Multiplate® platelet function analysis - application and interpretation

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The clinical data and algorithms presented in this compendium are based upon the experience of the authors and on the discussion with centres that use the Multiplate system in clinical routine. However, this data still requires prospective validation with respect to its clinical predictivity. Therapeutic interventions based on Multiplate analyses are the sole responsibility of the treating physician.

The authors thank Alby Pattison of Hart Biologicals for valuable discussions on this compendium.

The following terms are registered trademarks: Multiplate® (Dynabyte medical), Aspirin® (Bayer), Reopro® (Centocor) and Aggrastat® (MSD).
Introduction

This compendium is intended to give an overview over the device, its applications and limitations.

The Multiplate analyzer is a new platelet function analyzer utilising the analysis of whole blood.

Whole blood is the physiological environment, where platelet function takes place in vivo, and the use of whole blood for in-vitro testing eliminates the need for time-consuming centrifugation steps.

Several test reagents are available to allow triggering of different receptors / signal transduction pathways of the platelet in order to detect its function or drug effects.

The Multiplate detects the effects of the platelet inhibitors Aspirin®, clopidogrel and GpIIb/IIIa antagonists. Also the sensitivity for direct ADP receptor antagonists has been shown.

The use of a small amount of whole blood (0.3 ml per test) allows the differentiated assessment of platelet function without drawing large amounts of blood (as required for analysis of Born aggregation) and facilitates the analysis of blood from children and also in experimental settings (animals).

The term Multiplate is derived from the phrase "multiple platelet function analyzer". Multiplate stands for the multiple electrodes in the disposable test cell (4 electrodes form 2 independent sensor units), multiple channels of the instrument (5) as well as multiple test procedures available for a comprehensive assessment of platelet function.

The instrument

The Multiplate instrument is a compact device with 5 channels for parallel tests and an internal Windows XP based computer system. User inputs are performed with the mouse and keyboard. Using the optional electronic pipette the application of the device can be simplified using computer-assisted operation procedures.

Single use test cell

Multiplate analysis takes place in a single use test cell, which incorporates a dual sensor unit and a teflon-coated stirring magnet.

The principle of Multiplate analysis is based on the fact that platelets get sticky upon activation, and therefore have a tendency to adhere and aggregate on metal sensor wires in the Multiplate test cell.

The test cell has a pipetting inlet, a cup portion with the sensor wires, which protrude into the blood and a jack portion, which allows to connect the test cell to the instrument in order to record the electrical resistance between the sensor wires during the test.

The sensor wires are made of highly conductive copper, which is silver-coated. When activated platelets adhere onto the sensor wires the electrical resistance between the wires rises, which is continuously registered.
Multiplate® detection principle

Impedance aggregometry was developed by Cardinal and Flower1 and has been used since the 1980’s for the assessment of platelet function in whole blood.

Impedance aggregometry is based on the principle that blood platelets are non-thrombogenic in their resting state, but expose receptors on their surface when they get activated which allow them to attach on vascular injuries and artificial surfaces.

When platelets stick on the Multiplate sensor wires, they enhance the electrical resistance between them, which is continuously recorded. In order to enhance the resistance on the sensor wires a tight attachment of the platelets is required. On the scanning electron microscopy shown on this page a typical platelet aggregate on the Multiplate sensor wire is shown (picture by Armin Reininger of Munich University Clinic). Various states of platelet activation are visible, such as spreading of platelets on the surface as well as platelets aggregated with each other.

The fact that aggregation in Multiplate takes place on surfaces is a major difference compared to methods such as Born aggregometry and single platelet counting.

In Born aggregometry and single platelet counting methods, platelets aggregate with each other in the liquid phase. This presumably happens only in severely ill patients (e.g. during HIT type II and DIC) as coagulation and platelet aggregation in-vivo usually only take place on surfaces (vascular injuries / inflamed vessels / atheromatous plaques).

Dual sensor in Multiplate® analysis

Every Multiplate test cell incorporates two independent sensor units, each consisting of 2 silver-coated highly conductive copper wires with a length of 3.2 mm. The instrument detects the impedance change of each sensor separately. The impedance change is expressed in arbitrary „Aggregation Units“ (AU). Pearson’s correlation coefficient of the data points detected by each channels is calculated. If the correlation coefficient is lower than 0.98 a quality control flag is added to the measurement and the user is asked whether he wants to repeat the measurement. In addition the areas under the aggregation curve detected by each channels are compared and if the difference is higher than 20% (vs. the mean curve) again the user is prompted by the Multiplate software.

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Multiplate® test results:

Multiplate continuously records platelet aggregation. The increase of impedance by the attachment of platelets onto the Multiplate sensors is transformed to arbitrary aggregation units (AU) and plotted against time.

Three parameters are calculated: The most important parameter is the area under the aggregation curve (AUC). It is affected by the total height of the aggregation curve as well as by its slope and is best suited to express the overall platelet activity.

Two more parameters are calculated for research use: the aggregation is the height of the curve. The velocity is the maximum slope of the curve.

As seen on the diagram on the right, two curves are assessed using the two independent sensors in the test cell. The parameters calculated by the software are the mean values of the parameters determined with each curve.

When the option „print additional values“ in the service menu is selected, then the individual values of each curve are printed in addition to the mean values.

The scale of the y axis is shown both on the screen and on the printout. It can be set to 50, 75, 100 or 200AU.

The unit of the AUC is AU * min (as the y-axis is the aggregation, expressed in Aggregation units (AU) and the x-axis is the time, expressed in minutes). Alternately The AUC can be expressed in U (1 U corresponds to 10 AU*min).

The aggregation and velocity are calculated for research use.

The correlation coefficient (cc) between the values of the 2 individual curves is determined. The analysis is accepted when the cc is at least 0.98.

The difference from the mean curve (DIF) is calculated based on the AUC values of the 2 individual measured curves. The analysis is accepted when the difference is lower than 20% (vs. the mean value of the 2 curves).

new scale for the AUC → 10 AU*min = 1 U
**Multiplate® tests: Sensitivity to anti-platelet agents**

<table>
<thead>
<tr>
<th>Test</th>
<th>Activation</th>
<th>Sensitivity</th>
</tr>
</thead>
<tbody>
<tr>
<td>TRAPtest</td>
<td>direct activation of the thrombin receptor using the peptide TRAP (thrombin receptor activating peptide)</td>
<td>Aspirin® - Clopidogrel - GPIIb/IIIa Ant. +</td>
</tr>
<tr>
<td>ASPItest</td>
<td>activation via arachidonic acid. Arachidonic acid is the substrate of the cyclooxygenase (target enzyme of Aspirin®).</td>
<td>++ - +</td>
</tr>
<tr>
<td>ADPtest</td>
<td>activation by ADP</td>
<td>- + +</td>
</tr>
<tr>
<td>ADPtest HS</td>
<td>activation by ADP with the addition of the inhibitor Prostaglandin E1 (PG).</td>
<td>- ++ +</td>
</tr>
</tbody>
</table>

*For thrombin inhibitor blood. For heparin and citrated blood please inquire with the manufacturer. It is advised to determine locally own reference ranges.*

The main application of the Multiplate system so far is the monitoring of platelet function inhibitors. Several tests are available which possess a different sensitivity towards the various anti-platelet agents.

TRAPtest, by triggering the thrombin receptor is not sensitive towards a blockade of cyclooxygenase by Aspirin® or the ADP receptor by clopidogrel (there might be some minor effect on TRAPtest, however normally aggregation is still in the normal range). All Multiplate tests are affected by a blockade of the GpIIb/IIIa receptor. This shows that the binding of platelets to the metal sensors is dependent on the GpIIb/IIIa receptor.

