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REVIEW

Assessment of platelet function: Laboratory and point-of-care methods

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Abstract

In the event of blood vessel damage, human platelets are promptly recruited on the site of injury and, after their adhesion, activation and aggregation, prevent blood loss with the formation of a clot. The consequence of abnormal regulation can be either hemorrhage or the development of thrombosis. Qualitative and/or quantitative defects in platelets promote bleeding, whereas the residual reactivity of platelets, despite antiplatelet therapies, play an important role in promoting arterial thrombotic complications. Platelet function is traditionally assessed to investigate the origin of a bleeding syndrome, to predict the risk of bleeding prior surgery or during pregnancy or to monitor the efficacy of antiplatelet therapy in thrombotic syndromes that, now, can be considered a new discipline. "Old" platelet function laboratory tests such as the evaluation of bleeding time and the platelet aggregation analysis in platelet-rich plasma are traditionally utilized to aid in the diagnosis and management of patients with platelet and hemostatic disorders and used as diagnostic tools both in bleeding and thrombotic diathesis in specialized laboratories. Now, new and renewed automated systems have been introduced to provide a simple, rapid assessment of platelet function including point of care methods. These new methodologies are also suitable for being used in non-specialized laboratories and in critical area for assessing platelet function in whole blood without the requirement of sample processing. Some of these methods are also beginning to be incorporated into routine clinical use and can be utilized as not only as first panel for the diagnosis of platelet dysfunction, but also for monitoring anti-platelet therapy and to potentially assess risk of both bleeding and/or thrombosis.

Key words: Platelets; Method; Test; Point of care testing; Laboratory assessment; Bleeding; Thrombosis; Platelet function

Core tip: This review discussed the scenario of available platelet function laboratory and point-of-care methods suitable in different clinical setting. As this matter has become of crucial importance in the bleeding management and for monitoring antiplatelet therapies, improved ability to assess platelet function in a timely and efficient manner is essential. Traditional platelet function methods, requiring a fair degree of expertise, have been limited to specialized laboratory. Many efforts have been carried out for improving platelet function assays for centralized laboratory, such as different point-of-care testing methodologies have been developed. Moreover, different guidelines and recommendations for their method standardization are growing.

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INTRODUCTION

Platelets are multifunctional cells that play a role in many pathophysiological processes including haemostasis and thrombosis, clot retraction, vessel constriction and repair, inflammation including promotion of atherosclerosis, host defense and tumor growth/metastasis[1,2]. Bizzozero in the late 1800s first described platelets, identifying them as distinct cells and observing that aggregated platelets form thrombi into damaged parts of vessel[3].

Notwithstanding the multiple roles of platelets, the available platelet tests investigate those functions directly involved in haemostasis[4,5]. The fine relationships between platelets and the vessel wall, *i.e.*, the primary haemostasis, is the first phase of haemostatic process. At the injury of a vessel wall platelets are involved in sequential functional responses including adhesion, spreading, shape change, aggregation, release reaction, exposure of a procoagulant surface and clot retraction. The progression of these different steps conveys the activated platelets rapidly to form a hemostatic plug that occludes the site of lesion to prevent blood loss[6]. If one of these functions and/or platelet number are defective, then hemostasis is impaired and an associated increased risk of bleeding could be also present. On the other hand, an increase in platelet count or reactivity may lead to unsuitable thrombus formation. Upon and within atherosclerotic lesions platelets adhere, aggregate with the development of arterial thrombi that may result in stroke and myocardial infarction, two of the major causes of morbidity and mortality in the western world[7]. The prevention of arterial thrombotic complications, the antiplatelet therapy and its monitoring, can be beneficial, but their management should be carefully conducted without increasing the risk of bleeding[1,8-11].

To date, platelet function testing has been used to identify the possible causes of bleeding[12] to monitor pro-haemostatic therapy in patients at high risk of bleeding and to verify normal platelet function prior and during surgery[13,14]. Recently, different methodologies have progressively developed for monitoring the response to antiplatelet therapy and for the identification of patients with residual platelet reactivity at risk of thrombotic complications[15-18].

Development of platelet function testing

At the beginning of 1900s the bleeding time (BT) by Duke procedure[19], was the first test for evaluating the capacity of platelets to form a plug. For long time, this test has been considered a useful screening test to identify both congenital or acquired platelet disorders[20].

The cornerstone for the diagnosis of platelet function was the platelet aggregation in platelet-rich plasma (PRP) according to Born's studies[21]. This method measures the capacity of platelet to aggregate to each other in response to external aggregating agents-agonists, *i.e.*, adenosine-diphosphate (ADP), arachidonic acid (AA), collagen, epinephrine (EPI) and others[22]. Since the late 1980s new laboratory tests of platelet function have become available, such as flow cytometry as well as the evaluation of platelet nucleotides[23].

