

Platelet function tests: a comparative review

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Abstract: In physiological hemostasis a prompt recruitment of platelets on the vessel damage prevents the bleeding by the rapid formation of a platelet plug. Qualitative and/or quantitative platelet defects promote bleeding, whereas the high residual reactivity of platelets in patients on antiplatelet therapies moves forward thromboembolic complications. The biochemical mechanisms of the different phases of platelet activation – adhesion, shape change, release reaction, and aggregation – have been well delineated, whereas their complete translation into laboratory assays has not been so fulfilled. Laboratory tests of platelet function, such as bleeding time, light transmission platelet aggregation, lumiaggregometry, impedance aggregometry on whole blood, and platelet activation investigated by flow cytometry, are traditionally utilized for diagnosing hemostatic disorders and managing patients with platelet and hemostatic defects, but their use is still limited to specialized laboratories. To date, a point-of-care testing (POCT) dedicated to platelet function, using pertinent devices much simpler to use, has now become available (ie, PFA-100, VerifyNow System, Multiplate Electrode Aggregometry [MEA]). POCT includes new methodologies which may be used in critical clinical settings and also in general laboratories because they are rapid and easy to use, employing whole blood without the necessity of sample processing. Actually, these different platelet methodologies for the evaluation of inherited and acquired bleeding disorders and/or for monitoring antiplatelet therapies are spreading and the study of platelet function is strengthening. In this review, well-tried and innovative platelet function tests and their methodological features and clinical applications are considered.

Keywords: platelets, method, point-of-care testing, laboratory assessment, bleeding, thrombosis

Introduction

Human platelets are critically involved in both normal hemostasis and pathological bleeding and thrombosis. These particular cells contribute greatly to vessel constriction and repair, host defense, and tumor growth/metastasis.¹ In addition, platelets acting together with other cells – white, endothelial, or smooth muscle cells – play a part in inflammation, in related pathologies, and in the promotion of atherosclerosis.²⁻⁴ Notwithstanding this multiplicity of roles, the available platelet function tests explore principally the alterations directly related to the hemostatic process.⁵

Primary hemostasis consists of the interaction between platelets and vessel wall. At the site of damage of a vessel wall, platelets are rapidly engaged in a sequential functional response including various steps in platelet activation: adhesion, spreading, shape change, aggregation, release reaction, exposure of a procoagulant surface,

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and clot retraction. The rapid progression of these different capacities causes the activated platelets to form a hemostatic plug that occludes the site of injury to prevent blood loss.⁶ An increased risk of bleeding could be present when platelet count is reduced and/or one of their functions is defective. Conversely, improper thrombus formation could be due to a growth in platelet count or reactivity. In particular, activated platelets adhere and aggregate within atherosclerotic lesions, forming occluding arterial thrombi that may result in thromboembolic disease such as stroke or myocardial infarction, two of the major causes of morbidity and mortality in the Western world.^{4,7}

These different functions of platelets may be reliably detected with a wide spectrum of tests. The inherited or acquired platelet dysfunctions have to be essentially diagnosed because a rapid identification of patients at risk of bleeding is really necessary; the monitoring of antiplatelet therapy is becoming increasingly important for the identification of hypo- or hyperresponder patients at risk of both thrombosis or hemorrhage; the management of perioperative hemostasis and the different aspects of platelet banking (concentrates, transfusions, and evaluation of donor platelet function) is of vital importance; and – last but not least – the physiopathology of platelets should be critically recognized by basic research with these tests.^{8,9}

The history of platelet function testing (PFT) begins with the development of the evaluation of the bleeding time (BT) by the Duke procedure.¹⁰ This was the first test for the assessment of the capacity of platelets to form a plug. For many years, it has been the unique screening test to identify both congenital or acquired platelet disorders.¹¹ In the 1960s, the revolutionary platelet aggregation test in platelet-rich plasma (PRP) – ie, light transmission aggregometry (LTA) – according to Born's studies was the key method used to diagnose platelet function.¹² In this test, the ability of platelets to aggregate to each other in response to external aggregating agents – agonists, such as adenosine-diphosphate (ADP), arachidonic acid (AA), collagen, and epinephrine (EPI) – is measured *in vitro*.¹³ Since the late 1980s, other PFT methods, such as platelet aggregometry in whole blood (WB), the study of activated platelets *ex vivo* by flow cytometry, the measurement of specific compounds released by platelets, and the assessment of platelet nucleotides have become available.^{14–16} Hence, PFT has been narrowly employed for the diagnosis and management of patients suffering from hemorrhagic problems.¹⁷ In the last 2 decades, the clinical application of PFT has improved as it has increasingly come to be used for monitoring the antiplatelet treatment of

cardiovascular patients at risk of arterial disease.¹⁸ However, the increasing number of patients on antiplatelet drugs, with a higher risk of bleeding, especially during trauma and surgical procedures, has also led to the emergence of PFT as a useful tool in presurgical/perioperative settings for the prediction of hemorrhage and for monitoring the efficacy of different prohemostatic therapies. In this scenario, the development of new, simpler instruments for assessing platelet function at the point-of-care (POC) or bedside has led to better prospects of using PFT not only in specialized clinical or research laboratories, but also in general laboratories and in different clinical settings.^{9,18,19}

This review aims to describe the current status of available PFT that employs laboratory methodologies and point-of-care testing (POCT) with the pertinent instrumentation for different clinical settings. Different methodologies are employed both in the diagnosis of inherited and acquired bleeding disorders and in the monitoring of residual platelet reactivity of patients on antiplatelet treatment.

Platelet function methods

In recent years, the assessment of platelet (dys)function has become increasingly necessary in a variety of clinical settings: 1) for the identification of patients with bleeding disorders; 2) for monitoring the response to antiplatelet treatment; 3) in the evaluation of perioperative hemostasis; and 4) in transfusion medicine. Because there are diverse employment fields in which platelet function is studied and the platelets present a plethora of functions, different methodologies have been developed. Indeed, the platelet methods are based on different operating principles, and few assays are able to investigate “all in one device” platelet activation pathways. The different modalities of devices to operate may be based on the assessment of platelet adhesion and aggregation, on the submission of platelets under special shear conditions, on the analysis of physical properties of clot, and on the measurement of platelet compounds.^{8,9} In addition to the wide set of assays and devices for studying platelet function, because platelets are cells that may be easily activated during the blood sampling, a number of preanalytical variables can produce platelet artifacts affecting the different platelet functions. Therefore, a high degree of experience and expertise is necessary to perform and interpret PFT. In this sense, this review attempts to make a comparison of the various platelet function methodologies on the basis of the same principle that both are laboratory tests or POCT. The different platelet function tests reported in this review are grouped by the various methodological

principles in Table 1, and the pros and cons of some platelet tests are documented in Table 2.

