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CICLO XXIX

On-Treatment Platelet Reactivity in Peripheral and Coronary Arterial Blood in Patients Undergoing Primary PCI for ST-Segment Elevation Myocardial Infarction (STEMI).

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To my family,

for their constant support

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1- SUMMARY

BACKGROUND

Dual antiplatelet therapy is recommended in patients undergoing primary percutaneous coronary intervention (p-PCI) for ST-segment elevation myocardial infarction (STEMI). In the past few decades, oral antiplatelet agents have proved to significantly reduce the incidence of ischemic events in patients with atherothrombotic diseases. Nevertheless, recurrent ischemic events often occur in patients undergoing stent implantation. High platelets reactivity has been associated with a higher risk for major cardiovascular events in patients with acute coronary syndromes (ACS). Several pre-analytical variables may influence platelet function analysis. The aim of our study was to assess the on-treatment platelet reactivity in peripheral and coronary blood, in a group of patients receiving dual antiplatelet therapy undergoing primary percutaneous coronary intervention (p-PCI) for STEMI.

METHODS

Eligible patients for the study were considered as consecutively admitted patients to the emergency department of University-Hospital of Padua with a diagnosis of ACS with ST-segment elevation scheduled for an urgent procedure of coronary angioplasty. One hundred nine patients who consecutively underwent p-PCI (males: 72%, females: 28%; mean age: 64±13 years) were enrolled. Before the coronary angioplasty intervention, the patients were treated with dual antiplatelet therapy (aspirin 250mg I.V in association with one another oral thienopyridines; Clopidogrel 300/600mg, Prasugrel 60 mg or Ticagrelor 180 mg) and with anticoagulant therapy (unfractionated heparin 70U/Kg I.V). During the coronary angioplasty intervention two different samples were obtained, one from peripheral artery and the other from coronary blood. The platelet aggregation was studied using the impedance aggregometry Multiplate®, according to manufacturer's indications. For each patient the values of "Area Under the Curve" (AUC) in ADP-test and ASPI-test were considered, both in the peripheral and in coronary blood.

"Low responders of antiplatelet therapy" were considered when an AUC value of ASPI-test or ADP-test greater than or equal to a pre-established cut-off.

RESULTS

The Multiplate® analysis of ADP-test revealed that mean values were slightly higher in peripheral blood compared to coronary blood (peripheral blood: 41±28 U; coronary blood: 39±28 U), However these values with no statistically significant difference (p=0.68). Likewise, for the ASPI-test; no statistically significant difference between the mean values in the peripheral blood compared to the coronary blood (peripheral blood: 23±4 U; coronary blood: 17±2 U; p=0.06).

The percentage of low-responders to ADP-receptor inhibitors was significantly greater than the percentage of low-responders to acetylsalicylic acid at time of primary PCI both in the peripheral and in the coronary blood samples (peripheral ADP-test: 38%; peripheral ASPI-test: 14%; p<0.01, Coronary ADP-test: 36%; coronary ASPI-test: 11%; p<0.01). In peripheral blood, the prevalence of "low Clopidogrel responders" was higher (45%) than that observed for Prasugrel (36%) and Ticagrelor (33%). Similar results were observed in coronary blood, the prevalence of "low Clopidogrel responders" was higher (40%) than that observed for Prasugrel (36%) and Ticagrelor (29%) however these results were with no significant statistical difference (p >0.05). Finally, a positive and statistically significant linear correlation was observed for both ASPI-test and ADP-test in peripheral and coronary blood (r^2 0.23, p <0.001 and r^2 0.12, p <0.001; respectively). That means; those who are resistant to acetylsalicylic acid tend to be resistant to ADP receptor inhibitors, and vice versa; those who are sensitive to acetylsalicylic acid therapy tend to be sensitive to ADP inhibitor therapy also. Our observed data did not show a correlation between platelet function and clinical outcome both for in-hospital and 1-year clinical outcomes.

CONCLUSIONS

In this study we observed that the overall platelet reactivity in coronary blood is lower than in peripheral blood, though not statistically significant. This more likely appears to be due to high antiplatelet drugs effect at plaque ulceration/thrombus site, where the hemostatic process is highly active at onset of

STEMI. Larger studies are needed for better evaluation of these mechanisms in term of pharmacodynamic, pharmacokinetic and receptor kinetic properties of antiplatelet agents.

The other interesting result emerging from data processing is the high incidence (about 30%) of low response to thienopyridine type antiplatelet drugs at the time of primary angioplasty. This result, moreover known for Clopidogrel in addition our results include patients treated with Prasugrel and Ticagrelor also. An explanation of this phenomenon which also involves potent recent drugs, requires careful analysis and further studies. The significant direct correlation between platelet reactivity in peripheral and in coronary blood is still a matter of debate. Larger studies are needed for in-depth assessment of any correlation between on–treatment platelet reactivity measured in coronary blood and clinical outcome.

2- RIASSUNTO

INTRODUZIONE

La doppia terapia antiaggregante (DAPT) è raccomandata in pazienti sottoposti ad intervento di angioplastica coronarica primaria (p-PCI) per infarto miocardico acuto con sopraslivellamento del tratto ST (STEMI). Infatti, il trattamento con farmaci antipiastrinici orali ha dimostrato di ridurre significativamente l'incidenza di eventi ischemici nei pazienti con malattie aterotrombotiche sia in fase acuta che in fase cronica. Tuttavia, spesso si verificano eventi ischemici ricorrenti nei pazienti sottoposti ad angioplastica ed impianto di stent. È stato dimostrato che una delle cause di recidiva ischemica sia l'elevata reattività delle piastrine. Pertanto, lo studio della funzione piastrinica diventa un elemento sempre più importante per valutare questo tipo di pazienti. Diverse variabili pre-analitiche possono influenzare l'analisi della funzione piastrinica. Lo scopo del nostro studio è stato quello di valutare la reattività piastrinica del sangue periferico e coronarico in un gruppo di pazienti trattati con DAPT e sottoposti p-PCI per STEMI.

METODI

Abbiamo considerato eleggibili allo studio tutti i pazienti consecutivamente giunti in urgenza al Pronto Soccorso dell'Azienda Ospedaliera di Padova con diagnosi di sindrome coronarica acuta con sopraslivellamento del tratto ST per i quali fosse indicata l'esecuzione in urgenza di una procedura di angioplastica coronarica. Sono stati arruolati 109 pazienti (maschi: 72%, femmine: 28%; età media: 64±13 anni). I pazienti arruolati nello studio sono stati trattati, prima di essere sottoposti alla procedura di angioplastica primaria, con doppia terapia antiaggregante (aspirina 250 mg e.v in associazione con uno dei seguenti tre farmaci: Clopidogrel 300/600 mg per os, Prasugrel 60 mg per os o Ticagrelor 180 mg per os) e con terapia anticoagulante (eparina non frazionata 70 U/Kg e.v). Durante la procedura di angioplastica primaria sono stati eseguiti due tipi di prelievo, uno dal sangue arterioso periferico ed uno dal sangue arterioso coronarico. L'aggregazione piastrinica è stata studiata con l'aggregometro Multiplate®

secondo le indicazioni fornite dal costruttore. Per ogni paziente sono stati valutati i valori di “Area Under the Curve” (AUC) nell’ADP-test e nell’ASPI-test, ottenuti sul sangue periferico e sul sangue coronarico. “Low responders alla terapia antiaggregante” sono stati definiti quei pazienti con valori di “Area Under Curve” (AUC) all’ASPI test o all’ADP test sono maggiore o uguale a un range prestabilito.

RISULTATI

Non abbiamo osservato differenza statisticamente significativa tra i valori medi di ADP-test calcolati su sangue periferico e su sangue coronarico. I valori medi delle AUC sono risultati lievemente superiori nel sangue periferico che nel sangue coronarico (sangue periferico: 41±28 U; sangue coronarico: 39±28 U; p=0.68). Allo steso modo, non è stata riscontrata differenza statisticamente significativa tra i valori medi di ASPI-test calcolati su sangue periferico e su sangue coronarico. Anche in questo caso abbiamo osservato valori medi di AUC lievemente superiori nel sangue periferico che nel sangue coronarico (sangue periferico: 23±4 U; sangue coronarico: 17±2 U; p=0.06). Sia nel sangue periferico che nel sangue coronarico la percentuale di pazienti “low responders” al trattamento con inibitori del recettore per l’ADP è risultata essere statisticamente superiore alla percentuale di pazienti “low responders” alla terapia con acido acetilsalicilico al momento dell’angioplastica primaria (ADP-test periferico: 38%; ASPI-test periferico: 14%; p<0.01. ADP-test coronarico: 38%; ASPI-test coronarico: 11%; p<0.01). Nel sangue periferico la prevalenza di "low responders” al Clopidogrel era superiore (45%) a quella osservata rispettivamente per Prasugrel (36%) e Ticagrelor (33%). Risultati simili sono stati osservati nel sangue coronarico. In particolare, la prevalenza di "low responders” al Clopidogrel è stata superiore (40%) rispetto a quella osservata per Prasugrel (36%) e Ticagrelor (29%). Non è stata osservata alcuna differenza significativa (p> 0,05) nella prevalenza dei pazienti con valori di ADP-test superiori al cut-off prestabilito, considerando separatamente le tre diverse tienopiridine. Infine è stata individuata una correlazione lineare statisticamente significativa tra “low responders” all’acido acetilsalicilico e “low responders” agli inibitori del recettore dell’ADP. Questa osservazione indica come i pazienti resistenti al trattamento con acido

acetilsalicilico tendono ad essere resistenti anche al trattamento con inibitori del recettore per l'ADP e, viceversa, pazienti "sensibili" alla terapia con acido acetilsalicilico tendono ad essere "sensibili" anche al trattamento con inibitori del recettore per l'ADP. Questi resultati sono stati osservati sia su sangue periferico (r^2 0.23, $p<0.001$) che su sangue coronarico (r^2 0.12, $p<0.001$). I dati che abbiamo osservato non mostrano un'associazione tra funzione piastrinica e outcome clinico nè per quanto riguarda gli "in-hospital outcome" né per quanto riguarda gli outcome a distanza di 1 anno.

CONCLUSIONI

I dati analizzati ci hanno permesso di dimostrare che la reattività piastrinica nel sangue coronarico era inferiore rispetto a quella osservata nel sangue periferico. Sembrerebbe quindi che, la risposta alla terapia farmacologica con doppia antiaggregante prima della procedura sia maggiore proprio laddove il processo emostatico è più attivo, ossia a livello della placca aterosclerotica sede della formazione del trombo responsabile dell'insorgenza della STEMI. Questo meccanismo necessità di conferma in termini di farmacodinamica, farmacocinetica e di cinetica recettoriale. L'altro dato estremamente interessante emerso dall'elaborazione dei dati è l'elevata incidenza (circa 30%) dei pazienti "low responders" al trattamento con farmaci antiaggreganti di tipo tienopiridinico al momento della angioplastica primaria. Questo risultato, peraltro noto per il Clopidogrel, comprende anche pazienti trattati con Prasugrel e Ticagrelor. Una possibile spiegazione di questo fenomeno, che coinvolge anche i farmaci di "seconda generazione", necessita di un'attenta analisi. Abbiamo infine osservato una significativa correlazione tra reattività piastrinica nel sangue periferico e nel coronario. I nostri risultati, che alla luce dei limiti del nostro lavoro devono considerarsi come preliminari, necessitano di essere confermati su casistiche più numerose soprattutto per quanto riguarda la correlazione tra "on-treatment platelet reactivity" misurata nel sangue coronarico e outcomes clinici.

3- BACKGROUND

1- Pathogenesis of acute coronary syndrome in patients with ST-Elevation Myocardial Infarction.

Atherosclerotic plaque rupture or erosion and subsequent thrombus formation constitute the principal mechanisms leading to vessel occlusion in acute ST-elevation myocardial infarction (STEMI) (1-4). In fact, plaque rupture is by far the most frequent cause of arterial thrombosis, accounting for approximately 75% of coronary thrombi leading to myocardial infarction or death (5-7). Exposure of the thrombogenic lipid-rich core in plaque rupture may lead to thrombosis, which covers the rupture site and extends into the lumen (8). Inflammation and increased oxidative stress play important roles in the pathogenesis of atherosclerosis and plaque instability (9, 10). Several studies have focused on the platelet and/or fibrin component of coronary thrombi (11-13), and tissue factor has also been reported to be involved in the pathogenesis of atherosclerosis by promoting thrombus formation (14-17). Coronary thrombi developed as consequence of plaque erosion or rupture, are dynamic and usually evolve in stages (18-24). When the thin fibrous cap of atherosclerotic plaque is disrupted, collagen and tissue factor become exposed to flowing blood, which triggers for accumulation and activation of platelets promoting conversion of fibrinogen to fibrin, thereby initiating thrombus formation (9). Initially, platelets aggregate in the lipid core, and the thrombus begins to protrude into the lumen. The thrombus grows in association with the formation of a fibrin network until the entire vessel is eventually occluded. Fibrin may accumulate over the plaque, stabilizing the thrombus and entraping large numbers of erythrocytes and inflammatory cells, finally forming a red thrombus. The erythrocyte-rich thrombus in this final stage may propagate proximally and distally after the onset of STEMI (9).

The presence of an intracoronary thrombus at the culprit lesion site may increase the risk of distal embolization (DE), resulting in larger myocardial necrosis, microvascular damage and worse residual left ventricular function during primary percutaneous coronary intervention (p-PCI) (25-29). In the last decades, different aspiration thrombectomy and distal protection devices have been

developed in order to limit distal embolization during p-PCI. The evidences coming from many studies involving these devices provided conflicting results in terms of myocardial salvage and clinical outcome. However, the increased use of thrombus-aspiration in p-PCI has enabled the unique opportunity of antemortem evaluation of composition, architecture and dynamic process of coronary artery thrombi (9).

