Comparison of methods for monitoring residual platelet reactivity after clopidogrel by point-of-care tests on whole blood in high-risk patients

Rita Paniccia1,2; Emilia Antonucci1; Niccolò Maggini1,3; Marco Miranda1; Anna Maria Gori1,3; Rossella Marcucci1,2; Betti Giusti1,2; Daniela Balzi4; Domenico Prisco1,2; Rosanna Abbate1,2

1Department of Medical and Surgical Critical Care, Thrombosis Center, University of Florence, Italy; 2Department of Heart and Vessels, Azienda Ospedaliero-Universitaria Careggi, Italy; 3Fondazione Don Carlo Gnocchi ONLUS, Centro S. Maria degli Ullivi-IRCCS, Florence, Italy; 4Epidemiology Unit, Azienda Sanitaria Firenze, Florence, Italy

Summary
Cardiovascular events are more frequent in high-risk coronary artery disease (CAD) patients on dual antiplatelet therapy with a residual platelet reactivity (RPR) than in those showing inhibition of ADP-inducible platelet activation. It is known that post-interventional RPR is a clinically important entity confirming it as a risk factor for thrombo-ischaemic events. Multiple electrode platelet aggregometry (MEA) on whole blood has been recently proposed as a rapid tool to evaluate RPR in high-risk CAD patients on clopidogrel therapy. It was the aim of this study to detect RPR in 801 high-risk CAD patients on dual antiplatelet therapy comparing MEA with the VerifyNow P2Y12 assay on whole blood and classical light transmission aggregation (LTA) on platelet-rich plasma. ADP (10 μM) was employed as agonist for MEA and LTA. The prevalence of RPR was 20.6% by MEA, 16.1% by LTA and 30.8% by VerifyNow. MEA showed a significant correlation (rho=0.62, p<0.0001) with VerifyNow and a moderate agreement (k=0.52, p<0.001) with 81.5% of concordant values. A significant correlation was found between MEA and LTA (rho=0.71, p<0.0001) with a good agreement (k=0.63, p<0.001) and 88.8% of concordant values. MEA in relation to LTA showed a sensitivity of 80% and a specificity of 91%. MEA might represent a reliable method and valid alternative in comparison with other available platelet function assays. It might help to guide antiplatelet therapy and thus improve clinical outcome of high-risk CAD patients.

Keywords
Clopidogrel therapy, light transmission aggregometry, multiple electrode aggregometry, point-of-care testing, residual platelet reactivity

Introduction
Clopidogrel exerts its effect on platelets by irreversibly antagonising the platelet P2Y12 receptor and inhibiting ADP-induced platelet aggregation (1, 2). Several clinical studies reported that the cardiovascular events are more frequent in patients on dual antiplatelet therapy with a residual platelet reactivity (RPR) than in those showing inhibition of ADP-inducible platelet activation (3–8).

In order to obtain a rapid identification of RPR in coronary artery disease (CAD) patients on dual antiplatelet therapy, different “point-of-care” (POC) methods for evaluating platelet function on whole blood have become available (9–16). Recently, the multiple electrode platelet aggregometry (MEA) which implements the principle of impedance platelet aggregometry in whole blood by using a new device, the Multiplate analyser (Dynabyte, Munich, Germany), to assess platelet function has been introduced (17–20) and it has been shown able to identify CAD patients at risk of early stent thrombosis (21, 22).

This study was planned to compare MEA method on whole blood with light transmission aggregometry (LTA) on platelet-rich plasma (PRP) and the POC system VerifyNow on whole blood in high-risk CAD patients on dual antiplatelet therapy.

Materials and methods
Patients investigated

The study population included 801 adult patients referred to the Department of Heart and Vessels (Azienda Ospedaliero-Università Careggi, Florence, Italy) and enrolled in the AMI-Florence 2 Registry for acute coronary syndromes (ACS). The design of the AMI-Florence Registry has been detailed elsewhere (23). Coronary angiography and percutaneous coronary interventions (PCI) with stent implantation were performed. All patients received 500 mg i.v.
of acetylsalicylic acid (ASA), followed by 100 to 325 mg ASA daily. A loading dose of clopidogrel (300 to 600 mg) was administered, followed by 75 mg of clopidogrel daily. Patients receiving glycoprotein IIb/IIIa inhibitors or with a personal or family history of bleeding disorders, a platelet count <100x10^9/l or >450x10^9/l, haemoglobin levels <10 g/dl, or having a major surgery within one week of enrolment were excluded. None of the patients investigated received other platelet function inhibitors (dipyridamole, cilostazol or ticlopidine) or drugs likely to interact with the antiplatelet effect of aspirin. Risk factors – diabetes, hypertension, peripheral artery disease and dyslipidaemia – were defined in line with the current guidelines (24–27). The study was approved by the ethics review board and was in accordance with the Helsinki Declaration. A written informed consent was obtained from all patients.

