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Role of platelet reactivity and leptin in cardiovascular outcome of patients undergoing percutaneous coronary intervention

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….to my family…

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STATEMENT OF ORIGINALITY

The work described in this thesis was carried out at the University Campus Bio-Medico, Rome.

The author designed the studies that are reported in this thesis and/or analyzed and described the results.

I hereby state that this thesis entitled **"Role of platelet reactivity and leptin in cardiovascular outcome of patients undergoing percutaneous coronary intervention"** has not submitted for a degree or any other qualification at any other University.

Elisabetta Ricottini

ABSTRACT

RESEARCH PROJECT N.1

Correlation of Platelet Reactivity and C-Reactive Protein Levels to Occurrence of Peri-Procedural Myocardial Infarction in Patients Undergoing Percutaneous Coronary Intervention (from the ARMYDA-CRP Study)

Background. The incremental predictive value of high inflammatory status and high on-treatment platelet reactivity (HPR) on the occurrence of periprocedural myocardial infarction (PMI) after percutaneous coronary intervention (PCI) has not been characterized. The aim of this study was to evaluate the correlation of elevated C-reactive protein (CRP) level and/or HPR with the incidence of PMI in patients who undergo PCI.

Methods. Five hundred consecutive patients treated with clopidogrel who underwent PCI had preprocedural measurement of CRP levels and platelet reactivity using the point-of-care VerifyNow P2Y12 assay. Elevated inflammatory status was defined as CRP >3 mg/L and HPR as P2Y12 reactivity units ≥ 240. The primary endoint was the incidence of PMI in relation to platelet reactivity and/or inflammatory status.

Results. Rates of PMI were increased in patients with CRP levels >3 mg/L (10.9% vs 4.6% in those with normal levels, odds ratio 2.4, 95% confidence interval 1.2 to 4.5, P=0.015) and in patients with HPR (11% vs 5.5% in those without HPR, odds ratio 2.2, 95% confidence interval 1.2-4.4, P=0.018). The occurrence of PMI was highest in the subgroup with HPR and high inflammatory status (16.6% vs 3.6% in patients with

CRP ≤3 mg/L and P2Y12 reactivity units <240, odds ratio 4.3, 95% confidence interval 1.5 to 12.6, P=0.008). HPR in association with elevated CRP levels resulted in a significant increase in the discriminatory power of a model including clinical and procedural variables in predicting PMI (area under the curve 0.811, P=0.041).

Conclusions. In patients who undergo PCI, baseline stratification according to platelet reactivity and inflammatory status may identify those at higher risk for PMI.

ABSTRACT

RESEARCH PROJECT N.2

Incremental Value of Platelet Reactivity Over a Risk Score of Clinical and Procedural Variables in Predicting Bleeding After Percutaneous Coronary Intervention via the Femoral Approach.

Development and Validation of a New Bleeding Risk Score

Background. Growing evidence suggests that platelet reactivity (PR) may predict bleeding. We investigate the incremental value of PR in predicting bleeding after percutaneous coronary intervention (PCI) via the femoral approach over a validated bleeding risk score (BRS) of clinical and procedural variables.

Methods and Results. A total of 800 patients undergoing elective PCI via the femoral approach were included. PR was measured before PCI with the VerifyNow $P2Y_{12}$ assay and low PR was defined as a $P2Y_{12}$ reaction unit value ≤178. Calculation of the BRS included the following: age, sex, intra-aortic balloon pump, glycoprotein IIb/IIIa inhibitors, chronic kidney disease, anemia, and low-molecular-weight heparin within 48-hour pre-PCI. A new risk score including low PR (BRS-PR) was developed and validated in an independent cohort of patients (n=310). Bleeding events at 30 days after PCI were defined according to the thrombolysis in myocardial infarction, Randomized Evaluation in PCI Linking Angiomax to Reduced Clinical Events (REPLACE)-2, and Bleeding Academic Research Consortium criteria. Both BRS and PR showed high discriminatory power for bleeding (area under the curve [AUC] >0.7 for all definitions). Discriminatory power of BRS-PR (AUC=0.809 for thrombolysis

in myocardial infarction bleeding; AUC=0.814 for Bleeding Academic Research Consortium class ≥2 bleeding; AUC=0.708 for Bleeding Academic Research Consortium class ≥3 bleeding; and AUC=0.813 for REPLACE-2 bleeding) was significantly higher than that of BRS alone (P<0.001 for all bleeding definitions). In the validation set, BRS-PR showed higher discriminatory power for thrombolysis in myocardial infarction bleeding than BRS alone (AUC=0.788 versus 0.709; P=0.036).

Conclusions. PR has incremental predictive value on bleeding events after elective PCI via the femoral approach over a validated risk score of clinical and procedural variables. A risk score including PR yields significantly better prognostic performance compared with the original BRS.

ABSTRACT

RESEARCH PROJECT N.3

Relationship of platelet indices with platelet reactivity and periprocedural myocardial infarction in patients undergoing percutaneous coronary angioplasty.

Background. No comprehensive data are available on role of platelet indices (PI) in periprocedural risk stratification of patients undergoing percutaneous coronary intervention (PCI). Aim of this study was to investigate the relationship of PI with platelet reactivity (PR) and with periprocedural myocardial infarction (PMI) in patients receiving PCI.

Methods. 502 PCI patients had preprocedural measurement of PI and PR, the latter assessed by the point-of-care VerifyNow $P2Y_{12}$ assay and expressed as $P2Y_{12}$ reaction units (PRU). Study endpoints were incidence of high platelet reactivity (HPR) and PMI according to tertiles of different PI.

Results. Incidence of PMI in overall population was 6.6%. Rates of PMI were not different among PI tertiles: platelet count (I: 6.0%, II: 7.1%, III: 6.5%; P=0.74), mean platelet volume (MPV, I: 6.6%, II: 7.3%, III: 5.8%; P=0.86), platelet distribution width (PDW; I: 7.2%, II: 7.2%, III: 5.4%; P=0.74), MPV/P ratio (I: 6.6%, II: 6.0%, III: 7.1%; P=0.91). A significant difference in the occurrence of PMI was identified among PRU tertiles (I: 3 %, II: 5.4 %, III: 11.4 %; P=0.006). Only platelet count and

MPV/P ratio were significantly different in patients with and without HPR (respectively 221.8±58.6x10^3/μl vs. 207±59.4x10^3/μl, P=0.008; 51.73±15.17 vs. 56.7±18.3, P=0.002).

Conclusion. This study showed no relation between PI and PMI in PCI patients, but confirms association of HPR with increased incidence of PMI. Thus, PI alone seem to be not able to identify patients at higher periprocedural risk, but monitoring PR by a bedside assay remains a useful tool for risk stratification.

ABSTRACT

REASERACH PROJECT N.4

Hyperleptinemia as risk factor for high platelet reactivity and cardiovascular events in patients undergoing percutaneous coronary intervention

Background. Leptin is an adipose tissue derived hormone, which through a direct effect on the hypothalamus is involved in the regulation of food intake and energy balance. Previous investigations suggested a correlation between leptin and platelet aggregation, but no comprehensive data are available on relation of leptin levels and post treatment platelet reactivity (PR) and cardiovascular outcome in patients undergoing percutaneous coronary intervention (PCI).

Methods. 155 PCI patients were enrolled in the study and had preprocedural measurement of plasma leptin levels and preprocedural measurements of PR. Leptin plasma levels were assessed by ELISA (Leptin Sandwich - ELISA Standard Curve). Hyperleptinemia was defined as leptin plasma levels ≥14 ng/ml. PR was evaluated by the point-of-care VerifyNow $P2Y_{12}$ assay and expressed as $P2Y_{12}$ reaction units (PRU). Patients were divided in three groups based on PRU values: low platelet reactivity (LPR) for PRU ≤178; normal platelet reactivity (NPR) for PRU between 178 and 239; high platelet reactivity (HPR) for PRU ≥ 239. All patients were followed with office visit or telephone interview every 12 months for up 8 years.

Primary endpoint was evaluation of leptin plasma levels according group of platelet reactivity. Secondary endpoints were incidence of periprocedural myocardial

infarction (PMI) and incidence of MACE (cardiovascular death, MI and target lesion revascularization) at long-term follow up.

Results. Leptin plasma levels were significantly different among groups of PR (P=0.047). In particular leptin levels were significantly higher in patients with HPR (12.61±16.58 ng/ml) compared to LPR (7.83±8.87 ng/ml, P=0.044) and NPR (7.04±7.03 ng/ml, P=0.01) group. No significant difference was identified between leptin plasma level in LPR and NPR group (P=0.66). Incidence of PMI in general population of the study was 8% (13 patients). Rate of PMI was higher, even not significant, among hyperleptinemic patients compared with the other group (15.1% vs 6.5%, P=0.22). Clinical long-term follow-up was complete in 140 patients (90.3%). Incidence of MACE was 40% (12 patients) in hyperleptinemic group and 21% (23 patients) in the normoleptinemic group. Patients with hyperleptinemia experienced a significantly higher rate of MACE compared with those in the normoleptinemic group (HR 2.3; CI 95% 1.14-4.6, P=0.02). These results remained substantially unchanged after adjusting for BMI, hypertension and sex.

Conclusions. The present study suggests that high levels of leptin are associated with HPR and with a worse clinical outcome in patients treated with clopidogrel undergoing PCI. Further studies are needed to better define the pathophysiological pathways underlying this association and to evaluate the possible efficacy of targeting leptin as a measure of effectiveness of secondary prevention treatments in patients with CAD.

STATEMENT OF ATTRIBUTION

1 Authors of the following trial:

Correlation of platelet reactivity and C-reactive protein levels to occurrence of peri-procedural myocardial infarction in patients undergoing percutaneous coronary intervention (from the ARMYDA-CRP study).

Giuseppe Patti, Fabio Mangiacapra, Elisabetta Ricottini, Antonio Cannatà, Ilaria Cavallari, Vincenzo Vizzi, Andrea D'Ambrosio, Giordano Dicuonzo, Germano Di Sciascio.