Bleeding time

BT is the first test designed for evaluating *in vivo* primary hemostasis.¹⁰ BT assesses the platelet ability to develop a hemostatic plug by recording the time that the platelets take to occlude an *in vivo* skin wound for stopping the hemorrhage.²⁰ Although the technique is easy and quick to perform without any WB processing, it is influenced by different variables such as differences in skin thickness and temperature among patients and an incorrect management of the test procedure. Nevertheless, the use of available devices to standardize the test, a lack of accuracy, and unclear association with clinical patient state persist. No study has clearly established the

ability of BT evaluation to predict the risk of bleeding in patients, and a study only reported that BT could predict clinical bleeding in patients with acute myocardial infarction undergoing thrombolytic therapy.^{21,22} Moreover, this test is not used routinely to monitor the effect of antiplatelet therapy.²³ Nevertheless, BT is still regarded as a screening test to identify both congenital and acquired disorders of primary hemostasis in those laboratories that do not perform other platelet function tests.

Tests based on platelet aggregation

Light transmission platelet aggregometry on platelet-rich plasma

LTA, designed by Born in the 1960s, is performed by using the PRP as milieu.^{12,24} It is still deemed the gold standard test for the

Table 1 Different methodologies for assessment of platelet function

Method	Sample	Method application	Method principle
Bleeding time	Native WB	Screening test (obsolete)	In vivo measurement of bleeding block
Tests based on platelet aggregation			
Light transmission platelet aggregation (LTA)	Citrated PRP	Screening test for bleeding tendency Diagnostic for platelet defects Monitoring antiplatelet treatment effect	Photo-optical measurement of light transmission increase in relation to agonist-induced platelet aggregation
Impedance platelet aggregation	Citrated WB	Screening test for bleeding tendency Diagnostic for platelet defects Monitoring antiplatelet treatment effect	Measurement of electrical impedance increase in relation to agonist-induced platelet aggregation
Lumiaggregometry	Citrated WB	Detection of storage/release disorders	LTA or WB aggregometry combined with luminescence
Plateletworks	Citrated WB	Monitoring of the platelet response to antiplatelet agents	Platelet counting pre- and postactivation in whole blood
Tests based on platelet adhesion under shear stress			
PFA-100; Innovance PFA-200	Citrated WB	Assessment of bleeding risk and drug effects Searching severe platelet dysfunctions, revealing of VWD	Time evaluation of high shear WB flow blocked by platelet plug into a hole in activated surface
Impact; Cone and Plate(let) Analyzer	Citrated WB	Screening of primary hemostasis	Shear-induced platelet adhesion–aggregation upon specific surface
Global thrombosis test (GTT)	Native WB	Evaluation of platelet function and thrombolysis	Measurement of time cessation of WB flow by high shear-dependent platelet plug formation
Platelet function methods combined with viscoelastic test			
TEG/platelet mapping system	Citrated WB	Assessment of global hemostasis plus monitoring antiplatelet treatments effect	Evaluation of rate of clot formation based on low shear-induced and agonist addition
ROTEM platelet	Citrated WB	Assessment of global hemostasis plus diagnostic of platelet defects plus monitoring antiplatelet treatments effect	Measurement of electrical impedance increase in relation to agonist-induced platelet aggregation
Platelet analysis based on flow cytometry			
Flow cytometry	Citrated WB, PRP, W-Plt	Cell counting, detection platelet activation by extent of expression of surface and/or cytoplasmic biomarkers	Engineering laser-based detection of suspending fluorescent label platelets in a flowing solution
Evaluation of Thromboxane metabolites			
Radio- or enzyme-linked immune assays	Serum, urine, citrated Pls	Measurement of TxA ₂ metabolites (and Beta-TG, PF ₄ , soluble P-selectine) ^a	Ligand-binding assays

Note: ^aNot planned in this review.

Abbreviations: Beta-TG, beta-thromboglobulin; Pls, plasma; PRP, platelet-rich-plasma; ROTEM, rotational thromboelastometry; TEG, thromboelastography; TxA₂, thromboxane A₂; WB, whole blood; W-Plt, washed platelets; VWD, Von Willebrand disease.

Table 2 Pros and Cons of reviewed platelet function tests

Method	Pro	Contra
Bleeding time	In vivo test, quick No WB processing	Invasive Poorly standardized Dependent on many variables (skin thickness, and temperature operator's skills)
Light transmission platelet aggregation (LTA)	Historical gold standard Diagnostic method Flexible Different platelet pathways investigated Sensitive to anti-plt therapy	Manual sample processing Pre- and analytic variables High sample volume Time-consuming
Impedance Platelet Aggregometry	No sample processing Diagnostic method Flexible Different platelet pathways investigated Sensitive to anti-plt therapy Close to POCT (multiple system)	Limited HCT and platelet count range
VerifyNow system	Waived POCT No WB processing Easy, quick	Nonflexible Very expensive Monitoring antiplatelet therapy Limited HCT and platelet count
Plateletworks	POC system Minimal sample preparation Easy, rapid screening test	Indirect assay Required adjunctive platelet count Scarce data
PFA-100; Innovance PFA-200	In vitro standardized BT Easy, quick POCT Sensitive to severe platelet defects	Rigid closed system Platelet count – HCT-dependent Not sensitive to platelet secretion defects.
Impact Cone and Plate(let) Analyzer	WB assay Global platelet method Small sample volume	Expensive Experienced staff Lack of clinical studies Not widely available
Global thrombosis test (GTT)	WB assay Global platelet method Small sample volume	Lack of clinical studies Not widely available
TEG platelet mapping	POCT Global hemostasis test Reduces blood transfusions	Measure clot properties More studies are needed
ROTEM platelet	POCT Predicts bleeding Reduces blood transfusions Improves clinical outcome Global hemostasis test WB platelet aggregometry	Limited HCT and platelet count range (for platelet system) Lack of clinical studies

