The goal of platelet function tests used in cardiac catheterization laboratories and intensive cardiac units is to guide antiplatelet therapy to the optimal dose for the prevention or treatment of thrombosis, minimizing hemorrhagic side effects in acute coronary syndrome (ACS) patients undergoing percutaneous coronary intervention with stenting. Platelet function is traditionally assessed by light transmission aggregation (LTA) test in platelet-rich plasma (PRP). By using different agonists to induce aggregation, the extent of platelet inhibition can be monitored. As platelet LTA presents some drawbacks (manual sample preparation, selection of agonists and their concentration, time-consuming), new and renewed automated systems have been introduced to provide a simple, rapid assessment of platelet function, including point-of-care (POC) methods that are also suitable for use in non-specialized laboratories.

Conventional Platelet Function Tests

Bleeding Time

BT, the oldest test for measuring platelet function, evaluates the capacity of platelets to form a hemostatic plug. The test measures the time that the platelets employ to occlude a standardized skin wound by evaluating the ability of platelets to stop the bleeding. It is useful as a screening test to identify either congenital or acquired platelet disorders. Advantages are that it is quick and easy to perform without any whole-blood processing; drawbacks are that it is affected by a skilled operator, skin thickness, and temperature. Even if there was a device to standardize the size and the depth of the cut, a lack of precision and uncertain correlation with clinically significant factors remains. Therefore, this method is not routinely used to monitor the effect of antiplatelet therapy.

Despite this, a small study reported that BT could predict clinical bleeding in patients with acute myocardial infarction undergoing thrombolytic therapy. In addition, Jakubwosky et al. employed BT in trials of the novel thienopyridine prasugrel.
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Table 1: Platelet Function Tests in Cardiovascular Disease

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<th>Tests for Rapid Screening of Platelet Function—Suitable for Monitoring Effect of Antiplatelet Therapy</th>
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| **Platelet–Platelet Aggregation Studies** | **Principle of Method** |
| Light transmission platelet aggregation | Low shear platelet-to-platelet aggregation in response to agonists in platelet-rich plasma |
| Impedance platelet aggregation | Low shear platelet-to-platelet aggregation in response to agonists in whole blood |
| VerifyNow system | Fibrinogen–platelet agglutination in response to agonist in whole blood |
| Ichor-Plateletworks | Platelet counting pre- and post-activation in whole blood |
| VASP phosphorylation state (flow cytometry) | Monitoring of activation markers directly dependent on clopidogrel target P2Y12 |

| **Analysis of Clot Formation** | **Principle of Method** |
| Thromboelatography/thromboelastometry | Monitoring of rate and quality of clot formation in whole blood |
| Expression of platelet-specific surface makers* | Monitoring of CD41/61, CD42, CD62P by flow cytometry |
| Soluble markers* | Beta thromboglobulin, PF4, GP V soluble P-selectin, thromboxane(s) by radio- or enzyme-linked immune assays (plasma or urine) |

*Platelet function tests to investigate platelet activation that are not suitable for a rapid screening of antiplatelet therapy (not proposed in this report).

Platelet Aggregation

**Platelet Aggregation on Platelet-rich Plasma**

The current *de facto* ‘gold standard’ test of platelet function is the LTA method. The principal benefit of this method is that it measures platelet-to-platelet aggregation in a glycoprotein IIb/IIIa (GPIIb/IIIa)-dependent manner, the most important function of platelets. LTA, developed in 1962, is still the most extensively used test for investigating platelet function. The addition of different agonists to PRP allows information about many different aspects of platelet function to be obtained. Upon addition of the chosen agonist to optically dense PRP, platelets aggregate, resulting in an increase of brightness of the plasma sample. The aggregometer records the rate and percentage increase in light transmission from 0% (maximal optical density of PRP) to 100% (no optical density of autologous platelet-poor plasma) by a photometer. Recently, aggregometers have become easier to use with multichannel capability, simple automatic setting of 100 and 0% baselines, computer aid, and storage of results. The aggregating agents most commonly used to investigate platelet function in ACS patients on dual antiplatelet therapy are ADP, AA, collagen, and EPI.