2 Authors of the following trial:

Incremental Value of Platelet Reactivity Over a Risk Score of Clinical and Procedural Variables in Predicting Bleeding After Percutaneous Coronary Intervention via the Femoral Approach: Development and Validation of a New Bleeding Risk Score.

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ABBREVIATIONS

AA: arachidonic acid

ACS: acute coronary syndrome

ADP: adenosine diphosphate

AGE: Advanced Glycosylation End products

ARU: Aspirin Reaction Unit

ATP: adenosine trisphosphate

BMI: body mass index

BMS: bare metal stent

CAD: coronary artery disease

CABG: coronary artery bypass graft

CBC: complete blood count

CD40-L: CD40 ligand

CVD: cardiovascular disease

COX-1: cyclooxygenase-1

CRP: C reactive protein

CYP450: cytochrome 450

DAG: diacylglycerol

DAPT: dual antiplatelet therapy

DM: diabetes mellitus

DES: drug-eluting stent

GTT: global thrombosis test

GpIa: glycoprotein Ia

GpIb/V/IX: glycoprotein Ia/V/IX

GpIIb/IIIA: glycoprotein IIb/IIIa

GPI: glycoprotein IIb/IIIa inhibitors

HDL: high-density lipoprotein

HPR: high platelet reactivity

IL-1β: Interleukin-1β

IP_3: inositol triphosphate

JAK: Janus kinase family

LDL: low-density lipoprotein

LPR: low platelet reactivity

LTA: light transmission aggregometry

MACE: major adverse cardiovascular events

MAPK: mitogen activator protein kinases

MEA: multiple electrode aggregometry

MPV: mean platelet volume

MI: myocardial infarction

MMP: metalloproteinase

NSTE-ACS: non-ST elevation acute coronary syndrome

NSTEMI: non-ST elevation myocardial infarction

NO: nitric oxide

NPR: normal platelet reactivity

Ob-r: leptin receptor

Ox-LDL: oxidated low-density lipoprotein

PAD; peripheral artery disease

PAR: protease-activates receptor

PCI: percutaneous coronary intervention

PDW: platelet distribution width

PMI: periprocedural myocardial infarction

PFA: platelet function analyzer

PGE1: prostaglandin E1

PIP_2: phosphatidylinositol 4,5-bisphosphate

PLC: phospholipase C

PKC: protein kinase C

PR: platelet reactivity

PRP: platelet reach plasma

PRU: $P2Y_{12}$ Reaction Unit

ROS: reactive oxygen species

ROTEM: rotational Thromboelastography

ST: stent thrombosis

STAT: signal traducers and transcriptional activator

STE-ACS: ST elevation acute coronary syndrome

STEMI: ST elevation myocardial infarction

TEG: thromboelastography

TLR: target lesion revascularization

TXA_2: thromboxane A2

TXB_2: thromboxane B2

TVR: target vessel revascularization

VAPS: vasodilator-stimulated phosphoprotein

VCAM-1: vascular cell adhesion molecule-1

vWF: von Willendbrad Factor

WBA: whole blood aggregomtry

CHAPTER 1

Role of platelets in coronary artery disease and antiplatelet therapy

Platelets, also known as thrombocytes, are produced by megakaryocytes as anucleate cells that lack genomic DNA, but contain megakaryocyte-derived messenger RNA (mRNA) and the translational machinery needed for protein synthesis. Like the other blood cells, platelets originate in bone marrow from pluripotent stem-cells and after leaving the bone marrow they circulate for about 10 days. Their primary physiological function is to stop hemorrhages after vascular injury, but they play a central role also in inflammation and in some pathological response, like atherogenesis and atherothrombosis process [1-3].

Coronary artery disease (CAD) is the most important cause of mortality and morbidity in Western Countries. In particular, each year cardiovascular diseases (CVD) cause 3.9 million deaths (45% of all deaths) in Europe. Acute thrombotic events are responsible for most cardiovascular deaths and platelets are obviously a key actor of these events [4].

1.1 Platelets activation mechanisms

Hemostasis at sites of vascular injury is due by formation of a platelet plug, this process needs of 3 stages: 1) initiation phase characterized by platelet adhesion; 2)

extension phase that comprises activation, additional recruitment and aggregation; 3) perpetuation phase with stabilization of clot (Figure 1) [5].

The initial step in primary hemostasis is the platelet adhesion to the extracellular matrix. In normal conditions circulating platelets do not interact with endothelium; nevertheless, the exposure of subendothelial matrix caused by a vascular injury is the fundamental trigger for this first phase. Adhesion is mediated by interaction of glycoprotein (Gp) Ib/V/IX receptor, located on the platelet surface, to von Willebrand factor (vWF) and GpVI and GpPIa to collagen at sites of endothelial damage. Therefore, the interaction between vWF and GpIb/V/IX is crucial for the initial adhesion of platelets to the subendothelium, especially under conditions of high shear [3,5].

In the extension phase platelet activation and recruitment is stimulated by bound platelet secretion products and local prothrombotic factors (tissue factor), which leads to generation of haemostatic plugs. Multiple soluble agonists are involved in platelet activation: adenosine diphosphate (ADP), thromboxane A2 (TXA_2), epinephrine, serotonin, collagen and thrombin (Figure 2). TXA_2 is synthesized by activated platelets from arachidonic acid (AA) trough conversion by cyclooxygenase-1 (COX-1) and TX synthase [3, 6]. TXA_2 binds two variants of receptors: TPα and TPβ both couple to the G proteins which activate the phospholipase C (PLC). This enzyme is responsible for degradation of phosphatidylinositol 4,5-bisphosphate (PIP_2) and consequent release of the second messengers, inositol triphosphate (IP_3) and diacylglycerol (DAG). IP_3 increases cytosolic levels of Ca^{2+}; DAG activates intracellular protein kinase C (PKC), resulting in protein phosphorylation. ADP is released by platelets and red cells. Two different ADP receptors are expressed by platelets: $P2Y_1$ which is coupled to Gq, and

$P2Y_{12}$, which is coupled to G_i. The activation of $P2Y_1$ leads to an increase of the intracellular Ca^{2+}, while the activation of $P2Y_{12}$ causes an inhibition of adenylate cyclase with a consequent decrease in the cyclic AMP (cAMP) level [5,7]. The $P2Y_{12}$ receptor is the principal receptor for amplification of platelet activation. Furthermore, thrombin represents the most potent platelet activator: it is generated at the site of vascular injury from circulating prothrombin. Thrombin activates platelet trough stimulation of protease-activates receptors (PARs). Human platelets express PAR-1 and PAR-4. PAR-1 is activated at low thrombin concentration and couples some G proteins. In particular, the α-subunits of G_{12} and G_{13} are probably involved in the change of platelet shape trough Rho-mediated cytoskeletal responses. The G_q and G_i signaling pathways are responsible for the increase of intracellular Ca^{2+} and for the decrease of cAMP. The final effect of these antagonists, mediated by increase of intracellular Ca^{2+} and decrease of cAMP, results in a change in ligand-binding properties of glycoprotein IIb/IIIa (αIIβ3-integrin), which is able to bind soluble adhesive protein like fibrinogen and von Willebrand factors. All these mechanisms are finalized to the recruitment of platelets from the circulation and lead to several manifestations of platelet activation. These include platelet shape change, expression of pro-inflammatory molecules such as P-selectin and soluble CD40 ligand (sCD40L), expression of platelet procoagulant activity, and conversion of GpIIb/IIIa into an active form, which lets to platelet aggregation [1,5].

The perpetuation phase is characterized by the consolidation of the hemostatic plug and it is supported also by generation of fibrin from fibrinogen. The final common pathway of platelet agonists is the activation of GpIIb/IIIa (Figure 2). This integrin is the central receptor mediating platelet aggregation as it promotes platelet adhesion,

aggregation and spreading on the exposed extracellular matrix at the site of endothelial injury, as well as thrombus formation and stability. The bound of fibrinogen to GpIIb/IIIa bridges activated platelets and contributes to thrombus stabilization. The platelet-rich thrombus and coagulation cascades reinforce each other and finally lead to a stable platelet plug. When mechanisms of platelet activation are overly active, they can lead to pathological thrombosis. Typically, platelet-rich white thrombi are not completely obstructive and are associated with non-ST elevation acute coronary syndromes (NSTE-ACS). While fibrin-rich red clots generated by thrombin, may progress ultimately to an occlusive thrombus, which is usually associated to ST elevation (STE) ACS [3,6].

1.2 Platelets in inflammation and atherothrombosis

Platelets play a central role in inflammatory reactions and are involved in responses to a variety of inflammatory diseases. They are a storehouse for several inflammatory mediators and growth factors, which are involved also in mechanisms of atherothrombosis. These comprehend chemokines (eg, macrophage inflammatory protein-1α; epithelial neutrophil-activating protein 78, PF4) cytokines and cytokine-like factors (eg CD40 ligand, thromboglobulin-β and interleuchin-1β), adhesion proteins (eg, fibrinogen, fibronectin, thrombospondin, vitronectin, von Willebrand factor, P-selectin, integrin αIIbβ3), growth factors (eg, transforming growth factor-β, basic fibroblastic growth factor, platelet-derived growth factor, epidermal growth factor) and coagulation factors (eg, protein S, plasminogen, factor V, factor XI, plasminogen activator inhibitor type1). All these substances work in concert to mediate several functions, such as cell adhesion process, cell activation, chemotaxis, cell

proliferation, coagulation and proteolysis [1,7]. Below we will examine the main proinflammatory mechanisms associated with platelet activation.

