Comparison of Different Methods to Evaluate the Effect of Aspirin on Platelet Function in High-Risk Patients With Ischemic Heart Disease Receiving Dual Antiplatelet Treatment

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Abstract

Patients with coronary artery disease (CAD) receiving aspirin therapy with a residual platelet reactivity (RPR) may be at increased risk of ischemic vascular events. Point-of-care (POC) methods PFA-100 (Dade-Behring, Marburg, Germany) and VerifyNow (Accumetrics, San Diego, CA) assays have been suggested as rapid tools to evaluate RPR. We compared PFA-100 closure times by collagen/epinephrine and VerifyNow Aspirin assays with light transmission aggregation (LTA) induced by 1 mmol/L of arachidonic acid in 484 patients with CAD undergoing percutaneous coronary intervention and receiving dual antiplatelet therapy. RPR was detected in 30.0% of patients by LTA, in 32.4% by PFA-100, and in 14.3% by VerifyNow. Significant correlations were found among 3 methods (all P < .0001). In relation to the presence or absence of RPR by LTA and PFA-100, by LTA and VerifyNow, and by PFA-100 and VerifyNow, samples were significantly concordant (all P < .0001). Assuming LTA as the reference method, PFA-100 and VerifyNow showed sensitivity of 62.1% and 39.3% and specificity of 80.2% and 96.4%, respectively. The cutoff values for POC methods need to be defined for clinical use.

Platelet activation has a crucial role in the pathogenesis of coronary artery disease (CAD). Aspirin is a basilar support in CAD secondary prevention and has been found to reduce by about 25% the risk of major vascular events and vascular death in high-risk patients. A growing number of studies have demonstrated that residual platelet reactivity (RPR) measured by light transmission aggregation (LTA) on antiaggregating therapy is associated with a higher prevalence of clinical adverse events. This association is particularly relevant in high-risk patients such as patients with acute coronary syndrome (ACS) undergoing percutaneous coronary intervention (PCI). Indeed, the 2005 guidelines of the American College of Cardiology/American Heart Association Task Force on Practice Guidelines suggest that in patients at very high risk, in whom subacute thrombosis may be catastrophic or lethal, platelet aggregation studies may be considered and the dose of antiaggregating drug increased if less than 50% inhibition of platelet aggregation is demonstrated.

To evaluate RPR, different methods have been used, and wide variability in laboratory test response in assessing RPR exists. On this topic, in 2005, the Working Group of the Platelet Physiology Subcommittee of the Scientific and Standardization Committee of the International Society on Thrombosis and Haemostasis stated that the use of platelet function methods for monitoring the response to aspirin therapy is not appropriate, and the variation of aspirin therapy based on these tests is not recommended.

LTA is the classic method for the study of platelet function, but it requires a specialized laboratory and is a manual and time-consuming test. In the last years, 2 point-of-care (POC) devices evaluating platelet function became available,
the Platelet Function Analyzer-100 (PFA-100; Dade-Behring, Marburg, Germany) and the VerifyNow system (Accumetrics, San Diego, CA). These POC instruments could allow rapid identification of RPR. However, few comparisons of these POC tests with LTA are available. In addition, an extensive overview of available evidence from the use of platelet function systems, such as the PFA-100, has suggested that use be restricted to research studies and clinical trials.

There is intense research interest in finding platelet function tests that permit evaluation of RPR in patients with ACS receiving dual antiplatelet therapy (eg, clopidogrel and aspirin). The cyclooxygenase (COX)-1 pathway, strongly inhibited by aspirin, is not inhibited by clopidogrel because the mechanism of action of clopidogrel is related to the adenosine diphosphate (ADP) receptor P2Y12 pathway and is unrelated to that of arachidonate.

The aim of our study was to compare arachidonic acid (AA)-induced LTA (AA-LTA) with the PFA-100 and VerifyNow systems in a setting of very high-risk patients, ie, patients with ACS undergoing PCI and receiving dual antiplatelet therapy.

