Inconsistency of different methods for assessing *ex vivo* platelet function: relevance for the detection of aspirin resistance

Giulia Renda, Maria Zurro, Gelsomina Malatesta, Benedetta Ruggieri, and Raffaele De Caterina

The Institute of Cardiology and Center of Excellence on Aging at "G. d'Annunzio" University, Chieti, Italy

Manuscript received on April 28, 2010. Revised version arrived on August 25, 2010. Manuscript accepted on August 27, 2010.

Correspondence:
Raffaele De Caterina, MD, PhD
Institute of Cardiology
"G. d'Annunzio" University – Chieti, C/o Ospedale SS. Annunziata, Via dei Vestini
66013 Chieti, Italy
Phone: international +39-0871-41512
Fax: international +39-0871-402817
E-mail: rdecater@unich.it

ABSTRACT

Background
Assays to evaluate platelet function are often interchangeably used to assess "resistance" to aspirin. We compared different platelet function assays in patients treated or untreated with aspirin.

Design and Methods
Platelet function was evaluated in 162 subjects, 85 of whom were not being treated with any antiplatelet drug and 77 of whom were receiving chronic therapy with low-dose aspirin. Platelet Function Analyzer collagen/ADP- and collagen/epinephrine closure times, as well as light transmittance aggregometry in response to ADP, collagen and arachidonic acid (this last in 47 aspirin-treated patients) were determined. In 43 aspirin-treated patients, serum thromboxane B_2 levels were also measured.

Results
In untreated patients, collagen/ADP- and collagen/epinephrine-closure times were correlated with each other (r=0.5, *P*=0.0001), but did not correlate with ADP- or collagen-induced aggregation. In patients treated with aspirin, collagen/ADP-closure time values were not different from those in untreated patients, while the collagen/epinephrine-closure time was prolonged. ADP-induced aggregation was unaffected by aspirin, while collagen-induced aggregation was reduced. Arachidonic acid-induced aggregation was almost completely suppressed (% maximum light transmittance aggregometry=5±13%). There was, however, no correlation between the various platelet function tests. Serum thromboxane B_2, an index of platelet cyclooxygenase-1 activity, was almost completely suppressed (down to 8±17 ng/mL) in treated patients, and was not correlated with arachidonic acid-, ADP- and collagen-induced aggregation or with collagen/ADP-closure time, but was inversely correlated with collagen/epinephrine-closure time.

Conclusions
There is a high heterogeneity of results of tests evaluating inhibition of platelet function by aspirin, and the results of functional tests do not match biochemical measurement of cyclooxygenase-1 activity. Extreme caution should, therefore, be used in defining "resistance" to aspirin on the basis of the results of these tests.

Key words: Platelet function, platelet aggregation, serum thromboxane B2, aspirin resistance.

Citation: Renda G, Zurro M, Malatesta G, Ruggieri B, and De Caterina R. Inconsistency of different methods for assessing ex vivo *platelet function: relevance for the detection of aspirin resistance. Haematologica 2010;95(12):2095-2101. doi:10.3324/haematol.2010.027102*

Introduction

A great deal of attention has been paid recently to assessing potential reasons for the failure of aspirin in preventing thrombotic events.[1-3] Most studies defining aspirin "resistance"[4-7] have interchangeably used different *ex vivo* tests of platelet function, mostly light transmittance aggregometry (LTA) with different agonists and the Platelet Function Analyzer (PFA)-100®, reporting extremely variable frequencies of aspirin "resistance".[8,9]

LTA, carried out according to the method described by Born,[10] has been by far the most widely used *ex vivo* assay of platelet function. This assay is based on the physical principle of an increase in light transmittance proportional to the formation of platelet aggregates. Platelet response, most often expressed as maximum light transmittance from the aggregometric tracing (% max LTA), varies according to the different agonists used, such as thrombin, collagen, ADP, epinephrine, ristocetin and arachidonic acid, exploring different pathways of platelet activation.

The whole-blood PFA-100® has been developed as a bedside point-of-care device.[11] The system is an *in vitro* simulation of the process of platelet adhesion and aggregation following a vascular injury through an aperture in cartridges containing a membrane coated with collagen and ADP (collagen/ADP=CADP) or epinephrine (collagen/EPI=CEPI). In principle, the system determines the time to occlusion of this aperture (i.e., the closure time). The manufacturer advises that each laboratory establishes its own reference range: coefficients of variation between 6% and 13% for closure times with normal samples were reported in different studies.[12] Both the PFA-100 and LTA have been used to evaluate the individual response to antiplatelet therapy, in particular to low-dose aspirin.