Because platelet dysfunction may be due to a wide multiplicity of defects, to diagnose an affected platelet function is difficult and there are no pivotal screening tests. The current laboratory assessment of platelet defects usually investigates platelet adhesion/aggregation and/or measurement of granule content/release. However, these tests are labor intensive, costly, time consuming and require a fair degree of expertise and experience. These problems have mainly limited their extensive clinic use. Actually, these methodologies are available only in specialized clinical laboratories dedicated to the studies of pathophysiological processes including haemostasis and thrombosis. As the evaluation of platelet function has become of crucial importance in the management of severe bleeding, improved ability to assess platelet function in a timely and efficient manner is essential. During the last two decades, different point of care testing (POCT) instruments for the assessment of platelet function at the bedside of patients at high risk of bleeding or thrombotic complications have been developed. Now, simple platelet function tests on whole blood (WB), that may be employed as POCT at bedside or within non-specialized laboratories, have been proposed[24-28]. In Table 1 the different laboratory and point-of-care assays for the evaluation of platelet function are reported; in Table 2 advantages and disadvantages of these methodologies are indicated and in Table 3 the clinical value of the principal platelet tests is specified.

This report attempts to focus on the scenario of available platelet function POCT with the pertinent instrumentation more suitable for the use in different clinical setting of critical area such for the diagnosis of inherited and acquired bleeding disorders or for monitoring residual platelet reactivity of patients on antiplatelet treatment.

PLATELET FUNCTION LABORATORY TESTING

Bleeding time

The skin Bleeding time (BT) is the oldest test for assessing *in vivo* primary haemostasis[20]. BT assesses the capacity of platelets to form a haemostatic plug. The time, that the platelets employ to occlude an *in vivo* skin wound, is recorded by evaluating the ability of platelets to stop the bleeding[29]. BT still remains a useful test to identify both congenital and acquired disorders of primary haemostasis in those laboratories that don't perform other platelet function tests. The technique is easy and quick to per-

Table 1 Laboratory and point-of-care assays for evaluation of platelet function

Platelet function tests	Principle of method	Application of the methods
Platelet adhesion studies		
Bleeding time	*In vivo* stopping of blood flow	Screening test of platelet function on defects of primary hemostasis
Platelet Function Analyzer - PFA-100/InnovancePFA-200	*In vitro* stopping of high shear blood flow by platelet plug in whole blood	Assessment of bleeding risk, thrombotic risk, drug effects Sensitive to severe platelet dysfunctions Detection of VWD
Impact Cone and Plate(let) analyzer	Shear-induced platelet adhesion/aggregation onto surface in whole blood	Screening of congenital primary hemostasis abnormalities Evaluation of platelet response to aspirin and clopidogrel (scarce data).
Platelet-To-Platelet Aggregation Studies		
Light transmission platelet aggregation	Low shear platelet-to-platelet aggregation in response to agonists in platelet-rich-plasma	Screening test for bleeding behavior Diagnostic for platelet surface glycoprotein defects Monitoring of the platelet response to antiplatelet agents
Impedance platelet aggregation	Low shear platelet-to-platelet aggregation in response to agonists in whole blood	Screening test for bleeding behavior Diagnostic for platelet surface glycoprotein defects Monitoring of the platelet response to antiplatelet agents
VerifyNow system	Fibrinogen-platelet agglutination in response to agonist in whole blood	Monitoring of the platelet response to antiplatelet agents
Plateletworks	Platelet counting pre- and post-activation in whole blood	Monitoring of the platelet response to antiplatelet agents
Analysis of Clot Formation		
Thromboelastography/ Thromboelastometry	Monitoring of rate and quality of clot formation in whole blood based on viscoelastic blood changes	Assessment of global haemostasis Possible definition of different platelet and clotting abnormalities Diagnosis and treatment of bleeding after cardiac surgery, liver transplantation, trauma and PPH
Platelet function tests to investigate platelet activation		
Flow cytometry	Cell counting, cell sorting, biomarker detection and protein engineering laser-based detection of suspending fluorescent label platelets in a stream of fluid	Expression of platelet specific surface and/or cytoplasmatic markers; VASP phosphorylation state[1] (Monitoring of CD41/61, CD42, CD62P, *etc.* Activation markers directly dependent on thienopyridine target)
Radio- or Enzyme Linked-Immune Assays: Soluble markers determination[1]	Ligand binding assays	Measurement of Beta-thromboglobulin, PF4, GPV, Soluble P-Selectin, Thromboxanes

[1]Not planned in this report. GP: Glycoprotein; PPH: Post-partum hemorrhage; VASP: Vasodilator-stimulated phosphoprotein; VWD: Von Willebrand Disease.

form without any WB processing; but it can be affected by an inaccurate operator managing and by skin thickness and temperature. Notwithstanding, BT was fulfilled by the use of an available device to standardize the size and the depth of cut, a lack of precision and uncertain correlation with clinical patient state remain. No study has clearly established the ability of BT evaluation to predict the risk of bleeding in patients[30] and only a study reported that BT could predict clinical bleeding in patients with acute myocardial infarction undergoing thrombolytic therapy[31]. Moreover, this test is not used routinely to monitor the effect of antiplatelet therapy[32].

