Thrombotic events in high risk patients are predicted by evaluating different pathways of platelet function.

Thrombotic events in high risk patients are predicted by evaluating different pathways of platelet function

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Summary

A higher rate of clinical events in poor clopidogrel and/or aspirin responders was documented by using different methods to measure platelet function, but no conclusive data about the appropriate methodology to explore platelet reactivity are available. A total of 746 patients included in the cohort of the RECLOSE trial who had successful drug-eluting stent implantation were assessed for post-treatment residual platelet reactivity (RPR) in platelet-rich plasma by 10 μM adenosine 5'-diphosphate (ADP), 1 mM arachidonic acid (AA) and 2 μg/ml collagen-induced platelet aggregation and in whole blood by the PFA-100 system. At six-month follow-up, RPR by two stimuli (ADP and AA or ADP and collagen) and by three stimuli (ADP,AA and collagen) is significantly associated with higher percentage of primary (definite or probable stent thrombosis) and secondary (cardiac mortality and stent thrombosis) end-points than RPR by ADP, AA, collagen and PFA-100 system. According to the primary and secondary end points, the specificity values for RPR identified by two (ADP and AA: 94%; ADP and collagen: 97%) and three stimuli were higher with respect to RPR by ADP (88%), or RPR by AA (83%) or RPR by collagen (90%). The positive likelihood ratio values of RPR by three stimuli (9.55) or of RPR by ADP and collagen (8.08) were higher than those of RPR by ADP (2.59), by AA (2.05), by collagen (4.73), or by PFA-100 (2.63). This prospective study documents that the evaluation of platelet reactivity addressed to identify patients at risk of thrombotic events on dual antiplatelet treatment has to be carried out by methods able to explore different pathways.

Keywords

Residual platelet reactivity, stent thrombosis, cardiac mortality, platelet function tests, aspirin, clopidogrel

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Introduction

Clopidogrel treatment along with aspirin is presently considered the "gold standard" for attenuation of platelet activation and aggregation in patients undergoing percutaneous coronary intervention (PCI) (1–6).

However, despite the wide use of the antiplatelet therapy and the significant benefits reported with combined antiplatelet treatment in large clinical trials, adverse vascular events, including stent thrombosis, remain a serious clinical problem that occurs in a significant proportion of patients (7–8).

A growing body of evidence demonstrates that the presence of residual platelet reactivity (RPR) on antiplatelet therapy has a clinical relevance as it is associated with an increased risk of cardiovascular events (9–15). In particular, clopidogrel non-responsiveness as assessed by light transmittance aggregometry (LTA) induced by 10 μM adenosine 5'-diphosphate (ADP) was found to be an independent predictor of drug-eluting stent thrombosis (14).

LTA is considered as the standard method for the assessment of platelet function, but point-of-care assays of platelet function have become available, including PFA-100 (16).

Therefore, it would be necessary to develop platelet function test/s able to better identify patients at high risk of vascular events who could clearly benefit of a more aggressive antiplatelet strategy and to reduce the number of false positive patients who would be over-treated and exposed to a bleeding risk.

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To the best of our knowledge, no studies have evaluated whether the investigation of platelet function by different agonists – ADP, arachidonic acid (AA) and collagen – ameliorates the accuracy of LTA in predicting adverse clinical events.

In the cohort of RECLOSE trial we evaluated the accuracy of LTA by ADP, collagen and AA and of the point-of-care test PFA-100 in predicting clinical outcomes .

Methods

Patients

We previously performed a prospective study (RECLOSE Trial) evaluating the relationship between non-responsiveness to clopidogrel by ADP-induced platelet aggregation and the occurrence of stent thrombosis (14).

In the present study we evaluate whether the residual platelet reactivity to AA and collagen in addition to ADP is associated with clinical outcomes of patients receiving drug-eluting stent in 746 patients for whom complete AA- and collagen-induced platelet aggregation values were available.

As previously described (14), all patients were considered eligible for the study irrespective of clinical presentation or coronary anatomy. Thus, patients with acute coronary syndromes and ST-segment elevation acute myocardial infarction (AMI) were included, as well as patients with left main disease, chronic total occlusions, bifurcation lesions, or diffuse disease. The only exclusion criteria were: 1) in-hospital death that was not due to stent thrombosis; 2) anticipated noncompliance to dual drug antiplatelet treatment for at least six months; and 3) premature discontinuation of clopidogrel therapy. All patients gave informed consent. The study was approved by the local ethical committee.

PCI and antiplatelet management

All interventions were performed according to current standard guidelines, and the type of stent implanted and the use of IIb/IIIa inhibitors were at discretion of the operator. All patients received aspirin (325 mg) and a loading dose of 600 mg of clopidogrel followed by a maintenance dose of 75 mg daily. Patients on a maintenance dose of ticlopidine or clopidogrel at the time of admission received a loading dose of clopidogrel (600 mg).

Platelet reactivity assessment

Blood samples anticoagulated with 0.109 M sodium citrate (ratio 9:1) for platelet reactivity assessment was obtained 12 to 18 hours (h) after clopidogrel loading. For patients receiving in the catheterization laboratory both the loading dose of clopidogrel and a IIb/IIIa inhibitor, blood samples were obtained after six days while the patient was on the 75-mg maintenance dose of clopidogrel.

Platelet-rich-plasma, obtained by centrifuging whole blood for 10 minutes at 200 *g*, was stimulated with 10 μM ADP (Mascia Brunelli, Milan, Italy) with 1 mM AA (Sigma-Aldrich, Milan, Italy) and with 2 μg/ml collagen and residual drug aggregation was assessed using a APACT 4 light transmittance aggregometer (Helena Laboratories, Milan, Italy). The 100% line was set using platelet-poor plasma and the 0 baseline established with platelet-rich plasma (adjusted from 180×10^9/l up to 300×10^9/l). Platelet aggregation (according to the Born's method) was evaluated considering the maximal percentage of platelet aggregation in response to stimulus.

