Precision and Reliability of 5 Platelet Function Tests in Healthy Volunteers and Donors on Daily Antiplatelet Agent Therapy

Brad S. Karon,1* Nicole V. Tolan,2 Christopher D. Koch,1 Amy M. Wockenfus,1 Randall S. Miller,1 Ravi K. Lingineni,3 Rajiv K. Pruthi,1 Dong Chen,1 and Allan S. Jaffe1

BACKGROUND: Anticoagulation protocols used during mechanical circulatory support call for titration of antiplatelet agents. We compared the precision and reliability of 5 platelet function tests in healthy volunteers and donors on daily antiplatelet therapy to distinguish their efficacy for titrating antiplatelet therapy.

METHODS: We assessed arachidonic acid–induced platelet function by light transmission aggregometry (LTA), Multiplate impedance aggregometry, VerifyNow, and platelet mapping by thromboelastography (TEG PM). We assessed ADP-induced platelet function by the same methods and flow cytometry. Forty healthy volunteers and 10–13 volunteers on daily aspirin and/or clopidogrel therapy were evaluated. We compared tests for intraassay precision, interassay precision (samples from 2 separate blood draws), and reliability coefficient.

RESULTS: For arachidonic acid–induced platelet aggregation in healthy volunteers, intra- and interassay CVs were ≤10% for all methods. Intra- and interassay precision among donors on daily aspirin was ≤30% for all methods except LTA (38% interassay CV) and TEG PM (95% intra-assay and 104% interassay CV). For ADP-induced platelet function, intra- and interassay precision was ≤10% and ≤30% for all methods. Only Multiplate demonstrated moderate or greater (R > 0.40) reliability coefficients for arachidonic acid–induced platelet function among all subjects. All methods of ADP-induced platelet function, except TEG PM, demonstrated substantial or greater (R > 0.60) reliability among all subjects.

CONCLUSIONS: TEG PM is least suited to monitor effects of antiplatelet agents. Multiplate impedance aggregometry was the only method to demonstrate an acceptable reliability coefficient among healthy volunteers and donors on both aspirin and clopidogrel therapy.

© 2014 American Association for Clinical Chemistry

Long-term mechanical circulatory support (MCS)4 is used as a bridge to cardiac transplant, a bridge to recovery in patients with myocardial damage, and a destination therapy for end-stage heart failure patients who are not candidates for transplant (1). MCS, such as left ventricular assist device (LVAD) and total artificial heart, has dramatically increased survival to transplant in both adults and children (2, 3), although use of MCS has many complications. During the perioperative period, up to 60% of patients experience excess bleeding, and historically 5%–20% have experienced thromboembolism or stroke (1). Although some newer continuous-flow LVAD devices demonstrate lower rates of systemic thromboembolism and bleeding, device thrombosis continues to be a problem (4). Hemorrhage and thromboembolism are also common long-term complications after device implantation (5).

To balance the risk of perioperative bleeding with device thrombosis and thromboembolism, protocols for MCS placement call for titration of antiplatelet agents by use of laboratory tests of platelet function [tests of arachidonic acid–induced platelet function to titrate aspirin and adenosine diphosphate (ADP)–induced platelet function to titrate agents such as dipyridimole and clopidogrel (2, 6, 7)]. However, there is little evidence to suggest that laboratory tests of platelet function are precise or reliable enough to allow for titration of antiplatelet agents over short periods of time. Recent consensus guidelines have also been developed to define high on-treatment platelet reactivity by use of tests of ADP-
induced platelet function, although neither biologic nor analytic test variability was considered in recommendations for interpreting these results (8, 9).

Several studies have examined the analytic precision of platelet function tests (10–13). However, platelet activation occurs during blood sampling and processing (14–17), making replicate analysis of samples from a single blood draw an inaccurate estimate of total assay precision. Only a small number of studies have measured platelet function precision by use of multiple blood samples collected over a short period of time (13, 18, 19). One previous study used the reliability coefficient of platelet function tests to measure variability in platelet function associated with blood collection and processing (18). Those authors reasoned that platelet function tests that show a lower reliability coefficient (higher degree of within person variability) would be less likely to show significant correlations with risk factors or disease outcomes (18).