Platelet aggregation on platelet-rich plasma

The Light Transmission Aggregometry (LTA), method performed on PRP and developed in the 1960s[21,33], is still considered as the gold standard test for investigating platelet functions. This analysis measures *in vitro* the platelet-to-platelet aggregation in a glycoprotein(GP) Ⅱb Ⅲa-dependent manner, the most important function of platelets. PRP and platelet poor plasma (PPP), obtained after opportune centrifugation of citrated blood samples, are used to perform LTA. The addition of an agonist to optically dense PRP, promotes platelet aggregation resulting in an increase of brightness of plasma sample. The aggregometer records the rate and extent percentage of increase in light transmission from 0% (maximal optical density of PRP) to 100% (no optical density of autologous PPP) by a photometer. Multi-channel easy to use aggregometers are available to achieve platelet aggregation tests including automatic setting of 100% (PPP) and 0% (PRP) baselines of light transmission, computer aid and storage of results and disposable stirring bar-preloaded cuvettes. Different agonists can be added to PRP sample

Table 2 Advantages and disadvantages of different platelet function methodologies

Platelet tests	Advantages	Disadvantages
Bleeding time	Physiological	Operator dependent
	In vivo test	Invasive
	Easy, quick	Poorly standardized
	No WB processing	Dependent on different variables (skin thickness, t°C)
Light transmission platelet aggregation in PRP	Historical gold standard	Pre- and analytic variables
	Flexible	Time-consuming
	Diagnostic method	High sample volume
	Different agonists available	Sample preparation
	Sensitive for anti-plt therapy	
WB Impedance Platelet Aggregometry	No sample preparation	Limited HCT and platelet count range
	Flexible	
	Diagnostic method	
	Different agonists available	
	Sensitive for anti-plt therapy	
	Close to POCT (Multiple system)	
Flow Cytometry	Small blood volumes	Expensive
	Diagnosis *ex vivo* of platelet activation	Specialized equipment
	Evaluation of efficacy of thienopyridyne therapy	Experienced operator
		Careful sample processing
		Probable, possible artifacts
Platelet Function Analyzer -PFA-100 /Innovance PFA-200	*In vitro* standardized BT	Nonflexible
	POCT	Platelet count- HCT-dependent
	Easy, quick	Not sensitivity for platelet secretion defects.
	Sensitive to severe platelet dysfunctions	
VerifyNow system	POCT	Expensive
	WB assay	Nonflexible
	Easy, quick	Monitoring antiplatelet therapy only
	No WB processing	Limited HCT and platelet count
Impact Cone and Plate(let) analyzer	WB assay	Expensive
	Global platelet function	Experienced staff
	Small sample volume	Lacking of clinical studies
		Not widely available
Plateletworks	POC WB system	Indirect assay
	Minimal sample preparation	Required platelet count method
	Easy, rapid screening test	Not so well studied
Viscoelastic methods	POCT	Measure clot properties
	Global hemostasis test Anticoagulation monitoring	Depend on: platelet function, coagulation and fibrinolysis factors
	Predicts bleeding	More studies are needed
	Reduces blood transfusions	
	Improve clinical outcome	

HCT: Hematocrit; plt: Platelet; POCT: Point-of-Care Testing; PRP: Platelet-rich-plasma; WB: Whole blood.

in order to obtain information about many different aspects of platelet function. Different parameters can be obtained from the evaluation of the aggregation trace: lag phase, shape change, primary and secondary aggregation, slope, and the maximal aggregation (%) at a fixed time.

However, despite the widespread use of LTA test, it is poorly standardized and variation between laboratory practice has been evidenced[34,35]. Because LTA is recognized to be the most important and common assay that clinical laboratories can perform to diagnose platelet function disorders, its procedure is constantly substantiated by an ongoing standardization process. Recently, specific guidelines for LTA that want to stabilize/normalize the correct procedure, have been published[36-39].

Concisely, these guidelines discuss the possible problematic pre-analytical, analytical and post-examination aspects of LTA, in order to guide toward an accepted, agreed and standardized procedure. Regarding some principal pre-analytic aspects, a complete record of medication taken by patients should be done prior the blood sampling. Blood withdrawal should be atraumatically performed with the use of 19 and 21 gauge needles. Evacuated tube systems are accepted and the anticoagulant recommended is the buffered trisodium citrate at the concentration of 109 mmoL/L (described as 3.2%). Also the Anticoagulant-Citrate-Dextrose solution (formula) A (ACD-A), that maintains the pH at 7.2 may be used. The citrated blood specimens must be gently mixed, maintained at room temperature (RT) and softly, but rapidly, transferred to the laboratory. Samples should be tested no more than 4 h from withdrawals. Regarding some principal analytic aspects, the PRP should be obtained by centrifugation at RT at 170-200 g for 10 min, whereas the autologous PPP may be prepared by centrifugation (after removal of PRP or using whole samples) at 1500 *g* for at least 15 min at RT. The adjustment of platelet