### Blood sampling

Blood samples were obtained 24 or 48 hours (h) after the end of PCI that was performed 3–6 h after the myocardial infarction. Blood samples were anticoagulated by one-tenth volume 0.109 M buffered trisodium citrate within Vacutainer plastic tubes (Becton Dickinson, Plymouth, UK). An additional 1.8 ml aliquot of blood was taken into the special citrated Vacutainer tube (Vacuette, Grainer Bio-One, Monroe, NC, USA) for VerifyNow analysis. All assays were performed within 2 h after blood sampling.

### Platelet aggregation in whole blood by Multiplate analyser

Whole blood MEA was performed by using the Multiplate analyser that consists of five channels for contemporary tests, integrated computer and guided automatic pipetting. Disposable ready-to-use test cells with two independent sensor units are used. The method has been described in detail elsewhere (28). Briefly, the adhesion and aggregation of platelets on the sensor surface enhances the electrical resistance (impedance) between the two sensor electrodes. The increase of impedance due to the increasing platelet attachment to the electrodes is detected for each sensor unit separately and the electrical signal measured in Ohm is transformed to arbitrary aggregation unit (AU) that are plotted against time (AU*min). This device quantifies the aggregation measurements as area under curve (AUC) of AU*min. Multiplate aggregations were performed in citrated anticoagulated whole blood employing ADP as agonist (10 μM, final concentration-fc) (Dynabyte, Munich, Germany). Reference intervals (5th – 95th percentile of control distribution, n=23) obtained in our laboratory were 32 to 104 AUC for 10 μM ADP. Our laboratory imprecision measured as coefficient of variation (CV) of MEA was determined assessing (five times) samples from control healthy subjects and from ACS patients on dual antiplatelet therapy. The mean CVs were: ADP-MEA, 3.9% in controls and 5.8% in CAD patients.

### Platelet aggregation in PRP

Aggregation studies were performed by using LTA in PRP by Born method (29) on APACT-4004 aggregometer (LABITec, Ahrensburg, Germany) in 250 μM minicuvettes stirred at 1,000 rpm at 37°C, as previously described (11). Briefly, PRP was adjusted with autologous platelet-free plasma to reach a platelet count between 180,000 plt/μl to 300,000 plt/μl and was stimulated with ADP (Mascia Brunelli, Milan, Italy) (10 μM, fc). The aggregation was expressed as the percent change in light transmittance from baseline at maximum (maximal aggregation). Our CVs were determined assessing (five times) samples from 10 control healthy subjects and from 10 CAD patients on dual antiplatelet therapy. The mean CVs were: ADP-LTA, 3.7% in controls and 4.8% in CAD patients. Reference interval (5th – 95th percentile of control distribution, n=98) obtained in our laboratory was 64% to 100% for ADP-LTA.

### VerifyNow system

The VerifyNow P2y12 assay (Accumetrics Inc, San Diego, CA, USA) is a turbidimetric-based optical detection system that measures platelet-induced aggregation (30) in a system containing fibrinogen-coated beads. Into the cartridge of VerifyNow P2Y12 assay a channel is present in which the inhibition of the ADP P2Y12 receptor is measured. This channel contains ADP as platelet agonist and prostaglandin E1 (PGE1) as suppressor of intracellular free calcium levels to reduce the non specific contribution of the ADP-binding to P2Y1 receptors. Results are expressed as P2Y12 reaction units (PRU). Reference interval (5th – 95th percentile of control distribution, n=98) obtained in our laboratory was 244 to 382 PRU. Samples from five control subjects and five CAD patients on dual antiplatelet therapy and VerifyNow Assay Wet Quality Control (level 1, normal and level 2, abnormal) were assessed four times to determine our laboratory CV of VerifyNow P2Y12 test. The mean CV was 3.5% in controls, 3.2% in CAD patients, and 2.5% and 3.4%, for level 1 and 2 quality controls.

### Definitions

RPR was defined in the presence of MEA ≥37 AUC (17), VerifyNow ≥240 PRU (11) and LTA ≥70% (6).

### Statistical analysis

Statistical analysis was performed using SPSS (version 11.0). The relationship between different methods was evaluated by using Spearman correlation test. The 2 x 2 agreement tables between LTA and POC systems were used for the qualitative analysis. Agreement between the different tests was determined by kappa statistics and
95% confidence intervals (CI) were calculated. Statistical significance was considered as a two-tailed probability <0.05.

