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COORDINATORE Prof. Matucci Cerinic Marco

Funzione piastrinica e rischio di eventi avversi in pazienti con arteriopatia periferica sottoposti a rivascolarizzazione percutanea

(Platelet function and risk of adverse events in peripheral artery disease patients undergoing percutaneous revascularization)

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Dottorando

Dott. Grifoni Elisa

Tutore

Prof. Marcucci Rossella

Coordinatore

Prof. Matucci Cerinic Marco

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Summary

Background and Aim: Lots of studies demonstrated that a different entity of on-treatment platelet function inhibition is associated with different clinical outcomes in patients with acute coronary syndromes. In particular, high on-treatment platelet reactivity (HPR) has been associated with an increased risk of ischemic complications (especially stent thrombosis), and there is a growing body of evidence that, on the contrary, low on-treatment platelet reactivity (LPR) could be associated with bleeding risk. Few data are available in the literature on the association between a different entity of platelet inhibition on-antiplatelet treatment and clinical outcomes in patients with peripheral artery disease (PAD).

Aim of this study was to evaluate, in patients with PAD undergoing percutaneous revascularization, the degree of on-treatment platelet reactivity, and its association with ischemic and hemorrhagic adverse events at follow-up.

Methods: In this observational, prospective, single center study, consecutive patients with PAD undergoing percutaneous transluminal angioplasty (PTA) with or without stenting, were enrolled. All patients were treated with dual antiplatelet therapy with aspirin and a P2Y12 inhibitor. Platelet function was assessed by Light Transmission Aggregometry (LTA) using arachidonic acid (AA) and adenosine diphosphate (ADP) as agonists of platelet aggregation, on blood samples obtained within 24 hours from PTA. HPR was defined by LTA ≥20% if induced by AA, and LTA ≥70% if induced by ADP. Follow-up was performed in order to record the occurrence of ischemic and bleeding events.

Results: The study enrolled 177 patients [118 males, median age 75 (IQR 68-81) years]. HPR by AA was found in 52% of patients, and showed a non significant association with older age and a higher prevalence of renal failure, whereas HPR by ADP was found in 32% of patients, and was significantly associated with older age. During follow-up [median duration 23 (IQR 13-

27) months] 23 deaths (13%) were recorded; 27 patients (17.5%) underwent target limb revascularization, 2 (1.3%) amputation, and 6 (3.9%) myocardial revascularization. Twenty-four patients (15.6%) experienced a minor bleeding complication. At multivariate analysis HPR by AA and HPR by ADP were independent predictors of death [HR 3.75 (1.20-11.66), P=0.023 and HR 4.78 (1.57-14.52), P=0.006, respectively]. Moreover, patients with dual HPR both by AA and by ADP showed a significantly higher risk of death than those without (P<0.001). The median value of LTA by ADP was significantly lower in patients with bleeding complications than in those without [26.5 (22-39.2)% vs 62 (44.5-74)%, P<0.001). At ROC curve analysis the cut-off of platelet aggregation induced by ADP with the best sensitivity and specificity for increased risk of bleeding was 41%. LTA by ADP lower than 41% was independently associated with bleeding [HR 14.59 (2.55-24.01), P=0.001] at multivariate analysis.

Conclusions: In PAD patients undergoing PTA, HPR by ADP and AA were predictors of death, whereas LPR by ADP was predictor of bleeding complications. These results suggest the potential utility of assessing platelet function, even in the setting of PAD, in order to ensure the patient the best tailored antiplatelet therapy.

INDEX

1. INTRODUCTION

Lower extremity peripheral artery disease (PAD) encompasses a wide range of vascular diseases, which are most commonly caused by atherosclerosis. Symptomatic lower extremity PAD arises from inadequate blood flow, with consequent oxygen supply and demand mismatch. Critical limb ischemia (CLI), defined as lower extremity pain that occurs at rest or in the presence of ulcers or gangrene, secondary to severe blood flow compromise, is the most severe form of lower extremity PAD. Acute limb ischemia refers to subset of CLI, defined as a sudden decrease in limb perfusion that threatens limb viability.

The burden of PAD is considerable. The prevalence of PAD, defined as an ankle–brachial index (ABI) of 0.90, in adults aged 40 years or older, is estimated at about 4-29%. The incidence of CLI has been reported to be much lower: only 2% of patients with symptomatic PAD would progress to CLI. Recent data, however, suggest that CLI may be more common than previously realized. The REACH (Reduction of Atherothrombosis for Continued Health) registry showed that among patients with symptomatic PAD, 15% will eventually have a lower limb amputation.[1]

Patients with PAD are at increased risk of myocardial infarction, stroke, and cardiovascular death: the incidence of coronary artery disease (CAD) and stroke may be as high as 68% and 42%, respectively, and the relative risk of cardiovascular death is increased almost 6-fold. The prognosis of patients with CLI is even more severe: 1 year after the onset of symptoms, 25% of these patients will be dead, and 25% will have a major amputation.[1]

2. ANTITHROMBOTIC THERAPY IN PERIPHERAL ARTERY DISEASE

2.1 ANTIPLATELET THERAPY

Given the markedly elevated cardiovascular risk in patients with PAD, antiplatelet therapy would be expected to be of great benefit in this clinical setting. However, there is very little evidence that antiplatelet therapy alters the natural history of PAD.

There is consensus among major society guidelines that antiplatelet therapy with aspirin is indicated for secondary prevention of cardiovascular events in patients with symptomatic PAD or PAD associated with coronary or cerebral atherosclerotic disease. The same guidelines, however, are inconsistent and often contradictory in regard to asymptomatic PAD, CLI, or the addition of clopidogrel.[2-8]

2.1.1 Aspirin

In a meta-analysis conducted by the Antithrombotic Trialists' Collaboration on 287 studies involving 135000 patients at high risk of vascular disease to evaluate the effect of antiplatelet therapy (primarily aspirin) upon the primary end point of vascular death, non-fatal myocardial infarction, and non-fatal stroke, a significant reduction in the primary end point (10.7% vs 13.2%, $P<0.0001$) was shown in the overall population. Among the subgroup of 9214 patients with PAD across 42 trials, there was a similar reduction in serious vascular events of 23% (5.8% vs 7.1%, $P=0.004$), with similar trends among patients with intermittent claudication, those who underwent peripheral angioplasty, and those who underwent peripheral grafting.[9] A subsequent meta-analysis by the ATC on 16 trials of aspirin for secondary prevention showed a significant reduction in vascular death, non-fatal myocardial infarction, and non-fatal

stroke (6.7% vs 8.2% per year, P<0.0001), but outcomes in the subgroup of patients with PAD were not reported.[10] A meta-analysis by Berger et al. specifically examined aspirin therapy (with or without dipyridamole) in 5269 patients with PAD. Although aspirin therapy was associated with a significant reduction in nonfatal stroke (relative risk [RR] 0.66, 95% confidence interval [CI] 0.47-0.94, P=0.02), there was no significant effect upon cardiovascular or all-cause mortality, myocardial infarction, or major bleeding. Results were similar for the subgroup of 3019 patients taking aspirin alone versus control.[11] In the AAA (Aspirin for Asymptomatic Atherosclerosis) trial, a double-blind, randomized controlled trial of aspirin 100 mg daily versus placebo in patients with no known cardiovascular disease and an ABI of 0.95, at a mean follow-up of 8.2 years, there was no difference in the primary end point of non-fatal coronary event, stroke, or revascularization, but there was a trend for increased bleeding in the aspirin group which was borderline significant (hazard ratio [HR] 1.71; 95% CI 0.99-2.97). Major limitations of this study are its relatively low-risk PAD population, as patients were required to be asymptomatic and without a clinical history of PAD for inclusion, and suboptimal compliance with aspirin, with study drug taken for only 60% of trial person-years.[12]

Although there remains some controversy regarding effective aspirin dose, there is no question that low-dose aspirin (lower than 100 mg) is effective for the prevention of vascular events in patients with cardiovascular disease.[9,13] Furthermore, the relatively recent The CURRENT-OASIS 7 (Clopidogrel and Aspirin Optimal Dose Usage to Reduce Recurrent Events–Seventh Organization to Assess Strategies in Ischemic Syndromes) randomized trial demonstrated no benefit of high-dose versus low-dose aspirin in patients with acute coronary syndromes (ACS), but showed a small increase in gastrointestinal bleeding with high-dose aspirin.[14] Data regarding high- versus low-dose aspirin in patients with PAD are more limited. The strategy of high-dose aspirin after peripheral vascular intervention to prevent restenosis or reocclusion was investigated in four randomized studies.[15-18] High-dose aspirin was given as 900 to

1000 mg (combined with dipyridamole in one trial); low-dose aspirin ranged from 50 to 300 mg daily. A subsequent Cochrane review of the four trials showed no effect of high-dose aspirin upon reocclusion within one month (odds ratio [OR] 1.45, 95% CI 0.63-3.36; P=0.38), and at 6 months after intervention (OR 0.99, 95% CI 0.71-1.38; P=0.96), with more common gastrointestinal side effects versus low-dose aspirin (OR 1.85, 95% CI 1.15-2.98; P=0.01).[19]

The efficacy of aspirin in patients with CLI remains to be proven, and available data suggest that aspirin does not decrease cardiovascular end points in these patients. There are multiple potential reasons for the lack of aspirin efficacy in patients with CLI, including underrepresentation in clinical trials, inefficient aspirin metabolism and so-called ''aspirin resistance'', and inappropriate dosing. However, aspirin is recommended by all major professional societies for secondary prevention of cardiovascular events in patients with symptomatic PAD, and, by extension, to patients with CLI.

2.1.2 Thienopyridines

The thienopyridines selectively inhibit adenosine diphosphate (ADP)-induced platelet aggregation by irreversibly blocking the P2Y12 ADP receptor on platelets surface.[20] All thienopyridines are prodrugs that require hepatic metabolism by the cytochrome P450 (CYP) enzymatic system to be active; prasugrel has a more rapid onset of action and produces greater and more predictable inhibition of ADP-induced platelet aggregation.[21]

In the CAPRIE (Clopidogrel versus Aspirin in Patients at Risk of Ischemic Events) trial, which compared aspirin 325 mg to clopidogrel 75 mg in 19185 patients with symptomatic atherosclerotic disease, including recent myocardial infarction, ischemic stroke, and symptomatic PAD, clopidogrel significantly reduced the combined primary end point of stroke, myocardial infarction, and vascular death in the overall population, with a RR reduction of 8.7% (95% CI 0.3%-16.5%, P=0.043). The subgroup of 6452

patients with PAD included in CAPRIE trial showed a more impressive RR reduction of 23.8% (95% CI 8.9%-36.2%, P=0.003), and similar adverse event rates compared to ischemic stroke and myocardial infarction subgroups. Gastrointestinal bleeding was marginally less frequent with clopidogrel versus aspirin (2.0% vs 2.7%, P=0.05).[22] The CHARISMA (Clopidogrel for High Atherothrombotic Risk and Ischemic Stabilization, Management, and Avoidance) trial was a randomized, double-blind trial comparing the effect of clopidogrel 75 mg daily versus placebo, in addition to aspirin 75 to 162 mg, on he primary end point of cardiovascular death, myocardial infarction, or stroke, in a total of 7802 high-risk patients with stable coronary artery disease. At 2 years, no benefit from clopidogrel in regard to the primary end point was found; a marginal benefit was observed in the subgroup of patients with clinically evident (ie, symptomatic) cardiovascular disease (RR 0.88; 95% CI 0.77-0.998; P=0.046).[23] In a subsequent post hoc analysis of 3096 patients with symptomatic and asymptomatic PAD enrolled in the CHARISMA trial, the effect on the primary end point of cardiovascular death, myocardial infarction, or stroke in the overall trial was higher in patients with PAD than in those without PAD (8.2% versus 6.8%, HR 1.25, 95% CI 1.08-1.44; P=0.002), but, among the subgroup of patients with PAD, there was no significant difference between clopidogrel and aspirin versus placebo and aspirin in regard to the primary end point (7.6% vs 8.9%, P=0.18). Dual antiplatelet therapy (DAPT), however, was associated with a lower rate of myocardial infarction (2.3% vs 3.7%, P=0.029) and hospitalization for ischemic events (16.5% vs 20.1%, P=0.011). There was no difference in severe or fatal bleeding between the two groups.[24] The COOPER (Clopidogrel for Atherothrombotic Event Management in Patients with Peripheral Arterial Disease) study, which investigated the safety profile (ie, bleeding, blood disorders, and hepatic dysfunction) of clopidogrel 75 mg daily compared to ticlopidine 200 mg daily in 431 patients with PAD, demonstrated that clopidogrel has a better safety profile than ticlopidine, with an adverse event rate of 2.4% versus 13.6%, respectively (adjusted HR 0.16; 95% CI 0.06-0.42; P< 0.0001).[25]

2.1.3 Dipyridamole

Dipyridamole inhibits the activity of adenosine deaminase and phosphodiesterase, which causes an accumulation of adenosine, adenine nucleotides, and cyclic adenosine monophosphate (AMP); these mediators then inhibit platelet aggregation, and stimulate release of prostacyclin or prostaglandin (PG) D2, causing coronary vasodilation.