P-selectin and von Willebrand factor mediate platelets and leukocytes adhesion process. They are stored in the platelet α-granules and in endothelial organelles called Weibel-Palade bodies: following activation by thrombin, these granules are translocated on the surface of endothelial and platelet cells and fused with the cell membranes with a consequent release of P-selectin and von Willebrand factor in circulation. Together these two mediators favor leukocytes recruitment and macrophages accumulation in cell wall. Interaction between platelets, leukocytes and endothelium can occur not only by activation of leukocyte adhesion receptors, but also by chemoattract effect of platelets adherent on the endothelium, which provides a surface for neutrophil-endothelium interaction. The result is the infiltration of inflammatory cells into the vessel wall, which is crucial for atherosclerosis development [3, 9].

CD40L, originally identified on activated T cells, is a transmembrane protein of the tumor necrosis factor family. It is stored in cytoplasm of platelets and exposed on the surface after platelet activation. Once exposed on the cell membrane, the CD40L undergoes a cleavage with generation of a functional soluble fragment [1,10]. This can induce endothelial cells production of reactive oxygen species, tissue factors, chemokines and adhesion molecules, which are involved in inflammatory response. The blockage of the CD40-CD40L signaling pathway inhibits the formation and progression of atherosclerotic plaque, as it was demonstrated in an experimental model in LDL-receptor$^{-/-}$ mice [11]. Furthermore, there are evidences connecting cardiovascular risk factors, such as smoke habit and type 2 diabetes, with increased

release of CD40L. High plasma levels of CD40L are associated with increased risk of cardiovascular events also in a population of heathy women [12].

Interleukin-1β (IL-1β) is a central mediator of inflammation. It is not stored in platelets cytoplasm, but it is synthetized after platelet activation, by way of the signal-dependent translation of IL-1β mRNA. This interleukin induces endothelial cells to express genes that mediate the leukocyte adhesion and increase the release of chemokines and molecules implicated in inflammatory process [1].

Reactive oxygen species are other important actors in atherosclerosis process. These are release from vessel wall, but can also be generated by activated platelets. In particular, metabolism of AA through COX-1 pathway can induce the platelet isoform of NADPH oxidase and contributes to the production of reactive oxygen species and agonists, which are implicated in platelet activation. The increased generation of reactive oxygen species can favor enhanced lipid peroxidation of cell-membrane phospholipids or circulating LDL, resulting to an increased generation of F2-isoprostanes, a family of prostaglandin isomers, which can modulate the adhesion and the activation of platelets with low levels of other agonists [1]. The evidence of a consistent relationship of thromboxane and F2-isoprostanes in patients with type 2 diabetes, homozygous homocystinuria and hypercholesterolemia show that a low grade of inflammatory state associated with these metabolic disorders may be a trigger for platelet activation [12,13]. Therefore, through the above-mentioned mechanisms, the inflammatory processes mediated by platelets provide the basis for plaque formation. Nevertheless, evidences for the role of platelet in human atherogenesis are limited or in large part indirect. Platelet activation is associated with markers of atherosclerosis, like increased intima-media thickness of the carotid artery in patients

with type 2 diabetes [15]. As well persistent platelet activation has been associated with traditional risk factors, implicated in acceleration of atherogenesis [1]. Certainly, atherosclerosis is a slowly progressive disease with a long subclinical phase: if the role of platelets in the first stages is not yet completely understand, their action in the transition from a stable and silent disease to a symptomatic and life-threatening condition is surely more definite. Thrombosis occurs at site of denudation ad erosion of endothelial surface, in particular vulnerable sites are located were fibrous cap, separating lipid reach core from the lumen, is thin and at increased risk of breaks. The large majority of these lesions resolve spontaneously through a repair phenomenon, however in a substantial proportion of symptomatic episodes of acute coronary-plaque disruption, platelet activation progresses to persistent intraluminal thrombosis. The most convincing evidence for the participation of platelets in arterial thrombosis in humans comes from studies on platelet activation in patients with acute coronary syndromes (ACS) and from trial on antiplatelet drugs.

1.3 Antiplatelet therapy

The inhibition of platelet activation with antiplatelet drugs is the cornerstone of treatment of cardiovascular disease, in particular of both stable and unstable coronary artery disease. The mechanisms through which antiplatelet drugs inhibit the activation of platelets have as target some of the pathways, which as previously described, are crucial for hemostasis and thrombosis. Moving from these pathways, it is possible to identify four strategies for platelet function inhibition: 1) inhibition of enzymatic cascades; 2) receptors' inhibition; 3) Glycoproteins' inhibition; 4) inhibition of agonists' generation (Figure 2). Therefore, there is a large number of antiplatelet agent

approved for clinical use and an increasing number of new drugs, which are under development [16].

1.3.1 Inhibitors of TXA_2 pathways

Aspirin is an irreversible COX-1 inhibitor, which blocks TXA_2 production and reduces the correspondent activation pathways. Because of the absence of protein synthesis, in platelets TXA_2 inhibition persists for lifetime of the cell. Aspirin has been for over 50 years the foundation of antiplatelet therapy. It reduces cardiovascular death by 15% and non-fatal vascular events by 30% as shown by a meta-analysis of more than 100 trials [5,17]. Benefits of aspirin therapy have been documented also in primary prevention, even if with a more modest effect due to the excess of bleeding complications. Despite the efficacy of aspirin treatment, a consistent number of patients continue to experience recurrent cardiovascular events, especially in the setting of ACS. Furthermore, also the optimal dose with correct balance between ischemic and bleeding risk remains unclear. In the CURRENT-OASIS 7 (Clopidogrel Optimal Loading Dose Usage to Reduce Recurrent Events/Optimal Antiplatelet Strategy of Intervention) trial [18], it was demonstrated a similar outcome for efficacy, without difference in risk of bleeding complication for both dose of aspirin ($\geq$ 300 mg/day vs 75-100 mg/day).

1.3.2 Inhibitors of $P2Y_{12}$ Receptor

$P2Y_{12}$ receptor antagonists include ticlopidine, clopidogrel, prasugrel, ticagrelor and cangrelor. Ticlopidine, clopidogrel and prasugrel are three generations of drugs of

the same family, the thienopyridines. They are selective and irreversible inhibitors of the $P2Y_{12}$ receptor, while ticagrelor and cangrelor reversibly inhibit the receptor.

Ticlopidine was the first $P2Y_{12}$ inhibitor approved. It is a first generation thienopyridine, is metabolized by the cytochrome P450 (CYP450) and administered in the dose of 250 mg twice daily. Ticlopidine was demonstrated to be superior to aspirin in prevention of nonfatal stroke and death [5]. Subsequently, in patients undergoing coronary stenting, a better clinical outcome was shown for those treated with aspirin plus ticlopidine than aspirin alone or aspirin plus warfarin. Nevertheless, treatment with ticlopidine had two limitations: its inability to achieve a rapid platelet inhibition and a non-optimal safety profile (neutropenia, thrombotic thrombocytopenic purpura, rash). Therefore, ticlopidine was rapidly replaced by the next generation thienopyridine [5].

Clopidogrel is a second-generation thienopyridine. The parent compound, clopidogrel bisulfate, is an inert prodrug that is metabolized in the liver into the active metabolite by CYP450 enzyme CYP3A4. This process requires two intermediate oxidations to achieve the active agent, which finally is only the 15% of the prodrug. The remaining 85% is hydrolyzed into an inactive form. Even if clopidogrel presents a half-life of 8 hours, it has an irreversible effect on platelet that last 7-10 days. It is administered in chronic treatment with a dose of 75 mg/die. The CAPRIE (Clopidolgrel versus Aspirin in Patients at Risk of Recurrent Ischemic Events) trial [19] evaluated the efficacy of clopidogrel monotherapy versus aspirin monotherapy in secondary prevention in patients with a history of ischemic stroke, myocardial infarction (MI), symptomatic atherosclerotic peripheral arterial disease (PAD). Treatment with clopidogrel showed a significant, although marginal, greater efficacy

versus aspirin (5.32% vs 5.83% in aspirin group, P=0.043), while bleeding rates were comparable between two groups [19]. Furthermore, when administered in association with aspirin, clopidogrel has demonstrated a greater efficacy than aspirin monotherapy. In the CURE (Clopidogrel in Unstable angina to prevent Recurrent Events) trial [20], dual antiplatelet therapy (DAPT) with aspirin and clopidogrel had reduced the risk of composite endpoint of death from cardiovascular (CV) causes, nonfatal MI, or stroke versus aspirin alone, among patients with NSTE-ACS (11.4% vs 9.3% in clopidogrel group; P<0.001). At the same time, an increased risk of bleeding was identified among patients in DAPT, with no significant difference in rates of hemorrhagic stroke and life-threatening bleeding [20]. A sub-analysis [21] including patients treated with percutaneous coronary intervention (PCI-CURE) demonstrated a significant reduction of composite endpoint (CV death, MI, urgent target vessel revascularization-TVR) within 30 days with clopidogrel plus aspirin versus aspirin alone (4.5% vs 6.4%; RR 0.70; 95% CI, 0.50-0.97; P=0.03). No significant differences were identified between two groups in rate of major bleeding and blood transfusions (1.6% with clopidogrel vs 1.4% with placebo) [21]. In the CREDO (Clopidogrel for the Reduction of Events During Observation) trial [22], a clopidogrel loading dose 300 mg before PCI demonstrated a modest and not significant reduction of composite end point (Death, MI, TVR) at 28 days. In the same trial prolongation of treatment with clopidogrel plus aspirin beyond 28 days significantly reduced the rate of 1-year composite end-point (death, MI, stroke) vs aspirin alone. No differences in incidence of major bleedings were identified at 28 days and at 1 year [22]. Clinical benefit of clopidogrel plus aspirin versus aspirin monotherapy was also demonstrated in patients with STEMI treated with fibrinolytic therapy enrolled in the

CLARITY (Clopidogrel as Adjunctive Reperfusion Therapy) trial [23]. In this study patients were randomized to receive clopidogrel (300 mg loading dose followed by 75 mg daily) or placebo before undergoing coronary angiography 2 to 8 days (median 3 days). The sub-analysis of PCI-CLARITY [24] showed that pretreatment with clopidogrel reduced the risk of 30-day MACE (Major Adverse Cardiovascular Events comprehensive of CV death, MI, and stroke) following PCI by 46% (3.6% vs 6.2%; P=0.008) [24]. However, in the CHARISMA (Clopidogrel for High Atherothrombotic Risk and Ischemic Stabilization, Management and Avoidance) trial [25], there was no difference in rate in composite endpoint (CV death, MI, stroke) in patients treated with DAPT (aspirin plus clopidogrel) versus aspirin alone. At the same time, a non-significant increase in GUSTO severe bleeding and a significant increase in GUSTO moderate bleeding was registered in clopidogrel treated group. In the same study, the subgroup analysis including CAPRIE-like patients (prior MI, ischemic stroke, symptomatic PAD) showed that treatment with clopidogrel significantly reduced the incidence of composite end point (CV death, MI, stroke) at 28 months (8.8% in placebo vs 7.3% in clopidogrel group, P=0.01), without increase in GUSTO severe bleedings [26].