ASPItest is highly sensitive to Aspirin® but not towards a blockade of the ADP receptor by clopidogrel.

ADPtest again is sensitive towards the ADP receptor blockade, but not towards the inhibition of cyclooxygenase.

The addition of prostaglandin E1 to ADP enhances the sensitivity of the ADPtest towards clopidogrel, but leads also to an increased proportion of samples from non-clopidogrel treated individuals which show only a very weak aggregation (lower specificity compared to ADPtest).

The typical workup for monitoring of Aspirin is ASPItest or ASPItest + TRAPtest. In case of the performance of ASPItest + TRAPtest the difference of the two tests can help to assume the inhibitory function of Aspirin® (aggregation is normally quite similar in TRAPtest and ASPItest in the non-Aspirin® treated individual, while aggregation is typically much weaker in ASPItest when the patient is on Aspirin® treatment).

For the monitoring of clopidogrel we recommend to perform ADPtest + ADPtest HS. Also for this indication the additional performance of TRAPtest can help to assess the baseline aggregability of platelets.
**ASPltest: Expected values**

Values obtained with thrombin inhibitor blood.
For heparin or citrated blood inquire with the manufacturer.

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In ASPltest, platelets are activated by arachidonic acid, which is converted by the platelet cyclooxygenase (COX) to the potent platelet agonist Thromboxane A2. Arachidonic acid alone is not a platelet agonist. Therefore the platelet activation in ASPltest allows a very sensitive and specific detection of Aspirin action. Arachidonic acid is the physiological substrate of the platelet COX.

Aspirin and NSAID can block the platelet COX, resulting in a reduced aggregation in ASPltest. However, the potency of Aspirin is higher compared to NSAID.

ASPltest can also be reduced when other drugs interfering with platelet aggregation are taken, or in case of more global platelet disorders, e.g. in hematological disorders, thrombocytopenia, etc.

On the left the distribution of ASPltest in 5 cohorts is shown. Healthy blood donors showed a good aggregation in ASPltest with a median of almost 100 U. More than 90% had an aggregation of at least 75 U in ASPltest. In in-patients without aspirin treatment more than 75% of the patients had an ASPltest of at least 80 U. However some patients may also have a reduced platelet aggregation, due to other drugs interfering with COX activity or other comorbidities.

In all the three cohorts examined under aspirin treatment, the majority of patients showed strongly decreased aggregations in ASPltest with values lower than 30 U, which we therefore regard as preliminary cut-off. Patients with higher ASPltest values show an incomplete or no platelet inhibition and may thus have an increased risk for arterial thromboembolism compared to patients with aggregation values below the cut-off.\(^1\)

By definition Aspirin therapy should block arachidonic acid induced aggregation. Potential strategies to treat an Aspirin non-response as determined by this method\(^1\) would be either an increase of the aspirin dose (most likely treating Aspirin non-response because of reduced aspirin absorption), an increase in frequency of Aspirin\(^\circledR\) administration (in case of suspected elevated turn-over of platelets in the body), or switching the medication to a different drug (e.g. clopidogrel).

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1 Whether an increased ASPltest under Aspirin\(^\circledR\) treatment leads to increased thrombembolic risk has not yet been prospectively evaluated.

2 It is advised to determine locally own reference ranges.
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ADPtest: Expected values
Values obtained with thrombin inhibitor blood.
For heparin blood inquire with the manufacturer.
We do not recommend the use of citrated blood for the monitoring of clopidogrel.

In ADPtest platelets are activated by ADP, which triggers several receptors on the platelet surface. Clopidogrel and related drugs block the P2Y12 ADP receptor, which is believed to be the most important receptor for ADP on the platelet surface.

ADPtest can also be reduced when other drugs interfering with platelet aggregation are taken, or in case of more global platelet disorders, e.g. in hematological disorders, thrombocytopenia, etc. An ingestion of Aspirin leads typically to no or only a minor inhibition of aggregation in ADPtest.

On the left the distribution of ADPtest in 4 cohorts is shown. Healthy blood donors showed a good aggregation in ADPtest with a median of 85 U. 90% had an aggregation of at least 60 U in ADPtest. For in-patients without clopidogrel treatment more than 75% of the patients had an ADPtest of at least 50 U in cohort 1 and 60 U in cohort 2. However, some patients may also have a reduced platelet aggregation, due to other drugs interfering with platelet activity or due to comorbidities.

In the patients tested who were on clopidogrel treatment, approx. 75% of patients showed decreased aggregation in ADPtest with values lower than 50 U, which we therefore regard as a preliminary cut-off. Patients with higher ADPtest values show an incomplete or no platelet inhibition and may thus have an increased risk for arterial thromboembolism compared with patients with aggregation values below the cut-off.

Potential strategies to treat an clopidogrel non response as determined by this method would be either an increase of the clopidogrel dose, an increase in frequency of clopidogrel administration, switching the medication to a different drug (e.g. Aspirin®, Aggrastat for short-term use) or adding a second drug to the treatment.

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In the patients tested who were on clopidogrel treatment, approx. 75% of patients showed decreased aggregation in ADPtest with values lower than 50 U, which we therefore regard as a preliminary cut-off. Patients with higher ADPtest values show an incomplete or no platelet inhibition and may thus have an increased risk for arterial thromboembolism compared with patients with aggregation values below the cut-off.

Potential strategies to treat an clopidogrel non response as determined by this method would be either an increase of the clopidogrel dose, an increase in frequency of clopidogrel administration, switching the medication to a different drug (e.g. Aspirin® - Aggrastat for short-term use) or adding a second drug to the treatment.

1 Whether an increased ADPtest under clopidogrel treatment leads to increased thrombembolic risk has not yet been prospectively evaluated.

2 It is advised to determine locally own reference ranges.
ADPtest HS (high sensitivity):
Expected values

NB: ADPtest HS was previously referred to as ADP+PG

Values obtained with thrombin inhibitor blood.

ADPtest HS: reference range based on the results of healthy blood donors: 31-107

In ADPtest under clopidogrel therapy an incomplete blockade of aggregation is often seen. This may be due to the fact that ADP not only triggers the P2Y12 ADP receptor on the platelet surface (i.e. the receptor that is blocked by clopidogrel), but also other ADP receptors (which are not affected by clopidogrel).

Heptinstal, Fox et al. have shown that a combination of ADP and a physiological platelet inhibitor (prostaglandin E1 = PGE1) can be more sensitive for the detection of the action of clopidogrel than the use of ADP alone.

The binding of ADP to the P2Y12 receptor reduces the level of cAMP in the platelet which in turn enhances the release of calcium from endogenous sources. The enhancement of intracellular calcium then leads to the activation and aggregation of the platelets. PGE1 reduces the mobilisation of calcium and thus inhibits platelet aggregation. As clopidogrel also reduces the platelet activation by ADP, clopidogrel and PGE1 act synergistically.

On the left the distribution of ADPtest HS in 3 cohorts is shown. Healthy blood donors showed a good aggregation in ADPtest HS with a median of 68 U. 90% had an aggregation of more than 40 U in ADPtest HS. In in-patients without clopidogrel treatment almost 75% of the patients had an ADPtest HS of at least 40 U. However some patients may also have a reduced platelet aggregation, due other drugs interfering with platelet activity or other comorbidities.

In the patients examined under clopidogrel treatment, more than 75% of patients showed decreased aggregation in ADPtest with values lower than 25 U, which we therefore regard as a preliminary cut-off.