Table 3 Major platelet function tests: Clinical value

Platelet tests	Clinical value	Ref.
Light transmission platelet aggregation	Assessment of: (1) idiopathic bleeding behavior (primary hemostasis defective); (2) residual platelet reactivity of patients on antiplatelet treatment to stratify risk of ischemic events; (3) detection of VWD (RIPA test); (4) diagnostic for platelet surface glycoprotein defects.	Moffat *et al*[34] Hayward *et al*[41] Gadisseur *et al*[43] Breet *et al*[44] Buonamici *et al*[45] Paniccia *et al*[51,65] Gum *et al*[63] Rechner[107]
Whole blood platelet aggregation	Assessment of: (1) idiopathic bleeding behavior (primary hemostasis defective); (2) residual platelet reactivity of patients on antiplatelet treatment to stratify risk of ischemic events; (3) acquired bleeding risk: antiplatelet therapy, surgical coagulopathy; (4) detection of VWD (RIPA test); (5) diagnostic for HIT.	Paniccia *et al*[72] Paniccia *et al*[73] Sibbing *et al*[74] Sibbing *et al*[75] Würtz *et al*[77] Bolliger *et al*[78] Morel-Kopp *et al*[79] Ranucci *et al*[81] Görlinger *et al*[84]
PFA-100 Innovance PFA-200	Assessment of: (1) idiopathic bleeding behavior (primary hemostasis defective); (2) detection of VWD; (3) acquired bleeding risk: anti-plt therapy, surgical coagulopathy; (4) thrombotic risk also in relation to potential failure of anti-plt therapy; (5) platelet function in pregnancy, kidney or liver disease.	Hayward *et al*[25] Favaloro[94] Koessler *et al*[92] Marcucci *et al*[103] Reny *et al*[104] Crescente *et al*[105] Raman *et al*[108] Cammerer *et al*[109] Chauleur *et al*[113]
VerifyNow system	Assessment of: (1) residual platelet reactivity of patients on antiplatelet treatment to stratify risk of ischemic events; (2) low platelet reactivity of patients on antiplatelet treatment to stratify risk of bleeding events (scarce clinical data).	Breet *et al*[44] Paniccia *et al*[51,65] Tantry *et al*[116] Marcucci *et al*[119] Price *et al*[120] Angiolillo *et al*[121]

HIT: Heparin-Induced Thrombocytopenia; plt: Platelet; RIPA: Ristocetin-Induced Platelet Aggregation; VWD: Von Willebrand Disease.

count of PRP is still matter of debate. The need of adjustment of PRP with autologous PPP occurs in general for standardizing the platelet count between 200 and 300×10^9 platelets/L and in particular for lowering the platelet count for matching it with that of a thrombocytopenic patient[36]. Previous in house reference intervals (RI) for the % maximal aggregation response specific for each concentration of agonist used must be established on healthy adult volunteers (these RI can be applied to children older than neonates). LTA tracings should be studied and the final interpretative comment shall be organized by a laboratory physician. The principal agonists are commonly used at the following recommended final concentrations: ADP, 2.0-10 μmol/L; arachidonic acid, 0.5-1.64 mmol/L (usually 1.0 mmol/L); collagen, 1-5 μg/mL (typically 2 μg/mL); epinephrine, 5-10 μmol/L (typically 5.0 μmol/L); ristocetin, 0.5-0.6 mg/mL at low concentration and 1.2-1.5 mg/mL at high concentration.

To date, platelet aggregometry is still the most widely used method for identifying and diagnosing platelet function disorders or for monitoring antiplatelet therapies. Actually, this analysis is considered the first panel test to study hemorrhagic patient with inherited or acquired platelet dysfunctions[37,40-43]. When congenital/acquired bleeding disorders are suspected, apart from the most commonly agonists ADP, AA and collagen - used principally for monitoring antiplatelet therapies - other agonists should be also used: ristocetin, epinephrine, thrombin receptor activating peptide (TRAP), thromboxane A2 mimetic U46619, calcium ionophore A23187.

Monitoring antiplatelet therapies by using LTA allows to predict 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 ACS patients and in those with stable coronary artery disease[44-49].

ADP agonist is generally used to investigate congenital/acquired bleeding disorders by LTA[22]. In the presence of different platelet alteration, ADP induced platelet aggregation may result reduced (P2Y12 defects, storage pool deficiency of α and δ granules, and defects of α granules) or severely impaired (Glanzmann's thrombasthenia)[42,50]. ADP at high concentrations (*i.e.*, ⩾ 10 μmol/L) is used to monitor thienopyridines effect: ticlopidine, clopidogrel, prasugrel and ticagrelor act through the P2Y12 ADP receptor causing selective inhibition of responses to ADP[44,45,51-55]. For the classification of patients responsive or not to clopidogrel therapy, a collectively shared cut-off value of 70% for 10 μmol/L ADP-in-

