



REVIEW ARTICLE

Measuring antiplatelet drug effects in the laboratory

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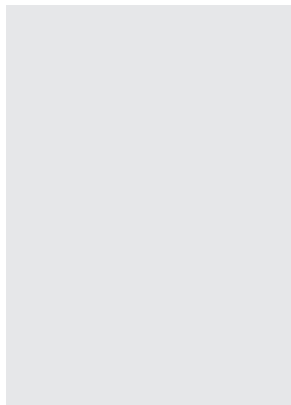
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Abstract This review discusses the advantages and disadvantages of currently available tests for the monitoring of antiplatelet therapy (especially aspirin and clopidogrel). Many tests of platelet function are now available for clinical use, and some of these tests have been shown to predict clinical outcomes after antiplatelet therapy. However, in most of these studies, the number of major adverse clinical events was low. No published studies address the clinical effectiveness of altering therapy based on the results of monitoring antiplatelet therapy. Therefore, the correct treatment, if any, of "resistance" to antiplatelet therapy is unknown and, other than in research trials, monitoring of antiplatelet therapy in patients is not generally recommended. A clinically meaningful definition of "resistance" to antiplatelet drugs needs to be developed, based on data linking drug-dependent laboratory tests to clinical outcomes in patients.
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Introduction

Most platelet function tests have been traditionally utilized for the diagnosis and management of patients presenting with bleeding problems rather than thrombosis [1]. However, as platelets are now implicated in the development of atherothrombosis, which is the leading cause of mortality in the Western world, [2,3] new and existing platelet function tests are increasingly being used for monitoring the efficacy of the antiplatelet drugs used to treat these conditions. This, coupled with the development of new, simpler tests and point-of-care (POC) instruments, has resulted in the increasing tendency of platelet function testing to be performed away from specialized hemostasis clinical or research laboratories, where the more traditional and complex tests are still performed [4,5]. Table 1 is a summary of the currently available tests for the monitoring of antiplatelet therapy, including their advantages and disadvantages.

History of platelet function testing and overview of currently available tests

Platelets were discovered in the 1880's [6]. Platelet function testing began with the application of the *in vivo* bleeding time by Duke in 1910 [7]. The bleeding time was still regarded as the most useful screening test of platelet function until the early 1990's [1,8,9]. Recently, the widespread use of the bleeding time has rapidly declined because its limitations have been recognized (see below) and other, less invasive, screening tests have become available [10–12].

Light transmission aggregometry (LTA) was invented in the 1960's and soon revolutionized the diagnosis of primary hemostatic defects [13,14]. LTA is still regarded as the gold standard of platelet function testing and by adding a panel of agonists to stirred platelets it is possible to obtain a large amount of information about many different aspects of platelet function [15]. This test, often now coupled with the measurement of stored and releasable platelet nucleotide content, is still utilized in most laboratories for the diagnosis of many platelet defects [16]. Recently, commercial aggregometers have become easier to use with multi-channel capability, simple automatic setting of 100% and 0% baselines, and computer operation and storage of results. For example, a new fully computerized 8-channel aggregometer has just

become available (Fig. 1). Some instruments can simultaneously measure luminescence, to monitor the release reaction of dense granular nucleotides during secondary aggregation. LTA is relatively non-physiological, as separated platelets are stirred under low shear conditions and only form aggregates after addition of agonists, conditions which do not accurately mimic platelet adhesion, activation and aggregation upon vessel wall damage. Also, conventional LTA using a full panel of agonists requires both large blood volumes and a significant expertise to perform the tests and interpret the tracings. In response to the problems with the bleeding time and LTA, a number of alternative tests have been developed, including impedance whole blood aggregometry (WBA), a fully automated cartridge-based instrument (VerifyNow®) that measures platelet LTA in anticoagulated whole blood, and a variety of tests that attempt to simulate primary hemostasis *in vitro* (Table 1).

WBA provides a means to study platelet function in anticoagulated whole blood [17]. The test measures the change in resistance or impedance between two electrodes as platelets adhere and aggregate in response to classical agonists. A new fully computerized two or four channel instrument has now become available (Fig. 2). Although the latter instrument can also be used for LTA of platelet-rich plasma (PRP), WBA has many significant advantages including the use of smaller sample volumes and the immediate analysis of samples without manipulation, loss of time or potential loss of platelet subpopulations or platelet activation during centrifugation. A new five channel computerized WBA instrument (Multiple Platelet Function Analyzer or Multiplate®) now has disposable cuvettes/electrodes with a range of different agonists for both diagnosis and monitoring antiplatelet therapy.

