A comparison of platelet function tests and thromboxane metabolites to evaluate aspirin response in healthy individuals and patients with coronary artery disease

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Summary
Individualised antiplatelet therapy and platelet function testing have attracted considerable clinical interest, but several aspects of test performance have not been thoroughly evaluated. We investigated repeatability and concordance of light transmission aggregometry (LTA) induced with arachidonic acid (AA) 1.0 mM, PFA-100® induced with collagen/epinephrine, multiple electrode aggregometry (MEA) induced with AA 0.5 or 0.75 mM and VerifyNow® Aspirin. Patients with stable coronary artery disease (n=43) and healthy individuals (n=21) were included. All tests were performed in duplicate at baseline in healthy individuals and in duplicate for four days in all study participants during aspirin treatment. Serum and urinary thromboxane metabolites were evaluated several times to evaluate cyclooxygenase-1 inhibition by aspirin. MEA was most sensitive for aspirin as treatment induced a 12-fold difference in AA-induced platelet aggregation. Coefficients of variation for duplicate measurements at baseline (0.4–12%), during aspirin treatment (3–46%) and for day-to-day variability (3–37%) differed markedly between tests and were lowest for VerifyNow®. The prevalence of aspirin low-responsiveness also differed between tests (0–9%) and the agreement was low: kappa ≤ 0.21 for all tests compared with AA-induced LTA (reference test), which correlated best with VerifyNow® (r=0.43, p<0.001). Urinary thromboxane metabolites did not correlate with any platelet function test, whereas serum thromboxane correlated with VerifyNow® Aspirin (r=0.41, p=0.001). Overall, repeatability was moderate and the correlation between tests was low. VerifyNow® Aspirin proved most reproducible, and this was the only assay showing a significant positive correlation with serum thromboxane. This study demonstrated that conclusions based on platelet function testing strongly depend on the assay used.

Keywords
Aspirin, aspirin resistance, cardiology, platelet function tests, thromboxanes

Introduction
Platelets are key players in atherothrombosis, and antiplatelet therapy reduces the risk of occlusive arterial events in patients with atherosclerosis (1). Aspirin irreversibly acetylates the platelet cyclooxygenase (COX) enzyme, thus blocking the transformation of arachidonic acid (AA) into the vasoconstrictor and activator of platelet aggregation, thromboxane A₂. Even low daily doses of aspirin (75–150 mg) suppress thromboxane biosynthesis, inhibit platelet aggregation and reduce the risk of ischaemic events and vascular death (1). However, a highly variable response to aspirin has been reported, and several studies indicate an association between low platelet inhibition and atherothrombotic events (2–7). Thus, a substantial number of patients suffer cardiovascular events despite aspirin treatment (1) and the terms “aspirin low-responsiveness” and “aspirin resistance” have emerged (8). Given the prevalence of atherothrombotic disease and wide use of aspirin, the potential impact of insufficient platelet inhibition is extensive, and identification of these patients may be important. Unfortunately, there is no consensus on how to measure the effect of aspirin on platelet aggregation. Light transmission aggregometry (LTA) is considered the gold standard for evaluation of platelet aggregation (9–11). Nevertheless, LTA is non-physiological and time-consuming and, consequently, whole blood tests have been developed. These tests are quick and easy to perform, but available comparative studies are scarce and hampered by the use of only single measurements not allowing appropriate evaluation of test repeatability.

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In the present study we compared several major platelet function tests to investigate the repeatability of each test and to evaluate aspirin response in healthy individuals and a cohort of stable patients with coronary artery disease (CAD). Furthermore, thromboxane metabolites were measured in serum and urine to obtain a pharmacologically specific measure of aspirin-induced COX-inhibition.

