MEASUREMENT OF PLATELET ACTIVATION MARKERS CD62P AND CD63 TO DETERMINE THE EFFICIENCY OF DUAL THERAPY WITH ASPIRIN AND CLOPIDOGREL: A PRELIMINARY STUDY

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Abstract

**Background:** A dual antiplatelet therapy with aspirin and clopidogrel is advised to prevent stent thrombosis. However, the most appropriate assay for evaluating the antiplatelet effects remains undefined.

**Objective:** This study aimed to assess the efficacy of measuring platelet surface activation markers, CD62P and CD63, using flow cytometry to determine the effectiveness of dual antiplatelet therapy.

**Methods:** Thirty patients who received aspirin plus clopidogrel before cerebral artery stent implantation and thirty unrelated healthy controls were enrolled. The expression of CD62P and CD63 was measured using flow cytometry. The diagnostic performances were evaluated and compared with the conventional light-transmitted aggregometry (LTA) using 5.0 and 20.0 µM of adenosine di-phosphate (ADP).

**Results:** The expression ratios of both markers were significantly lower in the patients receiving dual antiplatelet therapy than controls ($p < 0.001$). The area under the receiver operating characteristic (ROC) curve (AUC) of CD62P using flow cytometry and the LTA using 5.0 and 20.0 µM of ADP were comparable (0.97 vs. 0.95 and 0.97 vs. 0.96, respectively), whereas those of CD63 was lower than the LTA (0.88 vs. 0.96). The sensitivity of CD62P and CD63 were 71.4% and 60.0%, and the specificity of CD62P and CD63 were 100.0% and 96.7%, respectively.

**Conclusion:** Flow cytometry measurements of CD62P could be utilized to identify the efficacy of dual antiplatelet therapy. Additional studies are suggested to support this issue and its effects on clinical outcomes.

**Keywords:** antiplatelet monitoring, drug effectiveness, dual antiplatelet therapy, flow cytometry, platelet aggregation test

J Southeast Asian Med Res 2024: 8: e207
https://doi.org/10.55374/jseamed.v8.207

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Introduction

Antiplatelet therapy is the most important and effective management for preventing major clinical complications in several thrombotic vascular diseases, including coronary artery disease (CAD), ischemic cerebrovascular disease (CVD), and peripheral arterial disease (PAD), as well as in patients with coronary stents. Over the past decades, aspirin or acetylsalicylic acid (ASA), an inhibitor of cyclooxygenase-1, has been widely used as an antiplatelet drug to prevent cardiovascular events and long-term aspirin therapy could effectively lower the rate of disease recurrence. Clopidogrel, a P2Y12-receptor antagonist, has recently emerged as a novel antiplatelet agent and is more effective than aspirin in preventing secondary vascular events. Although several large-scale clinical trials have demonstrated these medications’ clinical efficacy and safety in reducing ischemic events and mortality in patients with high-risk factors, aspirin or clopidogrel monotherapy is not always sufficient to prevent thrombotic events.

In patients undergoing stent implantation, combined therapy with aspirin and clopidogrel is advised as the most appropriate intervention for stent thrombosis prophylaxis. Despite the benefits of dual antiplatelet therapy in lowering cardiovascular risk proven by several trials, related studies indicated unexpected drug-related adverse effects of this approach, such as bleeding episodes. In addition, even after receiving effective dual treatment, some patients continue to develop thrombotic episodes, a syndrome known as “antiplatelet resistance.” Antiplatelet resistance is defined as either progressive clinical symptoms (a thromboembolic event) or laboratory testing that determines normal platelet function during drug treatment. The prevalence of aspirin and clopidogrel resistance in patients with cardiovascular disease and ischemic stroke varied from 5% to 65% and 28% to 44%, respectively. A more potent antiplatelet is indicated to overcome these resistances; however, the risk of bleeding increases.

Various laboratory tests evaluating in vitro platelet function have been used to assess the effectiveness of antiplatelet drugs and determine antiplatelet resistance. Platelet function testing by light-transmitted aggregometry (LTA) is a gold standard assay to assess the antiplatelet effects. However, some limitations have been noted, including the large sample volume required, time-consuming procedures, poor reproducibility, low sensitivity, poor standardization between different laboratories, and skilled technicians required. Furthermore, LTA does not reflect physiological conditions because platelets are isolated from whole blood, swirling under low shear force conditions, and only form aggregates in the presence of agonists. As a result, this test is less realistic in in vivo settings.