The VerifyNow® (formerly known as the Ultegra Rapid Platelet Function Analyzer [RPFA]) instrument (Fig. 3) is a fully automated POC test that was originally developed to monitor glycoprotein (GP) IIb–IIIa antagonists within a self-contained cartridge (containing a platelet activator and fibrinogen-coated beads) [18–20]. Blood sample tubes are then simply mixed prior to insertion onto the cartridge that has been pre-mounted onto the instrument. Aggregation in response to the agonist is monitored by light transmission through two duplicate reaction chambers in each cartridge. Other specialized cartridges are now available for measuring platelet

Table 1 An alphabetical list of currently available tests for the monitoring of antiplatelet therapy

| Name of test | Principle | Advantages | Disadvantages | Frequency of use |
|---|--|---|---|--|
| AspirinWorks [®] | Immunoassay of urinary 11-dehydrothromboxane B ₂ | Measures stable thromboxane metabolite Dependent upon COX-1 activity | Indirect assay Not platelet-specific Renal function-dependent | Increasing use |
| Bleeding time | <i>In vivo</i> cessation of blood flow | <i>In vivo</i> test Physiological POC | Insensitive Invasive Scarring High CV | Decreasing popularity |
| Flow Cytometry | Measurement of platelet glycoproteins and activation markers by fluorescence (e.g. VASP phosphorylation to monitor P2Y ₁₂ inhibition) | Whole blood test Small blood volumes Wide variety of tests | Specialized operator Expensive | Widely used |
| HemoStatus [®] Device | Platelet procoagulant activity | Simple POC | Insensitive to aspirin and GPIb function | Used in surgery and cardiology |
| Ichor–Plateletworks [®] | Platelet counting pre- and post-activation | Rapid Simple POC Small blood volume | Indirect test measuring count after aggregation | Used in surgery and cardiology |
| Impact [®] cone and plate(let) analyzer | Quantification of high shear platelet adhesion/aggregation onto surface | Small blood volume required High shear Rapid Simple Research (variable) and fixed versions available POC | Instrument not yet widely available. | Little widespread experience as only recently commercially available |
| Light Transmission Aggregometry (LTA) | Low shear platelet-to-platelet aggregation in response to classical agonists | Historical gold standard | Time consuming Sample preparation Expensive | Widely used in specialized labs |
| PFA-100 [®] | High shear platelet adhesion and aggregation during formation of a platelet plug | Whole blood test High shear Small blood volumes Simple Rapid POC | Inflexible VWF-dependent Hct-dependent Insensitive to clopidogrel | Widely used |
| Platelet Reactivity Index | Measurement of platelet aggregates in whole blood (modified Wu and Hoak method) | Simple Rapid Inexpensive | Requires blood counter Indirect test measuring count after aggregation | Little widespread experience |
| Serum Thromboxane B ₂ | Immunoassay | Dependent upon COX-1 activity | Prone to artefact Not platelet-specific | Widespread use |
| Thromboelastography (TEG [®] or ROTEM [®]) | Monitoring of rate and quality of clot formation | Global whole blood test POC | Measures clot properties only; largely platelet-independent unless platelet activators are used | Used in surgery and anesthesiology |
| VerifyNow [®] | Fully automated platelet aggregometer to measure antiplatelet therapy | Simple POC 3 test cartridges (aspirin, P2Y ₁₂ and GPIIb–IIIa) | Inflexible Cartridges can only be used for single purpose | Increasing use |
| Whole Blood Aggregometry | Monitors changes in impedance in response to classical agonists | Whole blood test | Older instruments require electrodes to be cleaned and recycled | Widely used in specialized labs although less than LTA |

Abbreviations: COX-1, cyclooxygenase 1; CV, coefficient of variation; GP, glycoprotein; Hct, hematocrit; LTA, light transmission aggregometry; PFA-100, platelet function analyzer 100; POC, point-of-care; VASP, vasodilator-stimulated phosphoprotein; VWF, von Willebrand factor.

responses to either aspirin (VerifyNow® Aspirin) or clopidogrel and other P2Y₁₂ antagonists (VerifyNow® P2Y12). This instrument is a considerable advance, as the test is a fully automated POC test without the requirements of sample transport, time delays or a specialized laboratory, and it can provide immediate information.

It is also possible to monitor platelet aggregometry in whole blood by a simple platelet counting technique. After addition of an agonist to anticoagulated, stirred whole blood, platelets aggregate and the platelet count decreases when compared to a control tube [21–23]. The Plateletworks® aggregation kits (Helena Biosciences) are simply based upon comparing platelet counts within a control EDTA tube and after aggregation with either ADP or collagen within citrated tubes [24–27].