Abbreviations: BT, bleeding time; HCT, hematocrit; plt, platelet; POC, point-of-care; POCT, point-of-care testing; ROTEM, rotational thromboelastometry; TEG, thromboelastography; WB, whole blood.

assessment of the various platelet functions. Indeed, by adding a wide panel of agonists to PRP, a considerable amount of data are obtained about the various pathways of platelet activation.¹³

This test assesses in vitro the platelet-to-platelet clump formation in a glycoprotein (GP) IIb/IIIa-dependent manner, ie, the aggregation, the most important function of platelets. The assay is based on the measurement of the increase in light transmission through the optically dense sample of PRP after the addition of the exogenous platelet agonist. During the assay, the PRP after the addition of agonist becomes clearer because of the precipitation of platelet aggregates.

This determines an increase in light transmission through the plasma sample. The device records the rate and maximal percentage of this increase from 0% (maximal optical density of PRP) to 100% (no optical density of autologous platelet-poor plasma) by a photometer. This signal is converted automatically in a graphic curve that parallels the increase in light transmission during the platelet aggregation (Figure 1). The available aggregometers are easy-to-use devices equipped with automatic setting (100% and 0%), software for storing results and disposable cuvettes with a stirring bar. The slope of the curve, the maximal extent of aggregation (%), and the

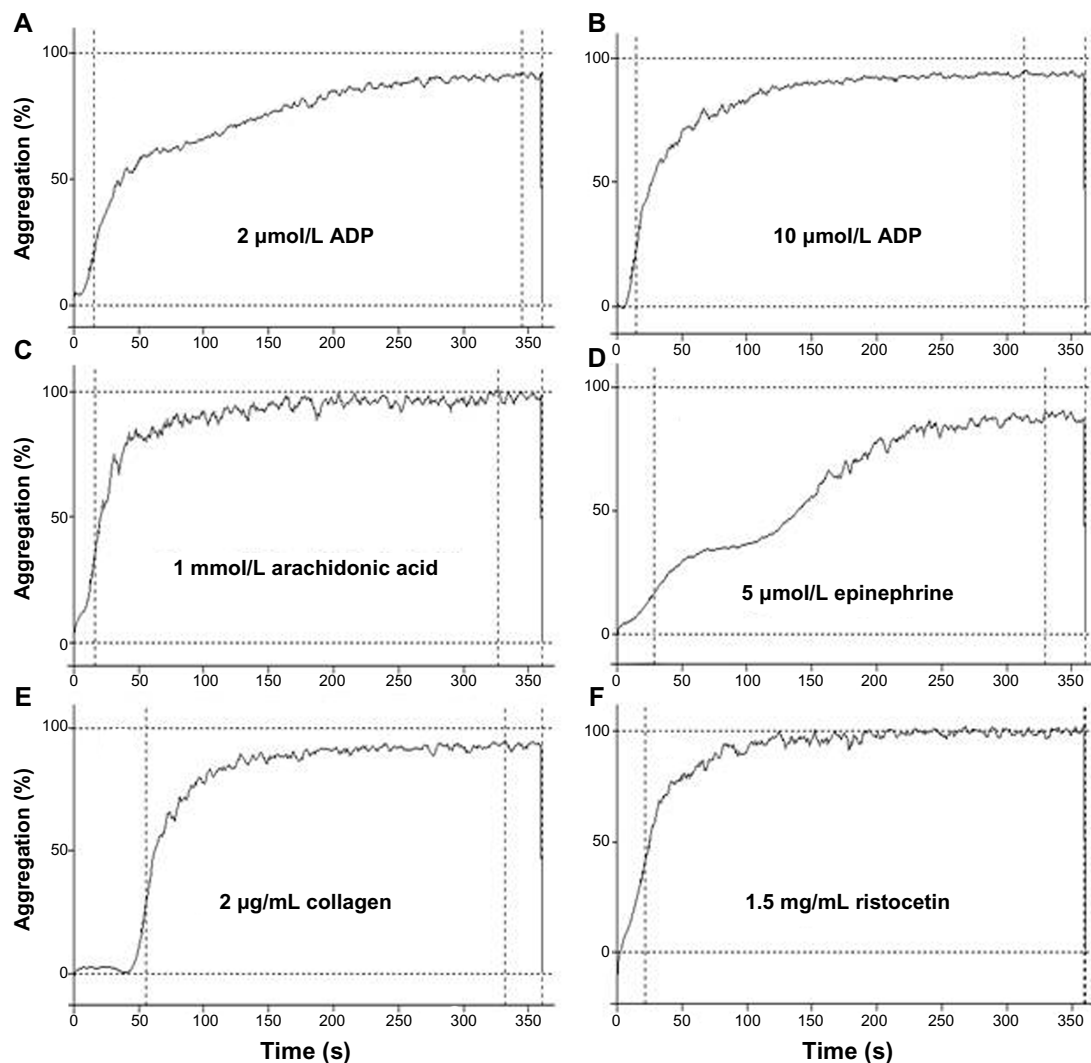


Figure 1 Light transmission platelet aggregation tracings from a healthy subject in response to different agonists.

Note: (A–F) Final concentrations are reported.

Abbreviation: ADP, adenosine-diphosphate.

latency time (lag phase) are the parameters automatically measured, and the shape change and primary and secondary aggregation may be seen graphically. To the PRP sample, different agonists are added to stimulate different platelet activation pathways, obtaining information about the several features of platelet function.