Methods

Study population and design

We enrolled 21 healthy individuals and 43 patients with stable CAD. Healthy individuals were eligible for the study if they were above the age of 18. Exclusion criteria were aspirin intolerance, any acute or chronic disease, use of anticoagulants or any drugs known to affect platelet function (including non-steroidal antiinflammatory drugs), smoking, pregnancy or a platelet count < 120 x 10⁹/l. The inclusion criteria for patients were CAD verified by angiography, age above 18 and chronic aspirin treatment for at least the previous seven days. In addition to the above-mentioned exclusion criteria for healthy individuals, patients were also excluded if they had had any ischaemic event or revascularisation procedure (angioplasty or coronary by-pass graft surgery) within the previous 12 months.

Although patients were on chronic aspirin treatment (all patients had received aspirin for more than one year), a one-week run-in phase (days 1–7) with 75 mg of non-enteric-coated aspirin (Nycomed Denmark Aps) was performed to maximise uniformity of pharmacokinetics. Subsequently, blood sampling was performed for four consecutive days (days 8–11) during continued aspirin treatment. Baseline samples (day 0) were obtained in healthy individuals prior to aspirin treatment (Fig. 1).

The study was performed in accordance with the principles outlined in the Declaration of Helsinki and was approved by the Central Denmark Region Committees on Biomedical Research Ethics. All study participants gave written informed consent.

Compliance

At inclusion all patients received a tablet dosage box containing aspirin tablets in separate compartments for each day. Compliance was further optimised by face-to-face interviews and pill counting at each visit. Additionally, serum thromboxane B₂ (S-TxB₂) was measured twice in all study participants during aspirin treatment and in healthy individuals at baseline.

Collection of blood samples and urine (Fig. 1)

At all visits, standardised blood sampling was performed one hour after aspirin intake at the same time of the day for each participant to avoid any diurnal variation in platelet function. Samples were collected in heparinised tubes for platelet function tests and serum tubes for thromboxane metabolite analysis. Urine samples were collected in the morning of the last sampling day.

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collected through 19 G needles with participants in the supine position after 30 minutes (min) of sitting rest. The first 2 ml of blood was drawn into a glass tube without anticoagulant for analysis of S-TxB₂; the remaining samples were collected into 3.2% citrate Terumo Venosafe tubes for platelet aggregation analyses performed in duplicate within 2 hours (h).

**Light transmission aggregometry (LTA)**

Classical LTA in platelet-rich plasma (PRP) was performed at 37°C using a PAP-4D aggregometer (Bio/Data Corporation, Alpha Laboratories Ltd, Horsham, PA, USA). PRP was produced by immediate centrifugation of whole blood for 15 min at 100 g without brake, and the remaining blood was recentrifuged for 15 min at 1,500 g with brake to obtain platelet-poor plasma (PPP). The final platelet count in PRP was not adjusted (12). The recorder was adjusted to ensure that the difference in light transmission between PRP and PPP was 100%. Results are given as the percentual change in light transmittance from baseline 5 min after addition of the agonist, using PPP as reference. A final agonist concentration of 1.0 mM AA (Medinova Scientific, Glostrup, Denmark) was used.

**Multiple electrode aggregometry (MEA)**

Platelet aggregation in whole blood was assessed by MEA using an impedance aggregometer (Multiplate®, Dynabyte, München, Germany & Triolab A/S, Brøndby, Denmark). Blood used for MEA was incubated at room temperature for a minimum of 30 min prior to analysis exactly according to manufacturer’s instructions. All blood samples were gently inverted five times immediately before analysis, and platelet aggregation was induced by AA (0.50 and 0.75 mM, Medinova Scientific A/S, Glostrup, Denmark). Each disposable test cell contains two pairs of electrodes, thus enabling two simultaneous measurements. In the present study, true duplicate measurements were performed. Aggregation was recorded for 6 min and reported as area under the curve (AUC, Aggregation Units • min), an integrated measure of aggregation velocity and maximal aggregation.