ADP is used to monitor the effect of thienopyridines: ticlopidine and clopidogrel act through the P2Y12 ADP receptor, causing selective inhibition of responses to ADP. The choice of ADP concentration and what result is to be reported—i.e. maximal or late aggregation—also play a part. Some authors use both 2 and 10µM of ADP, whereas others use 5 and 20µM. There is not a great difference between ADP 10 and 20µM because these two maximal concentrations of ADP produce similar curves. However, the collectively shared cut-off value is 70% for ADP 10 and 20µM maximal extent aggregation to classify patients on clopidogrel with or without RPR.

Arachidonic acid used as an agonist induces platelet aggregation because it is converted to thromboxane (TXA2). As the ASA-blocking platelet cyclooxygenase 1 (COX-1), which converts arachidonic acid to TxA2, is able to inhibit platelet aggregation, it is the agonist of choice to investigate ‘ASA resistance’. In the literature, concentrations of 1 and 1.3µM AA are usually used to monitor antiplatelet therapy, and the cut-off value of 20% is used to identify patients on ASA with or without RPR.

Other agonists used to monitor inhibition of platelet function by ASA are collagen and EPI. Collagen (1–5µg/ml) produces a characteristically prolonged lag phase before aggregation occurs. ASA and other drugs that block TxA2 formation are able to inhibit platelet aggregation in response to low levels of collagen. Recently, RPR identified by collagen aggregation in ACS patients on ASA has been reported to be associated with cardiovascular events, as well as the polymorphism C807T predisposing to major adverse cardiovascular events (MACE). EPI (5–10µM) is a weak agonist that aggregates platelets in PRP without a lag phase. ASA inhibits aggregation to any concentration of EPI.

Various studies have reported that platelet aggregation testing in PRP is able to predict MACE. The occurrence of RPR defined by AA and ADP-induced LTA has been associated with the development of ischemic events both in ACS patients and in those with stable coronary artery disease. Initially, Gum et al. demonstrated a 5% prevalence of aspirin resistance by using AA and ADP-induced LTA in patients with stable coronary artery disease. Additional studies reported the occurrence of ‘aspirin and clopidogrel resistance’ revealed by both AA- and ADP-induced LTA, which is related to selected patients on dual antiplatelet therapy. Gori et al. demonstrated that patients who exhibited a significantly higher extent of AA platelet aggregation also had higher levels of ADP and collagen-induced LTA. These data suggest that a generalized persistent platelet reactivity phenotype exists that might enhance thrombotic risk.

**Platelet Aggregation on Whole Blood**

Platelet aggregation on whole blood is performed by impedance platelet aggregometry (IPA) based on the principle that activated
platelets expose their surface receptors, which allows them to attach to artificial surfaces.46,47 IPA measures the change in electrical resistance or impedance between two electrodes set at a fixed distance into a blood sample. As the current passes, platelets adhere to electrodes and, in response to classic agonists, other platelets aggregate around those attached to the electrodes, increasing the electrical impedance. The extent of the increase in impedance is recorded in Ohm. The use of whole blood allows platelet function to be assessed under more physiological conditions that also take into account the contributions of other blood elements that may affect platelet function. Another important difference compared with LTA is that IPA takes place on surfaces. In LTA, platelets aggregate with each other in the liquid phase, which presumably happens only in severely ill patients (i.e. heparin-induced thrombocytopenia type II [HIT II] and disseminated intravascular coagulation [DIC]), whereas physiological coagulation and platelet aggregation in vivo usually take place only on surfaces (vascular injuries, inflamed vessels, atheromatous plaques).

