Platelet function testing in clinical diagnostics

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Keywords
Platelet function testing, clinical application, anti-platelet drugs, bleeding risk assessment

Summary
Although the utility of platelet function testing is still under debate, the necessity to inhibit platelets in patients suffering from cardiovascular and cerebrovascular disease is undoubted and well proven. The wide variety of available platelet function tests often using different methodologies, the apparent lack of standardization, and finally the emerging evidence on the clinical value of platelet function testing are resulting in a considerable uncertainty in the clinical practice, how to deal with the issue of platelet function testing. Platelet function testing might not only yield clinical benefits for the patients but also economical advantages by identifying the right drug at the right dose for the right patient.

This article intends to provide an overview of the current platelet function tests such as light transmittance aggregometry, whole blood impedance aggregometry, the PFA-100® system, the VerifyNow® system, flow cytometry, as well as other promising technologies like Plateletworks®, IMPACT-R®, PADA, thromboelastography, and the mean platelet component (MPC), briefly addressing strengths, weaknesses and clinical utility of these tests.

Platelet function testing is currently one of the most discussed topics within the field of haemostasis. Historically, platelet function was first measured to elucidate the reason for unexplained bleedings in patients. Most bleedings in patients are caused by

- defects of primary haemostasis such as von Willebrand disease (VWD),
- inherited platelet disorders, and
- drug-induced platelet dysfunction

Impairment of the plasmatic coagulation system such as factor VIII or factor IX deficiency is less frequent (75). Therefore, preoperative screening of coagulation and primary haemostasis including platelet function should be performed in patients with a potential bleeding risk, which can be identified by a standardized questionnaire (46, 47). For those patients and patients on anti-coagulation or antiplatelet therapy peri-operative monitoring and management of coagulation including primary haemostasis is considered to be mandatory. The role and importance of platelet function is undisputed (60) in diseases such as

- atherothrombosis,
- various vascular diseases,
- diabetes mellitus,
- inflammation,
- angiogenesis,
- tumour growth and metastasis.

Their key role in cardiovascular or cerebrovascular disease renders platelets to be the major pharmacological targets for the treatment of these diseases. Drugs such as acetylsalicylic acid (e.g. Aspirin®) or clopidogrel (Plavix®, Iscover®) are widely and successfully used in secondary prevention of coronary artery disease and stroke. It is well known that the inefficacy of these drugs increases the risk of recurrent thrombosis.

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Platelet function tests

LTA

Light transmittance aggregometry (LTA), invented by Born in the early 1960s (8), is still considered as the gold standard for the evaluation of platelet function (40). The standard medium is platelet-rich plasma (PRP), which is derived from centrifuged whole blood via a mild centrifugation. By adding an agonist to the stirred opaque PRP in a vial at 37°C, the platelets will become activated and start to form aggregates. The increase in light transmittance due to the formation of large aggregates is measured in an aggregometer typically at wavelengths around 600 nm. Platelet-poor plasma (PPP) is normally used as the baseline signal, which represents 100% light transmittance while PRP is defined as 0% light transmittance. LTA is fibrinogen-dependent. Common agonists are:
- collagen,
- ADP,
- epinephrine,
- arachidonic acid,
- thrombin,
- TRAP (thrombin receptor activating peptide),
- PMA (phorbol 12-myristate 13-acetate),
- ristocetin,
- U44619 (thromboxane A2 analog) and
- heparin.

LTA is either used to:
- monitor the efficacy of anti-platelet drugs or
- detect and characterize inherited platelet defects such as:
  - storage pool disease,
  - platelet secretion defects,
  - Glanzmann’s thrombasthenia, or
  - Bernhard-Soulier-syndrome.

Using ristocetin as agonist LTA can also be used to detect von Willebrand disease. In many laboratories a panel of ADP, collagen, epinephrine is typical for the assessment of platelet function, often accompanied by the more specific agonists arachidonic acid and ristocetin (12, 42, 66).

Collagen is typically used at concentrations ranging from 1–5 μg/ml to induce platelet aggregation in LTA. Collagen as a strong agonist is sensitive only at lower concentration to the effect of acetylsalicylic acid or P2Y12-receptor antagonists and platelet secretion deficiencies (45, 66).

The effect of P2Y12-receptor antagonists such as the active metabolites of clopidogrel or prasugrel are measured using ADP at final concentrations between 2 and 20 μmol/l as agonist. LTA induced by 5, 10, or 20 μmol/l ADP is applied for identification of clopidogrel non-responders or the detection of high on-treatment platelet reactivity of patients under clopidogrel therapy. The clinical value of the test results has been shown by an increased risk for recurrent ischemic events of those patients, which are not responding well or at all to administered clopidogrel (9, 10, 56, 87). LTA with lower concentration of ADP is not only more sensitive to the effect of clopidogrel but also more sensitive to the effect of acetylsalicylic acid, which has to be considered for result interpretation. The distribution of the results of LTA induced by 20 μmol/l ADP from 177 cardiovascular disease patients prior to and after ingestion of clopidogrel is shown in Figure 1a. A consensus on the optimal ADP concentration and the best cut-off for the segregation of clopidogrel responders and non-responders has not yet been reached.