A number of tests have also been developed that attempted to mimic the processes that occur during vessel wall damage. Many of these techniques have remained primarily research tools within expert laboratories because of their inherent complexity and technical difficulty. Commercially available instruments include the platelet function analyzer 100 (PFA-100®, Fig. 4) and the Impact® cone and plate(let) analyzer (Fig. 5). Both of these tests measure platelet adhesion and aggregation under conditions of high shear, in an attempt to simulate primary hemostatic mechanisms that are encountered *in vivo*.

The cone and plate(let) analyzer, originally developed by Varon and Savion, monitors platelet adhesion and aggregation to a plate coated with



Figure 1 An example of a modern 8-channel platelet aggregometer. The model shown is the Biodata PAP-8E. Reproduced with permission from Biodata and Biodis.



Figure 2 The Chrono-log Model 700 whole blood/optical 2 channel lumiaggregometer. Reproduced with permission from Chrono-log.

collagen or extracellular matrix (ECM) under high shear conditions of 1800 s^{-1} [28–30]. In the commercial version of the device, the Impact® (DiaMed), a plastic plate is utilized instead of a collagen or an ECM-coated surface. The test is now fully automated, simple to operate, uses a small quantity of blood (0.12 mL) and displays results in 6 min. The instrument contains a microscope and performs staining and image analysis of the platelets that have adhered and aggregated on the plate. Preliminary data suggest the test can be used in the diagnosis of platelet defects and monitoring anti-platelet therapy. Because the test has only just become commercially available, widespread experience is limited.

The PFA-100® device (Dade-Behring) has been available for a number of years and is now in widespread use within many laboratories, with over



Figure 3 The VerifyNow system. Reproduced with permission from Accumetrics.

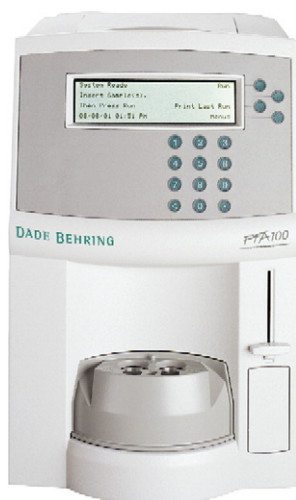


Figure 4 The PFA-100 instrument. Reproduced with permission from Dade-Behring.

200 papers published on various clinical applications [31,32]. The test was originally developed as a prototype instrument called the Thrombostat 4000 by Kratzer and Born [33,34]. The PFA-100® measures the fall in flow rate as platelets within citrated whole blood are aspirated through a capillary and begin to seal a 150 μm aperture within a collagen-coated membrane. This reaction takes place contained within one of two types of disposable cartridge (collagen–epinephrine or collagen–ADP). The instrument records the time (closure time or CT) it takes to occlude the aperture, along with the total volume of blood used during the test.

Platelets contribute significantly to the generation of thrombin and the dynamics of blood clotting including clot formation, clot retraction and lysis. Clot retraction can be easily measured in whole blood or PRP within glass tubes after the addition of calcium. The role of platelets in clot retraction was first described by Hayem in the late 19th century and Glanzmann famously described patients with poor clot retraction or thrombasthenia in 1918, who were subsequently shown to be defective in GPIIb–IIIa [35]. Modern tests are available that can study both the role of platelets in thrombin generation, clot formation and clot retraction. For example, thrombin generation tests can be used to measure thrombin generation in PRP and whole blood [36–38]. The HemoStatus® test (Medtronic Blood Management, Parker, CO, USA) can be used to detect the effects of GPIIb–IIIa antagonists [39–41]. There are also a number of instruments that measure the physical properties of clot formation. Thromboelastography® (TEG) was developed more than 50 years ago [42–44]. Anticoagulated whole blood is incubated in a heated sample cup in which a pin is suspended that is connected to a chart recorder or

computer. The cup oscillates 5° in each direction. In normal anticoagulated blood the pin is unaffected, but as the blood clots, the motion of the cup is transmitted to the pin. Whole blood or re-calcified plasma can be used, with or without activators of the tissue factor or contact factor pathways. The TEG provides a relatively rapid result (<30 min), can be conducted in a POC fashion and provides various data relating to clot formation and lysis (the lag time before the clot starts to form, the rate at which clotting occurs, the maximal amplitude of the trace and the extent and rate of amplitude reduction). Rotational TEG (ROTEG® or ROTEM®) is an adaptation of the TEG in which the cup is stationary and the pin oscillates [42,45]. Unlike platelet function tests, TEG instruments have been traditionally utilized within surgical and anesthesiology departments as POC tests for determining the risk of bleeding and as a guide to transfusion requirements. More recent developments include an expansion in the range of activators to initiate aggregation rather than coagulation e.g. the platelet mapping system® using ADP and arachidonic acid [46,47]. The Haemostasis Analysis System® (HAS) by Hemodyne is based upon the original technique developed by Carr [48–51]. The HAS® measures a number of parameters in clotting blood including platelet contractile force (PCF), clot elastic modulus and thrombin generation time (TGT) in a small sample (700 μL) of whole blood.



