

State of the Art in Platelet Function Testing

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Summary

Platelets perform many functions in hemostasis but also in other areas of physiology and pathology. Therefore, it is obvious that many different function tests have been developed, each one conceived and standardized for a special purpose. This review will summarize the different fields in which platelet function testing is currently in use; diagnostics of patients with bleeding disorders, monitoring patients' response to anti-platelet therapy, monitoring in transfusion medicine (blood donors, platelet concentrates, and after transfusion), and monitoring in perioperative medicine to predict bleeding tendency. The second part of the review outlines different methods for platelet function testing, spanning bleeding time, and platelet counting as well as determining platelet adhesion, platelet secretion, platelet aggregation, platelet morphology, platelet signal transduction, platelet procoagulant activity, platelet apoptosis, platelet proteomics, and molecular biology.

Introduction

Platelets are known to have a large variety of functions. They are main regulators of hemostasis, where they contribute to the hemostatic plug, and in addition accelerate the coagulation system [1]. Platelets also interact with a large variety of cell types, such as monocytes, neutrophils, endothelial cells and smooth muscle cells, and contribute to the pathogenesis of atherosclerosis and vascular inflammation [2–4]. Platelets also contribute to neurodegenerative diseases [5] and other

inflammation-mediated pathologies such as arthritis, systemic lupus and sepsis [7] as well as acute lung injury, including transfusion-related acute lung injury (TRALI) [8]. In addition platelets are involved in cancer metastasis [9] and in innate immunity [10] as well as in adaptive immunity [11, 12]. Platelet interact with microorganisms [13] and with neutrophils to provide protection against invasion by pathogens by forming neutrophil extracellular traps (NETs) [14]. Such NETs are involved in infection-mediated thrombosis, like disseminated intravascular coagulation and deep-vein thrombosis [15].

Platelet-derived microparticles and exosomes not only participate in hemostasis but also play a role in thrombotic and inflammatory diseases as well as in cancer progression and metastasis [16]. Accurate measurement of platelet functions is critical for basic research on the role of platelets in physiology and pathology, is necessary for identifying patients with platelet dysfunction or potential hyperfunction, is becoming probably important for the monitoring of antiplatelet therapy, and is in addition valuable to determine platelet function in donors, concentrates, and after transfusion as well as in managing perioperative hemostasis.

Platelets are sensitive to manipulation, and are prone to artificial in vitro activation. Therefore testing of platelet function requires a high degree of experience as well as expertise to perform and interpret. Unexpected, abnormal platelet function tests should always be repeated with a fresh blood sample and in parallel with a normal control sample. As there are so many different platelet functions and so many different application areas, such a thing as we call in German 'eierlegende Wollmilchsau', an all in one method/device suitable for every purpose, to study platelet function cannot exist. For example, special devices for the assessment of anti-platelet drugs, for studying platelet function under shear conditions, or for testing physical properties of the clot have been developed. Such assays are developed and standardized for a special purpose. Using these function tests for other purposes might lead to misinterpretation or over-interpretation of the results and harm to patients.

Platelet Function Testing in Patients with Bleeding Disorders

An evaluation of patients with abnormal bleeding requires objective clinical assessment of bleeding history, family history, and physical examination. When appropriate, it is followed by laboratory investigations. A carefully collected history provides the most effective tool for determining presence and significance of a bleeding disorder. If bleeding symptoms cannot be fully explained by standard laboratory investigation of coagulation, fibrinolysis, or von Willebrand's disease, laboratory investigations of platelet number and function are recommended [17]. To help to determine the cause of or potential for excessive bleeding and management of patients with platelet disorders, the multi-step process to assess platelet function starts with platelet count and size. If appropriate, platelet adhesion studies, platelet aggregation testing, platelet secretion studies, and specific testing including flow cytometry, electron microscopy, study of signal transduction pathways, support of thrombin formation, genetics, and proteomics will be done [18–20].

In most cases a platelet-mediated hemostatic disorder cannot be characterized by just a single function defect, but rather by a combination of platelet functional abnormalities. Platelet function disorders are varied, with a wide spectrum of known disorders [21, 22], and therefore many different function tests are necessary for a clear diagnosis. This is a significant challenge even for specialized laboratories. The different function tests which are in use for the assessment of platelet function in patients with bleeding problems are described below.

A diagnostic approach to platelet disorders in children has recently been published [23]. Children are not 'small adults', and platelet function differs profoundly [24–26]. Therefore, 'general standard values', according to the age of the children, are inevitable to generate for each single platelet function test in each different laboratory [27]. Molecular genetic diagnosis of heritable platelet disorders may offer valuable confirmation of diagnosis in affected individuals, in family members and for ante-natal diagnosis, and is valuable for the understanding of platelet function. Single-nucleotide polymorphism microarrays (SNP arrays) are able to map regions of homozygosity and to narrow areas of interest for further directed sequencing in families with shared genetic heritage and with proven consanguinity. Massively parallel DNA sequencing technologies (next-generation sequencing) have rendered the whole-genome re-sequencing of individual patients practical [28, 29], but such analyses are currently only performed in research laboratories and are very expensive. This repertoire can be extended by 2D-DIGE platelet protein analysis (whole platelets, phospho-proteome and platelet secretome) and even for a few cases complete platelet proteomics [30].