In this study, we measured intraassay precision (duplicate analysis from a single blood draw), interassay precision (analysis of samples from 2 blood draws performed within 24–28 h), and reliability coefficient of 5 platelet function tests. We measured platelet function in both healthy volunteers and donors on daily aspirin and/or clopidogrel therapy. By comparing the distribution of platelet function test results in healthy volunteers to those on platelet inhibitors, and, by calculating intraassay precision, interassay precision, and reliability coefficient among volunteers with both normal and inhibited platelet function, we compared the relative efficacy of 5 platelet function tests for titrating antiplatelet therapy over short periods of time.

Materials and Methods

STUDY POPULATION
Forty fasting healthy adult volunteers (20 men and 20 women) who denied taking over-the-counter medications for at least 4 days, aspirin-containing products for at least 10 days, or prescription medications before the study, were recruited. After 2 mL discard, blood was collected by venipuncture into one 3-mL plain syringe, four 2.0-mL Vacuette 3.2% citrate tubes (Greiner Bio-One), three 2.7-mL Vacutainer 3.2% citrate tubes (BD), one 4.0-mL lithium heparin (75 USP units) tube (BD), and one 3.0-mL, 25-µg/mL hirudin tube (Verum Diagnostica). Twenty-three of the 40 healthy volunteers returned within 24–28 h for a repeat fasting blood draw. An additional 13 treatment donors (8 men and 5 women) were solicited through “volunteers wanted” research advertising around the clinic. These donors were not hospitalized, acutely ill, or seeking medical care during the study period and were taking daily doses of aspirin (80–325 mg). Ten similar donors (6 men and 4 women) taking daily doses of clopidogrel were recruited for 2 blood draws within 24–28 h as described above. Eight donors were taking both aspirin and clopidogrel daily and were counted in both treatment groups.

Samples from all 40 healthy donors were used for assessment of arachidonic acid– and ADP-induced platelet function. For the treatment group, only those volunteers on aspirin were included in the assessment of arachidonic acid–induced platelet function, and only those on clopidogrel were assessed for ADP-induced platelet function. All platelet function tests were completed within 2 h of blood draw. The study design was approved by the Mayo Clinic Institutional Review Board.

PLATELET FUNCTION ASSAYS
Platelet function tests included light transmission aggregometry (LTA) by use of platelet-rich plasma on a PAP-8E aggregometer (Bio/Data Corp.), Multiplate whole blood impedance aggregometry (Multiplate, Dynabyte), VerifyNow whole blood aggregometry (VerifyNow, Accumetrics), TEG PM (Hemonetics), and flow cytometric assessment of vasodilator-stimulated phosphoprotein (VASP). For details of platelet function assays, see Supplemental Data 1, which accompanies the online version of this article at http://www.clinchem.org/content/vol60/issue12.

STATISTICAL ANALYSIS
We calculated mean (SD) values for tests of both arachidonic acid– and ADP-induced platelet function for healthy volunteers and donors on daily aspirin (arachidonic acid–induced function only) and clopidogrel (ADP-induced function only) therapy. Using average (of duplicate) values observed on initial blood sampling, we performed ROC sensitivity analysis to compare tests in the ability to discriminate between healthy volunteers and donors on daily aspirin or clopidogrel therapy.