duced maximal extent aggregation was found[45,51,56,57]. AA is the agonist of choice to investigate the efficacy of the ASA antiplatelet therapy[58-60]. ASA is able to inhibit platelet aggregation by irreversible inactivation of the COX-1 enzyme resulting in an inhibition of the TXA2 production[61]. The concentrations of 1 and 1.3 mmol/L AA are usually used to monitor antiplatelet therapy and the cut-off value of 20% is used to identify patients responsive or not to ASA treatment[62-67]. Platelet aggregation profile induced by collagen (1-5 μg/mL) is characterized by a lag phase before aggregation arises. Collagen binds to the GPVI and GP I a/ II a platelet receptors inducing granule release and TXA2 generation. Recently, RPR identified by collagen aggregation in ACS patients on ASA has been reported associated with cardiovascular events[67,68] and with the polymorphism C807T predisposing to MACE[69]. Collagen induced platelet aggregation can be impaired in different condition of platelet function disorders, such as: Glanzmann's thrombastenia, abnormalities of the signal-transduction pathways caused by COX-1 deficiency (aspirin like defect) or defects of platelet granules (α and/or δ storage pool deficiency)[42]. Epinephrine (5-10 μmol/L) is a weak agonist that binds to the α_2-adrenergic receptor on the surface of platelets leading to inhibition of adenylcyclase and the release of calcium ions. Platelet aggregation induced by epinephrine is similar to that obtained with ADP and characterized by an initial primary wave of aggregation, the release of stored ADP from the platelet dense bodies and second wave sustained aggregation[38]. ASA inhibits aggregation to any concentration of epinephrine[67]. Impaired response to epinephrine can be present in some congenital platelet disorders such as the Wiskott-Aldrich syndrome or the Quebec platelet syndrome. Ristocetin (1.2-1.5 mg/mL) causes platelet agglutination through the Von Willebrand Factor (VWF) and GPIb-IX-V complex. In the presence of Bernard-Soulier syndrome a severely impaired platelet agglutination induced by ristocetin is present. Moreover, LTA test performed by using different concentrations of ristocetin (0.6-1.2-1.5 mg/mL), exerts an important role to analyze possible VW Disease (VWD) and to differentiate the VWD variants[42,43].

In summary, LTA test is considered the first diagnostic step in the evaluation of platelet disorders. Since these platelet alterations are complex, in order to perform a diagnostic hypothesis, LTA results should be supported by further and more specific tests. Lumiaggregometry method for the identification of impaired platelet secretion (*i.e.*, the measurement of the platelet content of adenine nucleotide and serotonin), flow cytometry analysis or western blotting test for the identification of expression of specific platelet component and the evaluation of deficiency of α and δ granules by electron microscopy can specifically confirm the LTA results and should be performed as second diagnostic step[42].

Platelet aggregation on WB

Platelet aggregation on WB is achieved by impedance platelet aggregometry, based on the principle that activated platelets expose their surface receptors which allow them to bind to artificial surfaces[70,71]. This test measures the change in electrical resistance or impedance between two electrodes set at a fixed distance within WB sample. The platelet adhesion to electrodes and the response to classical agonists get other platelets aggregate to those stacked to the electrodes, increasing the impedance. The extent of the increase in impedance is normally recorded in Ohm. The use of WB allow to assess platelet function under more physiological conditions taking into account that also the contributions of other blood elements that may affect platelet function. In addition, another important aspect is that WB aggregometry takes place on surfaces. Platelet aggregation on WB has many advantages as well as the use of small sample volume, the immediate analysis without no sample manipulation, loss of time or possible failure of subpopulation of platelets.

Recently, a new multiple electrode aggregometry (MEA) by using a five channel computerized WB aggregometer (Multiple Platelet Function Analyzer - Dynabyte - Roche Diagnostics, Germany) equipped by disposable cuvettes ready to use with two independent sensor units and an automated pipetting has become available. The increase of impedance is detected for each sensor unit separately and calculated automatically as area under curve (AUC). By using this device with these advantages, MEA has acquired the high valence for being considered a POCT. Because MEA may use different agonists (similarly to LTA), it is suitable for diagnosis of bleeding and also for monitoring antiplatelet therapy[72-77]. Indeed, MEA has been used to investigate the presence of VWD in patient with severe aortic stenosis[78] and on the other hand the high thrombotic risk due to heparin induced thrombocytopenia (HIT)[79].

In particular, MEA, beyond the identification of cardiovascular patients at risk of MACE[72-75], is able to discriminate those patients that have a too much high inhibition of platelet function and at risk of bleeding[80]. In the same manner, Ranucci *et al*[81] reported that the use of MEA before cardiac surgery allowed to identify those patient at risk of bleeding. Different reports elucidated that MEA might be able to identify preoperatively those patients at risk of blood loss after cardiac surgery[82,83] and it is de facto entered as rapid and useful tool for the management of postoperative severe bleeding[84]. More recently, Malek *et al*[85] reported that low extent of TRAP-induced platelet aggregation by using this method was a factor independently associated with intramyocardial hemorrhage of patients with myocardial infarction.

Flow cytometry platelet analysis

Platelet analysis by using flow cytometry (FC) may offer information on the functional status *in vivo* of platelets[23]. This technique allows the evaluation of the physical and antigenic properties of platelets, *i.e.*, surface expression of receptors, bound ligands, secretion, presence of platelet aggregates and leukocyte-platelet aggregates. FC is

able to measure cell size and granularity of a large population of cells, not only the platelets and to quantify the fluorescence emitted by fluorochrome-labeled antibodies and ligands bound to the cells evaluated.