**VerifyNow® Aspirin**

The VerifyNow® Aspirin (Accumetrics, San Diego, CA, USA) is a turbidimetric-based optical detection system measuring platelet aggregation in whole blood (13). Test cartridges contain fibrinogen-coated beads and AA, and platelet aggregation is detected when activated platelets bind fibrinogen and agglutinate. The instrument measures light transmittance, reported as Aspirin Reaction Units. Due to technical fallbacks, complete VerifyNow® data were not available in three study participants.

**Platelet function analyser (PFA-100®)**

The PFA-100® (Dade Behring, Deerfield, IL, USA) measures whole blood platelet aggregation under high shear stress, mimicking flow conditions in a stenotic artery. Test cartridges contain a membrane coated with collagen and epinephrine (C-EPI). Citrated whole blood is aspirated through a small aperture in the agonist-coated membrane, inducing platelet activation and aggregation. PFA-100® records the time in seconds (s) until the platelet plug occludes the aperture (closure time, CT). The maximal CT is 300 s, and values higher than 300 s are reported as non-closure. Test results obtained with the PFA-100® have been shown to significantly correlate with creatinine kinase MB and cardiac troponin I levels in patients with myocardial infarction undergoing percutaneous coronary intervention (PCI) (14) and with the risk of cardiovascular events (15).

**Urinary 11-dehydro-thromboxane B₂ (U-TxM)**

U-TxM represents a time-integrated index of thromboxane A₂ biosynthesis in vivo (16). Urine was collected and stored at −20°C until analysis. U-TxM was extracted by chromatographic methods and analysed by radioimmunoassay techniques (16, 17) prior to normalisation for urinary creatinine concentrations. The assay variability is about 10% and the detection limit is 2–5 pg/ml urine.

**Serum thromboxane B₂ (S-TxB₂)**

S-TxB₂ is a stable metabolite of thromboxane A₂ and is the pharmacologically most specific test to evaluate the effect of aspirin on platelets (18, 19). Whole blood was allowed to clot at 37°C for 1 h before separating serum by centrifugation. S-TxB₂ was measured with an enzyme immuno-assay according to manufacturer’s instructions (Cayman Chemicals, Ann Arbor, MI, USA). All samples were measured in duplicate and in two dilutions. Samples with results outside the standard curve were re-assayed with appropriate dilutions.

**Detection of aspirin low-responders**

The evaluation of aspirin response was based on mean values of duplicate measurements. As LTA induced with AA is considered the gold standard for evaluation of platelet response to aspirin (9–11), this test (using AA 1.0 mM) was used to separate aspirin low-responders from normal-responders. This analysis was based on data from the same day (day 9) for all platelet function tests (Fig. 1). In accordance with previous studies, participants with residual platelet aggregation ≥ 20% were considered aspirin low-responders (3, 11, 20–26). Other definitions of aspirin low-respon-
siveness were a mean CT < 165 s using the PFA-100® (27); AA-induced MEA > 300 AU • min (28) and ≥ 550 aspirin reaction units as recommended by the manufacturer. In the literature, there are no validated cut-offs for these tests and even the above-mentioned limits are often discussed. Therefore, the results of all platelet function tests were both treated as categorical and continuous variables in the statistical analyses. Definitions of aspirin low-responsiveness are summarised in Table 4 and discussed below.