The coefficient of variation of ADP-LTA, AA-LTA and collagen-LTA were 6.8%, 5.8% and 5.6%, respectively.

The PFA-100 system (Dade-Behring, Marburg, Germany) simulates high-shear platelet function within test cartridges. Platelet function is measured as a function of the time (closure time [CT]) that platelets take to occlude an aperture in a membrane coated with collagen/epinephrine (CEPI) or with collagen/ADP (CADP). Citrated whole blood sample (0.8 ml) was pipetted into the sample reservoirs of CEPI or CADP cartridges (pre-warmed to room temperature) and then loaded into the PFA-100 device. Normal range (95^{th} percentile of control distribution) obtained in our laboratory was 65 to 203 seconds (s) for CT/CEPI cartridge and 62 to 139 s for CT/CADP cartridge. The mean CV was 5.4% for the CEPI CT in control subjects and 9.9% in patients with CAD. The mean CVs for C/ADP CT were 4.3% in controls and 9.3% in CAD patients.

Residual platelet reactivity (RPR)

We defined patients with RPR those with platelet aggregation by AA ≥20% and/or ADP ≥70% according to literature (17–18) and studies from our group (14, 19). RPR by collagen was defined in the presence of platelet aggregation induced by collagen above 90^{th} percentile of patients' distribution (56%). Patients with RPR by CEPI PFA-100 and by CADP PFA-100 were defined those subjects with a CT/CEPI below the 90^{th} percentile of controls (< 203 s) and with a CT/CADP below 68 s (19), respectively.

Endpoints

The primary endpoint of the study was definite or probable stent thrombosis during a six-month follow-up. Definite stent thrombosis was defined as acute coronary syndrome and either angiographic confirmation of thrombosis or pathological confirmation of thrombosis. Probable stent thrombosis was defined as unexplained death or myocardial infarction in the territory supplied by a stented vessel without angiographic confirmation. The diagnosis of myocardial infarction was based on either the development of new Q waves on two or more electrocardiographic leads or an increase of creatine kinase-myocardial band isoenzyme or troponin T >3 times the upper limit of normal. Event time was categorized as acute (within 24 h from stent implantation), subacute (from 1 day to 30 days), and late (30 days to 6 months). The secondary end point was the composite of cardiac mortality and definite or probable stent thrombosis.

Follow-up

All patients had scheduled clinical and electrocadiographic examinations at one, three, and six months. All other possible information derived from hospital readmission or by the referring physician, relatives, or municipality live registries was entered into the database.

Statistical analysis

Discrete data are summarized as frequencies, whereas continuous data as mean ± SD or median and range.

The chi-square test was used for comparison of categorical variables. A Mann-Withney test for non-parametric data or t-test for parametric data were used to test differences between stent thrombosis (or composite endpoint) and no stent thrombosis (or composite endpoint) groups.

The ability of platelet aggregation values by ADP, AA and collagen and the PFA-100 CT/CEPI and PFA-100 CT/CADP values (alone or in combination) to predict primary and secondary endpoints was examined by receiver operating characteristics (ROC) curves. ROC curves were constructed by plotting the sensitivity against the corresponding false-positive rate (FPR) which equals 1-specifity. Due to the low prevalence of primary and secondary end-points, we further calculated the likelihood-ratio values.

The optimal cut-off point was calculated by determining the post-treatment RPR that provided the greatest sum of sensitivity and specificity. Bootstrap validation was performed using R software.

Clinical characteristics (age, sex, cardiovascular risk factors, ejection fraction, number of vessel disease, renal failure, total stent length, chronic total occlusion, bifurcation lesion and glycoprotein IIb/IIa inhibitors) were included in the logistic regression analysis as independent variables in a model in which each RPR (RPR by ADP, RPR by ADP and AA, RPR by ADP, AA and collagen, RPR by CEPI PFA-100 and RPR by CADP PFA-100) was added separately. Variables that resulted not to be associated with the outcome were removed from the final most parsimonious regression model through a backward selection algorithm.

A p-value <0.05 was considered significant. Analyses were performed using the software package SPSS 8.0 (SPSS Inc., Chicago, IL, USA).

Table 1: Baseline clinical and procedural characteristics according to clinical outcomes.

