Monitoring anticoagulation of primary haemostasis

Estimation of platelet function in whole blood assays

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Summary
This article provides an overview on current commercially available methods to determine primary haemostasis as a target of drug-mediated anticoagulation. It focuses on whole blood methods only, and references the currently major achievements that have been reported with each method in respect to its clinical use. Advantages and disadvantages of the various methods are presented, based on considerations of platelet physiology, and on feasibility of the procedures.

Platelets comprise a variety of functional activities, like
• inflammation,
• cell proliferation,
• vasoconstriction,
• interaction with plasmatic coagulation,
• in addition to their well-known and established role in primary haemostasis.

These functions are distinct and maybe independent from each other. Traditionally, the role of platelets in primary haemostasis is considered in the term platelet function. However, primary haemostasis as a sole platelet function has also to be seen in the light of the interaction of platelets with plasma proteins, the prototypes being von Willebrand factor (VWF) and fibrinogen. Further, platelet-platelet interaction is also facilitated by fibrinogen and other proteins, and platelets provide other plasma coagulation factors (e. g. factor V), and their surface for coagulation.

Furthermore, there is growing evidence from clinical studies that platelets participate actively in venous thromboembolic disease, and thus are not only participating in coagulation under high shear conditions. Therefore, a purist view of platelet function in the context of so-called primary haemostasis cannot be maintained. Platelet function needs to be seen in the context of the entire coagulation system, where platelets play a specific role. It needs to be kept in mind, that impairment of the platelet role in primary haemostasis does not necessarily mean defects in other functions. For instance, platelets with impaired aggregation due to glycoprotein GPIIb/IIIa defects in Glanzmann’s thrombasthenia respond to agonists by expressing P-selectin and form platelet-leukocyte aggregates. Indeed, atherosclerosis is not less common in these patients with the inherited platelet function disorder than in the general population (3, 18, 25), but, of course, bleedings are the main concern. In keeping with the traditional view on platelet function and in order to keep the following review clinically simple and applicable, this article will focus on the estimation of traditionally termed primary haemostasis with respect to platelet function.

Primary haemostasis involves a tightly controlled cascade of events where under flow conditions activated platelets play a key role. Vascular damage induces the interaction with collagen, and VWF leading to platelet activation which then through release of numerous activators propagates
• platelet adhesion to the vascular tissue,
• platelet aggregation and
• formation of the platelet plug (12).

In the past, platelet function was assessed mainly for platelet haemostatic capacity to control bleeding. Only in the preceding years, the role of platelet activation became better understood in thromboembolic disease. Treatment with anti-platelet drugs has enhanced the demand to test for platelet function to control anti-platelet drug effects, thereby balancing between the induced increased risk for bleedings and thrombosis.
Bleeding time

The physiologically closest way to test platelet function is by inducing a lesion and to measure the time required to stop bleeding. The bleeding time was inaugurated by Milian in 1901, and again by Duke in 1910, further developed by Ivy and Mielke in 1935 and 1969 (5, 9, 16, 17). A standardized method to test the bleeding time has been developed, involving the subendothelium, tissue components and vessel wall contraction. Thereby, a lesion of defined length and depth is set, using a template for the injury of the microvasculature tissue to determine the skin bleeding time (Simplate, Surgicutt).

This in the 1990s rather popular measurement is not used very often nowadays because it is not very sensitive, nor is it specific, it hurts and leaves a scar. Variables that influence the result include skin thickness, temperature, blood vessel characteristics, orientation of the incision (horizontal versus vertical), localization of the incision, handedness, and others.

LTA

In order to estimate platelet function in vitro, agonists can be added to platelet rich plasma and the increase of light transmission is recorded as platelets start to aggregate. Light-transmission aggregometry (LTA) was first described by Born and O’Brien in 1961/1962 (1, 19).

LTA, however, has a variety of shortcomings, mainly the need to separate platelets from the other cellular components thus losing the physiological environment, some of the platelet population and the lack of significant shear. The clinical application of potent platelet inhibitors therefore forced the development of other means to assess platelet function. Nowadays, a variety of tools are available to test platelet function ex vivo. All these test systems are based on platelet aggregation, and their results are evaluated in comparison to LTA, which is still regarded the gold standard, even though LTA is not even close to physiological conditions.

Whole blood testing

Technically, whole blood systems are advantageous because they do not require preparation of the platelets. Thus, any whole blood system tests not platelets alone, but also their interaction with the other elements in the blood. This appears
- closer to physiological conditions,
- is easier to handle and
- can even be developed to point of care (POC) techniques.