Platelet Function Testing to Monitor Patient Response to Antiplatelet Therapy

Platelet responsiveness to anti-platelet drugs might carry direct clinical relevance, but the mechanisms leading to poor anti-platelet drug effects are not fully elucidated. Studies assessing the ability of platelet function tests to predict patients' clinical response to aspirin or clopidogrel have generated contradictory results. Therefore, the use of platelet function tests to monitor and guide anti-platelet therapy has provoked heated debates [31, 32].

On the one hand, Gurbel et al. [33] and Geisler et al. [34, 35] described that low response to clopidogrel in patients with symptomatic CAD treated by stenting significantly enhanced the occurrence of cardiovascular events and death. Several other authors share the positive view on the role of platelet function testing for making informed decisions on both initiation and titration of anti-platelet drug therapies, so-called tailored anti-platelet therapy. Sharma et al. [36] wrote recently: 'These platelet function assays are no longer regarded just as a laboratory phenomenon but rather as tools that have been shown to predict mortality in several clinical trials. It is believed that suboptimal response to an anti-platelet regimen (pharmacodynamic effect) may be associated with cardiovascular, cerebrovascular, and peripheral arterial events'.

On the other hand, a study on 2,440 patients, 1,227 in the monitoring group, reported recently [37] that patients, who received platelet function monitoring by the VerifyNow assay and dose adjustment after stent implantation, fared no better than those given standard care. Additional studies are needed to determine whether changes in therapy based on results of platelet function testing improve clinical outcomes. One of these studies will be the ongoing ANTARCTIC study which assesses the value of platelet function testing in patients aged >75 years with the focus on the prevention of major bleedings.

The absence of an association between stronger therapy with anti-platelet drugs and clinical outcomes has been described [38, 39]. In patients with a poor response to clopidogrel, intensification of platelet ADP receptor inhibition failed to improve outcomes [40, 41]. Therefore, the strategy of monitoring platelet function to yield better ischemic and safety outcomes might be still hampered by at least two uncertainty factors: What is the optimal platelet function test for this purpose, and what is the optimal therapy of identified high risk patients?

The prognostic value of high on-treatment platelet reactivity against ADP or arachidonic acid has been shown repeatedly, but that does not inevitably mean that inadequately inhibited ADP receptors or cyclooxygenase are the cause of patients' higher risk. Platelet response to ADP and/or arachidonic acid might just be a marker, and we still have to find out the cause of patients' higher risk. Therefore, other methods as the yet tested ones might be necessary to develop based on deeper understanding of pathology of thrombosis and ischemia. In addition, if high on-treatment platelet reactivity to ADP and/or ara-

chidonic acid is just a risk marker and not more, we might need to reconsider the proposed changes in treatment of these patients. This would mean that platelet function testing is still valuable, as we identify patients at higher risk, but we need to find new strategies to overcome these higher risks.

Several function tests have been used to monitor the presence and effectiveness of anti-platelet medications. Global assessments of 'hemostasis', such as the standard thrombelastograph, Sonoclot, Clot Signature Analyser and Hemodyne, are not specific for platelet function and are essentially insensitive to cyclooxygenase inhibitors and P2Y12 antagonists [42]. Of the platelet function tests assessed by Breet et al. [43], only light transmittance aggregometry with ADP as the agonist, VerifyNow P2Y12, and Plateletworks using ADP tubes, were significantly associated with the primary end point, and the predictive accuracy of these tests was only modest [43]. The VerifyNow assay, the best evaluated assay so far, is still limited by the poor positive predictive value (12.7%) [44].

Other methods assess activation-dependent changes on the platelet surface by flow cytometry (vide infra). These tests include measurement of levels of platelet surface P-selectin, CD63, activated GPIIb/IIIa, PAR-1 cleavage, microparticle formation and monocyte-platelet association. Flow cytometric measurement of platelet activation status and platelet reactivity to closely monitor anti-platelet drug therapy is hampered by the fact that the stabilization of the blood sample was a real challenge. This problem might in the future be overcome by the use of specially developed fixatives (Platelet Solutions Ltd, Nottingham, UK) that stabilize blood samples so that measurements can be performed up to 9 days after blood sampling. This allows blood samples to be transferred by post to a central laboratory where the analyses are performed and negates the need for any special equipment or technical expertise at the point of blood sampling.

Platelet Function Testing in Transfusion Medicine

In transfusion medicine platelet function is mainly determined in blood donors, platelet concentrates, and after transfusion [45].

Platelet Function Methods to Screen Donors

Platelet concentrates can be prepared as random-donor platelet concentrates from whole blood or as apheresis platelets from a single donor. Single donor apheresis concentrates more and more account for a large percentage of platelet concentrates. Therefore, the platelet quality of individual donations becomes increasingly important. The best 'function test' is, as mentioned in tests for screening of platelet disorders, a good anamnesis, including drug history. Platelet function screening can identify donors with diet-related platelet dysfunction or with poor recollection of aspirin use [46–48]. Platelet refractoriness induced by alloimmunization can be

managed by transfusion of matched platelets [49]. Assays like microarray-based genotyping or flow cytometric cross-match can be used for this purpose [50, 51].