Arachidonic acid (AA) induced LTA is typically performed to specifically and sensitively evaluate the effect acetylsalicylic acid on platelets (36, 72). The clinical value of AA-LTA measurements has been shown in prospective clinical studies (32, 37, 86, 87). The most widely used AA concentration is 0.5 mg/ml.

Epinephrine induced platelet aggregometry is either used:
- to measure the effect of acetylsalicylic acid on platelet function or
- to identify patients’ platelet hyperreactivity phenotype (96), storage pool deficiencies or the rare Quebec platelet disorder (66).

The thromboxane analogue U46619 is used at concentrations of 1–2 μmol/l to differentiate thromboxane response defects from congenital, or drug-induced, thromboxane generation defects (42, 66).

Ristocetin-induced platelet aggregation (RIPA) is the only LTA assay influenced by VWF and is therefore used for the detection of VWD typically using the antibiotic ristocetin at concentration of 1.5 mg/ml (31).

Heparin-induced platelet aggregometry (HIPA) is specifically used to detect heparin-induced thrombocytopenia (HIT), a common complication of patients treated with unfractionated heparin (89).

Reference intervals for LTA induced by variety of agonists and agonist concentrations have been reported (42) but most laboratories use self-derived cut-offs or reference intervals, which are often not appropriate (12). The flexibility of LTA is also one of its major problems: The lack of standardization and wide variability in test practices is summarized in the report from the platelet physiology subcommittee of the SSC of the ISTH (12). Efforts for the standardization of LTA have already been undertaken (CLSI guideline H58-A) or are currently developed (ISTH methodological guidelines for LTA).

It should be mentioned that the execution of LTA requires skilled and trained personnel and is rather time consuming.

These characteristics limit the use of LTA in clinical routine.

Impedance aggregometry

In addition to PRP-based LTA, whole blood platelet aggregation (WBA) was developed...
using the change of electrical impedance in the sample due to aggregates being formed at two noble metal electrodes following the addition of a platelet agonist. The use of whole blood as a more physiological sample medium is considered to be an advantage over LTA. Principally, the same agonists as for LTA can be used. Older instruments require a cleaning of the electrodes after each test. WBA detect congenital and acquired platelet disorder and can also be applied to monitor antiplatelet therapy (59).

In a study including 182 patients after PCI those identified as dual non-responders to acetylsalicylic acid and clopidogrel by WBA induced by ADP and collagen were at higher risk (relative risk 2.57; 95% CI 1.18–5.61; log-rank p = 0.03) to suffer from a combined primary endpoint of myocardial infarction, target vessel revascularization, late stent thrombosis, or cardiac death (43).

In contrast to most other platelet function tests a special hirudin blood collection tube has to be used to perform the tests. The Multiplate system transforms the increasing impedance due to platelet aggregation at the electrode into arbitrary aggregation units (AU). The Multiplate system requires no sample preparation, is rather simple to run, and has a quite short time to result (approx. 10 min).

The clinical utility of most tests has been shown in particular in the assessment of the effects of acetylsalicylic acid and clopidogrel (44, 67, 74, 90). Using the ADPtest a low response to a 600 mg loading dose of clopidogrel in 1608 cardiovascular disease patients prior to PCI was significantly associated with an increased risk of stent thrombosis within 30 days (odds ratio: 9.4; 95% CI 3.1–28.4; p < 0.0001) (83). The low response was defined as ADPtest result in the upper quintile of all results obtained. Interestingly, the risk for stent thrombosis in the upper ADPtest quintile of the study population decreased during a follow-up period of 6 months (OR 6.5; 95% CI, 2.4–17.0; p < 0.001), because after the first 30 days the event rate hardly differed from the control group (84). In a smaller study comprising 214 patients a dual non-response to clopidogrel and acetylsalicylic acid as defined by ADP test and ASPI test was independently associated with an increased risk for myocardial infarction, stent thrombosis, and death assessed during the periprocedural period and at 30 days (odds ratio 7.35, 95% CI 2.21–24.42, p < 0.001) (23). In a study on 416 patients with coronary artery disease undergoing PCI platelet hyperreactivity as defined by the hADPtest was predictive for the risk of stent thrombosis (85). The ADPtest was also successfully used to identify patients with enhanced risk of pre- or perioperative platelet concentrates transfusions (77) and preoperative prediction of postoperative bleeding risk using a cut-off of <31 U (78).

Platelet function analyzer (PFA)

The PFA system (Siemens, Marburg, Germany), which has been launched in 1995,
consists of an instrument and disposable test cartridges in which the process of platelet adhesion and aggregation following a vascular injury is simulated in vitro under arterial flow conditions (49). It is currently the most widely used and studied platelet function test in clinical routine with more than 600 publications in peer review journals. Three tests are available, the

- Dade® PFA Collagen/Epinephrine test cartridge (Col/EPI),
- Dade® PFA Collagen/ADP test cartridge (Col/ADP), and
- INNOVANCE® PFA P2Y.