The histopathological evaluations of aspirated intracoronary thrombi obtained from STEMI patients have shown heterogeneity in composition, architecture and chrono-biology of coronary thrombosis. (30-34). In particular, the early studies, investigating intracoronary thrombi obtained from STEMI patients within 6 hours of symptom onset by light microscopy, found that 50% of the thrombi were days or weeks old, before symptomatic presentation of infarct (30), indicating that plaque disruption and thrombus formation occur significantly earlier than the onset of symptoms (32). Moreover, Nagata et al. demonstrated that the dominant cell type of thrombi in the early phase of STEMI was platelets and that large thrombi contained more erythrocytes (31). Moreover, erythrocyte-rich thrombi contained more inflammatory cells and reflected high thrombus burden, resulting in impaired electrocardiographic and angiographic myocardial reperfusion and progression of LV remodeling (35). More recently, it has been demonstrated, by mean of electron microscopy, that the fibrin content increased in association with the ischemic time, whereas the platelet content decreased (36, 37), indicating that ischemic time was a powerful predictor of thrombus composition. Furthermore, thrombus structure clearly appeared to rapidly evolve with respect of ischemic time, paralleling in vivo animal studies, showing that in the very early stage of thrombus formation, just after endothelial injury, its structure is primary composed of activated platelets and rapidly stabilized by fibrin fibers with decreasing proportion of platelets over time (38, 39).

Taking together, all these observations reflect the fact that aspirated thrombi may be older than expected based on the duration of the ischemic time and indicate that partial vessel occlusion leading to acute coronary syndromes (ACS) frequently occurs days or weeks before symptom onset. In addition, another study including STEMI patients presenting within 12 hours of symptom onset revealed three

patterns of aspirated coronary thrombi: 1) platelet-rich, low-erythrocyte thrombi, 2) platelet and erythrocyte-mixed thrombi and 3) erythrocyte-rich, low-platelet thrombi. These three types were observed despite a similar time from symptom onset to reperfusion among the three groups (35). These findings suggest that thrombus components may be dependent on not only by ischemic time, but also by multiple factors influencing inflammation, oxidative stress and blood viscosity. There are some reports related to coronary thrombus and plaque morphology. The first one reported that fibrin was more abundant than platelets in the thrombi of ruptured plaques, whereas platelets tended to be more abundant than fibrin in the thrombi of eroded plaques (11). The second report was on the relationship between thrombus age classified as early (<1 day) or late characterized in phases of lytic (1 to 3 days), infiltrating (4 to 7 days) or healing (>7 days) and plaque morphology in sudden coronary death victims (40). The results showed that late-stage thrombi were present in the 69% of the culprit plaques and that the majority of early thrombi (<1 day) and lytic thrombi (1 to 3 days) were present in plaque ruptures as compared with erosions, whereas the majority of thrombi in erosions were infiltrating (4 to 7 days) or healing (>7 days) (40). The third study was a clinic-pathological study finding that the serum myeloperoxidase (MPO) levels were significantly higher in the patients with eroded plaques than in those with ruptured plaques (41).

In atherosclerotic plaques, macrophages and smooth muscle cells produce large amounts of tissue factor that is the main trigger of the coagulation system. Moreover, abundant tissue factor is expressed in advanced atherosclerotic lesions (11, 42, 43). Therefore, plaque disruption leading to tissue factor exposure to flowing blood, triggers coagulation activation and initiates platelet-fibrin thrombus formation. It has been suggested that abundant tissue factor within plaque is the major factor responsible for the formation of large-size fibrin-rich thrombi and that local/systemic activation of monocytes increases the thrombogenic potential of the blood flow, thus leading to the onset of ACS (44). Recently, also microparticles (MPs) have been reported to play a crucial role in pathogenesis of atherosclerotic plaque. MPs: are 0.1-1-μm membrane vesicles that are released into the extracellular space following cell activation or apoptosis (45). Several studies have proposed that MPs may contribute to atherosclerotic plaque development and

thrombus formation/progression (46-49). In addition, human atherosclerotic plaques contain high levels of MPs, and a large number of human plaque MPs express CD40 ligand and harbor tissue factor, increasing their procoagulant activity (46). Human plaque MPs are reported to be significantly prothrombogenic because they generate twice as much thrombin as circulating MPs in the same patient (50). These heterogeneities in the coronary thrombus composition and microbiology could reflect the thrombogenicity properties of coronary plaques, differing in composition, location and inflammatory pathways status.

2- Antiplatelet therapy in patients with ST-Elevation Myocardial Infarction

The main purpose to reduce myocardial necrosis is to restore coronary flow to the coronary culprit vessel as early as possible. When the clinical symptoms of STEMI occurred within the previous 12 hours and is associated with a persistent ST elevation or with a newly emerging left branch block, p-PCI must be done immediately, otherwise, thrombolytic therapy should be considered as alternative way of reperfusion (51). Several clinical evidences revealed a reduced mortality rate in patients undergoing primary angioplasty in centers that perform a high number of primary angioplasty procedures (52).

Patients undergoing primary angioplasty should receive dual antiplatelet therapy (DAPT) in combination with a parenteral anticoagulant. In general, the most commonly used antiplatelet drugs are Aspirin, ADP receptor antagonists or thienopyridine (Ticlopidine, Clopidogrel and Prasugrel), glycoprotein (GP) IIb / IIIa antagonists (such as Abciximab, Eptifibatide and Tirofiban), PAR-1 antagonists (SCH 530348 and 5555) and phosphodiesterase inhibitors (Dipyridamole, Dilostazol) (53) (Figure 1).

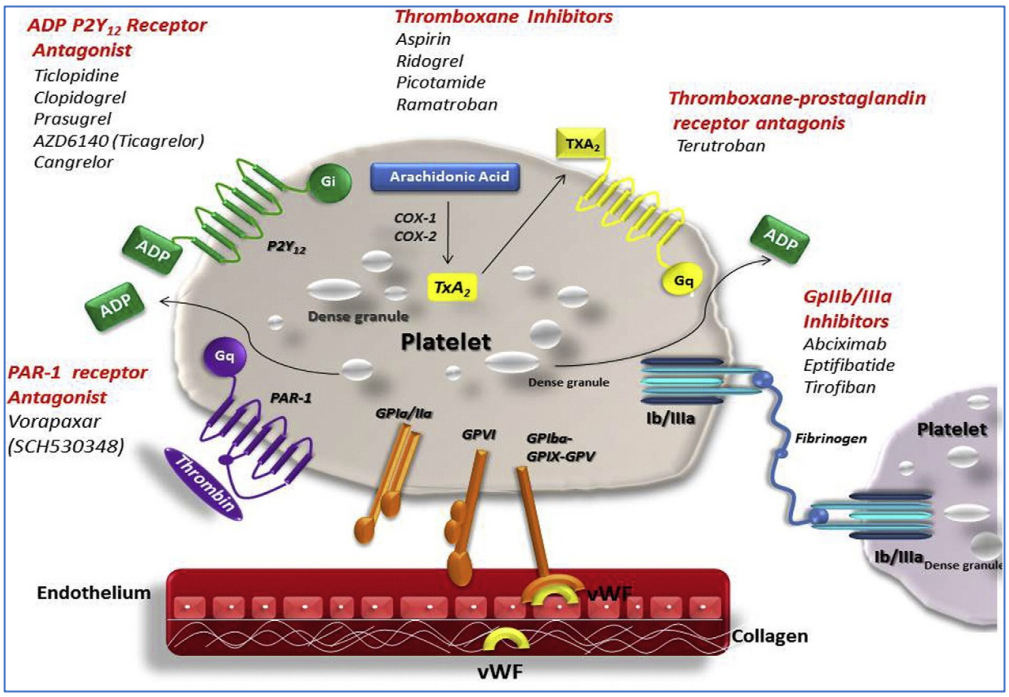

Figure 1. **Pathways of platelet activation and mechanism of action of antiplatelet agents.**

2-1 Thromboxane A2 receptor inhibitor (Figure 1):

In activated platelets, the phospholipase A2 produces thromboxane A2 (TxA2) from arachidonic acid through a series of reactions including an initial step mediated by cyclooxygenase**.** Acetylsalicylic acid inhibits the cyclo-oxygenase enzyme in platelets that converts arachidonic acid into the prostaglandin endoperoxides PGG2 and PGH2; and thus, the subsequent conversion of these (via thromboxane synthase) to TxA2 (54). Three isoforms of cyclooxygenase have been recognized for activation of platelets: COX-1 isoform is expressed ubiquitously and constitutively isoform in larger quantities in platelets; COX-2 is an inducible form that is expressed in cells at inflammation sites; COX-3 denotes a variant of splicing of COX-1 (53).

Acetylsalicylic acid, at low concentrations, inhibits irreversibly COX-1 and COX-3 but not COX-2. By this mechanism, aspirin inhibits platelet activation by reducing but not blocking the thromboxane A2 synthesis at the time of its action at COX-1 level (53).

2-2 ADP receptor inhibitors (Figure 1)**:**

Another important category of antiplatelet drugs are the ADP receptor inhibitors, thienopyridines. There are three forms of ADP receptors in the platelets: $P2Y_1$ and $P2Y_{12}$ are involved in the aggregation as metabotropic ADP receptors, while an ionotropic $P2X_1$ receptor of adenosine triphosphate receptor is involved in the calcium entry (53). Ticlopidine and Clopidogrel are pro-drugs, activated by cytochrome P450, that irreversibly inactivate $P2Y_{12}$ type of ADP receptor through dose-dependent aggregation inhibition (53, 55). Ticlopidine is a very effective drug in inhibition of platelet aggregation but is often associated with side-effects, particularly neutropenia in some patients; for this reason, it has been largely replaced by Clopidogrel (53). Approximately 15% of Clopidogrel is activated by cytochrome P450, while the remaining part is hydrolyzed into inactivate derivatives in circulation by hepatic carboxylesterase (56).

More recently ADP receptor inhibitors (Prasugrel and Ticagrelor) have been developed and always act at $P2Y_{12}$ level of ADP receptor.

Prasugrel is administered as Clopidogrel in the inactive prodrug form and likewise irreversibly inhibits the $P2Y_{12}$ receptor. However, Prasugrel, unlike Clopidogrel, is almost entirely transformed to its active form by allowing higher concentrations of active metabolites and better inhibition of $P2Y_{12}$ receptor.

Ticagrelor is a non-thienopyridine inhibitor of $P2Y_{12}$ receptor; in contrast to others thienopyridine drugs, it is already administered orally as active drug and also produces a reversible inhibition of the $P2Y_{12}$ receptor (56, 57). However, Ticagrelor, unlike Clopidogrel and Prasugrel, makes a reversible rather than irreversible inhibition of platelets function, which means that the drug can come off the receptor when treatment is curtailed and platelet function can be restored.

2-3 Dual antiplatelet therapy

Aspirin and ADP receptor antagonists act through independent and complementary mechanisms to inhibit platelet aggregation, and their combination has been proposed, particularly in patients with ACS who must undergo percutaneous coronary intervention (PCI). To date, the dual antiplatelet therapy with Aspirin and Clopidogrel represents the standard therapy for these patients, although recent studies have approved the use of either Prasugrel or Ticagrelor in combination with aspirin (57).

Aspirin is administered orally at a dose of 150-300 mg to ensure complete inhibition of thromboxane A2 dependent platelet aggregation, but may also be administered intravenously (250-500 mg) in patients unable to swallow (51). Clopidogrel is conventionally administered as loading dose with 300 mg orally or preferably 600 mg, followed by 75 mg as maintenance dose (51); Prasugrel is administered orally with a loading dose of 60 mg and a maintenance dose of 10 mg; Ticagrelor is administered with a 180 mg oral dose and a maintenance dose of 90 mg twice a day (51).

3- Study of platelet functional with Multiplate®, a new Point-of-Care (POC) technology.

Platelet function can be evaluated by platelet aggregation induced by different agonists. Frst of all ADP, the target of thienopyridines (Ticlopidine, Clopidogrel, Prasugrel) and arachidonic acid, which is predominantly affected by Aspirin. The historical method for studying platelet function is represented by platelet-rich plasma aggregation. There are also many systems, so-called "Point-of -Care", which allow the measurement of whole-blood platelet aggregation (58, 80). The different available methods to measure platelet function are reported in Table 1.

The majority of evidence in the field of on-treatment platelet reactivity to antiaggregant drugs refers to a very specific clinical setting: the acute phase of patients with acute coronary syndrome undergoing PCI with stent implantation. It was proposed cutoff values for high and low on-treatment platelet reactivity to antiplatelet drugs associated with post–percutaneous coronary intervention ischemic/bleeding events for various platelet function tests (PFTs). A relevant number of studies has shown that, in this clinical context, the presence of an ADP-induced high on-treatment platelet reactivity (HPR) is associated with a significantly increased risk of ischemic events, in particular of stent thrombosis, cardiac death and MI (59-62, 80) both at early and late follow-up.

	Method principle
Tests based on platelet aggregation	
LTA (light transmission aggregometry)	Photo-optical measurement of LA 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	
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	
TEG	Rate of clot formation based on low shear induced and agonist addition
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

Table 1. Laboratory methods of platelet funcion test.

3-1 Multiplate®analyzer.

The Multiplate® is an instrument that is used for measuring and studying platelet aggregation based on the principles of impedance measurement (63). The use of "impedance" in the study of platelet aggregation was introduced by Cardinal and Flower, who elaborated an instrument equipped with two platinum electrodes that were immersed in the sample and on which it occurred platelet adhesion, result in growing the electrical resistance between the two electrodes (64). Changes in electrical impedance were recorded continuously and varied proportionally as platelets are attached to the electrodes. The main limit of this system was represented by the fact that the electrodes were reusable and had to be cleaned between tests and this was a possible source of error (63). To overcome contamination problem, a new instrument that uses disposable cuvettes (Multiplate® analyzer, Dynabyte Medical, Munich) was introduced. Within each cuvette, there are four sensors, thus giving rise to two independent measurements that increase the reliability of the test. In addition, the Multiplate® has five channels that allow to run up to five tests at the same time for about 10 minutes, which dramatically shortens the time (64). The Multiplate® is a computerized instrument

Figure 2: **Multiplate® Machine.**

with internal Windows XP-based software; the operator, using the mouse and the keyboard, transmits the commands to test (Figure 2).