We calculated intraassay precision among healthy donors by averaging the standard deviation of all 63 (40 healthy donors on day 1 and 23 healthy donors who returned for a second blood draw) duplicate results, and dividing by the average value among the 63 duplicates. We calculated interassay precision from analysis of 4 samples each among the 23 healthy donors who had blood drawn for duplicate analysis on 2 occasions within 24–28 h, again by dividing the mean (SD) from quadruplicate analysis by the mean value. Interassay precision is an expression of average within-person variability (preanalytic and analytic, plus biologic variability). By repeating analysis within 24–28 h, biologic variability was minimized, to obtain an estimate of total assay (preanalytic plus analytic) variability.
In a similar manner, we calculated intraassay and interassay precision for 10 treatment donors taking daily clopidogrel and 13 treatment donors taking daily aspirin. By measuring platelet function among both healthy volunteers and donors receiving daily aspirin and/or clopidogrel, we calculated assay precision at levels of platelet function anticipated for patients with normal and inhibited platelet function. We calculated the reliability coefficient ($R$) by partitioning total variance ($\sigma_{TOT}^2$) from quadruplicate analysis (duplicates from each of 2 blood draws) among 23 healthy donors and 10–13 treatment donors into between-person ($\sigma_{BP}^2$) and within-person ($\sigma_{WP}^2$) variance, assuming a random effect in a linear mixed-effects model. Preanalytic (platelet activation during blood draw and processing), analytic, and biologic variability all contribute to within-person variance, whereas between-person variance reflects individual differences in both baseline platelet activity and response to antiplatelet agents. The proportion of total variance attributed to between-person variability, or reliability coefficient ($R = \sigma_{BP}^2/\sigma_{TOT}^2$), can be interpreted as the correlation between paired measurements (measurements obtained on 2 separate days). A lower $R$ implies that sources of within-person variance contribute more to total variance, such that observed differences between individuals are less likely to show significant correlations with risk factors or disease outcomes. Analogous to the calculation of intraclass coefficient, guidelines for interpretation of reliability coefficient are 0–0.20, poor reliability; 0.21–0.40, fair reliability; 0.41–0.60, moderate reliability; 0.61–0.80, substantial reliability; and 0.81–1.00, very good reliability (18). On the basis of a previous study (18), moderate or higher ($R > 0.40$) reliability was considered acceptable.

Results

DISTRIBUTION OF PLATELET FUNCTION RESULTS AMONG HEALTHY VOLUNTEERS AND DONORS ON ANTIPLATELET THERAPY

Four assays were used to assess arachidonic acid–induced platelet function on 64 samples (each in duplicate) obtained from 40 healthy volunteers, and 26 samples (each in duplicate) obtained from 13 donors taking daily aspirin therapy. For TEG PM, Multiplate, and LTA, mean values among healthy volunteers were at least 5-fold higher than mean values among donors taking daily aspirin (Fig. 1). In contrast, the mean VerifyNow value among healthy volunteers was <2-fold higher than the mean value among aspirin-treated donors (Fig. 1). ROC sensitivity analysis performed with average values from initial blood sampling demonstrated that the AUC for Multiplate and TEG was 1.000. By use of average initial values, the tests could distinguish healthy volunteers from donors on aspirin therapy. However, when using all individual values, there was overlap between healthy volunteers and aspirin-treated donors for TEG PM but not Multiplate (Fig. 1). AUCs for LTA and VerifyNow were 0.959 and 0.998, respectively. Differences in AUC values among tests were not statistically significant ($\chi^2 P$ value >0.05).

In a similar manner, ADP-induced platelet activity was assessed by TEG PM, LTA, VASP, VerifyNow, and Multiplate on 64 healthy volunteer samples and 20 clopidogrel donor samples. Mean (SD) TEG PM values for healthy donors [85% (14%)] and those on daily clopidogrel [72% (23%)] differed little. In contrast LTA, VerifyNow, and Multiplate mean values among healthy donors were approximately 2-fold higher than mean values among donors on daily clopidogrel. VASP flow cytometry mean value for healthy donors was approximately 5-fold higher than the clopidogrel group (Fig. 2). By ROC sensitivity analysis, the AUC for discriminating healthy volunteers from clopidogrel donors for TEG PM was 0.589, significantly lower ($\chi^2 P$ value <0.05) than the AUC for Multiplate (0.930), LTA (0.892), VerifyNow (0.950), and VASP (1.000). Although AUC values for Multiplate, LTA, VerifyNow, and VASP did not differ significantly, VASP was the only method that could distinguish healthy volunteers from donors on clopidogrel therapy by use of average initial values (AUC 1.000).

INTRA- AND INTERASSAY PRECISION OF ARACHIDONIC ACID-INDUCED PLATELET FUNCTION

VerifyNow was the only method to demonstrate intra- and interassay precision <10% among healthy volunteers and donors on daily aspirin therapy (Table 1). Multiplate had intra- and interassay CVs <10% among healthy volunteers, and intra- and interassay CVs <25% among donors on daily aspirin. Compared with the other methods, LTA (38% interassay CV) and TEG PM (95% intra-assay and 104% interassay CV) demonstrated poorer precision among donors on daily aspirin therapy (Table 1), owing in part to low absolute values for LTA and TEG PM among aspirin donors (Fig. 1).

