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 ($\kappa=0.16$, 95% CI, −0.08 to 0.39, $P=0.11$; and $\kappa=0.09$ −0.12 to 0.30, $P=0.32$, respectively). Agreement between the 2 POC tests was also poor ($\kappa=0.14$, −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.

Key Words: aspirin • platelets

Aspirin (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 therefore defined using the normal range cutoff of 164 seconds and ASA responders with a 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 (aperture. Platelet function is thus measured as a function of the time (closed time [CT]) it takes to occlude the aperture. When normal individuals ingest varying dosages (75 to 1000 mg) of ASA there is a dose-dependent prolongation of the CT on the CEPI cartridge but not on the CADP cartridge.21,22 The coefficient of variation of CTs has been reported as 10%.17

For analysis, 0.8 mL of the mixed whole blood was pipetted into the sample reservoirs of 1 CADP (batches 559555 and 569634) and 1 CEPI cartridge (batches 559634 and 579519; (prewarmed to room temperature) and then loaded into the PFA-100. In-house normal ranges are 55 to 112 seconds for CADP and 79 to 164 seconds for CEPI. Full blood counts were performed on the blood samples. An ASA response was therefore defined using the normal range cutoff of 164 seconds on the CEPI cartridge. ASA nonresponders were defined with a CEPI CT ≤164 seconds and ASA responders with a CEPI CT >164 seconds.

### RPFA

RPFA (Accumetrics Inc) is a turbidimetric-based optical detection system that measures platelet-induced aggregation.21 This device was originally developed as a POC testing instrument to provide a simple and rapid functional means of monitoring anti-Gp IIb/IIIa therapy.23–25 The test has been adapted to measure the effect of ASA within a modified cartridge (VerifyNow aspirin). The disposable cartridge contains fibrinogen-coated beads and a platelet activator (metallic cations and propyl gallate) to stimulate the COX-1 pathway 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. 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 diprydramidol 600 mg daily. None was taking regular nonsteroidal anti-inflammatory agents, although this had not been an exclusion criterion.

Three normal subjects (aged 40, 44, and 51 years) were tested by all 3 platelet function tests either before (n=3) and 2 hours after ingestion of 300 mg of ASA (n=2) or after addition and incubation for 30 minutes with 100 μmol/L ASA at 37°C in vitro (n=1) (Table 2). One hundred patients were analyzed by PFA-100 and RPFA; 99 of 100 and 98 of 100 were additionally analyzed by LTA using arachidonic acid and ADP, respectively. Results are given in Table 3.

**Table 1. Characteristics of the 100 Patients Studied**

<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>
<tr>
<td>Ex-smoker</td>
<td>45</td>
</tr>
<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.
Arachidonic acid–induced LTA identified 12 of 99 (12%) patients who were ASA nonresponders. ADP-induced LTA identified 14 of 98 (14%) patients who were ASA nonresponders. Five of 98 (5%) patients were classified as fully ASA nonresponsive because they were only detected by one of the tests, and 16 of 98 (16%) patients were classified as partially ASA nonresponsive because they were detected by both tests, and 12 of 98 (12%) patients who were ASA nonresponder. ADP-induced LTA identified 14 of 98 (14%) patients who were ASA nonresponsive by the CEPI CT alone and 8 by LTA alone. Overall agreement between the PFA-100 and arachidonic acid aggregation was poor (κ = 0.09, 95% CI, −0.12 to 0.30, P = 0.32).

Figure 3 shows the comparison of the PFA-100 with the RPFA. Of the 100 samples tested, 73 were concordant, with 67 ASA responders and 6 nonresponders. Twenty-seven samples were discordant, with 16 categorized as ASA nonresponders by the RPFA alone and 11 by the PFA-100 alone. Overall agreement between the 2 POC tests was poor (κ = 0.14, −0.08 to 0.36, P = 0.15).

Among those patients who were using another antiplatelet agent in addition to ASA, 4 of the 6 patients receiving clopidogrel and both patients receiving dipyridamole were ASA-responsive by all 3 tests. The 3 patients who were receiving 150 mg of ASA were also responsive according to all 3 tests.

### Discussion

The recent association of ASA nonresponsiveness with a possibly increased risk of major vascular events suggests that this phenomenon may be an important clinical entity\(^{11,12}\) and raises the possibility of routine screening of patients receiving ASA. However, LTA is poorly standardized, requires a specialist laboratory, and is unlikely to be used widely in routine clinical practice. Alternatives include urinary thromboxane measurements,\(^{30}\) the RPFA, and the PFA-100.\(^{13,14,17,18,22}\) The latter 2 tests are already approved by the Food and Drug Administration and are being used in some centers, but very few data are available comparing the results of the different tests and there has been no systematic comparison of the 2 POC tests with LTA in patients with transient ischemic attack or stroke.