In each of the five channels of the Multiplate®, a disposable cuvette is provided with two sensor units and a Teflon coated rotating magnet. The cuvette has a mouthpiece in which the blood and other reagents are inserted with the pipette and also has a cup portion in which the two pairs of electrodes long about 3.2 mm are embedded in silver coated. The electrodes protrude in the blood, and on the other hand, they connect to the instrument; so that it can be recorded the resistance between the two electrodes of each sensor unit.

Once the cuvette has been inserted in one of the channels and connected to the instrument cable, we added 300 µl of a specific diluent solution for the agonist to be tested, followed by 300 µl of blood. After three minutes of incubation, 20 µl of the desired agonist is added.

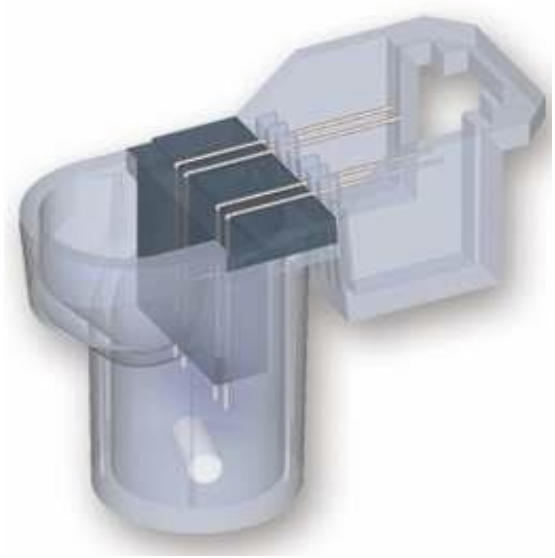

Figure 3. **Multiplate® Cuvette.**

The most commonly agonist reagent used are (65):

1- Thrombin receptor activating peptide-6 (TRAP-6), a thrombin analog that is actually a powerful platelet activator (TRAP test);

2- Arachidonic acid, the substrate of cyclooxygenase which in turn forms as a potent platelet activator thromboxane A2 (ASPI test);

3- Collagen, which binding to its receptor determines the release of arachidonic acid from which thromboxane A2 is produced (COL test);

4- The ADP that stimulates ADP receptors on the platelet surface (ADP test);

5- The ADP with the addition of 20 µl of prostaglandin E that in normal blood induces moderate platelet inhibition, while significantly increasing the sensitivity of the ADP test in patients treated with Clopidogrel (hence the name ADPHS test, high sensitivity) (66).

The activated platelets adhere to the sensor wires; so, the electrical impedance between them increases Figure 4.

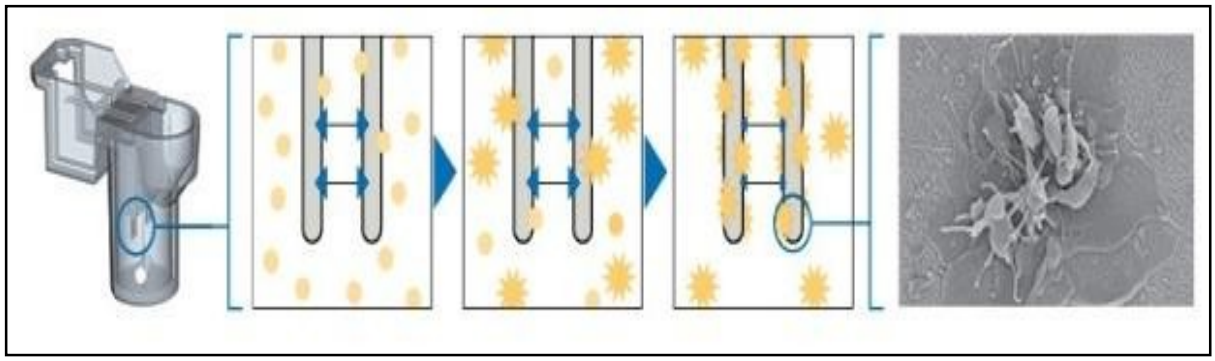

Figure 4. **Multiplate® functional principle:** The platelets adhere progressively to the electrodes resulting in an increase in the impedance.

The increase in impedance in each pair of wires sensor is recorded by the machine, it is transformed into arbitrary aggregation units and placed in relation to time. Thus, two aggregation curves (AU)/time, one for each pair of electrodes are obtained. From each of these, three parameters are obtained: the aggregation, given by the maximum height of the curve; the speed given by the maximum slope of the curve; the area under the curve (Figure 5).

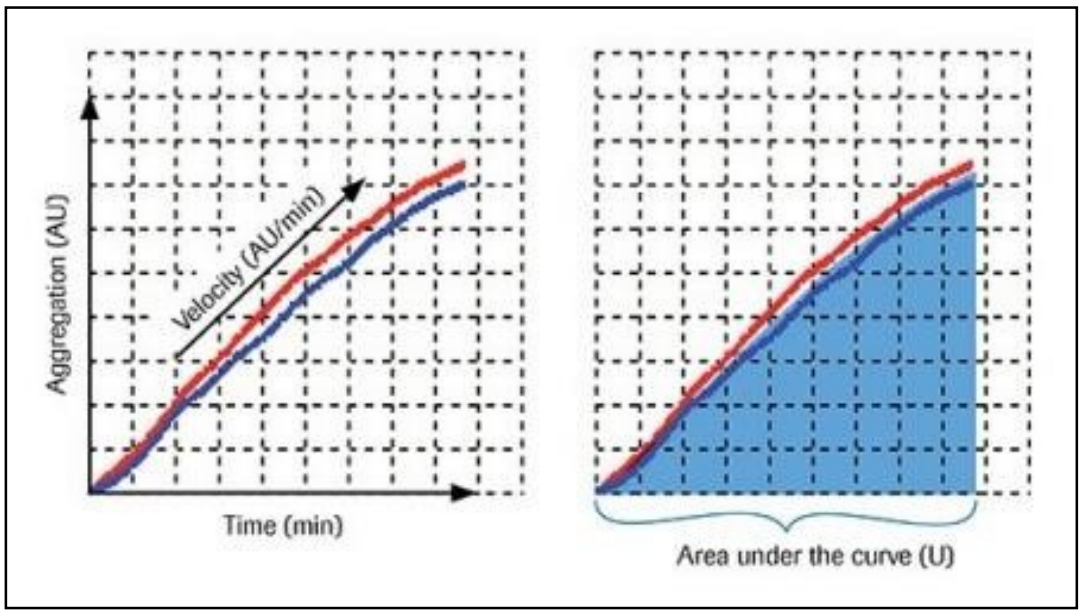

Figure 5. **Representing chart obtained through the Multiplate® and their main values**

The area under the curve (AUC) is the most complete parameter obtained by Multiplate® because it is conditioned by both the maximum height of the curve and the slope, and therefore can be considered as the best indicator of total platelet activity. The area under the curve can be expressed in AU/min or in U where 1U corresponds to 10 AU/min. The analysis is believed to be reliable if the Pearson correlation coefficient between the values of the two curves is less than 0.98 and if the difference between the AUC of each curve and the mean of AUC between the two curves is less than 10%.

3-2 Multiplate® clinical application.

The Multiplate® is applied as a Point-of-Care (POC) method as it's easy to use and rapid information are obtained. In particular, when we have lower AUC values in the absence of surgical bleeding may indicate a reduction in platelet aggregation, whether it is due to reduced platelet count or impaired platelet function (67, 68). In addition, in presence of normal values of Multiplate® can also avoid platelet transfusions, even with low platelet counts, as demonstrated by a study conducted by Rahe-Mayer during and after cardiac surgery (69). Another important use of Multiplate® is the ability to differentiate the effects of various antiaggregation agents by analyzing the various activation pathways (65). Dual antiplatelet therapy (DAPT) with Aspirin and ADP receptor antagonists represents the standard treatment of patients with ACS and patients undergoing angioplasty with stent implantation. Drug metabolism alterations, interactions with other drugs, or other factors may diminish the response to DAPT. Therefore, the Multiplate® is a useful method in monitoring and controlling the effectiveness of therapy. Aspirin resistance is higher in patients with ACS, particularly in those with STEMI (70). The main mechanisms of that have been reported to be: a) reduced bioavailability of aspirin and/or interference with other drugs (eg NSAIDs such as ibuprofen which can prevent the bond of aspirin to serine 530); b) accelerated platelet turnover; c) TXA2 production from COX2 in newformed platelets or other cells; d) lack of compliance, that is probably the most common cause (71).

Coronary angioplasty revascularization interventions could also induce temporary aspirin resistance. Zimmerman and others, in fact, demonstrated that inhibition of TxA2 biosynthesis by aspirin is compromised by a few days after PCI procedure. Although there was a 16-fold increase in platelet COX2, this would not be considered as the cause because specific COX-2 inhibitors do not lead to a reduction in resistance. On the other hand, Kearney and co-workers showed that coronary angioplasty is associated with an increase in TxA2 formation, but it would be completely inhibited by aspirin (72). Also, patients with Clopidogrel therapy had a variable response because of reduced bioavailability for a variable G protein-mediated intestinal absorption, interference with other drugs such as

benzodiazepines and selective serotonin inhibitors (73). However, the main mechanism involved is reported to be the existence of polymorphisms of cytochrome P450, particularly of CYP3A4 and 3A5 isoforms (74, 75).

In patients underwent percutaneous coronary angioplasty and treated with ADP receptor antagonists, ADP test values > 46 U indicate a high thrombotic risk as demonstrated by a study conducted by Sibbing and collaborators (76). In another study conducted by the same group, on patients with the same characteristics, it was observed that ADP test values <19 are indicative of high hemorrhagic risk (123). A study on aspirin-based patients showed that ASPI test values <40 U indicate inhibition of cyclooxygenase 1 (COX-1) by aspirin (77) and ASPI test values <30 U indicate a strong inhibition of COX -1 (78).

4- Clinical impact and variability of response of antiplatelet agents therapy in acute coronary syndrome

Rupture or erosion of an atherosclerotic plaque is by far the most common precipitating event of arterial thrombosis, accounting for approximately 75% of coronary thrombi leading to acute STEMI or death (1-5). Exposure of the thrombogenic lipid-rich core following plaque rupture leads to platelet activation that subsequently adhere to the damaged walls of blood vessels (79). Platelet activation is a key process in pathological thrombosis through the activation of multiple pathways by the binding of several agonists, such as thromboxane A2 (TxA2), adenosine diphosphate (ADP), and thrombin, to their receptors (Fig.1). 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 activates platelets by binding to specific receptors on the platelet ($P2Y_1$ and $P2Y_{12}$ receptors for ADP), whereas TxA2 activates platelets by binding to specific receptors on the platelet prostaglandin G2 and H2 (PGG2 and PGH2) for TxA2. These bindings result in reduced intracellular cyclic adenosine monophosphate levels and full activation of GPIIb/IIIa. Other factors, such as thrombin (most platelet agonist), epinephrine, PGE2, serotonin, and several chemokines, play a role in platelet activation, mainly potentiating platelet activation induced by other stimuli (Fig. 1) (79-83). Consequently, the perpetuation phase of thrombus formation is mediated by the cell to cell contact-dependent mechanisms that lead to change platelet morphology, expression of pro-coagulant and pro-inflammatory molecules, and platelet aggregation (79-83).

As the activation of multiple platelet pathways, in particular ADP and TxA2 platelet activation pathways, is the primary mechanism of thrombosis and ischemic events, their comprehensive inhibition has represented an attractive therapeutic approach for the treatment of athero-thrombotic diseases (Fig. 1). On the other hand, the potential clinical benefits of targeting various platelet activation pathways should be weighed against the likelihood of increased bleeding (80).

In the past few decades, oral antiplatelet agents that inhibit platelet activation by targeting cyclooxygenase 1 (COX-1) [acetyl salicylic acid (ASA) or

Aspirin] or ADP-induced $P2Y_{12}$ receptor pathway [thienopyridines, such as Clopidogrel, Ticlopidine and more recently Prasugrel, and Ticagrelor] have proved to significantly reduce the incidence of ischemic events in patients with atherothrombotic diseases and widely used in the clinical practice (80, 82, 84). While single antiplatelet agent with aspirin is crucial in management of chronic atherosclerotic disease, a dual antiplatelet therapy is highly needed in the presence of acute platelets and clotting activation, as in conditions like ACS unstable angina and myocardial infarction (MI) (80, 85).

The addition of Clopidogrel to Aspirin treatment had been demonstrated to reduce ischemic events in patients with cardiovascular disease. In the CURE trial, patients with non-ST-segment elevation ACS treated with Clopidogrel had a 20% relative risk reduction in the primary end point of cardiovascular death, MI or stroke at the expense of increased nonfatal major and minor bleeding (80, 86, 87). Current guidelines indicate Prasugrel or Ticagrelor as the first line-therapy, followed by Clopidogrel (88). These recommendations were derived from the results of the TRITON-TIMI 38 (89) and PLATO (90) trials, which have demonstrated a superiority of Prasugrel and Ticagrelor to Clopidogrel in terms of efficacy in reducing ischemic events (for Ticagrelor, a reduction was also was found in mortality) at the cost of a significantly higher proportion of bleeding events. These results fit in the situation of the high interindividual variability of Clopidogrel response. Recently, based on studies of Clopidogrel, it was demonstrated that the entity of platelet inhibition on Clopidogrel is a determinant of ischemic and, possibly, bleeding events during the follow-up in patients with ACS (91-93).

4-1 High on-treatment platelet reactivity (HPR).