The effect of dipyridamole plus aspirin has been studied in a number of randomized controlled trials for evaluation of graft patency in patients undergoing infrainguinal bypass surgery.[26-30] Data from six randomized controlled trials examining the effect of aspirin or aspirin plus dipyridamole versus placebo upon primary graft patency were analyzed in a Cochrane review, including a total of 966 patients, with 60% to 80% having CLI. In all trials, the study medication was started prior to bypass surgery, usually 48 hours before the intervention, and total daily doses ranged between 900 and 975 mg aspirin and 150 and 225 mg dipyridamole. Duration of treatment was 6 weeks in one trial but at least 12 to 24 months in the other trials. The results showed a statistically significant increase in infrainguinal primary graft patency for aspirin or aspirin plus dipyridamole at one year (OR 0.61, 95% CI 0.43-0.86; P=0.005), with a more profound impact upon prosthetic graft patency at one year (OR 0.22, 95% CI 0.12-0.38), and a marginal impact upon venous graft patency at 2 years (OR 0.71, 95% CI 0.51-1.00). Both major bleeding and gastrointestinal side effects were not more frequent in patients receiving aspirin or aspirin and dipyridamole.[31]

2.1.4 Prostanoids

Prostaglandins (PGE1, PGI2, and their derivatives) are potent inhibitors of platelet activation, adhesion, and aggregation, and have vasodilating and antithrombotic effects.

The currently available data support the use of prostanoids in patients unsuitable for lower limb revascularization or in patients in whom revascularization attempts have failed. In a meta-analysis of seven randomized, placebo-controlled trials including 643 patients, PGE1 therapy not only had significant beneficial effects over placebo on ulcer healing and pain relief (47.8% vs 25.2%; P=0.029), but also increased the rate of surviving with both legs after 6 months follow-up (22.6% vs 36.2%; P=0.015) in patients with Fontaine PAD stage III (rest pain) or IV (ischemic ulcers or gangrene).[32] A subsequent Cochrane review on thirteen trials comparing prostanoids to placebo for CLI, prostanoids appeared to be effective for relief of rest pain (RR 1.32, 95% CI 1.10-1.57) and ulcer healing (RR 1.54, 95% CI 1.22-1.96), but had no significant impact upon either amputation or mortality. In addition, there was a significant increase in side effects like headache, nausea, vomiting, and diarrhea (RR 2.35, 95% CI 1.99-2.78).[33] Overall, the evidence base for prostanoids is poor, and better quality studies with longer periods of follow-up are therefore required.

2.1.5 Cilostazol

Cilostazol selectively inhibits phosphodiesterase III, and therefore decreases the degradation of cyclic AMP, resulting in reversible inhibition of platelet activation and aggregation. It also improves endothelial cell function, decreases endothelial smooth muscle proliferation, and promotes vasodilation.[34-36]

In symptomatic patients with PAD, cilostazol has been shown to improve maximal walking distance and overall physical function. Pooled data from seven randomized controlled trials, comparing cilostazol with placebo to determine the effect of cilostazol on improving walking distance and in reducing vascular mortality and cardiovascular events in patients with stable intermittent claudication, showed that the weighted mean difference for the initial claudication distance was improved following treatment with cilostazol 100 mg twice daily (31.1 m; 95% CI 21.3-40.9 m) compared to placebo. In addition, the absolute claudication distance was improved in patients

receiving cilostazol 100 mg twice daily (49.7 m; 95% CI 24.2-75.2 m) and 50 mg twice daily (31.9 m; 95% CI 12.4-51.5 m) compared to placebo. Cilostazol 150 mg twice daily did not significantly improve either the initial or absolute claudication distance. No impact upon cardiovascular events or mortality was observed in patients receiving cilostazol compared with placebo.[37] In a multicenter, double-blind, randomized, placebo-controlled parallel study, a total of 394 patients of 40 years of age or older, with chronic, stable, symptomatic intermittent claudication received cilostazol 100 mg twice daily, 50 mg twice daily, or placebo for 24 weeks: only patients receiving cilostazol 100 mg twice daily experienced a significant improvement in maximal walking distance (P=0.0003), and in distance walked to the onset of symptoms (P=0.0015) compared with placebo. Quality of life and functional status assessments supported these objective results.[38] In a multicenter, prospective registry of 861 patients who underwent superficial femoral artery stenting with a self-expanding nitinol stent, the effect of cilostazol 200 mg daily on restenosis, reocclusion, all-cause mortality, and limb salvage in patients with CLI was investigated. Patients in both the study group and comparison group received DAPT (aspirin 100 mg and clopidogrel 75 mg daily) before the procedure; after the procedure, all patients were prescribed lifelong aspirin (100-200 mg daily), and at least one month of clopidogrel. Patients who had taken cilostazol before the procedure continued to receive cilostazol after the procedure: at 5 years, the binary restenosis rate was significantly lower in these patients (31.2% vs 42.9%; P=0.02), whereas in-stent reocclusion tended to be lower, but without reaching statistical significance (10.8% vs 18.2% at 5 years; P=0.09). No significant difference was found between the two groups in terms of all-cause mortality and limb salvage rate.[39] In a small prospective trial involving 20 lower limbs of 14 patients, the effect of one month of cilostazol 100 mg twice daily (or 50 mg twice daily in patients in case of side effects), in patients with Rutherford class 3 or 4 symptoms and whose skin perfusion pressure was less than 40 mmHg was studied:

after one month, cilostazol did not increase the ABI, but it significantly increased the skin perfusion pressure (24.5+8.9 to 42.8+21.0 mmHg; P<0.01).[40]

2.1.6 Novel antiplatelet agents

The role that inadequate platelet inhibition might play in the progression of PAD and CLI remains unknown. It is a reasonable hypothesis, however, that more potent platelet inhibition with novel agents (such as prasugrel, ticagrelor, cangrelor, elinogrel, atopaxar and vorapaxar) would improve overall cardiovascular, procedure-specific, and limb salvage outcomes in patients with PAD. Data on these novel drugs come mainly from the cardiology setting. Limitations, side effects and effectiveness of each of these agents are studied, but data on their use in PAD are limited.

- **Ticagrelor**

Ticagrelor is an orally active, direct-acting, and reversible inhibitor of P2Y12. The drug is given twice daily, it has a more rapid onset and offset of action, and produces greater and more predictable inhibition of ADP-induced platelet aggregation than clopidogrel.

The PLATO (Platelet Inhibition and Patient Outcomes) trial was a double-blind, randomized trial comparing ticagrelor to clopidogrel in 18624 patients with ACS: at 12 months, the primary combined end point of vascular death, myocardial infarction, or stroke occurred in 9.8% of the ticagrelor group versus 11.7% of the clopidogrel group (HR 0.84, 95% CI 0.77-0.92; P<0.001).[41] In a post hoc analysis of 1144 patients with PAD from the PLATO study, the primary end point occurred in 19.8% of patients with PAD versus 10.2% of patients without PAD (P<0.001). Among patients with PAD, those treated with ticagrelor had lower rates of vascular death or myocardial infarction than those treated with clopidogrel (16.7% versus 21.5%; P=0.045). Bleeding rates were similar in patients with PAD treated with ticagrelor or clopidogrel.[42]

Recently, the results of the EUCLID (Examining Use of Ticagrelor in Peripheral Artery

Disease) study, a randomized, double-blind, multicenter trial comparing ticagrelor with clopidogrel in regard to the risk of cardiovascular death, myocardial infarction, and ischemic stroke in patients with established PAD, were published. In this trial, 13885 patients with symptomatic PAD (with ABI of 0.80 or less or who had undergone previous revascularization of the lower limbs) were randomly assigned to receive monotherapy with ticagrelor (90 mg twice daily) or clopidogrel (75 mg once daily). At a median follow-up of 30 months, the primary efficacy end point (a composite of cardiovascular death, myocardial infarction, or ischemic stroke) occurred in 751 of 6930 patients (10.8%) receiving ticagrelor and in 740 of 6955 (10.6%) receiving clopidogrel (HR 1.02, 95% CI 0.92-1.13; P=0.65). In each group, acute limb ischemia occurred in 1.7% of the patients (HR 1.03, 95% CI 0.79-1.33; P=0.85), and major bleeding in 1.6% (hazard ratio, 1.10; 95% CI, 0.84 to 1.43; P=0.49). Therefore, in patients with symptomatic PAD, ticagrelor was not shown to be superior to clopidogrel for the reduction of cardiovascular events, and major bleeding occurred at similar rates among patients in the two trial groups.[43]

- **Vorapaxar**

Vorapaxar is an oral, reversible antagonist of the protease-activated receptor 1 (PAR-1), which is the primary receptor for thrombin on human platelets, and it is also present on vascular endothelium and smooth muscle. Vorapaxar competitively and selectively interferes with the interaction of PAR-1 and thrombin, thereby inhibiting thrombin-induced platelet activation. It does not interfere with thrombin-mediated cleavage of fibrinogen, thus not affecting the coagulation cascade.[44,45]

The TRA2P-TIMI 50 (Preventing Heart Attack and Stroke in Patients with Atherosclerosis) trial evaluated the efficacy and safety of vorapaxar 2.5 mg versus placebo for secondary prevention in 26449 patients with a history of prior myocardial infarction, ischemic stroke, or PAD. At 3 years, the primary end point of cardiovascular death, myocardial infarction, or stroke occurred in 9.3% of patients in the vorapaxar

group compared to 10.5% in the placebo group (HR 0.87, 95% CI 0.80-0.94; P<0.001). Moderate or severe bleeding, and intracranial hemorrhage were more common with vorapaxar (4.2% versus 2.5%, and 1.0% vs 0.5%, respectively; P<0.001), with the latter especially pronounced among patients with a history of stroke.[45] In contrast to the overall study population, vorapaxar did not significantly reduce the primary end point in the PAD subgroup of 3787 patients with a history of intermittent claudication and either an ABI <0.85 or previous revascularization for limb ischemia (11.3% vs 11.9%; P=0.53). However, vorapaxar significantly reduced the risk of hospitalization for acute limb ischemia (2.3% vs 3.9%; HR 0.58, 95% CI 0.39-0.86; P=0.006) and peripheral revascularization (18.4% vs 22.2%; HR 0.84, 95% CI 0.73-0.97; P=0.017). Moderate or severe bleeding was more common with vorapaxar (7.4% vs 4.5%; P<0.001), although there was no difference in the rates of intracranial or fatal hemorrhages.[45]

2.1.7 Antiplatelet therapy after peripheral endovascular intervention

The effect of antiplatelet therapy on patency after endovascular revascularization was investigated in two randomized trials. In a small study, Heiss et al. randomized 199 patients after femoropopliteal balloon angioplasty to placebo, dipyridamole 225 mg and aspirin 990 mg, or dipyridamole 225 mg and aspirin 300 mg. Evaluation of the combined angiographic and clinical results showed improvement or no deterioration in 37% of patients in the placebo group compared with 49% in the low-dose and 61% in the high-dose aspirin groups, respectively, with the only statistically significant difference between the placebo group and patients treated with dipyridamole and high-dose aspirin (P=0.01).[46] In a similar study after femoropopliteal or iliac angioplasty, 223 patients were randomized to placebo versus aspirin 50 mg and dipyridamole 400 mg: no differences were found in primary patency between the groups, although the study was limited by the low aspirin dose and a higher number of iliac angioplasties in the placebo group.[47] In the PARADISE (Preventing Amputations using Drug Eluting

Stents) trial, in which 106 patients with CLI were treated with tibioperoneal drug-eluting stents (DESs), and 90% of patients were maintained on DAPT (clopidogrel 75 mg and aspirin 81 mg daily) and close to 75% on statin therapy, the reported mortality rates were 13% at one year and 29% at 3 years, despite intensive medical therapy with DAPT and statins.[48]