Born's platelet aggregometry is the most widely employed methodology for detecting platelet function disorders and monitoring antiplatelet therapies. Actually, it is deemed the first step in the flowchart for the study of hemorrhagic patients with inherited or acquired platelet dysfunctions.^{25–29} At the first sign of congenital/acquired bleeding disorders, in addition to the most commonly used agonists ADP, AA, collagen, and epinephrine, other agonists should be employed: thrombin receptor activating peptide (TRAP), Thromboxane A2 (TXA2) mimetic U46619, calcium

ionophore A23187. The antibiotic ristocetin is another agent used as agonist in the assessment of platelet function. It facilitates the binding of Von Willebrand Factor (VWF) to the glycoprotein Ib/IX/V complex. Different concentrations of ristocetin are used: this way of dose–response allows one to investigate both increased and decreased sensitivity to ristocetin. For a normal result, both functional VWF and normal glycoprotein Ib/IX/V complex must be present. Hence, ristocetin-induced platelet aggregation is considered a test that can detect both VW disease and platelet dysfunctions such as Bernard–Soulier syndrome.²⁹

Monitoring antiplatelet therapies (eg, aspirin and thienopyridine) by using LTA has permitted the prediction of major adverse cardiovascular events (MACE) in cardiovascular patients at high risk. The rate of residual platelet reactivity defined by ADP-, AA-LTA, or both has been associated with the development

of ischemic events both in acute coronary syndrome (ACS) patients and in those with stable coronary artery disease. Not only the previous evaluation of degree of platelet inhibition in stable cardiovascular artery disease (CAD) patients, but also the laboratory and clinical research of a cut-off value for ADP and/or AA-induced platelet aggregation have been instrumental in identifying those ACS patients who are nonresponders to antiplatelet agents and are at high risk of MACE.³⁰⁻³³

Although LTA is accepted as the most important and complete assay that clinical laboratories can perform to diagnose platelet function disorders, this assay presents some peculiar problems. Indeed, the technique may be affected by different preanalytical conditions (ie, type of anticoagulant, lipid plasma, hemolysis, or low platelet count), and by different procedural conditions (ie, PRP preparation, use of different concentrations of agonists), and the laboratory staff should have a high degree of skill, experience, and expertise in performing and interpreting platelet function. Hence, this method is constantly verified by an ongoing standardization process,^{25,34,35} and recent specific guidelines for LTA aimed at stabilizing/normalizing the correct procedure have been published.³⁶⁻³⁹

Finally, LTA test is the first diagnostic step in the evaluation of platelet disorders as reported by different studies.^{25,28} Because platelet dysfunction may be caused by a plethora of factors, for achieving a diagnostic hypothesis, the results obtained by LTA should be confirmed by further specific tests. By using flow cytometry analysis for the identification of expression of specific platelet components and lumiaggregometry for the identification of impaired platelet secretion or the Platelet Function Analyzer-100 (PFA-100) system, performed as a second diagnostic, LTA as a first-step diagnostic tool can be specifically confirmed.²⁸

Impedance whole blood aggregometry

Impedance whole blood aggregometry (WBA) allows one to assess platelet function by using the anticoagulated WB as milieu without any sample processing.⁴⁰ It is based on the principle that activated platelets stick via their surface receptors to artificial surfaces of two electrodes within the WB sample positioned at a determined distance between them. Platelet aggregation is assessed by detecting the increase in electrical impedance generated by the aggregation of other platelets upon those fixed to the electrodes. Hence, by diminishing the current intensity, the electrical impedance increases. The degree of the increase in impedance is recorded in Ohms.¹⁴

By the utilization of WB aggregometry, platelet function is assessed under more physiological conditions given that the contributions of other blood elements also may affect

platelet function. Another key aspect is that WB aggregometry takes place on surfaces, which enables this assay to be more adherent to the actual capacity of platelets to adhere and aggregate upon a solid phase. Moreover, WBA presents two important advantages: 1) use of a small quantity of WB in which all subpopulations of platelets are present; 2) no manipulation of the sample without activation of platelets, resulting in a rapid analysis of platelet function.

Now, the Multiple Electrode Aggregometry (MEA) is a new methodology by which the WB platelet aggregation can be assessed by using a new pertinent device that allows one to consider this assay as a POCT.⁴¹ Recently, this new aggregometer (Multiple Platelet Function Analyzer – Dynabyte – Roche Diagnostics, Mannheim, Germany) has become available across the world. The device presents particular features that make it a valid tool for rapid and complete platelet function evaluation. It is a five-channel computerized instrument equipped with disposable cuvettes ready to use with two independent sensor units and an automated pipetting. The reagents – agonists and diluents – are ready to use. The platelet aggregation is simultaneously measured in duplicate by using each sensor unit separately and calculated automatically as area under curve (AUC). With these important advantages MEA has acquired a high clinical value like that of LTA, and the use of different agonists similar to those used for LTA has made it suitable for the diagnosis of bleeding diathesis and for monitoring antiplatelet therapies, defining cut-off values to discriminate cardiovascular patients with high on-treatment platelet reactivity.⁴²⁻⁴⁹ MEA has been used to study the occurrence of VWD in patients with severe aortic stenosis and, on the other hand, the high thrombotic risk due to heparin-induced thrombocytopenia (HIT).^{50,51}

In addition, MEA has not only been able to identify cardiovascular patients not responding to antiplatelet agents and at risk of MACE, but has been considered a skill assay to distinguish those patients with a high inhibition of platelet function and at risk of bleeding.⁵²⁻⁵⁴ Ranucci et al⁵⁵ reported that investigating platelet function by using MEA before cardiac surgery has made it possible to identify the hyper-responder patients at risk of postoperative bleeding. Recently, it was reported that low extent of TRAP-induced platelet aggregation by MEA was defined as a factor independently associated with intramyocardial hemorrhage in patients with myocardial infarction.⁵⁶ Actually, multiple evidence reveals that MEA might be able to identify pre-/postoperatively those patients at risk of blood loss. Hence, this platelet function test has been proposed as a rapid and useful tool for the management of postoperative severe bleeding.⁵⁶⁻⁵⁸

MEA, overcoming the traps that LTA presents, may allow the rapid assessment of the platelet function without the requirement of a specialized laboratory. This POC system requires minimal technical knowledge and training to perform accurately, the working steps are automatically accomplished and easily controlled, and only interpretation data and judgment are required. Probably, in the future this test will be able to have a similar clinical value as of LTA with the advantage of its use in general laboratories.