Subsequent studies have evaluated the optimal dose of clopidogrel in PCI setting. In the ARMYDA-2 (Antiplatelet Therapy for Reduction of Myocardial Damage During Angioplasty) trial [27], patients undergoing elective PCI, who were randomized to a 600-mg loading dose of clopidogrel, given 4 to 8 hours before PCI, had a lower risk of composite end-point (death, periprocedural MI, or TVR) compared with the group randomized to the 300-mg loading dose (4% vs 12%; RR, 0.48). Similarly, in the CURRENT-OASIS 7 trial [18], 25087 patients with ACS were

randomized to receive high dose (600 mg loading dose followed by 150 mg daily for seven days, followed by 75 mg daily) versus standard dose of clopidogrel (300 mg loading dose, the followed by 75 mg daily). In each clopidogrel arm, as previously mentioned, patients were randomized to receive low dose (75-100 mg daily) or high dose (300-325 mg daily) of aspirin. Rate of composite endpoint (CV death, MI, stroke) at 30 days of follow up did not significantly differ between two groups of treatment. In the subgroup of patients who underwent PCI, clopidogrel high-dose was associated with a 15% of relative reduction of composite end-point in the 30-days follow up (3.9% vs 4.5%; RR0.86; 95% CI, 0.76-0.99; P=0.039). No significant difference in the rates of Thrombolysis in MI (TIMI) major bleeding was observed between the high-dose and standard-dose clopidogrel groups [18].

Despite the significant benefit of DAPT with aspirin plus clopidogrel, a considerable number of patients has continued to experience cardiovascular events, which may in part be explained by interindividual variability in platelet response to clopidogrel. For this reason, new $P2Y_{12}$ antagonists were subsequently developed and are now available for use in clinical practice.

Prasugrel is a third generation thienopyridine. It is an oral, irreversible $P2Y_{12}$ antagonist with a faster onset of action time and a greater potency versus clopidogrel. The TRITON-TIMI 38 (Trial to Assess Improvement in Therapeutics Outcomes by Optimizing Platelet Inhibition with Prasugrel) trial [28] randomized 13608 patients with moderate-to-high-risk ACS with scheduled PCI to prasugrel (60 mg loading dose, 10 mg/d maintenance dose) or clopidogrel (300 mg loading dose, 75 mg/d maintenance dose). The study showed that prasugrel is superior to clopidogrel standard-dose in reducing ischemic events in patients with ACS treated with PCI. At 15 months,

prasugrel significantly reduced the primary composite endpoint (CV death, MI, stroke) compared with clopidogrel (9.9% vs. 12.1%, respectively; HR: 0.81; 95% CI, 0.73–0.90; P < 0.001) with a number needed to treat (NNT) within 15 months of 46. At the same time, prasugrel was associated with a significantly increase of major bleeding events in particular in non–CABG-related TIMI major bleeding (1.8% vs. 2.4%, HR: 1.32; 95% CI, 1.03–1.68; P = 0.03), including fatal bleedings. Patients with previous stroke or TIA, those ≥75 years old or those weighs <60kg had no benefit from prasugrel treatment [28]. Prasugrel demonstrated the greatest benefit among patients with diabetes mellitus (DM) and those presenting with STEMI undergoing primary PCI in whom there were no difference in major bleeding complications [28]. In the TRILOGY-ACS (Targeted Platelet Inhibition to Clarify the Optimal Strategy to Medically Manage Acute Coronary Syndromes) trial [29], 9326 medically managed patients (without revascularization) with unstable angina or non-ST elevation myocardial infarction (NSTEMI) were randomized to prasugrel 10 mg/die (5 mg/die if aged ≥75 years or with body weight <60 kg) or clopidogrel 75 mg/die. Clopidogrel-naive patients who underwent randomization within 72 hours after first medical contact received a loading dose of prasugrel 30 mg or clopidogrel 300 mg, followed by daily-blinded maintenance therapy. Patients who did not undergo randomization within 72 hours were treated with open-label clopidogrel before randomization and then received daily maintenance study drug. Prasugrel did not significantly reduce the frequency of the primary endpoint (CV death, MI, stroke) in patients <75 years (primary efficacy and safety cohort), as compared with clopidogrel, and similar risk of bleeding was observed [29]. Therefore, prasugrel in association with aspirin is more effective then clopidogrel plus aspirin in treatment of patients of ACS, when the

coronary anatomy is known and patients were scheduled for PCI. Treatment with prasugrel is not recommended in patients with ACS medically managed.

Ticagrelor is a cyclo-pentyltriazolo-primidine. It is direct-acting and selective inhibitor of the $P2Y_{12}$ receptor with rapid onset of action (2 hours to peak platelet inhibition) and it does not require any metabolic conversion. Ticagrelor binding to the $P2Y_{12}$ receptor is reversible, with partial recovery of platelet aggregation within 12 hours after discontinuation of treatment. For this reason, it was administered with a loading dose of 180 mg followed by the dose of 90 mg twice daily in chronic treatment. This feature of ticagrelor may be advantageous because rapid reversal of platelet inhibition after discontinuation of the drug could potentially minimize bleeding complications, particularly in patients who require surgery. Like prasugrel, also ticagrelor demonstrated greater potency and consistency of platelet $P2Y_{12}$ receptor inhibition versus clopidogrel. In the PLATO (Platelet Inhibition and Patient Outcomes) trial [30], 18624 patients with ACS were randomized to ticagrelor (180 mg loading dose, 90 mg twice-daily maintenance dose) or clopidogrel (300–600 mg loading dose, 75 mg/d maintenance dose). At 12 months, ticagrelor significantly reduced the primary composite endpoint (CV death, MI, stroke) compared with clopidogrel (9.8% vs. 11.7%, respectively; HR: 0.84; 95% CI, 0.77–0.92; $P < 0.001$). Ticagrelor did not increase the rate of overall major bleeding, but a statistically significant increase in non-coronary artery bypass grafting (non-CABG) major bleeding (4.5% vs. 3.8%; HR: 1.19; 95% CI, 1.02–1.38; $P < 0.03$) was observed. Dyspnea was more common in the ticagrelor group than in the clopidogrel group (13.8% of patients vs. 7.8%), although few patients discontinued treatment due to dyspnea (0.9% vs. 0.1%). Ticagrelor was associated, in the first week of treatment,

with a higher incidence of ventricular pauses. However, pauses were rarely associated with symptoms, and there was no difference between treatments groups in the incidence of syncope or pacemaker implantation. The NNT within 12 months was 54 [30]. More recently, efficacy of a prolonged DAPT with aspirin and ticagrelor was demonstrated among 21162 high risk patients with previous MI (1 to 3 years earlier) enrolled in the PEGASUS-TIMI 54 (Prevention of Cardiovascular Events in Patients with Prior Heart Attack Using Ticagrelor Compared to Placebo on a Background of Aspirin–Thrombolysis in Myocardial Infarction 54) trial [31]. High risk features were identified by presence of at list one of the following: age ≥ 65 years; diabetes requiring medication; second previous MI (> 1 years); multivessel CAD; renal failure (Creatinine Clearance < 60 ml/min). In this study, all patients were randomized to receive ticagrelor at a dose of 90 mg twice daily, or ticagrelor at a dose of 60 mg twice daily, or placebo. All the patients were treated with low-dose aspirin and were followed for a median of 33 months. The two ticagrelor doses each significantly reduced, as compared with placebo, the incidence of the primary composite end point (CV death, MI and stroke, 7.85% in ticagrelor 90 mg, 7.77% in ticagrelor 60 mg and 9.04% in placebo; HR for 90 mg of ticagrelor vs. placebo, 0.85; 95% CI, 0.75 to 0.96; P = 0.008; HR for 60 mg of ticagrelor vs. placebo, 0.84; 95% CI, 0.74 to 0.95; P = 0.004). The incidence of TIMI major bleeding was higher with the two ticagrelor doses than with placebo (respectively 2.6%, 2.3% and 1.06%, HR for 90 mg of ticagrelor vs. placebo, 2.69; 95% CI, 1.96 to 3.70; P<0.001; HR for 60 mg of ticagrelor vs. placebo, 2.32; 95% CI, 1.68 to 3.21; P<0.001), with no difference in rate of fatal bleeding or nonfatal intracranial hemorrhage [30]. Based on results of these two trials ticagrelor was indicated in treatment of ACS patients regardless treatment strategy, and for those

with high risk features also a prolongation beyond 12 months of treatment with ticagrelor 60 mg twice daily could be considered.