Platelet function analysis

Basically, there are three fields for testing platelet function in association with primary haemostasis:
- inherited platelet function defects,
- disease-associated altered platelet function (like in end-stage renal failure or immune-mediated thrombocytopenia), enhanced platelet activity (diabetes mellitus, metabolic syndrome),
- drug-induced impairment of platelet function (most common nowadays due to treatment with cyclooxygenase inhibitors and ADP receptor inhibitors, but also inhibitors of fibrinogen binding, inhibitors to thrombin receptors, inhibitors to the von Willebrand binding site).

Although this review is dealing with the application to test for the efficacy anti-platelet therapy in the laboratory with currently available whole blood systems (Tab. 1), any of these methods can also be used to estimate platelet function in any other clinical scenario.

Each of the following methods measures a very specific aspect of platelet function in vitro, which only partially mirrors in vivo conditions.

No or low shear systems

Flow cytometry and VASP assay

A variety of aspects of platelets’ contributions to coagulation can be studied by flow cytometry, and the most popular ones have been discussed in detail previously (20). In brief, the platelet glycoprotein composition can be quantified. Thus, major platelet disorders like Glanzmann thrombasthenia or Bernard-Soulier syndrome can be diagnosed with the appropriate monoclonal antibodies. For in vitro assessment of platelet function, a small amount of blood is diluted in buffer and the agonist-induced expression of platelet markers that become exposed upon activation is determined. These are the exposure of the fibrinogen-binding site by either labeling with the PAC-1 monoclonal antibody, which binds to platelets as they undergo the conformational change to expose the fibrinogen binding site, or staining with fluorochrome labeled fibrinogen. Alternatively or in addition, expression of platelet derived proteins from alpha granules, like P-selectin or thrombospordin-1, or lysosome granule content like lysosome-associated membrane proteins, or, degranulation of delta granules, which is associated with mepacrine release. A platelet marker is usually used for the identification of the platelet population. Further, the interaction of platelets with leukocytes and subpopulations thereof can be estimated by the quantification of leukocytes which are positive for a platelet-specific marker, like CD41 or CD61, (leukocyte-platelet-heterotypic aggregates). The residual capacity to activate platelets (activatability) can be assessed if suboptimal rather than optimal concentrations of the agonists are used. Thereby, hypo- and hyper-activatability may be distinguished. Some experience is required and the system is time and labor intensive.

The cAMP-dependent phosphorylation of vasodilator-stimulated phosphoprotein (VASP) assay allows direct measurement of P2Y12 receptor-mediated intracellular effects. Prostaglandin E1 stimulates phosphorylation which is inhibited by ADP through the P2Y12 receptor. Thienopyridines inhibit ADP activity on its receptor, thus allowing VASP phosphorylation which is known to be closely correlated with the inhibition of platelet and fibrinogen receptor (GP IIb/IIIa) activation. An important aspect of this method is its correct interpretation (27).
gation is counting. Once an agonist has been added to the blood sample platelets aggregate and are lost for counting in the upper fluid phase of the tube. A commercialized kit simply compares the number of platelets in a control tube with those after the addition of the agonist (2, 4, 13, 30).

**Impedance aggregometry (Multiplate)**

This system is based on the addition of platelet agonists, like for LTA. As platelets become activated they adhere to electrodes and form aggregates. Thus, the resistance between the electrodes changes and this change is recorded over time. Older systems required washing of the wires and were rather laborious. The new fully computerized system (Multiplate) has 5 channels for simultaneous measurements and uses disposable cuvettes with four silver-plated wires. Thus, each run is performed in duplicate to allow computerized control of the procedure and better reproducibility. The volume of blood and of the added agonists is controlled by the use of an automatic pipette. Furthermore, all samples undergo standardized warming before the blood is stirred and the agonist mixed to it. Any agonist can be used, and each run requires 0.3 ml of hirudin- or heparin-anti-coagulated blood.