Materials and Methods

Study Population

The study population included 484 consecutive adults admitted to the coronary care unit of the Azienda Ospedaliero-Universitaria Careggi (Florence, Italy) for ACS. All patients underwent coronary angiography performed by the Judkin technique and PCI. Before PCI, all patients received a loading dose of 500 mg of intravenous aspirin and 300 mg of clopidogrel orally, followed by 100 to 325 mg of aspirin daily and 75 mg of clopidogrel daily. During the procedure, 70 IU/kg of unfractionated heparin was used as the anticoagulant. None of the patients received antiplatelet glycoprotein IIb/IIIa drugs. Of 484 patients, 119 (24.6%) were already taking aspirin before entering the study. Patients with a personal or family history of bleeding disorders, with a platelet count of less than 100 × 10^3/µL (100 × 10^9/L) or more than 450 × 10^3/µL (450 × 10^9/L), with a hemoglobin level of less than 10 g/dL (100 g/L), or having a major surgery within 1 week of enrollment were excluded. The study was approved by the ethics review board, and signed informed consent was obtained from all patients.

Blood Sampling

Blood samples were obtained 18 to 48 hours after PCI. Blood samples were anticoagulated with one-tenth volume 0.109 mol/L of buffered trisodium citrate within Vacutainer plastic tubes (Becton Dickinson, Plymouth, England). An additional 1.8-mL aliquot of blood was taken into the special citrated Vacutainer tube (Vacutte, Grainer Bio-One, Monroe, NC) for VerifyNow analysis. All assays were performed within 2 hours of blood sampling.

Platelet Aggregation in Platelet-Rich Plasma

Platelet-rich plasma was prepared by centrifugation at 250g for 10 minutes. Platelet-rich plasma was removed, and then platelet-poor plasma was prepared by further centrifugation at 3,000g for 3 minutes. Aggregation studies were performed by an APACT-4 aggregometer (Helena Laboratories Italia, Assago, Italy) into 250-µL minicuvettes stirred at 1,000 rpm at 37°C. The 100% line was set using platelet-poor plasma and the 0% baseline established with platelet-rich plasma (adjusted from 180 × 10^3/µL [180 × 10^9/L] up to 300 × 10^3/µL [300 × 10^9/L]). A final concentration of 1 mmol/L of AA was used as the agonist. After 10 minutes, the maximal percentage of aggregation was recorded. Our laboratory imprecision (coefficient of variation [CV]) of AA-LTA was determined by measuring (5 times) samples from 10 healthy control subjects and from 10 patients with CAD receiving dual antiplatelet therapy. The mean CVs were 3.4% in control subjects and 5.8% in patients with CAD. Treated patients were considered to have an RPR when their platelets showed a more than 20% aggregation after stimulation by 1 mmol/L of AA (0.4 mg/mL).

PFA-100 System

The PFA-100 system simulates high shear platelet function within test cartridges. Platelet function is measured as a function of the time (closure time [CT]) that platelets take to occlude an aperture in a membrane coated with collagen/epinephrine (CEPI) or collagen/ADP. Because aspirin does not affect the CT with the collagen/ADP cartridge only the CEPI test was considered in this study.

Blood samples from 10 control subjects and 10 patients with CAD receiving dual antiplatelet therapy were measured 4 times to determine our laboratory CV for the CEPI CT test. The mean CV was 5.4% for the CEPI CT in control subjects and 9.9% in patients with CAD. Blood samples obtained during 5 consecutive days from 1 healthy subject with a low platelet count (152 ± 45 × 10^3/µL [152 ± 45 × 10^9/L]) were measured (4 times within the day). The within-day mean CV was 5.8%, and the day-to-day mean CV was 8.4%. Blood samples can be kept for at least 3 hours after collection at ambient temperature without significant changes in CT. A citrated whole blood sample, 0.8 mL, was pipetted into the sample reservoirs of the CEPI cartridge (prewarmed to room temperature) and loaded into the PFA-100 device. The normal range (5th-95th percentile of control distribution; n = 98) obtained in our laboratory was 85 to 203 seconds for CT with the CEPI cartridge. RPR was defined as a CEPI CT of less than 203 seconds.
VerifyNow System