INTRA- AND INTERASSAY PRECISION FOR ADP-INDUCED PLATELET FUNCTION

All methods had intra- and interassay precision <10% among healthy volunteers; and all assays with the exception of VASP flow cytometry had intra- and interassay precision <15% among clopidogrel-treated donors (Table 2).

RELIABILITY COEFFICIENT FOR PLATELET FUNCTION TESTS

For arachidonic acid–induced platelet function among healthy volunteers, Multiplate and LTA both demonstrated moderate reliability ($R = 0.41–0.60$), whereas
the VerifyNow demonstrated only fair and TEG PM poor reliability (Table 3). Among donors with reduced platelet function (aspirin-treated), both VerifyNow and Multiplate demonstrated substantial reliability ($R$/H110050.61–0.80) for arachidonic acid–induced platelet function, whereas LTA and TEG PM demonstrated only fair reliability (Table 3).

In a similar manner, Multiplate, LTA, VerifyNow, and VASP all demonstrated substantial or very good reliability for assessing ADP-induced platelet function among healthy volunteers, whereas TEG PM demonstrated only fair reliability (Table 3). Among clopidogrel-treated donors, all measures of ADP-induced platelet activation demonstrated substantial or very good reliability (Table 3).

**Discussion**

Protocols for MCS placement call for monitoring and titration of antiplatelet therapy in the postoperative period (2, 6, 7). Interpreting changes in platelet function over several days, whether to titrate antiplatelet therapy during MCS or monitor therapy for other acutely ill patients, requires knowledge of assay precision over short periods of time. Because platelet activation during blood collection (a source of preanalytic variability) is a function of collection tube type (15, 16); temperature and time of blood storage and processing (14–16); amount of vacuum in collection tube (17); and other factors, it is not possible to independently measure analytic and biologic variability for tests of platelet function.

In this study, we compared the distribution of platelet function results among healthy volunteers and donors on platelet inhibitors. We also measured intra-assay precision (analytic variability), interassay precision (preanalytic, analytic, and biologic variability), and reliability coefficient (the ratio of between-subject variance to total variance) for both arachidonic acid– and ADP-induced platelet function. Note that although interassay precision is an estimation of average within-subject variability, the within-subject variance used to calculate reliability coefficient is the sum of all within-subject variability. By performing repeat blood draws within 24–28 h, we purposefully minimized biologic variability to allow direct comparison of preana-
lytic and analytic variation in test results among subjects with normal and inhibited platelet function.

VerifyNow was the only method to demonstrate intra- and interassay precision <10% among both healthy volunteers and donors on daily aspirin (Table 1). Despite having the lowest intra- and interassay precision, VerifyNow demonstrated only fair reliability ($R = 0.23$) for arachidonic acid–induced platelet function among healthy volunteers. Lower VerifyNow reliability can be attributed to a subset of healthy volunteers (5 of 20) who had much higher interassay variability than the remaining 15 volunteers (data not shown). The result is that total within-person variability (used in the calculation of reliability coefficient) is substantially greater than average within-person variability (interassay precision). In contrast, VerifyNow demonstrated substantial reliability ($R = 0.78$) for arachidonic acid–induced platelet function among donors on daily aspirin therapy.

LTA demonstrated only fair reliability ($R = 0.25$) for arachidonic acid–induced platelet function among aspirin-treated donors, owing to higher interassay CV (37.6%) and in part to 1 treatment donor whose duplicate values on 1 testing day appear more consistent.

---

**Table 1.** Intra- and inter-assay precision for tests of arachidonic acid–induced platelet function among healthy volunteers and donors on daily aspirin therapy.

<table>
<thead>
<tr>
<th>Assay</th>
<th>Healthy volunteers</th>
<th></th>
<th></th>
<th>Aspirin-treated donors</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Intra-assay CV (%)</td>
<td>n</td>
<td>Interassay CV (%)</td>
<td>n</td>
<td>Intra-assay CV (%)</td>
<td>n</td>
</tr>
<tr>
<td>VerifyNow</td>
<td>1.4</td>
<td>120</td>
<td>2.4</td>
<td>22</td>
<td>3.7</td>
<td>24</td>
</tr>
<tr>
<td>Multiplate</td>
<td>5.2</td>
<td>128</td>
<td>9.7</td>
<td>24</td>
<td>16.3</td>
<td>26</td>
</tr>
<tr>
<td>LTA</td>
<td>2.7</td>
<td>128</td>
<td>7.2</td>
<td>24</td>
<td>10.1</td>
<td>26</td>
</tr>
<tr>
<td>TEG PM</td>
<td>3.4</td>
<td>124</td>
<td>6.4</td>
<td>23</td>
<td>95.3</td>
<td>26</td>
</tr>
</tbody>
</table>