Patients with higher ADPtest HS values show an incomplete or no platelet inhibition and may thus have an increased risk for arterial thromboembolism compared with patients with aggregation values below the cut-off.

1 It is advised to determine locally own reference ranges.

2 Whether an increased ADPtest HS under clopidogrel treatment leads to increased thromboembolic risk has not yet been prospectively evaluated.


NB: ADPtest HS was previously referred to as ADP+PG
COLtest leads to platelet activation via the platelet collagen receptor. For suitable platelet activation, the release of endogenous arachidonic acid from the platelet phospholipids is necessary, which is then transformed to the platelet agonist thromboxane A2 by the enzyme cyclooxygenase. Cyclooxygenase is blocked by Aspirin®, and therefore COLtest is sensitive to Aspirin® action.

However, this mechanism also explains why COLtest is less specific towards the action of Aspirin® compared to ASPItest. In ASPItest, a defined amount of arachidonic acid is used as the activator, while in COLtest, platelet are activated through endogenous TXA2 and other TXA2 independent mechanisms.

The distribution of COLtest values in the two groups shown on the left confirm the Aspirin® sensitivity of the assay. However, the distinction between the two groups (blood donors and Aspirin® treated patients) is weaker than with the use of ASPItest. Therefore, we prefer the use of ASPItest for the monitoring of Aspirin® responsiveness.

Collagen in aqueous solution has to be stored at 4-8°C and loses activity within few hours at room temperature. Appropriate storage of the reagent is therefore essential for the use in the test.

It is important to know that unlike arachidonic acid or ADP, collagen is a less clearly defined activator and there are large differences between different commercially available collagen preparations when used for whole blood aggregometry on Multiplate.

Other collagen preparations that work well on Multiplate are the collagen by Nycomed (Horm) or Chronolog. Using these collagens, it is possible to receive a very high aggregation signal when higher concentrations of collagen (e.g., 10 µg/ml) are used. Then, the platelet activation is not Aspirin®-sensitive.

It is advised to determine locally own reference ranges. Whether an increased COLtest in patients under Aspirin® treatment leads to increased thromboembolic risk has not yet been prospectively evaluated.
Screening for Aspirin Responsiveness After Transient Ischemic Attack and Stroke

Comparison of 2 Point-of-Care Platelet Function Tests With Optical Aggregometry

Paul Harrison, PhD; Helen Segal, PhD; Kevin Blasbery, BSc; Charlene Furtado, BSc; Louise Silver, MSc; Peter M. Rothwell, PhD, FRCP

Background and Purpose—Recent studies suggest that patients who do not respond to aspirin (ASA) therapy may be at increased risk of ischemic vascular events. The availability of simple to use point-of-care (POC) platelet function tests now potentially allows aspirin nonresponsiveness to be identified in routine clinical practice. However, there are very few data on whether the different tests produce consistent results. We therefore compared 2 POC tests (PFA-100 device and the Ultegra-RPFA [RPFA]) with conventional light transmission aggregometry (LTA).

Methods—Platelet function was assessed by all 3 tests in 100 patients receiving low-dose ASA therapy after transient ischemic attack (TIA) or ischemic stroke.

Results—The incidence of ASA nonresponsiveness was 17% by the RPFA and 22% by the PFA-100, compared with only 5% by LTA (ie, as defined with both arachidonic acid and ADP). Agreement between the RPFA and the PFA-100 and arachidonic acid induced LTA was poor (κ=0.16, 95% CI, −0.08 to 0.39, P=0.11; and κ=0.09 −0.12 to 0.30, P=0.32, respectively). Agreement between the 2 POC tests was also poor (κ=0.14, −0.08 to 0.36, P=0.15). Only 2% of patients were aspirin nonresponders by all 3 tests.

Conclusions—The prevalence of apparent ASA nonresponsiveness was higher with both the POC tests than with LTA. However, agreement between the tests was poor and very few patients were ASA nonresponsive by all 3 tests. Aspirin nonresponsiveness is therefore highly test-specific and large prospective studies will be required to determine the prognostic value of each of the separate tests. (Stroke. 2005;36:1001-1005.)

Key Words: aspirin • platelets

A spirin (ASA) reduces the relative risk of major vascular events and vascular death by ≈20% after ischemic stroke and acute coronary syndrome.1 However, the antiplatelet properties of ASA are not uniform between individuals and recurrent events in some patients may be caused by “ASA resistance” or ASA nonresponsiveness.2–10 The reported incidence of ASA nonresponsiveness varies widely (between 5% and 60%), partly because there is no accepted standard definition based on either clinical or laboratory criteria. Recently it has been proposed that the term “ASA resistance” should only be used as a description of the failure of ASA to inhibit thromboxane A2 production, irrespective of a nonspecific test of platelet function.9

There is now some evidence that ASA nonresponsive individuals as detected by platelet function tests may be at increased risk of ischemic vascular events.11,12 Although it could therefore be argued that the response to ASA should be monitored, the platelet function tests that has been shown possibly to be of prognostic value (light transmission aggregometry [LTA]) is time-consuming and difficult and cannot realistically be performed on large numbers of patients in routine practice. However, 2 simpler “point-of-care” (POC) tests of platelet function are now available, the PFA-100 and the Ultegra-RPFA-VerifyNow ASA test (RPFA),13,14 which could offer the possibility of the rapid and reliable identification of ASA nonresponsive patients, without the requirement of a specialized laboratory. Although some studies have suggested that these tests can detect ASA nonresponders and could be therefore clinically informative, there have been few validation studies and/or direct comparisons of these tests with LTA. We therefore compared LTA with both the PFA-100 and the RPFA in 100 patients with transient ischemic attack or stroke receiving daily low-dose ASA treatment.

Materials and Methods

100 patients were recruited from the Oxford Vascular Study (OXVASC). OXVASC is an ongoing population-based study of all patients with transient ischemic attack and stroke in a population of 92 000 in Oxfordshire, UK, the methods of which have been reported in detail previously.15,16 The 100 patients were recruited during 2
separate time periods a few months apart but were otherwise a consecutive series, with all eligible patients recruited during the 2 time periods. Patients with a personal or family history of bleeding disorders, with a platelet count <90×10^9/L or >450×10^9/L, a hemoglobin <8 g/dL, and having undergone major surgery within 1 week of enrollment were excluded. All patients were tested at their first follow-up assessment 1 month after initial presentation. All had been taking ASA 75 to 150 mg daily for at least 4 weeks. The study was approved by the Oxford Radcliffe Hospitals ethics committee and signed/informed consent was obtained from all patients. In addition, 6 control samples from normal volunteers (3 before and 2 after 300 mg aspirin in vivo and 1 sample before and after incubation with 100 μmol/L ASA in vitro) were also tested.

**Blood Sampling and Processing**

3×2.6 mL of blood was anticoagulated with one-tenth volume 3.2% buffered trisodium citrate within Vacutainer tubes (Becton Dickinson). An additional 1.8 mL of blood was taken into the special citrated Vacutainer tube for RPFA analysis (Accumetrics). All assays were performed within 2 hours of sampling.