<table>
<thead>
<tr>
<th>Medication</th>
<th>Healthy individuals (n = 21)</th>
<th>CAD patients (n = 43)</th>
<th>P-values</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, years</td>
<td>35 ± 11</td>
<td>65 ± 7</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Male, n (%)</td>
<td>14 (67)</td>
<td>35 (81)</td>
<td>0.2199</td>
</tr>
<tr>
<td>Body mass index, kg/m²</td>
<td>22 (21–25)</td>
<td>27 (25–31)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>B-Haemoglobin, mM</td>
<td>8.8 ± 0.8</td>
<td>9.1 ± 0.7</td>
<td>0.0735</td>
</tr>
<tr>
<td>Haematocrit, %</td>
<td>41.9 ± 0.03</td>
<td>43.7 ± 0.03</td>
<td>0.0406</td>
</tr>
<tr>
<td>B-Platelet count, 10⁹/l</td>
<td>263 (238–289)</td>
<td>234 (207–263)</td>
<td>0.0119</td>
</tr>
<tr>
<td>Mean platelet volume (fl)</td>
<td>10.5 ± 0.7</td>
<td>10.6 ± 0.7</td>
<td>0.5041</td>
</tr>
<tr>
<td>B-Leucocyte count, 10⁹/l</td>
<td>5.6 ± 1.2</td>
<td>7.2 ± 1.8</td>
<td>0.0003</td>
</tr>
<tr>
<td>P-Creatinine, μM</td>
<td>71 (63–85)</td>
<td>79 (68–88)</td>
<td>0.1223</td>
</tr>
</tbody>
</table>

Data are expressed as mean ± SD, median (interquartile range) or n (%).

<table>
<thead>
<tr>
<th>Medication</th>
<th>n</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Previous PCI</td>
<td>43</td>
<td>100</td>
</tr>
<tr>
<td>Previous MI</td>
<td>31</td>
<td>72</td>
</tr>
<tr>
<td>Previous CABG</td>
<td>2</td>
<td>5</td>
</tr>
<tr>
<td>Previous stroke</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>Angina</td>
<td>17</td>
<td>40</td>
</tr>
<tr>
<td>Claudicatio</td>
<td>4</td>
<td>9</td>
</tr>
<tr>
<td>Diabetes</td>
<td>10</td>
<td>23</td>
</tr>
<tr>
<td>Smokers</td>
<td>12</td>
<td>28</td>
</tr>
<tr>
<td>Medication</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Aspirin</td>
<td>43</td>
<td>100</td>
</tr>
<tr>
<td>Statins</td>
<td>41</td>
<td>95</td>
</tr>
<tr>
<td>Beta-blockers</td>
<td>35</td>
<td>81</td>
</tr>
<tr>
<td>ACE/AT2 antagonists</td>
<td>16</td>
<td>37</td>
</tr>
<tr>
<td>Diuretics</td>
<td>15</td>
<td>35</td>
</tr>
<tr>
<td>Calcium channel blockers</td>
<td>10</td>
<td>23</td>
</tr>
<tr>
<td>Proton pump inhibitors</td>
<td>2</td>
<td>5</td>
</tr>
</tbody>
</table>

ACE, angiotensin converting enzyme; AT2, angiotensin 2; CABG, coronary artery bypass grafting; CAD, coronary artery disease; MI, myocardial infarction; PCI, percutaneous coronary intervention.

### Statistics

Continuous variables are expressed as mean ± standard deviation (SD) or median and interquartile range (IQR) if not normally distributed. Categorical variables are reported as absolute counts and percentages. Using duplicate measurements, coefficients of variation (CV) were calculated as the SD of the log-transformed variables. ANOVA (analysis of variance) on log-transformed data was used for determination of the day-to-day variation. The agreement between aspirin response classification determined by LTA induced with AA 1.0 mM (reference method) and other platelet function tests was evaluated with kappa statistics, which specifically corrects for chance agreement in comparing classification by different methods. These analyses were performed on mean duplicate measurements on day 9. Irrespective of aspirin response classification, correlations between test results were calculated using Spearman’s rank correlation coefficient. Correlation coefficients and kappa statistics were not performed separately on patients and healthy individuals due to the relatively low number of study participants and the lack of difference in the frequency of low-responders with any of the platelet function tests performed. Student’s t-test and the Mann-Whitney test were used to compare normally and non-normally distributed data, respectively. The distribution of categorical variables was compared with Fisher’s exact test. A two-tailed p-value < 0.05 was considered statistically significant. The effect size (29) for each platelet function test was calculated by dividing baseline measurements (means of duplicate tests) with on-treatment measurements (means of duplicate tests, day 9). Statistics and figures were performed using STATA® version 10.0 (StataCorp., College Station, TX, USA) and GraphPad Prism 4 (GraphPad Software, San Diego, CA, USA).