Although DAPT with aspirin and clopidogrel is generally recommended after peripheral vascular intervention, this is largely based upon extrapolation of coronary percutaneous intervention data. Indeed, there are no dedicated studies that have shown convincing benefit of DAPT following peripheral vascular intervention. Nevertheless, recent studies comparing drug-eluting stenting to balloon angioplasty or bare-metal stenting for symptomatic PAD have shown impressive benefit. In the randomized Zilver PTX trial of 479 patients, sirolimus-eluting stenting for femoropopliteal disease proved superior to balloon angioplasty, with higher rates of event-free survival at one year (90.4% vs 82.6%; P=0.004). In both arms patients were treated for a minimum of 60 days of aspirin and clopidogrel.[49] Similarly, Rastan et al randomized 161 patients with infrapopliteal PAD to sirolimus-eluting versus bare-metal stenting. Event-free survival was higher with sirolimus-eluting stenting (65.8% vs 44.6%; P=0.02), in addition to an improvement in Rutherford class and a reduction in target limb amputation. Both arms received aspirin and clopidogrel for at least 6 months.[50]

2.1.8 Antiplatelet therapy after surgical bypass

The CASPAR (Clopidogrel and Acetylsalicylic Acid in Bypass Surgery for Peripheral Arterial Disease) trial investigated the effect of DAPT (clopidogrel 75 mg daily plus aspirin 75 to 100 mg daily) versus placebo plus aspirin 75 to 100 mg daily in patients undergoing unilateral, below-knee bypass grafting for atherosclerotic PAD. Study medications were started 2 to 4 days after surgery and continued for 6 to 24 months. The primary end point was a composite of graft occlusion or revascularization, above-

ankle amputation of the affected limb, or death. The primary safety end point was severe bleeding. The trial showed that the combination of clopidogrel plus aspirin did not improve limb or systemic outcomes in the overall population of patients with PAD requiring below-knee bypass grafting, but improved outcomes without a significant increase in major bleeding risk were observed in the subgroup of patients receiving prosthetic grafts (HR 0.65, 95% CI 0.45-0.95; P=0.025).[51] Similarly, a Cochrane review showed a benefit of aspirin or aspirin plus dipyridamole versus placebo in regard to prosthetic graft patency, with only a borderline benefit in regard to venous grafts.[23]

In patients with CLI, although DAPT with aspirin and clopidogrel is frequently used after endovascular or surgical revascularization, there is poor evidence for the efficacy of this strategy. Burdess et al. demonstrated that in patients with CLI undergoing surgery (infrainguinal revascularization or amputation), the use of perioperative DAPT with aspirin 75 mg and clopidogrel 75 mg decreased biomarkers of atherothrombosis compared to aspirin and placebo, without increasing major life-threatening or minor bleeding, although blood transfusions were increased.[52] The effect of statins, b-blockers, and aspirin upon survival, graft patency, and major adverse cardiovascular events was investigated in 1404 patients with CLI treated with infrainguinal vein bypass in a post hoc analysis of the PREVENT (Project or Ex-Vivo Vein Graft Engineering Via Transfection) III trial. Statin use was associated with a significant survival advantage at one year of 86% versus 81% (HR 0.71, 95% CI 0.52-0.98; P=0.03), but there was no difference with aspirin or b-blockers. None of the medications was associated with improved graft patency.[53]

2.2 ANTICOAGULANT THERAPY

The utility of an oral anticoagulant therapy in patients with PAD remains unclear. This was previously studied in the WAVE (Warfarin Antiplatelet Vascular Evaluation) trial, which compared warfarin and aspirin versus aspirin alone in patients with symptomatic PAD. There was no benefit to warfarin in regards to cardiovascular death, myocardial infarction, and stroke; however, a dramatic increase in life-threatening bleeding (4.0% vs 1.2%; $P<0.001$) was observed.[54]

The question has been readdressed in the recently published COMPASS (Rivaroxaban for the Prevention of Major Cardiovascular Events in Coronary or Peripheral Artery Disease) study, a double-blind superiority trial comparing rivaroxaban 2.5 mg twice daily combined with aspirin 100 mg once daily or rivaroxaban 5 mg twice daily vs aspirin 100 mg once daily for prevention of myocardial infarction, stroke, or cardiovascular death in patients with stable CAD or PAD. This trial demonstrated that, among patients with stable atherosclerotic vascular disease, those assigned to rivaroxaban (2.5 mg twice daily) plus aspirin had better cardiovascular outcomes and more major bleeding events than those assigned to aspirin alone, whereas rivaroxaban (5 mg twice daily) alone did not result in better cardiovascular outcomes than aspirin alone and resulted in more major bleeding events. The effects of rivaroxaban plus aspirin as compared with aspirin alone on the primary outcome and on major bleeding were consistent among subgroups of participants who met the eligibility criteria for CAD and in those who met the eligibility criteria for PAD.[55]

3. ON-TREATMENT PLATELET REACTIVITY

3.1 PLATELET ACTIVATION AND INHIBITION PATHWAYS

Platelet activation is a key process in both protective hemostasis and pathological thrombosis. Platelets adhere to the damaged walls of blood vessels at sites of endothelial cell activation, contributing to the development of chronic atherosclerotic plaques, and triggering the acute onset of arterial thrombosis in response to atherosclerotic plaque rupture.[56]

Platelet activation occurs via multiple pathways by the binding of several agonists, such as thromboxane A2 (TxA2), ADP, and thrombin, to their receptors. Platelets adhesion to subendothelium is mediated by direct interaction between the glycoprotein (GP)Ib/V/IX receptor complex on the platelet surface and the von Willebrand factor (vWF). Moreover, the interaction between exposed subendothelial collagen with platelet receptors GPVI and GPIa stimulates the release of platelet agonists ADP and TxA2 from the adherent platelets, as well as activation of GPIIb/IIIa receptor with high affinity for fibrinogen that mediates stable adhesion of platelets to the vessel wall, platelet-platelet cross-linking, and contact dependent signaling within platelet aggregates. Release of ADP and TxA2 promotes the recruitment of circulating platelets into the growing stable hemostatic plug. Thrombin-mediated cleavage of fibrinogen into fibrin further contributes to the formation of hemostatic plugs. ADP and TxA2 activate platelets by binding to specific receptors on the platelet [P2Y1 and P2Y12 receptors for ADP, and PGG2 and PGH2 endoperoxide receptors for TxA2]. These bindings result in reduced intracellular cyclic AMP levels and full activation of GPIIb/IIIa. ADP and TxA2 can also potentiate platelet activation induced by other ligands. Thrombin activates platelets primarily by binding PAR-1 on the platelet surface, cleaving the receptor, and exposing a tethered ligand, which binds and

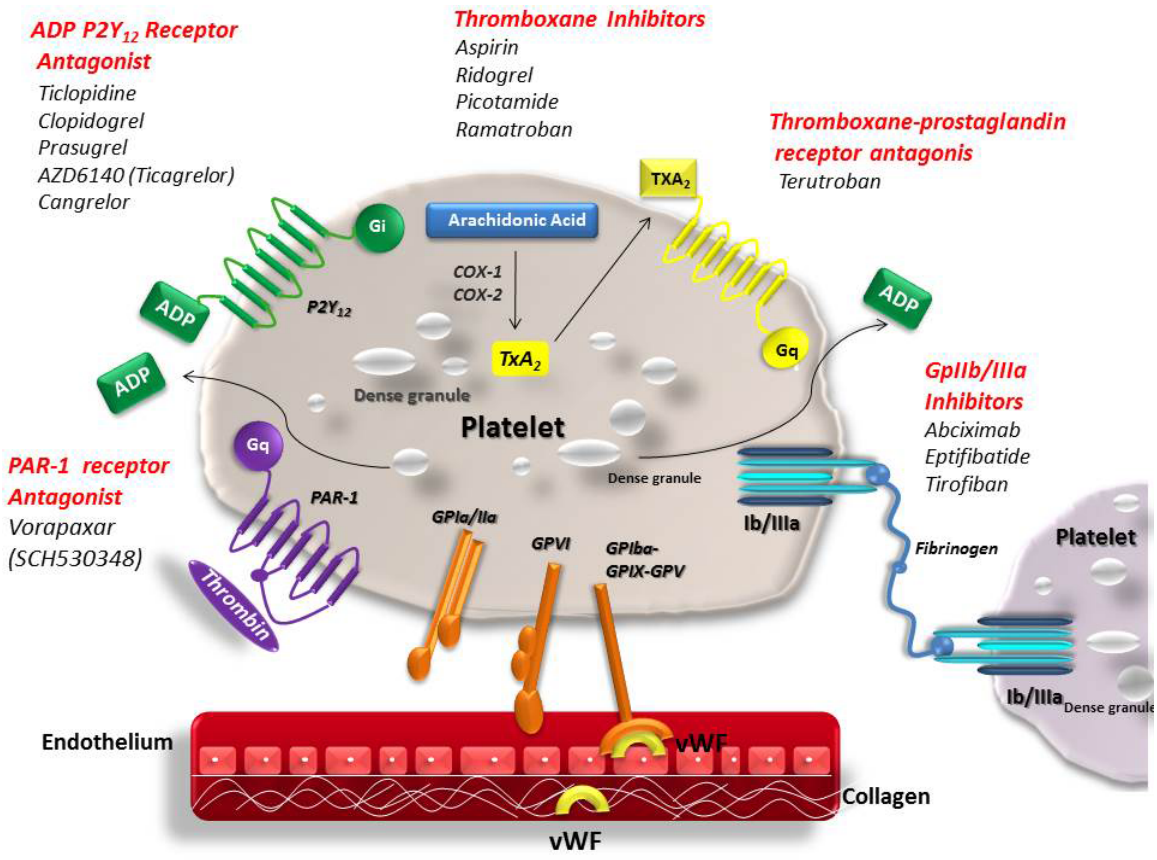
activates the receptor. Thrombin is the most potent platelet agonist, as it can stimulate platelet activation via the PAR-1 at very low concentrations, that are several orders of magnitude lower than those required for the activation of the coagulation cascade. Human platelets also express a secondary receptor for thrombin, PAR-4, which requires higher concentrations of thrombin for activation. Other factors, such as epinephrine, PGE2, serotonin, and several chemokines, play a role in platelet activation, mainly potentiating platelet activation induced by other stimuli. On the other hand, ligand-stimulated activation of platelets is inhibited by a number of endothelial-derived factors, nitric oxide and prostacyclin, that in physiological conditions, prevent uncontrolled platelet aggregation, and increase in the intracellular levels of cyclic nucleotides. In addition, the nucleoside adenosine, released as a result of cell damage or by endothelial ectonucleotidase CD39-mediated conversion of ADP, also inhibits platelet activation via Gs-coupled adenosine A2A receptor. Therefore, the perpetuation phase of thrombus formation is mediated by the cell-to-cell contact-dependent mechanisms that lead to changes in platelet morphology, expression of pro-coagulant and pro-inflammatory molecules, and platelet aggregation.[56]

Given that the activation of multiple platelet pathways, in particular the TxA2 and ADP ones, is the primary mechanism of thrombosis and ischemic events, their comprehensive inhibition has represented an attractive therapeutic approach for the treatment of atherothrombotic diseases (Figure 1). On the other hand, the potential clinical benefits of targeting various platelet activation pathways should be carefully weighed against the likelihood of increased bleeding, as both the TxA2 and ADP platelet activation pathways are also required for hemostasis.[57]

In the last decades, oral antiplatelet agents inhibiting platelet activation by targeting ciclooxigenase 1 (COX-1) inhibition of TxA2 formation [acetylsalicylic acid (ASA) or aspirin] or ADP-induced P2Y12 receptor pathway (ADP P2Y12 receptor inhibitors, such as clopidogrel, ticlopidine, prasugrel, and ticagrelor) have been widely studied and

used in clinical practice, demonstrating to significantly reduce the incidence of ischemic events in patients with atherothrombotic diseases (Figure 1).[57]

Figure 1. Pathways of platelet inhibition.



3.2 PLATELET FUNCTION ASSESSMENT

Platelet function may be reliably detected with a wide spectrum of laboratory tests (table 1).

Tests evaluating platelet aggregation are based on the use of different agonists, first of all ADP, the target of thienopyridines (ticlopidine, clopidogrel, prasugrel) and arachidonic acid (AA), which is predominantly affected by ASA.

The gold standard method for studying platelet aggregation is represented by light transmittance aggregometry (LTA) on platelet-rich plasma (PRP), developed by Born in the 1960s. This test assesses in vitro the platelet-to-platelet clump formation in a GP IIb/IIIa-dependent manner, ie. the aggregation, the most important function of platelets. The assay is based on the measurement of the increase in light transmission through the optically dense sample of PRP after the addition of exogenous platelet agonists. During the assay, the PRP after the addition of agonist becomes clearer because of the precipitation of platelet aggregates. This determines an increase in light transmission through the plasma sample. The device records the rate and maximal percentage of this increase from 0% (maximal optical density of PRP) to 100% (no optical density of autologous platelet-poor plasma) by a photometer. This signal is converted automatically in a graphic curve that parallels the increase in light transmission during the platelet aggregation. Different agonists can be added to the PRP sample to stimulate different platelet activation pathways, obtaining information about the several features of platelet function.

