

Novel strategies for assessing platelet reactivity

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Abstract

There are many approaches to assessing platelet reactivity and many uses for such measurements. Initially, measurements were based on the ability of platelets separated from other blood cells to aggregate together following activation with an appropriate 'aggregating agent'. Later, measurements of platelet aggregation in blood itself were performed, and this led to a point-of-care approach to platelet function testing. Measurement of secretory activity through the appearance of the activation marker P-selectin on platelets now provides an alternative approach, which enables remote testing. Measurement of vasodilator-stimulated phosphoprotein phosphorylation is also moving toward application in situations remote from the testing laboratory. Here we provide an overview of the various approaches that are now available, assess their advantages and disadvantages, and describe some of the clinical situations in which they are being used.

Keywords

aspirin, flow cytometry, light transmission aggregometry (LTA), multiplate electrode aggregometry (MEA), P-selectin, P2Y₁₂ antagonists, platelet aggregation, platelet reactivity, VASP phosphorylation, VerifyNow

Platelet reactivity is a broad term indicating the degree of the response of blood platelets to an external stimulus, usually an 'aggregating agent'. Platelet reactivity can be measured in many different ways and the purpose of this review is to summarize the approaches, with an emphasis on the newer approaches that are becoming available.

Platelets play an important role in the hemostatic process [1,2]. They contribute to the formation of a hemostatic plug that serves to prevent blood loss from injured blood vessels. The hemostatic plug contains thousands of platelets that have aggregated together within a network of fibrin. However, platelets also contribute to the generation of a thrombotic mass in a coronary or cerebral artery or in an artificial stent leading to blockage and resulting in conditions such as heart attack, stroke and stent thrombosis [3,4]. A thrombus is also composed of platelet aggregates within a fibrin network. So thrombus formation can thus be considered to be hemostasis in the wrong place.

It is believed that aggregating agents that are newly released into the arterial circulation or become newly exposed on damaged tissue or on atherosclerotic plaque bring about the platelet aggregation that contributes to the participation of platelets in hemostasis and thrombosis. Some important aggregating agents are collagen, thrombin, ADP and thromboxane A₂ (TXA₂). These activate platelets through receptors for these agents on the platelet surface. Measurements of platelet reactivity have served to quantify the effects of these and other aggregating agents on platelets. They have also served to quantify the degree of inhibition of platelet activity brought about by pharmacological agents known as 'antiplatelet agents', some of which are now used routinely as medicines [5].

There is a general concept that a pronounced platelet response to an aggregating agent is associated with an increased risk of thrombosis and that a low platelet response is associated with bleeding and bruising. Identification of congenital deficiencies in platelet function has helped explain bleeding diatheses and the importance of platelet function in the hemostatic process [6].

Patients with high residual platelet activity despite treatment with antiplatelet agents are at higher risk of future thrombotic events [7]. Also, excessive inhibition of platelet reactivity by antiplatelet agents can be associated with excessive bleeding with implications for surgical interventions. Consequently, there is now the concept of using measurements of platelet reactivity to identify a 'therapeutic window' in patients, so as to reduce the risk of a thrombotic event without increasing the risk of bleeding [8–10].

Approaches to assessment of platelet reactivity

Many years ago, it was discovered that adding ADP to platelet-rich plasma (PRP) caused platelets to clump together [11]. PRP is obtained when fresh blood, to which an anticoagulant such as sodium citrate has been added to stop the blood from clotting, is centrifuged slowly for about 10 min to remove the red cells and white cells. Within a few seconds of adding ADP or indeed any other aggregating agent to a stirred sample of PRP, the turbidity starts to reduce and within a few minutes the PRP becomes virtually clear. This is a consequence of the individual platelets clumping together to form aggregates of nearly all the platelets that are present. The clumps become so large that they can be seen easily with the naked eye.

Sometimes, the platelet aggregation is irreversible and sometimes it is reversible, in that the aggregation is followed by disaggregation. The type of response is dependent on the absence or presence of pathology in the volunteer or patient being investigated. For example, it can be reduced or absent in patients with bleeding disorders and can be excessive in some patients who are at high risk of thrombosis. It is also dependant on the type and concentration of the aggregating agent used, and on the presence or otherwise of a drug designed to inhibit the platelet response.

The 'clearing' of the PRP as aggregation progresses became the basis for measurement of platelet aggregation, hence 'light transmission aggregometry (LTA)' or 'turbidimetry' in which the 'clearing' of the PRP was monitored by detecting the amount of light transmitted through the stirred sample of PRP. The equipment that is used for stirring the PRP and for monitoring the changes in light transmission that occur is known as an

aggregometer. LTA is still referred to as the 'gold standard' for measurements of platelet aggregation or platelet reactivity [12]. This is despite additional approaches to measurement of platelet aggregation that have appeared over the years.

Measurement of platelet aggregation as a means of assessing platelet reactivity is attractive in that it is the formation of clumps of platelets (platelet aggregates) in response to an external stimulus that is a major contributor to the formation of a hemostatic plug formation or a thrombus. The aggregated platelets together with fibrin, the end product of the coagulation cascade, provide structure and stability.