The VerifyNow is a turbidimetry-based optical detection system that measures platelet-induced aggregation\(^9,24\) in a system containing fibrinogen-coated beads. The cartridges of the VerifyNow Aspirin Assay contain AA as the agonist. The instrument measures changes in light transmission and, thus, the rate of aggregation. Samples from 5 control subjects and 5 patients with CAD receiving dual antiplatelet therapy were measured 4 times to determine our laboratory CV for the VerifyNow Aspirin Assay. The mean CV was 3.5% in control subjects and 3.2% in patients with CAD. In our laboratory using the VerifyNow Aspirin Assay level 1 (normal) and level 2 (abnormal) wet quality controls, the mean CVs were 2.5% and 3.4%, respectively. Blood samples for the VerifyNow Aspirin Assay can be kept for at least 4 hours after collection at ambient temperature without significant changes in results. Results are expressed as aspirin reaction units (ARU), and the cutoff value assigned by the manufacturer to identify patients with RPR was more than 550 ARU.

Statistical Analysis

Statistical analysis was performed using SPSS (version 11.0, SPSS, Chicago, IL). A value of 300 seconds was attributed to all samples with a PFA-100 CEPI CT more than the maximum predefined value of 300 seconds. The 2 × 2 agreement tables between LTA, the PFA-100 CEPI, and the VerifyNow were used for the qualitative analysis. Agreement between different tests was determined by \(\kappa\) statistics, and 95% confidence intervals (CIs) were calculated. The relationship between different methods was evaluated by using the Spearman correlation test.

Results

In **Table 1**, the clinical characteristics of patients included in the study are reported. AA-LTA, PFA-100, and VerifyNow data are given in **Table 2**. The percentages of patients with RPR were 30.0% for AA-LTA, 32.4% for the PFA-100 CEPI CT, and 14.3% for the VerifyNow Aspirin Assay **Table 3** and **Table 4**.

**Figure 1** shows the correlation between PFA-100 CEPI CT and AA-LTA data. In relation to the presence or absence of RPR, of 484 samples tested, 362 (74.8%) were concordant, with 272 without RPR and 90 with RPR, whereas among the 122 discordant results (Table 3), 67 samples showed RPR by the PFA-100 CEPI CT alone and 55 by AA-LTA alone. A significant, moderate agreement between the PFA-100 CEPI CT and AA-LTA was observed (\(\kappa = 0.41; 95\% \text{ CI}, 0.3-0.49; P < .0001\)).

**Figure 2** shows the correlation between AA-LTA and VerifyNow Aspirin Assay data in 484 samples. These 2 methods gave concordant results in 384 samples (79.3%), with both tests indicating 327 without RPR and 57 with RPR (Table 3). A significant, moderate agreement was observed between results obtained by the 2 tests (\(\kappa = 0.42; 95\% \text{ CI}, 0.33-0.51; P < .0001\)).

**Figure 3** shows the correlation between VerifyNow Aspirin Assay and PFA-100 CEPI CT data. Of 484 samples tested, 364 (75.2%) were concordant, with 53 showing RPR,
whereas 120 samples were discordant, with 104 showing RPR only by the PFA-100 CEPI and 16 only by the VerifyNow assay (Table 4). The agreement between the 2 POC tests was $\kappa = 0.34$ (95% CI, 0.25-0.43; $P < .0001$).

All 3 methods agreed in 313 (64.7%) of 484 samples. However, the 3 tests gave discordant results in 171 (35.3%) of 484 samples

**Table 5.**

<table>
<thead>
<tr>
<th>Classification</th>
<th>PFA-100 RPR (n = 157)</th>
<th>No RPR (n = 327)</th>
</tr>
</thead>
<tbody>
<tr>
<td>VerifyNow RPR (n = 69)</td>
<td>53</td>
<td>16</td>
</tr>
<tr>
<td>No RPR (n = 415)</td>
<td>104</td>
<td>311</td>
</tr>
</tbody>
</table>

RPR, residual platelet reactivity.

$^*$ PFA-100, Dade-Behring, Marburg, Germany; and VerifyNow, Accumetrics, San Diego, CA.