Since the late 1980s, other methods, such as platelet aggregometry in whole blood, the study of activated platelets *ex vivo* by flow cytometry, the measurement of specific compounds released by platelets, and the assessment of platelet nucleotides have become available. Moreover, the development of new, simpler instruments for assessing platelet function at the point-of-care (POC) or bedside has led to better

prospects of using these tests not only in specialized clinical or research laboratories, but also in general laboratories and in different clinical settings.[58]

Table 1: Platelet function tests.

	Method principle
Tests based on platelet aggregation	
Light transmission aggregometry (LTA)	Photo-optical measurement of light trasmission increase in response to agonist-induced platelet aggregation
Impedance platelet aggregation	Measurement of electrical impedance increase in relation to agonist-induced platelet aggregation
VerifyNow system	Measurement of whole blood aggregation in response to agonist
Lumiaggregometry	Aggregometry combined with luminescence
Plateletworks	Platelet counting pre and post activation in whole blood
Tests based on platelet adhesion under shear stress	
Platelet Function Analyzer (PFA)-100/ Innovance PFA-200	Time evaluation for the formation of a platelet plug into a hole in activated surface under shear whole blood flow
Impact Cone	Shear-induced platelet adhesion-aggregation
Global Thrombosis test (GTT)	Time cessation of whole blood flow by high shear dependent platelet plug formation
Platelet function and viscoelastic test	
Thromboelastography (TEG)	Rate of clot formation based on low shear induced and agonist addition
Rotational thromboelastometry (ROTEM) platelet	Measurement of electrical impedance increase in response to agonist addition
Flow citometry	Engineering laser based detection of suspending fluorescent label platelets in a flowing solution
Evaluation of thromboxane metabolites	Measurement of TxA2 metabolites by radio or enzyme-linked immune assay

3.3 HIGH ON-TREATMENT PLATELET REACTIVITY

3.3.1 High on-clopidogrel platelet reactivity

Studies measuring platelet function in patients administered clopidogrel revealed that, unlike GP IIb/IIIa receptor blocker therapies that are associated with a uniform and high level of inhibition of their targets with appropriate dosing, clopidogrel treatment is associated with an overall variable and modest level of P2Y12 inhibition even when high loading doses are used. In addition to distinct response variability, a substantial percentage of patients will also exhibit complete nonresponsiveness (resistance) to clopidogrel.[59,60]

In recent years, data have been accumulating in the literature on the prognostic role of high platelet reactivity (HPR) on-clopidogrel treatment (HcPR) as a marker of vascular risk.[61-64]

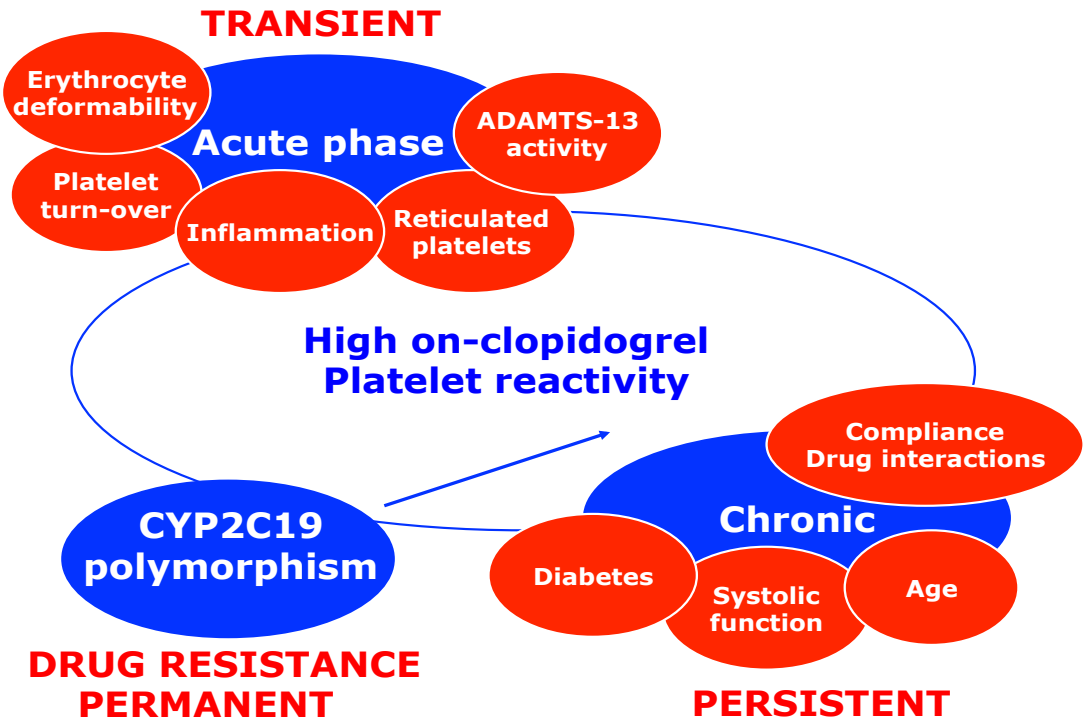
The majority of evidence in the field of HcPR refers to a very specific clinical setting: the acute phase of patients with ACS undergoing percutaneous coronary intervention (PCI) with stent implantation. A relevant number of studies showed that, in this clinical context, the presence of ADP-induced HPR is associated with a significantly increased risk of ischemic events, in particular of stent thrombosis, cardiac death and myocardial infarction, both at early and late follow-up.[65-72] In a prospective, observational, single-center cohort study, on 1789 consecutive patients with ACS undergoing an invasive procedure, platelet reactivity after clopidogrel loading was prospectively assessed, and anti-thrombotic treatment was adjusted according to the results of platelet function tests.[65] Patients with HcPR received an increased dose of clopidogrel or switched to ticlopidine. The results showed that HcPR remains high in 38% of patients after treatment adjustment with first-generation thienopyridines, and that HPR after clopidogrel loading is associated with increased risk of 2-year follow-up ischemic events, including stent thrombosis. [65] Sofi and coworkers, in a meta-analysis on the clinical consequences of clopidogrel non-responsiveness among over 4500 coronary

artery disease patients who underwent PCI, and followed for a period ranging from 2 weeks to one year, showed that poor responders to clopidogrel are at increased risk of cardiovascular clinical recurrences compared to those with a good response [OR 5.67, 95% CI 2.97-10.84; p<0.00001].[61]

HcPR: genetic and acquired determinants

HcPR can be due to multiple mechanisms, and both genetic and acquired determinants can be identified (figure 2).

Figure 2. High on-clopidogrel platelet reactivity: genetic and acquired determinants.



Although several genetic variables have been implicated, the strongest predictor of HcPR is the loss-of-function genetic variant in one of the isoforms of the cytochrome (CYP) P450, the CYP2C19*2 polymorphism.[73] Clopidogrel, a P2Y12 ADP-receptor antagonist, belongs to the thienopyridine class of chemical compounds and is administered as prodrug. Clopidogrel is absorbed in the duodenum and metabolized in the liver, by two consecutive steps of oxidation, to the active thiol metabolite. Each oxidation steps is catalyzed by members of the CYP P450 system; then the active metabolite irreversibly binds to the P2Y12 ADP platelet receptor. The CYP2C19 enzyme isoform is consistently involved in both the two oxidation steps, and therefore its non-defective activity is essential for the clopidogrel bioavailability and clinical efficacy. In 2009, Shouldiner et al. conducted a genome wide association study (GWAS), demonstrating that a major locus on chromosome 10q24 was associated with reduction in response to clopidogrel. This locus comprised the CYP2C18- CYP2C19-CYP2C9-CYP2C8 gene cluster. The follow-up genotyping indicated that CYP2C19*2 variant accounts for most of the association signal detected in the initial GWAS.[74] After the first demonstrations that the *2 loss-of-function allele was associated with a significant reduction of the response to clopidogrel in healthy subjects, and that the *2 allele was associated with higher platelet aggregability in patients undergoing PCI and stent implantation on DAPT, a large number of studies confirmed these findings.[73,75] Moreover, the CYP2C19*2 was consistently associated with major adverse cardiovascular events (MACE), and, in particular, with stent thrombosis in high risk vascular patients.[76-78] Different large meta-analyses were conducted, and showed that a significantly increased risk of MACE was evident in both carriers of one (HR=1.55) and 2 (HR=1.76) loss-of-function alleles, as compared with non-carriers.[79-81] The association was higher for stent thrombosis (HR=2.67 and HR=3.97 for one allele and 2 alleles, respectively).[81] Moreover, the CYP2C19 enzyme is responsible for the metabolism and clearance of numerous drug substrates, including proton pump

inhibitors, diazepam, phenytoin, and cyclophosphamide. Therefore, possible drug-drug interactions should be considered in complex patients assuming multiple medications.

CYP2C19*2 genetic polymorphism, however, does not fully explain the association between HPR and adverse events on clopidogrel: even within a population which is not carrying the CYP2C19*2 polymorphism, and which is therefore able to metabolize clopidogrel, the extent of ADP-induced inhibition of platelet function remains a risk marker of adverse events. These findings could be explained by the existence of other genetic determinants, even unknown, or by the presence of other 'non-genetic' determinants of HcPR.

Among genetic determinants, another widely studied polymorphism seems to influence the responsiveness to clopidogrel and patients' outcome: the C3435T in the gene ATP-binding cassette, subfamily B (MDR/TAP), member 1 (ABCB1), encoding the P-glycoprotein involved in clopidogrel intestinal absorption. For this polymorphisms, however, extremely conflicting data are available.[73]

The failure in clopidogrel response may be also due to multiple chronic conditions. Chronic mechanisms include inadequate drug compliance, drug–drug interactions, age, diabetes mellitus, elevated body mass index, female sex and reduced left ventricular ejection fraction (Figure 2). All these mechanisms could interact with the genetic predisposition in determining inadequate response to clopidogrel and consequently the increased risk of occurrence of MACE in high risk vascular patients.

A particularly important aspect of the possible existence of acquired determinants of HcPR is the role of 'acute phase': it was shown that inflammation and increased platelet turnover, that characterize the acute phase of ACS, are associated with an increased platelet reactivity and with a higher risk of HcPR.[82] Transient mechanisms include inflammation, accelerated platelet turnover, reticulated platelets, erythrocyte deformability, and the activity of ADAMTS13 (Figure 2).[83] Indeed, it has been demonstrated that the percentage of subjects with HcPR decreases significantly after one and 6 months from the acute event.[84] An indirect confirmation of this finding

derives from the outcome of CURRENT-OASIS 7 study, where, in the population of patients undergoing PCI, subjects randomized to receive a higher dose of clopidogrel (150 mg/day) in the first week of treatment experienced a significantly lower number of ischemic events, demonstrating a beneficial effect associated with an increased platelet inhibition in the acute phase of the disease.[14] Moreover, Martin et al. noted that platelets of patients with ACS had an increased mean volume, probably due to the presence of reticulated platelets, newly formed platelets with a higher granule content, and that this finding is a independent predictor of recurrent myocardial infarction and cardiac death.[85] Indeed, it has been demonstrated that a significantly higher percentage of reticulated platelets is present in patients with HcPR compared with those without, suggesting that the presence of these immature and more active platelets could be another mechanism involved in the variable response to antiplatelet therapy.[82]

Finally, a new mechanism of HcPR associated with post-receptor determinants has been recently proposed.[86,87] Intracellular signal transduction after P2Y12 receptor stimulation is mediated via Gi-protein linked suppression of adenylate cyclase. In turn, decreased adenylate cyclase activity results in diminished cyclic AMP formation and diminished phosphorylation of vasodilator-stimulated phosphoprotein (VASP).[88] VASP phosphorylation is also modulated by cGMP, a product of soluble guanylate cyclase upon activation by nitric oxide (NO).[89,90] Therefore, the effects of ADP binding to the P2Y12 receptor also reflect an attenuation of the physiological antiplatelet actions of adenylate cyclase activators. These mechanisms are potentially relevant as they might be present not only with the 'old molecule' clopidogrel, but also with the new and more potent agents, such as prasugrel and ticagrelor, and potentially related also to aspirin administration.

HcPR: which therapy?

Following the demonstration of a link between HPR in patients undergoing PCI and thrombotic/ischemic events, several studies have aimed to lower the level of platelet reactivity by modifying therapy.