Cangrelor is a reversible, potent, competitive inhibitor of the $P2Y_{12}$ receptor; it is administered intravenously and as for ticagrelor, it does not require any metabolic conversion to be active. The antiplatelet effects of cangrelor are almost immediate, with extensive platelet inhibition occurring within few minutes of drug administration. This level of inhibition of platelet aggregation is maintained throughout the duration of the infusion, with recovery of platelet aggregation occurring within 60 minutes in 80% of patients and within 90 minutes in 90% of patients [32]. Cangrelor efficacy was tested in the trials of CHAMPION (Cangrelor versus standard tHerapy to Achieve optimal Management of Platelet InibitiON) program. In the CHAMPION PLATFORM and CHAMPION PCI, cangrelor was randomly compared respectively with placebo or clopidogrel. Both trials did not show a superiority of cangrelor in term of primary composite endpoint (death, MI or ischemia driven revascularization in the first 48 following PCI procedure), probably results of these studies were affected by the definition of MI applied [33, 34]. The CHAMPION PHOENIX was a double-blind, double-dummy study which compared cangrelor with clopidogrel in 11145 patients underwent PCI. The composite primary endpoint, which include death, MI (in this case was applied the universal definition) or ischemia driven revascularization in the first 48 hours following PCI procedure, was significantly reduced in cangrelor group compared to clopidogrel (4.7% vs 5.9%, P=0.005). In particular, rate of stent thrombosis (ST) at 48 hours was lower than cangrelor group (0.8% vs 1.4%, P=0.0005) [33]. In CHAMPION trials rate of TIMI major bleedings and GUSTO severe-moderate bleedings were similar between two groups of treatment [33, 34, 35]. Outside the

CHAMPION trials, cangrelor was tested also in the context of patients on thienopyridines who require discontinuation of thienopyridine before coronary artery bypass grafting (CABG). The BRIDGE trial (Maintenance of Platelet inhiBition with cangRelor after dIscontinuation of thienopyriDines in patients undergoing surGEry) is a double-blind, placebo controlled trial enrolling 210 patients treated with thienopydirine and awaiting CABG (within 48 hours to 7 days from randomization). The primary endpoint is the maintance of inhibition of platelet reactivity which was achieved in 98.8% of patients treated with cangrelor vs 19% in the placebo group (P<0.0001). Interestingly CABG related bleedings were similar in two groups of treatment (11.8% vs 10.4%, P=0.76) [36].

1.3.3 Glycoprotein IIb/IIIa inhibitors

Unlike $P2Y_{12}$ receptor antagonists, which inhibit the phase of platelet activation, GpIIb/IIIa receptor antagonists exert their antiplatelet effect via blockade of the GpIIb/IIIa receptor, which is involved directly in binding fibrin and allows aggregation of adjacent platelets. Three parental GpIIb/IIIa inhibitors, abciximab, eptifibatide and tirofiban, are currently available. All of them can induce a nearly complete and rapidly (15 minutes) inhibition of platelet aggregation. Abiciximab, a fragment of a human-murine monoclonal antibody, irreversibly binds to and inactivates platelets; it has a short plasma half-life, but exerts its antiplatelet effect as long as abciximab-bound platelets remains in circulation. Tirofiban and eptifibatide are both reversible inhibitors of the GpIIb/IIIa receptor with short plasma half-life, with platelet aggregation returning to normal levels within 4 hours of cessation of the infusion [5, 37]. The efficacy of GpIIb/IIIa inhibitors was firstly tested in the EPISTENT trial (Evaluation

of Platelet IIb/IIIa Inhibition in Stenting Trial): patients with stable angina undergoing PCI were randomized to abciximab or placebo in addition to aspirin, ticlopidine and heparin. Abiciximab reduced the 30 days incidence of MACE (death, MI, urgent TVR) by 52% compared to placebo (5.3% vs 10.8%; HR, 0.48; 95% CI, 0.33–0.69; P<.001) without an increase in bleeding complications [38].

Subsequently, all three drugs have been tested both in the setting of non-ST-elevation MI and ST-elevation MI. The ISAR REACT-2 (Intracoronary Stenting and Antithrombotic: Regimen Rapid Early Action for Coronary Treatment 2) [39] confirmed the effectiveness of abciximab in patients with NSTE-ACS, but the effect was restricted to high-risk patients. While the ACUITY (Acute Catheterization and Urgent Intervention Triage Strategy)-Timing [40] and the EARLY ACS (Early Glycoprotein IIb/IIIa Inhibition in Non–ST-Segment Elevation Acute Coronary Syndrome) [41] trials showed no benefit from upstream GPI treatment, which should likely be ascribed to the presence of potent ADP receptor antagonism and improvements in devices technologies and PCI. Of note, bleeding complications are a major limitation of GPI treatment [40-41].

More recently in a meta-analysis comparing GPI with placebo in PCI treated patients, use of GpIIb/IIIa inhibitors significantly reduced 30-day mortality by 21% (0.92% vs 1.33%; RR, 0.79;95% CI, 0.64–0.97). Even if the benefit of reduction of ischemic events and mortality was attenuated by the increased risk of bleeding: 30 days major bleedings were increased by 39% (3.03% vs 2.23%; RR, 1.39; 95%CI, 1.21–1.61). This negative effect persisted regardless pretreatment with clopidogrel [42]. The availability of the newer oral antiplatelet agents and the trials showing better net clinical outcomes with the direct thrombin inhibitor bivalirudin (and use of

glycoprotein IIb/IIIa inhibitors for ischemic or angiographic bailout) has diminished the role of GpIIb/IIIa inhibitors in clinical practice, which is now confined to bailout strategies in patients with high thrombus burden during PCI [37].

1.3.4 PAR-1 Inhibitors

Despite the development of ever more potent antiplatelet drugs, the evidence of a residual risk for thrombotic events has prompted the research for new strategies in platelet inhibition. Among these, selective inhibition of the PAR-1 receptor for thrombin represent a promising novel strategy in treatment of CAD patients, with the possibility to reduce ischemic events without increasing the risk of bleeding [5]. PAR-1 inhibition could provide a more comprehensive platelet inhibition, when used in combination with current available oral antiplatelet drugs. Some preclinical studies have suggested that the PAR-1 platelet activation pathway may not be essential for hemostasis, therefore PAR-1 inhibition is not expected to increase bleeding risk [3,5]. PAR-4 in humans plays a secondary role and it may become active only at higher thrombin concentration, for this reason, new agents are selectively targeting the PAR-1. Until now two oral PAR-1 antagonists were developed: vorapaxar and atopaxar. The first one has been just tested in two phase III studies and was approved by US Food and Drug Administration (FDA) and European Medicines Agency (EMA). While, data on atopaxar come from four phase II trials.

The first trial evaluating vorapaxar was the TRACER (Thrombin Receptor Antagonist for Clinical Event Reduction in Acute Coronary Syndrome) trial [43]. This study enrolled 12944 patients with ACS and at least one of the following high-risk features: age older than 55 years, previous MI, PCI or coronary artery bypass graft

(CABG), diabetes, or PAD. Patients were randomized to receive vorapaxar or placebo in addition to standard treatment with aspirin and $P2Y_{12}$ inhibitor. The study was stopped by the Data and Safety Monitoring Board for an increased rate of intracranial hemorrhage in patients treated with vorapaxar. The rate of primary endpoint (CV death, MI, stroke, urgent revascularization and ischemia driven rehospitalization) was not significantly reduce d by vorapaxar (18.5% vs 19.9% in placebo group, P=0.07), but a key secondary endpoint (CV death, MI, stroke) was significantly lower in the vorapaxar group (14.7% vs 16.4%, P=0.02) [40]. At the same time, vorapaxar was also tested in the TRA2P-TIMI 50 (Thrombin Receptor Antagonist in Secondary Prevention of Atherothrombotic Ischemic Events – Thrombolysis in Myocardial Infarction 50) trial [44]. This study provided the evidences that led to FDA approval of vorapaxar for secondary prevention in high-risk patients. A total of 26449 patients were enrolled and randomized to vorapaxar or placebo and followed for a median time of 30 months. In this second study were enrolled patients with stable atherosclerotic vascular disease (spontaneous MI or ischemic stroke within 2 weeks to 12 months of enrollment or PAD with ankle-brachial index of less than 0.85 or previous limb ischemia). During the trial, the Data and Safety Monitoring Board recommended the interruption of treatment for patients with previous history of transient ischemic attack (TIA) or stroke for an excess in intracranial hemorrhage. The primary efficacy end-point (CV death, MI and stroke) was significantly reduce by vorapaxar (9.3% vs 10.5% in placebo group, P<0.001). This benefit was achieved at cost of an increase in bleeding rates: GUSTO moderate to severe bleedings were 4.2% with vorapaxar and 2.5% with placebo (P<0.001) and TIMI major and minor bleedings were 15.8% vs 11.1% (P<0.001) [44]. Of note, in patients with a previous MI and with diabetes,

vorapaxar significantly reduced the primary endpoint compared with placebo (11.4% vs 14.3%; HR = 0.73, 95% CI, 0.60 to 0.89; P = 0.002). At the same time, risk of moderate to severe bleeding was increased with vorapaxar compared to placebo (4.4% vs 2.6%; HR = 1.60, 95% CI, 1.07 to 2.40). Even if, the net clinical outcome was improved with vorapaxar (HR = 0.79, 95% CI, 0.67 to 0.93) [45].