FC can be a useful tool for the diagnosis of inherited or acquired platelet dysfunctions (*i.e.*, Bernard- Soulier Syndrome or HIT, respectively). In addition, FC is able to recognize the pathological activation state of platelets (*i.e.*, in the setting of acute coronary syndromes or cardiopulmonary bypass); the efficacy of antiplatelet drugs[86] and, finally, the state of stored platelet for the evaluation of efficacy of platelet transfusion[87,88].

A panel of antibodies may be used to study in detail the membrane glycoprotein receptors of platelets. To count binding, antibodies may be directly conjugated with different fluorochromes such as fluorescein isothiocyanate (FITC) or phycoerythrin (PE). But, also a specie-specific secondary antibody coupled to a fluorochrome can be used to recognize a primary antibody linked to surface antigens[23]. For FC both PRP and WB can be used. Prior fixation of platelets with paraformaldehyde stabilizes surface antigens and consents transport of reagent components. In the WB, the use of a double labeling binding allows the identification of platelets or mixed cell aggregates[89,90]. The results of FC are represented in the form of histograms with mean fluorescence intensity (MFI) plotted against cell number.

PLATELET FUNCTION POINT-OF-CARE TESTING

The Platelet Function Analyser - PFA-100/ Innovance PFA-200

This POC method PFA-100/Innovance PFA-200 (Siemens, Munich, Germany) assesses platelet function in WB and has been considered the standardization of BT[24,25,91]. The PFA-100 and the updated system Innovance PFA-200[92] by using apposite cartridges simulates primary haemostasis under shear stress conditions. Citrated WB is drew at high shear stress rate through a defined microscopic aperture (147 μm) into a collagen-coated membrane (C) filled with either epinephrine (EPI), CEPI cartridge, or ADP, CADP cartridge. In response to shear stress and agonists platelets undergo adhesion and aggregation upon the membrane forming a platelet clot which occludes the aperture. The time taken to occlude the hole is the closure time (CT), a measure of overall platelet-related haemostasis and this interval will be prolonged depending on the platelet activity. The use of two different cartridges with distinct agonists allows to distinguish the platelet function alterations due to intrinsic defects (principally by using CADP cartridge) or to antiplatelet therapy with ASA (CEPI cartridge)[93-97], whereas the new Innovance cartridge is affected by thienopyridine therapy[98]. In comparison to BT test, this method is revealed more sensitive[27,46] especially for diagnosis of VWD and platelet function defects[25].

The PFA-100 is sensitive to many variables that influence platelet function as well as low platelet count and haematocrit. Thus, to exclude thrombocytopenia or anemia, a WB count should always be performed prior test. In addition, it has been demonstrated that different determinants such as high levels of VWF, fibrinogen or erythrocytes tend to shorten CEPI CT[99,100]. Moreover, the PFA CT by CEPI cartridge could reveal high residual platelet reactivity despite aspirin therapy, and consequently predict the risk of ischemic events[101-103]. In ACS patients on ASA treatment, a high concordance between LTA and PFA-100 CEPI test results and a significant negative predictive value for the PFA system have been reported[65]. In addition, PFA CEPI shortened CT was demonstrated to be significant and independent predictor of MACEs in patients with AMI undergoing primary PCI[103-105].

Assessment of platelet dysfunction with PFA-100 in different clinical setting or in patients undergoing different kinds of elective surgeries, may provide useful information for postoperative blood transfusion management[106,107]. Especially in cardiac surgery PFA methodology showed a high predictive value of platelet function for management of intra- and postoperative blood loss[108-111]. In patients with biventricular assist device implantation on treatment with clopidogrel, the strict monitoring of impaired platelet function with this method (by using CADP cartridge) allowed them to go under successful transplantation with no major blood loss[112]. Prolonged CTs by CADP assay were found be independent risk factors for post-partum hemorrhage (PPH) severity[113] and prolonged CTs by CADP cartridge have been consistently described to be correlated in women with menorrhagia[114]. The pre-surgical correction of the prolonged PFA-100 CT with DDAVP treatment, allowed to maintain the number of postoperative blood transfusions not significantly different from that of patients with normal presurgical PFA CT[106].

It has been suggested that PFA system could be used as a screening tool that could be integrated into a panel of existing tests[38,42]. In particular, it is reported that this test presents a high negative predictive value[63,96]: so, in the presence of normal CT in a suspected platelet defect, further detailed analysis, *e.g.*, platelet aggregation, might be eliminated from the investigation[5].

VerifyNow system

The VerifyNow system (ITC, Edison, NJ, United States) is a POC turbidimetric-based optical detection device that measures platelet aggregation in a system cartridge containing fibrinogen-coated beads and specific agonist[115]. The instrument measures changes in light transmission and thus records the rate of aggregation in WB. This methodology originally was developed for monitoring antiplatelet therapy with to GP Ⅱb/Ⅲa antagonists. Now, the system provides other 2 different assays each sensitive to targeted drugs: Aspirin Assay with AA as agonist (sensitive to ASA) and P2Y12 Assay with ADP as agonist and PGE_1 as suppressor of intracellular free calcium lev-

els to reduce the non-specific contribution of the ADP-binding to P2Y1 receptors (sensitive to thienopyridines). The VerifyNow system allows a rapid assessment of the platelet function also without the requirement of a specialized laboratory. Since the VerifyNow is a cartridge-based WB assay, it is not necessary to perform tests with any blood manipulation and instrument handling. Actually, this methodology is so waived that it is largely used to monitor antiplatelet therapies[116].