The first attempt was to intensify clopidogrel therapy by enhancing the dosage. *Ex vivo* studies demonstrated that platelet aggregation in the acute phase could be better suppressed with 900 and 600 mg loading doses of clopidogrel than with the conventional dose (300 mg), even if the absorption of the drug may be saturated with the 600 mg dose.[91] The hypothesis that a high loading dose of clopidogrel may be more effective than a conventional dose was first demonstrated in the ARMYDA-2 (Antiplatelet Therapy for Reduction of Myocardial Damage During Angioplasty) trial: 255 stable CAD patients undergoing PCI and pretreated with 600 mg clopidogrel experienced a lower incidence of peri-procedural myocardial infarction with respect to those pretreated with 300 mg.[92] The CURRENT-OASIS 7 trial assessed whether doubling of the loading and maintenance dose of clopidogrel for 7 days was better than the standard dose, and whether high-dose aspirin was better than low-dose aspirin in patients undergoing PCI.[14] A total of 25086 patients with ACS were randomly assigned to a 600 mg loading dose of clopidogrel followed by 150 mg/day for 6 days and 75 mg/day thereafter or to conventional dosing with a 300 mg loading dose followed by 75 mg/day. The primary end point was cardiovascular death, myocardial infarction or stroke at 30 days. The trial failed to demonstrate a higher efficacy of the increased clopidogrel dosage in reducing ischemic events. However, in the 17232 patients who actually underwent PCI, double-dose clopidogrel reduced the rate of adverse events (P=0.039) at the expense of increased, albeit nonfatal, bleeding, while high-dose and low-dose aspirin did not differ for the primary outcome (P=0.76). *Ex vivo* studies documented that a clopidogrel dosage from 225 mg to 300 mg daily would be necessary to obtain an efficient platelet inhibition in carriers of CYP2C19*2 in heterozygous or homozygous form, respectively.[14]

Thereafter, a number of randomized trials evaluated different therapeutic strategies in HcPR: some trials had the level of platelet inhibition as end-point, and others included clinical end-points such as death, stent thrombosis and MACE.[93-115] Among the latter, GRAVITAS (Gauging Responsiveness With A VerifyNow P2Y12 Assay: Impact on Thrombosis and Safety)[94] and ARCTIC[98] trials enrolled the highest number of patients. Both trials did not demonstrate a reduced incidence of ischemic events in patients with HPR treated with a higher dose of clopidogrel and/or different drugs. Based on these results, many authors consider the issue of personalized antiplatelet therapy to be a dead-end street. Neither of these trials, however, were correctly designed to answer to the question of whether laboratory monitoring may help clinicians to choose the right antiplatelet therapy in ACS patients. The patients enrolled significantly differ from those in whom HPR was found to be associated with ischemic risk. Indeed, until now, a significant association between the entity of platelet inhibition and ischemic risk was found only in ACS patients, and not in stable CAD patients. In addition, both trials did not efficiently overcome HcPR. In the GRAVITAS trial, a strategy based on the double dosage of clopidogrel was used (150 mg/day) and, in approximately 40% of patients, HPR persisted after the introduction of the increased dosage.[94] In the ARCTIC trial, only 11% of patients were treated with an alternative antiplatelet drug, such as prasugrel, and, again, the majority of patients were treated with an increased dosage of clopidogrel or with an additional use of anti-IIb/IIIa inhibitors.[98] Finally, it was calculated that the number of patients enrolled in the ARCTIC trial was insufficient to adequately answer the question of whether a strategy based on laboratory monitoring is better than a standard strategy.[114] Recently, in the ANTARCTIC trial, a multicentre, open-label, blinded-endpoint, randomized controlled superiority study, 877 patients aged 75 years or older who had undergone coronary stenting for ACS were randomly assigned to receive oral prasugrel 5 mg daily with dose or drug adjustment in case of inadequate response (monitoring group) or oral prasugrel 5 mg daily with no monitoring or treatment adjustment (conventional group). No differences were

observed in the occurrence of the primary endpoint [a composite of cardiovascular death, myocardial infarction, stroke, stent thrombosis, urgent revascularisation, and Bleeding Academic Research Consortium-defined bleeding complications (types 2, 3, or 5) at 12 months' follow-up] between the monitoring and the conventional group, so suggesting that platelet function monitoring with treatment adjustment did not improve the clinical outcome of elderly patients undergoing coronary stenting for ACS.[115]

Moving from these negative, randomized trials to data from the real world, different groups documented that an antiplatelet therapy tailored on the basis of the results of platelet function testing is associated with a significantly lower risk of ischemic events, without a significantly higher bleeding risk. Notably, in all these registries published from Germany, Austria, and Italy, in patients treated with a tailored antiplatelet therapy, the incidence of ischemic events in HcPR patients was reduced to the same level of patients without HcPR.[116-126] In a meta-analysis by Aradi, ten clinical trials, that reported the clinical impact of using an intensified antiplatelet protocol on the basis of ADP-specific platelet reactivity testing, and comprising 4213 patients, were included. Compared with standard antiplatelet therapy, the intensified protocol was associated with a significant reduction in cardiovascular mortality, stent thrombosis and myocardial infarction. There was no difference in the rate of major bleeding events between intensified and standard groups. A meta-regression analysis revealed that the net clinical benefit of the intensified treatment significantly depended on the risk of stent thrombosis with standard-dose clopidogrel.[127] Finally, in the RECLOSE 3 (Responsiveness to Clopidogrel and Stent Thrombosis 3) study 1550 clopidogrel nonresponders after a 600 mg loading dose of clopidogrel were screened. Clopidogrel nonresponders (n=302) were switched to prasugrel (10 mg daily) the day of PCI, and ADP test (ADP 10 μmol/L) performed 6 days after PCI. The authors demonstrated that clopidogrel nonresponsiveness can be overcome by prasugrel (10 mg daily), and that optimal platelet aggregation inhibition on prasugrel treatment is associated with a low

rate of long-term cardiac mortality and stent thrombosis. Notably, no higher bleeding risk was documented in HcPR patients treated with prasugrel, underlining how this tailored antiplatelet strategy is able to obtain the maximal benefit from a more potent antiplatelet drug such as prasugrel, without paying a significantly higher bleeding risk.[128]

3.3.2 High on-aspirin platelet reactivity

Another area of research is investigating the possible role of HPR induced by agonists other than ADP, such as AA. This agonist predominantly mirrors the antiplatelet effect of aspirin. It is well known that in the majority of patients with HcPR, HPR induced by AA is present too. The presence of this dual nonresponsiveness seems to be associated with a significantly higher risk of ischemic events.[129] In addition, the clinical impact of HPR measured by LTA induced by AA, ADP and collagen on the occurrence of MACE in the setting of ACS patients (n=1108) was evaluated. A global HPR – induced by AA, ADP and collagen – was significantly associated with cardiovascular death and nonfatal myocardial infarction at a 12-month follow-up, whereas isolate HPR to only one agonist was not associated with a higher ischemic risk, thus suggesting that ACS patients with a recurrent ischemic event within 12 months of follow-up may have a global altered platelet function.[130]

Conflicting data are available about a possible correlation between HPR on-aspirin treatment (HaPR) and clinical outcome. Indeed, in contrast to HcPR, the phenomenon of "aspirin resistance" is less well defined, and its prevalence varies widely among published reports.[131-133] Some studies found a higher risk for ischemic events and stent thrombosis in patients with HaPR,[134-137] and recently, the results of the large-scale ISAR–ASPI (Intracoronary Stenting and Antithrombotic Regimen–Aspirin and Platelet Inhibition) registry showed that HaPR is associated with a higher risk for death or stent thrombosis during the first year after PCI.[138] In contrast, in the prospective multicenter ADAPT-DES (Assessment of Dual AntiPlatelet Therapy with Drug Eluting Stents)

registry no association was found between HaPR and ischemic events.[139] Recently, Gori et al. evaluated the possible role of on-aspirin HPR in 1789 ACS patients enrolled in the previous RECLOSE 2 (Responsiveness to Clopidogrel and Stent Thrombosis 2)–ACS study, and found that HaPR is an independent risk factor for cardiac death and stent thrombosis in ACS patients undergoing PCI.[140]

Diabetes is a clinical condition associated with a significantly higher risk of HaPR. It has been demonstrated that diabetic patients with ACS are at higher risk for recurrent events than non-diabetic ACS patients.[141] Despite sharp declines in restenosis rates with the use of drug-eluting stents, diabetic patients with ACS remain at the highest risk for recurrent ischemia.[142] Moreover, recent studies suggested that diabetes is an independent predictor of stent thrombosis and lower survival rates in patients treated with DES.[143,144] It has been suggested that diabetic patients not on aspirin therapy generally show HPR and elevated levels of platelet thromboxane synthesis, and that aspirin treatment is less effective in inhibiting thromboxane synthesis in diabetic patients than in non-diabetic ones.[145] A systematic review, which combined data from 31 studies with 2147 diabetic patients to examine the relationship between daily aspirin dose and prevalence of HaPR, showed that diabetic patients were 36% more likely to have on-aspirin HPR compared with non-diabetics, and that diabetic patients using 100 mg daily 70% more likely to have HaPR compared with those using 101-325 mg daily.[146]

Among the possible mechanisms underlying HaPR in diabetes, it was hypothesized that faster recovery of platelet COX-1 activity may explain incomplete thromboxane inhibition during the 24-hour dosing interval. In an elegant model, aspirin 100 mg twice daily completely reversed the abnormal TxB2 recovery in diabetic patients, suggesting that interindividual variability in the recovery of platelet cyclooxygenase activity during the dosing interval may limit the duration of the antiplatelet effect of low-dose aspirin in patients with and without diabetes.[147]

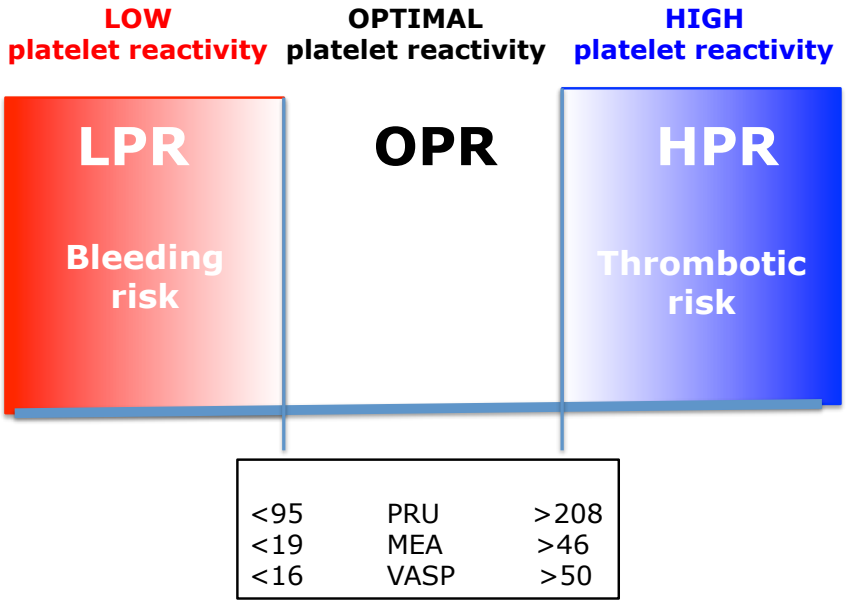
3.4 LOW ON-TREATMENT PLATELET REACTIVITY

In the clinical setting of DAPT in ACS, bleeding was often considered as an inevitable complication. Both TRITON TIMI-38 (Trial to Assess Improvement in Therapeutic Outcomes by Optimizing Platelet Inhibition with Prasugrel-Thrombolysis In Myocardial Infarction 38)[148] [12] and PLATO[41] demonstrated a superiority in the reduction of ischemic events of prasugrel and ticagrelor with respect to clopidogrel, but with a significant higher risk of bleeding in both cases. The balance between the absolute risk reduction in ischemic events and the absolute risk increase in bleeding events with more potent agents remains to be well defined. The focus is now shifting toward finding strategies that could avoid excessive bleeding while maintaining the benefit of reduced ischemic/thrombotic events. Furthermore, bleeding events have been associated with an increased risk of short- and long-term morbidity and mortality in coronary artery disease patients during long-term antiplatelet and anticoagulant therapy. In addition, the results of randomized trials of anticoagulants suggest that a survival benefit might be attributable only to reduction in bleeding.