Assessment of Platelet Function and Activation Status in Platelet Concentrates

The main application of platelet tests in transfusion medicine is to determine platelet function in concentrates. To produce platelet concentrates, platelets are mechanically manipulated, removed from the physiological environment, and stored. Therefore, it is important to evaluate platelets' hemostatic properties as well as changes, like platelet activation or platelet apoptosis, that might be an indicator for shortened survival after transfusion [52] or even 'prognostic indicators' for possible transfusion complications as longer storage of buffy coat-derived platelets was associated with inflammation and an increased risk of TRALI [53–56].

Platelet's hemostatic capability is often tested by application of the low-shear methods (light transmission aggregometry, impedance aggregometry, multiple electrode aggregometry, flow cytometry), and methods applying high shear (the platelet function analyzer-100 (PFA-100) and the cone and plate(let) analyzer). An overview on platelet function tests in stored concentrates is given in recent reviews [45, 57, 58].

Not everything that can be measured needs to be measured. In validation studies it is more important to get knowledge on the influence of changes in the procedure to gain and store platelet concentrates on the platelets as it is in the routine ongoing check of platelet concentrate quality. During platelet storage extracellular plasma proteins inside the concentrates are subjected to aging and other influences that might change their conformation and function. As such proteins influence hemostasis and inflammation, it might prove beneficial to quantify such altered proteins in stored platelet concentrates. This can be done by direct quantification using tools that recognize the altered protein structures or by platelet function tests using gel-filtered fresh donor platelets and plasma from concentrates as such proteins activate platelets (personal communication by the authors).

Testing of platelet activation status in concentrates is mostly done by observing platelet 'swirling', the shimmering of discoid platelets, and measuring ristocetin-induced aggregation (GPIIb-related function), lactate dehydrogenase (LDH) concentration, pH value, platelet factor 4 (PF4) release, and cell surface expression of activation markers like P-selectin [59, 60]. Measuring platelet apoptosis (vide infra) and/or microparticle formation (vide infra) in platelet concentrates are new emerging fields [61]. Understanding of platelet apoptosis and its role in the platelet storage lesion is an exciting challenge [62].

Assessment of Platelet Function Following Platelet Transfusion

To measure the outcome of platelet transfusion is still an unsolved challenge. Quantifying bleeding tendency as an outcome measure seems to have the highest clinical relevance,

but key variables like severity and site of bleeding and differences and methods trying to quantify bleeding make this approach not really reliable for studies [63]. Therefore, in most cases platelet recovery and survival is measured as a surrogate parameter to assess the clinical function of platelet concentrates. In vitro evaluation of stored platelets did not fully predict post-transfusion platelet survival and function [64]. Platelet recovery can be measured by radiolabelling an aliquot of platelet concentrate and re-infusion into the normal volunteer donor. Platelet recovery can also be measured in animals using flow cytometry and anti-human platelet antibodies [65]. These tests have been helpful to study the clearance mechanism of chilled platelets [66, 67].

Perioperative Assessment of Platelet Function

Whether objective measure of platelet function may aid in perioperative hemostatic control is still a matter of debate. It is unclear so far whether platelet testing in patients with no bleeding history adds to the care of patients at all and, if though, which patients may actually benefit from routine pre- and perioperative testing of platelet function. Several studies are in favor of perioperative platelet function testing. Poston et al. [68] for example described platelet aggregometry to be useful to predict bleeding and thrombosis after off-pump coronary artery bypass. Other authors described that preoperative screening does not add to patients' care and might even result in unnecessary medication [69, 70]. Current evidence does not suggest the use of platelet function tests to guide therapy with anti-platelet drugs in the perioperative period [71].

The association between on-treatment platelet reactivity measured by an ex vivo assay and the occurrence of bleeding events is low [72]. The bleeding time has been widely used as a preoperative screening test, but it is a completely non-informative test, and therefore not beneficial for this purpose [73, 74]. Carroll et al. [75] came to the conclusion that the most significant predictors of postoperative bleeding were a low body mass index (BMI), lowest core body temperature, and cross clamp time, but not perioperative platelet function tests (glass bead adhesion and thrombelastograph platelet mapping). Korte et al. [76] used preoperative fibrin monomer measurement for risk stratification for high intraoperative blood loss in elective surgery. Using the Enzymun Test FM, preoperative fibrin monomer concentration $< 3 \mu\text{g/l}$ excluded intraoperative blood loss $> 500 \text{ ml}$ with 92% sensitivity and 95% negative predictive value, while platelet count did not. Using other fibrin monomer assays, this remarkable result could not be repeated. The difference in methodology is not yet understood. In conclusion the authors agree with Peterson et al. [77]: 'The best preoperative screen to predict bleeding continues to be a carefully conducted clinical history that includes family and previous dental, obstetric, surgical, traumatic injury, transfusion, and drug histories'.

Methods to Test Platelet Function

Template Bleeding Time

The bleeding time is a crude test of hemostasis. Bleeding time, the time taken for bleeding to stop after a defined incision is made into the skin, to measure the interaction between platelets and blood vessels to form a clot has been developed by Duke in 1910 [78]. Ivy [79] made the method more reliable, by introducing a blood pressure cuff on the upper arm, inflation to 40 mm Hg and placing the incision into the anterior surface of forearm. Spring-loaded template devices are provided by different companies to make standard sized cuts within the skin.