For Aspirin Assay, results are expressed as Aspirin Reaction Units (ARU) and for the identification of responsiveness to ASA treatment a specific cut-off value of 550 ARU is recommended by manufacturers[117]. In stroke patients on low dose of ASA[96] and in coronary artery disease patients on dual antiplatelet therapy[65,46,118] a moderate agreement between VerifyNow system and LTA results was observed. For the VerifyNow P2Y12 assay results are expressed as P2Y12 Reaction Units (PRU). Different laboratory and clinical studies relative to patient on different thienopyridines have tried to choose a cut-off value for discriminating patients not responsive to drug[44,51,119-122].

The potential role of this system for prediction of postoperative bleeding in surgical practice remains placed for the evaluation of the extent of inhibition of platelet function in response to antiplatelet medication[123]. Actually, by using this system antiplatelet therapy for outpatients or patients immediately after surgery could be tailored to the individual depending on the results.

Plateletworks

Plateletworks system is a POC assay based on platelet aggregation on WB. This system consists of the Plateletworks aggregation kits and the Ichor blood counter (Helena Laboratories, Beaumont, TX, United States). The Plateletworks procedure compares the platelet count measured in the control sample (EDTA tube) with those obtained after aggregation in citrate blood with either collagen, ADP or AA (citrate tube plus agonist). Platelet aggregation is measured as the decrease of platelet count. Results are available in minutes and without any manipulation of blood sample[124]. This method has showed a relationship with LTA, VerifyNow system and Thromboelastography[125,126] and may be used to monitor antiplatelet therapy[127]. Plateletworks gives information about both platelet count and function within an acute care situation. However, it is still under consideration and has not reported to predict clinical outcomes.

IMPACT Cone And Plate(Let) Analyzer

IMPACT (Image Analysis Monitoring Platelet Adhesion Cone and Plate Technology) Cone and Plate(let) Analyzer (CPA) (DiaMed, Cressier, Switzerland) is a new POC completely automated system that evaluates platelet function simulating *in vitro* primary haemostasis[4,27,28,128]. Citrated WB is exposed to shear stress by the spinning of a cone in a standardized polystyrene plate. 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 by image analysis software.

This system is highly dependent on plasma VWF, fibrinogen binding the platelet glycoproteins GPIb and GP IIb/IIIa to the plastic surface. Therefore, this instrument methodology should be a reliable device for the diagnosis of platelet defects. Moreover, the addition of the agonists AA and ADP in the system allows to monitor dual antiplatelet therapy[128-130]. This system still needs an experienced use and additional studies must be conducted for assessing its possible role for monitoring inherited or acquired platelet dysfunctions.

Viscoelastic methods

These methods are global tests for the assessment of haemostatic process, based on the measurements of changes in viscoelastic forces in WB. These analyses are able to assess the extent of platelet count and function, clotting and fibrinolytic activation[131,132]. To date, three principal systems are available: Thromboelastography, performed on "old" renewed devices (TEG, Haemoscope, Niles, IL, United States), 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, Boulder, CO, United States). All these systems providing a graphic representation of clot formation and lysis, are now used as a bedside monitor in different clinical setting such as cardiac surgery, liver transplantation and trauma center[133,134]. For TEG and ROTEM, in a rotating system consisting of a pin suspended by a torsion wire in a cup the WB clot entraps the pin promoting a motion that increases as the clot strengthens and decreases when the clot lyses. In Sonoclot device in the cup the pin is moved up and down at ultrasonic rate.

Different studies[135-142] reported these systems to be predictive of risk of increased postoperative bleeding. Other reports have stated the use of different parameters provided by these tests are predictors of both postoperative bleeding and blood product use[143-145].

Thrombelastograph Platelet Mapping System has been developed to monitor antiplatelet therapy[146-149]. A weak clot is formed by the addition of reptilase and factor XIII, by adding AA or ADP the clot strength is increased allowing this assay to be sensitive to dual antiplatelet therapy.

However, further large prospective studies should be performed in order to define the possible role of these devices in monitoring antiplatelet therapy.

FUTURE PERSPECTIVES

As reported in this review, several *in vitro* tests for the assessment of platelet (dys)function in order to screen different idiopathic or acquired pathological conditions

-hemorrhagic and/or prothrombotic status - have been developed. Now, platelet testing is mostly used thanks also to the recent and constant standardization effort. These available tests allow to study global platelet function including the different steps of platelet activation. For example, the POC platelet tests simultaneously evaluate in vitro platelet adhesion and aggregation; platelet aggregometry in PRP and in WB (by using the new Multiplate system) is a comprehensive examination of platelet secretion and aggregation phenomena, also considering the role of other blood cells (platelet aggregation in WB); viscoelastic methods analyze the global hemostasis with the regard of clot retraction (Tables 2 and 3).