Atopaxar was tested in LANCELOT (Lesson from Antagonizing the Cellular Effect of Thrombin) phase II trials. Patients with ACS were enrolled in LANCELOT-ACS [46] and in J-LANCELOT-ACS [47]; this latter included only a Japanese population. These two trials were also dose-ranging studies to determine an optimal dose to minimize harm while preserving efficacy of atopaxar. Patients were enrolled to receive atopaxar in three different dose groups (400 mg loading dose followed by either a 50 mg, 100 mg, or 200 mg maintenance dose daily) or placebo. The primary endpoint was for both studies the incidence of patients with major bleeding according CURE bleeding classification. In the Japanese population, there was no increase in any CURE bleeding between the combined (50 mg, 100 mg, 200 mg) atopaxar group and the control group (0.6% versus 3.3%; P = 0.125) [47]. In the larger international trial (n=603), there was no statistically significant difference in major and minor CURE bleedings between the placebo and the combined atopaxar groups (2.2% versus 3.1%; I=0.63). There was no statistically significant difference between the combined atopaxar and control groups (8% versus 7.8%, respectively; P = 0.93) in the composite endpoint (CV death, MI, stroke, recurrent ischemia), but the study was not power for the ischemic clinical outcome [46]. LANCELOT-CAD [48] and J-LANCELOT-CAD [47] allowed atopaxar to be studied over a longer term (24 weeks) and looked at the safety and tolerability at various doses regimens (50 mg to 200 mg). Both studies

enrolled patients with a history of high-risk CAD (n=710 patients in LANCELOT-CAD and n=263 patients in J-LANCELOT-CAD) defined as: previous ACS (including MI or unstable angina) at least in the previous four weeks, PCI in previous 12 weeks, and angina with angiographically evident CAD (> 70%) or documented ischemia by provocative testing or and at least one high-risk indicator (high-sensitivity C-reactive protein > 3.0 mg/L, diabetes, PAD, stroke, or carotid arterial disease). In the Japanese population, there were no increases in any CURE bleeding events between the atopaxar group and the control group (1% vs 0%; P = 0.48) [43]. In the international trial, a statistically significantly lower rate of any CURE bleedings was observed in the placebo group compared with the combined atopaxar group (0.6% versus 3.9%; P = 0.03) [45]. Regarding safety of atopaxar, a dose-dependent increase in liver enzyme abnormalities and QTc prolongation has been observed in these phase II clinical trials and no phase III trials are available. Therefore, at this time vorapaxar is the only PAR-1 inhibitor approved for clinical use.

Up to now, many possible antiplatelet strategies are available for treatment of patients with coronary artery disease both in primary and secondary prevention. Despite this wide range of possibilities, a considerable number of patients still present ischemic and bleeding events, suggesting that response to antiplatelet agents could present an interindividual variability.

1.4 Figures

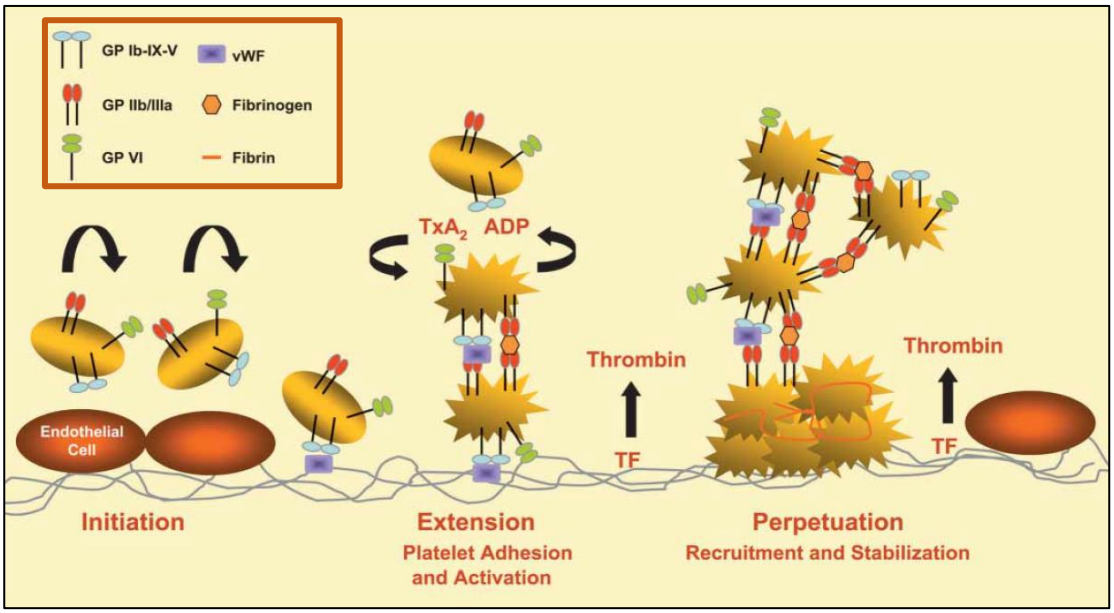

Figure 1. Phases of Platelet plug formation.

Adapted from Jennings LK. Mechanisms of platelet activation: need for new strategies to protect against platelet-mediated atherothrombosis. Thromb Haemost 2009;102:248-57

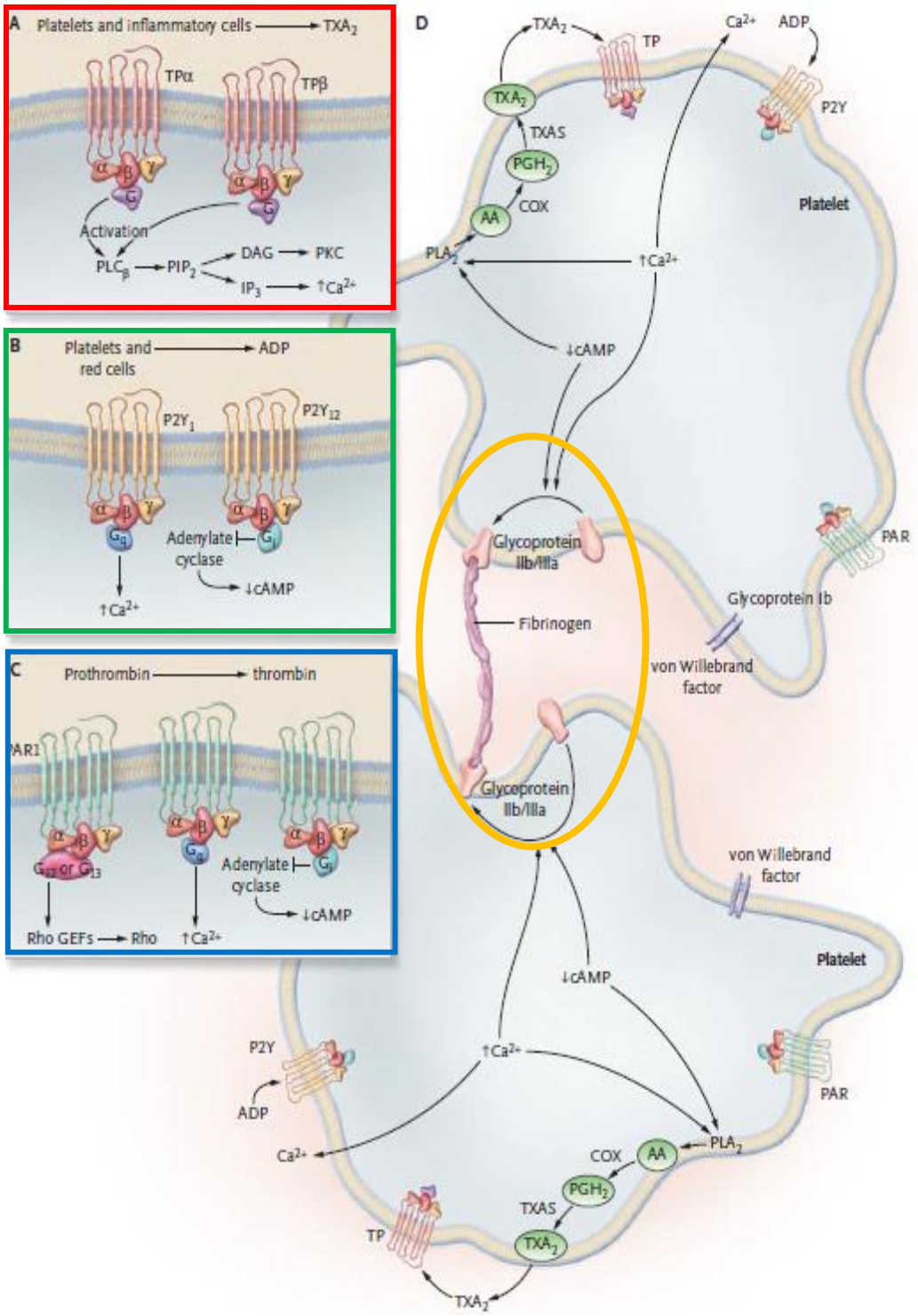

Figure 2. Agonists, Receptors, and Effector Systems in Platelet Activation.

Adapted from Davì G, Patrono C. Platelet activation and atherothrombosis. N Engl J Med. 2007;357:2482-94.

CHAPTER 2

Platelet reactivity and cardiovascular outcomes

Percutaneous coronary intervention (PCI) with stent implantation is currently the treatment of choice for coronary revascularization in patients affected by CAD coronary artery disease (CAD). The introduction of DAPT with aspirin and $P2Y_{12}$ inhibitors has resulted in a dramatic decrease in the incidence of recurrent ischemic complications after PCI and MI [21, 22]. The estimated number of patient requiring DAPT is increasing every year and it was estimated that in Europe 1400000 and 2200000 patients per year may have an indication for DAPT after coronary intervention or MI [49,50].

Nevertheless, cardiovascular events following stent implantation or ACS still occur in a clinically significant proportion of patients [51, 52]. This observation has stimulated intensive research on the pharmacodynamic and pharmacokinetic properties of antiplatelet drugs, focusing on aspirin and $P2Y_{12}$ inhibitors.

Studies measuring platelet function in patient receiving DAPT, revealed that treatment with these drugs is associated with an overall variable level of platelet inhibition. The recurrence of cardiovascular events has been in part attributed to variability in individual responsiveness to DAPT, also known as "resistance".

2.1 Resistance to antiplatelet drugs

The definition of "resistance" to antiplatelet therapy is frequently related to the condition of recurrence of cardiovascular events despite the use of DAPT. More precisely, resistance should be defined as a laboratory finding that consists of failure of an antiplatelet agent that blocks its specific target. In the case of aspirin, resistance is defined by inadequate inhibition or lack of inhibition of the cyclo-oxygenrase-1 (COX-1)-mediated TXA_2 pathway, and for clopidogrel, resistance involves $P2Y_{12}$ receptor signaling [53]. It should be always take in account that thrombotic events involve multiple signaling pathways, and it could be premature to ascribe an adverse outcome to antiplatelet agents' resistance in absence of testing it in the affected patient. When a confirmation by test is not available, it should be first able considered a treatment failure, not a resistance to an antiplatelet drug.