**Thrombelastography (ROTEM, TEG Haemoscope)**

Thrombelastography (29) (introduced in 1948 by Hartert) is a whole blood coagulation method that provides information about clot strength and stability, and indirectly about platelet function. Kaolin-added whole blood is placed into a pre-warmed cuvette. A suspended piston is then lowered into the cuvette which oscillates. The normal clot goes quite fast through acceleration and strengthening phase. The fiber strands which interact with activated platelets attach to the surface of the cuvette and the suspended piston. The clot forming in the cuvette transmits its movement onto the suspended piston which is recorded continuously. The resulting thrombelastogram is a continuous graphic record of the physical shape of a clot during fibrin formation and subsequent lysis. The specific contribution of platelets and fibrinogen to the clot formation is evaluated indirectly by the addition of the GPIIb/IIIa monoclonal antibody c7E3 Fab (REOPRO). The antibody eliminates the platelet contribution from the thrombelastogram which becomes a function of fibrinogen activity only. Therefore, subtraction of the results in the absence of the antibody from that obtained with the antibody allows determination of the platelet contribution to clot formation. The effect of aspirin or GPIIb/IIIa inhibitors cannot be detected using thrombelastography.

**Whole blood aggregometry (VerifyNow)**

The VerifyNow system is a turbidimetrically based optical detection system, which measures platelet-induced aggregation as an increase in light transmittance in whole blood. A cartridge containing a platelet activator and fibrinogen-coated beads is filled with the anticoagulated blood and the light transmission upon platelet activation is recorded in duplicate reaction chambers of the cartridge. Citrate anticoagulated whole blood is automatically dispensed from the blood collection tube into the assay device by the instrument. There are cartridges that are specific to test for GPIIb/IIIa inhibitors, aspirin, and P2Y12 antagonists.

**High shear assays**

**Platelet function analyzer (PFA-100)**

The system consists of disposable test cartridges (requires 800 μl of whole blood per run) where a platelet plug occludes a microscopic aperture cut into a membrane coated with collagen and epinephrine (CEPI, Epi-nephrine cartridge ) or collagen and ADP.
(CADP, ADP cartridge). The plug formation occurs under high shear flow conditions (5000/s to 6000/s) produced by a constant vacuum and controlled by a capillary. Platelets become activated, attach, and aggregate, building a stable platelet plug at the aperture. The time required for occlusion (closure time, CT) is indicative of platelet function and primary haemostasis capacity. The device is sensitive to screen for von Willebrand disease; on the other hand, high plasma ristocetin cofactor shortens the CT. The CEPI-CT is very sensitive to Aspirin. A new cartridge is currently evaluated for its sensitivity for monitoring P2Y12 antagonists.

**Cone and plate analyzer (Impact-R)**

The Impact-R which is a commercially available development of the cone and plate(let) analyzer, allows evaluation of platelet function under close to physiologic conditions in a whole blood assay. Adhesion and aggregation are tested. A total of 130 μl of citrate anticoagulated whole blood are placed on the polystyrene plate and shear stress is immediately applied (1800/s) using a specially developed cone. Plates are then washed with tap water and stained with May-Grünewald solution. The resulting adhering objects are analyzed with an inverted light microscope connected to an image analyzing system. Platelet adhesion and aggregation are recorded by examination of the percentage of total area covered with objects, designed surface coverage (SC, %), and average size (AS, μm²) of surface bound objects.

In the modified test (22, 24) whole blood samples are first incubated with an agonist at suboptimal concentration, and then subjected to the Impact-R well. During the incubation platelets will transiently aggregate and therefore not be available for adherence to the polystyrene surface as shear stress is applied. The SC will be significantly lower than without the agonist. However, if platelets do not respond to the respective agonist they will adhere as shear is applied and the SC will be in the range of that seen without the addition of the agonist.

**Major advantages and limitations of the test systems**

Citrate anticoagulated blood which is used for most coagulation assays can be used in all systems but the Multiplate. The latter requires either hirudin or heparin anticoagulated blood. Citrate induces platelet Ca++ flux. Therefore, the blood should rest after collection, so that platelets recover. All assays should be run either within a specific time frame after blood collection (immediately: VerifyNow, Multiplate, platelet-leukocyte interaction by flow cytometry), or about half to one hour after blood collection, but not later than four hours thereafter. These requirements may limit shipping of the samples. The VASP assay may be performed with paraformaldehyde fixed blood samples.

All methods are affected by low platelet counts. Particularly the platelet counting method becomes more inaccurate the lower the platelet count, and platelet-platelet interaction is affected at low platelet counts. Even in flow cytometry, platelet counts less than 50×10⁹/L result in inaccurate data, as platelet-platelet interaction is diminished. Both shear dependent systems, the PFA-100 and the Impact-R, need platelet counts >100×10⁹/L. However, using the Impact-R, platelet function can be estimated even at low platelet numbers by normalizing for the platelet count (21).

All methods except for flow cytometry and platelet counting are affected by low haematocrit. The shear dependent assays are sensitive to both low and high von Willebrand factor and ristocetin cofactor. Thus, due to the experience particularly with the PFA-100 this device may be used to screen for von Willebrand disease or global platelet competence, rather than for the effects of specific platelet inhibitors (10).