Bleeding time has been used most often to detect qualitative defects of platelets, vascular defects, or von Willebrand's disease. It is also prolonged in thrombocytopenia; therefore, it is important to check the platelet count before performing a bleeding time. Although it seems to be simple, bleeding time measurement is invasive, poorly reproducible, and relatively insensitive to platelet function defects. Therefore, it is of limited aid in evaluating individual patients and useless in prediction of the risk of abnormal bleeding from internal organs during and/or after an invasive procedure [73, 74], although is still in use in some centers.

Lehman et al. [80] wrote in their recently published article, showing that discontinuation of bleeding time tests at their University had no detectable adverse clinical impact: 'Unfortunately, the BT test became popularized in clinical practice without sufficient peer-reviewed evidence supporting its use, probably under the assumption that an in vivo test was needed and no other test was available'.

Platelet Counting Methods

Rapid and accurate platelet concentration measurements are crucial in all laboratories working with platelets. Platelet counting is clinically required in severe thrombocytopenia. Several techniques have been developed to count platelets. The manual count is still recognized as the gold standard or reference method especially for measurement of low platelet counts, but it is time-consuming and requires a high degree of technical skill [81]. Automated hematology analyzers can be used to obtain platelet counts over a wide range of values; but cell size analysis cannot discriminate platelets from other similar-sized particles like small erythrocytes or erythrocyte fragments. Optical counting methods like light scatter or fluorescence methods have been introduced for automated platelet counting. The RBC/Platelet Ratio International Reference Method (IRM) uses flow cytometry to count large numbers of cells rapidly, accurately, and precisely [82, 83]. Platelet count is derived from the ratio of fluorescent platelets, labeled with a monoclonal antibody, to collected red blood cells. Recently a flow cytometer, the compact BD Accuri™ C6, has been developed that can, due to a unique fluidic system driven by peristaltic pumps, report absolute cell counts based on direct

volume sampling. This new direct volume method requires no additional measurement of red blood cells or reference beads and therefore reduces costs and measurement time [84].

Platelet Adhesion and Spreading

The adhesive properties of platelets contribute to maintaining vascular integrity and defending from external pathogens [85]. Platelets patrol the blood vessels and adhere where alterations of the endothelial cell lining are detected. Alterations of the endothelium can be accompanied or not by exposure of subendothelial matrix components. Adhesion to the exposed subendothelial extracellular matrix (ECM) proteins is the first step in the formation of a hemostatic plug. In addition the adhesive property of platelets to foreign surfaces such as implants or catheters can be measured.

The different platelet adhesion assays can be divided in those which measure platelet adhesion performed under static conditions and those which measure platelet adhesion under flow conditions. A nice overview about platelet adhesion assays under static conditions is given by Joanne Stevens [86].

Platelet adhesion can be measured in vitro using cultured vascular cells [87] or foreign surfaces such as glass beads, but today mainly purified matrix proteins, like collagen, von Willebrand factor, thrombospondin [88] and fibrinogen, or complete ECM from cultured endothelial cells are used. Platelet adhesion can also be evaluated on cryostat cross-sections of human coronary arteries with or without atherosclerotic plaques [89] or on cholesterol sulphate [90]. Adhesion to glass beads has been introduced by Hellem [91] in 1960 and Salzman [92] in 1963. Today this method is out of fashion. Perfusion chambers with matrix proteins or complete ECM have been introduced in the 1970s [93–95]. Under flow conditions adherence of platelets is affected by rheological conditions such as shear rate, presence of red blood cells, red blood cell deformability, and viscosity of the medium [96, 97]. Later perfusion chambers were adapted to smaller volumes of blood [98]. New improved flow systems led to the introduction of blood flow pulsatility [99]. Nowadays techniques with a variety of microfluidic devices are in use to observe platelet adhesion. Biochips, like the Cellix Vena8 Fluoro-TM biochip, can be coated with collagen and platelet adhesion can be measured using fluorescence immunostaining and confocal microscopy at up to 60 dynes/cm². Such chips can contain several parallel microcapillaries for continuous flow that can be coated with different adhesion molecules. Down-scaling of the blood volume now allows tests to be done more quickly and with better standardization [100–102].

Spreading

Platelet spreading depends on platelet cytoskeletal rearrangement. The methods which are used today most frequently are fluorescence microscopy, using marker-conjugated phalloidin, or scanning electron microscopy [103, 104].

Mixed Adhesion/Aggregation und Shear Conditions

Several in vitro methods measure platelet adhesion together with aggregation under shear conditions. Among these methods are the platelet function analyzer (PFA 100[®]) and the Impact-R[®] system (Cone and Plate(let) Analyzer; CPA). A review on the CPA is given by Savion and Varon [105] in 2006. The method is very sensitive for von Willebrand factor-mediated platelet activation processes and has been described to allow differentiation between inherited and acquired thrombotic thrombocytopenic purpura [106].