They consist of a number of integrated parts including a capillary, a sample reservoir and a biochemically active membrane with a central aperture with a diameter of 150 μm (Col/ADP, Col/EPI) or 100 μm (P2Y). Whole blood with 0.109 or 0.129 mol/l buffered sodium citrate is aspirated from the sample reservoir through the capillary and the aperture, thereby exposing platelets to high shear flow conditions. The membrane is coated with specific physiologic reagents, collagen and ADP (Col/ADP), collagen and Epinephrine (Col/EPI), and ADP, prostaglandin I1, and calcium chloride (P2Y). At the beginning of a PFA test, trigger solution is dispensed on the membrane to dissolve the reagents. During the test, platelets adhere to the membrane under high shear. Due to the interaction with the membrane and the dissolved reagents, platelets become activated, release their granule contents, and start to aggregate. This process leads to platelet thrombus formation at the aperture thereby gradually diminishing and finally arresting the blood flow.

The PFA system determines the time from the start of the test until the platelet plug occludes the aperture, and reports that time interval as the closure time (CT). The CT is an indicator of platelet function in the analyzed whole blood sample. The PFA-100® system is easy to use and reliable, requires no sample preparation, and has a short time to result (approx. 4–8 min).

Col/EPI and Col/ADP are offered with validated reference intervals in 3.2% and 3.8% buffered sodium citrate, while for INNOVANCE PFA P2Y an analytical cut-off of 106 s valid for both citrate concentrations in cardiovascular disease on or off acetylsalicylic acid therapy is provided. Using specimens collected in 3.8% buffered sodium citrate typical reference intervals for Col/EPI and Col/ADP are 84–160 s and 68–121 s, respectively. With 3.2% buffered sodium citrate as anticoagulant closure times tend on average to be 12% shorter. The result of PFA system is more or less strongly affected by platelet count, von Willebrand factor levels and activity, haematocrit, blood group, and age of the patient tested (24). In addition, inflammation markers like monocyte count and c-reactive protein might also influence the closure time (22).

The Dade PFA Collagen/Epinephrine (Col/EPI) test cartridge is the primary cartridge used to detect platelet dysfunction induced by intrinsic platelet defects, VWD or exposure to platelet inhibiting agents, in particular acetylsalicylic acid (ASA). The Dade PFA Collagen/ADP (Col/ADP) test cartridge is used to indicate if an abnormal result obtained with the Col/EPI test cartridge may have been caused by the effect of ASA or medications containing ASA.

The clinical utility of Col/EPI results has recently been nicely summarized (24). One major application of the PFA-100 system is the detection of von Willebrand disease (VWD). The sensitivity for the detection of VWD has been calculated at 90% (24). Only patients suffering from mild type 1, type 2B, and 2M VWD are occasionally not detected, while the PFA system is principally insensitive to VWD type 2N.

The detection of ASA-related platelet dysfunction is one of the major clinical applications of the Col/EPI test cartridge. Non-responders to ASA as identified by Col/EPI have an increased risk for recurrent ischemic event, which was nicely summarized in three recent reviews (20, 21, 78). In their first review Crescente et al. (21) determined a pooled relative risk of 1.63 (95% CI: 1.16–2.28) for so-called ASA non-responders compared to responders for the occurrence of vascular events based 64 populations from 53 studies, comprising 6,450 subjects. Various independent factors were identified influencing ASA non-response as defined by Col/EPI, namely high age, an acute vascular event, chronic ASA usage, the ASA dosage, diabetes mellitus, the concentration of buffered sodium citrate, and the cut-offs used, which were spread over wide range, although a cut-off is provided by the manufacturer. The authors also concluded that full COX-1 inhibition is not always associated with an abnormal Col/EPI closure time, because platelet hyperreactivity or high of VWF levels might overcome the effect of ASA. In a second study focusing only on prospective studies Crescente et al. (20) calculated an overall fixed-effects odds ratio for the risk of vascular events of ASA non-responders compared to “responders” by Col/EPI of 2.35 (95% CI: 1.96–2.83) based on 19 studies including 3003 patients. This odds ratio was only slightly higher than the global odds ratio (OR) of 2.1 (95% CI 1.4 – 3.4, p < 0.001) for the recurrence of an ischemic event in ASA non-responder relative to responders by Col/EPI derived by Reny et al. (79), which was based on eight prospective studies including 1227 patients.