Some of the systems have been developed to specifically monitor the response to antiplatelet drugs. The VerifyNow system, designed as a POC assay to monitor GPIIb/IIIa inhibitors was further developed to test for ADP and COX inhibition. The rather high costs of the cartridges could be a limitation for its use in daily clinical practice. Likewise, the PFA-100 is almost a POC device. ROTEM and Multiplate are very easy to use and can therefore be operated after a short training. Both are therefore used in some institutions in the labs that are in proximity to the surgery room, the intensive care unit, and the cardiology catheter lab.

Flow cytometry needs an experienced operator, but the commercially available VASP assay is easy to perform. The currently available cone and plate analyzer Impact-R should be used for research only. The same operator should perform all investigations of a series, as variation due to the operator can affect the results; an automated version was evaluated, but did not meet the requirements for marketing.

**Clinical experience**

**Flow cytometry**

There is widespread experience to assess platelet function by flow cytometry. The more recent reviews that can be found in PubMed deal with drug-induced impairment of platelet function, i.e. monitoring of anti-platelet therapy in atherosclerosis. However, all aspects of platelet function have been addressed by flow cytometry, as the variation of possible applications is almost unlimited.

The VASP assay has been found to correlate nicely with clinical data in patients receiving P2Y12 antagonists (8, 28).

**Multiplate**

Multiplate is a rather new device, but some very important observations, particularly drug-drug interactions have been reported based on measurement with the Multiplate. Furthermore, successful monitoring of aspirin and clopidogrel has been reported, and platelet function under specific research related conditions have been evaluated by this system (26,28).

**ROTEM**

There are numerous papers on this probably oldest method to assess “total coagulation” in the laboratory, and the specific role of platelets’ contribution to clot formation has been addressed in some papers (23).
Data obtained with the VerifyNow have been found to correlate nicely with clinical outcome in patients receiving GPIIb/IIIa inhibitors, aspirin, and P2Y12 antagonists, respectively (8).

**PFA-100**

There are numerous publications with reference to the PFA-100. Indeed, of the easy-to-use assays, there is probably the largest experience with the PFA-100. It has been applied for testing responsiveness to aspirin in numerous papers, correlating these findings with clinical outcome (6, 7, 11). However, it should be emphasized that the data obtained with the PFA-100 are not specific for aspirin.

**Impact-R**

The cone and plate analyzer Impact-R has been evaluated mainly in research projects, and only few data on its applicability to monitor drug-induced platelet function have been published, so far (8, 14, 15).

**General remarks**

There are some studies that have retrospectively correlated the laboratory determined platelet inhibition with clinical outcome. However, none of the studies has compared all the different devices within the same patient population. Further, it is currently unclear if adjustment of therapy can be based on laboratory results to influence the clinical outcome. It may appear that the closer a test system is to the physiological mechanisms of the disease the better the test system.

However, direct COX1 dependent assays to measure aspirin effects are not POC assays, and even though they measure aspirin-induced COX1, aspirin may have additional effects on platelets that are not covered by the determination of thromboxane. Furthermore, thromboxane assays may indicate sufficient aspirin effects which may not be seen by other assays.

The most specific assay to determine blockade of the ADP receptor P2Y12 is the VASP assay. Again, this is not a POC assay, runs without shear, and is independent of platelet red cell and platelet leukocyte interaction. Moreover, ADP may be generated during platelet activation or it may come from red cells. Thus the physiological milieu may play a role in the specificity of any results. ADP may also activate platelets also via P2Y1 which remains undetected by the VASP assay.

A recently published study comparing four methods to determine clopidogrel-induced platelet inhibition with LTA demonstrate a weak concordance of these methods with LTA (8). Thus, LTA as the gold standard may be doubtful and is mainly based on its history as the first widely applicable method to determine platelet function.

**Conclusion**

Currently, there is no single best method to determine platelet inhibition by aspirin, or thienopyridines, or glycoprotein IIb/IIIa inhibitors. Furthermore, standardized assays have only been developed for these platelet antagonists. However, new methods have to be developed because of other anti-platelet drugs in the pipeline, like platelet thrombin receptor antagonists, antagonists to the binding of VWF.

**Conflict of interest**

Andreas Calatzis is co-inventor of the Multiplate® and ROTEM® analyzers and has commercial interests in both methods. Martina Leitner, Simon Panzer: no conflict of interest to disclose.

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