The main external stimuli involved in hemostatic plug and thrombus formation are collagen and thrombin. Collagen is exposed on damaged or diseased blood vessels. Thrombin forms as a consequence of tissue factor exposure and also of platelet adherence to leukocytes to produce platelet-leukocyte conjugates that display tissue factor. Interaction with collagen or thrombin also leads to synthesis of TXA₂ from arachidonic acid (AA) liberated from phospholipids within the activated platelets, and also secretion of the contents of storage granules that include ADP. The TXA₂ and the ADP contribute further to the platelet aggregation [2,5].

The problems with measurement of platelet aggregation using the LTA approach are threefold. First, the measurements are made in PRP; this is considered to be less physiological compared with blood itself. Second, the number of platelets in the PRP (and hence the turbidity of the sample) varies from patient to patient. Third, the approach requires prompt access to a laboratory with the appropriate equipment and expertise for the measurements to be performed.

Newer approaches to measuring platelet aggregation focus on whole blood, rather than PRP, and include Multiplate Electrode Aggregometry (MEA; a type of impedance aggregometry), VerifyNow (a turbidimetric approach that involves adding fibrinogen coated beads to whole blood) and approaches based on platelet counting.

Platelet aggregation is only one platelet response to an external stimulus. Platelets are complex organelles present in blood in high numbers that, as already mentioned, can be induced to synthesize molecules such as TXA₂ and to secrete the contents of intracellular granules including ADP. P-selectin, that forms part intracellular granules known as α -granules, appears on the platelet surface following platelet activation and mediates platelet-leukocyte conjugate formation leading to enhanced generation of thrombin and subsequent fibrin formation. In addition, following an external stimulus there are biochemical changes in platelets that can be quantitated, such as phosphorylation of vasodilator-stimulated phosphoprotein (VASP). Some of these approaches have the advantage of being performed remotely from the testing laboratory with no need for specialist equipment or technical expertise close at hand.

Methods based on measurements of platelet aggregation

Light transmission aggregometry

Although LTA can no longer be considered a novel strategy, it formed the basis for the methods that came later on, and as previously mentioned, is still considered to be the

gold standard for measurement of platelet reactivity. The aggregating agent under investigation is added to a stirred sample of PRP in an aggregometer, and the aggregation is quantitated by the increase in light transmittance that occurs. Typical aggregating agents tested include ADP, epinephrine, collagen, AA and thrombin. With regard to thrombin, this is often replaced by a thrombin receptor activation peptide that has the advantage of activating the platelet without also initiating the coagulation cascade by converting fibrinogen to fibrin. The antibiotic, ristocetin is sometimes used for some diagnostic purposes.

For many years, the test has been used to look for defects in platelet aggregation that might explain enhanced bruising or bleeding in a patient. For example, deficiencies in the aggregation response to ADP and most other aggregating agents are indicative of a condition known as Glanzmann's thrombasthenia brought about by absent or abnormal glycoproteins (GPs) known as GPIIb and GPIIIa on platelets; the GPs need to be present as a complex (the GPIIb/IIIa complex) for platelet aggregation to occur. Platelet activation is accompanied by a rearrangement in the 3D structure of GPIIb/IIIa such that it binds fibrinogen, which then links adjacent-activated platelets together. An absent response to the antibiotic ristocetin is indicative of the Bernard-Soulier syndrome, brought about by the absence or change in another GP complex GPIb/IX/V that is needed for a normal interaction of platelets with the von Willebrand factor in blood plasma; interaction with the von Willebrand factor forms one of the mechanisms through which platelets adhere to exposed collagen [13].

Numerous studies have identified the pharmacological effects of different agents and drugs on platelets. For example, aspirin inhibits aggregation brought about by AA as it blocks the cyclo-oxygenase involved in converting this into TXA₂. Clopidogrel and newer P2Y₁₂ antagonists, such as prasugrel, ticagrelor and cangrelor reduce the extent of the platelet aggregation brought about by ADP by interfering with the ability of ADP to interact with P2Y₁₂ receptors on the platelet surface [5].

An example of the use of LTA to assess the impact of different doses of the newly developed drug, AZD6140, in comparison with a standard dose of clopidogrel in patients with atherosclerosis is the DISPERSE study [14]. This is mentioned specifically here because this was a landmark study that clearly demonstrated differences in the rate of onset of inhibition of platelet aggregation by a new orally administered P2Y₁₂ antagonist compared with clopidogrel, differences in degree of inhibition of platelet aggregation, and also differences in the rate of recovery when the drug was withdrawn. The drug in question became known later as ticagrelor. Both prasugrel and ticagrelor are now deemed to be much more effective overall than clopidogrel as inhibitors of platelet aggregation and this translates into more effective antithrombotic therapy, albeit with an increased risk of depressed hemostasis and thus, an increased risk of bleeding [7].