### Results

Baseline characteristics for study participants are shown in Tables 1 and 2. The gender distribution among healthy individuals and patients did not differ, but patients were significantly older than healthy individuals (Table 1). All patients had stable CAD previously treated with PCI and were treated with aspirin prior to the run-in phase (Fig. 1). The mean duration of aspirin treatment in CAD patients was 54 months. Risk profile including previous events and current medication is described in Table 2.

All study participants claimed to be fully compliant and returned pill boxes were all empty. Compliance was confirmed by S-TxB2 measurements: the mean concentration of S-TxB2 was 318 ± 119 (range 49–522) ng/ml at baseline and during aspirin treatment on days 9 and 10 it was 1.3 ± 0.9 (range 0.4–4.2) and 1.2 ± 0.8 (range 0.4–4.0) ng/ml, respectively, for the entire study population.

Repeatability was evaluated by calculating CVs for duplicate measurements prior to and during treatment with aspirin and CVs for day-to-day variation (Table 3). CVs were higher during aspirin treatment than at baseline for all platelet function tests. At baseline as well as during aspirin treatment, CVs were lowest for
VerifyNow\textsuperscript{®} Aspirin and highest for MEA. CVs for day-to-day variation during aspirin treatment were also lowest for VerifyNow\textsuperscript{®} Aspirin and high for MEA. However, day-to-day variation was equally high for AA-induced LTA, which is often considered as the reference test (9–11).

As a measure of sensitivity for aspirin treatment, the effect size (29) was calculated for each platelet function test using baseline and on-treatment as described above. Results are shown in Table 3.

Using LTA with AA 1.0 mM as the agonist, six aspirin low-responders were identified with no difference between healthy individuals and CAD patients (1/21 vs. 5/43, p=0.65). Based on cut-off levels from the literature, Table 4 shows the number of healthy individuals during aspirin treatment (for all comparisons: r<0.20, p>0.05). Urinary thromboxane metabolites did not correlate with any platelet function test, and the only significant positive correlation between S-TxB\textsubscript{2} and platelet function tests was found with the VerifyNow\textsuperscript{®} Aspirin assay (r=0.41, p=0.001).

### Discussion

This study compared important platelet function tests that are commonly used to evaluate the antiplatelet effect of aspirin. In-

<table>
<thead>
<tr>
<th>Test (units) (agonist)</th>
<th>Median and range (baseline) Healthy individuals</th>
<th>Median and range (on aspirin) Healthy individuals</th>
<th>Median and range (on aspirin) CAD patients</th>
<th>Effect size fold: median (min-max)</th>
<th>CV\textsuperscript{duplicate measurements} (baseline, %) Healthy individuals</th>
<th>CV\textsuperscript{duplicate measurements} (on aspirin, %) healthy / patients</th>
<th>CV\textsuperscript{day-to-day} (on aspirin, %) healthy / patients</th>
</tr>
</thead>
<tbody>
<tr>
<td>LTA (%) (AA 1.0 mM)</td>
<td>85 (76–105)</td>
<td>8.5 (2.5–60)</td>
<td>12.5 (3.5–67)</td>
<td>10.5</td>
<td>5</td>
<td>19 / 17</td>
<td>37 / 31</td>
</tr>
<tr>
<td>MEA (AU min) (AA 0.5 mM)</td>
<td>574 (461–976)</td>
<td>44 (20–79)</td>
<td>62 (21–219)</td>
<td>11.8</td>
<td>8</td>
<td>46 / 46</td>
<td>12 / 24</td>
</tr>
<tr>
<td>PFA-100\textsuperscript{®} (sec) (C-EPI)</td>
<td>113 (83–146)</td>
<td>289 (118–301)</td>
<td>279 (79–301)</td>
<td>2.2</td>
<td>8</td>
<td>8</td>
<td>37 / 31</td>
</tr>
</tbody>
</table>