Observational studies involving patients undergoing PCI have suggested a possible link between low on-treatment platelet reactivity (LPR) and bleeding [149-161] A link between platelet reactivity and bleeding was further observed in prasugrel-treated patients. Parodi et al. reported that patients undergoing PCI with LPR on prasugrel therapy had more frequent access site bleeding.[160] A recent collaborative analysis on 17 studies with 20839 patients with LPR showed a 1.7-fold higher risk for major bleeding complications, without any further reduction in the risk of stent thrombosis compared to patients with optimal platelet reactivity.[161]

These results suggest the existence of an optimal range of P2Y12-inhibition that can be considered as a therapeutic window, within which the predicted risk of stent thrombosis and major bleeding is the lowest after PCI (Figure 3).

Figure 3. On-treatment platelet reactivity and the concept of "therapeutic window".



3.5 ON-TREATMENT PLATELET REACTIVITY IN PERIPHERAL ARTERY DISEASE

Few data are available in the literature about the role of HPR in the setting of PAD.[162] Linnemann et al demonstrated that in the absence of background aspirin antiplatelet therapy, patients with PAD treated with clopidogrel as the sole antiplatelet drug have high variability of residual platelet function as measured by LTA. HcPR was observed in 35.2% of patients at baseline, and in 17.6% at a mean of 18 months of follow-up. During the observation period, 26.6% switched from responders to nonresponders or vice versa, thus suggesting unpredictable platelet inhibition with clopidogrel monotherapy in patients with PAD. Among nonresponders, clopidogrel or its metabolites were detected in 89.5% of patients; this suggests the findings were not related to medication compliance.[163]

The phenomenon of HcPR in PAD patients undergoing percutaneous transluminal angioplasty (PTA) was first reported in the MIRROR randomized controlled trial, which compared DAPT with aspirin and clopidogrel with aspirin monotherapy in 80 patients undergoing femoro-popliteal interventions due to intermittent claudication or CLI. Patients randomized to the DAPT group received a loading dose of 300 mg of clopidogrel 6–12 hours before the procedure. The incidence of HcPR was 30% assessed with an *ex vivo* flow model, and the two patients who required a clinically driven revascularization procedure during the 6-month follow-up were "resistant" to clopidogrel.[164] More recently, Pastromas et al., investigated the incidence and clinical significance of HcPR in patients undergoing peripheral endovascular procedures using the VerifyNow P2Y12 point-of-care assay in 113 patients returning for regular follow-up or clinical relapse after infrainguinal (femoropopliteal or infrapopliteal or both) angioplasty or stenting. HcPR, defined as platelet reaction unit (PRU) values ≥235, was found in 53.9% of patients, and was associated with diabetes mellitus, CLI, and renal disease. HcPR was identified as the only independent predictor of decreased target-

limb revascularization-free survival at ≤7-year follow-up (HR 0.536, 95% CI 0.31–0.90; P=0.01). Of note, concomitant aspirin intake did not affect outcomes according the multivariable model.[165] Following this pilot study, the same group designed a prospective trial, the PRECLOP (Platelet REsponsiveness to CLOpidogrel treatment after Peripheral endovascular procedures) study, for determination of the optimal cut-off PRU value influencing clinical outcomes, as well as the clinical significance of HcPR in peripheral endovascular procedures.[166] In total, 100 consecutive patients undergoing femoro-popliteal angioplasty or stenting were enrolled and stratified into four quartiles according to their PRU value (progressively increased PRU from 1st to 4th quartile), and platelet responsiveness testing with the VerifyNow assay was performed after at least one month of antiplatelet therapy. According to ROC analysis, the optimal cut-off value for the composite end point was PRU ≥234, and was identical to that proposed for PCI patients according to a recently published international consensus document.[62] The incidence of HcPR based on the estimated cut-off value (PRU ≥234) was 51%, and CLI, diabetes mellitus, and chronic renal disease were associated with HcPR. The one-year composite end point (death, major stroke, major amputation, target vessel revascularization, bypass) showed a significant difference between successive quartiles because patients in the first two quartiles had significantly fewer adverse events than those in the last two quartiles. At multivariable regression analysis HcPR was the only independent predictor for increased number of adverse events (HR 16.9, 95% CI 5–55; P<0.0001). Patients with PRU lower than the cut-off value showed one-year event-free survival of approximately 90% regardless of lesion length or grade, stent use, or baseline clinical presentation as intermittent claudication or CLI). In contrast, the one-year event-free survival in the HcPR patient subgroup was <40%. No significant difference in bleeding events was detected between the study quartiles.[166] In both studies, the incidence of HcPR was superior to the nearly 30% reported in the MIRROR study, and in trials investigating patients undergoing coronary procedures PCI, but in line with previous studies investigating patients with advanced intracranial

atherosclerotic disease, and with more recently reported data in patients with advanced PAD.[167-169] The increased incidence of HcPR in patients with severe PAD undergoing endovascular treatment is currently of unknown etiology. Nevertheless, it could be attributed, at least in part, to factors, such as marked endothelial decrease and/or multiple drug intake due to various comorbidities, which are common in patients with advanced atherosclerotic arterial disease.

Recently, in a study by Bernlochner et al., platelet function was assessed on a Multiplate analyzer in 385 patients with PAD undergoing percutaneous endovascular procedure, and treated with aspirin as a long-term therapy in addition to clopidogrel for at least one month. HcPR was defined as the upper quintile (20%) of platelet aggregation values (≥ 420 AU×min). No difference in the primary endpoint (target lesion revascularization) or mortality was observed at one year follow-up.[170]

Moreover, an analysis from the prospective, multicenter ADAPT-DES (Assessment of Dual Antiplatelet Therapy With Drug-Eluting Stents) registry was recently performed in order assess the relationship between platelet reactivity assessed by the VerifyNow point-of-care assay and clinical outcomes after PCI (stent thrombosis, all-cause mortality, myocardial infarction, and clinically relevant bleeding) among subjects with and without PAD. Among 8582 patients, those with PAD (10.2%) had higher 2-year rates of all-cause mortality, myocardial infarction, stent thrombosis, and clinically relevant bleeding. Associations between HcPR (PRU)>208 and adverse events were similar in PAD and no PAD groups, without evidence of interaction; however, adverse event rates were highest among subjects with both PAD and HPR, and in a propensity-adjusted multivariable model, both PAD and HPR were independent predictors of myocardial infarction at 2 years.[171]

The role of the so called "aspirin resistance" in PAD has been not completely elucidated. Available studies are not homogenous in terms of clinical presentation of the disease, time of blood sampling, and methods used for the determination of HaPR. It was demonstrated that HaPR is a rare phenomenon in PAD patients, and it is not

stable over time, due to changing disease activity or related differences in platelet activation pathways.[172-174] In 100 patients with intermittent claudication, Mueller et al. reported a 40% prevalence of aspirin resistance, assessed by whole blood aggregometry, and demonstrated that it was associated with an 87% increase in the risk of arterial re-occlusion at a 18-month follow-up.[175] At variance, the intra-individual variability in response to aspirin over time was not significantly correlated either with re-stenosis/re-occlusion after one year or with adverse long-term outcomes (occurrence of death for cardiovascular cause, stroke or myocardial infarction up to 8 years of follow-up) in 109 symptomatic PAD patients.[176]

4. PROJECT

4.1 TITLE

Platelet function and risk of adverse events in peripheral artery disease patients undergoing percutaneous revascularization.

4.2 BACKGROUND AND AIM

Peripheral artery disease is most commonly caused by atherosclerosis, and it is characterized by an increased risk of myocardial infarction, stroke, and cardiovascular death. Given the markedly elevated cardiovascular risk among patients with PAD, antiplatelet therapy would be expected to be of great benefit. However, there is little evidence that antiplatelet therapy could alter the natural history of PAD, both in asymptomatic and symptomatic patients, and which antiplatelet agent could be the best option is not well defined. Although DAPT is generally recommended after peripheral vascular intervention, this is largely based upon extrapolation of coronary percutaneous intervention data. We know from this setting that a different entity of on-treatment platelet function inhibition is associated with different clinical outcomes. In particular, lots of studies demonstrated that HPR is associated with an increased risk of ischemic complications (especially stent thrombosis), and there is a growing body of evidence that, on the contrary, LPR could be associated with bleeding risk. Concerning the role of HPR in PAD, few data are available in the literature. HcPR has been associated with an increased risk of adverse events (cardiovascular death, major amputation and re-intervention) during follow-up in patients with PAD undergoing PTA. The role of the so called "aspirin resistance" in PAD is not completely elucidated; indeed, available studies are not homogenous in terms of clinical presentation of the

disease, time of blood sampling, and methods used for the determination of HPR on-aspirin therapy.

Aim of this study was to evaluate, in patients with PAD undergoing PTA with or without stenting, the degree of on-treatment platelet reactivity, and the association between the entity of platelet inhibition and ischemic and hemorrhagic adverse events at follow-up.

4.3 METHODS

Study design and population

This was an observational, prospective, single center study, which enrolled 177 consecutive patients with PAD undergoing PTA, with or without stenting, referred to the University Hospital of Florence, Italy. Inclusion criteria were ABI <0.9 or >1.3 and age >18 years. Patients aged <18 years and/or unable to sign the informed consent were excluded. A platelet count <100 $\times 10^9$/l, a hemoglobin level <9 g/dl, and a haematocrit level <25% were additional exclusion criteria. The study was approved by the local Ethic Committee. All patients gave written informed consent.

Percutaneous transluminal angioplasty and antiplatelet management

All interventions were performed according to current standards, and the use and type of stent implanted was at the discretion of the operator. All patients, unless they were already on antiplatelet therapy for a previous coronary revascularization, underwent a P2Y12 inhibitor loading dose. Patients were discharged on DAPT with a P2Y12 inhibitor and aspirin. Clopidogrel was the first-choice P2Y12 inhibitor (according to the guidelines) unless patients were on treatment with prasugrel or ticagrelor for a recent ACS. Aspirin, 100-325 mg once daily, was recommended for an indefinite period; a P2Y12 inhibitor for at least 6 months.

Platelet function assessment

Platelet function was assessed by LTA (APACT4, Helena Laboratories, Milan, Italy), performed on platelet rich plasma, using AA and ADP as agonists of platelet aggregation. Blood samples anticoagulated with 0.109 M sodium citrate (ratio, 9:1) were obtained within 24 hours from PTA. Platelet rich plasma, obtained by centrifuging whole blood for 10 minutes at 200 g, was stimulated with 1 mM AA and 10 μM ADP.

According to literature data, HPR was defined by LTA ≥20% if induced by AA, and LTA ≥70% if induced by ADP.[177]

Follow-up

Follow-up was performed in order to record the occurrence of ischemic and bleeding events. All other possible information derived from hospital readmission or by the referring physician, relatives, or municipality live registries was collected.

Outcomes

The study's outcomes were: death, target limb revascularization, major amputation, acute myocardial infarction and/or myocardial revascularization, stroke/transient ischemic attack (TIA), and bleeding, classified as major and minor according to TIMI (Thrombolysis in Myocardial Infarction) classification.[178]

Statistical analysis

Statistical analysis was performed using the software package SPSS 20 (SPSS Inc., Chicago, Illinois). Discrete data were summarized as frequencies, and continuous data were expressed as means and standard deviations or medians and interquartile ranges (IQRs), as appropriate. The $\chi 2$ test was used for comparison of categorical variables, and the unpaired 2-tailed Student t test or Mann–Whitney rank sum test were used to test differences among continuous variables. The ability of platelet aggregation values by ADP and AA to predict outcomes was examined by receiver operating characteristics (ROC) curves. ROC curves were constructed by plotting the sensitivity against the corresponding false-positive rate which equals 1-specifity. Survival curves were generated with the use of the Kaplan-Meier method, and the difference between groups was assessed by log-rank test. A multivariable Cox proportional hazard model was performed to evaluate the independent contribution of clinical and laboratory variables to the outcomes. Variables known to be related with prognostic outcome or

variables with a P value <0.05 at univariate Cox analysis were forced into the final multivariate model. A landmark analysis was computed by the Kaplan-Meier method for mortality using a starting point of 6 months after the index procedure. All tests were two-sided, and a P value <0.05 was considered significant.

4.4 RESULTS

Baseline characteristics of the study population

Baseline characteristics of the 177 patients enrolled in the study are reported in table I. One hundred and eighteen patients (66.7%) were males. Median age was 75 (IQR 68-81) years.

Table I. Baseline characteristics of the study population.