IPA has many advantages, including small sample volume and immediate analysis with no sample manipulation, loss of time, or possible failure of subpopulation. Until now, the aggregometers for IPA (Chronolog device, US) utilized reusable electrode units, so after each test the electrodes had to be carefully rinsed and wiped. The need to clean and ensure the integrity of electrodes made IPA difficult to use in clinical practice. Now, a five-channel computerized IPA device (Multiple Platelet Function Analyzer, Multiplate-Dynabyte, Germany) has become available that has disposable ready-to-use cuvettes with two independent sensor units. The increase in impedance is detected in each sensor unit and calculated as area recorded in Ohm. The use of whole blood allows platelet function to account the contributions of other blood elements that may affect platelet function alterations due to intrinsic defects (CADP) and those due to therapy with antiplatelet drugs (CEPI). Even if this method is less specific for ASA monitoring than other assays, PFA-100 has been widely used in clinical studies for identification of ASA responsiveness in cardiovascular patients.45 Several studies have reported a high prevalence of ASA-resistant patients.44,45 This assay provides a global assessment of non-vascular primary hemostasis, and therefore has the advantage of taking different mechanisms for platelet activation into account. It has been demonstrated that different determinants, such as high levels of von Willebrand factor (vWF), fibrinogen, or erythrocytes, tend to shorten CEPI CT.46,47 So, the PFA-100™ CT could potentially serve to detect high RPR despite aspirin therapy, and thereby predict the risk for ischemic events.48 In a comparison study performed in ACS patients, Paniccia et al.49 reported a high concordance between LTA and PFA-100 CEPI test results, identifying the same patients with or without RPR. In addition, Marcucci et al.49 demonstrated that RPR revealed by CEPI test is a significant and independent predictor of MACEs in patients with AMI undergoing primary PCI. In a recent review, Remy et al.50 reported that in cardiovascular patients on ASA RPR measured with CEPI, CT is associated with recurrent ischemic events (with an odds ratio [OR] of 2.1). In addition, in a systematic review of 53 studies Crescente et al.51 reported a similar result—notwithstanding the heterogeneity of the studies analyzed—but concluded that better standardization and control of methodological variables for this test might reinforce the observed association with clinical vascular events.

VerifyNow System

The VerifyNow system (Accumetrics, US) is a POC turbidimetric-based optical detection device that measures platelet aggregation in a system cartridge containing fibrinogen-coated beads and a specific

**Data suggest that a generalized persistent platelet reactivity phenotype exists that might enhance thrombotic risk.**

**New Choices for Platelet Function Tests**

**Platelet Function Analyzer-100**

The Platelet Function Analyzer-100 (PFA-100) (Dade Behring, US) assesses platelet function in whole blood, simulating primary hemostasis under shear stress conditions by using proper cartridges.48,49 Blood is aspirated at a high shear stress rate through a defined aperture cut into a collagen-coated membrane (C) filled with either EPI (CEPI cartridge) or ADP (CADP cartridge). Platelets undergo adhesion and aggregation in response to shear stress and agonists in the membrane forming a platelet plug that occludes the aperture. The time taken to occlude the hole is the closure time (CT), a measure of overall platelet-related hemostasis, and this interval will be elongated depending on platelet activity. CEPI CT is sensitive to ASA therapy, while the CADP CT is unaffected by ASA and clopidogrel.49,50 The use of two different cartridges with distinct agonists allows us to distinguish between platelet function alterations due to intrinsic defects (CADP) and those due to therapy with antiplatelet drugs (CEPI). Even if this method is less specific for ASA monitoring than other assays, PFA-100 has been widely used in clinical studies for identification of ASA responsiveness in cardiovascular patients.45 Several studies have reported a high prevalence of ASA-resistant patients.44,45 This assay provides a global assessment of non-vascular primary hemostasis, and therefore has the advantage of taking different mechanisms for platelet activation into account. It has been demonstrated that different determinants, such as high levels of von Willebrand factor (vWF), fibrinogen, or erythrocytes, tend to shorten CEPI CT.46,47 So, the PFA-100™ CT could potentially serve to detect high RPR despite aspirin therapy, and thereby predict the risk for ischemic events.48 In a comparison study performed in ACS patients, Paniccia et al.49 reported a high concordance between LTA and PFA-100 CEPI test results, identifying the same patients with or without RPR. In addition, Marcucci et al.49 demonstrated that RPR revealed by CEPI test is a significant and independent predictor of MACEs in patients with AMI undergoing primary PCI. In a recent review, Remy et al.50 reported that in cardiovascular patients on ASA RPR measured with CEPI, CT is associated with recurrent ischemic events (with an odds ratio [OR] of 2.1). In addition, in a systematic review of 53 studies Crescente et al.51 reported a similar result—notwithstanding the heterogeneity of the studies analyzed—but concluded that better standardization and control of methodological variables for this test might reinforce the observed association with clinical vascular events.