We performed a study aimed at comparing different *ex vivo* platelet function assays in patients treated or untreated with aspirin. In order to assess how the results obtained with different *ex vivo* platelet function assays correlate with the inhibition of a biochemical index of platelet cyclooxygenase (COX)-1, the target enzyme of aspirin, in patients treated with aspirin we evaluated levels of serum thromboxane B_2 (TXB_2), the serum metabolite of thromboxane A_2, expected to be almost completely suppressed by chronic aspirin therapy.[13]

Design and Methods

Patients

We examined 200 consecutive subjects referred to the outpatient clinic of our institution over 12 months for a pre-operative screening of hemostasis. The screening included a complete blood cell count and differential, and measurement of the prothrombin time, activated partial thromboplastin time, fibrinogen concentration and von Willebrand factor (vWF) level. Whole blood samples were collected by venipuncture. We took two additional test-tubes of blood to perform platelet function tests by LTA (Born's method) in response to collagen and ADP, and by measuring the PFA-100® closure times using the CADP and CEPI cartridges (CAPD-CT and CEPI-CT, respectively). Finally, in a subgroup of aspirin-treated patients, we measured arachidonic acid-induced LTA (n=47) and serum TXB_2 generation (n=43).

All subjects answered a written questionnaire on current therapies (in the previous 7 days), with the help of a written list of the commercial names of all medications containing aspirin, thienopyridines, other antiplatelet drugs, anticoagulants or non-steroidal anti-inflammatory drugs.

We excluded patients with any hemorrhagic disorder, a platelet count less than 100×10^9/L or greater than 500×10^9/L, ongoing treatments with anticoagulants, or antiplatelet drugs other than aspirin, or non-steroidal anti-inflammatory drugs in the preceding 10 days. Out of the 200 subjects initially enrolled, 38 were excluded because of these criteria. Among the remaining 162 patients, 85 were not on any antiplatelet drug (serving as the control group), while 77 had been on low-dose aspirin (100-160 mg/day) for 1 month or more (aspirin group).

Since the study protocol only required two blood samples without venipunctures additional to those taken for the clinical screening, it did not require approval by the hospital Ethics Committee at the time when data were accrued (years 2000-2001). Written informed consent was obtained from all subjects.

Hematologic parameters

Blood cell counts (including red blood cell, white blood cell and platelet counts) were performed with a routine automated hemocytometer (System 9000; Baker Diagnostic, Bethlehem, PA, USA). Fibrinogen was measured by the Fibrinogen-Clauss coagulometric kit (Instrumentation Laboratories, Milan, Italy). The concentrations of vWF antigen were measured with an enzyme-linked immunosorbent assay from American Diagnostica Inc. (Greenwich, CT, USA).

Platelet function tests

Whole blood was collected into standard sodium citrate tubes (3.8% w/vol), with a blood:citrate ratio of 9:1. All platelet function tests were performed within 60 min of blood withdrawal.

PFA-100®

Whole blood specimens were mixed by inversion immediately before assessment of the closure time with a PFA-100® apparatus (Dade Behring, Deerfield, IL, USA) using both CADP and CEPI cartridges.[11] Duplicate measurements were performed, and mean closure times were recorded and reported. The PFA-100® system measures a closure time up to a maximum of 300 s, this upper value reflecting no closure. Any values greater than 300 s were included as 300 s in the data analysis. Based on our own laboratory assessment of normal subjects, we defined the upper normal limit of the PFA-100® closure times as the mean + one standard deviation: 120 s for CADP-CT and 165 s for the CEPI-CT.

Born's aggregation

Turbidimetric platelet aggregation was evaluated by the increase in light transmittance in a Chrono-Log platelet aggregometer (Mascia Brunelli, Milan, Italy), after the addition of ADP (Mascia Brunelli, Milan, Italy), 10 μmol/L final concentration, or collagen (Mascia Brunelli, Milan, Italy), 25 μg/mL final concentration, or arachidonic acid (Sigma-Aldrich, St. Louis, Missouri, USA), 1 mmol/L final concentration, to platelet-rich plasma, as previously described.[10,14]

Based on our own laboratory assessment of normal subjects, we defined the upper normal limit of LTA as the mean + one standard deviation: greater than 50% max LTA for ADP-induced aggregation, greater than 75% max LTA for collagen-induced aggregation and greater than 20% max LTA for arachidonic acid-induced aggregation.

Serum thromboxane generation

Whole blood samples were collected into glass Vacutainer tubes containing no anticoagulant and immediately allowed to clot for 1 h at 37 °C. At the end of the incubation time, serum was collect-

ed following centrifugation at 3000 x g for 10 min and stored at -80 °C until assayed for TXB_2, an index of maximally-stimulated platelet COX-1 activity in response to endogenously formed thrombin.[15] Serum TXB_2 was measured by a commercial enzyme immunoassay (Cayman Chemical, Ann Arbor, Michigan, USA).

To define the inhibition of platelet COX-1 activity through this biochemical index, we chose a cut-off value of 10 ng/mL, considered the uppermost value of serum TXB_2 generated in the presence of 98% or more inhibition of platelet COX-1 activity by long-term therapy with aspirin.[16]

Statistical analysis

Discrete variables were described as absolute values and percentages; continuous variables were described as mean ± standard deviation (SD). For duplicate measurements of CADP-CT and CEPI-CT the coefficient of variation was calculated as the ratio of the standard deviation to the mean value of the two measurements. After checking the normality of value distributions with the one-sample Kolmogorov-Smirnov test, two-group comparisons of hematologic parameters, CADP- and CEPI-CT, as well as ADP- and collagen-induced LTA values were performed with the Student's t-test for unpaired data. Pairs of platelet function tests were correlated with each other using the Pearson's test in the presence of normal distributions, or with the Spearman's non-parametric correlation test otherwise. *P* values less than 0.05 were considered statistically significant.