Notwithstanding the dual antiplatelet therapy is the current standard of care for ACS patients and patients undergoing stent implantation, recurrent ischemic events often occur during dual-antiplatelet therapy (94). Up to 25% of patients with acute MI undergoing PCI were found to have variable response to Clopidogrel, predisposing them to recurrent events of ACS (95). However, Gori et al. reported that the incidence of dual nonresponsiveness to Aspirin and Clopidogrel is a relatively infrequent (about 6%) condition and the incidence definite/probable drug-eluting stent thrombosis was significantly higher in dual Aspirin and Clopidogrel nonresponders than in Clopidogrel and Aspirin responders, isolated Clopidogrel nonresponders, or Aspirin nonresponders (96). The possibility to study platelet function, formerly with complex laboratory techniques (e.g. light transmission aggregometry) and more recently with Point-of-Care devices (e.g. multiple electrode aggregometry) has made it possible to identify patients with high platelet reactivity while taking one or multiple antiplatelet drugs (97). In particular, high on-aspirin platelet reactivity (HaPR) and high on-clopidogrel platelet reactivity (HcPR) have been associated with a higher risk for major cardiovascular events in patients with ACS (91, 98-100) (Figure 6). However, whole blood platelet function tests are influenced by several pre-analytical variables (e.g. platelet count, haematocrit, method and site of sample collection, temperature, anticoagulant used, etc.) leading to a lack of standardization and ultimately to clinical misinterpretation (101). There are conflicting evidences in current literature with respect to the influence of the site of sample collection on platelet function (102-106).

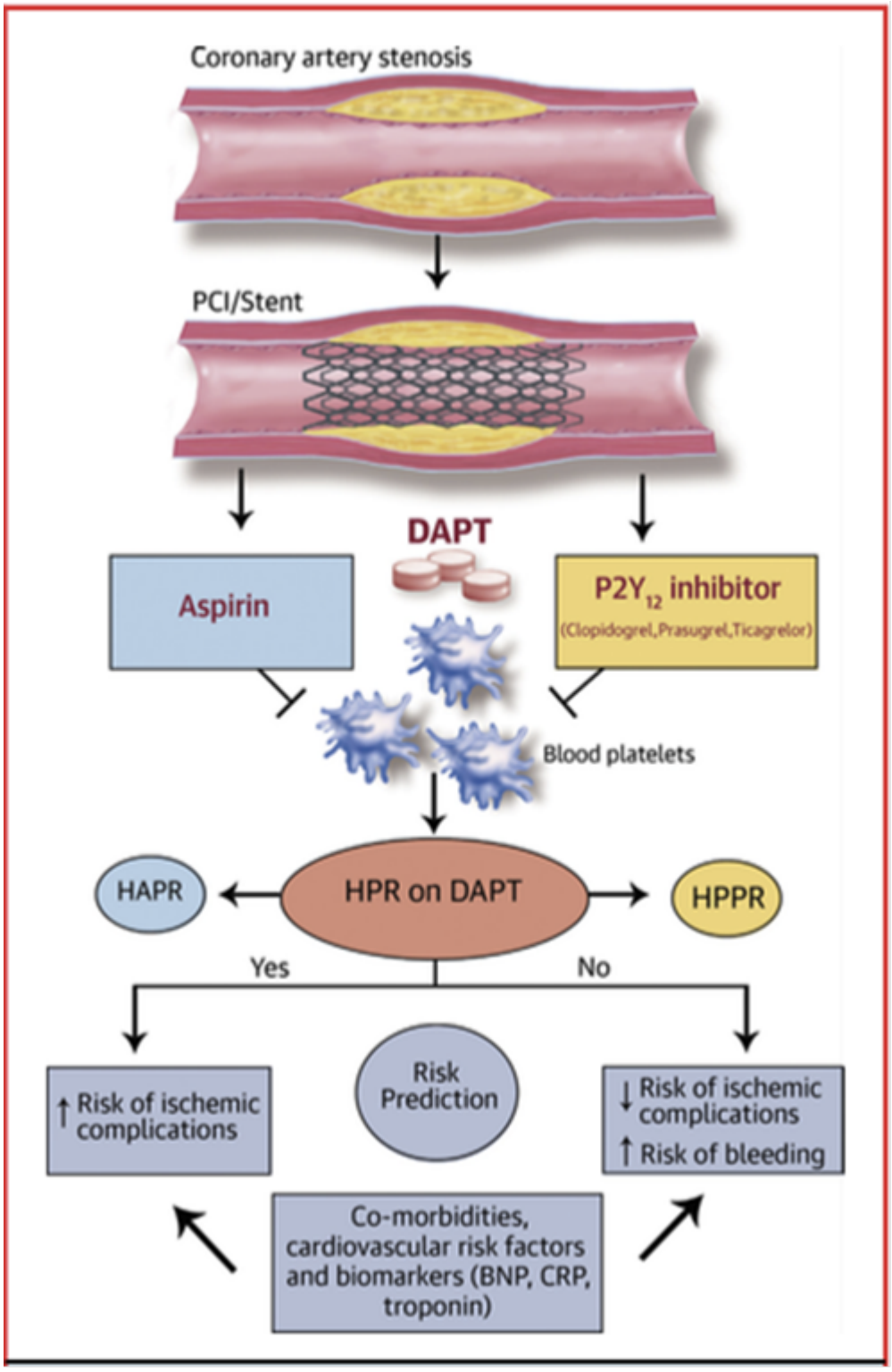

Figure 6. **Platelet test function and risk of adverse outcome after PCI.** BNP: brain natriuretic peptide; CRP: C-reactive protein; DAPT: dual antiplatelet therapy; HPR: high on-treatment platelet reactivity; HPPR: high on-$P2Y_{12}$ inhibitor platelet reactivity; PCI: percutaneous coronary intervention.

4-1-1 High on-aspirin reactivity (HaPR):

It has been demonstrated the role of Aspirin reactivity by the ISAR-ASPI registry (94) in which PCI treated patients with HaPR showed a significantly higher risk of death or stent thrombosis at 1 year. HaPR, measured at the time point of the PCI, was associated with a higher risk for death or stent thrombosis during the first year after PCI. It has been established that diabetic patients with ACS associated with significant higher risk of HaPR and recurrent events than nondiabetic ACS patients (80, 107).

In spite of sharp declines in restenosis rates with the use of drug-eluting stents (DES), diabetic patients with ACSs remain at the highest risk for recurrent ischemia (80, 108). Recently, multiple studies have suggested that diabetes is an independent predictor of stent thrombosis and lower survival rates in patients treated with DES (80, 109, 110). It has been proposed that diabetic patients not treated with aspirin therapy generally display HaPR and elevated levels of platelet thromboxane synthesis, and on other hand, in diabetic patients, Aspirin is less effective in inhibiting thromboxane synthesis than in nondiabetic patients (80, 111). In the ASPECT randomized study, it was shown that, diabetic patients had a higher platelet function and consequently Aspirin resistance than nondiabetic patients. In general, increasing the dose of Aspirin in the diabetic patient to reduced platelet function and thus the prevalence of Aspirin resistance to levels observed in the nondiabetic patient, suggests that low-dose aspirin therapy may not provide adequate platelet inhibition in selected diabetic patients and that higher Aspirin dosing reduces the prevalence of aspirin resistant patients (80, 112). A systematic review combined data from multiple studies to examine the relationship between daily aspirin dose and prevalence of HaPR showed diabetic patients were 36% more likely to have HaPR compared with nondiabetic ones (80, 113).

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 another study with an 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 (80, 114). Others possible causes of high on Aspirin platelet reactivity are listed in figure 7.

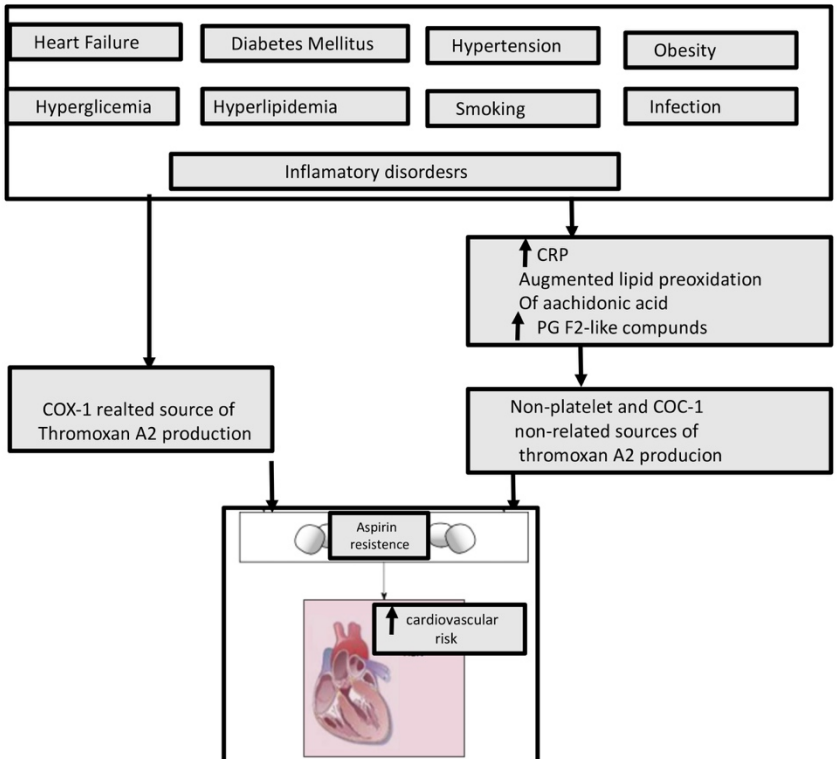

Figure 7. **The possible causes of aspirin failure response.**

4-1-2 High on–clopidogrel platelet reactivity (HcPR).

Clopidogrel has a peculiar metabolism which has been ameliorated by the other new antiplatelet agents. First of all, the hepatic conversion by cytochrome P450 is required. Studies measuring platelet function in patients administered Clopidogrel showed that this treatment is associated with an overall variable response and modest level of $P2Y_{12}$ inhibition even when high loading doses are used (80, 115-117) In addition to distinct response variability, a considerable percentage of patients will also exhibit complete non-responsiveness to Clopidogrel (80). However, genetic play important role in the phenomenon HcPR, which is associated with a number of other clinical and laboratory parameters, in addition to the possible interaction with other drugs. Different CYP450 isoenzymes involved in Clopidogrel metabolism have been associated with inactivation of the enzyme and impaired metabolism of Clopidogrel. CYP2C19*2 genetic polymorphism, however, does not fully explain the association between HcPR and adverse events observed in patients taking Clopidogrel: even within a population which is not carrying the CYP2C19*2 polymorphism, which is consequently able to metabolize Clopidogrel, the extent of ADP-induced inhibition of platelet function remains a marker of risk of adverse events (80, 118). The failure in Clopidogrel response may be due to others chronic conditions, such as, inadequate drug compliance, drug–drug interactions, age, diabetes mellitus, elevated body mass index (BMI), female sex and reduced left ventricular ejection fraction (LVEF). All these mechanisms could interact with the genetic predisposition in determining inadequate response to Clopidogrel and consequently the increased risk of occurrence of major adverse cardiovascular events (MACE), including stent thrombosis. A particularly important aspect of the possible existence of acquired determinants of HcPR is the role of ‘acute phase’: it was shown how inflammation and increased platelet turnover that characterize the acute phase of ACS are associated with increased platelet reactivity and with a higher risk of HcPR during treatment (119). Others transient mechanisms include inflammation, accelerated platelet turnover, reticulated platelets (RPs), erythrocyte deformability, and the activity of ADAMTS13 (80, 120). Certainly, re-evaluating the patients away from the early phase of an acute inflammation event, the percentage of subjects with HcPR

decreases significantly, suggesting that, when the inflammation related to the acute phase is extinguished, the degree of platelet reactivity, and therefore the percentage of patients with an inadequate inhibition, are also significantly reduced (80). Moreover, Martin et al. noted that platelets of patients with ACS had an increased mean volume, probably due to the presence of RPs, newly formed platelets with a higher granule content. An increased plasma level of RPs is also claimed to be independent predictor of recurrent MI and cardiac death (121). Definitely, it has been demonstrated that a more significantly elevated percentage of RPs is present in patients with HcPR compared to 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 (119). Others possible causes of high on Clopidogrel platelet reactivity are shown in figure 8.

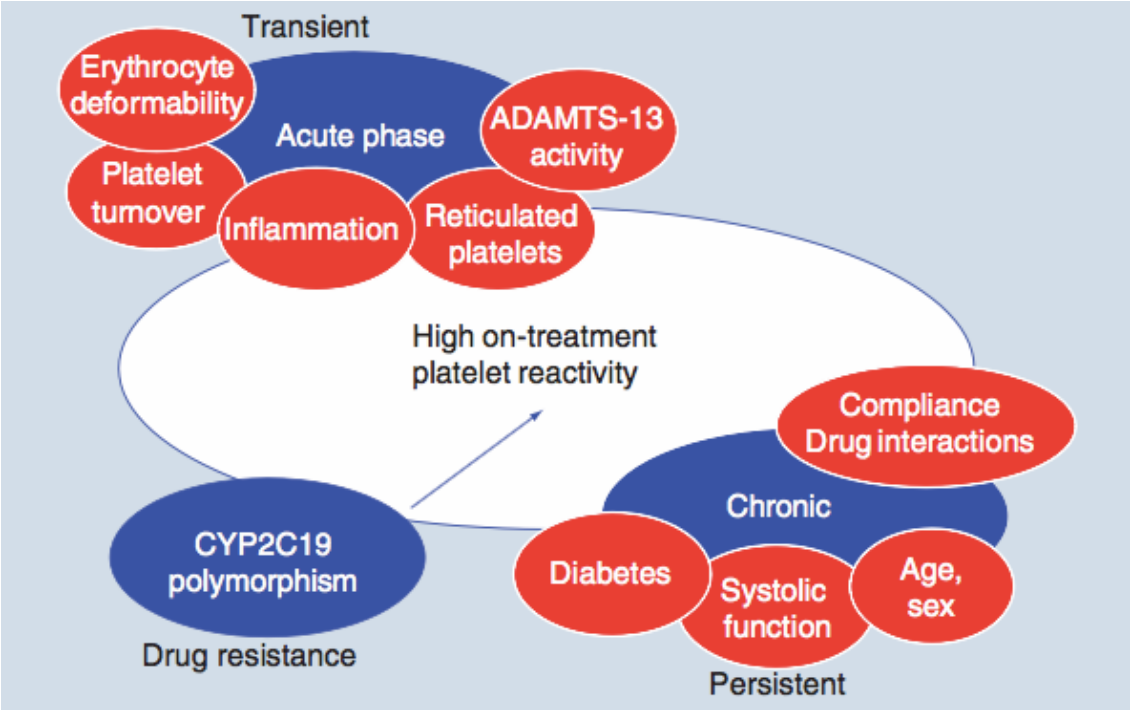

Figure 8. **The possible causes of Clopidogrel failure response.**

4-2 Low on-treatment platelet reactivity (LPR):

In the clinical setting of dual antiplatelet therapy in ACS, bleeding was often considered as an expectable complication. Both TRITON TIMI-38 (89) and PLATO (90) demonstrated a superiority in the reduction of ischemic events of Prasugrel and Ticagrelor on Clopidogrel, but both with a significant higher bleeding risk. 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-term and long-term morbidity and mortality in cardiovascular patients during long-term antiplatelet and anticoagulant therapy (80, 122). Observational studies involving patients undergoing PCI have suggested a possible link between LPR and bleeding (80, 123-128). Parodi et al. reported that patients undergoing PCI with LPR on Prasugrel therapy had more frequent access site bleeding (129). A recent collaborative analysis on 17 studies, 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 (80, 130). Hypothesized preliminary concept of therapeutic window of platelet reactivity on dual antiplatelet therapy, that similar to the international normalized ratio (INR) range used for warfarin therapy, is shown in figure 9 (97).