Lumiaggregometry

Lumiaggregometry allows simultaneous measurement of the release of adenine nucleotides from platelet granules and platelet aggregation.^{16,59–61} The method is based on the evaluation of adenosine triphosphate (ATP) released from activated platelets by different agonists by using a luminescence technique in PRP, washed platelets (WP), or WB.^{62–65} The assay is based on the conversion of ADP, released from the platelet dense granules, to ATP that reacts with the luciferin–luciferase reagent. The light emitted, proportional to the ATP concentration, is quantified by the lumi-aggregometer. This technique is used for evaluating specific deficiencies in the number and content of dense granules: the so-called storage pool defects or specific defects in degranulation.^{8,9,62–65} As with LTA, this analysis might be used as a first screening test of patients with clinical suspicion of platelet function defects such as abnormalities of platelet granule secretion and/or content, plasma membrane receptor defects, and to evaluate platelet functions during thrombocytopenia.^{28,66,67}

VerifyNow system

The VerifyNow system (ITC, Edison, NJ, USA) is a platelet function-waived POCT consisting of a device that assesses in WB platelet aggregation by a turbidimetric-based optical detection and using a system cartridge containing fibrinogen-coated beads and platelet agonists.⁶⁸ The method is based on the capacity of activated platelets to bind to fibrinogen: platelets aggregate upon the fibrinogen-coated beads within the assay cartridge in proportion to the number of activated GPIIb/IIIa receptors. The crossing WB optical signal increases as the activated platelets aggregate to the fibrinogen attached to the spheres. The use of this closed system requires no blood manipulation and instrument handling: actually, the methodology is very waived and it is largely employed to monitor antiplatelet therapies in the emergency cardiac operating room without the help of a specialized laboratory.⁵⁴ Formerly, the method (called Ultegra) was used to verify the inhibitory effect of platelet GPIIb/IIIa antagonists (abciximab or eptifibatide)

in cardiovascular patients undergoing percutaneous coronary intervention (PCI): in this assay (VerifyNow IIB/IIIA Test), the thrombin receptor activating peptide (iso-TRAP) is used as agonist to maximally stimulate platelet aggregation.⁶⁹ The device reports patient results as Platelet Aggregation Units (PAU) calculated as rate and degree of aggregation. Now, two more assays each sensitive to targeted drugs are available: Aspirin Test with AA as agonist (sensitive to aspirin therapy), whose results are expressed as Aspirin Reaction Units (ARU); and PRUTest (sensitive to thienopyridine treatment), whose results are expressed as P2Y₁₂ Reaction Units (PRU), by using ADP as agonist and PGE₁ as suppressor of intracellular free calcium levels for diminishing the nonspecific influence of the ADP-binding to P2Y₁ receptors. The VerifyNow system showed a moderate agreement with other platelet function tests. The assessment of the sensitivity and specificity of the method and the definition of a cut-off value for classifying patients at high risk of MACE and not responsive to acetylsalicylic acid (ASA) or thienopyridines have been debated and different laboratories and clinical studies have reported the clinical value of this system.^{70–77} Recently, in surgical settings, the use of the VerifyNow system in patients on antiplatelet agents has been expanding for attempting to predict postoperative hemorrhage.^{78,79}

Plateletworks system

This system is a WB POC assay based on the measurement of platelet count before and after aggregation. This system consists of the Plateletworks aggregation kit formed by EDTA tubes and citrate tubes implemented with agonists (ADP or AA) and the Ichor blood counter (Helena Laboratories, Beaumont, TX, USA).⁸⁰ The Plateletworks method matches the platelet count assessed in the control sample (EDTA tube) with those obtained after aggregation in citrate blood with ADP or AA (citrate tube plus agonist). Since platelets aggregate, the fall in their count measured in citrate tubes is the extent of activated platelets that have aggregated themselves. This rapid test does not need any manipulation of blood sample, and results are ready in minutes. A disadvantage of the test is that it should be performed within a few minutes of blood sampling. The need for this strict time interval has relegated this analysis to restricted operating areas such as cardiac surgery and cardiac operating room. Relationships between Plateletworks and LTA, VerifyNow system and Thromboelastography have been reported as validation test for monitoring antiplatelet therapy.^{81–83} In acute care condition, Plateletworks shows the clinical value of giving information about both platelet count and function.^{84,85}

Nevertheless, it is still under consideration and has not been reported to predict clinical outcomes.

Tests based on platelet adhesion under shear stress

The Platelet Function Analyzer – PFA-100/Innovance PFA-200

The Platelet Function Analyzer – PFA-100/Innovance PFA-200 is a PFT that uses a device called the PFA-100 or the new available and updated Innovance PFA-200 (Siemens, Munich, Germany). At the beginning, PFA-100 has been considered as the standardization of BT.^{86,87} The method is based on the property of platelets to adhere upon shear stress conditions and aggregate in consequence of agonist presence in the system. This methodology is considered a POCT that assesses platelet function in WB using appropriate cartridges in which primary hemostasis is simulated.^{88–90} Two different test-cartridges containing either collagen (C) plus ADP – CADP cartridge – or collagen plus epinephrine (EPI) – CEPI cartridge – are available. Within the cartridge, the citrated WB is drawn at a high shear stress rate through a capillary that has at its end a collagen-coated membrane, in which a defined microscopic aperture (147 μm) is present, filled with either ADP or EPI. A platelet clot, which fills the hole, occurs because of shear stress and agonists. The time taken by platelets to occlude the orifice and to block the WB flow is defined as closure time (CT), a measure of overall platelet-related hemostasis. The CT is as much prolonged as the platelets are able to act.

The use of two different assay cartridge allows to differentiate platelet defects due to intrinsic or platelet defects (prolonged times of CADP cartridge alone or plus CEPI cartridge) such as VW defects or Bernard–Soulier syndrome or Glanzmann's thrombasthenia from those due to antiplatelet therapy with ASA (prolonged time of CEPI cartridge).^{70,90–93} In comparison with BT test, this assay has been shown to be more sensitive,^{70,89} especially for diagnosis of VWD and platelet function defects.⁹¹ Recently, a third diagnostic, the new Innovance cartridge, sensitive to the thienopyridine therapy, has become available, but information on its clinical value is poor and limited.⁹⁴