Analyses were performed with the SPSS statistical software (Chicago, IL, USA) .

Results

Demographic, clinical, and hematologic parameters

The subjects' demographic, clinical and hematologic data, including red blood cell, white blood cell and platelet counts, together with plasma concentrations of fibrinogen and vWF, are reported in Table 1. Age and clinical characteristics were different between the control group and the aspirin-treated group because patients taking aspirin were older, had more vascular disease and a higher thrombotic risk. All hematologic parameters were, however, similar between the two groups.

Table 1. Demographic characteristics of the patients studied.

	Untreated controls	Aspirin
Number	85	77
Age, years (mean±SD)	38±18	*63±11
Males/females	61/24	51/26
Vascular disease: n (%)		
Ischemic heart disease	0 (0)	*41 (53)
Cerebrovascular disease	0 (0)	*5(6)
Peripheral arterial disease	1 (1)	*7 (9)
Hypertension: n (%)	0 (0)	*9 (11)
Atrial fibrillation: n (%)	0 (0)	*5(6)
Hematologic parameters: mean±SD		
Red blood cells ($\times 10^{12}$/L)	4.77 ± 0.63	4.75 ± 0.47
White blood cells ($\times 10^{9}$/L)	6.2 ± 1.76	6.6 ± 1.65
Platelets ($\times 10^{9}$/L)	214 ± 65	212 ± 59
Fibrinogen (mg/dL)	294 ± 68	292 ± 56
von Willebrand factor (%)	99 ± 40	102 ± 28

**P<0.0001 versus control.*

Platelet function tests

Overall population

Data were first analyzed in the entire population (n=162), irrespective of the use of aspirin, to assess the overall consistency between methods. The correlations between platelet function tests are reported in Table 2. Overall, CADP-CT correlated with CEPI-CT, but neither correlated with ADP-induced LTA. CADP-CT did not correlate with collagen-induced LTA, but CEPI-CT was inversely, albeit weakly, correlated with collagen-induced LTA. ADP-induced LTA also correlated with collagen-induced LTA.

The coefficients of variation for CADP-CT and CEPI-CT duplicate measurements were 5% and 15%, respectively.

In the entire population, CADP-CT was inversely correlated with red blood cell, white blood cell and platelet counts, while CEPI-CT was not correlated with these parameters (Table 2). No correlation was observed between plasma concentrations of fibrinogen and vWF and the various tests of platelet function (Table 2). Moreover, when we normalized CADP-CT and CEPI-CT values by red blood cell, white blood cell and platelet counts, we observed that the correlations between different tests did not change substantially (*data not shown*).

We next separately analyzed the data in the two groups of patients untreated or treated with low-dose aspirin.

Untreated patients

Among the patients not being treated with aspirin (n=85), CADP-CT values correlated with the CEPI-CT values (r=0.5, *P*=0.0001). CADP-CT did not correlate with ADP- or collagen-induced LTA (r=0.26 and 0.25, respectively, *P*=NS). CEPI-CT values also did not correlate with ADP- or collagen-induced LTA (r=0.05 and -0.009, respectively, *P*=NS). ADP- and collagen-induced LTA were not correlated between each other (r= 0.03, *P*=NS). The coefficiencts of variation for CADP-CT and CEPI-CT duplicate measurements were 4% and 15%, respectively.

In untreated patients no correlation was observed between red blood cell count or plasma concentration of fibrinogen or vWf and the different methods of platelet function (*data not shown*). CADP-CT and CEPI-CT were inversely correlated with white blood cell count (r=-0.29, *P*=0.01 and r=-0.3, *P*=0.01, respectively) and platelet count (r=-0.38, *P*=0.001 and r=-0.25, *P*=0.03).

Table 2. Correlations between platelet function tests in the overall population.

	CADP r (*P*)	CEPI r (*P*)	LTA ADP r (*P*)	LTA Collagen r (*P*)
LTA ADP	0.19 (0.06)	0.02 (0.8)	/	/
LTA Collagen	0.02 (0.8)	**-0.39 (0.001)**	**0.28 (0.01)**	/
CEPI	**0.39 (0.0001)**	/	/	/
RBC	**-0.28 (0.002)**	-0.05 (0.5)	/	/
WBC	**-0.19 (0.04)**	-0.04 (0.6)	/	/
Platelets	**-0.26 (0.003)**	-0.16 (0.06)	/	/
Fibrinogen	0.22 (0.2)	0.19 (0.2)	0.005 (0.9)	-0.26 (0.1)
vWF	0.3 (0.2)	0.33 (0.1)	0.3 (0.2)	0.09 (0.7)

LTA: light transmittance aggregometry; CADP: collagen/ADP; CEPI: collagen/epinephrine; RBC: red blood cell count; WBC: white blood cell count; vWF: von Willebrand factor. Values marked in bold are statistically significant.