Figure 5 The Impact cone and plate(let) analyzer. Reproduced with permission from DiaMed.

In the last 20 years, flow cytometric analysis of platelets has also developed into a popular means to study many aspects of platelet biology and function. Preferred modern methods now utilize diluted anticoagulated whole blood incubated with a variety of reagents including antibodies and dyes that bind specifically to individual platelet proteins, granules and lipid membranes [52–54]. Flow cytometric analysis of platelet function is discussed in detail in Refs. [52–54].

Monitoring of anti-platelet therapy

Most platelet function tests have been traditionally utilized to either screen for or diagnose platelet defects. Most traditional tests are not only difficult to perform but are expensive, time consuming, and require relatively large volumes of fresh blood. They are therefore usually performed within specialized hemostasis laboratories, often in close proximity to associated clinics. Many of these tests are limited in their capacity to predict bleeding or thrombosis. These limitations have largely restricted their widespread clinical use within other disciplines (e.g., cardiology, stroke and surgery). However, this is now beginning to change as simpler tests of platelet function become available that can potentially be utilized as POC tests or at least within non-specialized laboratories. With the increasing development of new classes of antiplatelet drugs and the known heterogeneity in their biological effects between patients, it may become useful to monitor an individual's response to antiplatelet therapy so that either the dosage and/or the type of drug(s) administered can be titrated or optimized within individual patients to help control and minimize the risk of either thrombosis or bleeding.

The antiplatelet drug aspirin has traditionally been administered at a standard dose with no monitoring of effect, on the assumption that usual doses are two to three times those required to inhibit all cyclooxygenase-1 (COX-1) activity. However, the lack of a simple, convenient, reliable and clinically relevant test of platelet function has meant that lack of effect in individual patients has gone undetected. With the availability of other classes of antiplatelet drugs (e.g. thienopyridines, new P2Y₁₂ antagonists and GPIIb–IIIa antagonists) there is renewed interest in the potential utility of platelet function tests to monitor the efficacy of platelet inhibition. The development of GPIIb–IIIa antagonists in particular resulted in the development of a number of new assays to monitor a patient's response (e.g., VerifyNow® IIb/IIIa) mainly because of their narrow therapeutic window with associated increased risk of bleeding. The still

poorly-defined phenomenon of “drug resistance” has led to an explosion of interest, research and availability of a variety of tests that can potentially monitor an individual's response to antiplatelet therapy [55]. The question remains as to whether these tests will be clinically useful in the prediction of bleeding and/or thrombosis. Patient non-compliance to their therapy is also an important but relatively common confounding problem in many studies [55].

It is well known that there is considerable variation in the response of individuals (either patients or normal controls) to aspirin, clopidogrel and GPIIb–IIIa antagonists as measured by various platelet function tests. Those individuals who respond poorly to a given drug are therefore termed “resistant”. However, this is a poorly-defined phenomenon and a precise definition of resistance should only relate to the action of a specific drug to inhibit its biochemical target [56]. Many platelet function tests are non-specific and do not meet this criterion. Resistance may simply represent natural biological variation in a given drug response or may be due to specific or more complicated mechanisms [57]. Is resistance specific to an individual class of drug related to its mechanism of action, or are there common inherited and/or acquired mechanisms that may influence an individual's response to not just one but potentially all antiplatelet drugs [57]? Recent data indeed suggests that aspirin resistant patients as a group also have a reduced response to clopidogrel [58]. Whatever the mechanisms, the key question is whether a laboratory test that detects resistance, or non-response, predicts future clinical events – and whether changing therapy based on the test is beneficial to the patient. Until these links are firmly proven within large trials, resistance in the laboratory cannot necessarily be ascribed as a cause of thrombosis. Therefore, except in research trials, it is still not yet clinically useful to test for resistance and change a patient's therapy on the basis of a laboratory test [55,57,59]. The following sections discuss the specific laboratory tests for the three main antiplatelet drugs.

Monitoring of aspirin

Aspirin irreversibly inhibits COX-1 resulting in the inhibition of thromboxane (TX) A₂ generation for the entire lifespan of the platelet [60]. Aspirin is an effective antiplatelet agent because it reduces the relative risk of major vascular events and vascular death by about 25% after ischemic stroke and acute coronary syndrome [61]. Regular low doses of aspirin (e.g., 81 mg/day) will usually result in >95% inhibition of thromboxane generation.