The PFA 100 test is a rapid, accurate test of monitoring shear-dependent platelet function, as anticoagulated blood is passed through a membrane coated either with collagen plus epinephrine (Col/Epi) or collagen plus ADP (Col/ADP) at a high shear rate. Platelets adhere to the membranes and gradually occlude a small aperture in the center of each membrane. The time to occlude the aperture in the membrane is the so-called closure time. As for all shear-dependent methods, knowledge of the full blood count is critical for interpreting results from the PFA-100 and Impact R. Abnormal platelet adhesion using both methods is typical for types 2A, 2B, 2M, and 3 VWD [107]. A normal PFA 100 closure time result should not be used to rule out platelet function defects [108]. The PFA-100 does not predict delta-granule platelet storage pool deficiencies [109].

Intravital Imaging of Thrombus Formation

Recently intravital imaging of thrombus formation has been reviewed by Kuijpers and Heemskerk [110] as well as by Furie and Furie [111]. As these function tests are restricted to animals, they are not discussed in this article.

Measurement of Total and Released Nucleotides, Released Serotonin, and Released PF4

Measurement of total and released nucleotides (ATP, ADP) is used for determining specific deficiencies in the number of dense granules and in dense granular content as in the so-called storage pool defects or specific defects in degranulation as in release defects [112].

In the past it has most often been done in combination with aggregometry using a lumi-aggregometer, but this method does not allow distinguishing between storage pool and release defects. Therefore, in several laboratories the total amount of ADP and ATP in lysates of resting platelets and in platelet releasates is measured in addition. The methods for ATP/ADP measurement are either based on luminescence [113] or on HPLC [114]. ADP is measured as ATP after phosphorylation by pyruvate kinase (PK) with phosphoenolpyruvate (PEP).

Serotonin (5-HT) is actively taken up and stored within the platelet dense granules. It is measured for example at suspicion of storage deficiency like in Hermansky-Pudlak Syndrome (HPS) platelets or storage pool disease. 5-HT uptake and release has been measured using radiolabelled 5-HT [115]. This assay has specially been used for heparin-induced

thrombocytopenia II (HIT II) diagnostics [116]. Platelet 5-HT content today is also measured by ELISA HPLC or by a fluorescence-based assay using a fluorescence microscope or a flow cytometer [117]. Recently, a technique capable of measuring real-time 5-HT release has been described based on fast scan cyclic voltammetry using carbon-fiber microelectrodes [118]. Platelet content and release of alpha granule proteins (e.g. PF4, beta-thromboglobulin and thrombospondin-1) can be measured by commercially available ELISAs.

Platelet Aggregometry

Aggregometry and flow cytometry are fundamentals in platelet function testing today. Light transmission aggregometry has been developed by the group of Gustav Born. Born's paper [119] is one of the most cited among platelet researchers. In light transmission aggregometry platelet rich plasma (PRP) is stirred in a cuvette which sits between a light source and a photocell. Agonists induce platelet adhesion to platelets and light transmission increases. Platelets will only aggregate if they are in contact with each other. So they must be stirred while testing is taking place. In routine practice ADP, collagen, ristocetin, arachidonic acid, adrenaline, and PAR-1-activating peptide (TRAP-6, SFLLRN) are used. But the panel of agonists can be extended. Other agonists are for example gamma thrombin or alpha thrombin (in the presence of GPRP to prevent fibrin polymerization), TRAP-4 (AYPGKF), thromboxane mimetic U46619 (stable analogue of the endoperoxide prostaglandin H₂) [120], calcium ionophore A23187, polymerized immunoglobulins, collagen-related peptide (CRP) [121], misfolded proteins like AGE-proteins and oxLDL [122], alpha defensins [123], proteins from microorganisms like EAP [124], and snake venoms like convulxin [125]. The aggregation parameters to consider are the lag phase, maximal amplitude, primary aggregation slope, and disaggregation for each used agonist concentration [126, 127]. Several types of mild platelet defects, (e.g. primary secretion defects) may be missed by aggregation. Reversible first-wave aggregation is seen with ADP and adrenaline and suggests a failure of granule release as in platelet storage pool disorder or a defect in nucleotide release [128, 129]. A lack of agglutination with ristocetin is consistent with the possible diagnoses von Willebrand disease or Bernard Soulier syndrome [130], while possible diagnoses with no aggregation with ADP, adrenaline, TRAP-1, and collagen are consistent with Glanzmann's thrombasthenia or afibrinogenemia.

Laser light scatter aggregometer are especially suitable to measure the formation of small aggregates [131]. Spontaneous platelet aggregation, defined as formation of small aggregates under constant stirring in the absence of any agonists, is rare in healthy individuals but seen in some cases of von Willebrand disease [132], in some patients with diabetes [133] and in some lipid disorders [134], and may be an indicator of an ongoing active atherosclerotic process [135].

Impedance aggregometry, developed in the early 1980s [136], allows the use of whole blood which is stirred at 37 °C; aggregation is detected by the adhesion of platelets to the surface of two fine, metal wire electrodes [137]. The electrical impedance between the electrodes can be displayed as a wave of aggregation. Although the use of whole blood makes impedance aggregometry highly valuable as it measures platelet function within whole blood without the requirement of sample processing, there are several drawbacks of impedance aggregometry in comparison to light transmission aggregometry that researchers and clinicians need to have in mind. The reversibility and a biphasic response to ADP cannot be demonstrated by whole blood impedance [138, 139], and platelet response to epinephrine/adrenaline tends to be absent or very weak [140]. The development of disposable electrodes, standardized reagents, and the availability of a 5-channel multiple electrode platelet aggregometer has solved several standardization problems [141].