For TEG PM, LTA, and VASP, each point represents 1 platelet function value expressed as percent activity or platelet reactivity index (VASP); for VerifyNow and Multiplate, values are expressed as arbitrary response units (ARUs) or arbitrary aggregation units (AAUs), respectively. Each individual measurement taken during the study (days 1 and 2) are plotted. Below the figure, mean (SD) values for healthy volunteers and donors on daily clopidogrel treatment are shown.

---
with reference values (Fig. 1). Rare healthy volunteer samples also demonstrated abnormally low values for both arachidonic acid– and ADP-induced aggregation (Fig. 1 and 2), a phenomenon that has been observed previously (19). LTA requires preparation of platelet-poor plasma from whole blood, which may result in preparation artifacts, leading to lower interassay precision and reliability. Our results for VerifyNow and LTA interassay precision are similar to those of a previous study that found 3% day-to-day CV for VerifyNow among donors on aspirin compared to 33% day-to-day CV for LTA (13).

TEG PM demonstrated only fair reliability ($R = 0.26$) among donors on daily aspirin, consistent with the high intra- and interassay CVs (Table 1). One previous study found that interassay precision for most TEG PM parameters [maximum amplitude (MA) thrombin, MA ADP, MA AA] was <10%, whereas interassay precision of the fourth parameter (MA fibrin) was much higher at 45.8% (20). Another study described “thrombin breakthrough,” wherein MA fibrin continues to increase over time rather than plateau rapidly, as one potential cause of imprecision in MA fibrin (21). Among 91 pairs of duplicate TEG PM tracings, we observed thrombin breakthrough in 4 tracings and observed imprecision between duplicates (>5 mm difference in MA fibrin) not due to thrombin breakthrough in another 6. Thrombin breakthrough and other causes of variability in MA fibrin are likely the reason for the poor precision and reliability of the TEG PM test.

Multiplate was the only measure of arachidonic acid–induced platelet function to demonstrate moderate or substantial reliability for both healthy volunteers and donors on daily aspirin therapy (Table 3). Multiplate impedance aggregometry had reasonably good precision in both healthy volunteers and aspirin-treated donors, and could distinguish values in all healthy volunteers from those on aspirin (Fig. 1), making it the single best test for measuring the effects of aspirin at the initiation of treatment. In contrast, VerifyNow might be the optimal test to monitor arachidonic acid–induced platelet function in patients with highly inhibited platelet function.

In a similar manner, we measured distribution of results, intra- and interassay precision, and reliability coefficient for 5 tests of ADP-induced platelet function among healthy volunteers and donors on daily clopidogrel therapy (Table 3). Multiplate impedance aggregometry had reasonably good precision in both healthy volunteers and aspirin-treated donors, and could distinguish values in all healthy volunteers from those on aspirin (Fig. 1), making it the single best test for measuring the effects of aspirin at the initiation of treatment. In contrast, VerifyNow might be the optimal test to monitor arachidonic acid–induced platelet function in patients with highly inhibited platelet function.

In a similar manner, we measured distribution of results, intra- and interassay precision, and reliability coefficient for 5 tests of ADP-dependent platelet function among healthy volunteers and donors on daily clopidogrel therapy (Table 3). TEG PM values differed little between healthy volunteers and clopidogrel-treated donors (Fig. 1), and it was the only method that did not demonstrate substantial or very good reliability in both groups (Table 3). TEG PM also had a significantly lower AUC (0.589) for distinguishing healthy volunteers from donors on clopidogrel. Thus TEG PM is least suitable for monitoring platelet function over short periods of time in patients on ADP inhibitors.

LTA, Multiplate, and VerifyNow all demonstrated intra-assay precision <10% and interassay precision

---

### Table 2. Intra- and inter-assay precision for tests of ADP-induced platelet function among healthy volunteers and donors on daily clopidogrel therapy.