Therapeutic monitoring was therefore considered to be unnecessary. However, the antiplatelet properties of aspirin have been shown to vary between individuals and recurrent events in some patients could be due to “aspirin resistance” or aspirin non-responsiveness [56,57]. The reported incidence of aspirin non-responsiveness varies widely (between 5–60%). There are also many possible mechanisms for aspirin resistance which have been discussed in detail elsewhere [57,62]. Recently it has been proposed that the term “aspirin resistance” should only be utilized as a description of the failure of aspirin to inhibit TXA₂ production, irrespective of a non-specific test of platelet function [56]. This is because there are many other biochemical pathways that can potentially bypass COX-1 even if this enzyme is inhibited. Depending upon the test system employed, “aspirin resistance” may therefore be detected even if COX-1 is fully blocked [56]. Recent studies also suggest that, in compliant patients, the incidence of aspirin resistance is rare using methods dependent on COX-1 activity [46,63,64]. Addition of *in vitro* aspirin to samples followed by retesting should also be an important consideration for testing compliance [64,65].

Many tests have been used to assess the influence of aspirin on platelets and aspirin resistance, including arachidonic acid- and ADP-induced LTA, ADP- and collagen-induced impedance aggregation, VerifyNow® Aspirin, PFA-100®, Thromboelastography® (TEG – platelet mapping system®), flow cytometry using arachidonic acid stimulation, Impact cone and plate(let) analyzer, and serum and urinary thromboxane [55]. Tests should ideally be performed pre- and post-drug. Some of the tests have been reported to be predictive of adverse clinical events [55]. However, the large majority of these studies are small and often statistically underpowered to completely answer whether each test can reliably predict the small number of adverse outcomes that were observed [57,62].

Although preliminary results from some studies could suggest that responses to aspirin should be monitored, there are additional problems in that LTA is time-consuming, difficult and cannot realistically be performed on large numbers of patients in routine practice. However, the simpler tests of platelet function (e.g. PFA-100®, VerifyNow® Aspirin, TEG platelet mapping®, Impact®, and urinary thromboxane) could offer the possibility of rapid and reliable identification of aspirin non-responsive patients. The PFA-100® usually gives a prolongation in the Collagen/Epinephrine (CEPI) CT in response to aspirin, with the Collagen/ADP (CADP) CT usually remaining within the normal range [66,67]. A

number of studies have observed that an appreciable number of both normals and patients fail to respond in terms of prolongation of their CEPI CT in response to aspirin [68–75]. Because the PFA-100® is a global high-shear test of platelet function, many variables have been shown to influence the CT including VWF levels, platelet count and hematocrit [32]. In patients identified with “aspirin resistance” by the PFA-100®, a number of studies have shown that VWF levels are elevated in non-responders [73,74,76]. As the CEPI CT is highly dependent upon VWF and other variables, pre- and post-aspirin CTs should ideally therefore be determined, because the true aspirin response may be masked before the drug is given [56]. Also, CADP CTs are lower in these patients, which may be caused by a combination of high VWF but also increased sensitivity to collagen and ADP [73,77,78]. It is therefore not surprising that the incidence of aspirin non-responders is reportedly much higher with the PFA-100® than with other tests [79,80]. The question remains whether or not this group of patients are at increased risk for thrombosis. Preliminary data suggests that PFA-100® CEPI CTs were non-informative in patients with stable coronary artery disease, in contrast to LTA [81–84]. However, another study suggests that the PFA-100® could be informative, [85] and that shortened CTs with the CADP cartridge (which is not affected by aspirin) may also be predictive [77,86–88].

The VerifyNow® Aspirin assay offers the possibility of rapid and reliable identification of aspirin resistance or non-responsiveness without the requirement of a specialized laboratory. Indeed, the test has United States Food and Drug Administration (FDA) approval for monitoring aspirin therapy and is being used by some cardiologists and general practitioners in the USA. The original VerifyNow® Aspirin cartridge contains fibrinogen-coated beads and a platelet activator (metallic cations and propyl gallate) to stimulate the COX-1 pathway and activate platelets [89]. Ideally, the test should produce similar results to those obtained by arachidonic acid-induced LTA. One study showed an 87% agreement with epinephrine-induced LTA [90]. Previous data comparing propyl gallate and other agonists by platelet aggregometry suggest that this agonist detects a lower number of responders in volunteers receiving either 100 or 400 mg of aspirin [89]. A more recent study compared LTA with VerifyNow® Aspirin and PFA-100® and demonstrated that aspirin non-responsiveness was not only higher in both POC tests but that agreement between the tests was poor and few patients were non-responsive by all 3 tests [79]. Nevertheless, the VerifyNow® Aspirin test can