Table 3: Coefficients of variation (CV) for duplicate measurements and day-to-day variation (duplicate measurements on four days) for light transmission aggregometry (LTA), multiple electrode aggregometry (MEA), VerifyNow\textsuperscript{®} and PFA-100\textsuperscript{®}. Effect size was calculated for each platelet function test as the ratio between baseline and on-treatment measurements. Calculations are based on mean values of duplicate measurements for healthy individuals (n = 21) and patients (n = 43) with coronary artery disease (CAD).

Table 4 shows the number of healthy individuals during aspirin treatment (for all comparisons: r<0.20, p>0.05). Urinary thromboxane metabolites did not correlate with any platelet function test, and the only significant positive correlation between S-TxB\textsubscript{2} and platelet function tests was found with the VerifyNow\textsuperscript{®} Aspirin assay (r=0.41, p=0.001).

### Discussion

This study compared important platelet function tests that are commonly used to evaluate the antiplatelet effect of aspirin. In-

<table>
<thead>
<tr>
<th>Test (agonist)</th>
<th>Definition of aspirin low-responsiveness</th>
<th>*Low-responders (n, %)</th>
<th>Kappa</th>
</tr>
</thead>
<tbody>
<tr>
<td>LTA (AA 1.0 mM)</td>
<td>residual platelet aggregation ≥ 20 %</td>
<td>6 (9)</td>
<td>- (reference method)</td>
</tr>
<tr>
<td>MEA (AA 0.5 mM)</td>
<td>AUC &gt; 300 AU*min</td>
<td>0 (0)</td>
<td>0.0000</td>
</tr>
<tr>
<td>MEA (AA 0.75 mM)</td>
<td>AUC &gt; 300 AU*min</td>
<td>0 (0)</td>
<td>0.0000</td>
</tr>
<tr>
<td>VerifyNow\textsuperscript{®} (AA)</td>
<td>≥ 550 ARU</td>
<td>0 (0)</td>
<td>0.0000</td>
</tr>
<tr>
<td>PFA-100\textsuperscript{®} (C-EPI)</td>
<td>Closure time ≥ 165 sec.</td>
<td>2 (3)</td>
<td>0.2131</td>
</tr>
</tbody>
</table>

*calculations based on mean values of duplicate measurements performed after at least one week of aspirin treatment.
cluding all agonists, a total of five platelet function tests were performed 8–10 times in each study participant and, additionally, thromboxane metabolites were measured in urine and serum on several occasions to obtain a pharmacologically specific measure of aspirin-induced COX-inhibition. Inhibition of S-TxB2 levels confirmed that study participants were 100% compliant.

Repeatability and effect size of platelet function tests

Well-designed studies investigating biological variation and the repeatability of commonly used platelet function tests are scarce. We have shown that significant imprecision is seen in results obtained with different platelet function tests. This finding is important to the current discussion of "aspirin resistance", since classification of patients is usually based on single measurements with the underlying assumption that this measurement represents a stable phenotype.

For all tests, CVs were higher during aspirin treatment than at baseline. This finding is in accordance with a previous study showing that aspirin increases the variation in test results (10). The repeatability of the traditional reference method according to the literature was not impressive. Thus, CVs for duplicate measurements with AA-induced LTA during aspirin treatment were 17–19% and CVs for day-to-day variation were 31–37%. Previously, CVs ranging from 2.8% to 63% have been reported for day-to-day variation with LTA (31).