The Plateletworks methodology involves using a cell counter to measure total platelet count in a whole blood sample and then to re-determine the platelet count on a second sample that has been exposed to a known platelet agonist. Aggregated or agglutinated platelets will not be counted as platelets in the second sample. The test has especially been used for assessment of anti-platelet drugs [142].

Another methodology, the VerifyNow assay that has been developed for monitoring of anti-platelet drugs in whole blood is based on platelet aggregation [143]. The VerifyNow P2Y₁₂ assay evaluates platelet aggregation of fibrinogen-coated beads in response to ADP and prostaglandin E₁. Light transmittance increases as activated platelets bind and aggregate fibrinogen-coated beads. The cut-off value for defining suboptimal response to ADP-platelet aggregation despite treatment with clopidogrel has not been firmly established [144]. The VerifyNow Aspirin assay incorporates the agonist arachidonic acid to activate platelets, while in the VerifyNow IIB/IIIa assay the reagent iso-TRAP is incorporated to induce platelet activation without fibrin formation.

Flow Cytometric Analysis of Platelet Functional Capability and Activation Markers

The ex vivo or in vitro testing of functional platelet properties using conventional techniques reflects the overall behavior of the whole platelet population in the sample under investigation. Flow cytometry allows the analysis of individual platelet functional capability and the measurement of the expression of platelet activation markers on individual platelets as well as the quantitation of associates between platelets and other blood cells [145, 146]. Overviews on the methodology of platelet flow cytometry are given by Michelson [147, 148] and Knight [149].

A step-by-step instructions manual to perform platelet flow cytometry written by Brodde et al. in 2012 is given in a booklet provided by Becton Dickinson [150]. It includes instructions

and samples of results from patients with inherited thrombocytopathies and examples of results for monitoring of anti-platelet drugs. Flow cytometry allows detecting platelet function defects even in patients with very low platelet count and therefore is the methodology of choice to determine whether a thrombopenic patient has in addition a thrombocytopathy.

Platelet procoagulant activity can be measured by flow cytometry at different steps, by the expression of phosphatidyl serine on activated platelets using labeled annexin V [151], and by the binding of coagulation factors using directly labeled coagulation factors or the combination of factor and specific antibody [152–154]. The determination of platelet-specific autoantibodies by flow cytometry has been reviewed by Tomer [155]. As diagnostic aid in the evaluation of thrombocytopenic disorders a flow cytometric analysis of thiazole orange to measure reticulated platelets has been developed [156]. Flow cytometry also allows to measure steps in signal transduction as intracellular methods have been developed. The most often used method is the intracellular quantification of VASP phosphorylation [157]. In addition flow cytometry is the method of choice to measure platelet microparticles as well as microparticles from other cells [158].

Quantitation of Shedded Platelet Receptors

Metalloproteinase-mediated ectodomain shedding of platelet receptors has emerged as a new mechanism for modulating platelet function [159]. Interestingly 5-HT stimulates platelet receptor shedding by ADAM17 [160]. Quantitation of shedded soluble platelet receptors in plasma has been used to analyze platelet function in different diseases. The first protein known to be shedded from the platelet surface was GPIb. The soluble shedded part has been named glyocalicin [161]. Quantitation of glyocalicin has especially been used in thrombocytopenic patients [162]. Recently it was described that diesel-exhaust particles are associated with GPIb shedding [163]. Shedding of CD36 from platelets and other blood cells has been associated with type II diabetes [164]. Plasma levels of shedded platelet glycoprotein V have been linked to type 2 diabetes, stroke, and myocardial infarction [165–167]. Shedded GPVI is evaluated as marker for acute coronary syndrome [168], while soluble CD40L, an immunostimulatory ligand for CD40 released from platelets, is associated with adverse reaction to platelet transfusion [169].

Signaling Pathways

Many different methods have been developed for the evaluation of platelet signal transduction. A series of reviews has been published recently for these methods. Nesbitt et al. [170] described a live cell microimaging technique to examine platelet calcium signaling dynamics under blood flow. Measurement and manipulation of Ca²⁺ in suspensions of platelets as well as in single platelets and megakaryocytes has been outlined by Ohlmann et al. [171] and Mason et al. [172], respectively. An up-to-date overview on signaling receptors on

platelets and megakaryocytes is given by Woulfe et al. [173]. How to study protein phosphorylation and dephosphorylation is presented by Gibbens [174], by Jones and Poole [175], and by Gorter and Akkerman [176]. Shrimpton et al. [177] described the isolation and analysis of platelet lipid rafts. The evaluation of redox-signaling is described by Naseem et al. [178]. Receptor tyrosine kinases (RTKs) and G-protein-coupled receptors (GPCRs) can form platforms in which protein signaling components specific for each receptor are shared to produce an integrated response upon engagement of ligands [179]. Evaluation of these signaling platforms allows a different view on platelet signaling in comparison with the model of cross-talk between RTKs and GPCRs.

In several techniques to study platelet signaling megakaryocytes are used as surrogate for platelets. These techniques include patch clamp, interference RNA, quantitative PCR, Ca²⁺-sensitive fluorescent indicators, and transgenic murine models. Experimental and computational methods can be combined to study platelet signaling [180]. A good example is the PlateletWeb [181, 182].