potentially identify a correlation between aspirin non-responders, adverse clinical outcomes and aspirin dose [91–94]. Since the end of 2004 the VerifyNow® Aspirin cartridge has been modified and arachidonic acid has replaced propyl gallate as the principle agonist. Further studies are therefore warranted to relate adverse clinical outcomes to the new VerifyNow® Aspirin assay and to see whether changing therapy based upon the result can also improve outcomes. A recent study suggests that aspirin resistant patients, as defined using =2 out of 3 tests (VerifyNow® Aspirin Assay, LTA with ADP, LTA with arachidonic acid), also exhibit reduced responsiveness to clopidogrel, suggesting that alternative antiplatelet drugs may not be effective in aspirin resistant patients [58]. In apparent contrast, we have recently reported that clopidogrel, in addition to its direct antiplatelet effect via the P2Y₁₂ ADP receptor, may decrease aspirin resistance (as defined by a residual arachidonic acid-induced increase in platelet surface P-selectin) in both the presence and absence of coronary artery disease [64].

Because aspirin inhibits COX-1, measurement of TXA₂ and its metabolites either within serum or urine provides a relatively simple potential way to monitor aspirin therapy. *In vivo*, TXA₂ is rapidly converted into the more stable and inert metabolite TXB₂ which is further metabolized to 11-dehydro TXB₂, the major product found in urine. Measurement of TXB₂ by various immunoassays can facilitate an indirect assessment of the capacity of platelets to form TXA₂. Assays can be standardised so that TXB₂ is measured either within serum derived from whole blood clotted for 30 min at 37°C or in supernatants derived from PRP or purified platelets (with standardized platelet counts) activated by agonists to stimulate COX-1 activity. The metabolite 11-dehydro TXB₂ can also be measured within urine samples and the assay is also commercially available as the AspirinWorks® test. This assay has the advantage that it is non-invasive and one large study suggested that high levels of urinary 11-dehydro TXB₂ are associated with adverse clinical events [95].

Monitoring of clopidogrel

Clopidogrel (Plavix) is a prodrug that is metabolized by cytochrome P450 in the liver to an active metabolite that specifically and irreversibly blocks the platelet ADP P2Y₁₂ receptor [96]. Platelet inhibition by clopidogrel is both dose- and time-dependent and patients are usually give a loading dose of 300–600 mg and then maintained on 75 mg/day. The CAPRIE trial showed that clopidogrel

prevented more thrombotic vascular events than aspirin (RRR 8.7%) in patients with known atherosclerosis [97]. The CURE trial showed aspirin plus clopidogrel was 20% more effective than aspirin alone in acute coronary syndromes,[98] but the MATCH study showed that adding aspirin to clopidogrel in high-risk patients with recent ischemic stroke or transient ischemic attack (TIA) was associated with a non-significant difference in reducing major vascular events [99]. Combination therapy is regarded as the gold standard during percutaneous coronary intervention (PCI) [100]. However, inter-individual variability in platelet response to clopidogrel has been observed,[101] and 5–10% of patients still experience acute or subacute thrombosis after coronary stent implantation [56,102–104]. The phenomenon of “clopidogrel resistance” has been estimated to be between 4% and 30%. The definition of clopidogrel resistance is even more complex than aspirin resistance because the physiological degree of inhibition detected by ADP-induced LTA can vary widely between individuals, especially as ADP can also activate platelets via a second receptor, P2Y₁, and there is inter-individual variability of cytochrome P450 activity [96]. There is an inverse correlation between P450 3A4 activity and platelet aggregation, and other drugs can either promote or inhibit metabolism to a certain degree [105]. Pre-existing variability in ADP responsiveness is also an important variable and may provide an explanation for response variability [106]. Many mechanisms of clopidogrel resistance have been proposed [55].

Laboratory responses to P2Y₁₂ inhibitors are largely based upon monitoring ADP-stimulated responses [107]. Platelets are stimulated with ADP and responses are monitored using either LTA, the VerifyNow® P2Y₁₂ assay, TEG platelet mapping system®, Impact, Plateletworks® flow cytometric analysis of activation-dependent markers (e.g., P-selectin, PAC-1), or flow cytometric analysis of intracellular signaling by monitoring the phosphorylation of vasodilator-stimulated phosphoprotein (VASP) [25,96,107,108]. Ideally responses are monitored pre- and post-drug. LTA using 5 or 20 μM ADP can be used to arbitrarily classify patients based upon measuring the change in (delta) aggregation at baseline and post-drug [109]. Non-responders can be defined with a delta aggregation of <10%. Studies have shown that there is considerable variation in patient response to clopidogrel and up to 30% of patients may be non-responders. The largest analysis so far has found 4% of 544 patients to be hypo-responsive to clopidogrel [101]. More recent data suggest that a proportion of patients are probably under-dosed and that a 600 mg loading

dose significantly reduces the number of non-responders when compared to 300 mg [109–111]. There is still the critical unresolved question as to whether *in vitro* lack of responsiveness to clopidogrel correlates with the an increased incidence of adverse events.