Some antiplatelet agents appear to be much more effective as inhibitors of platelet aggregation after administration to some patients compared with others, and it is this variability in effectiveness that may make the drug clopidogrel less effective as a medicine compared with other P2Y₁₂ antagonists. Despite this, clopidogrel is still widely used as a means of treating patients with a high risk of thrombosis [15]. One reason for variability in the patient response to clopidogrel is that there are genetic differences in

the CYP450 enzymes responsible for converting clopidogrel (which is a prodrug) into its active metabolite [16,17]. Others may include accelerated platelet turnover, upregulation of the P2Y₁₂ pathways, high baseline platelet reactivity, poor compliance, underdosing and drug–drug interactions [18]. Many clinical studies have demonstrated that high residual platelet aggregation in response to ADP, despite treatment with the P2Y₁₂ antagonist clopidogrel, is associated with further ischemic outcomes [7,16].

As stated above, the newer versions of this class of drug are much less variable in their effectiveness as inhibitors of platelet reactivity. The frequency of ‘nonresponse’ to aspirin, another drug that is very widely used in patients with atherosclerosis, as judged by a lack of effect of the drug on AA-induced platelet aggregation, is also much lower than that seen with clopidogrel [19].

Usually, LTA is used to study platelet aggregation in small samples of PRP stirred in an aggregometer. More recently, LTA has been successfully used in the Optimul Assay in which the effects of multiple platelet agonists have been tested in parallel in PRP obtained from patients with bleeding disorders. The approach involves agitating small volumes of PRP in a 96-well platelet with measurement of the changes in light absorbance using a platelet reader. The approach proved to be comparable with other testing procedures [20].

Multiplate electrode aggregometry

Impedance aggregometry was introduced by Cardinal and Flower [21]. It is based on the measurement of the electrical resistance between two electrodes immersed in stirred whole blood. As platelets aggregate and bind to the electrodes, there is a change in electrical impedance that corresponds to the degree of aggregation that has occurred. A clear advantage of using whole blood rather than PRP is that a centrifugation step is not required, thus simplifying the measurement and opening up the possibility of a point-of-care approach to measurement of platelet reactivity.

The version of impedance aggregometry that has been used most in recent years is MEA (Multiplate Analyzer, Roche, Munich, Germany), which uses two sets of electrode pairs, so that a double determination is performed on each sample to ensure measurement repeatability. The advantage compared with LTA is that the measurements are performed in blood rather than PRP. The main disadvantage is that, as for LTA, prompt access to a laboratory and appropriate expertise is still needed.

In the literature, there are many positive examples of the use of MEA. For example, MEA was used to investigate the inherited bleeding disorder Glanzmann’s thrombasthenia where markedly reduced responses to a number of platelet-aggregating agents were evident, in the same way as seen with LTA [22,23]. The approach also proved as useful as LTA for measuring the degree of inhibition of platelet aggregation by the antiplatelet agents aspirin and clopidogrel in preoperative patients scheduled for elective cardiac surgery [24].

Clopidogrel is one of the most commonly used P2Y₁₂ antagonists. However, as discussed above, the pharmacodynamics response varies with a significant number of patients presenting with high platelet reactivity despite the treatment. And as with

measurements performed using LTA, high residual platelet reactivity as measured with MEA is associated with increased risk of further ischemic outcomes. The practical usefulness of the MEA approach was demonstrated in a study performed by Hazarbasanov *et al.* [25] in which MEA was used to identify patients undergoing a percutaneous coronary intervention (PCI) who still demonstrated high platelet reactivity despite treatment with clopidogrel; in this case, increasing the dose of clopidogrel seemed to bring about favourable outcomes.

In another study, the MADONNA study, patients in one hospital received additional antiplatelet therapy (guided by investigations using the MEA) while those in another hospital did not. The study demonstrated the benefit of tailored antiplatelet therapy in that stent thrombosis and acute coronary syndrome occurred significantly less often in the guided group [26]. In another study, low platelet reactivity identified using the MEA seemed to be as effective as assessment of CYP450 variants in identifying patients undergoing coronary stent placement with an increased bleeding risk, although in this case the expected relationship between high platelet reactivity and stent thrombosis was not evident [27].

Currently, the benefit of MEA testing to guide treatment is being tested in the TROPICALACS trial that is investigating the clinical and health-economic impact of tailored antiplatelet therapies in PCI [28].

VerifyNow

VerifyNow (Accumetrics, Inc, CA, USA) is a testing approach that is specifically used for point-of-care measurement of inhibition of platelet aggregation by aspirin, P2Y₁₂ antagonists such as clopidogrel, and also antiplatelet agents known as GPIIb/IIIa antagonists. As with LTA and MEA, ADP is used to activate the platelet to monitor clopidogrel therapy, while AA is used to monitor aspirin therapy. A thrombin receptor activation peptide is used to monitor the effects of a GPIIb/IIIa antagonist. Following activation by the agonist, fibrinogen-coated beads are able to bind to the activated platelet through GPIIb/IIIa on the platelet surface to form an agglutinate and the resulting 'clearing' of the sample is measured by turbidimetry. The tests are fully automated for measurements made at the point of blood collection and there is no requirement for a specialized laboratory and/or specially trained expert personnel.