Platelet Proteomics

Initial platelet proteomic research focused on general proteome mapping. Exploration of subcellular compartments, the membrane proteome, and signaling pathways followed. Proteomics technology is based on mass spectrometry in combination with several separation techniques such as two-dimensional gel electrophoresis or multidimensional liquid chromatography. Differential proteomics can be used as a discovery tool to identify functionally important proteins in platelet activation as in platelets several proteins change in abundance upon platelet activation. This technique helps to identify activation networks. Two-dimensional difference gel electrophoresis (2D-DIGE) is a brilliant method to overcome the problem of inter-gel variability, connected with classical two-dimensional gel electrophoresis and therefore is an ideal discovery tool [183]. These techniques allow also the comparison of platelet proteome between different patients and between patient groups [184, 185].

The strategy to combine 2D-DIGE with matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometer analysis has been a useful approach for a quantitative analysis of the effect of thrombin on the secretome profile of human platelets [186]. To identify platelet surface proteins, these can be enriched using lectin affinity chromatography, biotin/NeutrAvidin affinity chromatography and free flow electrophoresis prior to identification [187]. Proteome analysis is useful in deciphering signaling cascades in human platelets [188, 189]. Combination of techniques to identify posttranslational modifications with techniques of proteomics led to the identification of palmitoylated platelet proteins and platelet phospho-proteom [190]. Platelet proteomics can also be applied to study pathologies where platelets play a fundamental role, e.g., acute coronary syndrome,

neurodegeneration, and diabetes [191–193]. Last but not least, platelet proteomic techniques are useful to study platelets with functional defects in more detail [194, 195].

Genetic Testing

Due to their enucleate structure, platelets are not accessible to large-scale genomic screens, but genetic testing is frequently used in patients with inherited platelet disorders (see above). The techniques used are above the scope of this review, and the reader is referred to the excellent review of Nurden et al. [196].

Measurement of Platelet Apoptosis

Similar to nucleated cells, platelets may undergo suicidal death, which is characterized by cell shrinkage, cell membrane blebbing, microparticle formation, cell membrane phospholipid scrambling, activation of caspases, cytochrome C release, and depolarization of the mitochondrial transmembrane potential ($\Delta\Psi_m$) [197]. Platelets have a functional intrinsic apoptotic signaling pathway. They contain the pro-apoptotic mitochondrial protease Omi/HtrA2 and Smac/Diablo as well as their target the X-linked inhibitor of apoptosis XIAP [198]. Detection of markers of platelet apoptosis is reviewed by Gylkhandanyan et al. [61]. In transfusion medicine, platelet apoptosis is a promising new field as platelets undergo apoptosis under nonoptimal storage conditions [199–201]. Platelet apoptosis can be provoked by suppression of thrombopoiesis, by injection of tumor necrosis factor, by high concentrations of thrombin [202], by anti-platelet antibodies and by contact with high concentrations of misfolded proteins (Brodde, Kehrel unpublished) and is seen in malaria infection.

Methods commonly used to study platelet apoptosis markers include flow cytometry, Western blot analysis, and electron microscopy. Depolarization of $\Delta\Psi_m$, an important parameter of mitochondrial function, can be measured by flow cytometry. The cytofluorimetric, lipophilic cationic dye, 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolylcarbocyanine iodide (JC-1) can selectively enter into mitochondria and change color from green fluorescence to intense red fluorescence as the membrane potential increases. In apoptotic platelets, JC-1 stays monomeric with green fluorescence [203].

Platelet-Mediated Thrombin Generation

Platelets play a major role in localizing and controlling the burst of thrombin generation leading to fibrin clot formation [204]. Fibrin at the vessel wall is built by the extrinsic coagulation pathway, but fibrin inside the body of the thrombus and fibrin in thrombus tails that form on the downstream margin of the thrombus is believed to be primarily dependent on the procoagulant function of platelets [205]. Platelet procoagulant function is tightly linked to two distinct processes: the induction of platelet activation and the induction of platelet cell death pathways, apoptosis, and necrosis. Two distinct pathways regulate platelet phosphatidylserine (PS) exposure and

procoagulant function [206]. In addition to platelets also red blood cells actively contribute to thrombin generation in whole blood [207]. Procoagulant tenase and prothrombinase complexes assemble on activated platelets to support cleavage of FX and prothrombin into the proteolytically active forms FXa and thrombin. In addition, platelets provide a scaffold for the formation of fibrin fibers and regulate clot retraction [208–210]. The GPIb thrombin binding site is essential for thrombin-induced platelet procoagulant activity [153]. Three steps in platelet procoagulant activity are usually measured: the exposure of surface PS, the binding of coagulation factors, and thrombin generation parameters. PS exposure is most often measured using labeled annexin V. This can be done fluorimetrically or by a flow cytometer [211]. By exposure of PS, platelets display high-affinity binding sites for vitamin K-dependent coagulation factors and coagulation inhibitors (protein C, protein S). Protein S may counterbalance platelets' procoagulant activity [212].