**Platelet Aggregation**

Platelet-rich plasma was prepared by centrifugation at 250 g for 10 minutes. The platelet-rich plasma was removed and then platelet-poor plasma prepared by further centrifugation at 2000 g for 20 minutes. Aggregation was performed using a Biodata-PAP-4 aggregometer (Alpha Laboratories) within 300 μL minicuvettes stirred at 900 rpm at 37°C. The 100% line was set using platelet-poor plasma and a 0% baseline established with platelet-rich plasma (adjusted to 20×10^9/L) before addition of 1 of 2 different agonists—arachidonic acid and ADP (final concentrations of 1 mg/mL and 10 μmol/L, respectively). The percent aggregation after 10 minutes was recorded. An aspirin response was defined as <20% aggregation with 1 mg/mL arachidonic acid and <70% aggregation with 10 μmol/L ADP in a similar fashion as reported by Gum et al.3

**PFA-100**

The PFA-100 (Dade-Behring) simulates high shear platelet function within test cartridges.17–20 Blood is aspirated under constant vacuum from the sample reservoir through a capillary and a microscopic aperture (147 μm) cut into a membrane. The membrane is coated with collagen/epinephrine (CEPI) or collagen/ADP (CADP). Platelet adhesion, activation, and aggregation result in formation of a platelet plug within the aperture. Platelet function is thus measured as a function of the time (closure time [CT]) it takes to occlude the aperture. When normal individuals ingest varying dosages (75 to 1000 mg) of ASA there is a 450–450 μmol/L ASA at 37°C in

**RPFA**

RPFA: Accumetrics Inc (a turbidimetric-based optical detection system that measures platelet-induced aggregation).11 This device was originally developed as a POC testing instrument to provide a result <550 ARU and there is an assigned cutoff of 550 ARU. Therefore, control samples or ASA-nonresponsive individuals would be expected to give values >550 ARU. Therefore, control samples or ASA-nonresponsive individuals would be expected to give values <550 ARU.

### Statistical Analysis

All statistical analysis was performed using SPSS (version 10.0) and Analyze-it. Agreement between the different tests was determined by kappa statistics and 95% confidence intervals (CIs) were calculated. Kappa values of <0.20 are taken to indicate poor agreement, 0.21 to 0.40 indicate fair agreement, 0.41 to 0.60 indicate moderate agreement, 0.61 to 0.80 indicate good agreement, and >0.81 indicate very good agreement.

### Results

Of the 100 patients studied, 50 were male and ages ranged from 40 to 105 years (Table 1). All had taken daily ASA (75 mg daily in 97 cases and 150 mg daily in 3 cases) for at least 4 weeks, 6 were also taking clopidogrel 75 mg daily, and 2 were taking dipivydamole 600 mg daily. None was taking regular nonsteroidal anti-inflammatory agents, although this had not been an exclusion criterion.

<table>
<thead>
<tr>
<th>Presenting Event</th>
<th>Number</th>
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<tbody>
<tr>
<td>Minor ischemic stroke</td>
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<td>TIA</td>
<td>41</td>
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<td>Possible TIA</td>
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<td>Mean age, y (SD)</td>
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<tr>
<td>Aspirin before most recent event</td>
<td>38</td>
</tr>
<tr>
<td>Statin therapy</td>
<td>69</td>
</tr>
</tbody>
</table>

MI indicates myocardial infarction; SD, standard deviation; TIA, transient ischemic attack.

and activate platelets.29 The instrument simply measures changes in light transmission automatically and thus the rate of aggregation. Precision testing using VerifyNow ASA level 1 (n=3×20) and level 2 wet quality controls (n=3×20) gave coefficients of variation of 3.2%, 3.5%, and 5.4%, and 4.3%, 2.6%, and 4.5%, respectively (Accumetrics VerifyNow ASA product information sheet).

Blood tubes were mixed and placed into RPFA cartridges (lot numbers WD0021 and W17507 preloaded into the RPFA instrument; Accumetrics). Results are expressed as aspirin reaction units (ARU) and there is an assigned cutoff of 550 ARU. Therefore, control samples or ASA-nonresponsive individuals would be expected to give values >550 ARU. ASA responders give values <550 ARU.

<table>
<thead>
<tr>
<th>Presenting Event</th>
<th>Number</th>
</tr>
</thead>
<tbody>
<tr>
<td>Minor ischemic stroke</td>
<td>56</td>
</tr>
<tr>
<td>TIA</td>
<td>41</td>
</tr>
<tr>
<td>Possible TIA</td>
<td>3</td>
</tr>
<tr>
<td>Mean age, y (SD)</td>
<td>72</td>
</tr>
<tr>
<td>Male</td>
<td>50</td>
</tr>
<tr>
<td>Previous MI</td>
<td>6</td>
</tr>
<tr>
<td>Previous stroke</td>
<td>11</td>
</tr>
<tr>
<td>Previous TIA</td>
<td>15</td>
</tr>
<tr>
<td>Previous angina</td>
<td>15</td>
</tr>
<tr>
<td>Hypertension</td>
<td>53</td>
</tr>
<tr>
<td>Diabetes mellitus</td>
<td>15</td>
</tr>
<tr>
<td>Current smoker</td>
<td>12</td>
</tr>
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<td>Ex-smoker</td>
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TABLE 1. Characteristics of the 100 Patients Studied

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Bleeding Time

Gale Encyclopedia of Medicine, 3rd ed. | 2006 | Lohr, John |

Bleeding Time

Definition

Bleeding time is a crude test of hemostasis (the arrest or stopping of bleeding). It indicates how well platelets interact with blood vessel walls to form blood clots.

Purpose

Bleeding time is used most often to detect qualitative defects of platelets, such as Von Willebrand's disease. The test helps identify people who have defects in their platelet function. This is the ability of blood to clot following a wound or trauma. Normally, platelets interact with the walls of blood vessels to cause a blood clot. There are many factors in the clotting mechanism, and they are initiated by platelets. The bleeding time test is usually used on patients who have a history of prolonged bleeding after cuts, or who have a family history of bleeding disorders. Also, the bleeding time test is sometimes performed as a preoperative test to determine a patient's likely bleeding response during and after surgery. However, in patients with no history of bleeding problems, or who are not taking anti-inflammatory drugs, the bleeding time test is not usually necessary.

Precautions

Before administering the test, patients should be questioned about what medications they may be taking. Some medications will adversely affect the results of the bleeding time test. These medications include anticoagulants, diuretics, anticancer drugs, sulfonamides, thiazide, aspirin and aspirin-containing preparations, and nonsteroidal anti-inflammatory drugs. The test may also be affected by anemia (a deficiency in red blood cells). Since the taking of aspirin or related drugs are the most common cause of prolonged bleeding time, no aspirin should be taken two weeks prior to the test.

Description

There are four methods to perform the bleeding test. The Ivy method is the traditional format for this test. In the Ivy method, a blood pressure cuff is placed on the upper arm and inflated to 40 mM Hg. A lancet or scalpel blade is used to make a stab wound on the underside of the forearm. An automatic, spring-loaded blade device is most commonly used to make a standard-sized cut. The area stabbed is selected so that no superficial or visible veins are cut. These veins, because of their size, may have longer bleeding times, especially in people with bleeding defects. The time from when the stab wound is made until all bleeding has stopped is measured and is called the bleeding time. Every 30 seconds, filter paper or a paper towel is used to draw off the blood. The test is finished when bleeding has stopped completely.
The three other methods of performing the bleeding test are the template, modified template, and Duke methods. The template and modified template methods are variations of the Ivy method. A blood pressure cuff is used and the skin on the forearm prepared as in the Ivy method. A template is placed over the area to be stabbed and two incisions are made in the forearm using the template as a location guide. The main difference between the template and the modified method is the length of the cut made.

For the Duke method, a nick is made in an ear lobe or a fingertip is pricked to cause bleeding. As in the Ivy method, the test is timed from the start of bleeding until bleeding is completely stopped. The disadvantage to the Duke method is that the pressure on the blood veins in the stab area is not constant and the results achieved are less reliable. The advantage to the Duke method is that no scar remains after the test. The other methods may result in a tiny, hairline scar where the wound was made. However, this is largely a cosmetic concern.