As with LTA and MEA, the VerifyNow system has been used to demonstrate the prognostic significance of high residual platelet function in patients treated with clopidogrel. For example, a higher risk of stent thrombosis and myocardial infarction was evident in association with high residual platelet reactivity in the ADAPT-DES study [29]. In a further analysis of the same study, low residual platelet reactivity in clopidogrel treated patients was demonstrated to be associated with increased bleeding [30]. Low residual platelet reactivity was also shown to be associated with bleeding in a study of patients undergoing PCI [31]. In view of such findings, VerifyNow has also been tested as a means of predicting bleeding in clopidogrel-treated patients undergoing coronary artery bypass grafting and a relationship between lower platelet reactivity and increased bleeding has been established in two pilot studies that have been performed [32,33].

In view of higher rates of thrombotic consequences in patients with high residual platelet reactivity in clopidogrel-treated patients, two large studies were performed in which the intention was to use the VerifyNow system to demonstrate the benefit of adjusting antiplatelet therapy in patients undergoing PCI for stable coronary artery disease. Unfortunately, the GRAVITAS study did not demonstrate any reduction in serious cardiovascular outcomes in patients with high on-treatment platelet reactivity who received a higher dose of clopidogrel consequent to testing [34]. It has been argued that simply increasing the dose of clopidogrel is insufficient to achieve the effect that was desired, and that this is the main reason for the negative outcome of this study [35]. In the second study, TRIGGER-PCI, the more potent P2Y₁₂ antagonist prasugrel was used in place of clopidogrel to effect greater inhibition of platelet function in patients with high residual platelet reactivity, but this trial also failed to meet the desired outcome. The study was terminated prematurely due to a lower than the expected event rate in the relatively low-risk patients who were recruited into this study [36].

Very recently, it was reported that VerifyNow testing to optimize treatment with prasugrel in elderly patients with acute coronary syndromes in the ANTARCTIC trial failed to provide any benefit in terms of a composite of cardiovascular death, myocardial infarction, stroke, stent thrombosis, urgent revascularization and bleeding complication at 1 year [37].

More positively, clopidogrel non-responders identified using the VerifyNow system who then received the GPIIb/IIIa antagonist tirofiban in addition to standard therapy with aspirin and clopidogrel went on to experience fewer subsequent incidents of myocardial infarction [38,39].

Platelet counting

When platelets aggregate together the number of single platelets in the blood falls and this fall in platelet number can be used as a measure of the extent of platelet aggregation that occurs. Platelet counting can be performed using a commercial whole blood cell counter that utilizes impedance counting technology, or using a flow cytometer [40]. The use of a fixative that stops the aggregation at the point at which a measurement is required, is an advantage if a platelet counting device is not immediately available for the counting to be performed [41,42,43].

Plateletworks (Helena Laboratories, TX, USA) is an approach to the measurement of platelet aggregation in which platelets in blood are stimulated with ADP or collagen following which platelet counting is performed. Counts are compared with those prior to adding the aggregating agent. Immediate platelet counting is essential as no fixative is employed. The advantages over other approaches are that no sample preparation is needed; the measurements are performed on whole blood using preloaded baseline and platelet agonist tubes. Also it is a simple and rapid procedure and platelet count and aggregation results are available immediately provided a platelet counting device needs to be available. In a comparison with LTA, there was good agreement when used for monitoring the inhibitory effects of clopidogrel, although the necessity for immediate platelet counting was noted [44], which would have been due to the instability of the unfixed platelet aggregates in the blood.

While not yet as widely used as other approaches to studying platelet reactivity in clinical situations, high on-treatment ADP-induced platelet reactivity in patients taking clopidogrel demonstrated using the Plateletworks approach has been shown to be predictive of subsequent myocardial infarction and of rehospitalization within 3 months of testing [45].

Adding a fixative to the blood at the precise point at which a measurement of platelet aggregation is required, has the advantage of introducing a period of time between experimentation and platelet counting that obviates the need for the immediate availability of a platelet counting device. It also provides the opportunity for remote testing with the actual platelet counting being performed up to several days after experimentation, thus opening up the number of different types of location for the testing. The fixative AGGFix (Platelet Solutions Ltd, Nottingham, UK) offers such an opportunity.

A good example of the use of AGGFix to measure platelet aggregation remotely is a study that was performed in Iceland with the platelet counting performed in the UK. The circumstance was a clinical trial in which a new antiplatelet agent, an EP3 receptor antagonist, was administered to volunteers with and without clopidogrel or clopidogrel and aspirin, after which the effectiveness of the drugs as inhibitors of platelet aggregation was assessed [46]. More recently, AGGFix has also been used successfully for assessment of platelet aggregation using the platelet counting approach in blood placed in 96-well plates and also, in parallel, to assess the degree of platelet-leukocyte conjugate formation that occurred, assessed by flow cytometry [47].

Methods based on measurements of platelet activation markers

As mentioned above, platelet aggregation is only one platelet response to an external stimulus. Synthesis of TXA₂, release of ADP and the appearance on the platelet surface of activation markers such as P-selectin also provide means of assessing platelet reactivity. Measurements of TXB₂, the stable breakdown product of TXA₂ and also of TXA₂ metabolites have provided a direct means of assessing the activity of the cyclooxygenase in platelets and particularly the effect of aspirin on this. Measurements of secreted ADP have been performed in parallel with LTA in a device known as a lumiaggregometer and this has proved particularly useful in studies of defects in the platelet storage pool in patients leading to pronounced bruising and bleeding. Once again, however, the ready availability of specialist equipment and expertise are required.