The PFA-100 is sensitive to many variables that influence platelet function – prolonging the CT – as well as low platelet count and hematocrit, conditions associated with thrombocytopathies. Thus, the clinicians should take these limitations into account. On the other hand, factors such as high levels of VWF, fibrinogen, or erythrocytes have been reported to shorten CEPI CT.^{95,96} In addition, a short CEPI CT could reveal high residual platelet reactivity despite aspirin therapy, thereby predicting the risk of thromboembolic

events.^{97,98} Indeed it has been demonstrated in ACS patients on ASA treatment that a high concordance exists between LTA and the PFA-100 CEPI test, giving significant negative predictive value to the PFA system.⁶⁵ In addition, PFA CEPI CT shortened results were demonstrated to be a significant and independent predictor of MACEs in patients with acute myocardial infarct (AMI) undergoing primary PCI.^{99–101} In patients undergoing different kinds of elective surgeries, the assessment of platelet dysfunction with PFA-100 may provide useful information for postoperative blood transfusion management.^{102,103} Especially in cardiac surgery, PFA methodology showed a high predictive value of platelet function for management of intra- and postoperative blood loss.^{104–107} In patients with biventricular assist device implantation, on treatment with clopidogrel, the use of CADP diagnostics for monitoring the degree of platelet inhibition allowed them to undergo transplantation without major blood loss.¹⁰⁸ In addition, prolonged CADP CTs were found to be independent risk factors for postpartum hemorrhage (PPH) severity and were significantly related to menorrhagia.^{109,110} Presurgical prolonged CTs corrected with 1-deamino-8-D-arginine vasopressin (DDAVP), ie, desmopressin treatment, made it possible to keep unchanged the quantity of postoperative blood transfusions with respect to those provided to patients with normal presurgical CTs.¹⁰²

It has been recommended that the PFA system be employed as a screening tool integrated into a panel of existing tests.^{28,38} This assay is characterized by a high negative predictive value: when a normal CT result is found in a suspected platelet disease, further PFT should not be performed.⁵

IMPACT: Cone and Plate(Let) Analyzer

IMPACT: Cone and Plate(Let) Analyzer (Image Analysis Monitoring Platelet Adhesion Cone and Plate Technology) (CPA) (DiaMed, Cressier, Switzerland) is a novel and innovative POC test that fully assesses platelet function by using an automatic and computerized system that evaluates in vitro primary hemostasis.¹¹¹ The test is based on the in vitro activation and adhesion of platelets that lie all over a standardized plate covered by a substrate of polystyrene. The shear stress force is impressed to platelets by the spinning of a cone on the plate. Citrated WB is used in the system that is formed by the reaction device – cone and plate – an automatic staining device and image analysis software. After automated staining, the percentage of the well surface covered by platelet aggregates – representing platelet adhesion – and the average size of the aggregates (per μm^2) – representing platelet aggregation – are measured.

This system is highly dependent on plasma. This instrument methodology could be a consistent system for the diagnosis of platelet defects. Moreover, the addition of the agonists AA and ADP in the system has made it possible to monitor and evaluate the efficacy of dual antiplatelet therapy.^{112,113} However, additional studies should be undertaken to assess its possible role in the diagnosis of inherited or acquired platelet dysfunctions.

The global thrombosis test

The global thrombosis test (GTT) (Montrose Diagnostics Ltd, London, UK) is a recent test that assesses platelet function in a manner close to physiological conditions because this technique is performed by using native nonanticoagulated WB, without adding agonists.^{114–116} The principle on which it is based is platelet activation due to high shear stress, opportunistically provoked, similar to that in coronary artery stenosis. In this new system, the WB flows into a plastic tube that holds a conical part in which two ceramic balls are placed. The complete occlusion of the tube lumen due to the presence of the spheres is prevented by the flat segment created along the inner wall of the tube. WB flows under the force of gravity through the four narrow gaps of the first ball where high shear stress activates platelets. In the space underlying the first ball, low shear and turbulent flow support platelet aggregation. The platelet plug occludes the gaps at the level with second ball at the end tube and the WB exits always more slowly in the form of drops. In the first phase, the occlusion of the tube system, expressed as occlusion time (OT) in seconds, occurs. The time of interval (d) between two consecutive drops is recorded until an arbitrary endpoint ($d \geq 15$ seconds) is reached. In the second phase, the blood flow resumes at the end of the tube owing to spontaneous lysis of occlusion expressed as lysis time (LT) in seconds. This rapid POC test provides results on the patient's thrombotic status, and it is thus employed in acute and critical care settings, as well as in more general screening.^{116–118} The evaluation of GTT results in association with clinical outcomes is still in progress. However, because GTT presents a double clinical value, this test might play a role in the future for the assessment of platelet function.^{115,119}

Platelet function methods in conjunction with viscoelastic methodologies

TEG platelet mapping system and ROTEM platelet test

Thromboelastography and thromboelastometry are methodologies employed for the global assessment of the hemostatic process. These assays investigate the entire development

of clot formation and are based on the analysis of WB modifications in viscoelastic forces during the clotting formation. Indeed, the potential of this methodology is related to assessing the extent of platelet count and function, clotting, and fibrinolytic activation.¹²⁰ In these tests, platelets play all their functional roles in hemostasis: thrombin generation, clot formation, clot retraction, and lysis.

At present, three principal systems are available: Thromboelastography, performed on “old” renewed devices (TEG; Haemoscope, IL, USA), Thromboelastometry, formerly called Rotational Thromboelastography, performed on a new device (ROTEM; TEM Int, Munich, Germany), and Sonoclot analysis performed on a new device (Sonoclot Signature; Sienco, CO, USA). The principle underlying the method for TEG and ROTEM consists in a rotating system inclusive of a pin suspended by a torsion wire in a cup. In the WB sample, owing to the addition of reagents and the shear stress of the rotating system, the forming clot entraps the pin promoting a motion that increases as the clot strengthens and decreases as the clot lyses. In the Sonoclot device in the cup, the pin is moved up and down at an ultrasonic rate.¹²¹