**Preparation**

There is no special preparation required of the patient for this test. The area to be stabbed should be wiped clean with an alcohol pad. The alcohol should be left on the skin long enough for it to kill bacteria at the wound site. The alcohol must be removed before stabbing the arm because alcohol will adversely affect the tests results by inhibiting clotting.

**Aftercare**

If a prolonged bleeding time is caused by unknown factors or diseases, further testing is required to identify the exact cause of the bleeding problem.

**Normal results**

A normal bleeding time for the Ivy method is less than five minutes from the time of the stab until all bleeding from the wound stops. Some texts extend the normal range to eight minutes. Normal values for the template method range up to eight minutes, while for the modified template methods, up to 10 minutes is considered normal. Normal for the Duke method is three minutes.

**Abnormal results**

A bleeding time that is longer than normal is an abnormal result. The test should be stopped if the patient hasn't stopped bleeding by 20-30 minutes. Bleeding time is longer when the normal function of platelets is impaired, or there are a lower-than-normal number of platelets in the blood.

A longer-than-normal bleeding time can indicate that one of several defects in hemostasis is present, including severe **thrombocytopenia**, platelet dysfunction, vascular defects, Von Willebrand's disease, or other abnormalities.
Low Responsiveness to Clopidogrel and Sirolimus or Paclitaxel-Eluting Stent Thrombosis

David Antoniucci *

Angela Migliorini, Piergiovanni Buonamici, Rossella Marcucci, Gian Franco Gensini, Alberto Santini, Rita Paniccia, Guia Moschi, Anna Maria Gori, Rosanna Abbate

Department of Cardiology, Careggi Hospital, Florence, Italy

Abstract

Condensed Abstract
A total of 804 patients who had sirolimus or paclitaxel-eluting stent had the assessment of residual platelet reactivity after a loading dose of 600 mg of clopidogrel. The incidence of stent thrombosis was 8.6% in patients non-responders to clopidogrel and 2.3% in responders (p < 0.001). By multivariate analysis the predictors of stent thrombosis were: non-responsiveness to clopidogrel (P = 0.009), left ventricular ejection fraction (p = 0.001), total stent length (p = 0.010), and ST-segment elevation acute myocardial infarction (p = 0.041). Non-responsiveness to clopidogrel is a strong independent predictor of stent thrombosis in patients receiving drug-eluting stents.

Abbreviations
PCI = percutaneous coronary intervention
LVEF = left ventricular ejection fraction
MLD = minimum lumen diameter
AMI = acute myocardial infarction
ADP = adenosine 5’-diphosphate

Dual antiplatelet regimen of aspirin and clopidogrel is the standard treatment for the prevention of stent thrombosis [1-3], and retrospective studies have shown that discontinuation of clopidogrel, even after six months or later after stent implantation, is associated with an increased risk of thrombotic events in patients with drug-eluting stents [4-7]. However, stent thrombosis can occur also in patients assuming clopidogrel and aspirin, and it has been shown that patients who suffered stent thrombosis had a high in vitro residual platelet reactivity despite the dual antiplatelet treatment suggesting platelet aggregation non-responsiveness to clopidogrel as a main cause of the thrombotic event [8-12]. The definite demonstration of the association between low in vitro responsiveness to clopidogrel and thrombotic events is still lacking because the large majority of previous studies were retrospective, or underpowered. Moreover, residual platelet reactivity may interact with one or more established clinical and procedural predictors of stent thrombosis, making difficult the definition of its role in precipitating thrombosis, and studies used different platelet reactivity assessments and definitions for determining the platelet responsiveness to clopidogrel. This prospective study sought to determine the impact of low responsiveness to clopidogrel on the clinical outcome of patients receiving drug-eluting stents.

Methods
Patients
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Patients
This study is based on a cohort of 804 consecutive patients who received successful sirolimus- or paclitaxel-


Abstract

Objective To determine whether non-responsiveness to clopidogrel as revealed by high in vitro residual platelet reactivity is predictive of drug-eluting stent thrombosis.

Background No data exist about the impact of non-responsiveness to clopidogrel on the risk of drug-eluting stent thrombosis.

Methods Prospective observational cohort study conducted from July 2005 to August 2006 in an academic hospital. A total of 804 patients who had successful sirolimus or paclitaxel-eluting stent implantation had the assessment of residual platelet reactivity after a loading dose of 600 mg of clopidogrel. Patients with platelet aggregation by 10 µmol ADP ≥ 70% were defined as non-responders. All patients received chronic dual antiplatelet treatment (aspirin 325 mg and clopidogrel 75 mg daily) for 6 months. The primary endpoint was the incidence of definite/probable early, subacute and late stent thrombosis at 6-month follow-up.

Results The incidence of 6-month definite/probable stent thrombosis was 3.1%. All stent thromboses were subacute or late. Out of 804 patients, 105 (13%) were non-responder to clopidogrel. The incidence of stent thrombosis was 8.6% in non-responders, and 2.3% in responders (p < 0.001). By multivariate analysis the predictors of stent thrombosis were: non-responsiveness to clopidogrel (HR 3.08, 95% CI 1.32-7.16; p = 0.009), left ventricular ejection fraction (HR 0.95, 95% CI 0.92-0.98; p = 0.001), total stent length (HR 1.01, 95% CI 1.00-1.02; p = 0.010), and ST-segment elevation acute myocardial infarction (HR 2.41, 95%CI 1.04-5.63; p = 0.041).

Conclusions Non-responsiveness to clopidogrel is a strong independent predictor of stent thrombosis in patients receiving sirolimus- or paclitaxel-eluting stents.

CV of the author

- Author of several articles published in the top rank medical journals mainly on coronary artery disease and interventional cardiology. Impact factor of the last year 2005 (> 80 points).
- Frequently invited speaker in the most important international meetings on Cardiology (ACC, ESC, TCT, PCR, JIM).
- Member of the Italian Federation of Cardiology, European Society of Cardiology, Sociedad Argentina de Cardiologia.
- Member of the Scientific Board of the Journal EuroIntervention.
- Member of the Editorial Board of the Journal of the American College of Cardiology.
- Member of the steering committee of the following international randomized trials: ACE
- ASSENT 4, BRAVE 2, and JETSTENT trials.

Publication: October 2007
Impact of Platelet Reactivity After Clopidogrel Administration on Drug-Eluting Stent Thrombosis

Piergiorgi Buonamici, MD, Rossella Marcucci, MD, Angela Migliorini, MD, Gian Franco Gensini, MD, Alberto Santini, MD, Rita Paniccia, MD, Guia Moschi, MD, Anna Maria Gori, MD, Rosanna Abbate, MD, David Antoniucci, MD

Florence, Italy

Objectives
We sought to determine whether nonresponsiveness to clopidogrel as revealed by high in vitro post-treatment platelet reactivity is predictive of drug-eluting stent (DES) thrombosis.

Background
No data exist about the impact of nonresponsiveness to clopidogrel on the risk of DES thrombosis.

Methods
We conducted a prospective observational cohort study from July 2005 to August 2006 in an academic hospital. A total of 804 patients who had successful sirolimus- or paclitaxel-eluting stent implantation were assessed for post-treatment platelet reactivity after a loading dose of 600 mg of clopidogrel. Patients with platelet aggregation by 10 μmol adenosine 5'-diphosphate ≥70% were defined as nonresponders. All patients received chronic dual antiplatelet treatment (aspirin 325 mg and clopidogrel 75 mg daily) for 6 months. The primary end point was the incidence of definite/probable early, subacute, and late stent thrombosis at 6-month follow-up.