The appearance on the surface of platelets of markers of platelet activation can be demonstrated and quantitated using flow cytometry [48] and these include P-selectin that is translocated to the platelet surface from α -granules within platelets. Platelets can be activated in whole blood using an appropriate aggregating agent, either in blood that is left unstirred or that contains an additional agent such as EDTA to prevent platelet aggregation occurring. The amount of P-selectin on activated single platelets is then measured by flow cytometry. The use of a fixative added to the blood following platelet activation successfully extends the time between experimentation and P-selectin analysis to several days, thus allowing for remote platelet function testing. A useful

fixative for this purpose that is commercially available is PAMFix (Platelet Solutions Ltd) [49].

There is now good progress in developing tests for particular purposes based on measurement of P-selectin and these include Aspirin Kits (for determining the effects of aspirin on platelet reactivity), P2Y₁₂ kits (for determining the effects of drugs such as clopidogrel, prasugrel, ticagrelor and cangrelor on platelet reactivity) and kits for screening patients for evidence of platelet dysfunction without the need for referral to a specialist facility [49,50].

An assessment of platelet functional defects in patients with easy bruising and bleeding based on the appearance of activation markers on platelets was performed as part of the Genotyping and Phenotyping of Platelets study. In this study, both P-selectin (as a marker of α -granule secretion) and CD63 (as a marker of dense body secretion) were measured on platelets stimulated with three different aggregating agents, and the results compared with those using lumiaggregometry. There was a good agreement between lumiaggregometry and the results of the P-selectin testing, with diagnosis being concordant in 84% of cases [51,52].

In another study, P-selectin measurement using the P2Y₁₂ kit identified a group of patients with acute coronary syndromes in whom high platelet reactivity was retained despite treatment with clopidogrel and were demonstrated to be at significantly higher risk of experiencing a further acute thrombotic event [53], thus demonstrating concordance between P-selectin measurements and other approaches to the measurements of platelet reactivity. Also it was demonstrated that treatment of patients with acute coronary syndromes with the P2Y₁₂ antagonist prasugrel generates a higher degree of inhibition of P-selectin expression than clopidogrel except, interestingly, in patients with hypertension [54]. The P-selectin approach has also been used to assess platelet reactivity in patients with acute stroke [55]. The study demonstrated the effects of antiplatelet therapy being taken on admission with acute stroke on the measurements performed as part of the TARDIS study [Bath PM *et al.* Remote assessment of platelet function in patients with acute stroke or transient ischaemic attack, Submitted (2016)]. The approach has also been used to assess the residual effects of aspirin in surgical patients from whom this antiplatelet agent had been withdrawn [56]. The approach is also finding a use for determining the efficacy of antiplatelet agents in veterinary medicine [57].

Platelet activation leads to formation of platelet–leukocyte conjugates in whole blood, largely consequent to P-selectin on the surface of activated platelets interacting with a protein known as PSGL-1 on the leukocytes. Such conjugates can be quantitated using flow cytometry. For testing sites that do not have immediate access to such equipment, the use of a fixative such as AGGFix (Platelet Solutions Ltd) enables testing to be conducted remotely and then transferred to an appropriate laboratory for the flow cytometric analysis. Early results demonstrated increased levels of particularly platelet–monocyte conjugates in association with myocardial infarction [58]. Also the approach has been used to quantitate the effects of antiplatelet drug therapies in volunteers and in patients with stroke [59]. More recently, measurements of platelet aggregation and also platelet–leukocyte conjugate formation have been measured in parallel in the same blood samples in studies involving 96-well plate technology [40].

Parallel studies have also been performed on blood from mice [60]. In a recent review, it was considered that further studies are needed before platelet-leukocyte conjugate formation becomes routinely used in clinical practice [61].

Methods based on measurements of a cell metabolite

A particular cell metabolite, the levels of which have been exploited as a means of studying platelet activation, is VASP. The level phosphorylated VASP (VASP-P) reflects the level of cyclic nucleotides in platelets (and other cells); in particular, it has been used as a means of determining the effects of agents that both increase and lower the level of cyclic AMP (cAMP) in platelets. cAMP is an important intracellular modulator of platelet reactivity with low levels supporting the platelet response and high levels being inhibitory. An important use of measurement of VASP phosphorylation has been in generating an assay for determining the effectiveness of clopidogrel acting as an antagonist at the P2Y₁₂ receptors on platelets.

One assay (Biocytex, Marseille, France) involves stimulating platelets with prostaglandin E₁ (PGE₁) to raise cAMP and then determining the ability of added ADP to lower that raised level of cAMP acting via the P2Y₁₂ receptor. Good blockade of the receptor following clopidogrel administration, and thus high levels of VASP-P, implies effective treatment with clopidogrel. Because the level of VASP-P is controlled through ADP acting at the P2Y₁₂ receptor and not by any other ADP receptor on the platelet, this approach is deemed to be the only current approach that selectively determines the effectiveness of clopidogrel acting via the P2Y₁₂ receptor. In this particular assay, measurement of VASP-P involves permeabilization of platelets followed by flow cytometric assessment of the level of a fluorescent antibody directed at VASP-P inside the platelets. So, like many other approaches to the measurement of platelet reactivity, direct access to specialist laboratory facilities is required.