In a larger prospective study on 700 ASA-treated patients presenting for angiographic evaluation of coronary artery disease, residual platelet COX-1 function measured by serum thromboxane B(2) and COX-1-independent platelet function measured by PFA-100 Col/ADP CT, but not indirect COX-1-dependent assays (arachidonic acid-stimulated platelet markers, shortened PFA-100 Col/EPI CT), correlated with subsequent major adverse cardiovascular events (MACE) (25). After adjustment for covariates (sex, ASA dose, thrombolysis in myocardial infarction risk score, clopidogrel use), both serum thromboxane B2 >3.1 ng/ml (OR 2.399, 95% CI 1.053–5.463, p = 0.0372) and Col/ADP CT <65 s (OR 3.5, 95% CI 1.2–10.4, p = 0.0265) were significantly associated with major adverse cardiovascular events.

A very short or shortened Col/ADP CT has also been identified as a potential marker for platelet hyperreactivity or hyperfunction, which is present in patients suffering from an acute myocardial infarction, an acute coronary syndrome or stable angina, stable cardiovascular disease patients with increased risk of major adverse events, ASA non-responders or patients with genetic polymorphisms associated with a heightened platelet reactivity (16, 29, 30, 34, 35, 50, 96).
Pre-operative screening of patients with a potentially increased bleeding risk is another application for the Col/EPI test cartridge (46, 47). Prior to surgery, patients 5649 unselected adult patients undergoing elective surgery completed a questionnaire to establish a bleeding history. Patients also underwent pre-operative haemostatic testing that included a platelet count, prothrombin time, activated partial thromboplastin time, bleeding time, VWF:Ag assay and PFA-100 Col/EPI and Col/ADP CT. Of 628 patients with a positive bleeding history, 256 had at least one abnormal haemostatic test. Of these 256 patients, 252 had Col/EPI or Col/ADP CTS above the local normal range with blood collected in 3.2% buffered sodium citrate.

Patients with CTS above the reference range had the following disorders:
- 54 VWD,
- 87 associated with ASA use and
- 113 due to other acquired or inherited problems of primary haemostasis.

Sensitivity, specificity, positive (PPV) and negative predictive values (NPV) were determined from ROC analysis (Tab. 1).

The 250 patients identified with prolonged Col/EPI CTS were treated with DDAVP prior to surgery and then tested again with Col/EPI. Previously it was already shown that Col/EPI and Col/ADP can both be used to monitor the effect of DDAVP (28). In 229 patients (91.6%), the Col/EPI CTS were within the laboratory reference range. After the DDAVP treatment blood transfusion needs among these patients were not significantly different than those of patients (n = 5393) with a negative bleeding history and normal primary haemostasis. In a related study of 3102 patients 317 were identified pre-operatively with having abnormal haemostasis, of which 311 demonstrated prolonged Col/EPI CTS. None of these patients were treated with DDAVP prior to surgery. The frequency of blood transfusion associated with surgery was significantly higher (p < 0.001) in these patients than in those with Col/EPI CTS within the normal range (n = 4785). Pre-surgical correction of a prolonged PFA-100 CT as a result of DDAVP treatment, especially with the Col/EPI cartridge, may provide useful information for blood transfusion management in patients undergoing different kinds of elective surgeries.

The recently launched new cartridge for the PFA-100 system INNOVANCE PFA P2Y is intended for the detection of platelet P2Y12-receptor blockade in patients undergoing therapy with a P2Y12-receptor antagonist using citrated human whole blood. This cartridge type has a universal analytical cut-off of 106 s and is provided with guidance for use for better analytical accuracy and easy result interpretation. The distribution of INNOVANCE PFA P2Y closure times prior to and after ingestion of clopidogrel (300 or 600 mg) are shown in Figure 1b. The cartridge is insensitive to the effect ASA on platelet function.

First published data indicates a good sensitivity for the effect of clopidogrel ingestion on platelet function (52). Unfortunately, the first clinical data available appeared to be erroneous (9). In this large clinical study platelet function tests were compared for their ability to predict clinical outcome in patients undergoing coronary stent implantation. INNOVANCE PFA P2Y was included but only became available half-way through the study. Thus, the sample size of INNOVANCE PFA P2Y was too small to have sufficient statistical power to detect the relationship between high on-treatment platelet reactivity and clinical outcome. Following the publication of the study it became apparent that the authors misinterpreted the raw data of the test system during statistical ROC curve analysis (short CTS were classified as being abnormal and prolonged CTS as being normal, which is opposite to the correct interpretation), which resulted in the calculation of a wrong cut-off value and consequently of wrong odds ratios for the clinical endpoints studied. The publication of the corrected paper (an erratum has been submitted to the Journal) will have to show if high on-treatment platelet reactivity as defined by INNOVANCE PFA P2Y using the correct clinical cut-off is associated with the combined clinical endpoint in cardiovascular disease patients (composite of myocardial infarction, death, stent thrombosis, and stroke). If the analytical cut-off provided by the manufacturer (＞106 s) might result in a significant association with the primary end point was not investigated.

The new INNOVANCE PFA-200 with improved connectivity, increased internal memory space and a state-of-the-art user interface including a touch screen has been launched in February 2011. The new software introduces new tools such as different levels of user authorization, a maintenance interface including a touch screen has been launched in February 2011. The new software introduces new tools such as different levels of user authorization, a maintenance record, colour-coded status and error messages, search tool for results stored onboard, and an on-board QC program.