**Results**

In Table 1, clinical and laboratory characteristics and data by MEA, LTA, and VerifyNow are reported. By using the above mentioned cut-off values, the percentage of patients with RPR was 20.6% for MEA, 16.1% for LTA; and 30.8% for VerifyNow. Figure 1 shows the correlation between MEA and LTA (rho=0.71, p<0.0001). In relation to the presence or absence of RPR, 711 (88.8%) samples tested were concordant, with 609 patients without RPR and 102 with RPR, whereas among the 90 discordant results, 27 patients had RPR by the LTA alone and 63 by MEA alone (Table 2). A significant good agreement between the two tests was observed (κ=0.63; 95% CI, 0.56–0.70; p<0.0001).

Figure 2 shows the correlation between MEA and VerifyNow P2Y12 assay data (rho=0.62, p<0.0001). In relation to the presence or absence of RPR, 653 (81.5%) samples tested were concordant, with 521 patients without RPR and 132 with RPR; whereas among the 148 discordant results, 33 patients had RPR by the MEA alone and 115 by VerifyNow P2Y12 assay alone (Table 2). A significant moderate agreement between the two tests was observed (κ=0.52; 95% CI, 0.46–0.58; p<0.0001). Figure 3 shows the correlation between VerifyNow P2Y12 assay and LTA (rho=0.70, p<0.0001). In relation to the presence or absence of RPR, 635 (79.2%) samples tested were concordant, with 530 patients without RPR and 105 with RPR; whereas among the 166 discordant results, 24 patients

Table 1: Clinical and laboratory characteristics of 801 patients studied.

<p>| | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>801</td>
</tr>
<tr>
<td>M/F, n</td>
<td>575/226</td>
</tr>
<tr>
<td>Age, years</td>
<td>72.5 ± 9.4</td>
</tr>
<tr>
<td>Smoking habit %</td>
<td>37.5</td>
</tr>
<tr>
<td>Hypertension %</td>
<td>63.9</td>
</tr>
<tr>
<td>Diabetes %</td>
<td>25.0</td>
</tr>
<tr>
<td>Dyslipidaemia, %</td>
<td>23.2</td>
</tr>
<tr>
<td>Peripheral artery disease, %</td>
<td>13.0</td>
</tr>
<tr>
<td>Erythrocytes x10¹²/l</td>
<td>4.0 ± 1.2*</td>
</tr>
<tr>
<td>Leucocytes, x10⁹/l</td>
<td>8.9 ± 2.8*</td>
</tr>
<tr>
<td>Haemoglobin, mg/dl</td>
<td>12.6 ± 2.0*</td>
</tr>
<tr>
<td>Platelets, x10⁹/l</td>
<td>215 ± 85*</td>
</tr>
<tr>
<td>ADP-MEA (AUC)</td>
<td>52 (2–100)**</td>
</tr>
<tr>
<td>ADP-LTA (%)</td>
<td>52 (2–100)**</td>
</tr>
<tr>
<td>VerifyNow P2Y12 assay (PRU)</td>
<td>190 (4–471)**</td>
</tr>
</tbody>
</table>

MEA, multiple electrode aggregometry; LTA, light transmission aggregometry; PRU, P2Y12 reaction unit. *Values expressed as mean ± standard deviation. **Values expressed as median and (range).

Table 2: Classification of results (n=801) by multiple electrode aggregometry (MEA) and VerifyNow P2Y12 in relation to light transmission aggregometry (LTA).

<table>
<thead>
<tr>
<th></th>
<th>10 μM ADP-LTA</th>
</tr>
</thead>
<tbody>
<tr>
<td>LTA&lt;70%</td>
<td>LTA≥70%</td>
</tr>
<tr>
<td>10 μM ADP-</td>
<td></td>
</tr>
<tr>
<td>AUC&lt;37</td>
<td>609</td>
</tr>
<tr>
<td>MEA AUC≥37</td>
<td>63</td>
</tr>
<tr>
<td>Total</td>
<td>672</td>
</tr>
<tr>
<td>VerifyNow vs. LTA</td>
<td></td>
</tr>
<tr>
<td>VerifyNow P2Y12 assay</td>
<td></td>
</tr>
<tr>
<td>PRU&lt;240</td>
<td>521</td>
</tr>
<tr>
<td>MEA AUC≥240</td>
<td>33</td>
</tr>
<tr>
<td>Total</td>
<td>554</td>
</tr>
</tbody>
</table>

ADP, adenosine-5 diphosphate; AUC, area under curve; LTA, light transmission aggregometry; MEA, multiple electrode aggregometry; PRU, platelet reactivity unit.
had RPR by the LTA alone and 142 by VerifyNow P2Y12 assay alone (Table 2). A significant moderate agreement between the two tests was observed ($\kappa=0.44$; 95% CI, 0.38–0.50; $p<0.0001$).