Early studies were adjudged to compare very favorably with the VerifyNow and LTA approaches to assess the degree of inhibition brought about by clopidogrel or prasugrel administered to patients with acute coronary syndromes [62,63]. A recent successful application of this technology was to time the onset of P2Y₁₂ receptor blockade after administration of prasugrel and ticagrelor in patients with acute coronary syndromes [64]. Very recently, a study was performed to directly compare the rate of formation of the active metabolite of clopidogrel in treated patients with the effects of the clopidogrel on platelet reactivity determined using the VASP assay and also MEA. It was concluded that the pharmacodynamic effects of the drug determined by the VASP measurements entirely reflected the pharmacokinetic measurements but that the results with MEA were disappointing in this regard [65].

More recently, another approach to measurement of VASP-P has been developed that utilizes VASPFix (Platelet Solutions Ltd), which provides a one-step approach to the measurement [66]. VASPFix includes a lytic agent, glass beads that bind the VASP from the lysed platelets and an appropriate fluorescent antibody to determine the level of VASP-P on the beads. The stability of the treated sample is such that after adding VASPFix to blood or PRP, the level of VASP-P is either determined directly using flow cytometry or the sample is frozen and analysed at a later time. Indeed the measurement

is stable for several months after sample preparation. The approach has been used to assess platelet reactivity in patients taking clopidogrel or prasugrel with similar results being obtained to those using the Biocytex assay. In contrast to the use of PGE₁ in the Biocytex assay, the VASPFix assay uses iloprost to raise cAMP in platelets. This is because it is now known that PGE₁ acts at EP₃ receptors on platelets, as well as the IP receptor, which acts to reduce VASP-P in the same way as ADP acting at the P₂Y₁₂ receptor, and this appears to reduce the specificity of a test designed purely to investigate the effects of drugs acting at the P₂Y₁₂ receptor [66].

Other approaches to assessing platelet reactivity

The Platelet Function Analyser (PFA)-100 involves passing whole blood at high shear stress through a capillary tube toward an aperture in a collagen-coated membrane, which contains either ADP or epinephrine. The time it takes to occlude this opening is defined as closure time. A high closure time is seen in patients with von Willebrand's disease reflecting the importance of platelet adhesion to collagen in this particular assay. The assay that includes epinephrine is affected by aspirin treatment but, surprisingly the assay that includes ADP is relatively insensitive to P₂Y₁₂ antagonists [67,68]. The approach was deemed useful for determining inhibition of platelet function by aspirin but not clopidogrel when compared with the MEA [69].

To circumvent this lack of sensitivity to P₂Y₁₂ antagonists, an additional test cartridge (the INNOVANCE PFA P₂Y test cartridge) was introduced in which the ADP is supplemented with PGE₁. At least as judged by experiments performed in which a P₂Y₁₂ antagonist was added to whole blood *in vitro*, the INNOVANCE PFA P₂Y cartridge appears to be comparable to other currently available tests of platelet reactivity [70,71]. Also the test was able to identify some non-responders to clopidogrel, identified initially using other approaches, in some groups of volunteers and patients of treated with this drug [71,72]. A further report indicated its usefulness in identifying patients with congenital defective responses to ADP [73] and there is also a correlation with *CYP450* genotypes in clopidogrel-treated patients [74,75]. The sensitivity of the test is markedly influenced by the nature of the anticoagulant used in obtaining the blood for analysis [70] and it is likely that this should be taken account of in any future studies. Incidentally, a focus on the implications of the anticoagulant used in testing of platelet reactivity globally is a matter that still requires close attention. For example, the MEA currently recommends the use of hirudin in preference to citrate for testing the effectiveness of P₂Y₁₂ antagonists where citrate seems to reduce the degree of inhibition of platelet reactivity brought about by such agents [76]. Also, very recently, potential problems have been identified in the routine use of citrate in the VerifyNow assay for measurements performed at a time point very close to venepuncture, which appear to be caused by a 'stunning' effect of the citrate on platelet reactivity [77].

Thrombelastography (TEG; Haemonetics Corporation, MA, USA) is a global test of thrombosis, which measures aspects of clot formation in a specially designed device. There were some early encouraging results when ADP-induced platelet-fibrin clot strength was used to serve as a predictor of either ischemic events or bleeding in clopidogrel-treated patients poststenting [78]. However, the approach is still not widely used for studies of platelet reactivity and in a recent study, the TEG results were found not to correlate well with the VerifyNow approach [79]. On the other hand, an

adaptation known as short TEG has recently been used to assess the impact of aspirin and clopidogrel in patients with stent thrombosis and to modify treatments on the basis of the results obtained [80,81]. In addition, the first report of the TEG-6S system has just been published, which is claimed to offer a point-of-care approach to TEG [82]. So far the studies are not large enough for the full impact of these new approaches to be assessed, but there does appear to be some promise here.