**VerifyNow**

The VerifyNow® System (Accumetrics, San Diego, USA) measures platelet function by the rate and extent of light changes in citrated whole blood as platelets aggregate in the presence of fibrinogen coated latex particles over time in response to different agonists. Within the assay device wells, the instrument measures the increase in light transmittance over time, which is low if platelet function is inhibited and high if platelet function is normal. The measurement is based on principles of LTA and has shown to correlate well with that method. Three different assays for the VerifyNow instrument are currently available, VerifyNow GPIIb/IIIa, VerifyNow Aspirin, and VerifyNow P2Y12. Testing is simple and possible at the point of care, and has a fast time to result (2–5 min).

The VerifyNow GPIIb/IIIa assay was the first to be marketed. The determination of the effect of GPIIb/IIIa-receptor antagonists on platelet function with the assay agrees well with other platelet function tests such as LTA, flow cytometry or platelet counting (57). The clinical utility of the assay was shown in a large prospective study it became apparent that the authors misinterpreted the raw data of the test system during statistical ROC curve analysis (short CTS were classified as being abnormal and prolonged CTS as being normal, which is opposite to the correct interpretation), which resulted in the calculation of a wrong cut-off value and consequently of wrong odds ratios for the clinical endpoints studied. The publication of the corrected paper (an erratum has been submitted to the Journal) will have to show if high on-treatment platelet reactivity as defined by INNOVANCE PFA P2Y using the correct clinical cut-off is associated with the combined clinical endpoint in cardiovascular disease patients (composite of myocardial infarction, death, stent thrombosis, and stroke). If the analytical cut-off provided by the manufacturer (＞106 s) might result in a significant association with the primary end point was not investigated.

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<th>Clinical performance characteristics for PFA-100® Col/EPI and Col/ADP CT in detection of primary haemostasis defects in 628 preoperative patients having a positive bleeding history</th>
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study on 500 patients undergoing PCI treated with GPIIb/IIIa-receptor antagonists (88). The level of platelet function inhibition as measured by the assay was an independent predictor for the risk of MACEs after PCI. The VerifyNow Aspirin assay uses arachidonic acid as agonist and is a qualitative assay to aid in the detection of platelet dysfunction due to acetylsalicylic acid ingestion in a citrated whole blood for the point of care or laboratory setting (54). Results below 550 ARU (ASA reaction units) indicate aspirin-induced platelet dysfunction, while results above 550 ARU indicate the inefficacy or absence of ASA. The test correlates well with AA-induced LTA (68) and is associated with an increased risk of adverse clinical outcomes in stable patients with coronary artery disease (15, 92).

The VerifyNow P2Y12 assay measures platelet aggregation in the presence of ADP and prostaglandin 1 (PG1). In a second baseline channel TRAP (thrombin receptor activating peptide) with PAR4-AP (PAR4 activating peptide) are the platelet activators. The results of each channel are reported as platelet reactivity units (PRU). Malinin et al. (53) report a reference interval for the ADP/PGE1 channel of 194–418 PRU (147 patients). Post-loading of 450 mg clopidogrel the results ranged from 0–368 PRU showing the ability of the assay to measure the effect of P2Y12-receptor blockade on platelet function. In prospective study the PRU values on clopidogrel (12 h post-loading of 600 mg) of 380 patient undergoing PCI were used to derive an optimal cut-off value of ≥235 PRU for post-treatment reactivity in predicting six month out-of-hospital cardiovascular (CV) death, non-fatal MI, or stent thrombosis (76). Using the clinical cut-off patients with high post-treatment reactivity had significantly higher rates of CV death, stent thrombosis, and the combined endpoint. These results were confirmed by a study in 683 patients with acute coronary syndrome undergoing dual-antiplatelet therapy during a percutaneous coronary intervention (55). With the optimal cut-off of ≥240 PRU derived in this study the assay was able to detect acute coronary syndrome patients at risk of 12-month cardiovascular death and nonfatal MI.

Flow cytometry
Flow cytometry rapidly measures the specific characteristics of a large number of individual cells using one or multiple laser beams with distinct wavelength(s). Prior to analysis in the flow cytometer, cells in suspension are typically labeled with monoclonal antibody (MoAb) conjugated with fluorescent dyes. In the instrument the suspended cells pass through a narrow flow chamber at a rate of 1000 to 10,000 cells per minute, where they cross the focused beam of the laser(s). After fluorescent activation of the fluorophore at the excitation wavelength, a detector processes the emitted light scattering properties of each cell.