<table>
<thead>
<tr>
<th>Assay</th>
<th>Healthy volunteers</th>
<th>Clopidogrel-treated donors</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Intra-assay CV (%)</td>
<td>Interassay CV (%)</td>
</tr>
<tr>
<td></td>
<td>n</td>
<td>Interassay CV (%)</td>
</tr>
<tr>
<td>VASP</td>
<td>1.9</td>
<td>120</td>
</tr>
<tr>
<td>VerifyNow</td>
<td>4.4</td>
<td>118</td>
</tr>
<tr>
<td>Multiplate</td>
<td>4.3</td>
<td>128</td>
</tr>
<tr>
<td>LTA</td>
<td>3.3</td>
<td>128</td>
</tr>
<tr>
<td>TEG PM</td>
<td>6.7</td>
<td>122</td>
</tr>
</tbody>
</table>

### Table 3. Reliability coefficient ($R$) for tests of arachidonic acid–induced and ADP-induced platelet function in healthy volunteers and donors on daily aspirin (arachidonic acid–induced platelet function only) or daily clopidogrel (ADP-induced platelet function only) therapy.

<table>
<thead>
<tr>
<th>Assay</th>
<th>Healthy volunteers</th>
<th>Treatment (aspirin) donors</th>
<th>Healthy volunteers</th>
<th>Treatment (clopidogrel) donors</th>
</tr>
</thead>
<tbody>
<tr>
<td>VASP</td>
<td>NA</td>
<td>0.64</td>
<td>0.83</td>
<td>0.66</td>
</tr>
<tr>
<td>VerifyNow</td>
<td>0.23</td>
<td>0.78</td>
<td>0.89</td>
<td>0.66</td>
</tr>
<tr>
<td>Multiplate</td>
<td>0.48</td>
<td>0.68</td>
<td>0.61</td>
<td>0.89</td>
</tr>
<tr>
<td>LTA</td>
<td>0.60</td>
<td>0.25</td>
<td>0.66</td>
<td>0.89</td>
</tr>
<tr>
<td>TEG PM</td>
<td>0.06</td>
<td>0.26</td>
<td>0.35</td>
<td>0.93</td>
</tr>
</tbody>
</table>

* Values in bold have less than moderate ($R \leq 0.40$) reliability for a given test and population. NA = not applicable.
<15% for ADP-induced platelet function among both healthy volunteers and clopidogrel-treated donors (Table 2). Similarly, these methods all demonstrated substantial or very good reliability for ADP-induced platelet function among healthy volunteers and donors on daily clopidogrel (Table 3). LTA, Multiplate, and VerifyNow may all be appropriate for monitoring the short-term effects of drugs such as dipyrimidole and clopidogrel that inhibit ADP-induced platelet function.

VASP flow cytometry best differentiated platelet function among healthy volunteers from those on clopidogrel therapy (Fig. 2) and was the only test in ROC sensitivity analysis that could distinguish healthy volunteers from donors on clopidogrel by use of average initial values (AUC 1.00). Although interassay CVs among clopidogrel-treated donors were higher for VASP than for other tests (Table 2), the reliability coefficient for VASP was still substantial and very good for healthy and clopidogrel-treated donors (Table 3). VASP may be the optimal test in situations where ADP-induced platelet function is anticipated to change from normal to inhibited. However, VASP requires fixation and staining of whole blood and expertise in flow cytometry, limiting applicability.

One limitation to our study is that we did not include a measure of accuracy. Other investigators have measured accuracy of platelet function tests relative to LTA. However, LTA has never been demonstrated to be suitable for use as a reference standard for quantitative platelet function. The lack of a consensus reference method for assessment of arachidonic acid– and ADP-induced platelet function precludes assessment of accuracy for these tests.

Conclusions

Only the Multiplate device demonstrated moderate or better reliability for measurement of arachidonic acid–induced platelet activity in healthy volunteers and donors on daily aspirin therapy. VASP flow cytometry, Multiplate impedance aggregometry, VerifyNow, and LTA all demonstrate substantial to very good reliability for measuring ADP-dependent platelet activation in healthy volunteers and donors on daily clopidogrel therapy. TEG PM is least appropriate for use in short-term monitoring of patients started on antiplatelet therapy.

References