	All patients (n=177)	**Males (n=118)**	**Females (n=59)**	**P**
Age, years, median (IQR)	75 (68-81)	75 (67-78)	77 (71-85)	0.004
BMI, Kg/m^2, median (IQR)	25.2 (22.8-27.6)	25.9 (23.1-28.6)	24.6 (20.8-26.8)	0.012
BMI ≥30 Kg/m^2, n (%)	25 (14.1)	20 (16.9)	5 (8.5)	0.094
Smokers, n (%)	35 (19.9)	22 (18.6)	13 (22)	0.950
Hypertension, n (%)	151 (85.3)	101 (85.6)	50 (84.7)	0.999
Dyslipidemia, n (%)	163 (92.3)	111 (94.2)	52 (88.5)	0.202
Diabetes, n (%)	57 (32.3)	40 (38.8)	10 (19.2)	0.018
Renal failure, n (%)	21 (11.8)	14 (11.9)	7 (11.9)	0.999
Ejection fraction <45%, n (%)	36 (20.2)	27 (22.9)	9 (15.3)	0.448
Leriche-Fontaine Class, n (%)				
I	2 (1.3)	2 (1.8)	0	0.181
IIa	14 (7.9)	9 (7.6)	5 (8.5)	
IIb	103 (57.9)	75 (63.6)	25 (42.9)	
III	7 (3.9)	6 (5.5)	0	
IV	51 (28.9)	26 (21.8)	29 (48.6)	

IQR, Interquartile Range; BMI, Body Mass Index.

Females were significantly older than males (P=0.007), whereas a significantly higher prevalence of diabetes was found among males than females (P=0.018 and P=0.005, respectively). As regard the severity of PAD, most patients were in class IIb according to Leriche-Fontaine classification.

In about 90% of patients PTA was followed by stent implantation (table II). No significant differences in procedural characteristics were found between males and females. All patients were discharged on DAPT with aspirin and a P2Y12 inhibitor, which was clopidogrel in 130 ptients (73.5%) (table II).

Table II. Percutaneous transluminal angioplasty characteristics and antiplatelet therapy at discharge.

	All patients (n=177)	**Males (n=118)**	**Females (n=59)**	**P**
Clopidogrel loading dose, n (%)	77 (44.3)	51 (43.2)	26 (44.1)	0.915
Stent implantation, n (%)	158 (89.3)	107 (90.7)	51 (86.4)	0.391
Number of stents, median (IQR)	2 (1-3)	2 (1-3)	2 (1-3)	1.000
Stent lenght, mm, median (IQR)	150 (79-270)	150 (80-263)	170 (65-350)	0.347
Clopidogrel, n (%)	130 (73.5)	84 (71.2)	46 (78)	0.336
Prasugrel, n (%)	39 (22)	29 (24.6)	10 (16.9)	0.248
Ticagrelor, n (%)	8 (4.5)	5 (4.2)	3 (5.1)	0.798

IQR, Interquartile Range.

Platelet function evaluation

At platelet function evaluation, HPR by AA was found in 52% of patients, and showed a non significant association with older age and a higher prevalence of renal failure (table III). HPR by ADP was found in 32% of patients, and was significantly associated with older age (table III). Thithy-seven patients (20.9%) had dual HPR both by AA and by ADP.

Table III. High on-treatment platelet reactivity by AA and ADP.

	No HPR by AA (n=98)	**HPR by AA (n=79)**	**P**	**No HPR by ADP (n=121)**	**HPR by ADP (n=56)**	**P**
Males/Females, n (%)	65/33 (66.3/33.7)	53/26 (67.1/32.9)	0.999	82/39 (67.8/32.2)	36/20 (64.3/35.7)	0.732
Age, years, median (IQR)	73.5 (67-79)	77 (70-83)	0.058	73 (67-78)	79 (70.5-84)	0.001
BMI, Kg/m^2, median (IQR)	25.5 (23-28)	25 (22-27.6)	0.419	25.2 (22.4-27.3)	25.7 (23.1-29.3)	0.338
BMI ≥30 Kg/m^2, n (%)	16 (16.3)	9 (11.4)	0.237	17 (14)	8 (14.3)	0.567
Smokers, n (%)	19 (19.4)	16 (20.2)	0.954	27 (22.3)	8 (14.3)	0.204
Hypertension, n (%)	86 (87.8)	65 (82.3)	0.306	106 (87.6)	45 (80.4)	0.448
Dyslipidemia, n (%)	92 (93.9)	71 (89.9)	0.241	113 (93.4)	50 (89.3)	0.255
Diabetes, n (%)	32 (32.6)	25 (31.6)	0.887	39 (32.2)	18 (32.1)	0.990
Renal failure, n (%)	8 (8.2)	13 (16.4)	0.090	14 (11.6)	7 (12.5)	0.859
Ejection fraction <45%, n (%)	17 (17.3)	19 (24.1)	0.271	23 (19)	13 (23.2)	0.518

HPR, High on-treatment platelet reactivity; AA, Arachidonic Acid; ADP, Adenosine Diphosphate; IQR, Interquartile Range; BMI, Body Mass Index.

Follow-up and outcomes

The median follow-up duration was 23 (IQR 13-27) months. During follow-up 23 deaths (13%) were recorded. Among survivors, 27 patients (17.5%) underwent target limb revascularization, 2 (1.3%) underwent amputation, and 6 (3.9%) myocardial revascularization. No patients experienced stroke or TIA. Twenty-four patients (15.6%) had a bleeding complication, which was minor in all cases (16 epistaxis, 3 mouth bleedings, 2 hematuria, 1 gastro-intestinal bleeding, 2 limb bruisings).

Patients who died were significantly older than survivors, and had a significantly higher prevalence of renal failure and left ventricular systolic dysfunction.

The median value of LTA by both AA and ADP was significantly higher in patients who died than in survivors [43 (16-75)% vs 18 (14-75)%, P=0.005 for LTA by AA; 75 (56-83)% vs 56 (38-83)%, P=0.001 for LTA by ADP, respectively]. Patients who died had a significantly higher prevalence of HPR by AA and HPR by ADP than survivors (73.9% vs 40.3%, P=0.002; 65.2% vs 26.6%, P<0.001, respectively). Kaplan-Meier analysis showed a significantly higher risk of death in patients with HPR by AA and HPR by ADP than in those without. At multivariate analysis HPR by AA and HPR by ADP remained independent predictors of death [HR 3.75 (1.20-11.66), P=0.023 and HR 4.78 (1.57-14.52), P=0.006, respectively] (table IV).

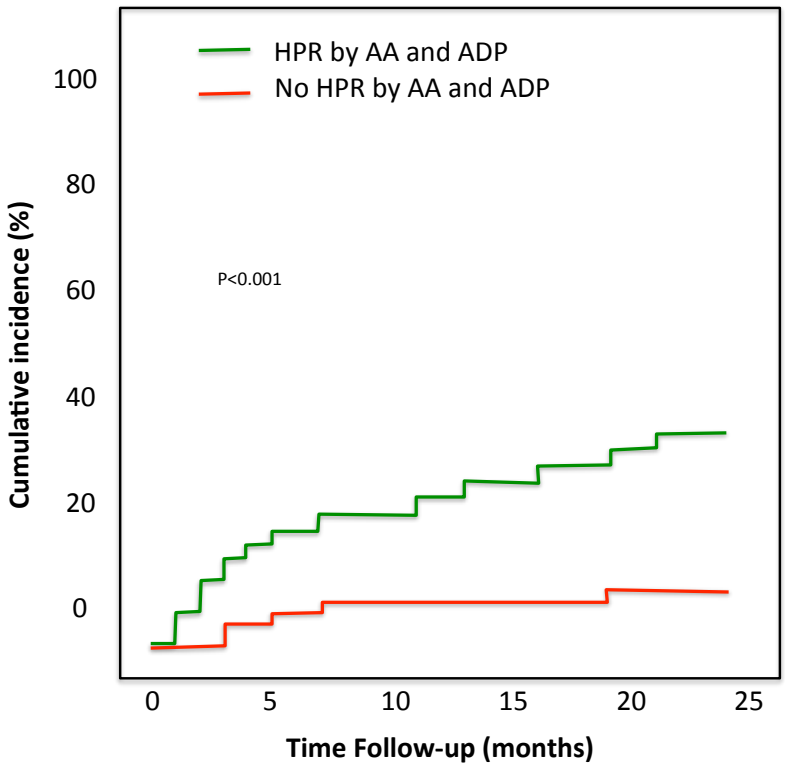
Table IV. Univariate and multivariate analysis for death.

	Univariate HR (95% CI)	P	Multivariate HR (95% CI)	P
Males/Females	0.67 (0.25-1.81)	0.432	0.44 (0.13-1.50)	0.189
Age	1.10 (1.03-1.16)	0.002	1.12 (1.03-1.22)	0.009
BMI ≥30 Kg/m^2	0.54 (0.12-2.47)	0.429	0.249 (0.03-1.94)	0.294
Smoking	0.16 (0.02-1.23)	0.079	0.26 (0.03-2.58)	0.249
Hypertension	0.32 (0.12-0.88)	0.028	0.19 (0.05-0.77)	0.019
Dyslipidemia	0.33 (0.09-1.16)	0.083	0.49 (0.10-2.35)	0.492
Diabetes	1.14 (0.45-2.88)	0.777	1.15 (0.34-3.83)	0.826
Renal failure	3.27 (1.12-9.56)	0.030	2.07 (0.55-7.77)	0.279
Ejection fraction <45%	3.79 (1.50-9.56)	0.005	3.16 (1.05-9.87)	0.049
HPR by AA	4.20 (1.57-11.26)	0.004	3.75 (1.20-11.66)	0.023
HPR by ADP	5.17 (2.04-13.09)	0.001	4.78 (1.57-14.52)	0.006

HR, Hazard Ratio; CI, Confidence Interval; BMI, Body Mass Index; HPR, High platelet reactivity; AA, Arachidonic Acid; ADP, Adenosine Diphosphate.

Moreover, patients who died had a significant higher prevalence of dual HPR both by AA and by ADP than survivors (56.5% vs 15.6%, P<0.001). Kaplan-Meier analysis showed a significantly higher risk of death in patients with dual HPR by AA and by ADP than in those without (figure I).

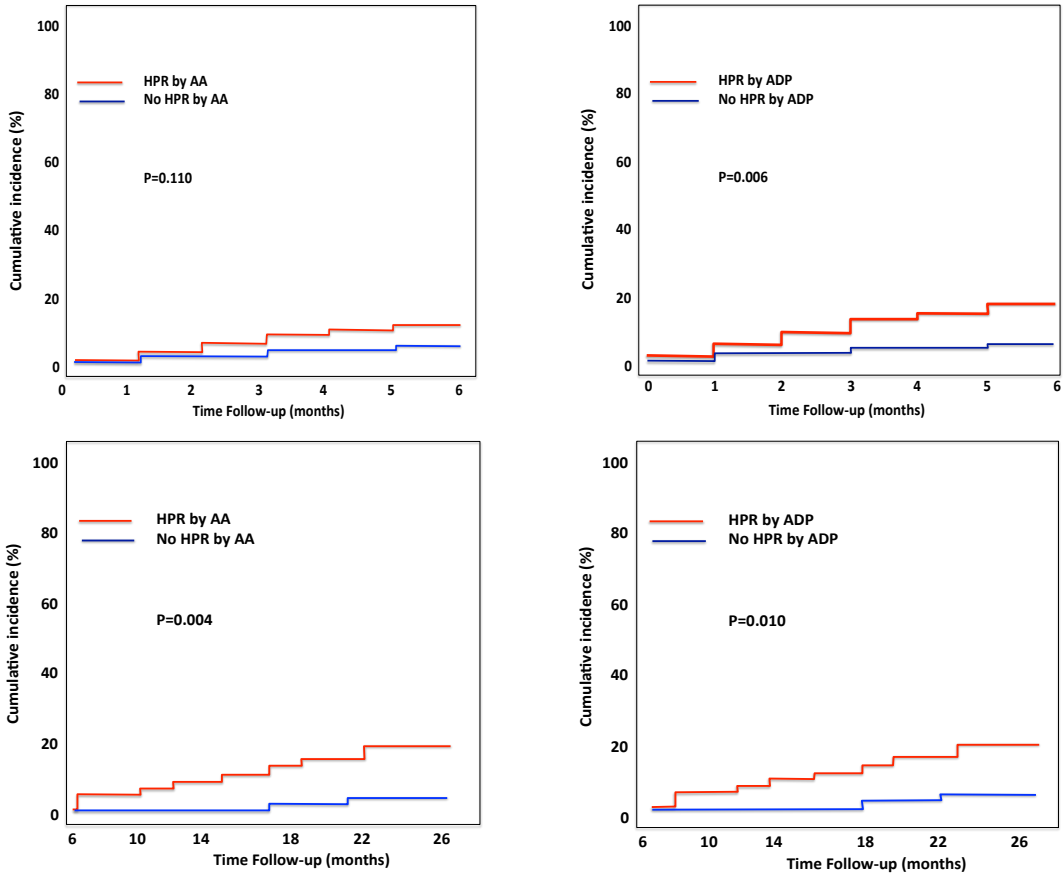
Figure I. Kaplan-Meier survival curves for death in patients with and without dual HPR by AA and by ADP.