	No stent thrombosis (n=726)	Stent thrombosis (n = 20)	No cardiovascular death or stent thrombosis (n = 721)	Cardiovascular death or stent thrombosis (n = 25)
Age (years)	68 ± 12	74 ± 9 *	75 ± 10	68 ± 11*
Male gender, n (%)	550 (75.8)	13 (65)	546 (75.7)	17 (68.0)
Current smokers, n (%)	165 (22.7)	5 (25)	164 (22.7)	6 (24.0)
Arterial hypertension, n (%)	450 (62.0)	15 (75.0)	447 (62.0)	18 (72.0)
Diabetes mellitus, n (%)	147 (20.2)	5 (25.0)	147 (20.4)	5 (20.0)
Hypercholesterolemia, n (%)	359 (49.4)	10 (50)	357 (49.5)	12 (48.0)
Previous myocardial infarction, n (%)	174 (24.0)	9 (45.0)§	171 (23.7)	12 (48.0)§
Previous PCI, n (%)	165 (22.7)	5 (25.0)	163 (22.6)	7 (28.0)
Previous coronary artery surgery, n (%)	44 (6.1)	1 (5.0)	43 (6.0)	2 (8.0)
Stable angina, n (%)	245 (33.7)	4 (20.0)	245 (33.7)	4 (20.0)
Unstable angina, n (%)	289 (39.8)	9 (45.0)	286 (39.8)	12 (48.0) *
Acute myocardial infarction, n (%)°	187 (25.8)	7 (35.0)	185 (25.7)	9 (36.0)
Renal failure, n (%)	80 (11.0)	3 (15 .0)	79 (11.0)	4 (16.0)
Multivessel disease, n (%)	407 (56.4)	16 (80)*	403 (55.9)	20 (80)*
LVEF (%)	47 ± 12	34 ± 13**	48 ± 11	32 ± 14**
Multivessel PCI, n (%)	407 (56.4)	16 (80) §	403 (56.2)##	20 (80)§
Thrombus-containing lesion, n (%)	155 (21.3)	3 (15.0)	153 (21.2)	5 (20.0)
Bifurcation lesion, n (%)	257 (35.4)	11 (55.6)	255 (35.4)	13 (52.0)
Chronic total occlusion, n (%)	77 (10.1)	6 (30.0)§	76 (10.5)	7 (28.0)§
Total stent length (mm)	37 ± 28	61 ± 41**	35 ± 27	52 ± 36**
Sirolimus-eluting stent, n (%)	399 (55.0)	10 (50.0)	398 (55.2)	11 (44.0)
Paclitaxel-eluting stent, n (%)	280 (38.6)	8 (40.0)	279 (38.7)	9 (36.0)
Both stent types, n (%)	47 (6.5)	2 (10.0)	47 (6.5)	2 (8.0)
Post-PCI MLD (mm)	2.88 ± 0.45	2.84 ± 0.46	2.81 ± 0.45	2.82 ± 0.52
Glycoprotein IIb/IIIa, n (%)	312 (43.0)	10 (50.0)*	311 (43.1)	11 (44.0)*

* p<0.05 vs. no stent thrombosis or no cardiovascular death or stent thrombosis. § p<0.01 vs. no stent thrombosis or no cardiovascular death or stent thrombosis. **p<0.001 vs. no stent thrombosis or no cardiovascular death or stent thrombosis. °ST-segment elevation acute myocardial infarction.

Table 2: Platelet aggregation induced by 10 µM ADP, 1 mM AA, 2 µg/ml collagen and closure time (CT) of collagen/epinephrine cartridges PFA-100 system.

	No stent thrombosis (n=726)	Stent thrombosis (n=20)	No cardiovascular death or stent thrombosis (n=721)	Cardiovascular death or stent thrombosis (n=25)
10 µM ADP-LTA (%) n=746	45 (1–98)	56 (11–82)*	45 (1–98)	54 (11–82)*
1 mM AA-LTA(%) n=746	10 (1–92)	11.5 (2–83)	10 (1–92)	11 (2–93)
2 µg/ml collagen-LTA (%) n=176	22 (1–84)	42 (1–79)**	22 (1–84)	34 (1–79)**
CT/CEPI PFA-100 (sec)n=746	300 (75–300)	269 (85–300)**	300 (75–300)	300 (85–300)**
CT/CADP PFA-100 (sec) n=398#	106 (51–300)	79.5 (61–86)**	106 (51–300)	82.5 (61–110)**

these data derived from 398 patients: 390 patients without stent thrombosis and 8 with stent thrombosis; 388 patients without stent thrombosis or cardiovascular death and 10 with stent thrombosis or cardiovascular death. * p<0.05; ** p<0.01 vs no stent thrombosis or vs no composite end point (stent thrombosis and cardiovascular death).