Platelet activation in the absence or presence of platelet agonists can be measured by determining the expression of granula membrane proteins such as P-selectin on the platelet surface (62). Other measures of platelet activation are PAC1-binding (only binds to the activated GPIIb/IIIa-receptor) and the number of Leukocyte-platelet aggregates (61). The currently most used flow cytometric assay in platelet function testing is the VASP (vasodilator-stimulated phosphoprotein) phosphorylation assay (Biocytex, France), which is used to specifically measure the inhibition of the P2Y12-receptor (81). The result of the assays is expressed as platelet reactivity index (normal range: approx. 65–90%) (3). The assay is considered to be the biochemical gold standard to assess the effect of P2Y12-receptor antagonists (13). Recent studies have demonstrated PRI cut-off values of 48–53% as prognostic indicators for stent thrombosis following coronary percutaneous interventions (5, 7, 27). Bonello et al. (6) reported that clopidogrel loading doses adjusted according to PRI values <50% decreased the rate of major adverse cardiovascular events after PCI. The assay is laborious and rather time-consuming and requires specialized and well-educated staff.

Plateletworks
Plateletworks® (Helena Laboratories, USA) is based on special blood collection containing collagen, ADP, or arachidonic acid. First, a baseline platelet count from a sample of whole blood in a tube containing EDTA is measured with a cell counter. Then, to assess platelet function, the platelet count is again measured with a second sample of whole blood containing the reagents. In the presence of the reagents, uninhibited platelets will activate and aggregate. Aggregated platelets exceeding the threshold limits for platelet size will not be counted as individual platelets in the second sample. The difference in platelet counts between the two samples provides a measure of aggregation, whereas the ratio of the two counts provides a percent inhibition. The method requires a cell counter but is fast and simple, can be performed at point-of-care, and has low reported coefficient of variation.

All assays correlate well according to the LTA assay (19, 93). Breet et al. (9) found an association of the Plateletworks ADP test with the primary endpoint (composite of all-cause death, nonfatal acute myocardial infarction, stent thrombosis, and ischemic stroke) in patients taking clopidogrel undergoing elective coronary stent implantation.

Impact-R
The research use only device Impact-R or Cone and Plate(let) Analyzer (Matis Medical Inc., Belgium) uses a disposable cone, which rotates in a polystyrene plate filled with citrated whole blood. The rotation creates shear rates mimicking the physiological flow condition within the human arteries. Platelet adhesion and aggregation is measured by analyzing the deposition of platelet on the polystyrene surface of the plate. Its clinical utility has been proposed (82) but also doubted (92). Breet et al. (9) did also not find a significant correlation between the result of ADP-induced surface coverage and clinical outcome.

PADA
The platelet adhesion assay (PADA; HaemoSys GmbH, Germany) measures the adhesion of platelets on special polymer
particles by which PADA provides quantitative measurements of platelet adhesive-ness (69). At defined shear rates 0.5 ml of freshly drawn citrated whole blood is mixed with the polymer particles. Proteins of the blood sample, especially fibrinogen, and thereafter also activated platelets, bind to the specific polymer surface. Following platelet counts both in the sample and in a control (blood without particles), the adhesion index (AI) is calculated as a quantitative measure of platelet adhesive-ness. Currently the assays PADA-RASS (70) for the evaluation of the responsiveness to ASA and PADA-HIT for the detection of heparin-induced thrombocytopenia are available (71). Clinical studies demonstrating the utility of the assay(s) are to be performed.

Thromboelastography

Thromboelastography covers the whole process of haemostasis by measuring the mechanical properties of a developing clot in whole blood sample. The two most commonly used thromboelastographs are the

- TEG® system (Haemoscope Corp., USA) and
- ROTEM® system (Tem International GmbH, Germany).

Both systems use an oscillating pin in sample cup with a small volume of citrated whole blood. The resistance during the oscillation is translated into a thromboelastogram, from which the lag time to the start of clot formation, the kinetics of clot formation, the strength of the clot and finally also the dissolution of clot or fibrinolysis can be derived. Thromboelastography is mainly applied to prevent bleedings during general surgery (i.e. trauma), cardiac surgery, or organ transplantations (e.g. liver) by monitoring haemostasis directly at the point of care. The monitoring of platelet function is performed by adding a specific platelet agonist (e.g. arachidonic acid, ADP) to the whole blood sample just prior to the thromboelastographic measurement. Three “platelet mapping™” assays are available for the TEG system,

- a full assay kit (ADP & AA),
- an ADP assay kit (ADP only), and
- an AA assay kit (AA only).

The effect of anti-platelet drugs can be monitored using thromboelastography but the clinical utility of the tests remains to be convincingly shown (3, 26, 17, 80).

Mean platelet component (MPC)

The haematology systems ADVIA 120 / ADVIA 2120 (Siemens Healthcare diagnostics, USA) can measure the mean platelet component (MPC), which is a measure of platelet density. Upon activation platelet density or MPC decreases due to the release of platelet granula content. A low MPC is therefore considered to be an indicator for platelet hyperreactivity and might be used to identify patients with this phenotype (1, 14). A clinical study including 324 patient of which 253 had coronary artery disease did not show a difference (p = 0.467) in MPC between patients with CAD and those with normal coronary arteries 25.8 versus 26.0) (18). A potential reason for this negative result might be the use of EDTA as anticoagulant, which in contrast to CITAD (citrate theophylline dipyridamole adenosine) is apparently not suitable for the determination of platelet activation (2).