Results
The incidence of 6-month definite/probable stent thrombosis was 3.1%. All stent thromboses were subacute or late. Of 804 patients, 105 (13%) were not responsive to clopidogrel. The incidence of stent thrombosis was 8.6% in nonresponders and 2.3% in responders (p < 0.001). By multivariate analysis, the predictors of stent thrombosis were as follows: nonresponsiveness to clopidogrel (hazard ratio [HR] 3.08, 95% confidence interval [CI] 1.32 to 7.16; p = 0.009), left ventricular ejection fraction (HR 0.95, 95% CI 0.92 to 0.98; p = 0.001), total stent length (HR 1.01, 95% CI 1.00 to 1.02; p = 0.010), and ST-segment elevation acute myocardial infarction (HR 2.41, 95% CI 1.04 to 5.63; p = 0.041).

Conclusions
Nonresponsiveness to clopidogrel is a strong independent predictor of stent thrombosis in patients receiving sirolimus- or paclitaxel-eluting stents. (J Am Coll Cardiol 2007;49:2312–7) © 2007 by the American College of Cardiology Foundation

A dual antiplatelet regimen of aspirin and clopidogrel is the standard treatment for the prevention of stent thrombosis (1–3), and retrospective studies have shown that the discontinuation of clopidogrel, even after 6 months or later after stent implantation, is associated with an increased risk of thrombotic events in patients with drug-eluting stents (DES) (4–7). However, stent thrombosis also can occur in patients taking clopidogrel and aspirin, and it has been shown that patients who suffer stent thrombosis have a high in vitro post-treatment platelet reactivity despite the dual antiplatelet treatment, suggesting that platelet aggregation nonresponsiveness to clopidogrel is the main cause of the thrombotic event (8–12). The definite demonstration of the association between low in vitro responsiveness to clopidogrel and thrombotic events is still lacking because the large majority of previous studies were retrospective or underpowered. Moreover, post-treatment platelet reactivity may interact with 1 or more established clinical and procedural predictors of stent thrombosis, making it difficult to define its role in precipitating thrombosis. In addition, studies have used different platelet reactivity assessments and definitions for determining the platelet responsiveness to clopidogrel. This prospective study sought to determine the impact of low responsiveness to clopidogrel on the clinical outcome of patients receiving DES.

See page 2318

Methods

Patients. This study is based on a cohort of 804 consecutive patients who received successful sirolimus- or paclitaxel-eluting stent implantation and for whom platelet reactivity after clopidogrel treatment was prospectively as-
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Relationships Between Optical Aggregometry (Type Born) and Flow Cytometry in Evaluating ADP-Induced Platelet Activation

Silverio Sbrana,1# Francesca Della Pina,1 Antonio Rizza,2 Manuela Buffa,1 Rossella De Filippis,1 Jacopo Gianetti,1 and Aldo Clerico3

1Laboratory of Hematology and Flow Cytometry, CNR Institute of Clinical Physiology, Massa, Italy
2Cardiology and Hemodynamics Department, CNR Institute of Clinical Physiology, Massa, Italy
3Scuola Superiore S. Anna, Pisa, Italy

Background: Platelet response to activating agents is used to monitor the efficacy of anti-aggregation therapies. The aim of our study has been to demonstrate the existence of relationships between early events of ADP-induced platelet activation, measured by flow cytometry and platelet-rich plasma aggregation, quantified by optical aggregometry.

Methods: We evaluated peripheral blood of 12 donors. The following parameters were quantified by cytometry after stimulation with adenosine diphosphate (ADP) (0.5, 1, 2, 5, 10, 20 μM): CD62P (P-selectin) and PAC-1 expression, and cytosolic Ca2+ mobilization. Aggregation was measured by optical aggregometry. We also studied 13 patients, undergoing coronary stenting, treated with aspirin (before procedure) or with aspirin plus clopidogrel (after procedure). We evaluated CD62P and PAC-1 expression, aggregation, and vasodilator-stimulated phosphoprotein phosphorylation (platelet reactivity index, PRI).

Results: Flow procedures were more sensitive than aggregometry, with a lowest interindividual variability. Linear relationships existed in donors between CD62P expression and Ca2+ mobilization (P < 0.0001), and between aggregation and Ca2+ mobilization (P < 0.0001). Linear relationships existed between aggregation and CD62P expression, as percentage (P < 0.0001), or relative fluorescence intensity (RFI) (P < 0.0001). Exponential equations related aggregation and PAC-1 expression, as percentage (P < 0.0001), or RFI (P < 0.0001). Linear relationships between aggregation and CD62P expression (as percentage) existed in the patients before (P = 0.0022) and after procedure (P = 0.0020). Exponential relationships between aggregation and PAC-1 expression (as percentage) existed before (P = 0.0012) and after procedure (P = 0.0024). Linear correlations related aggregation response predicted on CD62P expression, and measured aggregation inhibition after clopidogrel (P = 0.0013) as well as predicted aggregation and PRI inhibition (P = 0.0031).

Conclusions: Tight relationships between aggregation and cytometric quantification of platelet markers in whole blood, in particular CD62P, allow to predict aggregation response to ADP from flow data in patients treated with aspirin alone or with aspirin plus clopidogrel.

Key terms: platelet; ADP; optical aggregometry; flow cytometry; anti-aggregation therapies

How to cite this article: Sbrana S, Pina FD, Rizza A, Buffa M, De Filippis R, Gianetti J, Clerico A. Relationships between optical aggregometry (type born) and flow cytometry in evaluating ADP-induced platelet activation. Cytometry Part B 2008; 74B: 30–39.

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Multiplate® instrument

- 5 channels for parallel tests
- Easy to use Windows XP based software
- Automatic analysis and documentation
- Duplicate sensor for internal quality control
- Electronic pipetting
<table>
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<tr>
<th>test</th>
<th>activation</th>
<th>sensitivity</th>
<th>not sensitive for</th>
</tr>
</thead>
<tbody>
<tr>
<td>ASPItest</td>
<td>arachidonic acid: is converted to TXA2 by platelet-own cyclooxygenase</td>
<td>aspirin, IIb/IIIa antagonists</td>
<td>clopidogrel, vWF</td>
</tr>
<tr>
<td>ADPtest</td>
<td>ADP: binds onto platelet ADP receptors</td>
<td>clopidogrel, IIb/IIIa antagonists</td>
<td>aspirin, vWF</td>
</tr>
<tr>
<td>ADPtest HS</td>
<td>ADP + prostaglandin E1 (Prostaglandin is a natural inhibitor and enhances the sensitivity of the assay for clopidogrel)</td>
<td>clopidogrel, IIb/IIIa antagonists</td>
<td>aspirin, vWF</td>
</tr>
<tr>
<td>TRAPtest</td>
<td>TRAP-6 (thrombin receptor activating peptide): TRAP-6 is a potent agonist which mimicks the platelet-activating action of thrombin</td>
<td>IIb/IIIa antagonists</td>
<td>vWF, aspirin, clopidogrel (weak effect on TRAPtest)</td>
</tr>
<tr>
<td>COLtest</td>
<td>collagen: collagen activates platelet and triggers a release of arachidonic acid from the platelet membrane, which is converted to TXA2 by the Cyclooxygenase</td>
<td>aspirin, IIb/IIIa antagonists</td>
<td>clopidogrel, vWF</td>
</tr>
<tr>
<td>RISTOtest</td>
<td>Ristocetin: vWF dependent platelet activation via the GpIb receptor</td>
<td>Bernard-Soulier syndrome, severe vWD, aspirin</td>
<td>mild vWD</td>
</tr>
</tbody>
</table>
multiple electrode aggregometry = MEA

- one Multiplate test cell incorporates two independent sensor units.
- the increase of impedance due to the attachment of platelets to the electrodes is detected for each sensor unit separately and transformed to arbitrary aggregation units (AU) that are plotted against time.
- the duplicate sensors serve as an internal control
- during each measurement Pearson´s correlation coefficient of single measurements of the curves assessed by the two electrode pairs and the difference of the two AUCs is calculated. The result is flagged if the values are outside of the acceptance range (correlation coefficient <0.98, difference to the mean curve >20%).