As a measure of sensitivity for aspirin treatment, the effect size was calculated for each platelet function test. Effect size was greatest for MEA, which is in accordance with previous findings (29). In our opinion, the effect size should be interpreted with caution, since these calculations are only based on 21 healthy individuals and, importantly, this measure is obviously dependent on the scale used by each platelet function test, especially the PFA-100® (Fig. 2). The difficulty in comparing tests is also highlighted by the relatively high CVs obtained with MEA, which might partly be explained by low AUC values and relatively large SDs (32).

CVs for duplicate measurements at baseline, during aspirin treatment and for day-to-day variation were all lowest for VerifyNow® Aspirin. A high repeatability of the VerifyNow® Aspirin system has also been reported in other studies (33–35), and CVs ≤ 3% are indeed impressive. However, in our laboratory we have performed > 1,100 tests in > 500 study participants and have never seen ARU (aspirin reaction unit) values below 350. This might suggest that a fixed factor is added to the baseline of the ARU scale resulting in artificially low CVs (= SD/mean ARU). To further explore this hypothesis, we have recalculated all CVs for the VerifyNow® device in Table 3 after subtracting 350 from the actual values obtained. The adjusted CV for duplicate measurements at baseline was 0.9%, and the adjusted CVs for duplicate measure-
ments during aspirin treatment in healthy individuals and CAD patients and day-to-day variation in both groups were 13–15%. Overall, although CVs for day-to-day variation with the PFA-100® were slightly lower, the VerifyNow® Aspirin assay remained the most reproducible test.

**Agreement between platelet function tests and thromboxane metabolites**

The imprecision of various platelet function tests is likely to affect the evaluation of aspirin response and may partly explain the relatively low agreement between tests (26, 36). Categorising individuals as normal or low-responders to antiplatelet therapy is convenient if platelet function testing is to be implemented in clinical decision algorithms. While awaiting the results of ongoing clinical trials, the proven benefit of such strategy is limited. Accordingly, data were both analysed as continuous and dichotomous variables, resulting in several interesting observations.

In accordance with a recent study (37), the highest correlation between AA-induced LTA and other platelet function tests was observed with the VerifyNow®. However, LTA did not correlate particularly well with any other platelet function test when employing the conventional detection limit of ≥20% aggregation (3, 11, 20–25). This finding is in accordance with previous studies (10, 23–25) and could be explained by test imprecision and the fact that LTA and other platelet function tests differ. Importantly, LTA measures platelet function in PRP that, contrary to whole blood testing, necessitates centrifugation during which large, haemostatically active platelets may be lost. These circumstances might also at least partly explain why VerifyNow® whole blood aggregometry showed a better correlation with S-TxB₂ than LTA.

The low number of aspirin low-responders in our study may be explained by the thorough compliance control and the standardization of the time (1 h) from aspirin intake to blood sampling. Most previous studies did not standardise this time interval or performed blood sampling either in the middle (25) or at the end (22, 38) of the 24-h dosing interval. This is likely to result in a higher number of aspirin low-responders, since even 960 mg of aspirin is not sufficient to fully inhibit AA-induced platelet aggregation after 24 h (39).

The aspirin-induced inhibition of COX-1 should be evaluated with methods measuring the capacity of platelets to synthesise thromboxane A₂, and urinary levels of the thromboxane B₂ metabolite represents a time-integrated index of thromboxane A₂ synthesis in vivo. However, about 30% of this metabolite derives from non-platelet sources (40) and, therefore, many authors argue that S-TxB₂ is the best way of measuring the platelet response to aspirin (18, 41–44). S-TxB₂ is a stable metabolite of thromboxane A₂ and, since the contribution of other blood cells to its synthesis is marginal, it reflects the total platelet capacity to synthesise thromboxane A₂. The levels of S-TxB₂ and U-TxM did not correlate with the reference method or any other platelet function test, except that a significant positive correlation was seen between S-TxB₂ and the VerifyNow® device. The lack of correlation between thromboxane metabolites and LTA has been observed in other studies (26, 43) and is not surprising. Indeed, LTA is a turbidimetric assay performed in platelet-rich plasma with several analytical limitations, whereas S-TxB₂ is a specific metabolite directly reflecting the aspirin-induced inhibition of platelets.