### Tables

#### Table 2

<table>
<thead>
<tr>
<th>Control Subject</th>
<th>PFA-100 CADP, CT, sec</th>
<th>PFA-100 CEPI, CT, sec</th>
<th>RPFA ARU</th>
<th>10 mmol ADP, % Agg</th>
<th>1 mmol/L AA, % Agg</th>
</tr>
</thead>
<tbody>
<tr>
<td>PH before ASA</td>
<td>85</td>
<td>134</td>
<td>641</td>
<td>83</td>
<td>80</td>
</tr>
<tr>
<td>PH 2 hours after 300 mg ASA</td>
<td>&gt;80</td>
<td>&gt;300</td>
<td>517</td>
<td>51</td>
<td>9</td>
</tr>
<tr>
<td>KS before ASA</td>
<td>84</td>
<td>96</td>
<td>560</td>
<td>78</td>
<td>79</td>
</tr>
<tr>
<td>KS 2 hours after 300 mg ASA</td>
<td>96</td>
<td>&gt;300</td>
<td>420</td>
<td>59</td>
<td>8</td>
</tr>
<tr>
<td>HS before ASA</td>
<td>71</td>
<td>109</td>
<td>583</td>
<td>82</td>
<td>77</td>
</tr>
<tr>
<td>HS 100 mmol ASA final concentration in vitro</td>
<td>77</td>
<td>&gt;297</td>
<td>393</td>
<td>59</td>
<td>8</td>
</tr>
</tbody>
</table>

Agg indicates aggregometry.

#### Table 3

<table>
<thead>
<tr>
<th>Classification</th>
<th>Arachidonate Aggregation (n=99)</th>
<th>ADP Aggregation (n=98)</th>
<th>Combined Aggregation (n=98)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Nonresponsive</td>
<td>Responsive</td>
<td>Nonresponsive</td>
</tr>
<tr>
<td>RPFA (n=100)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nonresponsive</td>
<td>17</td>
<td>87</td>
<td>14</td>
</tr>
<tr>
<td>Responsive</td>
<td>83</td>
<td>8</td>
<td>10</td>
</tr>
<tr>
<td>PFA-100 (n=100)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nonresponsive</td>
<td>22</td>
<td>4</td>
<td>6</td>
</tr>
<tr>
<td>Responsive</td>
<td>78</td>
<td>8</td>
<td>8</td>
</tr>
</tbody>
</table>
We found that the prevalence of ASA nonresponsiveness was 12% and 14% by LTA with arachidonic acid and ADP, respectively. Only 5% of patients were nonresponsive by both tests. The prevalence of ASA nonresponders by either the RPFA (17%) or the PFA-100 CEPI cartridge (22%) were higher than with LTA. Interestingly, only 2% of patients were aspirin nonresponders by all 3 tests, and overall agreements between each of the tests were poor. Using 3 normal volunteers, we showed that each test was able to detect the expected influence of ASA on platelet function using the established cutoffs, which is consistent with previous studies but the use of specific cutoffs to define aspirin nonresponsiveness could in theory have reduced agreement in the clinical study. However, expression of the test results as continuous measurements (Figure 1 to 3) did not suggest that agreement had been underestimated.

Previous studies of the RPFA identified ASA nonresponsiveness in 23% of 422 patients with coronary artery disease, and 19.2% of 151 patients scheduled for nonurgent percutaneous coronary intervention were classified as ASA nonresponders. However, very few data are available comparing the test with LTA or PFA-100.

The RPFA cartridge contains fibrinogen-coated beads and a platelet activator (metallic cations and propyl gallate) to stimulate the COX-1 pathway and activate platelets. The test should therefore theoretically produce similar results to those obtained by arachidonic acid LTA. Yet the incidence of ASA nonresponsiveness was higher by the RPFA test than with either arachidonic acid alone (17.0% versus 12.0% including 13 false-positives and 8 false-negatives) or both agonists (17.0% versus 5%). Previous data comparing propyl-gallate and other agonists by platelet aggregometry demonstrated that this agonist detects a lower number of nonresponders in volunteers receiving either 400 mg or 100 mg of ASA. Because the majority of patients in this study were receiving low-dose ASA, this may explain the discrepancy.

One previous study of the PFA-100 reported that 37% of stroke patients using low-dose ASA were nonresponsive. This rate is higher than the 22% prevalence using the CEPI CT in our study but the sample sizes in both studies were relatively small. The PFA-100 has been shown to be more sensitive than LTA at detecting ASA nonresponsiveness, and our data confirm this. In theory, because the PFA-100 is a high-shear system, it may be more physiologically relevant. It is interesting therefore that although CADP CTs were also significantly lower in the ASA nonresponsive group than the responders, their VWF levels were not significantly higher. However, this may be because of our relatively small sample size because higher VWF levels have previously been reported in patients who are ASA nonresponders. Both platelet hyperfunctional response(s) and VWF levels could both contribute to the occurrence of normal CTs in ASA nonresponders.

Our study does have potential shortcomings. It could be argued that we should have derived more control data, rather than relying on previous studies. It could also be argued that we should have determined the reproducibility of the differ-
ent tests in our hands rather than relying on previous reports of coefficients of variation. However, longitudinal studies will be necessary to determine intra-individual reproducibility over longer periods of time.

In conclusion, we found that the prevalence of apparent ASA nonresponsiveness was higher with both the POC tests than with LTA, that agreement between the tests was poor, and that 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, if any, of each of the separate tests.

Acknowledgments
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References
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