Considering the results obtained by the VerifyNow in patients with an RPR by AA-LTA of more than 20% of maximal aggregation ($n = 145$), the ARU median value was 495 (reference interval, 356-676 ARU). Assuming the value of 495 ARU as the cutoff for identifying patients with RPR by using the VerifyNow, the percentage of patients was 21.9%. AA-LTA and the VerifyNow using the cutoff of 495 ARU gave concordant results in 379 samples (78.3%; 306 patients without RPR and 73 patients with RPR). A significant, moderate agreement was observed between results obtained by the 2 tests ($\kappa = 0.44$; 95% CI, 0.39-0.49; $P < .0001$). Assuming LTA as the reference method and the cutoff of 495 ARU for the VerifyNow, this method showed a sensitivity of 49.7% and a specificity of 90.6%, with 73 false-negative and 32 false-positive results, a positive predictive value of 69.2%, and a negative predictive value of 80.8%.

**Discussion**

The results of this study demonstrate moderate but significant concordance between AA-LTA and the 2 POC systems used in evaluating platelet function in patients receiving dual antiplatelet therapy. Given the main role of platelets in determination of arterial thrombosis, there has been intense research interest in the usefulness of platelet function tests as additional tools in cardiac risk stratification. Actually, these methods permit evaluation of the prevalence of RPR in patients with CAD.
To our knowledge, this is the first study of the new VerifyNow Aspirin Assay in high-risk vascular patients such as patients with ACS by using cartridges preloaded with AA as an agonist of platelet aggregation instead of propyl gallate, used in the past.\textsuperscript{11,12,18} As a reference method to study platelet function, we used AA-LTA, which has been found to be associated with an increased risk of clinical events in high-risk patients.\textsuperscript{2-5} Different studies using AA-LTA to investigate the inhibition of platelet function by aspirin reported different prevalence of subjects with RPR (5.5\%-36\%).\textsuperscript{2-14,18,25-27} However, this variability was due, at least in part, to different designs of the studies and clinical characteristics of the patients included. Results can also be influenced by differences in agonist concentration. For example, the AA concentration used in our study was slightly lower than in the study by Gum et al.\textsuperscript{2}

Previous studies using the PFA-100 to evaluate platelet function on aspirin reported that the prevalence of patients with RPR varies from 9.5\% to 60\%. In our study performed in a large sample of patients with ACS, this value was 32.4\%. The prevalence of patients with RPR was calculated by using an in-house reference range (CEPI CT, 85-203 seconds) that is, however, similar to the reference ranges reported in other studies.\textsuperscript{28} A moderate, significant concordance was found between the results obtained by the PFA-100 and AA-LTA. Assuming LTA as the reference method, the PFA-100 showed moderate sensitivity and specificity for the detection of RPR.

In 13.8\% of patients, CEPI CT values were not prolonged (<203 seconds), whereas AA-LTA was inhibited. The CEPI CT may be shortened by high WBC counts and elevated von Willebrand (vWF) plasma levels.\textsuperscript{13,18} Chakroun et al\textsuperscript{29} reported that patients with RPR had increased vWF levels. Also, Fontana et al\textsuperscript{14} suggested that high vWF levels cause a persistent ability of platelets to aggregate despite adequate inhibition of thromboxane A\textsubscript{2} production by aspirin. In addition, RPR by the PFA-100 has been related to altered RBC deformability, altered whole blood viscosity, and an increased WBC count.\textsuperscript{30-32}

For the 57 patients (11.8\%) classified with RPR by AA-LTA but not by PFA-100 (>203 seconds), we can exclude that prolonged CEPI CT values were due to a low platelet count (<100 \times 10^{3}/μL [100 \times 10^{3}/L]) or a low hematocrit value (<30\% [0.30]). Thus, other possible explanations should be considered, such as the lack of adhesion of the platelet surface to the collagen caused by genetic predisposition or the presence of a mild vWF disease (see review by Wang et al\textsuperscript{23}).

