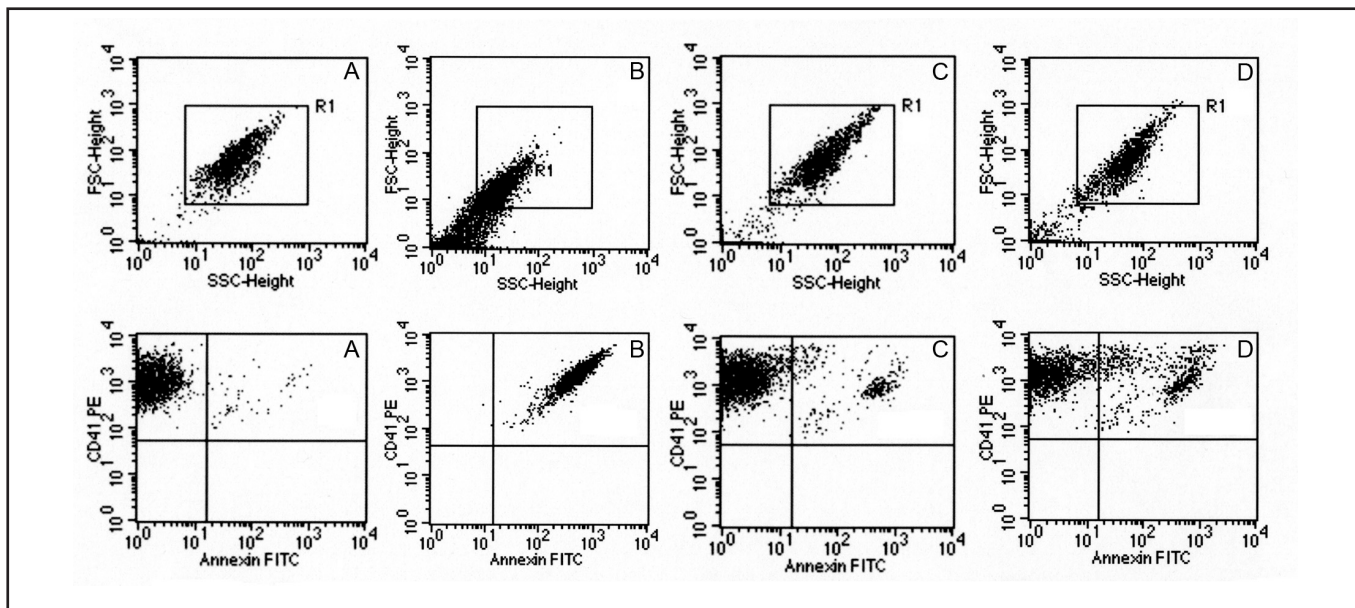
Figure 3

Representative dot plots of a complex flow cytometry analysis of platelet glycoproteins in a GT (A) and a BS (B) patient. Platelets were identified by anti-CD42a (GPIX), and GPIIb receptors (CD41; 1.5%) were hardly detectable in GT (A). In contrast, in a BS individual, markedly less GPIX (CD42a) receptors could be detected (67%), and there was no GPIb receptors (2%; CD42b) compared to a healthy person (CD42a: 99%; CD42b: 91%). Results from these patients were depicted with "P", while data of healthy controls were marked with "C". These dot plots were adapted with permission after some minor modifications from [69,70].

## V. Heparin-induced thrombocytopenia (HIT)

HIT is one of the most common immune-mediated reactions caused by platelet activating IgG antibodies, which usually bind to heparin/PF4 complexes after heparin administration. Heparin/PF4/IgG complexes may induce platelet aggregation with increased thrombin generation resulting in a prothrombotic state [77]. Three types of HIT can be distinguished according to the onset of thrombocytopenia. Typically, thrombocytopenia begins between 4 and 15 days after the start of heparin therapy. Sometimes HIT develops within the first 24 hours of heparin administration (rapid- onset), or several days after the discontinuation of heparin (delayed-onset) [77]. Functional test for HIT laboratory diagnosis is available on flow cytometers as well [78,79]. Oláh and co-workers recently analyzed a patient sample from a rapid-onset HIT with the following methodology [80]. Normal platelets were incubated with the serum of a HIT patient and the therapeutic concentration of heparin (0.3 IU/mL). Annexin V binding on the surface of platelets and microparticle release were measured, and platelets were identified by CD41 positivity. For instance, in a negative control, PRP were incubated with heparin alone, while Ca-ionophore-stimulated sample (10 µM) was used as a positive control. Then, PRP with the patient plasma was studied, and finally PRP with plasma plus heparin (0.3 IU/ml). Due to the presence of HIT, a significantly increased Annexin V positivity could be measured compared to samples with heparin or plasma alone (Figure 4).