Other point-of-care platelet function assays, including the platelet function analyzer (PFA)-100 or the upgraded version Innovance PFA-200 (Siemens, Marburg, Germany) and the VerifyNow system (Accumetrics, San Diego, CA, USA), were employed to screen for platelet dysfunction and to determine antiplatelet resistance. However, there is currently no reliable or gold standard for point-of-care testing due to the difficulty of discriminating between normal and abnormal ranges and finding antiplatelet resistance with different cut-offs. As mentioned above, platelet function tests are not equally effective at measuring antiplatelet effects, and poor correlations are reported between them.

A feasible alternative assay, flow cytometry, can be used to measure platelet surface activation indicators such as CD62P (P-selectin), CD63 (a lysosomal glycoprotein; granulophysin), CD40 ligand, and glycoprotein (GP) I Ib/IIIa, which help measure platelet function and antiplatelet effect. Higher proportions of platelets expressing CD62P and CD63 were observed in patients with acute- and previous-cerebrovascular ischemia compared to controls. In addition, increased expression of CD62P was associated with clopidogrel resistance in patients with ischemic stroke.
In this preliminary study, we measured platelet surface activation markers, CD62P and CD63, using flow cytometry to determine the efficiency of dual therapy with aspirin and clopidogrel in patients having cerebral stent insertion.

Methods

Study population

In this prospective observational study, 30 patients with unruptured cerebral aneurism who were planning for flow-diverting intracranial vascular stent implantation at the Unit of Neurological Surgery, Department of Surgery, Faculty of Medicine, Chulalongkorn University and King Chulalongkorn Memorial Hospital, Bangkok, Thailand, from January 2022 to August 2023, and 30 unrelated healthy controls were enrolled. All patients were treated with Clopidogrel 75 mg per day in combination with aspirin 300 mg per day over 5-7 days before blood collection and testing. The subjects under 18 years old and with a whole blood platelet count of less than 150 x 10^9/L were excluded.

The study was approved by the Institutional Review Board of the Faculty of Medicine, Chulalongkorn University, Bangkok, Thailand (COA No. 0083/2023, IRB No. 896/64) and the Human Research Ethics Committee of Thammasat University (HREC-TUSc), Pathumthani, Thailand (COA No. 048/2565). Written informed consent was obtained from all subjects following the principles of the Declaration of Helsinki.

Sample preparation

Blood samples were collected in 3.2% trisodium citrate tubes (Vacuette®, Greiner Bio-One Ltd., UK) at a 9:1 ratio. Platelet-rich plasma (PRP) was prepared by single centrifugation of whole blood at 180x g for 15 minutes and was maintained at room temperature for 30 minutes. After removing PRP, samples were centrifuged at 1500x g for 15 minutes to obtain autologous platelet-poor plasma (PPP).

Platelet aggregation test

The platelet aggregation test was evaluated using light transmission aggregometry (LTA) on the Chronolog aggregometers Model 700 (Chronolog Corporation, Havertown, PA, USA) using 100% PPP as a reference. Platelet agonist reagents, including 5.0 mM and 20.0 mM of adenosine diphosphate, ADP (Sigma Aldrich, St. Louis, USA) and 1.5 mM of arachidonic acid, AA (Sigma Aldrich, St. Louis, USA), were employed to evaluate the platelet function and determine the antiplatelet drug responsiveness.