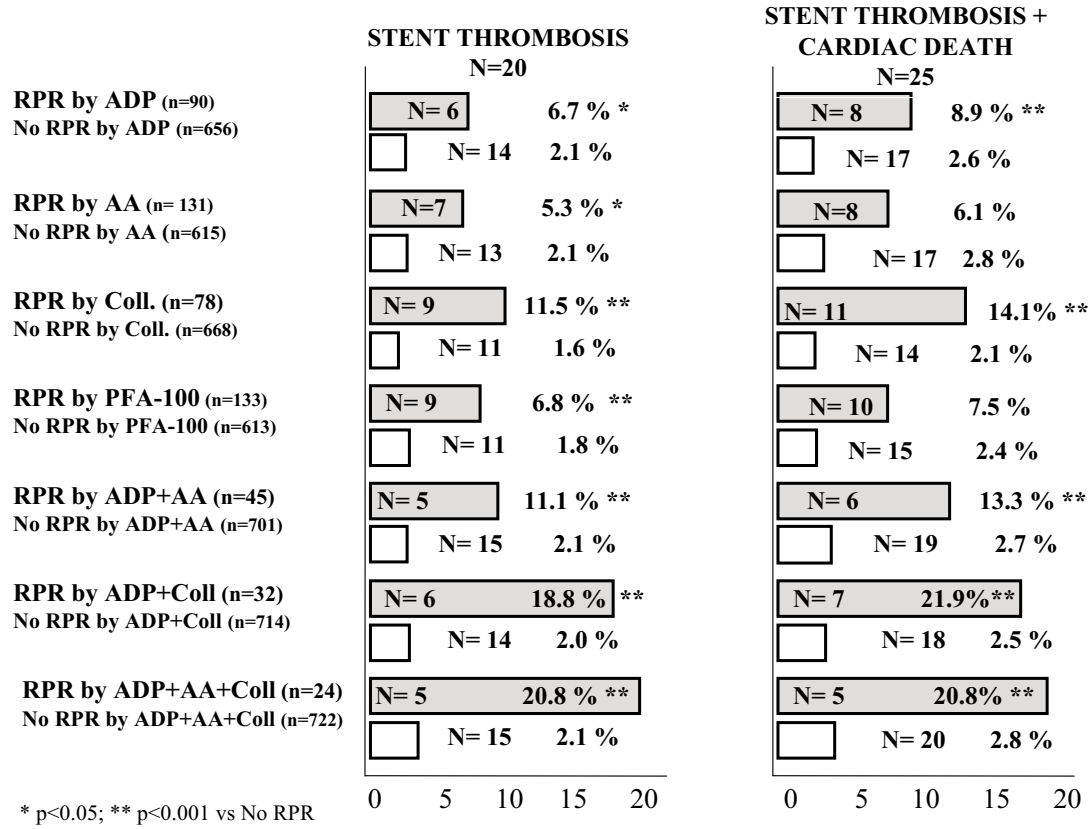

Figure 1: Clinical outcomes according to RPR by ADP, RPR by AA, RPR by collagen, RPR by PFA-100, RPR by AA and AA, RPR by ADP and collagen, RPR by ADP, AA and collagen.

Results

Demographic and clinical characteristics according to clinical outcomes

Demographic, clinical and procedural characteristics of the patients investigated according to clinical outcomes are presented in Table 1.

Platelet aggregation and RPR by ADP, AA, collagen and PFA-100 and clinical outcomes

ADP-induced platelet aggregation was significantly related with collagen- and AA-induced platelet aggregation (rho=0.55, p<0.001 and rho= 0.54, p<0.0001, respectively). CEPI PFA-100 values significantly correlated with ADP-, AA-, and collagen-induced platelet aggregation (rho=-0.26, p<0.001; rho= –0.36, p<0.0001 and rho= –0.34, p<0.0001, respectively). Similarly in

	RPR by ADP (≥70%) (N=90)	RPR by AA (≥20%) (N=131)	RPR by collagen (≥56%) (N=78)	CEPI PFA-100 (≤203) (N=133)	RPR by ADP and AA (N=45)	RPR by ADP and collagen (N=31)	RPR by ADP, AA and collagen (N=24)
Stent thrombosis							
Sensitivity (%)	30 (11–54)	35 (15–59)	45(23–69)	45(23–69)	25(23–67)	30 (10–50)	25(6–44)
Specifity (%)	88 (86–91)	83 (80–86)*	90(88–92) ***	83(80–86)*,***	94(93–96)**	97(95–98)**	97(96–99)**
Positive predictive value (%)	7 (3–14)	5 (2–10)	12 (5–20)	7(3–13)	11 (2–20)	19 (5–33)	21(5–37)
Negative predictive value (%)	98 (96–99)	98 (97–99)	98 (97–99)	98(97–99)	98(97–99)	98(97–99)	98 (97–99)
LR+	2.59(1.29–5.22)	2.05(1.10–3.80)	4.73(2.78–8.08)	2.63 (1.58–4.38)	4.54 (2.0–10.27)	8.08(4.02–18.86)	9.55 (3.96–23.01)
LR-	0.79(0.59–0.97)	0.78(0.57–0.97)	0.61(0.41–0.79)	0.66 (0.44–0.87)	0.79(0.62–0.94)	0.75 (0.54–0.88)	0.7(0.60–0.91)7
Accuracy (%)	87	82	89	82	93	94	95
Composite endpoint							
Sensitivity (%)	32(15–54)	32(14–54)	44(25–63)	40(21–61)	24)7–41)	28(10–46)	20 (4–36)
Specifity (%)	89(86–91)	83(80–86)*	91 (88–93)***	83(80–86)*,***	95(93–96)**	97(95–98)**°	97(96–99)**§#
Positive predictive value (%)	9(4–17)	6(3–11)	14(7–24)	8(4–13)	13 (3–23)	23(8–37)	21(5–37)
Negative predictive value (%)	97(96–99)	97(96–98)	98(97–99)	98(96–99)	97(96–98)	97(96–99)	98 (96–99)
LR+	2.81(1.53–5.16)	1.88(1.04–3.40)	4.73(2.88–7.88)	2.34(1.41–3.88)	4.44(2.75–9.5)	8.08(4.0–17.67)	8.64(3.08–18.68)
LR-	0.77(0.59–0.92)	0.82(0.63–0.98)	0.62(0.44–0.78)	0.72(0.52–0.90)	0.80(0.64–0.93)	0.75(0.58–0.88)	0.73(0.68–0.94)
Accuracy (%)	87	81	89	82	91	94	95

*P<0.01 vs RPR by ADP; ** p<0.01 vs RPR by ADP, or by AA or by collagen; # p<0.05 vs RPR by ADP and collagen; § p<0.0001 vs RPR by ADP and AA; ° p<0.01 vs RPR by ADP and collagen; *** p<0.0001 vs RPR by collagen.

Table 3: Specificity, sensitivity, positive predictive value, negative predictive value, positive likelihood (LR+), negative likelihood (LR-) and accuracy of RPR by ADP, RPR by AA, RPR by collagen and RPR by PFA 100 in identifying patients at risk of stent thrombosis and composite endpoint.

398 patients in whom CADP PFA-100 was available, a significant correlation between CADP PFA-100 values and ADP-, AA-, and collagen- induced platelet aggregation was found (rho=-0.28, p<0.001; rho= –0.18, p<0.0001 and rho= –0.25, p<0.0001, respectively). Platelet aggregation by ADP, collagen and CT/CEPI and CT/CADP values by PFA-100 system were significantly different in patients who developed stent thrombosis or secondary endpoints with respect to patients in whom no adverse cardiovascular event was recorded (Table 2).