Aspirin-treated patients

Among the patients being treated with aspirin (n=77), CADP-CT was not different from that in controls (Figure 1A), while CEPI-CT was significantly longer (by approximately 30%) than in controls (CEPI-CT=241±75 s *versus* 160±58 s, *P*<0.0001; Figure 1B). ADP-induced LTA in aspirin-treated patients was not different from that in controls (Figure 1C), while collagen-induced LTA was significantly lower in aspirin-treated patients than in controls (% max LTA=61±26% *versus* 85±10%, *P*<0.0001; Figure 1D). Arachidonic acid-induced LTA was measured in 47 of the 77 patients treated with aspirin and was almost completely suppressed (% max LTA=5±13%), although with a wide variability between patients, as reflected by the standard deviation, mostly due to the lack of suppression of arachidonic acid-induced LTA in two patients. CEPI-CT did not correlate with LTA induced by arachidonic acid (r=-0.18, *P*=NS), ADP (r=0.07, *P*=NS) or collagen (r=-0.27, *P*=NS). Likewise, CADP-CT did not correlate with LTA induced by different agonists (*data not shown*), nor did it correlate with CEPI-CT (r=0.14, *P*=NS). ADP-, collagen- and arachidonic acid-induced LTA were not correlated with each other (*data not shown*).

The coefficients of variation for CADP-CT and CEPI-CT duplicate measurements were 7% and 14%, respectively.

The percentage of patients showing normal platelet aggregation in spite of aspirin therapy varied in relation to the different assays and the different thresholds chosen for each assay. Only 4% of patients treated with aspirin had an arachidonic acid-induced LTA within the normal range (% max LTA >20%), 24% had an ADP-induced LTA within the normal range (% max LTA >50%), 38% had collagen-induced LTA within the normal range (% max LTA >75%), as shown in Figure 2A, 25% had a CEPI-CT within the normal range (<165 s), and 66% had a CADP-CT within the normal range (<120 s), as shown in Figure 2B.

Among the patients treated with aspirin, no correlation was observed between red blood cell, white blood cell and platelet counts, and fibrinogen concentration and the differ-

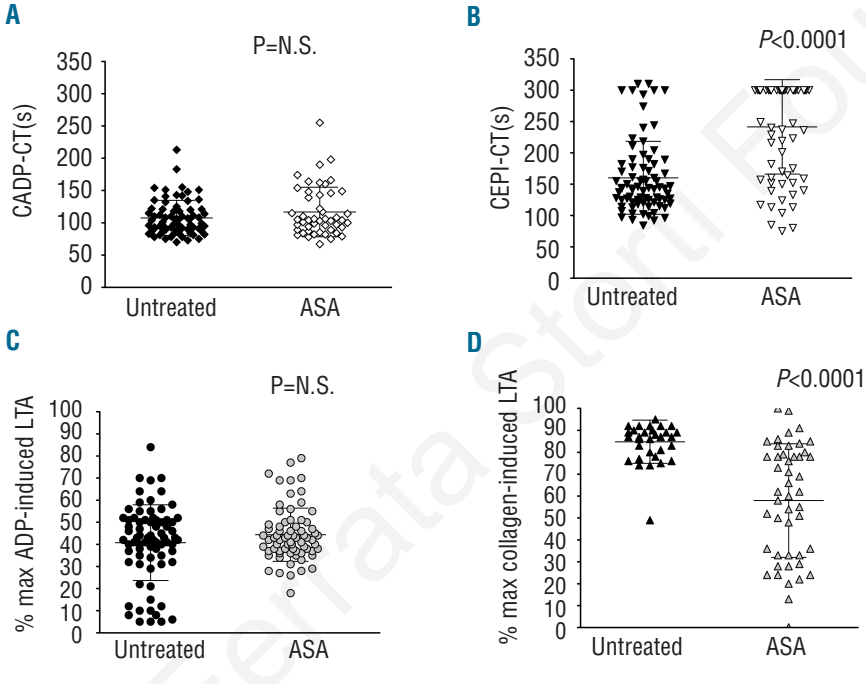

Figure 1. (A) CADP-CT in aspirin (ASA)-treated patients *versus* untreated patients. (B) CEPI-CT in ASA-treated patients *versus* untreated patients. (C) ADP-induced aggregation in ASA-treated patients *versus* untreated patients. (D) Collagen-induced aggregation in ASA-treated patients *versus* untreated patients. Mean and SD are shown in all panels.

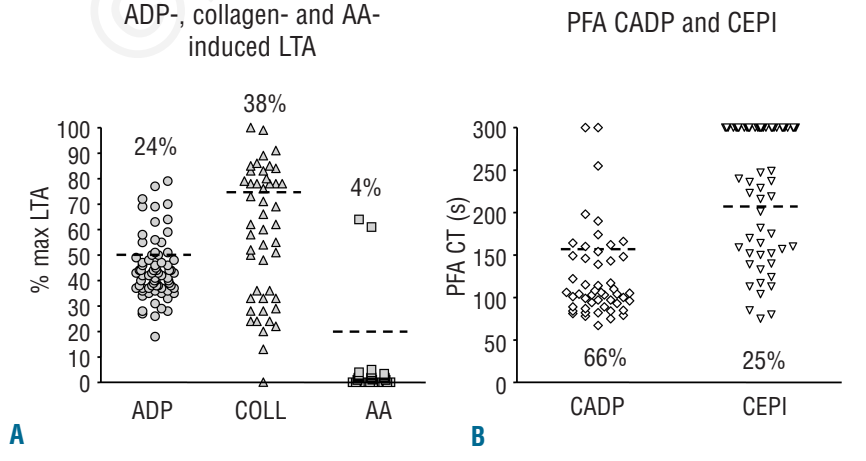

Figure 2. (A) ADP-, collagen- and arachidonic acid (AA)-induced platelet aggregation in aspirin (ASA)-treated patients. (B) CADP-CT and CEPI-CT in ASA-treated patients. Dotted lines indicate the cut-offs of normal ranges chosen for each assay. Percentages refer to patients with values in the normal range despite being treated with ASA.