ADP-induced LTA is probably not very practical to test on large numbers of clinical samples. Also, as residual P2Y₁ function can potentially widely vary despite P2Y₁₂ inhibition, this could not only explain some of the heterogeneity observed with LTA but suggests that ADP alone may not be specific enough to measure the effect of clopidogrel and other P2Y₁₂ antagonists [112]. Despite these problems, Matetzky et al. found evidence that ADP-induced LTA predicted adverse events [113]. The VerifyNow instrument was originally designed to overcome the major limitations of LTA and can be used as a POC test. The VerifyNow® P2Y₁₂ cartridge has become available for monitoring clopidogrel and other P2Y₁₂ antagonists. The assay uses prostaglandin (PG) E₁ in addition to ADP to increase intracellular cyclic adenosine monophosphate (cAMP), theoretically enhancing the sensitivity and specificity of the test for ADP-induced activation of platelets *via* P2Y₁₂ [114,115]. The PGE₁ should suppress the activation of platelets by P2Y₁. The VERITAS (The Verify Thrombosis Risk Assessment) trial will determine if the VerifyNow® P2Y₁₂ test is a reliable and sensitive measure for monitoring clopidogrel therapy. The combination of ADP and PGE₁ is also used in the flow cytometric-based VASP assay (BioCytex, Marseilles, France) [112,116]. The principle of this assay is to measure the phosphorylation of VASP, which is theoretically proportional to the level of inhibition of the P2Y₁₂ receptor. Comparison of the VASP assay with LTA shows that the level of inhibition is higher in the flow cytometry assay, because non-specific aggregation can occur *via* ADP stimulation of P2Y₁ [112]. Recent data indeed show that the phosphorylation of VASP correlates with inhibition of LTA [107]. The PFA-100 is considered unsuitable for monitoring clopidogrel [32,117–119]. Theoretically the PFA-100® CADP cartridge may be suitable for monitoring P2Y₁₂ but both collagen activation and ADP acting through the P2Y₁ receptor, along with the high shear conditions, may be normally sufficient to largely overcome P2Y₁₂ blockade [96]. A recent small study provides evidence that the P2Y₁ receptor may be an important variable in determining the CT in response to P2Y₁₂ blockade [120]. There may be also be a degree of time- and dose-dependence. It has also been observed that there is synergy with clopidogrel/aspirin combination therapy, reflected as prolongation of both CADP and CEPI CTs [121,122].

Assessment of platelet function by a variety of tests in correlation with clinical outcomes will be necessary to define responsiveness to clopidogrel and other P2Y₁₂ antagonists. Preliminary data from the CREST study (Clopidogrel Resistance and Stent Thrombosis study) by Gurbel et al. show differences with VASP, LTA and activated GPIIb–IIIa responsiveness to ADP between patients with and without subacute stent thrombosis (SAT) [123]. Comparing data from patients with (*n*=20) and without SAT (*n*=100) suggests that clopidogrel response variability to ADP is significantly associated with an increased risk of SAT [123]. This, coupled with other studies on post-discharge and post-PCI events, suggests that high post-treatment *ex vivo* reactivity to ADP may indeed be an important risk factor for adverse clinical events [113,116,124].

Carefully controlled, large randomized trials will be required to define an inadequate response to P2Y₁₂ inhibition for an individual test and to show that this correlates with adverse clinical events. Without such data, therapy should not be altered based upon the results of any of the tests that purport to determine responsiveness to a P2Y₁₂ antagonist. The RESISTOR (*Research Evaluation to Study Individuals who Show Thromboxane or P2Y₁₂ Resistance*) trial that is currently underway in 600 PCI patients may determine if the level of P2Y₁₂ inhibition correlates with clinical outcome and if changing therapy in resistant patients improves outcome.

The development and clinical application of thienopyridines such as clopidogrel has proven that the P2Y₁₂ receptor is an attractive target for new drug development. Because thienopyridines are metabolized to their active derivatives by the liver, a number of direct antagonists are in development (*e.g.*, cangrelor and AZD6140) [96]. Some new thienopyridines (*e.g.*, prasugrel) have also been developed which exhibit superior properties (*e.g.*, higher efficacy, faster onset and longer duration of action) compared with clopidogrel [96]. As some of the observed inter-individual heterogeneity of clopidogrel responsiveness may be caused by differences in liver metabolism, it will be interesting to determine whether the incidence of non-responsiveness is lower or even eradicated with these new drugs and whether high post-treatment reactivity to ADP remains a potential significant problem.