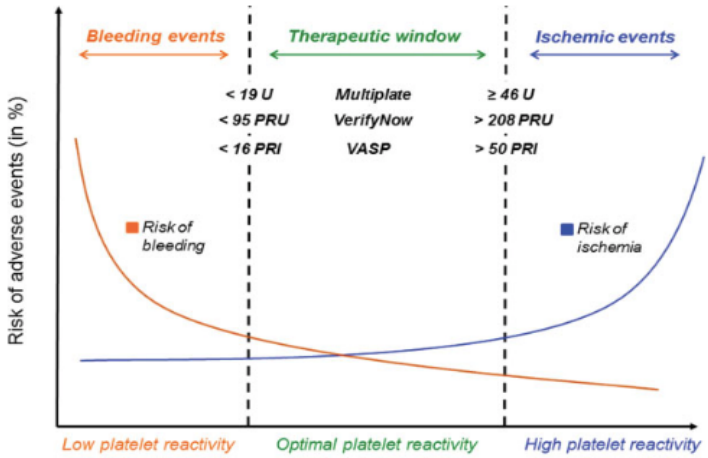

Figure 9. **On-treatment platelet reactivity and therapeutic window concept.** PRI: platelet reactivity index, PRU: P2Y12 reaction units, U: unite and VASP: vasodilator-stimulated phosphoprotein

4- AIM OF THE STUDY

The aim of our study was to assess the on-treatment platelet reactivity in peripheral and coronary arterial blood, in a group of patients receiving dual antiplatelet therapy following primary percutaneous coronary intervention (p-PCI) for STEMI.

5- MATERIAL AND METHODS

5-1 Study design

This is a cohort observational study based on prospective registry of patients undergoing primary percutaneous coronary intervention (p-PCI) for STEMI.

5-2 Patients eligibility

All consecutive patients who underwent p-PCI for STEMI at the Cardiology Unit of Padua University Hospital between June 2014 and June 2015 were considered for enrollment.

5-2-1 Inclusion criteria

Inclusion criteria were: continuous chest pain for at least 20 min within 12 h from onset and (i) ST-segment elevation ≥1 mm (0.1 mV) in two or more contiguous leads on the 12-lead electrocardiogram or (ii) persistent ST-segment depression in precordial leads V1-V4, with or without ST-segment elevation in inferior or lateral leads or (iii) new onset left bundle branch block.

5-2-2 Exclusion criteria

Exclusion criteria were: (i) patients who reperfused with thrombolytic therapy before PCI, (ii) contraindication for antiplatelet therapy, (iii) variant angina (iv) prior coronary bypass surgery, (v) severe renal dysfunction (creatinine clearance < 30 mL/min/1.73 m2), (vi) concomitant chronic inflammatory or malignant disease, (vi) pregnancy, (vii) thrombocytopenia (platelet count ≤100 x109/L) and (viii) refusal to sign written informed consent.

5-3 Biochemical analysis and timing of blood sampling

A group of 109 healthy volunteers age- (±3 years) and sex-matched with cases were selected as controls to establish reference values of the laboratory parameters measured in the study. Before the procedure, all patients, at emergency room or coronary care intensive unit, received: a) intravenous unfractionated heparin (starting dose 70 U/kg) to maintain an activated clotting time ≥ 250 s; b) intravenous aspirin 250 mg; c) oral Clopidogrel 300/600 mg, Prasugrel 60 mg or Ticagrelor 180 mg.

Primary percutaneous coronary intervention (P-PCI) was performed following the standard radial approach, or femoral approach in patients with unsuitable radial arteries.

5-3-1 Preparation of blood samples

Peripheral blood samples were collected from patients right before the p-PCI procedure, using a 19-Gauge needle and 4 BD vacutainer tubes (Becton Dickinson, Franklin Lakes, New Jersey) containing sodium citrate 109 Mmol/l (3.8% sodium citrate) and 1 BD Vacutainer containing Ethylenediaminetetraacetic acid 5.4 mg (EDTA) were filled. Another blood sample was drawn from the coronary arterial catheter guide after cannulation of the infarct-related artery (IRA). Specifically, 2 BD Vacutainer tubes (Becton Dickinson, Franklin Lakes, New Jersey) containing sodium citrate 109 Mmol/l (3.8% sodium citrate) and 1 BD Vacutainer containing Ethylenediaminetetraacetic acid 5.4 mg (EDTA) were filled. Peripheral and coronary arterial blood samples from each patient were used, in accordance with standard procedures, to measure the following parameters: hemoglobin (Hb, gr/dl), hematocrit (Htc, %), platelet count (x109/L), activated partial thromboplastin time (aPTT, sec.), prothrombin time (PT, %), d-dimer (ug/L), fibrinogen (g/L) and antithrombin (AT, %).

One-hundred samples of peripheral and coronary blood were collected, with the same measurement mentioned above, as control group from group of patients underwent coronary angiography for reasons other than ischemic heart disease.

5-3-2 Point-of-Care platelet reactivity assays

Both peripheral and coronary arterial blood samples were used to determine impedance aggregometry via the Multiplate electrode aggregometry (MEA, Roche Diagnostics SpA, Monza, Italy), in accordance with the manufacturer's protocol. Specifically, aggregation in response to arachidonic acid (ASPI-test) and ADP (ADP-test) was tested within 30 minutes of blood collection. The apparatus was heated in order to maintain the measurement temperature above 36 °C. We placed

300 µl of a diluent solution inside designated cuvettes and then added 300 µl of citrated blood from the patient at room temperature. The diluent solution was physiological saline (0.9% NaCl) at 37° C for the ASPI-test and NaCl-CaCl2 (0.9% NaCl with 3 mM CaCl2) at 37° C for the ADP test. Following a 3-minute incubation, we added 20 µl of 15 mM arachidonic for the ASPI-test and 20 µl of 0.2 mM ADP for the ADP-test.

After adding the reagent, the instrument automatically measures the change in electrical impedance between two sensor electrodes (e.g. aggregation curves, AU/time). Each cuvette contains two pairs of electrodes. The measurement ends after a standard time of 6 minutes. The parameter used to describe the curve was area under the curve (AUC) corresponding to the first derivative of the mean area under the curves measured by the two pairs of electrodes. The higher the AUC value the higher the capacity of platelets to aggregate, the lower the AUC value the lower the capacity of platelets to aggregate (131). "Low responders of antiplatelet therapy" were considered when an AUC value of ASPI-test or ADP-test greater than or equal to a pre-established normal range. The samples with a coefficient of variation greater than or equal to 10% between the two curves obtained in each cuvette were rejected and the test repeated. The control group samples underwent validation test with the same measurement mentioned above for both ASPI-test and ADP-test.

5-4 Interventional procedure

P-PCI were performed with standard technique by radial approach, or femoral approach in patients with unsuitable radial catheterization. After guide-wire positioning in the distal IRA, one or more manual thrombus aspiration attempts were made by Export catheter. Thrombus aspiration were based on step-by-step technique, starting just proximally to culprit lesion, and advancing the aspiration catheter very slowly through the lesion, to avoid distal embolization of debris removed by catheter tip. Repetition of this maneuver was accomplished according to angiographic results obtained. In particular, if the thrombus was not

reduced/removed additional attempts were performed using the same technique. The IRA was the only target of the procedure, and deployment of coronary bare metal stents or drug-eluting stents was left at operator's discretions. The procedure was considered successful if Thrombolysis In Myocardial Infarction (TIMI) 3 in the target vessel and a residual stenosis ≤ 20% at target site were obtained. Before the procedure, all patients, at emergency room or coronary care intensive unite, received: a) intravenous unfractionated heparin (starting dose 70 U/kg) to maintain an activated clotting time ≥ 250s; b) intravenous Aspirin 250 mg; c) oral Clopidogrel 300/600 mg, Prasugrel 60 mg or Ticagrelor 180 mg. All patients had been given written informed consent to the procedure.

5-5 Angiographic analysis

Coronary angiograms were acquired by using digital technique (Integris 5000, Philips Medical Systems, Best, The Netherlands). In order to better define the thrombus burden in case of IRA occlusion, the angiographic pattern of coronary occlusion, when present, was defined on baseline angiogram as follows: (i) cut-off pattern, when there was an abrupt occlusion of the epicardial vessel; (ii) tapered occlusion, when there was a vessel tapering just before the occlusion; (iii) persistent dye pattern, when there was a dye staining just proximally and/or distally to the occlusion (28). Intracoronary thrombus well is angiographically identified and scored in 5 grades as previously described (132). According to this classification, in thrombus grade 0 (G0), no cineangiographic characteristics of thrombus were present; in thrombus grade 1 (G1), possible thrombus were present, with such angiography characteristics as reduced contrast density, haziness, irregular lesion contour, or a smooth convex meniscus at the site of total occlusion suggestive but not diagnostic of thrombus; in thrombus grade 2 (G2), there were definite thrombus, with greatest dimensions ≤ 1/2 the vessel diameter; in thrombus grade 3 (G3), there were definite thrombus but with greatest linear dimension > 1/2 but <2 vessel diameters; in thrombus grade 4 (G4), there were definite thrombus, with the largest dimension ≥ 2 vessel diameters; and in thrombus grade 5 (G5), there was total occlusion (unable to assess thrombus burden due to total vessel occlusion). Subsequent PCI was performed for total occlusive lesions or lesions with >75%

diameter stenosis (DS), even with TIMI grade 3 flow. Thromboaspiration was performed whenever possible (when the anatomy of the coronary artery-curve and size-allowed it) in all patients with a Thrombolysis In Myocardial Infarction (TIMI) flow grade 0 and in all patients with a visible thrombus if TIMI flow grade was 1 or more.

Thrombosis In Myocardial Infarction (TIMI) flow and myocardial blush were assessed as previously reported (133). Distal embolization (DE) was defined as a distal filling defect with an abrupt 'cut-off' in one or more peripheral coronary branches of IRA, distal to the PCI site; in particular, we would not consider as DE the occurrence of TIMI flow impairment, at any stage of the procedure, without evidence of distal filling defect (28, 134). Angiographic no-reflow was defined as substantial coronary antegrade flow reduction (less than Thrombolysis in Myocardial Infarction [TIMI] flow grade 3) without mechanical obstruction (135). All angiographic and procedural parameters, including thrombus classification, were assessed by 2 experienced interventional cardiologists reviewing the angiograms together, who were unaware of the patients' characteristics. Both reviewers were blinded to clinical outcomes. Consensus were achieved in all patients. Half of the films were randomly selected and reanalysis by the same analysts for intraobserver variability, and by a third experienced interventional cardiologist for interobserver variability of the proposed large thrombus burden lesion (LTBL) and small thrombus burden lesion (STBL) classification. Quantitative coronary analysis was performed at baseline and after procedure, using the Coronary Quantification Package (Philips Medical Systems): minimal lumen diameter, reference vessel diameter, percent of stenosis in diameter will have provided; lesion length will also be assessed at baseline angiography, or after vessel reopening (by either wire positioning or balloon pre-dilatation) in patients with occluded IRA at baseline.

5-6 Electrocardiographic analysis

In each patient, a 12-lead electrocardiogram was recorded at admission and 60 + 30 min after the procedure. The sum of ST-segment elevation was assessed at

20 ms from J-point in the leads V1 – V6, I, and aVL for anterior infarction and in the leads II, III, aVF, V5, and V6 for non-anterior infarction; in the latter, the ST-segment depression in leads V1 – V4 was also analyzed, as a sign of transmural ischemia of the posterior wall. The two electrocardiograms were compared and the ST-segment elevation was classified as normalized if there was no residual ST-segment elevation after the procedure, improved if a regression ≥ 50% was seen and unchanged if the ST-segment elevation sum appeared unchanged, worsened, or regressed ≤50% (136).

5-7 Measures of outcome

Major adverse events occurring during the hospitalization, including death, non-fatal re-infarction, heart failure and stroke, stent thrombosis, bleeding and need to urgent intervention were collected. Diagnosis of non-fatal re-infarction bases on typical chest pain and/or new ST-segment changes with Troponin I level re-elevation (28, 137). The Troponin I levels were reported as micrograms per liter, and assessed every 6 hours during the first 48 after admission, and then twice daily up to discharge (28, 137). Peak value release from eight serial measurements up to 48 hours after admission was reported (28, 137). Stroke defined as development of new cognitive or neurological deficit confirmed by computed tomography or magnetic resonance imaging (28, 137). All patients underwent 2D-transthoracic echocardiographic examination before discharge.

5-8 Clinical follow-up

All patients were discharged on aspirin (100 mg) indefinitely and Clopidogrel 75 mg once a daily, Prasugrel 10 mg once a day or Ticagrelor 90 mg twice a day for 6–12 months. The follow-up protocol included an evaluation at hospital discharge and at 12-month follow-ups. The 1-year clinical outcomes included all-cause death, MI, target vessel revascularization, stent thrombosis and bleeding. Major adverse cardiac events (MACE) were a composite of all-cause death, MI, and TVR during a 1-year follow-up.