ent methods of platelet function (*data not shown*). ADP- and collagen-induced LTA correlated with vWF plasma concentration (r=0.69, *P*=0.04 and r=0.96, *P*<0.0001, respectively).

Serum thromboxane B_2 generation

Serum TXB_2 was measured in 43 of the 77 patients treated with aspirin, and was almost completely suppressed (8±17 ng/mL), being below the cut-off of 10 ng/mL. However, TXB_2 levels were not correlated with arachidonic acid- (Figure 3), ADP- or collagen-induced LTA (r=0.03, r=0.17, r=0.16, respectively; *P*=NS for all), or with CADP-CT (r=-0.2, *P*=NS), while they were inversely correlated with CEPI-CT (r=-0.48, *P*=0.001) (Figure 4). We observed that the mean TXB_2 value in patients with normal CEPI-CT (<165 s) was about 75% higher than the mean value in patients with prolonged CEPI-CT (20+23 ng/mL and 5+14 ng/mL, respectively) (Figure 4). Moreover, dividing patients according to TXB_2 inhibition, only about 11% of patients with inhibited TXB_2 had a CEPI-CT in the normal range; and about 77% of patients with inhibited TXB_2 also had a prolonged CEPI-CT (Figure 4). However, considering arachidonic acid-induced LTA, we observed that, of the two patients with non-inhibited arachidonic acid-induced LTA, both also had a high serum TXB_2 concentration, and TXB_2 values in patients with normal arachidonic acid-induced LTA (>20%) were 80% higher than those in patients with inhibited LTA (37±6 ng/mL and 7±16 ng/mL, respectively) (Figure 3). Moreover, no patient with inhibited TXB_2 had an arachidonic acid-induced LTA, and about 89% of patients with inhibited TXB_2 also had inhibited arachidonic acid-induced LTA (Figure 3).

Eliminating patients with a serum TXB_2 concentration greater than 10 ng/mL (possibly not fully compliant), we observed no correlation between TXB_2 concentration and either arachidonic acid-induced LTA (r=0.06, *P*=NS) or CEPI-CT (r=0.3, P=NS).

Discussion

The main findings of our study are that: (i) there is a high heterogeneity of outcomes in the tests used to evaluate platelet function and inhibition of platelet aggregation by aspirin; (ii) the results of these functional tests do not match with serum TXB_2 levels, considered the gold-standard biochemical index for the evaluation of COX-1 activity suppressed by aspirin.

In the overall population, PFA-100® CADP-CT and CEPI-CT correlated with each other, but neither correlated with measurements obtained with ADP-induced aggregation. A weak correlation between CEPI-CT, but not CADP-CT, and collagen-induced LTA was observed, as well as between ADP-induced and collagen-induced LTA. Part of this variability could be explained by the influence of hemorheologic variables in our series. Other reports have shown that PFA-100® closure times are prolonged by a significant reductions in platelet count or hematocrit.[17-20] For example, for blood samples with a platelet count of less than 100×10^9/L, an inverse relationship between CEPI-CT and platelet count was observed.[18] In our patients, none of whom was thrombocytopenic, we observed this negative correlation also within a range of normal platelet counts. An inverse correlation had also been found between hematocrit and closure time in another study.[20] In our patients, none of whom was anemic or polycythemic, we observed that the higher the number of red blood cells, the shorter the CADP-CT was. White blood cell count may also affect platelet function assessment by the PFA-100®,[21] as observed by the CADP-CT results in our entire population. The CEPI-CT did not seem to be much affected by these hemorheologic variables in our patients, while it was in other studies.[17-20] The influence of hemorheologic variables on a whole blood test may limit the test's reproducibility and undermine the reliability of the results. However, even normalizing closure time values for red blood cell, white blood cell and platelet counts, the corre-