Monitoring of GPIIb–IIIa antagonists

The identification of the importance of the GPIIb–IIIa complex in mediating platelet aggregation (*i.e.*, the final common pathway of platelet activation) suggested that this receptor would be an attractive

target for antithrombotic therapy. The FDA-approved GPIIb–IIIa antagonists (abciximab, tirofiban and eptifibatide) have now become an important class of antiplatelet agents that are widely used for the prevention of thrombotic complications in patients undergoing PCI or presenting with acute coronary syndromes. Early observations on the inhibition of thrombus formation within animal models not only established a strong correlation between the level of GPIIb–IIIa blockade and the prevention of thrombus formation but demonstrated steep dose-response curves [125,126]. It became rapidly apparent that a certain level of GPIIb–IIIa inhibition was required for the optimal efficacy of GPIIb–IIIa antagonists. This strongly suggested that monitoring of platelet inhibition could be important in patients treated with these agents. Monitoring GPIIb–IIIa antagonists can be performed by a variety of tests including LTA, WBA, flow cytometry, and radiolabeled antibody binding assays [127]. However some of these tests are time-consuming, expensive and are usually performed within specialized laboratories. Given the widespread clinical use of these GPIIb–IIIa antagonists in cardiology, there existed the potential demand for a simple, inexpensive and rapid method that could be utilized as a POC test either at the bedside or in the clinic, so that the degree of GPIIb–IIIa blockade could be also be potentially determined by non-specialists. The VerifyNow® system was originally developed to meet this demand. The assay principle was developed based upon experiments using fibrinogen-coated beads and TRAP which facilitated the rapid visual analysis of the degree of GPIIb–IIIa blockade [20]. The basis of the VerifyNow® IIb/IIIa assay is that fibrinogen-coated beads will agglutinate in whole blood in direct proportion to the degree of platelet activation reflected by exposure of the fibrinogen binding site on GPIIb–IIIa [18]. The presence of a GPIIb–IIIa antagonist will therefore decrease the amount of agglutination in proportion to the level of inhibition achieved.

Initial *in vitro* evaluations of the VerifyNow® IIb/IIIa assay demonstrated good correlations with both LTA in PRP and radiolabeled receptor binding assays [18]. Studies in patients receiving abciximab or other GPIIb–IIIa antagonists also demonstrated good correlations with LTA [128,129]. GOLD (AU – Assessing Ultegra), a large prospective multicenter study, showed a significant association between the level of platelet inhibition by the VerifyNow® IIb/IIIa assay and clinical outcomes [130]. This suggests that the device has clinical utility, although no study has yet been performed to determine whether titration of GPIIb–IIIa therapy based upon the VerifyNow® IIb/IIIa test result decreases adverse events.

A slightly modified Plateletworks® POC assay was recently reported to correlate more strongly than VerifyNow® IIb/IIIa with LTA in measuring platelet inhibition by GPIIb–IIIa antagonists [26]. The PFA-100 has also been utilized to monitor GPIIb–IIIa blockade and correlates well with LTA and receptor occupancy measurements [131–133]. Although many patients give non-closure or >300 s CT in the PFA-100 following GPIIb–IIIa antagonist treatment, one study suggests that failure to observe non-closure may be associated with an increased risk of cardiac events [133].

Conclusions

As summarized in this review article many tests of platelet function are now available for clinical use, and some of these tests have been shown to predict clinical outcomes after antiplatelet therapy. However, in most of these studies, the number of major adverse clinical events was low, and additional studies are therefore needed. Most importantly, no published studies address the clinical effectiveness of altering therapy based on the results of monitoring antiplatelet therapy. Therefore: 1) the correct treatment, if any, of “resistance” to antiplatelet therapy is unknown; 2) other than in research trials, it is not currently appropriate to monitor antiplatelet therapy in patients or to change therapy based on such tests [55–57]. A clinically meaningful definition of “resistance” to antiplatelet drugs needs to be developed, based on data linking drug-dependent laboratory tests to clinical outcomes in patients. Nevertheless, the 2006 American College of Cardiology/American Heart Association guidelines for PCI provided a Class IIB recommendation (based on level C evidence) that, in patients in whom subacute stent thrombosis may be catastrophic or lethal, platelet aggregation studies may be considered and the maintenance dose of clopidogrel increased from 75 mg to 150 mg per day if less than 50% inhibition of platelet aggregation is demonstrated [134].

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