Conclusions

A large number of platelet function assays are available. They not only strongly differ in their assay principles but also in the availability of performance and clinical data. The most commonly used techniques or assays are the

- PFA-100 system,
- light transmittance aggregometry,
- whole blood impedance aggregometry in its modern version (Multiplate), the
- VerifyNow system, and flow cytometry.

Clinical outcome studies for these methods show the potential platelet function testing, in particular in secondary prevention of major adverse events due to anti- thrombotic therapy failure.

Method comparison studies often reveal poor agreement between methodologies (9, 41, 58, 73, 94), which nicely highlights the necessity of either standardization or larger prospective clinical studies proving the value of platelet function testing in general and certain techniques or assays in particular. This can only be achieved if not only one platelet function test but a relevant number of tests are included in such large studies.

The identification of patient with a too weak (thrombotic risk) or too strong (bleeding risk) response to anti-platelet drugs could well be the main future application of platelet function testing. For example, using new P2Y-receptor antagonists such as prasugrel and ticagrelor the absolute event rate in patients with acute coronary syndrome can be roughly reduced by 2% (11, 95). That benefit for 2% of all patients comes with the price of an increased bleeding risk and/or higher costs due to the most likely higher sales prices of the new drugs relative to clopidogrel, which is already available in some countries as generic drug. Identifying those patients really benefiting from these new drugs might thus be not only medically but also economically valuable.

New types of anti-thrombotic drugs such as the very promising thrombin receptor (PAR-1) receptor antagonist SCH530348 (Schering-Plough; USA) or the aptamer ARC1779 (Archemix Corp., USA), which blocks binding of the von Willebrand factor to the platelet GPIb receptor, are on the horizon. Like clopidogrel or acetylsalicylic acid this next generation of anti-platelet drugs might also require monitoring or diagnostic guidance by testing their effect on platelet function to optimize therapeutic success. Another major and old problem of platelet function testing besides its diversity is the lack of stable control material allowing state-of-the-art quality control. But a solution for this problem is currently not in sight.

Conflict of interest

Dr. Andreas R. Rechner is an employee of Siemens Healthcare Diagnostics.