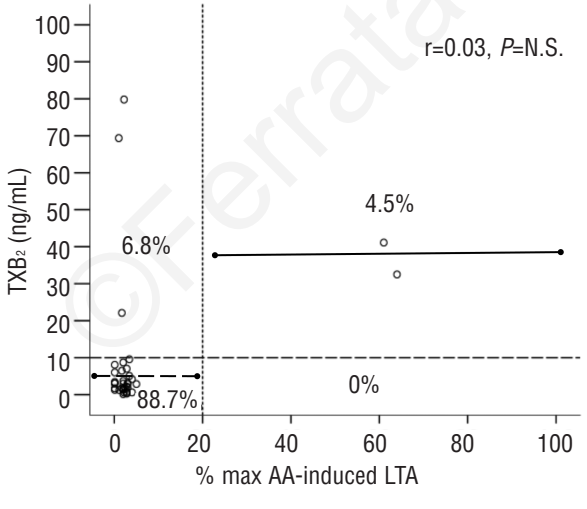

Figure 3. Correlation between TXB_2 values and arachidonic acid (AA)-induced LTA in patients treated with aspirin. The vertical dotted line indicates the cut-off of normal range chosen for AA-LTA (20% max LTA). The horizontal dashed line indicates the cut-off of TXB_2 inhibition (10 ng/mL). The continuous dark line indicates the mean TXB_2 value in patients with normal AA-induced LTA; the dashed dark line indicates the mean TXB_2 value in patients with inhibited LTA. Percentages of patients below or above threshold values for AA-LTA as a function of TXB_2 inhibition are also indicated.

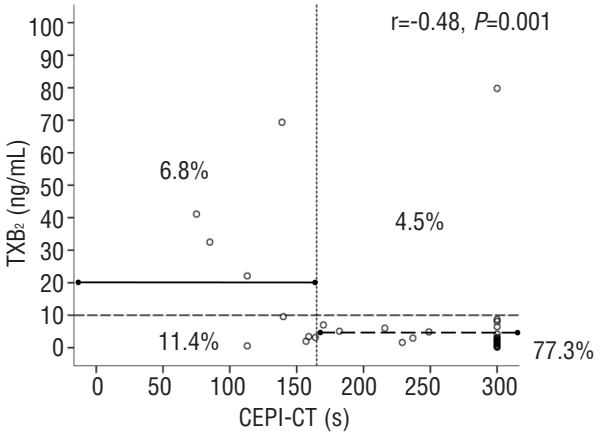

Figure 4. Correlation between TXB_2 values and CEPI-CT in patients treated with aspirin. The vertical dotted line indicates the cut-off of the normal range chosen for PFA-100® CEPI-CT (165 s). The horizontal dashed line indicates the cut-off of TXB_2 inhibition (10 ng/mL). The continuous dark line indicates the mean TXB_2 value in patients with normal CEPI-CT. The dashed dark line indicates the mean TXB_2 value in patients with prolonged CEPI-CT. The percentages of patients below or above threshold values for CEPI-CT as a function of TXB_2 inhibition are shown.

lation between the different assays did not change in our population.

We found no differences in vWF levels between patients treated and not treated with aspirin. It has been described that vWF levels increase during acute coronary syndromes, and vWF is considered an independent predictor of adverse clinical outcome in these patients.[22,23] Our patients, however, although being on aspirin in most cases for coronary heart or cerebrovascular disease, were all clinically stable, and this may explain the similarity of vWF levels between the two groups.

The lack of correlation between different methods evaluating platelet function was also present in untreated control patients and in patients treated with aspirin when analyzed separately. The percentage of "responders" to aspirin varied in relation to the different assays and to the different agonists used. Although it was already known that CEPI-CT is more sensitive than CADP-CT in assessing the effect of aspirin,[5] still one quarter of patients receiving aspirin did not show any prolongation of closure times beyond the normal range even using CEPI-CT. This confirms that this technique has a low performance and poor reproducibility, as indicated in some recent papers.[24,25] Interestingly, less than half (42%) of the patients were aspirin-responders by all tests, and only 5.7% of patients were aspirin non-responders by all tests.

Moreover, we found a lack of correlation between the results obtained by functional tests of platelet aggregation and measurements of serum TXB_2, the biochemical index of enzymatic COX-1 activity suppressed by aspirin, as also observed in other recent studies,[26,27] indicating that functional assays cannot predict which individuals have effective inhibition of platelet thromboxane production in response to aspirin.

Based on the 10 ng/mL cut-off value of TXB_2, considered an appropriate biomarker to identify patients with inadequate inhibition of platelet COX-1 activity by aspirin,[16,28,29] there were five "non-responders" to aspirin (about 11%) in our population, a very small fraction of patients exposed to the drug, in agreement with other reports.[8] Since the only information about the compliance of these patients was the written questionnaire on current therapies, we cannot definitely exclude that the observed phenomenon depended on poor compliance rather than a true lack of inhibition of platelet COX-1. However, when patients with serum TXB_2 greater than 10 ng/mL, judged as potentially poorly compliant, were excluded, the results were not greatly different from the main findings.

Among the functional tests of platelet aggregability, ADP- and collagen-induced LTA are less affected by aspirin than are responses to arachidonic acid, as previously observed,[27] while arachidonic acid-induced LTA should be in principle the most appropriate way to identify "non-responders" to aspirin (arachidonic acid being the substrate of the COX-1 enzyme blocked by aspirin). In fact, the variability in aspirin response observed by this assay in our study was very low, and the majority of patients on aspirin had inhibited platelet aggregation by this functional test: only two patients (4% of all treated) had normal platelet aggregation despite being on aspirin. Both these patients also had high serum TXB_2. However, three other patients with high serum TXB_2 (60% of patients with high TXB_2) had inhibited arachidonic acid-induced LTA, explaining the lack of correlation found here between arachidonic acid-induced LTA and serum TXB_2. Therefore, compared with the "gold-standard" biochemical test, the inhibition of arachidonic acid-induced LTA appears to have good specificity, but poor sensitivity. This may be explained by the several pre-analytical and analytical variables affecting LTA.[30] Accordingly, in a recent study, no correlation was observed between serum and urinary thromboxane metabolites and LTA induced by arachidonic acid.[26] Moreover, no correlation was observed between thromboxane metabolites and the PFA-100® CEPI-CT.