To date, platelet function tests are available to address the different phases of platelet activation. Platelet assays, evaluating platelet adhesion under static or flow conditions and platelet spreading have been developed[107,150]. Platelet adhesion tests in static condition, using a large number of different surfaces - glass beads, cultured vascular cells, purified matrix proteins or complete subendothelial extracellular matrix from cultured endothelial cells - that goes to the detriment of univocal results and standardized procedure, might be achieved[151]. Under flow conditions, platelets adhesion is affected by rheological conditions such as shear rate, presence of red blood cells, red blood cell deformability, and viscosity of the medium. In this multitude of conditions, platelet adhesion can be evaluated by using microfluidic devices for example biochip containing several different adhesion molecules[152,153]. Platelet spreading tests, using fluorescence microscopy or scanning electron microscopy are frequently employed[154]. Platelet secretion may be evaluated measuring the concentration of several compound released - nucleotides (ATP, ADP), serotonin (5-HT), Platelet Factor 4 (PF4), beta-thromboglobulin , thrombospondin-1) by using different methodologies such as: ELISA, HPLC, fluorescence microscopy or flow cytometry[42,155,156]. The assessment of these distinct steps - platelet adhesion, secretion and interactions with circulating cells - might be helpful to better define pathological conditions related to different platelet dysfunction. However, most of these assays, prevalently aimed for research studies show different clinical impact and methodological challenges. Main limitations of the application of these assays in clinical practice are the scarcity of clinical and laboratory data, often divergent each other, and the lack of clear indications or guidelines for a correct use of such tests. In the future, specific, standardized, more rapid and easy tests - whose clinical value has been well defined - for the study of single steps of platelet function or for the definition of clinical value of new platelet biomarkers by using new tests showing high sensitivity and specificity, are desirable for routinely laboratory analysis.

New potential biomarkers of platelet activation

Recent studies have shown that the interaction of activated platelets with CD34+ cells might potentially contribute in the differentiation of CD34+ cells to endothelial progenitor cells (EPCs)[157] and mature endothelial cells (EC)[158]. The identification of cellular mediators, tissue specific chemokines, factors and molecular determinants involved in this interactions could be useful to identify new strategies for the vascular repair and tissue regeneration in ischemic organs[159,160].

In this contest, the chemokine CXCL12 (stromal cell-derived factor-1α, SDF-1α), principally produced by platelet and stored in α granules, but also released from endothelial cells, is directly involved[161]. The principal role of CXCL12 is related to the platelet activation accompanied with P-selectin expression and release of different platelet chemokines[162,163]. In the site of vascular injury, CXCL12 stimulates the differentiation of CD34+ cells into EPCs and ECs, so exerting an important role in neointima formation[157,162]. Actually, the measurement of CXCL12 and/or the rapid identification of platelet-CD34+ cell complexes in the future might be used, on hand, as assessment of a predictive biomarker of ischemic events in combination with other vascular parameters and, other hand, to early detect CD34+ cells as biomarkers for cardiovascular diseases or for tissue renewal and/or repair.

CONCLUSION

New guidelines for platelet function testing have been written in the 2011[38] and, recently, new procedures for improving the ongoing standardization of LTA have been reported in the 2013[39]. From the late 1980's to nowadays, the study effort, in the field of application of platelet function methods as diagnostic tool for evaluating bleeding disorders and monitoring the efficacy of antiplatelet therapies, is at this time again in progress. However, the increasing number of new POC methods for the assessment of platelet function is making possible the introduction of these tests into the routine laboratory and opening the door for the their application in different clinical settings such as inherited bleeding disorders, cardiovascular intensive care, trauma coagulopathy, liver transplantation and obstetric care for the prediction of bleeding.

To date, the improvement of reliable, advanced and innovative, but simple to use WB methodologies, that simulate primary hemostasis, is allowing to screen rapidly patients and to guide the clinicians for an appropriate diagnosis of bleeding risk or for tailoring correctly the antiplatelet therapy. Surely, the general consensus is that the *in vivo* BT should be replaced. On the other hand, the use of platelet aggregometry in PRP of WB at the light of new instruments should be implemented into routine laboratories. Similarly, some POC platelet function tests could also be, actually, used as instruments for evaluating bleeding risk, thrombotic risk and monitoring antiplatelet therapy not only at the bedside, but also in centralized or in satellite laboratories. Conversely, platelet function testing is become increasingly used in critical area outside of the specialized laboratory. Although the presence of

these new methodologies represents an important improvement, a validation procedure, the study of reliability and quality control testing of these point of care tests is becoming an increasingly important issue[38].

In conclusion, old and new platelet function tests are now available. Many tests are beginning to prove to be useful supplements to the existing set of platelet function tests, but large prospective well designed clinical trials are necessary for defining the true applications of these tests. In the future, the developments in platelet genome and proteome may lead advances in the field of platelet function testing which may have a significant impact upon the diagnosis and management of patient affected by hemorrhagic or thrombotic defects.

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