5-9 Statistical methods

Statistical analysis was performed using the PASW Statistics 17.0.2 (SPSS Inc.) for Windows. Continuous variables were expressed as mean ± standard deviation (SD). Differences between frequency distribution according to qualitative variables were calculated using Chi-square test. The parametric t-Student test or the non-parametric Mann–Whitney U was used to test for differences between variables, where appropriate. For multiple comparisons (differences between three groups), the Kruskall-Wallis test was used. Spearman's correlation was used to detect significant associations between variables. For the clinical outcomes during the follow-up a descriptive statistical analysis was used (i.e. prevalence, percentage and chi-square test). Upper and lower limits of reference range in contolswere calculated as mean+2SD and mean-2SD. P value <0.05 was considered statistically significant.

6- RESULTS

During the study period one hundred eighteen patients were considered for enrollment. Four patients were excluded because they did not receive thienopyridines before the procedure, three refused to give their informed consent and two had severe renal insufficiency. Eventually, 109 patients (age 64±13 years, Male/Female 79/30) were enrolled. On the issue of cardiovascular risk factors, 56 (51%) patients were active smokers, 71 (65%) suffered from hypertension, 60 (55%) with dyslipidemia and 26 (24%) with diabetes. The main baseline characteristics of the studied population are summarized in the Table 2.

The elapsed time (mean ± SD) between the administration of aspirin and thienopyridines and the start of procedure was 81±63 min and 61±53 min, respectively.

Subjects involved in the study		
Pt. enrolled, n.		109
Male/female, n. (%)		79 (72) / 30 (28)
Age (years), mean age ± standard deviation		64 ± 13
Age range , years		34-94
Active smoker, %		51
Comorbidities	Arterial hypertension, %	65
	Diabetes mellitus II, %	24
	Dyslipidemia,%	55

Table 2: Main baseline characteristics.

6-1 ASPI-test and ADP-test

In ASPI-test, normal AUC values were considered between 35U and 87U. On peripheral blood, the mean values were 23 ± 4 U, however below the normal range, as well as at the coronary level, where the mean values were even lower (17 ± 2 U). Comparison between the two medians by student T test showed no statistically significant difference between mean values for peripheral and coronary blood, with a p-value equal to 0.06 (Table 3, Figure 10 a).

In ADP-test, normal AUC values range between 39U and 79U. On peripheral blood test, the mean values were 41 ± 28 U, i.e. a value within the normal range. The same was observed in the ADP-test performed for coronary blood, where, however, the mean values were lower (39 ± 28 U), however within the normal range. In particular, the comparison between the two averages through student's T test showed that the difference between the two means was not statistically significant, with a p-value =0.68 (Table 3, Figure 10 b).

Thus, no statistically significant differences were observed in ASPI-test between peripheral (23±4 U) vs coronary (17±2 U, p=0.06) blood and in ADP-test between peripheral (41±28 U) vs coronary (39±28 U, p=0.68) blood.

	Peripheral blood	Coronary blood	P
AUC in ASPI- test (U)			
Mean	23±4	17± 2	0.06
Median (10-90 Percentiles)	19 (3-49)	12 (1-40)	
AUC in ADP-test (U)			
Mean	41± 28	39±28	0.68
Median (10-90 Percentiles)	40 (14-82)	33 (7-79)	

Table 3: Mean and Median AUC values obtained in ASPI-test and in ADP-test by Multiplate® methods. Median (10-90 Percentiles)

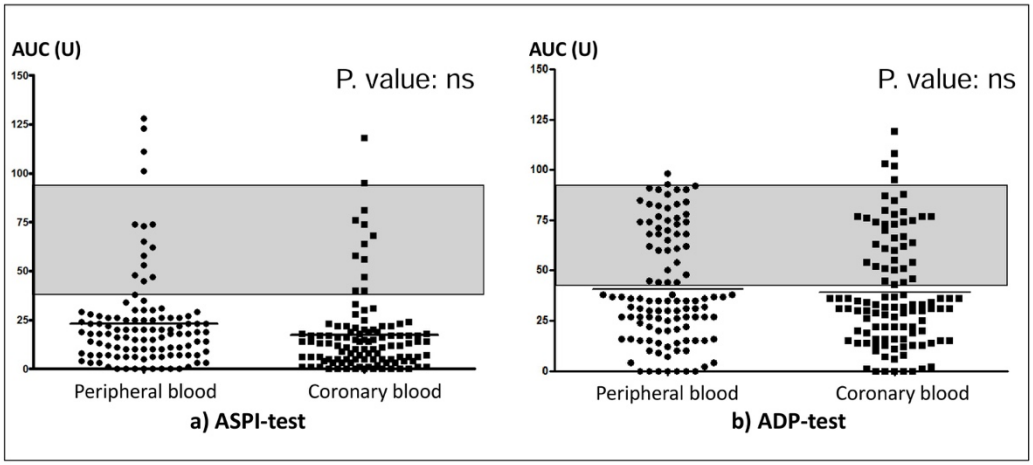

Figure 10: Distribution of mean AUC in peripheral and coronary blood: a) ASPI-test and b) ADP-test. The horizontal line inside the box represents the median AUC levels. Grey area refers to normal range.

These data show that the overall platelet reactivity in coronary blood is lower than in peripheral blood, though without statistical significance, it more likely appears due to highly DAPT effect at atherosclerotic plaque ulceration/thrombus formation site where the hemostatic process is highly active at onset of STEMI.

In consideration of normal mean AUC values of ASPI-test observed in healthy subject control were (61±13 U), with an observed statistically significant difference in ASPI-test between patients (either in peripheral and in coronary blood) and controls (Figure 11). Similarly, in ADP-test, being the normal AUC values (59±10 U), we observed a significant difference in ADP-test between patients (either in peripheral and in coronary blood) and healthy individual controls (Figure 11).

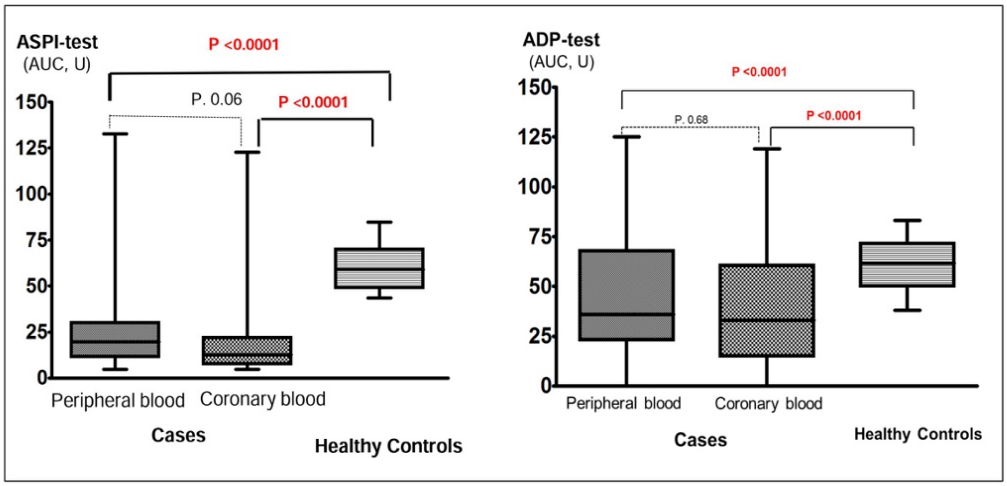

Figure 11: ASPI-test and ADP-test in diseased patient blood (peripheral and coronary) in comparison with control group.

Further data emerged from the study, we found statistically significant linear correlation between low responders to acetylsalicylic acid (evaluated by ASPI-test) and low responders to ADP receptor inhibitors (assessed by ADP-test) (Figure 12). That means, patients with an AUC value in ASPI test within or above the normal range tend to have an AUC value in ADP-test either within or above the range in the same sample of blood; as well, patients with AUC below the normal ASPI-test values tend to have values below the range in ADP-test in same sample of blood.

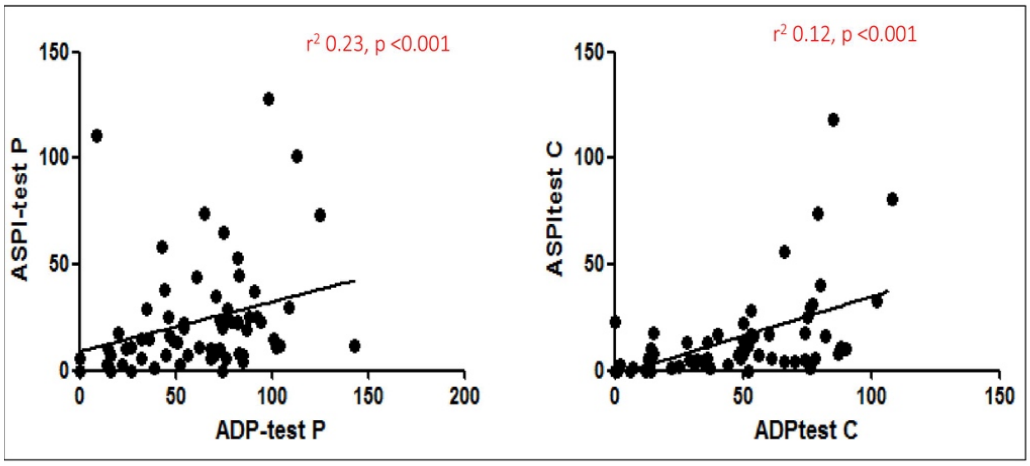

Figure 12: Linear correlation relationship between ASPI-test values and ADP-test values in peripheral blood and coronary blood. C= Coronary blood; r^2= Coefficient of correlation; P=peripheral blood.

In summery; a positive and statistically significant linear correlation was observed for both ASPI-test and ADP-test in peripheral and coronary blood (r^2 0.23, p <0.001 and r^2 0.12, p <0.001; respectively) (Figure 12).

No statistically significant correlations were observed with respect to age, hematocrit, antithrombin, PT, aPTT, fibrinogen and D-Dimer plasma levels for either ASPI-test or ADP-test in peripheral and coronary blood except for platelet count (Table 4). In both peripheral and in coronary blood, no significant correlation was observed in regard to the elapsed time between the administration of aspirin and the ASPI-test, and between the administration of thienopyridines and the ADP-test (Table 4)

	ADP-test P	ASPI-test P	ADP-test C	ASPI-test C
Age (years)	r 0.02; p 0.84	r 0.033; p 0.73	r 0.05; p 0.60	r 0.02; p 0.84
Hematocrit, %	r 0.09; p 0.35	r 013; p 0.17	r 0.14; p 0.15	r 0.08; p 0.40
Platelet count x109/L	r 0.33; p 0.0001	r 0.34; p 0.0001	r 0.26; p 0.063	r 0.33 p 0.0001
Fibrinogen, g/L	r 0.14; p 0.15	r 0.18; p 0.06	r 0.14; p 0.15	r 0.14; p 0.15
AT, %	r 0.32 p 0.48	r 0.02 p 0.55	r 0.15 p 0.63	r 0.19 p 0.08
D-DIMER, ug/L	r 0.20 p 0.37	r 0.01 p 0.08	r 0.04 p 0.84	r 0.58 p 0.14
APTT, sec	r 0.41 p 0.56	r 0.29 p 0.21	r 0.02 p 0.09	r 0.01 p 0.74
PT, %	r 0.26 p 0.07	r 0.062 p 0.39	r 0.06 p 0.48	r 0.16 p 0.25
Time between ASA administration and ASPI-test, min	-	r 0.21; p 0.28	-	r 0.06; p 0.53
Time between Thienopyridines administration and ADP-test, min	r 0.11; p 0.25	-	r 0.12; p 0.21	-

Table 4: Comparison of classic coagulation parameters & elapsed time between the administration of antiplatelet drugs and ASPI/ADP tests.

APPT= activated partial thromboplastin, AT= antithrombi, PT= prothrombin time, r= coefficiente of correlation.

In a subgroup analysis of 15 patients <50 years (range 34-49), we observed no significant difference neither in peripheral nor in coronary blood between this subgroup of patients and the overall study population in regard to ASPI-test and ADP-test results or low responders to antiplatelet therapy (Table 5).

	Peripheral blood			Coronary blood		
	Age < 50	**Age > 51**	**P value**	**Age < 50**	**Age > 51**	**P value**
ASPI-test, U (mean±SD)	22±2	23±7	0.87	19±8	17±4	0.72
ADP-test, U (mean±SD)	41±26	44±29	0.74	38±22	40±30	0.79
"low responders", n (%)						
Aspirin	2 (13)	12 (13)	0.71	1 (6)	9 (10)	0.95
Thienopyridines	5 (33)	27 (29)	0.65	5 (33)	26 (28)	0.71

Table 5: Subanalysis according the age of population.

6-2 Low Aspirin/Thienopyridines responders

In our study, the values of AUC of ASPI-test and ADP-test (mean ± SD) in the group of controls were 61±13 U and 59±10 U, respectively. Having established cut-off values of 35 U for ASPI-test and 39 U for ADP-test, corresponding to the respective lower limits of the normal range measured in controls (from 35 U to 87 U for ASPI-test and from 39 U to 79 U for ADP-test). We observed, in peripheral blood 15 (14%) patients with an ASPI-test value (e.g. "low aspirin responders") and 41 (38%) patients with an ADP-test value (e.g. "low thienopyridines responders") above their respective cut-offs (Table 6). In the coronary blood, we observed 12 (11%) patients with an ASPI-test value and 39 (36%) patients with an ADP-test value above their respective cut-off (Table 6).