VI. Coated-platelets

Coated-platelets are produced by a simultaneous activation of collagen and α-thrombin, and represent a subpopulation of activated platelets with high PS exposure and a substantial prothombinase activity [58]. In addition, coated-platelets are characterized by the retention of several α-granule-derived coagulation factors e.g. factor V, vWF, thrombospondin, and fibrinogen on their surface [58], which are covalently bound together via serotonin creating a potentially procoagulant surface matrix [81]. Elevated levels of coated-platelets were measured in patients with TIA and ischemic stroke compared to healthy subjects [82,83]. In contrast, significantly lower levels of coated- platelets were also shown in spontaneous cerebral bleeding, severe hemophilia A, and asymptomatic ET versus healthy cohorts [17,84,85]. Dale and his coworkers previously set a standardized methodology [58]. Accordingly, subsequent immunostaining and platelet activation were assessed in gel-filtered platelets by biotinylated-fibrinogen and anti-CD41 antibody with convulxin and α-thrombin. Coated-platelets were then indirectly labeled with streptavidin-PE to detect enhanced fibrinogen binding compared to the rest of platelets. Detection of P-selectin percent positivity was simultaneously performed (Figure 5).

Figure 4
Representative dot plots of HIT investigation by flow cytometry. Normal platelets were incubated with the serum of a HIT patient and heparin (0.3 IU/mL).
Platelets were identified by their CD41 positivity. In a negative control, PRP were incubated with heparin alone (2%; A), and Ca-ionophore-stimulated sample (99%; 10 µM) was used as a positive control (B). PRP with the patient plasma was studied (12%; C), and PRP with plasma plus heparin (0.3 IU/ml) (25%; D). Due to the presence of HIT, a significantly increased Annexin V positivity could be measured compared to samples with heparin or plasma alone.
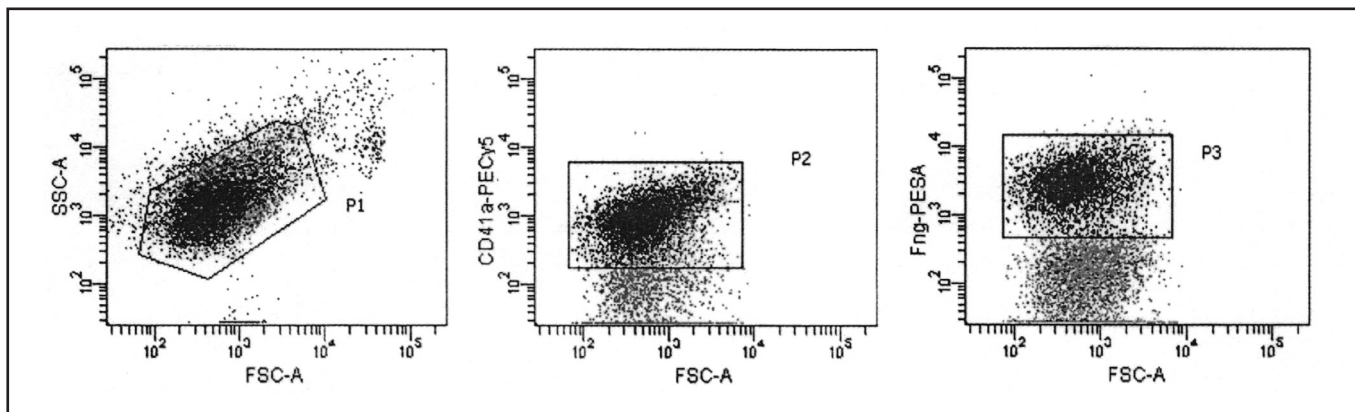
Figure 5
Representative dot plots of coated-platelet measurement in a normal sample on flow cytometer. Platelets were gated (P1) based on FSC-SSC parameters, and then these events were further analyzed in P2 gate where only CD41-positive cells were counted (92%). Finally, coated-platelets were separated from the rest of platelets according to their increased fibrinogen binding detected by biotinylated- fibrinogen and streptavidin-PE (38%; P3).

Conclusions

Investigation of platelet biomarkers has not been only an approach to study platelet reactivity in variable diseases, but also provided new insights for a better understanding of the complexity of platelet physiology. For instance, modulating the activity of intracellular proteins (e.g. protein phosphatases) via activation signaling with potential anti-platelet drugs could be easily tested through platelet biomarkers by flow cytometry [86]. Novel aspects can be also studied, i.e. two distinct subpopulations of procoagulant platelets have been recently described after high concentrations of thrombin or collagen- related peptide based on the quantification of PS positivity, PAC-1 binding, and intracellular Ca^{2+} concentration [87,88]. The relative function of these platelet subpopulations needs further analysis.

In summary, platelet activation markers generally show good sensitivity and specificity even in the detection of lower degree of change in platelet reactivity, and except for PMP analysis, these biomarkers provide a good reproducibility as well.

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