The in vitro addition of specific reagents and activator to WB provokes the hemostatic activation followed by modifications in hematic viscoelastic forces: the entire process is instantly recorded during all clotting formation and also visualized in a typical curve. Different tests are available depending on the reagent used: tissue factor for extrinsic pathway, different activators (glass, silica, kaolin, ellagic acid) for intrinsic pathway. Those additional compounds are used for revealing particular aspects of hemostasis: heparinase for evaluating the degree of anticoagulation of heparin, aprotinine for evaluating the activation of fibrinolysis, and antiplatelet agents (cathepsin G or GPIIb/IIIa antagonists) for eliminating the contribution of platelets to clotting formation. For each test, different parameters, indicating the different steps of hemostasis, are shown after the recognition and computation by the integrated software detection: K index (TEG) and Clot Formation Time (CFT, ROTEM) recognizes platelet function, Angle alpha (for both) indicates the rate of fibrin-formation, Maximal Amplitude (MA, TEG) and Maximum Clot Firmness (MCF, ROTEM) indexes show the platelet contribution to clot formation. In addition, by the selective activation of extrinsic pathway without and with antiplatelet agents (ie, clotting by fibrinogen alone), it is possible to evaluate the extent of platelet contribution to the clot formation. Because all this instrumentation promptly delivers a graphic representation of clot formation and lysis, they are now used as POC devices in different clinical settings such as

cardiac surgery, liver transplantation, obstetrical emergency, and trauma center.^{122–135} To date, the clinical value of this technology has consisted in offering a predictive evaluation of the risk of increased postoperative bleeding. For surgical and emergency settings, different studies have documented flow charts in which are reported when to use these systems and how to interpret their results in patients at risk of bleeding, such as those on antiplatelet treatment. Actually, it has now been identified how the use of the different parameters provided by these tests may be predictors of both postoperative bleeding and blood product use.^{136–138}

Currently, new applications for the study of platelet function are available. The TEG platelet mapping system is a modification of the original TEG and has been developed for monitoring antiplatelet therapy.^{139–142} First, a kaolin-activated test is performed to evaluate the maximal hemostatic activity. Second, a test in the presence of reptilase and Factor XIIIa (activator F) is used to produce a cross-linked fibrin clot. The addition of ADP or AA stimulates differentially the role of platelet ADP or TXA2 receptors in clot formation. The effect of therapy with aspirin (AA addition) or thienopyridines (ADP addition) is evaluated by comparing the TEG kaolin-activated test curve with the AA or ADP-stimulated TEG curve. The method has been shown to be reliable with low analytical variation.^{143,144} However, further large prospective studies should be performed in order to define the possible role of these devices in monitoring antiplatelet therapy.

On the other hand, the ROTEM platelet system is a new module that could be added to the ROTEM. A WB aggregometer as an integrated module has been added to the ROTEM system. This allows a deeper analysis of the primary hemostasis of the patient along with that of coagulation. The measurement is based on true impedance aggregometry (such as the MEA system), which allows the measurement of platelet aggregation in WB samples. The software for the analysis data is the same as of the ROTEM system. In this manner, the platelet function analysis is visualized together with the thromboelastometric measurements, altogether on the same screen; in fact, the two different assays (both thromboelastometry and platelet aggregometry) can be run together in the same time on the same system. Some characteristics of the ROTEM platelet are similar to those of MEA, such as single-use cuvettes, time of test, measured parameters, and agonists used. Because the availability of this assay is very recent – it goes back to a few months ago – only a few data are currently available about its laboratory and clinical value.¹⁴⁵

Platelet analysis based on flow cytometry

Flow cytometry (FC) is a technique of rapidly measuring specific characteristics of many different cells such as platelets, measuring the cell size, and granularity. FC analysis of platelets may offer information on their functional status in vivo, and this technique encompasses multiple assays for several purposes such as the assessment of the activation state (platelet membrane-associated IgG), evaluation of thrombopoiesis, diagnosis of specific disorders, and antiplatelet agent monitoring.^{146–148}

This analysis is based on the optical and fluorescence evaluation of physical – such as size and internal complexity – and antigenic properties of platelets, ie, determination of surface receptors including conformational changes related to the receptor activation, platelet granule secretion, presence of platelet aggregates, and leukocyte–platelet aggregates.¹⁴⁹ It consists of the measurement of antibodies conjugated to fluorescent dyes that may bind specific proteins on cell membranes or inside cells, revealing their presence. A light source, crossing labeled platelets, excites to a higher energy state the fluorescent molecules of dyes. When the fluorochromes return to a resting state, they emit light energy at different wavelengths. The use of multiple fluorochromes with similar excitation wavelengths, but different emission wavelengths (or “colors”), allows several cell properties to be measured simultaneously. Different fluorochromes such as fluorescein isothiocyanate (FITC) or phycoerythrin (PE) can be used, but also a specie-specific secondary antibody coupled to a fluorochrome can be employed to recognize a primary antibody linked to surface antigens.¹⁵ In the WB, the use of a double labeling binding allows the identification of platelets, platelet microparticles, or mixed cell aggregates.^{150–152} The results of FC are represented in the form of histograms with mean fluorescence intensity (MFI) plotted against cell number.

A plethora of platelet defects and features can be investigated by FC. Indeed, it can be a useful tool for the diagnosis of inherited (ie, Bernard–Soulier syndrome, Glanzmann’s thrombasthenia) or acquired platelet dysfunctions (ie, HIT) and storage pool disease. As this analysis is able to assess platelet function defects in patients with very low platelet count, it is considered the test of choice to define the presence of thrombocytopathy in a thrombocytopenic patient.⁹ In this regard, an FC for the diagnosis of thrombocytopenic disorders has been developed to measure reticulated platelets.¹⁵³ In addition, FC allows one to recognize the procoagulant capacity and activation state of platelets (eg, in the setting of acute coronary syndromes or cardiopulmonary bypass),

by highlighting the expression of phosphatidylserine on activated platelet membranes using labeled annexin V.¹⁵⁴

Different steps in intracellular signal transduction can be investigated by FC. Indeed, the intracellular quantification of Vasodilator Stimulated Phosphoprotein (VASP) phosphorylation by FC is the most used method for monitoring the efficacy of antiplatelet drugs.^{155,156} Finally, FC is used to evaluate the state of stored platelets in blood banks. For example, it is possible to assess the platelet activation state and identify leukocyte contamination of platelet concentrates for the evaluation of the efficacy of platelet transfusion.^{157–159}

FC has many advantages, such as the use of small volumes of blood samples and the analysis of platelets in physiological milieu (WB). On the other hand, FC analysis is an expensive test, requiring specialized operators and devices, and the pre-analytical phase can be affected by the delicate preparation prone to artifact unless carefully prepared.^{8,9}