**Discussion**

This study compares for the first time in a large population of high-risk CAD patients the performance of MEA and VerifyNow P2Y12 assay and confirms the relationships previously studied among LTA, MEA and VerifyNow P2Y12 assay (11, 18, 20, 22). The main result of this study is the comparability of MEA with the other platelet function methods for monitoring clopidogrel therapy. MEA significantly correlates with both ADP-LTA on PRP and VerifyNow P2Y12 assay on whole blood in agreement with our previous work (20) and other authors (18, 22, 31–33). The feasibility of this method and most importantly its predictive value for the occurrence of stent thrombosis has previously been shown (18, 22, 31).

This large study confirms the high concordance of values found between MEA and LTA (20). These two tests demonstrated the same prevalence of patients with RPR with a good significant agreement. Actually, when LTA is considered the reference method, MEA has the capability of identifying true negative and true positive values for patients with and without RPR showing very high specificity and high sensitivity.

The agreement between ADP-MEA and VerifyNow P2Y12 assay was moderate. The percentage of discordant values could be in part explained by the presence into VerifyNow cartridge of ADP plus PGE1 as suppressor of the additional contribution of ADP-induced aggregation via the P2Y1 receptor. VerifyNow P2Y12 assay is specific for monitoring ADP P2Y12 receptor inhibition and consists of an agglutination on fibrinogen coated beads, whereas Multiplate system measures platelet aggregation in which the response to all ADP receptors is assessed.

Another important issue to be stressed is that in this investigation the platelet function has been assayed on citrate samples on all three tests. To our knowledge in previous studies (18, 21, 22, 31–35) conducted by Multiplate device hirudin samples were used, but the ideal anticoagulant for accurate platelet aggregometry remains controversial because turnaround time of specimens and

---

**What is known about this topic?**
- Light transmission aggregation method is considered the reference method to identify residual platelet reactivity in coronary artery disease patients on clopidogrel treatment.
- In this clinical setting the assessment of different platelet function methods and the evaluation of their cut-off values are still ongoing.

**What does this paper add?**
- This study provides new data on the use of platelet function point-of-care tests on whole blood for the identification of residual platelet reactivity in a large population of acute coronary artery disease patients on antiplatelet treatment.
- Evaluation of platelet function by using Multiplate whole blood aggregometry shows highly correlated results and good agreement with light transmission aggregometry.
- Multiplate whole blood aggregometry, used as a point-of-care method, might be a reliable tool to test platelet function for research purpose, in daily clinical practice and in non specialised laboratories.
pre-analytical procedures could affect platelet aggregability in whole blood. In our previous study, we compared platelet function of citrate specimens with that of hirudin in CAD patients (n=68) and in 10 healthy subjects (36) and no difference was found between the two types of anticoagulants. This fact might play an important role, because in the real world citrated blood samples are commonly used to assess haemostasis tests.

**Strengths and limitations of the study**

It is important to note that the platelet function was assessed in the dedicated laboratory of Thrombosis Centre so maintaining uniformity of procedures; patients investigated were from a unique Institution; the same concentration of ADP was employed for MEA and LTA; for all venous blood samples trisodium citrate was only used as anticoagulant. In addition, each blood sampling for platelet function assay was obtained 24 or 48 hours after the end of PCI and hand delivered by an ad hoc special transport service to reduce turnaround time (37).

The ultimate target of the test which fits platelet function in high-risk patients would be the potential identification of a therapeutic window around the cut-off value for identification of RPR; but, a large gap remains in the understanding of the association between antiplatelet therapies and bleeding. For this reason it is important to have information on the degree of imprecision around aggregation cut-offs: in this study in-house within-run imprecision remained below 6% for all tests assessed.

In conclusion, the use of different POC systems might allow a rapid and easy screening of platelet function in CAD patients at high risk of thrombotic events (38). MEA on whole blood is feasible in daily clinical practice in non-specialised laboratories or in cardiological wards. MEA seems a valid alternative to the other systems for the rapid detection of RPR in clinical investigations, because it is a standardised method, is not expensive or time-consuming and require small volumes of blood. The reliability of MEA to assess RPR might theoretically help to guide antiplatelet therapy ad thus improve clinical outcome in high-risk CAD patients. Other studies need to be performed to confirm the ability of MEA to become a clinically used diagnostic test.

**Acknowledgments**

Multiple aggregate meter, cuvettes and ADP reagent have been kindly offered by Dynabyte (Germany). This organisation had no role in study design, data collection, analysis and interpretation and manuscript preparation or (dys)approval.

**References**