	Peripheral blood	Coronary blood	Healthy controls
ASPI-test, U (mean±SD)	23±4	17±2	61±13
ADP-test, U (mean±SD)			59±10
in patients taking thienopyridines	41±28	39±28	-
- in patients taking lopidogrel	47±31	44±33	-
- in patients taking prasugrel	39±27	39±28	-
- in patients taking ticagrelor	39±28	37±32	-
"low responders", n (%)			
Aspirin	15 (14)	12 (11)	-
Thienopyridines	41 (38)	39 (36)	-
- Clopidogrel	10 (45)	9 (40)	-
- Prasugrel	25 (36)	25 (36)	-
- Ticagrelor	6 (33)	5 (29)	-

Table 6: Mean values of ASPI-test/ADP-tests and the prevalence of "low aspirin/thienopyridines responders" in the study population. SD, standard Deviation.

No significant difference (p >0.05 in all comparisons) between this group of patients with both ASPI-test and ADP-test results above the predefined cut-offs and the overall study population with respect to age (63±14 vs 65±13 years), sex (Male/Female 9/3 vs 70/27; respectively), platelet count (229±175 vs 272±130 x109/L), fibrinogen (3.7±1.3 vs 3.9±1.10mg/dl) and elapsed time between drugs administration and aggregometry evaluation (ASPI-test 82±65 vs 69±42 min; ADP-test 63±55 vs 42±36 min) (Table 7). Moreover, there was no significant difference on the prevalence of smoking (67 vs 51%), hypertension (67 vs 66%),

hypercholesterolemia (75 vs 53%) and diabetes (25 vs 24%) in subjects with both ASPI-test and ADP-test results above the predefined cut-offs compared with the study population (p >0.05 in all comparisons) (Table 7).

	Low Responder		
	ASPI-test	ADP-test	P value
Age (years)	63±14	65±13	0.66
Smoking	67%	51%	0.53
Hypertension	67%	66%	0.62
DM tipo II	25%	24%	0.81
Dyslipidemia	75%	53%	0.91
Hematocrit, %	39.4±4.0	40.9±3.8	0.23
Platelet count x10^9/L	229±175	272±130	0.28
aPTT, sec	63±80	56±51	0.48
PT, %	6.9±1.5	6.9±1.4	0.98
Fibrinogen, g/L	3.70±1.30	3.90±1.10	0.57
D-Dimer, ug/L	141±92	109±29	0.13
AT, %	87±10	86±11	0.77
Elapsed time between DAPT administration & p-PCI, min	82±65	63±55	0.36

Table 7: Comparison of low responders to antiplatelet therapy observed in ADP-test and ASPI-testing according to baseline characteristics of study population.

By definition, patients who are taking antiplatelet drugs and have mean AUC values within or above the normal range are considered to be low responders to antiplatelet therapy. Regarding the ADP-test, where the normal AUC values between 39U and 79U, it was observed that in peripheral blood, 38% patients did not respond as expected to ADP inhibitor therapy. At a coronary level, patients who had AUC values greater than 39U at the ADP-test were 36%. By comparing percentages through the chi-square test, it was found that the difference in low responder to ADP inhibitors in peripheral and coronary blood was not statistically significant (Figure 13).

In relation to the ASPI test, normal AUC values are between 35U and 87U. In peripheral blood analysis, 14% patients were found to be low responders to acetylsalicylic acid treatment. On coronary blood, the number of low responders to treatment was 11%. Applying the chi-square test for the comparison of percentages,

there was no statistically significant difference between the percent of low responders to acetylsalicylic acid between peripheral and coronary blood (Figure 13).

Finally, we directly compared the mean values obtained in ADP-test and ASPI-test initially in the peripheral blood then in coronary blood, based on the fact that the normal range for both tests is approximately similar (39-79 U and 35-87 U, respectively) and we observed a statistically significant difference between low responders to ADP receptor inhibitors (evaluated with ADP-test) and low responders to acetylsalicylic acid therapy (evaluated with ASPI–test) in both peripheral blood and coronary blood. In particular, it was found that the prevalence of low responders to thienopyridine was significantly greater than to acetylsalicylic acid, both in peripheral blood and in coronary blood, with a p-value less than 0.01 (Figure 13). Twelve (11%) in peripheral blood and ten (9%) patients in coronary blood had both ASPI-test and ADP-test results above the cut off values.

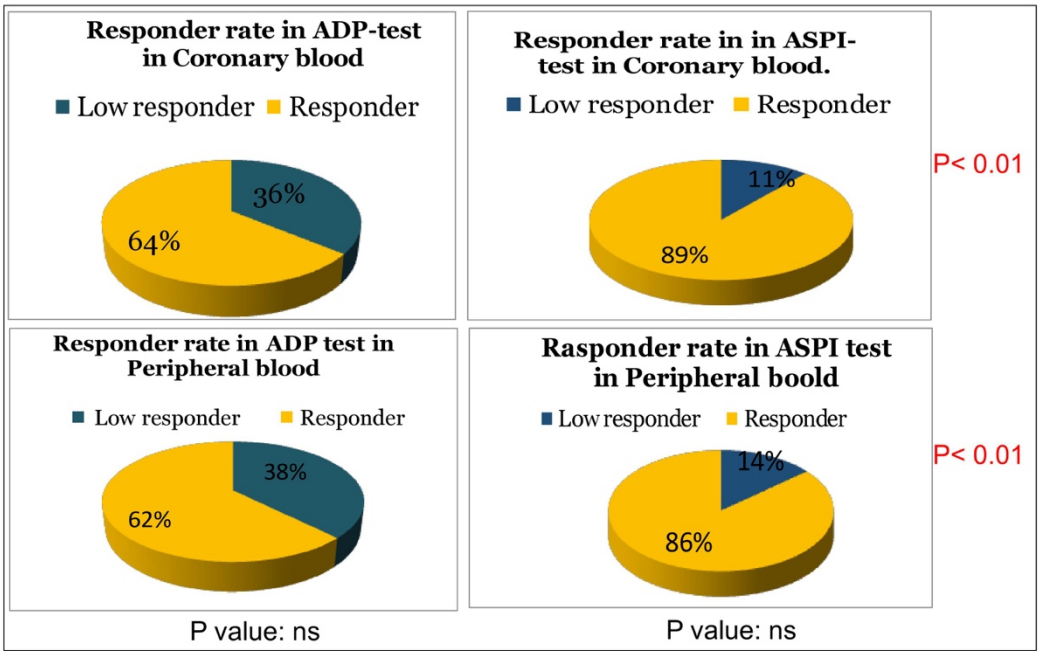

Figure 13: Percentage of low responders to antiplatelet therapy assessed by ADP-test and ASPI-test in both peripheral and coronary blood.

The aggregometry Multiplate® results showed responsiveness failure to antiplatelet treatment with $P2Y_{12}$ inhibitors, in at least one third of cases, at time of p-PCI.

We found statistically significant linear correlation between low responders to acetylsalicylic acid (evaluated by ASPI-test) in coronary blood and low responders to the same test in peripheral blood. Likewise, for low responders to ADP receptor inhibitors (assessed by ADP-test) in coronary blood had significant linear correlation relationship to the same test in peripheral blood. That means; interestingly enough almost all patients with a coronary ASPI-test value above the cut-off also had a peripheral value of the same test above the cut-off as well as for ADP-test (Figure 14). This significant direct correlation between platelet reactivity in peripheral and in coronary blood is still a matter of debate.

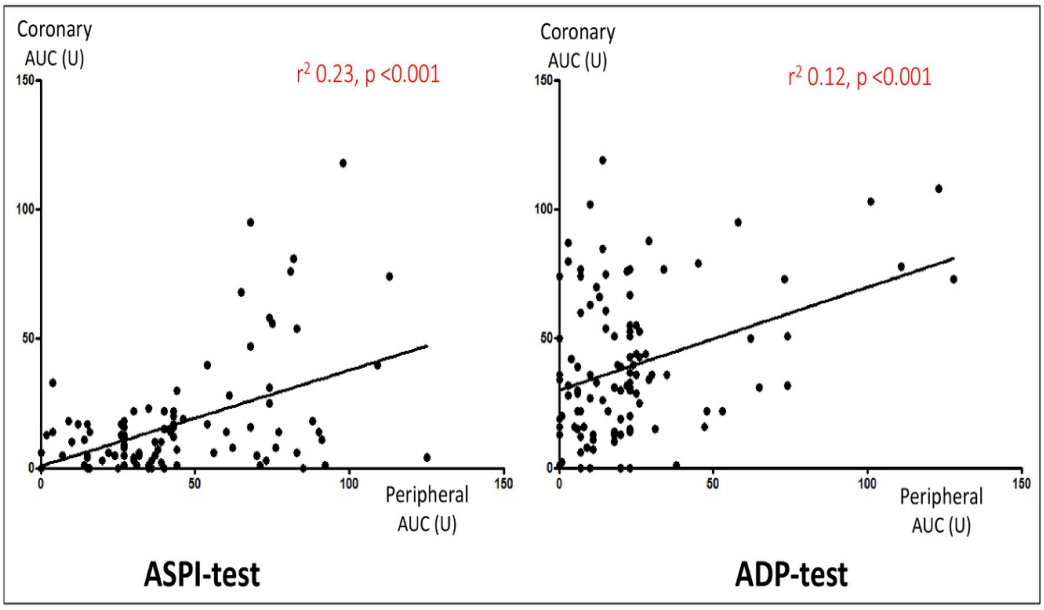

Figure 14: Linear correlation between ASPI-test and ADP-test in peripheral vs coronary blood.

6-3 Clopidogrel, Prasugrel and Ticagrelor

Before the PCI procedure, antiplatelet therapy was performed in all cases with the use of Aspirin 250mg I.V in combination with one of the following ADP receptor inhibitors: Clopidogrel 300/600 mg, Prasugrel 60 mg or Ticagrelor 180 mg. The prevalence of thienopyridines was distributed as follow: Clopidogrel n 22 (20%), Prasugrel n 69 (63%) and Ticagrelor n 18 (17%) (Figure 15).

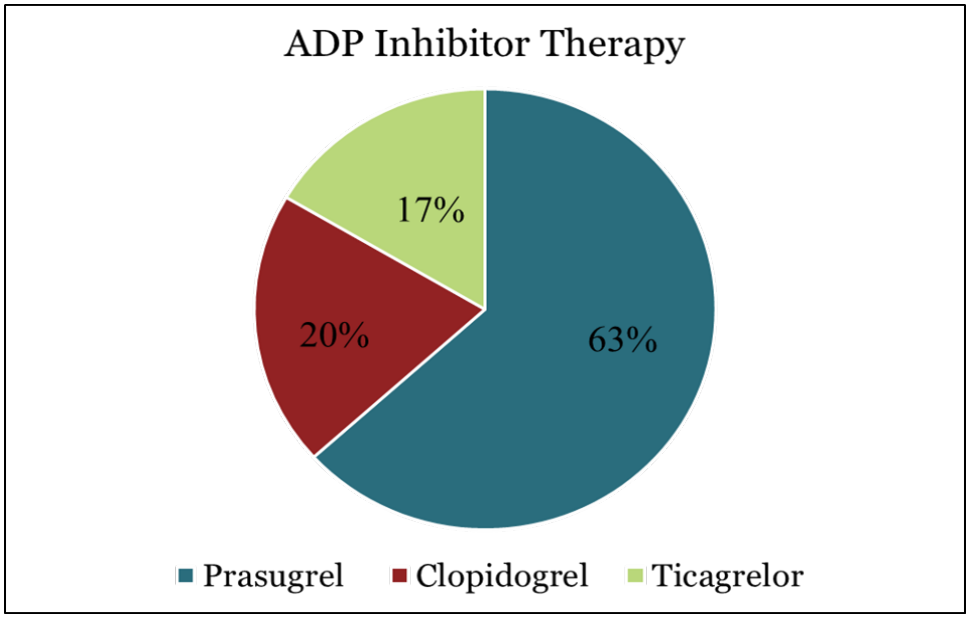

Figure 15: Choice of ADP receptor inhibitor in dual antiplatelet therapy.

The main baseline characteristics of the studied population regarding the use of ADP receptor inhibitors (Clopidogrel, Prasugrel and Ticagrelor group) are summarized in the Table 8.

	Clopidogrel	Prasugrel	Ticagrelor
Patients enrolled (109)	22	69	18
Age, (years)	60±11	60±11	71±12
Sex, no	13 M/9 F	56 M/13 F	10 M/8 F
Smoker, %	11	39	7
Arterial Hypertension, %	18	39	14
Diabetes Mellitus II, %	6	17	3
Dyslipidemia, %	13	34	13

Table 8: Main baseline characteristics of population according to thienopyridine drugs. M: male, F: Female

The study attempted to identify a relationship between "low responders to antiplatelet therapy" and the type of drug used. In particular, mean values of AUC in ADP test were compared among the three groups of patients, according to the thienopyridine used. In peripheral blood, ADP-test results were 47±31 U in patients taking Clopidogrel, 39±27 U in patients taking Prasugrel and 39±28 U in those taking Ticagrelor (Table 6). In coronary blood, ADP-test results were 44±33 U in patients taking Clopidogrel, 39±28 U in patients taking Prasugrel and 37±32 U in those taking Ticagrelor (Table 6). Irrespective to the thienopyridine administered, no statistically significant difference was found comparing ADP-test results in peripheral vs coronary blood (Figure 16). Regarding peripheral blood among patients taking Clopidogrel 10 (45%) had ADP-test values above the predefined cut-off; a lower prevalence of "low responders" was observed among patients taking Prasugrel 25 (36%) and Ticagrelor 6 (33%) (Table 6).

No significant difference (p =0.69) was observed on the prevalence of patients with ADP-test values above the cut-off considering the three different thienopyridines separately. As for coronary blood, 9 (40%) taking Clopidogrel, 25 (36%) patients taking Prasugrel and 5 (29%) patients taking Ticagrelor had ADP-test values above the predefined cut-off (Table 6). However, these findings were not statistically significant (p >0.05). That means, the antiplatelet therapy during a STEMI seems to be equally effective in inhibiting platelet function both in peripheral and in coronary blood.