HPR, High platelet reactivity; AA, Arachidonic Acid; ADP, Adenosine Diphosphate.

The landmark analysis using the prespecified starting point of 6 months showed that the differences in mortality between patients with and without HPR by ADP emerged both in the short-term follow-up as well as from 6 months to long term, whereas HPR by AA was significantly associated only with long-term mortality (figure II).

Figure II. Kaplan-Meier landmark analysis survival curves for death in patients with and without HPR by AA and HPR by ADP using the prespecified starting point of 6 months.



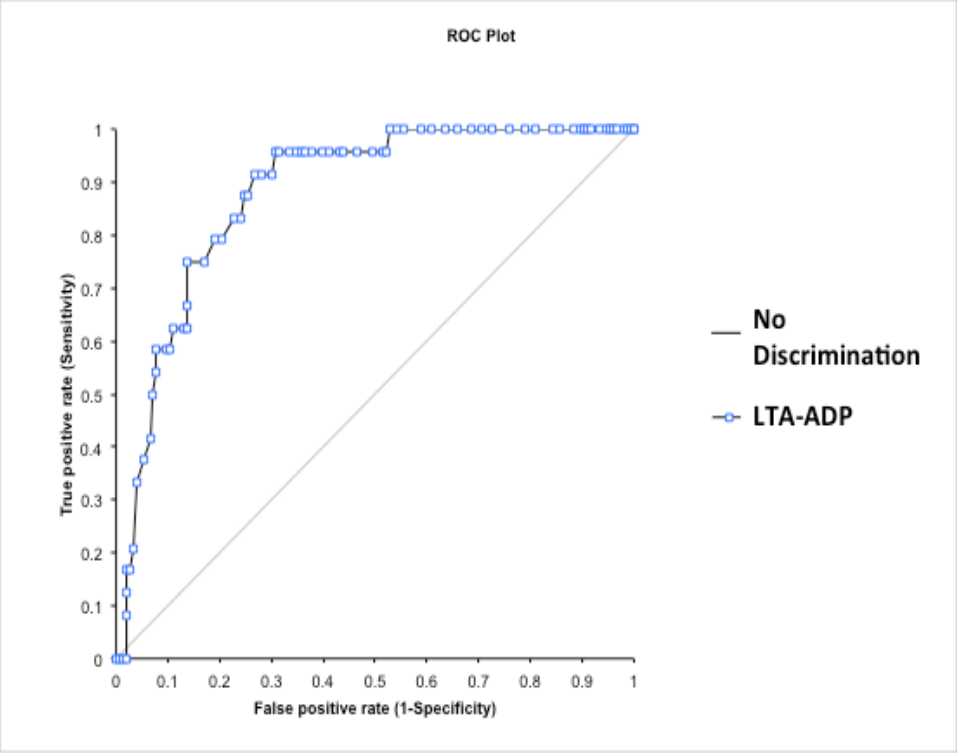
HPR, High platelet reactivity; AA, Arachidonic Acid; ADP, Adenosine Diphosphate.

No significant association was observed between target limb revascularization and HPR by AA or HPR by ADP.

As regard bleeding complications, they were significantly associated with younger age (table V).

The median value of LTA by ADP was found to be significantly lower in patients who experienced bleeding complications than in those who didn't [26.5 (22-39.2)% vs 62 (44.5-74)%, P<0.001). At ROC curve analysis the cut-off of platelet aggregation induced by ADP with the best sensitivity and specificity for increased risk of bleeding was 41% (figure III).

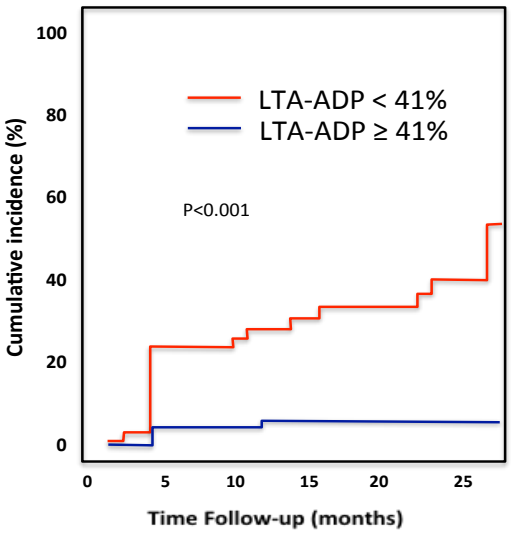
Figure III. ROC curve analysis evaluating the cut-off of platelet aggregation induced by ADP with the best sensitivity and specificity for increased risk of bleeding.



LTA-ADP, Light Transmission Aggregometry by Adenosine Diphosphate.

Using this value as cut-off of LPR, we found a significant association with bleeding (figure IV).

Figure IV. Kaplan-Meier survival curves for bleeding in relation to LTA-ADP.



LTA-ADP, Light Transmission Aggregometry by Adenosine Diphosphate.

At multivariate analysis LTA by ADP lower than 41% remained independently associated with bleeding [HR 14.59 (2.55-24.01), P=0.001] (table V).

Table V. Univariate and multivariate analysis for bleeding.

	Univariate HR (95% CI)	**P**	**Multivariate HR (95% CI)**	**P**
Males/Females	0.80 (0.31-2.05)	0.642	0.89 (0.19-4.07)	0.879
Age	0.95 (0.92-0.99)	0.019	0.92 (0.86-0.98)	0.009
BMI ≥30 Kg/m^2	0.51 (0.11-2.34)	0.389	0.16 (0.01-2.20)	0.172
Smoking	1.43 (0.52-3.91)	0.491	0.37 (0.07-1.95)	0.241
Hypertension	1.24 (0.34-4.49)	0.745	1.26 (0.22-7.07)	0.793
Dyslipidemia	2.14 (0.27-17.11)	0.475	0.94 (0.08-11.35)	0.960
Diabetes	1.06 (0.43-2.65)	0.899	0.42 (0.10-1.74)	0.235
Renal failure	1.07 (0.29-3.96)	0.918	1.18 (0.18-7.60)	0.864
Ejection fraction <45%	1.76 (0.67-4.64)	0.252	3.11 (0.89-10.89)	0.075
LTA-ADP<41%	14.96 (5.18-43.21)	<0.001	14.59 (2.55-24.01)	0.001

HR, Hazard Ratio; CI, Confidence Interval; BMI, Body Mass Index; LTA-ADP, Light Transmission Aggregometry by Adenosine Diphosphate.

4.5 DISCUSSION

In this study the role of platelet hyper- or hypo-reactivity in predicting mortality or bleeding events during a 2-year follow-up was assessed in a cohort of PAD patients undergoing PTA on DAPT. HPR by ADP and AA were found to be predictors of death, whereas LPR by ADP was predictor of bleeding complications.

This study showed a high prevalence of HPR by ADP and AA in the acute phase of the disease, and the assessment of HPR by ADP and AA after PTA was able to identify patients who died during follow-up. These findings are consistent with those obtained in the clinical setting of ACS, where the presence of ADP and/or AA-induced HPR has been associated with a significantly increased risk of ischemic events, and cardiac death.[61,65,133]

Few studies exploring the role of HPR, and in particular of the so-called "clopidogrel resistance", in the occurrence of adverse events in PAD patients on DAPT are available. Pastromas and coworkers demostrated that, in patients with PAD treated with clopidogrel and aspirin for 6 months, the post-PTA evaluation of HPR by ADP (assessed by the point-of care test VerifyNow) provided a prognostic information on the occurrence of target limb reintervention.[165] In the PRECLOP study, the assessment of HPR before PTA by the same point-of care test provided the optimal PRU cut-off value in order to identify PAD patients at high risk of developing the combined end-point of death, target vessel reintervention, and amputation during 1-year follow-up.[166] Consistently with these previous observations, PAD patients carriers of at least one CYP2C19 loss-of-function allele had a diminished pharmacodynamic response to clopidogrel (measured as platelet aggregation induced by ADP), and those with both HPR and a CYP2C19 loss-of-function allele had a significantly higher risk of ischemic events.[179]

In this study, not only HPR by ADP, but also HPR by AA was found to be an independent predictor of death in PAD patients treated with PTA. Moreover, the risk of

death was significantly higher in patients with dual HPR both by AA and by ADP, than in those without. Three previous studies on PAD patients treated with PTA failed to demonstrate a significant association between HPR by AA and adverse events during follow-up.[175,176,180] Although the clinical setting of these studies was similar, i.e. symptomatic PAD patients undergoing PTA, the timing of HPR evaluation was different. In this study, HPR was evaluated 24 hours after a P2Y12 loading dose, unless patients were already on antiplatelet therapy for a previous coronary revascularization, whereas in the above metioned studies platelet function was assessed before PTA.[175,176,180] In the clinical setting of ACS, only platelet function assessment after PCI was significantly associated with the occurrence of cardiovascular events. It is likely that platelet reactivity detected in the acute phase of the disease (both ACS and PAD) reflects the presence of an "aggressive" blood, which may play a key role in making the patient a "vulnerable" one. A complex network of pro-inflammatory and anti-inflammatory cytokines,[181] and an increased platelet turn-over with a higher number of reticulated platelets may be the determinants of HPR in the acute phase. Indeed, reticulated platelets, which are the youngest platelets released into the circulation from the bone marrow, rich in mRNA content and particularly hyper-reactive, have been associated with adverse outcomes in ACS patients.[182] The different methods used to determine HPR should also be taken into account in order to explain the different results. In this study, HPR was assessed by LTA on platelet rich plasma, which is considered the gold standard test for platelet function assessment. Moreover, LTA was used to define the HPR by AA cut-off of 20%, which was the value associated with adverse cardiovascular outcomes in ACS patients.[15] The definition of the best cut-off value for identifying PAD patients at higher risk of clinical outcomes is a crucial point too. It was found that in PAD patients treated with aspirin, the whole platelet aggregation induced by AA was completely inhibited, whereas only 40% of patients showed the expected effect of aspirin on whole platelet aggregation induced by ADP or collagen.[175] Interestingly, only patients with HPR by ADP, but not by AA were at high risk of reocclusion following

peripheral angioplasty. In a prospective study which enrolled 98 PAD patients treated with PTA and followed for 12 months, the point of care assay PFA-100 was used, and the authors defined as non-responders to antiplatelet therapy those patients who had epinephrine-closure time <170 seconds or ADP-closure time <120 seconds.[180] There was no evidence for greater incidence of complications in aspirin non-responders, whereas patients with clopidogrel resistance experienced a higher incidence of restenosis or reocclusion after PTA compared with clopidogrel responders. However, the small number of aspirin non-responders observed in this study (5 patients) reduces the significance of these data. Furthermore, the choice of PFA-100 as method to investigate clopidogrel and aspirin resistance, and the cut-off values used for HPR definition are questionable. On the contrary, a more recent study demonstrated that aspirin resistance assessed by means of the VerifyNow Aspirin Assay was highly prevalent (25.8%), and was an independent predictor of death, myocardial infarction, or ischemic stroke in symptomatic PAD patient.[183]

Finally, an interesting finding of this study was the significant association between lower values of LTA by ADP and bleeding complications, and the identification of a cut-off value independently associated with bleeding during follow-up. A possible link between LPR and bleeding has also been reported in ACS patients undergoing PCI.[160] The finding of excessive inhibition of platelet function as risk factor for bleeding suggests the hypothesis that a "therapeutic window", i.e. an optimal range of P2Y12-inhibition, exists, within which the predicted risk of ischemic and bleeding complications is the lowest.

The strenghts of the study are the duration of the follow-up (about 23 months), which is longer than in the other published studies, and the evaluation of both platelet hyper- and hypo- reactivity in PAD patients. However, it suffers from some limitations. First, the observational and single center design, and the small sample size. Moreover, the lack of further blood sampling does not allow to determine if platelet hyper- or hypo-reactivity detected in the acute phase of the disease is maintained over the time, and,

secondly, if changes in platelet response during follow-up could be associated with clinical outcomes.

In conclusion, the results of this study strengthen and extend to PAD patients the evidence that an impaired inhibition of platelet function by clopidogrel and aspirin in the acute phase of the disease is associated with subsequent worse clinical outcomes, underlining the importance of optimal platelet inhibition, and suggesting the potential utility of assessing platelet function, even in the setting of PAD, in order to ensure the patient the best tailored antiplatelet therapy.

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