Evaluation of Thromboxane metabolites

In physiological conditions, when platelets are stimulated by agonists, TXA₂ is rapidly synthesized and released from platelets at the site of vascular injury. TXA₂ amplifies the platelet activation, recruits additional platelets to the site of clot formation, and induces vasoconstriction, accelerating the hemostasis process.¹⁶⁰ TXA₂ is the major product of the platelet metabolism of AA. AA, released from platelet membrane phospholipids via phospholipase A₂ (PLA₂), is converted in the endoperoxides, prostaglandin G₂ (PGG₂) and PGH₂, via cyclooxygenase-1 (COX-1) and finally transformed into TXA₂ by thromboxane synthase. TXA₂ is rapidly transformed by hydrolysis into TXB₂, a biologically inactive and stable product.^{160,161} TXB₂ is excreted unchanged in urine in a small percentage being prevalently transformed into two major metabolites: 2,3-dinor-TXB₂ and in 11-dehydro-TXB₂ by β -oxidation and dehydration, respectively. These metabolites circulate in the plasma at low concentrations and are excreted in urine, and it has been established that 11-dehydro-TXB₂ is more abundant in urine than 2,3-dinor-TXB₂.^{162,163} In inflammatory conditions, TXA₂ can be produced by other biochemical pathways independently from platelets and extraplatelet sources of TXA₂ can become relevant.¹⁶⁴ Conversely, ASA interfering with COX-1 activity causes a decrease in the concentration of serum TXB₂ and urinary 11-dehydro-TXB₂.^{165,166}

The measurement of TXA₂ metabolites allows evaluation of the activation state of platelets.^{8,9} Serum and urine TXB₂ metabolites concentrations are related to the entity of TXA₂ biosynthesis, and their dosage may be useful to assess platelet

function in different diseases, to detect defects of thromboxane production, and to monitor aspirin therapy.^{8,9,167,168} Different ligand-binding assays based on the binding of the analyte to specific receptors by using different detection methods can be used to assess serum TXB₂ and urinary 11-dehydro-TXB₂ concentration, such as: 1) the “old” radioimmunoassays (RIA) or immunoradiometric assays (IRMA) that are now outdated;^{169–171} 2) enzyme-linked immunoassays (ELISA) that are now the most commonly utilized assays.^{172,173}

Future perspective

Different methodologies for the assessment of platelet (dys) function in order to screen different idiopathic or acquired pathological conditions – hemorrhagic and/or prothrombotic status – are currently available. At present, platelet tests are mostly used, thanks to the work put into the standardization of these tests, in the drawing up flowchart and to the introduction of primary hemostasis POCT. These available tests allow either the study of global platelet function or the evaluation of particular aspects of platelet activation. Primary hemostasis POC tests simultaneously may assess in vitro platelet adhesion and aggregation; platelet aggregation analysis is inclusive of platelet secretion and aggregation phenomena. In particular, in WB platelet aggregation, the role of other blood cells is considered; viscoelastic methods analyzing global hemostasis are able to individuate platelet dysfunction (Tables 1 and 2). Despite this versatility and their important clinical value, platelet assays pertaining to the same category (eg, those based on platelet aggregometry) are again affected by a wide range of methodological aspects. The numerous conditions involved in them cause an interlaboratory variability of results and can delay a clear standardized procedure. This major gap is partly overcome by the introduction of platelet function POCT, which presents a basilar standardized procedure. In light of this, a new platelet adhesion test using microfluidic devices, for example, a biochip containing several different adhesions, is under evaluation.^{174,175} Now, platelet spreading tests, using fluorescence microscopy or scanning electron microscopy, are frequently employed.¹⁷⁶ On activation, platelets are able to release or express several compounds such as ATP, ADP, 5-HT, CXCL12, CD34, P-selectine, or thromboxanes, which may be assessed by using different methodologies such as immunological assay, High-performance liquid chromatography, fluorescence microscopy, or flow cytometry.^{28,177,178} The major drawback of the application of these assays in clinical practice is the scarcity and high variability of clinical and laboratory data and the absence of clear indications or guidelines for the correct use of such tests. Either for the

study of global or single step of platelet function or for the definition of the clinical value of new platelet biomarkers, new or renewed platelet assays with high sensitivity and specificity are considered necessary, especially for routine laboratory analysis. In the future, it is desirable that specific, standardized, more rapid and easy tests – whose clinical value has been well defined – are available.

Conclusion

To date, the effort of translating the different PFTs as diagnostic tools for evaluating bleeding disorders and monitoring antiplatelet therapies is in progress. Actually, the available platelet function POCT is making possible the institution of platelet tests in laboratories and intensive care units, allowing their use in different clinical settings such as inherited bleeding disorders, cardiovascular intensive care, trauma coagulopathy, liver transplantation, and obstetric care for the prediction of bleeding. Similarly, the use of these POC tests could be extended not only at the bedside in critical areas outside of the specialized laboratory, but also in centralized and satellite laboratories. Indeed, the upgrading of the WB test, such as MEA, or platelet mapping or the new ROTEM platelet device, may guide clinicians in making the correct diagnosis of bleeding risk or in properly tailoring the antiplatelet therapy directly in pre-/postoperative care.

Nevertheless, for the amelioration of the ongoing standardization of LTA, new procedures were reported in 2010, 2011, and 2013.^{37–39} For this, PRP or WB platelet aggregometry by using new systems might be realized into routine laboratories. Because the availability of platelet tests is an important improvement, the drafting of validation procedures and the study of reliability and quality control testing of PFT are becoming an increasingly important issue.

In conclusion, the new POCTs available may be considered useful supplements to the existing well-known platelet function tests, but further prospective studies are necessary to define the applications of these tests. In the future, the improvements in the study of platelet genome and proteome may encompass knowledge of platelet function testing with a noteworthy impact on the diagnosis and management of patients affected by hemorrhagic or thrombotic defects.

Author contributions

Paniccia R conceived and designed the review, prepared the manuscript, and surveyed the literature; Priora R contributed to the literature review and preparation of the manuscript; Alessandrello Liotta A contributed to the preparation of the manuscript; and Abbate R approved the version to be

published. All authors contributed toward data analysis, drafting and revising the paper and agree to be accountable for all aspects of the work.

Disclosure

The authors report no conflicts of interest in this work.

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