A moderate although significant concordance was also found between the results obtained by the VerifyNow and AA-LTA. Actually, they study the same pathway of platelet activation. The VerifyNow Aspirin Assay mimics AA-LTA because the cartridge of this assay contains AA to activate the COX-1 pathway. Assuming LTA as the reference method, VerifyNow showed high specificity but low sensitivity for identifying patients with RPR. A possible explanation for this difference is the choice of the cutoff value for the VerifyNow to separate patients with and without RPR. The 550 ARU value, according to the manufacturer’s suggestion, derives from studies by Malinin et al\textsuperscript{11} and Coleman et al.\textsuperscript{12} These authors, differently from us, calculated this cutoff by comparing values obtained by LTA induced by epinephrine with those obtained by VerifyNow cartridges preloaded with propyl gallate in subjects assumed to be taking 325 mg of aspirin. This makes their data hardly comparable with ours. Thus, this cutoff might be not adequate because the new cartridge contains a different platelet agonist (AA instead of propyl gallate), LTA is induced.

\begin{table}[h]
\centering
\begin{tabular}{|c|c|c|}
\hline
 & AA-LTA & VerifyNow \\
\hline
PFA-100, no RPR; VerifyNow, no RPR & 44 & 267 \\
PFA-100, no RPR; VerifyNow, RPR & 11 & 5 \\
PFA-100, RPR; VerifyNow, no RPR & 44 & 60 \\
PFA-100, RPR; VerifyNow, RPR & 46 & 7 \\
\hline
\end{tabular}
\caption{Classification of 484 Results in Relation to RPR by Platelet Aggregation and the PFA-100 and VerifyNow Assays Combined\textsuperscript{*}}
\end{table}

\textsuperscript{*} PFA-100, Dade-Behring, Marburg, Germany; and VerifyNow, Accumetrics, San Diego, CA.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure3.png}
\caption{Comparison and agreement of 484 results obtained by the PFA-100 (Dade-Behring, Marburg, Germany) CEPI CT and VerifyNow Aspirin (Accumetrics, San Diego, CA) assays. The horizontal arrow represents the cutoff value for RPR by the PFA-100 CEPI CT (<203 seconds). The vertical arrow represents the cutoff value for RPR by the VerifyNow (>550 aspirin reaction units [ARU]). Concordant results, 75.2\%; $\kappa = 0.34$, $P < .0001$; $\rho = -0.43$, $P < .0001$. CEPI CT, closure time by the collagen/epinephrine cartridge; RPR, residual platelet reactivity.}
\end{figure}
by AA, and clinical characteristics of patients are different. We identified a value of 495 ARU, which has higher sensitivity for RPR by AA-LTA. As a consequence, this value is associated with increased agreement (κ = 0.44) between the 2 methods.

Another explanation for the different behavior of LTA and the VerifyNow is the different physiologic environment in which the tests are performed. A whole blood sample is used for the VerifyNow, and whole blood platelet aggregation is influenced by WBC-platelet interaction, whereas LTA is performed in platelet-rich plasma.

Another issue that must be discussed is the dual antiplatelet therapy administered to patients included in the study. Patients with CAD were taking clopidogrel and aspirin. It could be argued that the prevalence of patients with RPR might have been misinterpreted owing to the influence of the inhibition of the ADP receptor P2Y12 on tests used in this study. However, previous studies reported that the addition of clopidogrel did not affect laboratory measurements of platelet function by AA-LTA.21,22 Indeed, the mechanism of action of clopidogrel did not affect laboratory measurements of platelet inhibition of the ADP receptor P2Y12 on tests used in this study. Previous studies reported that the addition of clopidogrel did not affect laboratory measurements of platelet function by AA-LTA. Indeed, the mechanism of action of clopidogrel did not affect laboratory measurements of platelet function by AA-LTA. The prevalence of patients with RPR might have been misinterpreted owing to the influence of the inhibition of the ADP receptor P2Y12 on tests used in this study. Previous studies reported that the addition of clopidogrel did not affect laboratory measurements of platelet function by AA-LTA.

Advancing an early detection of RPR has to be elucidated. The importance of evaluating different methods to measure platelet function derives from the growing evidence that RPR may indicate an increased risk of future cardiovascular events. In particular, because subacute thrombosis in patients at high risk may be critical or lethal, platelet function studies may be considered in the patient’s management.6 The introduction of POC devices might give the opportunity to perform rapid screening to evaluate platelet function in patients with CAD at higher risk. Because the study of RPR in these patients is, at least in part, test-dependent, the prognostic value of each test has to be defined and the clinical relevance of an early detection of RPR has to be elucidated.

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