RPR by ADP, AA, collagen and PFA-100 system was found in 90/746 (12.1 %), 131/746 (17.6%), 78/746 (10.5 %) and 133/746 (17.8%) patients, respectively. In 398 patients we also evaluated CT/CADP values by PFA-100: 22/398 (5.5%) patients had RPR by CADP PFA-100.

In Figure 1 the clinical outcomes during six-month follow-up according to RPR by ADP, RPR by AA, RPR by collagen, RPR by CEPI PFA-100 system and combined RPR are shown.

The presence of RPR by two stimuli, ADP and AA or ADP and collagen, as well as by three stimuli (ADP, AA and collagen) was significantly associated with a higher percentage of stent thrombosis and secondary endpoints (stent thrombosis and cardiovascular mortality) than in patients with RPR after stimulation by ADP, AA, collagen and CEPI PFA-100 system (Fig. 1). No significant association was found between RPR by CADP PFA-100 and stent thrombosis and secondary endpoints (data not shown).

The analysis of the aggregometry tests in relation to the primary and secondary endpoints (Table 3) demonstrated that the sensitivity and specificity and positive predictive value of RPR by collagen were higher than RPR by ADP, by AA or by PFA-100 system, whereas negative predictive values were similar.

According to the primary and secondary endpoints, the specificity values for RPR identified by two (ADP and AA; ADP and collagen) and three stimuli were significantly higher with respect to RPR by ADP, or RPR by AA or RPR by collagen. Conversely, the sensitivity values were lower, but not statistically significant, when RPR was defined after three stimuli or by two stimuli (ADP and AA) than only by ADP, by AA or by collagen (Table 3). The positive predictive value significantly increased for RPR identified by three stimuli with respect to the other groups of RPR, where negative predictive values were similar among different groups of RPR (Table 3).

Due to the low prevalence of primary and secondary endpoints, we further calculated the likelihood ratio (LR) values of RPR by ADP, AA, collagen and PFA-100. As shown in Table 3, according to primary and secondary endpoints, the detection of RPR by three stimuli or of RPR by two stimuli (ADP and collagen) is associated with a higher value of positive likelihood ratio (Table 3).

As shown in Table 4, RPR identified by collagen, RPR by ADP and collagen and RPR by three stimuli remained signifi-

cantly associated with both primary and secondary endpoints after adjustment for age, sex, cardiovascular risk factors, ejection fraction, number of vessel disease, renal failure, length of stent > 20 mm, chronic total occlusion and bifurcation lesion (Table 4).

Evaluation of different platelet aggregation cut-off values for identifying patients at high risk of adverse events

The analysis of ROC curves of the different platelet aggregation tests showed that areas under the curves of ADP-, AA- and collagen-induced platelet aggregation were 0.65 ± 0.06, 0.56 ± 0.08 and 0.50 ± 0.07, respectively.

The optimal cut-off values for platelet aggregation tests for identifying patients at high risk of adverse events derived from ROC curve analysis (Fig. 2) were 46% for ADP, 69% for AA, 55% for collagen, 238 s for CT/CEPI PFA-100 and 105 s for CT/CADP PFA-100.

Sensitivity, specificity, negative, positive predictive LR+, LR- and accuracy values, according to these cut-off values, are shown in Table 5.

The combination of RPR by ADP (> 46%), RPR by AA (>69%) and RPR by collagen (>55%) consistently increased the LR+ value with respect to the "previous" cut-off values (70%, 20% and 56%).

Sensitivity, specificity and negative predictive, LR- and accuracy values were similar, whereas positive predictive value increased from 21 % to 26%.

Bootstrap analyses

By bootstrap analysis using 10,000 replicates, the optimal cut-off values of ADP-, AA-, collagen-induced platelet aggregation and PFA-100 system were 50 (95% CI 34–71), 49 (95% CI 9–79), 56 (95% CI 55–60) and 106 (95% 58–194) for primary endpoint, and 50 (95% CI 34–72), 36 (95% CI 9–79), 55 (95% CI

Table 4: Predictors of stent thrombosis and composite endpoint (cardiac death and stent thrombosis).

	Odds ratio (95% CI) Stent thrombosis	P-value	Odds ratio (95% CI) Composite endpoint	P-value
Univariate analysis				
RPR by ADP	**3.28 (1.23–8.75)**	**0.018**	**3.67 (1.53–8.76)**	**0.003**
RPR by AA	2.61 (1.02–6.69	0.045	2.29 (0.97–5.42)	0.060
RPR by collagen	7.79 (3.12–19.46)	0.0001	7.67 (3.35–17.57-)	0.0001
RPR by CEPI PFA-100	3.97 (1.61–9.79)	0.003	3.24 (1.42–7.38)	0.003
RPR by ADP and AA	5.72 (1.98–16.52)	0.001	5.52 (2.09–14.61)	0.001
RPR by ADP and collagen	12.01 (4.26–33.87)	0.0001	11.29 (4.31–25.59)	0.0001
RPR by ADP, collagen and AA	12.40 (4.09–37.64)	0.001	9.24 (3.13–27.22)	0.001
Chronic total occlusion	3.16 (1.34–9.67)	0.011	3.30 (1.34–8.16)	0.010
Multivessel disease	3.10 (1.02–9.35)	0.045	3.12 (1.16–8.40)	0.025
Previous myocardial infarction	2.60 (1.06–6.37)	0.037	2.97 (1.33–6.63)	0.008
Bifurcation lesion	2.23 (0.91–5.45)	0.079	1.98 (0.89–4.40)	0.094
Age (years)	1.06 (1.01–1.11)	0.013	1.07 (1.02–1.12)	0.003
Total stent lenght, mm	1.02 (1.00–1.03)	0.0001	1.02 (1.01–1.03)	0.0001
LVEF per 1% increase	0.93 (0.90–0.96)	0.0001	0.92 (0.89–0.94)	0.0001
Multivariate analysis				
RPR by ADP	2.41 (0.99–6.85)	0.090	2.86 (1.13–7.03)	0.022
RPR by AA	1.93 (0.72–5.16)	0.100	1.63 (0.65–4.09)	0.303
RPR by collagen	5.45 (2.05–14.53)	0.001	4.99 (2.05–12.17)	0.0001
RPR by PFA-100	3.25 (1.26–8.39)	0.025	2.60 (1.08–6.21)	0.031
RPR by ADP and AA	4.23 (1.36–13.10)	0.012	3.96 (1.38–11.32)	0.010
RPR by ADP and collagen	7.50 (2.40–23.43)	0.001	6.45 (2.24–18.58)	0.001
RPR by ADP, collagen and AA	6.91 (2.08–23.0)	0.002	6.42 (2.23–14.32)	0.011
Age (years)	1.05 (1.01–1.10)	0.05	1.06 (1.01–1.11)	0.014
Total stent lenght, mm	1.01 (1.00–1.02)	0.018	1.02 (1.00–1.03)	0.003
LVEF per 1% increase	0.94 (0.91–0.97)	0.0001	0.92 (0.89–0.95)	0.0001