Platelet function test by flow cytometry

Sample preparation and fixation of platelets were performed after resting for 1 hour and completed within 2 hours following the protocol previously described. Briefly, 200 µL of PRP was diluted with 1x phosphate-buffered saline, PBS (Eurobio Scientific, Courtaboeuf, France) to achieve the final platelet count ranging from 10 to 30 x 10^9/L. The diluted PRP was aliquoted into three tubes (500 µL per each). Platelets in the first tube were activated by adding 20.0 µM ADP and incubated for 5 minutes at room temperature, while platelets in the second tube were not stimulated. Both tubes were stained in the dark for 15 minutes with Brilliant Violet 421™ anti-human CD62P (Biolegend, CA, USA) and PE anti-human CD63 (Biolegend, CA, USA) antibodies, respectively. The third tube was an unstimulated and unstained control for calculating the CD62P and CD63 expression ratios. Flow cytometry analyses were performed using a BD Biosciences LSRFortessa X-20 Flow Cytometer (Becton Dickinson, San Jose, CA, USA) and 1x10^5 events were recorded. Cell debris at the left side of the forward scatter (FSC)-A/ side scatter (SSC)-A plot and clumped cells in the FSC-A/SSC-A plot were excluded, ensur-
ing that only single cells were included in the analysis. An unstained sample was utilized to set voltages and gating. Histograms were generated from measurements of 1x10^5 cells and the data were analyzed using Kaluza software version 1.2 (Becton Coulter, IN, USA). The results were expressed as the median fluorescence intensity (MFI) of the surface markers on platelets and the percentage of marker-positive platelets (%-gated). Moreover, CD62P and CD63 expression ratios were calculated as follows:

\[
\text{Expression ratio} = \frac{(\text{MFI}_{\text{stimulated}} - \text{MFI}_{\text{unstained}})}{(\text{MFI}_{\text{resting}} - \text{MFI}_{\text{unstained}})}.
\]

Statistical analysis

Statistical analysis was performed using IBM SPSS Statistics for Windows, Version 20.0 (IBM Corp., Armonk, NY, USA) and GraphPad Prism, Version 9 (GraphPad Software, CA, USA). Demographic characteristics were analyzed using descriptive statistics. Continuous variables were presented as mean and standard deviation (SD) or median with interquartile range (IQR) and compared using the Student’s t-test and Mann-Whitney’s U-test, depending on the data distribution. To assess the diagnostic performance of each test, sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV) were determined. The area under the receiver operating characteristic (ROC) curve (AUC) for each analytical assay was also investigated. A p-value of less than 0.05 was considered statistically significant.

Results

Baseline characteristics of the patients and controls

The baseline characteristics of 30 patients and 30 controls are shown in Table 1. The average ages of the patients and controls were 55.7±14.6 and 54.9±15.0 years. Male-to-female (M: F) ratios among the patients and controls were 1: 2 and 1: 3.3, respectively. There were no significant differences in age and sex between the patients and controls (p > 0.05). The platelet counts ranged from 213 x 10^9/L to 696 x10^9/L in the patient group, and their ranges were 141 x 10^9 to 682 x 10^9/L in the control group, and no significant difference was observed (p > 0.05).

Patients who received clopidogrel in concomitant with aspirin demonstrated a significantly lower percentage of max aggregation (%Max) when activated by ADP 5.0 µM, ADP 20.0 µM, and AA 1.5 mM than the controls (p < 0.001).

CD62P and CD63 expressions in patients vs. controls

Expression of CD62P and CD63 was measured using flow cytometry and reported as MFI of the surface markers on platelets and %-gated. The expression of both markers in ADP-activated and not-activated platelets is shown in Figure 1. The study showed that expressions of CD62P and CD63 after ADP stimulation were higher than in the non-stimulated state in controls (Figure 1A). On the contrary, no difference in expressions of both markers before and after ADP stimulation was observed in the patient group (Figure 1B).

Next, the expression ratios of CD62P and CD63 were calculated and compared between the patients and controls (Figure 2). The expression ratios of CD62P and CD63 in the patients receiving aspirin combined with clopidogrel treatment were significantly lower than controls (p < 0.001), with median expression ratios of 2.1 vs. 4.3 and 1.2 vs. 1.5, respectively.

The patients were separated into response and resistant groups based on clinical manifestations and laboratory measurements. Aspirin resistance was defined as high-responsive platelet aggregation induced by AA (cut-off > 20.0%), while clopidogrel resistance was characterized as high platelet aggregation induced by 5.0 and 20.0 µM ADP (cut-offs > 43.0% and > 61.0%, respectively). No aspirin resistance was detectable, while clopidogrel resistance was found in 7 of 30 patients (23.3%). All seven patients with clopidogrel resistance developed thrombotic events after stent implantation. We evaluated the efficacy of CD62P and CD63 expression ratios determined by flow cytometry to conventional LTA using ADP concentrations of 5.0 and 20.0 µM in identifying drug responsibility in patients and controls. The flow cytometry results showed that the CD62P and CD63 ratios were compatible with LTA (Figure 3).
Table 1. Baseline characteristics of 30 patients with cerebral aneurism and 30 healthy controls