Also the binding of coagulation factors to activated platelets can be measured by flow cytometry as described by Doermann et al. [152]. Platelet factor V is suffice to ensure thrombin generation in patients with severe inherited plasma factor V deficiency [213]. Feedback activation of thrombin generation on the platelet surface does depend on the activity of platelet disulfide isomerase (PDI) as surface-associated PDI is an important regulator of coagulation factor ligation to thrombin-stimulated platelets [154]. Not all activated platelets become procoagulant as seen by flow cytometry. Using collagen and thrombin (coat) as platelet agonist, the procoagulant platelet population can be increased. Dale et al. [214] showed that this procoagulant population, called 'coated platelets' use 5-HT to enhance the retention of procoagulant proteins on the cell surface. In addition to 5-HT, 'coated platelets' retain on their surface high levels of several procoagulant proteins, including fibrinogen, von Willebrand factor, fibronectin, amyloid beta, factor V, and thrombospondin that can be measured by flow cytometry.

Dale et al. [215] developed an easy-to-use method to identify 'coated platelets' by flow cytometry. PRP is activated by convulxin (a specific agonist for collagen receptor GPVI) plus thrombin in the presence of GPRP to inhibit fibrin polymerization and FITC-abciximab and biotin-fibrinogen are added. Biotin is recognized by phycoerythrin-streptavidin. The percentage of abciximab-positive platelets with bound biotin-fibrinogen is quantitated by flow cytometry. 'Coated platelets' are interesting biomarkers for prothrombotic diseases and in neurodegeneration [216–220].

The Thrombinoscope (CAT assay) (Thrombinoscope BV, Maastricht, The Netherlands) allows to measure parameters of thrombin generation in the presence of platelets (lag-time, maximum concentration of thrombin (Cmax), time required to reach Cmax (Tmax), and endogenous thrombin potential (ETP)). In the Thrombogram-Thrombinoscope assay thrombin measurement is carried out in a 96-well plate fluorometer. A thrombin

calibrator can be added to plasma. This calibrator is measured in the Thromboscope and compared to the measurement of fluorescence in another well containing the same plasma.

The replacement of the chromogenic substrate with fluorogenic substrates made it easier to study the role of platelets in coagulation. All platelet inhibitors tested thus far appear to influence thrombin generation in PRP. The test system is based on work by Hemker et al. [221, 222], who introduced the EPT as a term for the total amount of thrombin generated during the test.

Interestingly extracellular histones from dying cells and polyphosphates promote thrombin generation through platelet-dependent mechanisms [223]. Using assays for platelet-dependent thrombin formation might be useful to recognize new mechanisms in pathology and to find new avenues for anti-thrombotic drugs.

Assessing Protein Synthesis by Platelets

Platelets possess a translational machinery that can direct protein synthesis. A variety of protocols that can be employed to assess protein synthesis by platelets has been summarized by Schwertz et al. [224] in 2011. The interested reader is referred to this comprehensive instruction manual.

Measuring Platelet Contractile Force

Physical forces play a critical role in hemostasis by regulating the mechanobiology of platelets. Platelet contractions are driving the retraction and stiffening of clots and contribute in strengthening platelet adhesions through integrin-related mechanotransduction. The archetypical platelet disorder Glanzmann's thrombasthenia got its name from the Greek words 'thrombos' (thrombus, clot) and 'astheneia' (weakness) as clot retraction is disturbed in this disease. According to the review of Fegghi and Sniadecki [225], five different conventional platelet force assays have been developed, the traditional clot retraction assays, the in vitro observation of thrombus consolidation, the thrombelastography/-metry, the clot strip assay, and the clot retractometry [225–227]. Today techniques exist to measure even the physical forces of single individual platelets [228, 229]. Upon activation, G-actin monomers in platelets polymerize into F-actin filaments. This step

which is crucial for platelet mechanobiology can be measured by several assays. In platelet suspensions like PRP the DNase I inhibition assay can be used [230]. A method to measure platelet content of F-actin using rhodamine-phalloidin binding has been described by Cassimeris et al. [231]. A pyrenyl-actin assay of actin nucleation has also been described [232]. Today platelet cytoskeletal rearrangement is mostly analyzed flow cytometrically by measuring F-actin content with NBD- or bodipy-phalloidin or FITC-phalloidin [233, 234].

Measuring Circulating Platelet-Derived Microparticles

Circulating platelet – and other cell-derived microparticles have been implicated in several disease processes. Elevated levels are found in many pathological conditions. Flow cytometry is considered the 'gold' standard of microparticle detection [235], but also commercially available assays on 96-well plates that based on the procoagulant activity are in use [236]. Preanalytical parameters have a high impact on the measurement of microparticles [237, 238].

Morphological Methods

Electron microscopy has proven to be very useful for defining ultrastructural abnormalities associated with platelet defects and in basic understanding of platelet physiology [239–241]. A technical review is given by Clauser and Cramer-Borde [242]. Cheaper and compact table electron microscopes are currently developed.

As new techniques arrive at the horizon, they are also applied to platelets. Atomic force microscopy is one of these techniques. This technique has been already used, e.g., to study platelet interaction with biomaterials [243], for validation of flow cytometric detection of platelet microparticles [244], and even for the analysis of platelet GPIIb/IIIa receptor [245]. Differential interference contrast microscopy, also named Nomarski interference contrast microscopy, is an optical microscopy technique used to enhance the contrast in unstained, transparent samples. It has been used in several platelet laboratories with great success [246].

Disclosure Statement

The authors declared no conflict of interest.

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