Adjusted for traditional cardiovascular risk factors, bifurcation lesion, chronic total occlusion, multivessel disease, previous myocardial infarction, left ventricular ejection fraction and glycoprotein IIb/IIIa inhibitors. Variables with a p value <0.20 that were entered in the multivariate model.

55–55) and 108 (95% CI 58–194) for secondary endpoint. According to primary endpoint the area under the curve (AUC) of ADP-, AA-and collagen-induced platelet aggregation were 0.68 (95% confidence interval [CI] 0.58–0.88), 0.58 (95% CI 0.43–0.73) and 0.61 (95% CI 0.48–0.77), whereas sensitivity and specificity were 59% (95% CI 40–80) and 60% (95% CI 37–43) for ADP-, 25% (95% CI 10–40) and 93 (95% CI 92–94) for AA-, 50% (95% CI 30–65) and 85% (95% CI 83–87) for collagen-induced platelet aggregation. Similar results were obtained for secondary endpoints (data not shown).

Discussion

To the best of our knowledge, this is the first prospective study which has searched for the best platelet function evaluation among three platelet-rich plasma aggregation tests and one point-of-care test in relation to clinical events and which documents that the evaluation of platelet reactivity carried out by stimuli exploring different pathways represents an accurate tool for identifying patients at risk of thrombotic events in spite of dual antiplatelet treatment.

Our results show that the positive likelihood ratio (9.55) of aggregometry test induced by three stimuli (ADP, AA and collagen) is higher than that of ADP or ADP and AA induced aggregation test, even though the sensitivity is 25%. This association was stronger than that observed in patients with two stimuli and in patients with RPR by only ADP.

Present findings are not inconsistent with those we previously obtained in the same patients showing that a high platelet reactivity to ADP is a predictor of stent thrombosis (14), as in the previous study the group of patients with RPR to ADP brings in patients with RPR to AA and collagen.

Present results underpin the concept that no single test encompasses the complexity of platelet biology and function, but that a "combination" of different stimuli has to be used to achieve a fair detection of patients at high risk to develop adverse events.

In this study we evaluated the platelet function by using three agonists – AA, collagen and ADP. AA-induced platelet aggregation is mainly influenced by the inhibition of the thromboxane synthesis and ADP-induced platelet aggregation is mainly sensitive to the inhibition of ADP receptors, both $P2Y_1$ and $P2Y_{12}$. However, evidence exists of the role of the $P2Y_{12}$ receptor, target of clopidogrel, as a functional regulator of TxA2 generation consequent to protein-activated receptor stimulation (20) and/or, conversely, the fact that ADP- and collagen-induced platelet aggregation are affected to some extent by aspirin (21). Collagen acts on GPVI and directly activates the receptor function of GpIIb/IIIa to induce a maximal platelet aggregation response (22). Signalling by these receptors causes the secretion of ADP, which also participates in glycoprotein IIb/IIIa activation.