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Healthy controls (n=30)</th>
<th>Patients (n=30)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Age, mean±SD</strong></td>
<td>54.9±15.0</td>
<td>55.7±14.6</td>
<td>ns</td>
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<tr>
<td><strong>Sex, n (%)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Female</td>
<td>23 (76.7)</td>
<td>20 (66.7)</td>
<td>ns</td>
</tr>
<tr>
<td>Male</td>
<td>7 (23.3)</td>
<td>10 (33.3)</td>
<td></td>
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<tr>
<td><strong>Platelet count (x10^9 cells/L), mean±SD</strong></td>
<td>401±106.7</td>
<td>393±117.8</td>
<td>ns</td>
</tr>
<tr>
<td><strong>Platelet aggregation test (%Max), Median (IQR)</strong></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>ADP, 5.0 mM</td>
<td>74.0 (69.0–81.0)</td>
<td>36.5 (36.5–40.0)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>ADP, 20.0 mM</td>
<td>87.0 (79.0–91.0)</td>
<td>47.0 (37.0–58.0)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>AA, 1.5 mM</td>
<td>86.5 (81.0–92.0)</td>
<td>3.5 (2.0–5.5)</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

%Max; the percentage of max aggregation, AA; arachidonic acid, ADP; adenosine diphosphate, IQR; interquartile range, ns; not significant, SD; standard deviation

![Figure 1](image)

**Figure 1.** The representative of platelet activation markers, CD62P and CD63, expressions measured by flow cytometry in (A) controls and (B) patients
The ROC curve with AUC analyses

The ROC analysis assessed the overall performance of discriminating between the patients on aspirin and clopidogrel treatment and controls. The ROC curves of LTA using ADP 5.0 µM and 20.0 µM and the surface markers, CD62P and CD63, were plotted, as shown in Figure 4. The optimal cut-offs for each assay’s highest sensitivity and specificity were determined. Moreover, the diagnostic indices for each assay, including AUC, sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV), were calculated, as shown in Table 2. The AUC of CD62P using flow cytometry and the LTA results using ADP 5.0 µM and 20.0 µM were comparable (0.97 vs. 0.95 and 0.97 vs. 0.96, respectively), whereas the AUC of CD63 was lower than the LTA (0.88 vs. 0.96). The sensitivity and specificity of CD62P were 71.4% and 100.0%, respectively. The sensitivity and specificity of CD63 were 60.0% and 96.7%, respectively.

Discussion

Despite the benefits of dual antiplatelet therapy in preventing cardiovascular events being evidenced by multiple trials,6, 7, 12, 13 the emergence of unexpected drug-related side effects remains a concern.7, 14, 15 Consequently, rigorous monitoring of its therapeutic efficacy is recommended. The current gold standard assay, LTA, has been employed for decades and has demonstrated efficacy in predicting clinical outcomes among patients undergoing antiplatelet therapy.17, 19 Nevertheless, it is encompassed by several limitations, including poor standardization, high blood specimen volume required, and the requirement for skilled technician involvement.17, 19 While several commercially available tests for evaluating platelet function are now accessible, there remains a lack of correlation among them.21 Additionally, no currently established assay for evaluating the antiplatelet effect in patients receiving dual antiplatelet therapy is available.17, 18, 21 Here, we performed a preliminary study to assess the efficacy of measuring platelet surface activation markers, CD62P and CD63, using flow cytometry to determine the effectiveness of dual antiplatelet therapy with aspirin and clopidogrel.

This study enrolled 30 patients who received dual antiplatelet therapy with aspirin and clopidogrel before cerebral stent implantation and 30 unrelated healthy controls. No significant differences were observed in age, sex, or baseline platelet counts between the patients and controls. As expected, the percentages of max platelet aggregation induced by 5.0 and 20.0 µM of ADP were significantly lower in the patients than in the healthy controls. Regarding the AA-induced platelet aggregation results, among the 30 patients, no aspirin resistance was observed in this study.

CD62P, also known as P-selectin, is an abundant glycoprotein in platelets’ alpha granule membranes. It becomes exposed on the cell
Figure 3. The expression ratios of CD62P and CD63 determined by flow cytometry compared to conventional platelet aggregation by LTA in patients and controls.