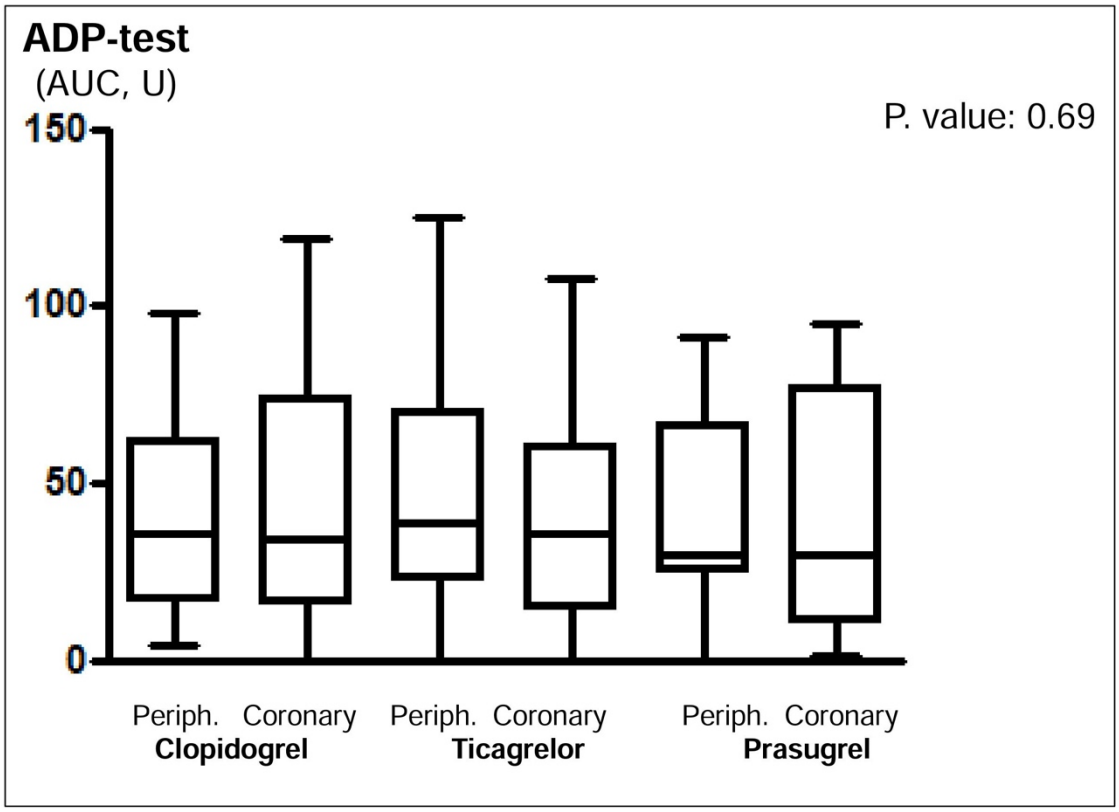

Figure 16. ADP-test results in peripheral and coronary blood in relation to Clopidogrel, Prasugrel and Ticagrelor treatment. In each box plot, the lower and upper bars represent the 10th and 90th percentiles, respectively; the lower and upper ends of the box represent the 25th and 75th percentiles, respectively; and the horizontal line inside the box represents the median AUC levels

6-4 Clinical outcome.

Three cardiovascular deaths (2.8%) occurred during hospitalization, two of which due to cardiogenic shock and one due to cardiac rupture. Cardiogenic shock occurred in 8 patients (7.3%) whereas two cases (1.8%) had congestive heart failure. Myocardial re-infarction needing to urgent re-intervention occurred in 3 cases (2.8%); one of them underwent coronary artery by-pass grafting and two cases underwent re-PCI. There were three cases (2.8%) of sub-acute stent thrombosis and two patients (1.8%) had a non-fatal ischemic stroke. Five patients had major bleeding that needed urgent blood transfusion. The overall incidence of in-hospital major bleeding events in these patients was 4.6%. Five patients (4.6%) had combined major adverse cardiovascular events (death, non-fatal MI and target vessel revascularization) (Table 9).

Death	3 (2.8%)
Due to cardiac rupture	1 (33,3%)
Due to cardiogenic shock	2 (66.6%)
Cardiogenic Shock	8 (7.3%)
CHF	2 (1.8%)
Re-MI	3 (2.8%)→ 1 CABG & 2 PCI
TVR	2 (1.8%)
Stroke	2 (1.8%)
Subacute stent thrombosis	3 (2.8%)
Major Bleeding	5 (4.6%)--> 4 transfusion
Minor bleeding	6 (5.5%)
MACE (death, Re-MI, TVR)	5 (4.6%)

Table 9. In-hospital outcome. CHF= Congestive heart failure; MACE= Major adverse cardiovascular events; Re-MI= Re-myocardial infarction; TVR= Target vessel revascularization.

Out-of-hospital clinical outcomes at 12 months (Table 10)

Clinical follow-up at 12 months was completed in all of patients. After discharge, there was one cardiovascular death (0.9%). Non-fatal MI occurred in two patients (1.8%) whereas angina/NSTEMI happened in six patients (5.5%). Out of them, 3 patients (2.8%) underwent target vessel revascularization. Atypical non cardiac chest pain with evaluated in emergency room occurred in 5 cases (4.6%). None of these patients had late stent thrombosis nor major or minor bleeding. All other events like congestive heart failure, tachyarrhythmia and hypertensive crisis had occurred in about 2% of cases and needed re-hospitalization. As a total six patients (5.5%) had combined major adverse cardiovascular events (death, non-fatal MI and target vessel revascularization).

Death	1 (0.9%)
Re-MI	2 (1.8%)
Angina /NSTEMI	6 (5.5%)
Non cardiac chest pain	5 (4.6%)
CHF	2 (1.8%)
Tachyarrhythmia	2 (1.8%)
Hypertensive crisis	2 (1.8%)
Stroke/Bleeding	0 (0%)
Subacute stent thrombosis	0 (0%)
TVR	3 (2.8%) → PCI
MACE (death, Re-MI, TVR)	6 (5.5%)

Table 10. Out-of-hospital clinical outcomes. CHF= Congestive heart failure; MACE= Major adverse cardiovascular events; MI= Myocardial infarction; TVR= Target vessel revascularization.

Our data showed no correlation between platelet reactivity function and clinical outcome neither for in-hospital outcome nor for clinical outcomes at 1-year, most probably due to the small size of the population of the study.

7- DISCUSSION

The increasing, and sometimes conflicting evidence in current literature describing the peculiar aspects of regional (e.g. venous vs arterial blood) differences in platelet reactivity motivated the endeavor to study the intracoronary platelet function using a whole blood Point-of-Care test based on the principle of impedance aggregometry (102-106). In particular, we aimed to assess on-treatment platelet reactivity in peripheral and coronary blood in a group of patients treated with dual antiplatelet therapy following p-PCI for STEMI.

The role of coagulation in the onset and evolution of ACS has recently been the subject of various studies aimed to detect a possible causal relationship between the presence of systemic hypercoagulability state and clinical consequences. In this study, patients underwent dual antiplatelet therapy and anticoagulant therapy prior to the sample withdrawal, so the values obtained by Point-of-Care methods, in particular the Multiplate® aggregometer, were influenced by both acute clinical condition of the patient and antiplatelet drug therapy. Accordingly, we expected presence of ASPI-test and ADP-test AUC values below the normal range and we considered "Low responders" to antiplatelet therapy when an AUC value of ASPI-test or ADP-test greater than or equal to a pre-established normal range. In our study; a greater tendency of low platelet reactivity in coronary blood, however differences between values of AUC of both ASPI-test and ADP-test in coronary blood and peripheral blood were not statistically significant. This appears more likely to be due to high antiplatelet drugs effect at plaque ulceration/thrombus site, where the hemostatic process is highly active at onset of STEMI.

Our findings suggest that coronary arteries could provide an adequate and reliable site of blood sampling to study platelet function with MEA. Moreover, antiplatelet therapy during a STEMI seems to be equally effective in inhibiting platelet function in peripheral or in coronary blood. The results of our study are in line with previously published work by other groups that found similar results in platelet function measured in venous and in arterial blood (104-106). We found no correlation between platelet function and hematocrit (138). Likewise, there was no significant correlation between higher on-treatment reactivity and gender (139,140)

or cardiovascular risk factors - mainly diabetes - as previously reported (141,142). The most plausible explanation could be related to the relatively small sample size unsuitable to prove the existence of any such differences.

In addition, the platelet reactivity in coronary blood was not significant inferior to that observed in peripheral blood. We detected a significant correlation between platelet activity in peripheral and in coronary blood, i.e. the presence of a linear correlation between the "low Aspirin responders" observed by the ASPI-test and the "low ADP receptor inhibitors responders" observed by the ADP test. Moreover, it has been observed that those who are low responders to acetylsalicylic acid tend to be low responders also to the ADP receptor inhibitors and vice versa, and who is sensitive to acetylsalicylic acid therapy with curtain to be sensitive to receptor inhibitors for ADP. These data remain a matter of debate, but could identify a subgroup of patients with "cross non-responsiveness" to the two drugs, which may have a worse prognosis in terms of clinical outcomes (e.g. acute or subacute thrombosis of the stent) compared to the group of subjects not responding to one of the two treatments and even more to the patients responsive to both drugs.

The novelty of our study resides in measuring the platelet function in subjects taking Prasugrel and Ticagrelor, two drugs not taken into account by previous studies. In particular, we studied a group of controls and determined an "in-house" threshold (e.g. the lower limit of the normal range) both for the ASPI-test and the ADP-test to identify patients with a reduced response to antiplatelet therapy (e.g. subjects with a platelet function in ASPI-test or ADP-test above the aforementioned cut-off). With that in mind, the prevalence of "low aspirin responders" was approximately 14% both in peripheral and in coronary blood, a similar number (11%) to that previously reported (104,143). The prevalence (38%) of "low thienopyridines responders" observed in our study, both in peripheral and in coronary blood, was in line with the data reported in literature to date (144). To come to the point, we observed the failure of responsiveness to antiplatelet treatment with $P2Y_{12}$ inhibitors in at least one third of cases, at time of p-PCI. The most reasonable explanation may be the different route of administration of both types of drugs. In particular, being intravenous for aspirin and oral for ADP receptor

inhibitors, it could contribute to explain the major prevalence of low responders observed in both peripheral blood and coronary blood to ADP-test in compared to the ASPI test. In fact, it is likely that the bioavailability of aspirin, in terms of rapid onset of action and high plasma concentration, is greater than that of ADP receptor inhibitors. Remarkably, the prevalence of "low Prasugrel/Ticagrelor responders", though smaller than that observed in patients taking Clopidogrel, was higher than previously reported (145,146). In our study, thienopyridines administrated during a STEMI underwent emergent p-PCI, appeared to be equally effective in inhibiting platelet function both in peripheral and in coronary blood. Moreover, all patients who exhibited higher on-treatment platelet reactivity in coronary blood also had higher on-treatment platelet reactivity in peripheral blood.

In this study, we observed no correlation between platelet reactivity function and clinical outcome neither for in-hospital nor clinical outcomes at 1-year, most probably due to small size population of the study. The clinical significance of laboratory "aspirin and/or thienopyridines resistance" remains a matter of debate and larger studies are needed for in-depth assessment of any correlation between on–treatment platelet reactivity measured in coronary blood and clinical outcome. If a definitive close correlation will be established between platelet function in peripheral and/or coronary blood and possible clinical outcomes (e.g. intrastent thrombosis), all patients on antiplatelet therapy for acute coronary syndrome might benefit from a routine monitoring of the platelet activity in order to tailor the antiplatelet therapy to the patient's test results and unique needs. In this regard, features such as the use of whole blood, automatic method, and bedside Point-of-Care device would make MEA more suitable than the other platelet function assays. The possibility to measure the platelet function with a bedside Point-of-Care device could also play a crucial role in identifying patients with a high responsiveness to antiplatelet therapy and therefore at a higher risk for bleeding complications (147).

Needleless to say, one very important issue to consider while comparing the results of different studies on platelet function assessment is the pre-analytic conditions of each study which can strongly influence the results (148-152). Such pre-analytic conditions include, but are not limited to, methods of sample

collection, temperature and anticoagulant used. Consequently, we set out to curb variability in pre-analytics between venous and arterial blood samples by following strict procedures in both the collection and the management of samples. In particular, trained personnel managed blind samples swiftly and each sample was managed at room temperature. The use of an "in-house" reference range established in a group of healthy individuals, whose samples were obtained and managed as mentioned above, contributed to reducing pre-analytic bias.

Some limitations need to be acknowledged, the sample of patients enrolled while homogeneous, was too small to lose the significance of the differences that emerged in some comparisons (e.g. between ASPI-test values peripheral vs coronary or ADP-test values peripheral vs coronary blood). Moreover, despite impedance aggregometry test performed through MEA being a widely validated methodology in the study of platelet function in patients with coronary artery disease (153), it carries a high inter-individual variability that prevents standardization. Similarly, the citrate as anticoagulant in the tubes lacks standardization even though its use has been suggested in literature as a possible alternative way to draw samples for aggregometry (154,155). Finally, the direct comparison between peripheral and coronary blood could be affected by the ex vivo platelet activation in the latter setting. Young A et al. (156) have previously argued that this is not the case, but we cannot rule out this bias with certainty.

8- CONCLUSIONS

The study observed that the overall platelet reactivity in coronary blood is lower than in peripheral blood, though without statistical significance. This more likely appears to be due to high antiplatelet drugs effect at plaque ulceration/thrombus site, where the hemostatic process is highly active at onset of STEMI. Larger studies are necessary to better evaluate these mechanisms in terms of pharmacodynamic, pharmacokinetic and receptor kinetic properties of antiplatelet agents.

Antiplatelet therapy during a STEMI seems to be equally effective in inhibiting platelet function both in peripheral and in coronary blood. The other interesting data emerging from data processing is the high incidence (about 30%) of low response thienopyridine type antiplatelet drugs at the time of primary angioplasty. These results, moreover known for Clopidogrel, also include patients treated with Prasugrel and Ticagrelor. An explanation of this phenomenon, which also involves very potent drugs, requires careful analysis of further studies. The significant direct correlation between platelet reactivity in peripheral and in coronary blood is still a matter of debate. Larger studies are needed for in-depth assessment of any correlation between on–treatment platelet reactivity measured in coronary blood and clinical outcome.

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