On the other hand, our results showed that the CEPI-CT was inversely correlated with serum TXB_2, and only one patient (20%) out of five with high TXB_2 levels still had inhibited platelet function as shown by a prolonged closure time. However, even when statistical significance was achieved, the R value was very low, indicating that only a minimal part of the variability observed in the CEPI-CT can be explained by changes in serum TXB_2 concentrations. In fact, many patients (about 25% of all treated) had normal platelet function according to this assay, some of whom even with inhibited TXB_2. A prolongation of the closure time in this assay may, therefore, be considered to have good sensitivity, but poor specificity with respect to the biochemical test. The Born method is time-consuming and requires specialized laboratories; however, the availability of a simple and rapid platelet function test might be very important to evaluate the response to antiplatelet agents in a Coronary Care Unit or in a catheterization laboratory, where the definition of aspirin "poor response" may be related to the probability of a detrimental clinical outcome, as suggested in a recent study,[31] and therefore be used for guiding therapeutic decision. However, the unreliability of the PFA-100®, as well as of LTA, precludes the translation of these test results into therapeutic decisions at present.

Overall, our results clearly indicate a high heterogeneity of outcomes in the tests used to evaluate platelet function. This is probably due, on the one hand, to the various physical principles underlying the methods used, to the different agonists initiating *ex vivo* platelet activation (some of which are known to have a very low sensitivity for aspirin-induced platelet inhibition), to the low reproducibility of many tests, and to the possible influence of hemorheologic variables on PFA-100® results, and, on the other hand, to the complexity of the target phenomenon (platelet aggregation) involving many biochemical pathway and agonists, with different relative relevances in different subjects and in different clinical conditions. For all these reasons it seems hazardous to assess the functional efficacy of aspirin therapy or to define patients as "responders" or "resistant" to aspirin based on the results of a single test. In particular, the definition of "aspirin resistance" based on any such assays is likely to be a laboratory result not reflecting a single identifiable and clinically relevant mechanism of aspirin failure.

Authorship and Disclosures

The information provided by the authors about contributions from persons listed as authors and in acknowledgments is available with the full text of this paper at www.haematologica.org.

Financial and other disclosures provided by the authors using the ICMJE (www.icmje.org) Uniform Format for Disclosure of Competing Interests are also available at www.haematologica.org.