Aggreguide (AggreDyne, Inc., TX, USA) is a new point-of-care testing procedure based on light scattering technology that is in the very early stages of evaluation. While it appears to be attractive and easy to use, the first published study in cardiovascular patients showed poor reproducibility with other tests of platelet reactivity [83].

Final considerations

There are many different approaches to assessing platelet reactivity. Here, the focus has been on measurements of platelet aggregation using variety of different approaches and on the use of platelet activation markers and a particular cell metabolite. Although not discussed in detail in this review, it is generally considered that the comparability of different testing procedures is sometimes quite disappointing [84]. However, this is against a background of a huge number of variations in the detailed methodologies employed within any one of the approaches used, and uncertainties about the positioning of cutoffs between values that are deemed acceptable and those that are not. Our own experience in developing the newer remote assays based on measurement of P-selectin is that these appear to compare reasonably well with the other technologies for identification of patients with platelet dysfunction [51,52] and also when comparing results obtained for patients taking aspirin, clopidogrel or prasugrel [49].

One of the main problems with platelet function testing in general is the need for specialist equipment and expertise in close proximity to the blood sampling. However, some progress is now being made in the use of fixatives that provide the option of remote testing with no requirement for equipment and expertise at the testing site.

Future perspective

Platelet reactivity is measured in freshly prepared blood and historically has relied on the availability of specialized equipment and technical expertise for the measurements to be performed. But recent advances mean that measurement of platelet reactivity is becoming easier. Blood can be activated, stabilized and sent away for analysis without any need for equipment or special expertise at the point of blood sampling. It is anticipated that this will make it easier to assess platelet function in relation to bleeding diatheses consequent to congenital or drug-induced platelet hypoactivity, and also to determine high residual platelet reactivity in patients who are at risk of conditions such as heart attack and stroke, often despite drug treatment. Effective titration of drug treatment could then be a real possibility with more effective P2Y₁₂ antagonists such as prasugrel or ticagrelor used in place of clopidogrel in all patients who need this, and possibly clopidogrel used in place of the more effective agents to avoid bleeding.

Conclusion

An overall assessment of current and future approaches to assessing platelet reactivity is provided in Table 1. The table summarizes the various approaches available and identifies their main advantages and disadvantages. It can be seen that measurements of platelet aggregation are gradually giving way to alternative userfriendly methodologies that provide similar information on the degree of platelet activation in a blood sample from individual patients that can be used for diagnostic and drug monitoring purposes. The use of flow cytometry with fixation, which enables remote platelet testing, is moving forward rapidly, while some of the very new approaches require further validation before they will find a place in routine practice.

Financial & competing interests disclosure

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Table 1

Approaches to assessment of platelet reactivity.

Abbreviations: LTA: light transmission aggregometry; MEA: multiple electrode aggregometry; PFA: platelet function analyser; PRP: platelet-rich plasma; TEG: thrombelastography; VASP: vasodilator-stimulated phosphoprotein

Approach	Method/ equipment	Advantages	Disadvantages
LTA	Aggregometer	Considered to be the gold standard. Formation of visible platelet aggregates accords with hemostatic plug and thrombus formation. Wide range of uses.	Performed in PRP. Centrifugation required. Platelet counts vary. Prompt access to laboratory facilities and technical expertise required.
	Optimul assay	96-well format enables large number of samples to be assessed in parallel Potential wide range of uses.	As for LTA
Platelet aggregation in whole blood	MEA	Use of whole blood considered to be more physiological than use of PRP. No centrifugation required. Wide range of uses.	Prompt access to laboratory facilities and technical expertise required
	VerifyNow	Use of whole blood considered to be more physiological than use of PRP. Measurements require availability of special equipment but	Specifically designed to monitor efficacy of antiplatelet agents and thus not designed for other uses. Disappointing clinical outcomes

	Platelet counting	<p>minimal technical expertise is required. Point-of-care testing.</p> <p>Use of whole blood considered to be more physiological than use of PRP. Fixation of blood samples can negate the need for immediate platelet counting and can enable remote testing. Wide range of uses.</p>	<p>when used to tailor treatment with antiplatelet agents.</p> <p>A commercial version of the test (Plateletworks) requires immediate access to equipment for platelet counting.</p>
Platelet activation markers	Flow cytometry, e.g., P-selectin	Fixation of blood samples can negate the need for immediate flow cytometric analysis and can enable remote testing. No stirring or agitation of the blood is needed. Wide range of uses.	When used without fixation immediate access to flow cytometry facilities is required.
	Flow cytometry, e.g., platelet-leukocyte conjugates	<p>Fixation of blood samples can negate the need for immediate flow cytometric analysis and can enable remote testing</p> <p>A 96-well format can be used to agitate the blood samples</p> <p>Wide range of uses</p>	When used without fixation immediate access to flow cytometry facilities is required.
Cell metabolites	Flow cytometry, e.g., VASP phosphorylation	VASP phosphorylation reflects cyclic nucleotide levels in Platelets. Can be used to assess efficacy of P2Y12	One approach (use of the Biocytex assay) requires immediate access to flow cytometry.

		antagonists. One approach (use of VASPFix) allows frozen samples to be stored to enable remote testing.	
Other approaches	PFA	An assay based on platelet adhesion to collagen under high shear. Measurements require availability of special equipment but minimal technical expertise is required. Point-of-care testing. The INNOVANCE PFA P2Y cartridge appears to provide an improved means of testing for effects of P2Y12 antagonists.	Further studies are needed with the INNOVANCE PFA P2Y cartridge to define the usefulness of the approach.
	TEG	Deemed to provide a global test of thrombosis based on clot formation. Newer adaptations of the methodology show promise.	Further studies are needed to define the usefulness of the approach.
	Aggreguide	Measurement of platelet aggregation based on light scattering technology.	Still in the early stages of Development.