**Study limitations**

Although it was not a primary study aim to compare healthy individuals and patients with CAD, the inclusion of age-matched groups would have been preferable. For ethical reasons, it was not possible to withdraw aspirin treatment in patients and baseline

### Table 5: Correlation between platelet function tests and thromboxane (TxA) metabolites.

<table>
<thead>
<tr>
<th></th>
<th>LTA 1.0 mM</th>
<th>LTA 0.5 mM</th>
<th>LTA 0.75 mM</th>
<th>VerifyNow®</th>
<th>PFA-100® (C-EPI)</th>
<th>U-TxM</th>
<th>S-TxB₂</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>AA 1.0 mM</strong></td>
<td>1.00</td>
<td>0.22</td>
<td>0.36*</td>
<td>0.43*</td>
<td>–0.56</td>
<td>0.12</td>
<td>0.02</td>
</tr>
<tr>
<td><strong>AA 0.5 mM</strong></td>
<td>–</td>
<td>1.00</td>
<td>0.65*</td>
<td>–0.05</td>
<td>–0.16</td>
<td>0.00</td>
<td>–0.39*</td>
</tr>
<tr>
<td><strong>AA 0.75 mM</strong></td>
<td>–</td>
<td>–</td>
<td>1.00</td>
<td>0.32*</td>
<td>–0.43*</td>
<td>–0.08</td>
<td>–0.21</td>
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<td><strong>Point-of-care tests</strong></td>
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</tr>
<tr>
<td>VerifyNow®</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>1.00</td>
<td>–</td>
<td>0.09</td>
</tr>
<tr>
<td>PFA-100® (C-EPI)</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>1.00</td>
<td>–</td>
<td>0.00</td>
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<tr>
<td><strong>TxA metabolites</strong></td>
<td></td>
<td></td>
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<tr>
<td>U-TxM</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>1.00</td>
<td></td>
</tr>
<tr>
<td>S-TxB₂</td>
<td>–</td>
<td>–</td>
<td>–</td>
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<td>–</td>
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</tr>
</tbody>
</table>

*P < 0.05. AA, arachidonic acid; C-EPI, collagen-epinephrine; LTA, light transmission aggregometry; MEA, multiple electrode aggregometry; S-TxB₂, serum thromboxane B₂; U-TxM, Urinary 11-dehydro-thromboxane B₂.
values could thus not be obtained. Observed ingestion of aspirin and additional platelet function testing in low-responders could have extended our findings. The use of citrate as anticoagulant might have attenuated platelet aggregation, thus masking aspirin low responsiveness assessed by MEA and other platelet function tests (45). Many recent studies on MEA have been performed with hirudinised blood (29, 46), but the use of citrate was still standard, when this study was performed, and we have only recently started using hirudin to further investigate the importance of the anti-coagulant employed (47). The use of repeated duplicate measurements with all tests being performed 8–10 times in each individual did not allow the inclusion of a large study population; consequently, the frequency of aspirin low-responders should be interpreted with caution.

Conclusions

This study shows that conclusions drawn from platelet function measurements are highly dependent on the assay used; overall, repeatability is moderate and the correlation between different tests is low. Performance parameters for the conventional reference test, LTA with AA as the agonist, were not impressive. The strongest positive correlation was seen with the VerifyNow® Aspirin system measuring whole blood platelet aggregation. Among several major platelet function tests, this assay produced the lowest CVs for duplicate measurements both at baseline and during aspirin treatment, including day-to-day variation. Furthermore, this was the only assay showing a significant positive correlation with S-TxB2. Although the VerifyNow® Aspirin system has been shown to correlate with clinical outcomes (4, 5), large-scale prospective studies are required to determine whether any platelet function test should be implemented in clinical decision making to individualise and optimise antiplatelet therapy.

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References