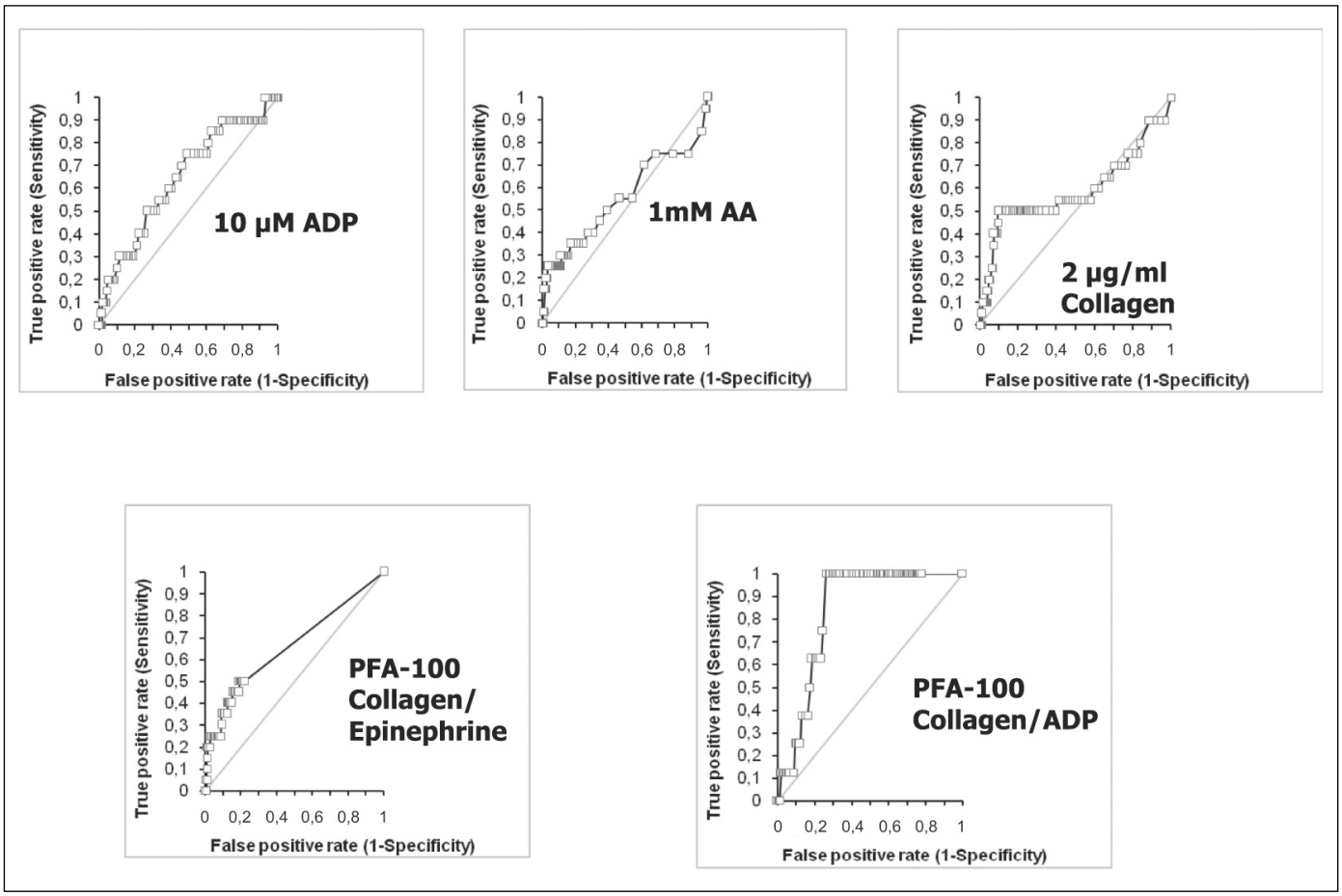

Figure 2: Receiver-operating characteristics (ROC) curves for different platelet function tests according to stent thrombosis.

Table 5: Specificity, sensitivity, positive predictive value, negative predictive value, positive likelihood (LR+), negative likelihood (LR-) and accuracy of RPR by ADP, RPR by AA, RPR by collagen and RPR by CEPI-PFA 100 and CADP-PFA-100 in identifying patients at risk of stent thrombosis and composite endpoints.

	RPR by ADP (>46%) (N= 352)	RPR by AA (>69%) (N= 28)	RPR by collagen (>54%) (N=78)	RPR by CEPI-PFA (< 238 s) (N=238)	RPR by CADP-PFA ^(<105 s) (N=196)	RPR by ADP and AA (N=24)	RPR by ADP and collagen (N=61)	RPR by ADP, AA and collagen (N=19)
Stent thrombosis								
Sensitivity (%)	70(50–90)	25(6–44)	50(28–72)	40(19–61)	#100	*25(6–44)	45(23–67)	*25(6–44)
Specifity (%)	§53(50–57)	§,**97(96–98)	**91(88–93)	**81(78–89)	#52(47–57)	§,**97 (96–99)	§,**93(91–95)	§,**98 (97–99)
Positive predictive value (%)	4 (2–6)	18(4–32)	13(5–20)	5(2–9)	4 (1–5)	21 (5–37)	13(6–24)	26(7–46)
Negative predictive value (%)	98(97–100)	98(97–99)	99(98–99)	98(97–99)	100	98 (97–99)	98(97–99)	98 (97–99)
LR+	1.50(1.12–2.04)	7.89(3.34–18.63)	5.34(3.26–8.74)	2.09(1.1–3.64)	2.07(1.9–2.3)	9.55 (3.96–23.01)	6.28(3.62–10.90)	12.96 (5.17–32.5)
LR-	0.56(0.29–0.88)	0.77(0.6–0.91)	0.55(0.39–0.74)	0.74(0.52–0.94)	0	0.77 (0.6–0.91)	0.59(0.4–0.77)	0.76 (0.59–0.91)
Accuracy (%)	53	95	89	80	53	95	94	96
Composite endpoint								
Sensitivity (%)	64(45–83)	°,*20(4–36)	48(28–68)	*36(17–55)	80(55–100)	°,*20(4–36)	*40(21–59)	°,*20(4–36)
Specifity (%)	§53(50–57)	§,**97(96–98)	91(89–93)	§,**81(78–84)	#52(467–57)	§,**97(96–99)	**93(91–95)	§,**98(97–99)
Positive predictive value (%)	5(2–7)	18(4–32)	14(7–23)	6(2–10)	4(1–7)	21(5–37)	16(7–26)	26 (7–46)
Negative predictive value (%)	96(94–98)	97(96–98)	98(97–99)	97 (96–99)	99 (98–100)	97(96–98)	98(97–99)	97 (96–98)
LR+	1.37(1.01–1.86)	6.27(2.6–15.13)	5.24(3.28–8.38)	1.88(1.09–3.23)	1.65(1.19–2.29)	7.59(3.08–18.68)	5.65(3.27–9.78)	10.3 (4.02–26.37)
LR-	0.67(0.4–0.96)	0.83(0.68–0.94)	0.57(0.39–0.74)	0.79(0.59–0.97)	0.39(0.11–0.98)	0.82(0.68–0.94)	0.65(0.47–0.8)	0.82 (0.67–0.93)
Accuracy (%)	54	94	89	79	52	95	91	95

* p<0.01 vs. RPR by ADP. ° p<0.01 vs. RPR by collagen. ** p<0.0001 vs. RPR by ADP. § p<0.0001 vs. RPR by collagen; # p<0.001 vs CEPI –PFA-100; ^data derived from 398 patients.