Analysis and Regulation of Vasodilator-stimulated Phosphoprotein Serine 239 Phosphorylation in Vitro and in Intact Cells Using a Phosphospecific Monoclonal Antibody*

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The development and functional analysis of a monoclonal antibody (16C2) are reported; the antibody recognizes vasodilator-stimulated phosphoprotein (VASP), an established substrate of both cAMP- and cGMP-dependent protein kinase (cAPK) only when serine 239 is phosphorylated. VASP serine 239 represents one of the best characterized cGMP-dependent protein kinase phosphorylation sites in vitro and in intact cells. Experiments with purified, recombinant human VASP and various VASP constructs with mutated phosphorylation sites (S157A, S239A, T278A) and experiments with intact cells (human/rat platelets and other cells) treated with cyclic nucleotide-elevating agents demonstrated the specificity of the monoclonal antibody 16C2. Quantitative analysis of the VASP shift from 46 to 50 kDa (indicating VASP serine 157 phosphorylation) and the appearance of VASP detected by the 16C2 monoclonal antibody (VASP serine 239 phosphorylation) in human platelets stimulated by selective protein kinase activators confirmed that serine 239 is the VASP phosphorylation site preferred by cGMP-dependent protein kinase in intact cells. Immunofluorescence experiments with human platelets treated with cGMP analogs showed that the 16C2 monoclonal antibody also detects VASP serine 239 phosphorylation in situ at established intracellular localization sites. Analysis of VASP serine 239 phosphorylation by the 16C2 antibody appears to be the best method presently available to measure cGMP-dependent protein kinase activation in intact cells. Also, the 16C2 antibody promises to be an excellent tool for the evaluation of VASP function in intact cells.

Although cGMP-dependent protein kinases (cGPKs)1 have been recognized as important components of major signal transduction pathways (1–3), quantitative analysis of cGPK activation in intact cells has been very difficult (1–4). This is because of the relatively low expression of cGPK in most cell types compared with the relatively high expression of its closest functional homolog, the cAMP-dependent protein kinase (cAPK), and the scarcity of specific cGPK substrates. Unfortunately, the mediating role of cGPK for a given effect/function is often implied or excluded by the use of cGPK activators and/or inhibitors alone, which is clearly insufficient to establish or rule out functional roles of cGPKs (1–4). One of the few established cGPK substrates is the 46-kDa/50-kDa vasodilator-stimulated phosphoprotein (VASP), which was initially discovered and characterized as a substrate of both cAPK and cGPK in human platelets (5–8). VASP phosphorylation in response to cyclic nucleotide-regulating vasodilators (i.e. cAMP-elevating prostaglandins and cGMP-elevating nitric oxide donors) closely correlates with platelet inhibition and in particular with the inhibition of fibrinogen binding to the integrin αIIbβ3 of human platelets (9–11). Molecular cloning of human, canine, and mouse VASP predicted highly homologous proteins and revealed a proline-rich protein that is organized into three structural segments of different sequence complexity (12, 13). VASP is the founding member of a new family of proline-rich proteins, which includes Enabled (Ena), a dose-dependent suppressor of Drosophila Abl- and Disabled-dependent phenotypes, its mammalian homolog Mena, and the Ena-VASP-like protein Evl (14–16). These proteins all share an overall domain organization consisting of highly homologous NH₂-terminal and COOH-terminal domains (Ena-VASP homology domains 1 and 2, EVH1 and EVH2), which are separated by a proline-rich central domain of low complexity (12–16). In platelets and many other cells including vascular smooth muscle cells, endothelial cells, and fibroblasts, VASP has been found to be associated with stress fibers, focal adhesions, cell-cell contacts, and highly dynamic membrane regions (16, 17). VASP colocalizes with profilins and binds directly to their poly(proline) binding site (18), binds to and colocalizes with zyxin and vinculin (16, 19), and also directly binds to Listeria monocytogenes surface protein ActA, which is essential for the actin polymerization-based intracellular motility of this pathogen (20). Functional evidence indicates that VASP is a crucial factor involved in the enhancement of spatially confined actin filament formation (16, 20, 21). Three distinct phosphorylation sites were biochemically identified in VASP (serine 157, serine 239, and threonine 278) which are used in vitro and in intact human platelets by both cAPK and cGPK and by the serine/threonine protein phosphatas 2A and 2B with overlapping selectivity (8, 22). Phosphorylation of serine 157, the site preferred by the cAPK, leads to a marked shift in apparent molecular mass of VASP in SDS-PAGE from 46 kDa to 50 kDa (6, 8). In experiments with

1 The abbreviations used are: cGPK(s), cGMP-dependent protein kinase(s); cAPK, cAMP-dependent protein kinase; VASP, vasodilator-stimulated phosphoprotein; PAGE, polyacrylamide gel electrophoresis; Fmoc, N-(9-fluorenyl)methoxycarbonyl; PBS, phosphate-buffered saline; VSV, vesicular stomatitis virus; FRP, platelet-rich plasma; PGI₂, prostaglandin I₂ (prostacyclin); PGE₂, prostaglandin E₂; SNP, sodium nitroprusside; 8-pCPT-cGMP, 8-(4-chlorophenylthio)-cGMP; DCL-cBIMPS, Sp-5,6-dichloro-1-nitroprusside; 5,6-DCL-cBIMPS, Sp-5,6-dichloro-1-β,γ-dinitro-4-tert-butyloxymethylbenzimidazole-3,5–monophosphorothioate.
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Will Measuring Vasodilator-Stimulated Phosphoprotein Phosphorylation Help Us Optimize the Loading Dose of Clopidogrel?*

Neal S. Kleiman, MD, FACC

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Thienopyridines have robust effects on the ischemic manifestations of coronary artery disease. Randomized clinical trials have shown that treatment with thienopyridines dramatically lowers the rates of intracoronary stent thrombosis (1); reduces the rate of cardiovascular death, stroke, or reinfarction in patients with non-ST-segment elevation acute coronary syndromes (2); and reduces the likelihood of death after treatment for ST-segment elevation myocardial infarction (3). Yet, for the past 5 years we have known that the biological response to clopidogrel, the best-tolerated and most widely used thienopyridine currently available, is heterogeneous (4). Patients in whom platelet reactivity remains persistently elevated after treatment with clopidogrel may be at elevated risk for serious ischemic events, such as thrombosis of an intracoronary stent or myocardial infarction.

Although the heterogeneity of the response to clopidogrel is fairly well characterized, it is poorly understood. A variety of explanations have invoked genetic variation of its target receptor, variability in the hepatic conversion of clopidogrel to its active metabolite, and drug–drug interactions. It is also unclear to what extent increasing the dose of clopidogrel used as an initial load can increase the biological effect of the drug on platelets. Use of an initial loading dose of clopidogrel, as compared with a maintenance dose only, shortens the time required to achieve maximum inhibition of platelet aggregation from about 24 to 6 h; increasing the loading dose further to 600 mg shortens this period to about 2 h. Whether global implementation of a 900-mg loading dose shortens this time further and leads to a more profound antithrombotic effect is controversial.