References

1. McKee S, Sane D, Dellargyris E. Aspirin resistance in cardiovascular disease: a review of prevalence, mechanisms, and clinical significance. Thromb Haemost. 2002;88(5): 711-5.
2. Michelson AD, Cattaneo M, Eikelboom JW, Gurbel P, Kottke-Marchant K, Kunicki TJ, et al. Aspirin resistance: position paper of the Working Group on Aspirin Resistance. J Thromb Haemost. 2005;3(6): 1309-11.
3. Patrono C, Rocca B. Drug insight: aspirin resistance – fact or fashion? Nat Clin Pract Cardiovasc Med. 2007;4(1):42-50.
4. Crowe B, Abbas S, Meany B, de Haan J, Cahill MR. Detection of aspirin resistance by PFA-100: prevalence and aspirin compliance in patients with chronic stable angina. Semin Thromb Hemost. 2005;31(4):420-5.
5. Gum PA, Kottke-Marchant K, Poggio ED, Gurm H, Welsh PA, Brooks L, et al. Profile and prevalence of aspirin resistance in patients with cardiovascular disease. Am J Cardiol. 2001;88(3):230-5.
6. Harrison P, Segal H, Blasbery K, Furtado C, Silver L, Rothwell PM. Screening for aspirin responsiveness after transient ischemic attack and stroke: comparison of 2 point-of-care platelet function tests with optical aggregometry. Stroke. 2005;36(5):1001-5.
7. Alberts MJ, Bergman DL, Molner E, Jovanovic BD, Ushiwata I, Teruya J. Antiplatelet effect of aspirin in patients with cerebrovascular disease. Stroke. 2004;35(1): 175-8.
8. Hovens MM, Snoep JD, Eikenboom JC, van der Bom JG, Mertens BJ, Huisman MV. Prevalence of persistent platelet reactivity despite use of aspirin: a systematic review. Am Heart J. 2007;153(2):175-81.
9. Kuliczkowski W, Witkowski A, Polonski L, Watala C, Filipiak K, Budaj A, et al. Interindividual variability in the response to oral antiplatelet drugs: a position paper of the Working Group on antiplatelet drugs resistance appointed by the Section of Cardiovascular Interventions of the Polish Cardiac Society, endorsed by the Working Group on Thrombosis of the European Society of Cardiology. Eur Heart J. 2009;30 (4):426-35.
10. Born GV. Aggregation of blood platelets by adenosine diphosphate and its reversal. Nature. 1962;194:927-9.
11. Mammen EF, Comp PC, Gosselin R, Greenberg C, Hoots WK, Kessler CM, et al. PFA-100 system: a new method for assessment of platelet dysfunction. Semin Thromb Hemost. 1998;24(2):195-202.
12. Hayward CP, Harrison P, Cattaneo M, Ortel TL, Rao AK. Platelet function analyzer (PFA)-100 closure time in the evaluation of platelet disorders and platelet function. J Thromb Haemost. 2006;4(2):312-9.
13. Patrignani P, Filabozzi P, Patrono C. Selective cumulative inhibition of platelet thromboxane production by low-dose aspirin in healthy subjects. J Clin Invest. 1982;69(6): 1366-72.
14. Pedersen AK, FitzGerald GA. Cyclooxygenase inhibition, platelet function, and metabolite formation during chronic sulfinpyrazone dosing. Clin Pharmacol Ther. 1985;37(1):36-42.
15. Patrono C, Ciabattoni G, Pinca E, Pugliese F, Castrucci G, De Salvo A, et al. Low dose aspirin and inhibition of thromboxane B2 production in healthy subjects. Thromb Res. 1980;17(3-4):317-27.
16. Capone ML, Sciulli MG, Tacconelli S, Grana M, Ricciotti E, Renda G, et al. Pharmacodynamic interaction of naproxen with low-dose aspirin in healthy subjects. J Am Coll Cardiol. 2005;45(8):1295-301.
17. Harrison P, Robinson MS, Mackie IJ, Joseph J, McDonald SJ, Liesner R, et al. Performance of the platelet function analyser PFA-100 in testing abnormalities of primary haemostasis. Blood Coagul Fibrinolysis. 1999;10(1):25-31.
18. Kundu SK, Heilmann E, Sio R, Garcia C, Ostgaard R. Characterization of an in vitro platelet function analyzer - PFA-100. Clin Appl Thromb Hemost 1996;235(2):134-40.
19. Ortel TL, James AH, Thames EH, Moore KD, Greenberg CS. Assessment of primary hemostasis by PFA-100 analysis in a tertiary care center. Thromb Haemost. 2000; 84(1):93-7.
20. Eugster M, Reinhart WH. The influence of the haematocrit on primary haemostasis in vitro. Thromb Haemost. 2005;94(6):1213-8.
21. Mannini L, Marcucci R, Paniccia R, Antonucci E, Giglioli C, Valente S, et al. Erythrocyte deformability and white blood cell count are associated with aspirin resistance in high-risk vascular patients. Clin Hemorheol Microcirc. 2006;35(1-2):175-81.
22. Gori AM, Cesari F, Marcucci R, Giusti B, Paniccia R, Antonucci E, et al. The balance between pro- and anti-inflammatory cytokines is associated with platelet aggregability in acute coronary syndrome patients. Atherosclerosis. 2009;202(1):255-62.
23. Spiel AO, Gilbert JC, Jilma B. von Willebrand factor in cardiovascular disease: focus on acute coronary syndromes. Circulation. 2008;117(11):1449-59.
24. Harrison P, Segal H, Silver L, Syed A, Cuthbertson FC, Rothwell PM. Lack of reproducibility of assessment of aspirin responsiveness by optical aggregometry and two platelet function tests. Platelets. 2008;19(2):119-24.
25. Mortensen J, Poulsen TS, Grove EL, Refsgaard J, Nielsen HL, Pedersen SB, et al. Monitoring aspirin therapy with the Platelet Function Analyzer-100. Scand J Clin Lab Invest. 2008;68(8):786-92.
26. Grove EL, Hvas AM, Johnsen HL, Hedegaard SS, Pedersen SB, Mortensen J, et al. A comparison of platelet function tests and thromboxane metabolites to evaluate aspirin response in healthy individuals and patients with coronary artery disease. Thromb Haemost. 2010;103(6):1245-53.
27. Santilli F, Rocca B, De Cristofaro R, Lattanzio S, Pietrangelo L, Habib A, et al. Platelet cyclooxygenase inhibition by low-dose aspirin is not reflected consistently by platelet function assays: implications for aspirin "resistance". J Am Coll Cardiol. 2009; 53(8):667-77.
28. Reilly IA, FitzGerald GA. Inhibition of thromboxane formation in vivo and ex vivo: implications for therapy with platelet inhibitory drugs. Blood. 1987;69(1):180-6.
29. Sciulli MG, Renda G, Capone ML, Tacconelli S, Ricciotti E, Manarini S, et al. Heterogeneity in the suppression of platelet cyclooxygenase-1 activity by aspirin in coronary heart disease. Clin Pharmacol Ther. 2006;80(2):115-25.
30. Nicholson NS, Panzer-Knodle SG, Haas NF, Taite BB, Szalony JA, Page JD, et al. Assessment of platelet function assays. Am Heart J. 1998;135(5 Pt 2 Su):S170-8.
31. Marcucci R, Paniccia R, Antonucci E, Gori AM, Fedi S, Giglioli C, et al. Usefulness of aspirin resistance after percutaneous coronary intervention for acute myocardial infarction in predicting one-year major adverse coronary events. Am J Cardiol. 2006;98(9):1156-9.