The relevance of platelet hyperreactivity identified by collagen stimulation "per se" is underlined by the observation that the specificity and LR+ values are higher for RPR identified by collagen, RPR by collagen and ADP and/or AA with respect to RPR by only ADP or AA stimuli.

In our study, the definition of high platelet reactivity as platelet aggregation by ADP-LTA ≥70%, AA-LTA ≥20% and collagen-LTA ≥56%, according to the literature and ourselves (14, 17–19) had low sensitivity but high specificity. By using different cut-off values derived from the analysis of ROC curves for RPR by ADP, AA and collagen a sensitivity higher than the values referred in the literature and in our own studies was obtained, without remarkable changes in positive predictive and LR+ values of the single aggregometry test. Anyway, the highest LR+ value (12.96) was obtained when RPR was detected by three stimuli (ADP, AA and collagen).

As concerns the sensitivity, players other than haemostatic factors participate in determining clinical outcomes. In fact several factors including procedural characteristics (length of stent, chronic total occlusion, bifurcation lesion), ejection fraction and risk factors (diabetes and age) are relevant for the occurrence of stent thrombosis in drug-eluting stents (14–15). In our study, patients with stent thrombosis, but without high platelet reactivity, were characterized by low left ventricular ejection fraction, older age, bifurcation lesion, and chronic total occlusion, which may account for a significant proportion of adverse events independently of platelet function.

In the present study we also evaluated the platelet function by using a point-of-care test – PFA-100 system – in relation to clinical outcome during the follow-up, and we found that PFA-100 test had a lower specificity with respect to the other platelet aggregation tests. This assay is a global function test, which explores the capability of platelets to occlude an aperture in a membrane coated with collagen/epinephrine in the presence of other blood components, such as erythrocytes and leukocytes, that are also involved in platelet function non-responsiveness (23–24). In clinical trials, RPR by PFA-100 was associated with clinical events in patients undergoing PCI (16, 25, 26). Among patients who experienced stent thrombosis 3/20 (15%) patients had only RPR by PFA-100, thus suggesting that this point-of-care test gives a different picture of the hyperreactive blood which causes thrombosis.

Recently, by using another point-of-care test designed to measure platelet $P2Y_{12}$ receptor blockade a sensitivity of 78% and a specificity of 68% for detecting clinical events was found (15).

The clinical relevance of availability of accurate tests for platelet function is related to the concern of the risk of bleeding complications in relation to the administration of higher anti-

What is known about this topic?
– Higher rate of clinical events in poor clopidogrel and/or aspirin responders was documented by using different methods to measure platelet function, but no conclusive data about the appropriate methodology to explore platelet reactivity are available.
– In particular, no studies have evaluated whether the investigation of platelet function by different agonists – ADP, arachidonic acid and collagen – ameliorates the accuracy of light transmittance aggregometry in predicting adverse clinical events.

What does this paper add?
– This is the first prospective study which has searched for the best platelet function evaluation among three platelet-rich plasma aggregation tests and one point-of-care test in relation to clinical events.
– It documents that the evaluation of platelet reactivity carried out by stimuli exploring different pathways represents an accurate tool for identifying patients at risk of thrombotic events on dual antiplatelet treatment.

aggregating drug dosage or to the availability of more potent antiaggregating drug.

De facto in TRITON TIMI 38, it was found that the lower rate of thrombotic events was charged with higher rate of haemorrhagic complications in prasugrel than in patients with clopidogrel (27). These findings make urgent the need of a more precise identification of patients likely to profit from additional antiplatelet treatments, thus avoiding more aggressive treatment of those patients who are not prone to experience stent thrombosis. Interestingly, the number-needed-to-treat (NNT) calculation from our findings indicated that the recognition of patients at high risk to develop stent thrombosis by using the definition of RPR by three stimuli gave a NNT of about 5, whereas the definition of RPR by ADP, AA and collagen and by PFA-100 gave a NNT of 22, 31, 10 and 20 respectively.

Limitations

In this study we did not explore thrombin-induced platelet activation pathway. Platelet surface expression of activated glycoprotein IIb/IIIa and P-selectin after TRAP stimuli assessed by flow cytometry could provide further insight into the mechanisms involved in the different response to antiplatelet drugs. Second, we did not investigate platelet function by using other whole blood-assays (28) such as the flow cytometric vasodilator-stimulated phosphoprotein (VASP) phosphorylation assay and VerifyNow P2Y12, which has been evaluated in detecting a reduced response to clopidogrel and for a platelet function-driven antiplatelet therapy (29). Third, our study cannot provide data about whether platelet hyperreactivity is responsible of acute cardiovascular death (from 0 to 6 days after clopidogrel loading) in patients who had GPIIbIIIa treatment. Finally, we do not provide information about the duration of the aspirin and/or clopidogrel resistance.

Future perspectives

New point-of-care tests aimed to identify global platelet function should use also collagen, a stimulus not specifically drug-related, but which is able to mirror a platelet hyperactive state which renders the blood aggressive, and the patient vulnerable. Adequately powered studies aimed to evaluate the safety of more aggressive antiplatelet therapy should use an algorithm which includes multiple platelet aggregation tests.

In conclusion, this prospective study documents that the evaluation of RPR is associated with thrombotic complications and that the recognition of platelet reactivity requires the exploration of different platelet multi-receptor pathways.

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