In this issue of the *Journal*, Bonello et al. (5) studied a novel variant of personalized medicine to determine whether a more profound antiplatelet effect could be achieved by further augmenting the loading dose in selected patients (i.e., those in whom the antiplatelet effect of clopidogrel is minimal). Understanding this approach requires understanding the action of clopidogrel on the platelet. Clopidogrel is converted (as are the other thienopyridines) by members of the hepatic cytochrome P450 family from a prodrug to an active metabolite that has only recently been identified (4,6). The prodrug antagonizes the action of adenosine diphosphate (ADP) (released by other activated platelets and by damaged cells) on P2Y12, 1 of the 3 purinergic receptors on the human platelet. Platelet aggregation and activation responses to ADP are initiated by another receptor, P2Y1, but cannot be completed without activation of P2Y12. The ADP-induced activation of P2Y12 causes, through a G protein-based intracellular signaling pathway, a decrease in adenyl cyclase resulting in lower levels of intracellular cyclic adenosine monophosphate (cAMP). Cyclic AMP is a necessary co-factor for phosphorylation of a protein known as vasodilator-stimulated phosphoprotein (VASP) (7). Vasodilator-stimulated phosphoprotein is important in the platelet for regulation of the actin cytoskeleton and for conversion of glycoprotein IIb/IIIa to its active conformation, thus permitting platelets to aggregate; VASP exists in both phosphorylated and dephosphorylated states. The phosphorylated form is inactive. Inhibition of cAMP activity after activation of P2Y12 by ADP leads to an increase in VASP dephosphorylation, whereas blockade of P2Y12 by the active metabolite of a thienopyridine leads to an increase in phosphorylated VASP. Thus, the ratio of phosphorylated to dephosphorylated VASP reports the degree of P2Y12 blockade (i.e., activity of the active thienopyridine metabolite that has bound P2Y12) (8). Whereas measurement of VASP phosphorylation originally required Western blotting techniques, flow cytometry now allows this measurement to be performed with considerably greater ease and precision (9,10). This technique has the additional advantage that samples remain stable for about 24 h after they are collected. Artifacts of platelet activation after sample collection (commonly seen in platelet aggregation studies) are thus avoided. Most investigators who study this field have adopted a measure known as the platelet reactivity index (PRI), which compares maximally stimulated VASP phosphorylation with the amount of phosphorylation present after ADP stimulation of the platelet. When P2Y12 is blocked, the index decreases (Fig. 1). In volunteer subjects given clopidogrel, close correlation between light transmittance aggre-
expression. However, activation of P2Y12 by ADP initiates other signaling pathways involving molecules such as phosphoinositide-3-kinase (18) and Rap1b (19) that activate and stabilize glycoprotein IIb/IIIa independent of VASP phosphorylation. Furthermore, the antibody 16C2 used for VASP measurement reports phosphorylation at a serine residue that is also phosphorylated by cyclic guanosine monophosphate-dependent kinases that are not regulated by P2Y12 (20).

The use of a flow cytometric assay also introduces logistic and economic concerns. Although well regarded as a tool for differentiating cell types, flow cytometry is expensive, requires the availability of experienced personnel, and can hardly be considered a bedside tool. As any researcher who has fought for time on a flow cytometer can attest, results do not become available quickly. The median time between administration of the first clopidogrel dose and performing the first VASP assay was 24 h in the Bonello et al. (5) study, and 33% of patients required 3 assays and subsequent clopidogrel doses to achieve a PRI <50. These considerations are difficult to disregard, particularly in the U.S., where only a small minority of patients receive a clopidogrel loading dose 2 h or more before placement of a stent. Such an approach must also be viewed in light of competing technologies, some of which, like bedside platelet aggregation technologies, lend themselves more easily to point-of-care application. Currently, a large randomized trial is poised to test whether a bedside aggregation device can be used to achieve similar results in the chronic phase of clopidogrel therapy.

Although widespread measurement of the PRI before PCI may be impractical, the current study provides an early view into the potential utility of directing therapy based on a pathway-specific assay of the biological activity of an antiplatelet drug, and suggests that once a target level of inhibition is appropriately validated, an individualized approach to dosing may ultimately be preferable to a shotgun approach to dose selection.

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REFERENCES

through the activation of adenylate cyclase (with a subsequent rise in cAMP concentrations), which in turn prevents calcium mobilization. The clinical application of PGE\textsubscript{1} and PGI\textsubscript{2} has been limited by their effect on vascular tone, producing substantial systemic hypotension (Emmons et al., 1967; Terres et al., 1989), and by extensive first-pass metabolism in the lungs (70% of the active compound is rapidly cleared) (Kleiman et al., 1994).

The prostanoid analogs (i.e., iloprost, beraprost, cicaprost, ciprostene) are more stable compounds than are PGE\textsubscript{1} and PGI\textsubscript{2}; however, their development has focused primarily on potential use in patients with primary pulmonary hypertension (Okano et al., 1997).

### Thromboxane/Endoperoxide Receptor Antagonists

This class of compounds is designed to prevent platelet activation in response to TXA\textsubscript{2} and other endoperoxides. There is limited experience with the thromboxane receptor antagonists sulotroban and SQ30741 in patients with myocardial infarction (MI) treated with streptokinase (Kopia et al., 1989) and tissue plasminogen ac-

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<th>Table 11.1 A Platelet Biology-Based Approach to Inhibition</th>
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<td><strong>Agents That Inhibit Platelet Secretion</strong></td>
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<td>Calcium channel antagonants</td>
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GP, glycoprotein; PAF, platelet-activating factor.
Clopidogrel anti-platelet effect: An evaluation by optical aggregometry, impedance aggregometry, and the Platelet Function Analyzer (PFA-100™)

Anna Dyszkiewicz-Korpanty¹, Horatiu Olteanu², Eugene P. Frenkel¹ and Ravindra Sarode, MD²†

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Abstract

Platelet aggregation inhibition by clopidogrel may be suboptimal in 4–30% of patients. Traditionally, optical aggregometry is used to assess clopidogrel's anti-platelet effects by inhibition of ADP-induced aggregation in platelet rich plasma. Red blood cells are an important source of ADP and, thus, are known to modulate platelet function. Because the whole blood aggregation by impedance method assesses platelet function in a physiological milieu, we compared clopidogrel response by this method with the optical method in platelet rich plasma (PRP) and the Platelet Function Analyzer (PFA-100™). Platelet function studies were performed in 17 healthy subjects at baseline and after 10 days of clopidogrel intake (75 mg/day). Optical and impedance aggregometry were performed after addition of ADP (10 and 20 µM) and collagen (1 and 2 µg/mL). For PFA-100™ analysis, whole blood closure time was measured in collagen-coated cartridges with ADP and epinephrine. All subjects except one showed a decrease in ADP-induced aggregation using both aggregation methods. However, ADP-induced platelet aggregation was significantly inhibited when assessed in whole blood as compared to the optical method (71 ± 34% vs. 34.2 ± 23%, p = 0.0002); this suggests that whole blood aggregometry is more sensitive in the detection of clopidogrel effect in the presence of red cells, which are known to modulate platelet function. The PFA-100™ ADP closure time was slightly prolonged above the reference interval in only 5/17 (29%) subjects, suggesting that this instrument is not able to detect clopidogrel effect. We conclude that whole blood aggregation appears to be more sensitive in detecting clopidogrel effect compared with the platelet rich plasma method; the PFA-100™ was unable to detect clopidogrel effect in the majority of the subjects.
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