The beneficial antithrombotic effect of aspirin was recognized since 1950s. Nevertheless, 10-20% of patients treated with aspirin experienced another event in long term follow up, indicating that not all patients benefit equally from aspirin treatment. First reports on variability in response to aspirin were available from 1960s. Subsequently it has been shown that patients with elevated degree of platelet reactivity while on aspirin therapy were at increased risk of ischemic events after ACS [54]. Several studies have correlated the aspirin resistance with clinical outcomes, however most of them are studies on patients treated with monotherapy and the prevalence of resistance varied considerably [53, 55]. In particular when it was used a test that specifically access COX-1 activity (serum thromboxane B_2 levels), aspirin resistance is unfrequently reported. It has been reported that the frequency of aspirin resistance or high on aspirin reactivity could be associated to an increased platelet turnover.

Immature platelets empress both COX-1 and COX-2, while mature platelets express only COX-1. This latter is sensible also to very low dose of aspirin, COX-2 is inhibited only by aspirin high doses. So, the presence of a greater amount of newly formed platelets could be explain the phenomenon of aspirin resistance. Other studies suggest that the real aspirin resistance should be restricted to the inability of aspirin to inhibit the COX-1 enzyme to produce platelet dependent TXA_2. In this case the resistance has to be referred only to blocking of COX-1 active site, as in the case of drug-to-drug interaction (as reported in the case of ibuprofen) [56]. Beyond definitions used and possible mechanisms, the problem of aspirin resistance seems to not play a critical role in term of clinical outcome, when considered in the context of DAPT. The ADAPT-DES (Assessment of Dual AntiPlatelet Therapy with Drug-Eluting Stents) registry [57], found no difference in response to aspirin between patients with and without stent thrombosis. Furthermore, data from the ASCET (Aspirin Nonresponsiveness and Clopidogrel Endpoint Trial) Study [58] suggested that high on treatment platelet reactivity to AA is not associated with cardiovascular events. Because of the inconsistency of these results, current evidences do not support the prognostic utility of screening for resistance to aspirin.

The occurrence of thrombotic events despite DAPT suggests the inadequate response to treatment of some patients. Criteria for individual responsiveness to clopidogrel have not been yet standardized. The principal limitation to standardization is related to the multiplicity of available assays for measuring drug's antiplatelet effect, to differing ways in which laboratories use these assays, and to the lack of a standard definition of non-responsiveness. The mechanisms underlying variability in response to clopidogrel are not fully characterized and are probably multifactorial [53, 59]. It is

possible to individualized three groups of factors involved in clopidogrel response variability (Figure n. 1): clinical factors; cellular factors and genetic factors. Increased baseline platelet reactivity may be more frequently observed in specific clinical settings such as ACS, increased body mass index (BMI), and diabetes mellitus (DM), in particular insulin-dependent DM. Other clinical factors that may lead to reduced clopidogrel effects are related to inappropriate dosing, poor compliance and lack of drug prescription. Differences in individual absorption of clopidogrel as well as levels of its active metabolite may also cause clopidogrel response variability. Drugs that are substrates or inhibit the CYP isoenzyme 3A4 can potentially interfere with the oxidation of clopidogrel in liver and reduce the availability of the active metabolite. In particular, studies have shown that lipophilic statins, such as atorvastatin and simvastatin, which require CYP3A4 metabolization, hamper clopidogrel-induced antiplatelet effects [60]. However, these data are quite controversial as larger studies have shown the lack of any interaction between lipophilic statins and clopidogrel [61]. In addition, most studies do not show any negative clinical interaction with co-administration of these drugs. Beyond possible interactions, baseline metabolic activity of this enzyme may also contribute to variability of clopidogrel-induced antiplatelet effects. In fact, individuals with low baseline CYP3A4 activity, which decreases clopidogrel activation, have been shown to have suboptimal clopidogrel responsiveness. [62]. Pharmacogenomic analysis have identified CYP2C19 as the predominant isoenzyme in both oxidative steps of clopidogrel bisulfate [63]. Both loss-of-function (*2) and gain-of-function (*17) variant alleles of CYP2C19 are common in the population and are associated with a variable generation of active metabolite. Therefore, carriers of the *2 allele have been shown to present a less potent

platelet inhibition and an elevated risk of ischemic events following PCI. It is necessary to underline that this genotype accounts for 2-12% of the interindividual variability in response to clopidogrel. Despite this small incidence and the existence of a large number of factors influencing clopidogrel responsiveness, the rapid inactivation after absorption could explain the magnitude of influence of this genetic polymorphism in the formation of the active metabolite of clopidogrel, but does not impact substantially in prasugrel and ticagrelor treatment (Figure n.2) [63-66].

Some studies have also suggested that polymorphisms of other targets not directly involved in clopidogrel metabolism may be involved in response variability. The impact of other genetic polymorphisms on clopidogrel response has also been evaluated. A minor haplotype of the $P2Y_{12}$ receptor was found to be associated with increased platelet reactivity in non-treated healthy volunteers [59, 67, 68]. A genetic polymorphism of the GpIIb/IIIa receptor has been shown to be involved in modulation of clopidogrel response in the acute phase, but it seems to not play a critical role in patients on chronic clopidogrel therapy [69]. Other polymorphisms are under study, these include platelet membrane receptors, such as GPIa, which are pivotal for aggregatory response [70]. While, other mechanisms involved in variability in clopidogrel responsiveness may include increase exposure to ADP, up-regulation of the $P2Y_{12}$ pathway, and up-regulation of $P2Y_{12}$-independent pathways [53,59]. The latter includes enhanced ADP-induced platelet aggregation through the $P2Y_1$ pathway as well as up-regulation of pathways independent of ADP, such as collagen, epinephrine, TXA_2, and especially thrombin (Figure n. 1) [59].

2.2 Methods for measurement of platelet function

The assessment of platelet function has become over years increasingly necessary in various clinical settings: 1) transfusion medicine; 2) evaluation of perioperative hemostasis; 3) identification of patients with bleeding disorders; 4) monitoring to response to antiplatelet treatment. This latter is obviously the setting of major interest for cardiologist, considering the critical role of antiplatelet therapy in treatment of stable and unstable CAD. The platelet function tests are based on different operating principles, and few assays are able to investigate "all in one device" platelet activation pathways. The different modalities of devices to operate may be based on the assessment of platelet adhesion and aggregation, on the submission of platelets under special shear conditions, on the analysis of physical properties of clot, and on the measurement of platelet compounds. In addition to the wide set of assays and devices for studying platelet function, a number of preanalytical variables can produce platelet artifacts affecting the different platelet functions, because platelets are cells that may be easily activated during the blood sampling [71].

2.2.1 Bleeding time

Bleeding Time (BT) is the first test designed for evaluating in vivo primary hemostasis. The test is relatively simple; it evaluates the platelet ability to develop a hemostatic plug by recording the time that the platelets take to occlude an in vivo skin wound for stopping the hemorrhage. Although the technique is easy and quick to perform without any needs for whole blood processing, it is influenced by different variables (i.e. differences in skin thickness and temperature among patients and an incorrect management of the test procedure). Despite the use of available devices to

standardize the test, a lack of accuracy, and unclear association with clinical patient state persist. This test is not generally used to monitor the response to antiplatelet drugs, since no evidence are available in this field. Moreover, the accuracy of BT in prediction of risk of bleeding is not yet clearly established. Nevertheless, BT is still regarded as a screening test to identify both congenital and acquired disorders of primary hemostasis, when other platelet function tests are not available [71].

2.2.2 Light transmission platelet aggregometry

Light transmission platelet aggregometry on platelet-rich plasma (LTA) was designed by Born in 1960 and is still considered the gold standard test for evaluation of platelet function. The test consists in adding a wide panel of agonists to platelet-rich plasma and allows to get a considerable amount of data on various pathways of platelet activation. This assay evaluates in vitro platelet-to-platelet clump formation in a GpIIbIIIa-dependent manner, which is the phase of aggregation, the most important for plug stabilization. The test is substantially based on the measurement of increase in light transmission through the optically dense sample of platelet-rich plasma after addition of the exogenous platelet agonist (i.e. AA, ADP). After the addition of agonists, the platelet-reach plasma becomes clearer because of the precipitation of platelets aggregates. This determines the increase in light transmission through the sample. With a photometer, it is possible to record the rate and maximal percentage of this increase from 0% (maximal optical density of plasma-reach platelets) to 100% (no optical density). Then the signal is automatically converted in a graphic curve that parallels the increase in light transmission during the platelet aggregation. The latency time, the slope of the curve and, the maximal extent of aggregation are the parameters

automatically measured. Also, the shape change and both primary and secondary aggregation may be seen graphically. The possibility of add different agonists allows to obtain information about several features of platelet function.

Until now the LTA is one the most employed methodology for detection of platelet function disorder and for monitoring the efficacy of antiplatelet treatments. It was demonstrated that the monitoring of antiplatelet therapies by LTA has permitted the prediction of MACE in high risk patients, both in the setting of ACS and stable CAD. Although the relevance of LTA as the most complete assay for diagnosis of platelet function disorders, this test presents some problems. The technique may be affected by different preanalytical conditions (ie, type of anticoagulant, lipid plasma, hemolysis, or low platelet count), and by different procedural conditions (ie, PRP preparation, use of different concentrations of agonists), and the laboratory staff should have a high degree of skill, experience, and expertise in performing and interpreting platelet function [71]. Therefore, these features do not make the assay a useful method in emergency conditions, which may require a rapid evaluation of platelet function.