Executive summary

Platelet reactivity: what is it & why study it?

- Platelet reactivity is a broad term indicating the response of blood platelets to an external stimulus, usually an 'aggregating agent'.

- Platelets play a role in the hemostatic process and also contribute to generation of a thrombus that can block a blood vessel leading heart attack or stroke.
- A pronounced platelet response is associated with an increased risk of thrombosis and a low platelet response is associated with bleeding and bruising.
- Detection of high residual platelet activity despite treatment with antiplatelet agents helps predict future ischemic events.
- Identification of deficiencies in platelet function explains why some individuals are subject to enhanced bleeding or bruising.
- Excessive inhibition of platelet reactivity in patients treated with antiplatelet agents can also be associated with excessive bleeding with implications for surgical interventions in those patients.
- There is now the concept of using measurements of platelet reactivity to identify a 'therapeutic window' in patients receiving antiplatelet agents, so as to optimize a reduction in risk of a thrombotic event without increasing the risk of bleeding.

Approaches to assessment of platelet reactivity

- Measurement of platelet aggregation in platelet-rich plasma using light transmission aggregometry (LTA) is considered to be the 'gold standard' for measurement of reactivity but is complex and technically demanding.
- Measurement of platelet aggregation in whole blood using Multiplate Electrode Aggregometry (MEA), the VerifyNow approach and by platelet counting is deemed to be more physiological than studies performed in platelet-rich plasma.
- Measurement of platelet activation markers including P-selectin provides an alternative approach to measuring platelet reactivity and this introduces the concept of 'remote' platelet testing.
- Phosphorylation of vasodilator-stimulated phosphoprotein (VASP) provides another approach that has found value particularly in assessing the impact of P2Y₁₂ antagonists on platelet reactivity. Methods based on measurements of platelet aggregation.

Light transmission aggregometry:

- Used in identifying deficiencies in platelet aggregation in association with enhanced bruising and bleeding.
- Used in quantitating the pharmacological effects of antiplatelet agents.

- There are positive examples of the use of LTA in determining the variable effects of the P2Y₁₂ antagonist clopidogrel and in considering the implications for re-thrombosis and bleeding.

Multiplate electrode aggregometry:

- Generally data obtained using MEA compare favorably with LTA.
- There are positive examples of the use of MEA for guiding antiplatelet therapy.

VerifyNow:

- Generally data obtained using VerifyNow compare favorably with LTA and MEA, and the advantage of VerifyNow is that little technical expertise is required.
- Clinical trials of using VerifyNow to guide the use of P2Y₁₂ antagonists as antiplatelet therapy have been unsuccessful so far in terms of improving clinical outcomes.

Platelet counting:

- Plateletworks provides a commercially available means of measuring platelet aggregation in whole blood by platelet counting and there are some positive comparisons with LTA.
- There are potential benefits of fixation of platelets prior to platelet counting that have already proved useful in platelet research.

Methods based on measurements of platelet activation markers

- Measurements of P-selectin as a means of studying platelet reactivity include studies in cardiology, stroke, surgery and veterinary medicine.
- The P-selectin approach offers the advantage of remote platelet testing with no need for a laboratory or special technical expertise available close to the point of blood testing.
- A consequence of P-selectin expression on platelets in whole blood is platelet-leukocyte conjugate formation, which can be quantified in parallel with measurements of platelet aggregation.

Methods based on measurements of a cell metabolite

- Measurement of the phosphorylation of VASP provides a convenient means of measuring the effects on platelets of agents that modify cyclic AMP, including P2Y₁₂ antagonists.
- The Biocytex approach involves permeabilizing platelets and measuring VASP-P by flow cytometry.

- The VASPFix approach is a one-step approach that can be used remotely without the immediate availability of a flow cytometer.

Additional approaches to assessing platelet reactivity

- The platelet function analyzer (PFA)-100 is easy to use device that is based on platelet adhesion to collagen.
- The introduction of the INNOVANCE PFA P2Y test cartridge for use in the PFA is being tested with some positive outcomes in assessment of the effects of ADP on platelets and the inhibitory effects of P2Y₁₂ antagonists.
- Thrombelastography measures platelet–fibrin clot formation and has found some application in the studies of platelet reactivity.
- Newer approaches to thrombelastography are being introduced and assessed for measurement of platelet reactivity.
- Aggreguide is a new approach to testing that is based on the light scattering properties of aggregating platelets that is now starting to be assessed for its usefulness in the research and clinical practice.