Figure 4. The receiver operating characteristic (ROC) curve analyses of the platelet function test using ADP concentrations of 5.0 and 20.0 µM and expression ratios of CD62P and CD63 measured by flow cytometry
surface following degranulation after platelet activation. (28, 29) Similarly, CD63 is a member of the tetraspanin superfamily, initially present in the dense granule and lysosomal membranes of platelets at rest and translocates to the plasma membrane during platelet activation. (30) Hence, measuring the surface CD26P or CD63 is considered a feasible and reliable method for assessing granule secretion and, thus, platelet activation in ex vivo patient samples. (28, 29) In the patients receiving antiplatelet treatments affecting the ADP-dependent platelet activation pathway, including aspirin, clopidogrel, or combined therapy with these drugs, it was reported that the expressions of surface CD26P and CD63 were decreased. (29) In this study, the expressions of the platelet activation markers, CD62P and CD63, were measured using flow cytometry in both patients and controls. Subsequently, each marker’s expression ratios for stimulated and non-stimulated platelets were calculated. Consistent with the previous studies, (28, 29, 30) our results revealed that the expression ratios of both markers were significantly lower in the patients receiving dual antiplatelet therapy than in the controls.

The AUCs of the ROC curve were analyzed to determine the optimal cut-offs and evaluate the performance of discriminating between the receiving dual treatment of aspirin and clopidogrel and controls. Our analysis revealed that the LTA using 5.0 and 20.0 mM of ADP and CD62P expression ratio demonstrated excellent performance, while the CD63 expression ratio exhibited very good performance. Based on our optimal cut-offs, CD62P and CD63 expression ratios provided inferior sensitivities; however, their specificities were higher than those of the conventional LTA. It was suggested that the CD62P expression ratio could be a helpful marker for monitoring the effect of antiplatelet therapy since it provided less false positive results in untreated individuals. This flow cytometry technique required a small amount of blood volume, provided high reproducibility, and was simple to standardize. Furthermore, flow cytometry based on single-cell analysis, independent of platelet counts, could be utilized to assess the efficacy of antiplatelet medication in patients with thrombocytopenia.

Some issues merit careful consideration. First, the sample size in this study was insufficient to provide the statistical power to suggest implementation in clinical practice. Further studies with larger sample sizes are recommended. Second, the proportion of patients receiving dual antiplatelet medication who developed antiplatelet resistance was relatively low. As a result, the capacity to distinguish between patients with response and resistance to antiplatelet drugs was not evaluated.

**Conclusion**

In conclusion, due to their superior sensitivity and specificity, flow cytometry measurements of the platelet surface activation marker CD62P

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**Table 2. The diagnostic performance of the platelet function measured by aggregometry and flow cytometry**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Light transmitted aggregometry</th>
<th>Flow cytometry</th>
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<tr>
<td></td>
<td>ADP 5.0 mM</td>
<td>ADP 20.0 mM</td>
</tr>
<tr>
<td>Optimal cut-offs</td>
<td>43.0</td>
<td>61.0</td>
</tr>
<tr>
<td>AUC (95% CI)</td>
<td>0.95 (0.90–1.00)</td>
<td>0.96 (0.91–1.00)</td>
</tr>
<tr>
<td>p-value</td>
<td>&lt; 0.001</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Sensitivity, % (95% CI)</td>
<td>83.3 (66.4–92.7)</td>
<td>100.0 (88.7–100.0)</td>
</tr>
<tr>
<td>Specificity, % (95% CI)</td>
<td>90.0 (74.4–96.5)</td>
<td>86.7 (70.3–94.7)</td>
</tr>
<tr>
<td>NPV, % (95% CI)</td>
<td>96.2 (78.3–99.4)</td>
<td>96.2 (78.3–99.4)</td>
</tr>
<tr>
<td>PPV, % (95% CI)</td>
<td>85.3 (72.2–92.8)</td>
<td>85.3 (72.2–92.8)</td>
</tr>
</tbody>
</table>

ADP, adenosine di-phosphate; AUC, area under the curve; CI, confident interval; NPV, negative predictive value; PPV, positive predictive value
could be utilized to identify the efficacy of dual therapy with aspirin and clopidogrel. Additional studies are suggested to support this issue and its effects on clinical outcomes.

**Disclosures**

The authors declared they have no conflicts of interest.

**References**