**VerifyNow System**

The VerifyNow system (Accumetrics, US) is a POC turbidimetric-based optical detection device that measures platelet aggregation in a system cartridge containing fibrinogen-coated beads and a specific agonist.51 The instrument measures changes in light transmission, and thus the rate of aggregation in whole blood. The VerifyNow system allows the rapid assessment of RPR without the requirement of a specialized laboratory because no laboratory instrument handling or blood manipulation is required. This methodology is specific to the monitoring of antiplatelet therapy, comprising three different assays each sensitive to targeted drugs: lib/Ila assay with thrombin receptor
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agonist peptide (TRAP) as agonist (sensitive to GPIIb/IIIa antagonists); aspirin assay with AA as agonist (sensitive to aspirin); and P2Y12 assay with ADP as agonist (sensitive to thienopyridines) and prostaglandin E1 (PGE1) as a suppressor of intracellular free calcium levels to reduce the non-specific contribution of the ADP-binding P2Y1 receptors.

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 550ARU is recommended by manufacturers. This value was chosen by Malinin et al., who reported a significant correlation between this method and EPI-induced LTA. In addition, various studies compared this method with AA-LTA and PFA-100 CEP I CT. In stroke patients on low doses of ASA, and coronary artery disease patients on dual antiplatelet therapy, a moderate agreement was observed. Recent small comparisons with ADP-induced LTA have been performed, so this method may be suitable to monitor and, in time, to change ASA therapy.

The advantages of this method are that it is dependent on the P2Y12 receptor target of clopidogrel and uses low whole blood samples; drawbacks are that it is expensive and requires sample preparation, skilled technicians and the availability of a flow cytometer. Aleil et al. demonstrated that the VASP assay finds a higher inhibition by thienopyridine compared with LTA, probably because LTA still takes place via ADP stimulation of P2Y1 in the presence of thienopyridine. Adjustment of the clopidogrel loading dose according to this method may improve the clinical outcome after PCI in patients who are non-responsive to clopidogrel.

Plateletworks
Plateletworks (Helena Laboratories, US) is a POC whole-blood test constituting an aggregation kit and an Ichor blood counter. The aggregation test is based on measuring the platelet count before and after inducing platelet aggregation, using as agonists collagen, ADP, or AA, consisting of a baseline tube and an agonist reagent tube (citrate tube plus agonist). Platelet aggregation is measured as the loss of single platelets. Results are available in minutes and without any manipulation of a blood sample. This assay correlates with LTA, IAPA, TEG, and VerifyNow® and may be used to monitor antplatelet therapy. However, it is still under consideration and has not reported clinical outcomes.

IMPACT Cone and Plate Technology Cone and Plate(let) Analyzer
Image Analysis Monitoring Platelet Adhesion Cone and Plate Technology (IMPACT) Cone and Plate(let) Analyzer (CPA) (Diamed, Switzerland) is a new POC fully automated system that measures platelet adhesion and aggregation under high shear stress conditions simulating in vitro primary hemostasis. Whole blood 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 μm²) are measured by image analysis software. The addition of AA and ADP ex vivo allows us to evaluate dual antiplatelet therapy. This system still needs an experienced user, and additional studies must be performed to establish its possible role in monitoring antiplatelet therapy.

Conclusions
Many platelet function tests are now available for clinical use, including monitoring antiplatelet therapy, and some of these tests have been shown to be associated with clinical outcomes in patients on dual antiplatelet therapy. In ACS patients, who present a high variability of the response, the ultimate goal of these tests is to guide antiplatelet therapy to the optimal dosage for the prevention or treatment of thrombosis by minimizing side effects. The development
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of rapid but simple-to-use whole-blood tests provides the opportunity of screening critical for ACS patients. Therefore, platelet function testing is becoming increasingly utilized. As this represents an important advance, the reliability and the quality control testing of these tests is an important issue.

In the future, the developments in platelet genome and proteome may lead to advances in the field of platelet-specific microarrays, which may have a significant impact on the diagnosis and management of patients affected by hemostatic or thrombotic defects.