References


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Hämostaseologie 2/2011


52. Linnemann B, Schwonberg J, Rechner AR et al. Asses-
ment of clopidogrel non-response by the PFA-100 system using the new test cartridge INNOVANCE PFA P2Y. Ann Hematol 2010; 89:
597–605.
53. Malinin A, Pokov A, Spergling M et al. Monitoring
platelet inhibition after clopidogrel with the Verify
Now-P2Y12(R) rapid analyzer. Thromb Res 2007;
54. Malinin AI, Atar D, Callahan KP et al. Effect of a
single dose aspirin on platelets in humans with mul-
tiple risk factors for coronary artery disease. Eur
55. Marucci R, Gori AM, Paniccia R et al. Cardiovascu-
lar death and nonfatal myocardial infarction in ac-
ute coronary syndrome patients receiving coro-
rary stenting are predicted by residual platelet reac-
tivity to ADP detected by a point-of-care assay. Cir-
56. Matetzky S, Shenkman B, Guetta V et al. Clopido-
grel resistance is associated with increased risk of
derurrent atherothrombotic events in patients with ac-
ute myocardial infarction. Circulation 2004; 109:
3171–3175.
57. Matzdorff AC, Kühnel G, Kernkes-Matthes B, Voss
R. Comparison of GP IIb/IIa inhibitors and their
action as measured by aggregometry, flow cyt-
ometry, single platelet counting, and the rapid pla-
etelet function analyzer. J Thromb Thrombolysis
58. McGlasson DL, Fritsma GA. Comparison of four
laboratory methods to assess aspirin sensitivity.
59. McGlasson DL, Fritsma GA. Whole blood platelet
aggregometry and platelet function testing. Semin
60. Michelson AD (ed.). Platelets. Boston: Academic
61. Michelson AD, Bardnar MR, Krueger LA et al.
Circulating monocyte-platelet aggregates are a
more sensitive marker of in vivo platelet activation
than platelet surface P-selectin. Circulation 2001;
104: 1533–1537.
62. Michelson AD, Linden MD, Bardnar MR et al. Flow
cytometry. In: Michelson AD (ed.). Platelets. Bos-
63. Michelson AD. Flow cytometry: a clinical test of
64. Michelson AD. P2Y12 antagonism: promises and
challenges. Arterioscler Thromb Vasc Biol 2008;
65. Michiels JJ, Gawaz M. Preface: platelets in inflam-
mation and atherothrombosis. Semin Thromb Hemost
2005; 31: 549–553.
66. Nowak G, Wiesenburg A, Schumann A, Bucha E.
Platelet adhesion assay—a new quantitative whole
67. Nowak G. From aspirin to aspirin resistance—His-
try, biochemical background, diagnosis and
clinical relevance. Transfus Med Hemother 2007;
34: 413–419.
68. Nielsen HL, Kristensen SD, Thygesen SS et al. As-
pirin resistance evaluated by the VerifyNow Aspirin
69. Nowak G, Wiesenburg A, Schumann A, Bucha E.
Platelet function testing—a new quantitative whole
70. Nowak G. From aspirin to aspirin resistance—His-
try, biochemical background, diagnosis and clinical relevance. Transfus Med Hemother 2007;
34: 413–419.
71. Nowak G. Heparin-induced thrombocytopenia
(HIT II) — a drug-associated autoimmune disease.
72. Pacini R, Antonucci E, Gori AM et al. Compari-
sion of different methods to evaluate the effect of
aspirin on platelet function in high-risk patients with
ischemic heart disease receiving dual antiplatelet
73. Pacini R, Antonucci E, Maggini N et al. Compari-
sion of methods for monitoring residual platelet
reactivity after clopidogrel by point-of-care tests on
74. Pfanner G, Kociech J, Pernerstorfer T et al. Resus-
citation and intensive care. Preoperative evaluation of
the bleeding history. Recommendations of the
working group on perioperative coagulation of the
Austrian Society for Anaesthesia, Resuscitation and
75. Price MJ, Endemann S, Gollapudi RR et al. Prog-
nostic significance of post-clopidogrel platelet reac-
tivity assessed by a point-of-care assay on throm-
botic events after drug-eluting stent implantation.
76. Rahe-Meyer N, Winterhalter M, Boden A et al. Pla-
etelet concentrations transfuse in cardiac surgery and
platelet function assessment by multiple elec-
53: 168–175.
77. Ranucci M, Baryshnikova E, Soro G et al. Multiple
platelet-peroxidase aggregation and bleeding in
adult cardiac surgery patients receiving thienopyri-
78. Reny JL, De Moorloose P, Daoutz MH, Fontana P. Use
of the PFA-100 closure time to predict cardiovascu-
lar events in aspirin-treated cardiovascular patients:
a systematic review and meta-analysis. J Thromb
79. Schmarbert G, Auer A, Kozek-Langenecker S. Evalu-
ation of the platelet mapping assay on rotational
thromboelastometry ROTEM. Platelets 2009; 20:
125–130.
80. Schwarz UR, Geiger J, Walter U, Eigenthaler M.
Flow cytometry analysis of intracellular VASP
phosphorylation for the assessment of activating
and inhibitory signal transduction pathways in
human platelets. Thromb Haemost 1999; 82:
1145–1152.
81. Shenkman B, Einav Y, Salomon O et al. Testing
agonist-induced platelet aggregation by the Im-
82. Sibbing D, Braun S, Morath T et al. Platelet reactiv-
ity after clopidogrel treatment assessed with point-
of-care analysis and early drug-eluting stent throm-
83. Sibbing D, Morath T, Braun S et al. Clopidogrel
response status assessed with Multiplate point-of-
care analysis and the incidence and timing of stent
thrombosis over six months following coronary
84. Siller-Matula JM, Christie G, Lang IM et al. Multiple
electrode aggregometry predicts stent thrombosis
better than the vasodilator-stimulated phospho-
85. Snoep JD, Hovens MM, Eikenboom JC et al. Associ-
ation of laboratory-defined aspirin resistance with
a higher risk of recurrent cardiovascular events: a
systematic review and meta-analysis. Arch Intern
86. Thielemann M, Bunschlosk M, Tossios P et al.
Perioperative thrombocytopenia in cardiac surgical
patients — incidence of heparin-induced thrombo-
cytopenia, morbidities and mortality. Eur J Car-
87. Toth O, Calatays A, Penz S et al. Multiple electrode
aggregometry: a new device to measure platelet
aggregation in whole blood. Thromb Haemost 2006;
A comparison between the Plateletworks-assay and
light transmittance aggregometry for monitoring the
inhibitory effects of clopidogrel. Int J Cardiol
2010; 140: 123–126.
89. Von Beckerath N, Sibbing D, Jawansky S et al. As-
essment of platelet response to clopidogrel with
multiple electrode aggregometry, the VerifyNow
P2Y12 analyzer and platelet Vasodilator-Stimulated
Phosphoprotein flow cytometry. Blood Coagul Fi-
90. Vyvotic JD, Straathof EJW, van Zundert P et al.
Dose-dependence of the VerifyNow system to monitor anti-
91. Wiviott SD, Braunwald E, McCabe CH et al. TRI-
TON-TIMI 38 Investigators. Prasugrel versus clopi-
dogrel in patients with acute coronary syndromes.
92. Yee DL, Bergeron AL, Sun CW et al. Platelet hyper-
reactivity generalizes to multiple forms of stimu-