2.2.3 Lumiaggregometry

Lumiaggregometry lets simultaneous measurement of release of adenine nucleotides from platelet granules and platelet aggregation. The assay is based on the evaluation of adenosine triphosphate (ATP) released from activated platelets by different agonists by using a luminescence technique in platelet-rich plasma, washed platelets or whole blood. The test evaluates the conversion of ADP released by platelets in ATP that interacts with the luciferin–luciferase reagent. The light emitted, proportional to the ATP concentration, is quantified by the lumi-aggregometer. This

test is used to identify specific deviancies in content of number of granules. This analysis could be useful as first screening in patients with clinical suspicion of platelets function abnormalities [71].

2.2.4. Impedance whole blood aggregometry

Platelet aggregation can be measured in anticoagulated whole blood by the use of impedance whole blood aggregometry (WBA). This test is based on the principle that activated platelets stick with their surface receptors to the artificial surfaces of two electrodes within the whole blood sample positioned at a fixed distance between them. Platelet aggregation in this case is measured by detection of the increase in electrical impedance generated by aggregation of other platelets upon those fixed to electrodes. Thus, by diminishing the current intensity the electrical impedance increases. The degree of this increase is recorded in Ohms. With this assay, it is possible to evaluate platelet function under more physiological conditions considering the contributions on platelet aggregation also of other elements of the blood. The assay presents two key advantages: 1) there is no manipulation of the sample without activation of platelets allowing a rapid analysis of platelet function; 2) the quantity of whole blood required is small and all subpopulations are present [71,72].

Nowadays, the multiple Electrode Aggregometry (MEA), is a new methodology that allows to assess the whole blood platelet aggregation with a new device working like a point of care test. The device (Multiple Platelet Function Analyzer- Dynabyte-Roche diagnostics, Manheim, Germany) shows featured that make it a valid assay for a rapid and complete platelet function evaluation. It is a five-channel computerized instrument equipped with some disposable cuvettes ready to use with two independent

sensor units and an automated pipetting. The platelet aggregation is simultaneously measured in duplicate by using each sensor unit separately and calculated automatically as area under curve (AUC). MEA has acquired a high clinical relevance like LTA: the use of different agonists similar to those used for LTA has made it suitable for the diagnosis for both bleeding diathesis and monitoring antiplatelet therapies, defining cut-off values to discriminate cardiovascular patients with high on-treatment platelet reactivity [71,72]. Therefore, MEA is not only able to identify patients not responding to antiplatelet drugs and at risk of cardiovascular events, but it a useful tool in identification of patients with high level of platelet function inhibition and at risk for bleeding events [73, 74]. The advantages of MEA compared to LTA are several: it is not necessary a specialized laboratory, it requires only minimal technical knowledge and training to use it, the working steps are automatically accomplished and easily controlled, and only interpretation data and judgment are required.

2.2.5 VerifyNow System

The VerifyNow system (Accriva Diagnostics, San Diego, CA, USA), is a point of care test consisting in a device that assess in whole blood platelet aggregation by a turbidimetric-based optical detection and using a system cartridge containing fibrinogen-coated beads and platelet agonists. The assay is based on the capacity of activated platelets to bind to fibrinogen: platelets aggregate upon the fibrinogen-coated beads within the assay cartridge in proportion to the number of activated GpIIb/IIIa receptors. The crossing whole blood optical signal increases as the activated platelets aggregated to the fibrinogen attached to the spheres. The system is very easy to use, it does not require manipulation or instrument handling or specialized laboratory. It is

used especially in emergencies room because it provides a rapid result. The method is initially used to evaluate the inhibitory effect of GpIIb/IIIa antagonists in patients undergoing PCI (VerifyNow IIb/IIIa Test): the thrombin receptor activating peptide (iso-TRAP) is the agonist used to stimulate the platelet aggregation. The results are reported as Platelet Aggregation Units (PAU). At this time other two tests are available: Aspirin Test with AA as agonist and PRU test with ADP and prostaglandin E1 (PGE_1) as agonists. PGE1 is a suppressor of intracellular free calcium levels of diminishing the non-specific influence of the ADP-binding to $P2Y_1$ receptors (Figure n. 3). The results of these last two tests are respectively expressed as Aspirin Reaction Unit (ARU) and $P2Y_{12}$ Reaction Unit (PRU). Several studies have reported the clinical value of this test for evaluation of response to antiplatelet treatment in patients treated with PCI. Indeed, sensitivity and specificity rather than optimal cut-off value of the tests were objects of controversies among different study groups [71,72,74].

2.2.6 Plateletworks System

The Plateletworks System in based on the measurement of platelet count before and after aggregation. The system consists of EDTA tubes and citrate tubes implemented with agonists (AA or ADP) and the Inchor blood couter (Helena Laboratories, Beaumont, TX USA). This test matches the platelet count (control sample in EDTA tube) with those obtained after aggregation induced by ADP or AA (citrate tubes). The fall of platelet count in citrate tube, because of aggregation, is a measure of the extent of platelet activation. This test is rapid and results are available in a few minutes. It is also relatively sample because it does not require any blood manipulation. The real disadvantage is that it should be performed few minutes after blood sampling. This

assay gives information on platelet count and activation, but no data are available on its role in prediction of clinical outcomes [71].

2.2.7 The Platelet Function Analyzer – PFA-100/Innovance PFA-200

The Platelet Function Analyzer – PFA-100/Innovance PFA-200 (Siemes, Munich Germany) is based on the property of platelets to adhere upon shear stress conditions and aggregate in presence of agonists. This is a point of care test that evaluate platelet function from whole blood samples using appropriate cartridges in which primary hemostasis is simulated. There are two test-cartridges: collagen plus ADP – CADP cartridge – or collagen plus epinephrine (EPI) – CEPI cartridge. Within the cartridge, the citrate whole blood sample is drawn at a high shear stress rate through a capillary that has at its end a collagen-coated membrane, in which a defined microscopic aperture (147 μm) is present, filled with either ADP or EPI. A platelet clot, which fills the hole, occurs because of shear stress and agonists. The time taken by platelets to occlude the orifice and to block the whole blood flow is defined as closure time and it is a measure of overall platelet-related hemostasis. The closure time is as much prolonged as the platelets are able to act [71]. The use of two different cartridge is functional for the differentiation of platelets dysfunction derived by intrinsic platelets defects (von Willenbrand defects, Bernard-Soulier Syndrome etc.) from those induced by antiplatelet therapy. The principal limitation of PFA-100 is that it is sensitive to variables that influence platelet function (closure time prolongation) as well as low platelet count and hematocrit. In addition, short CEPI closure time could reveal high residual platelet reactivity despite antiplatelet treatment with aspirin and was demonstrated to be a predictor of ischemic events in patients with STEMI undergoing

primary PCI [71,75]. Finally, this test is characterized by a high negative predictive value. Therefore, in case of a normal closure time in a patient with a suspected platelet disorder, no other platelet tests should be performed.

2.2.8 Phosphorylation of vasodilator-stimulated phosphoprotein

Phosphorylation of vasodilator-stimulated phosphoprotein (VASP) for the measurement of $P2Y_{12}$ antagonism is available commercially as a flow cytometry kit (BioCytex, Marseilles, France).

This test was based on the activation of platelet signal by prostaglandin. PGE1 binds to its inositol phosphate receptor on the platelet surface and signals through a G stimulatory protein and adenylyl cyclase to convert ATP to cAMP then, through protein kinase A, to convert VASP to phosphorylated VASP (VASP-P). ADP binds to its $P2Y_{12}$ receptor and signals through a G inhibitory protein to inhibit PGE1–induced signaling through adenylyl cyclase. $P2Y_{12}$ antagonists (active metabolite of clopidogrel) inhibit this ADP-induced effect. Therefore, in the presence of both prostaglandin E1 and ADP, VASP-P is directly proportional to the degree of $P2Y_{12}$ antagonism. VASP-P is measured by whole blood flow cytometry, using permeabilization and a monoclonal antibody specific for the phosphorylated form of VASP.

The advantages of this test are that it is dependent on the target of clopidogrel ($P2Y_{12}$), and it involves low sample volume of whole blood. The disadvantages are sample preparation and the requirement for a flow cytometer and an experienced technician.

2.2.9 IMPACT: Cone and Plate(Let) Analyzer

IMPACT: Cone and Plate(Let) Analyzer (Image Analysis Monitoring Platelet Adhesion Cone and Plate Technology) (CPA) (DiaMed, Cressier, Switzerland) is a point of care test that fully assesses platelet function by a computerized system, evaluating primary hemostasis. The test is based on adhesion and activation of platelets lying on plate covered by polystyrene. The shear stress is impressed to platelets by a spinning cone on the plate. The test needs of whole blood sample with citrate. The measurement of platelet function is based on the percentage of the well surface covered by platelet aggregates (representing platelet adhesion) and the average size of the aggregates (representing platelet aggregation). Advantages of this test are the simplicity, low sample volume, rapid readout, no sample preparation. The disadvantages are that it is not a true point-of-care instrument, because it requires pipetting. The possibility to add agonists (AA or ADP) allows to monitor the efficacy of antiplatelet treatment. Nevertheless, larger studies are needed to address the possible role of this test for prediction of clinical outcome of patients with CAD, and to date the instrument is not widely used in clinical practice [71,72]

2.2.10 The global thrombosis test

The global thrombosis test (GTT) (Montrose Diagnostics Ltd, London, UK) is a relatively recent assay which evaluates platelet function in a way closer to physiological conditions, because it is performed by using native nonanticoagulated whole blood, without adding agonists. The test is based on the evaluation of platelet activation in high shear stress condition, similar to that in the coronary artery stenosis. The whole blood was inserted in plastic tube with a conical part in which are placed

two ceramic balls. Also in this case the assay is based in occlusion time and provides information on patient's thrombotic status, but its association with clinical outcome is still under evaluation [71].

2.2.11 Thromboelastography and thromboelastometry

Thromboelastography and thromboelastometry are methodologies employed for the global assessment of the hemostasis. These tests evaluate the entire development of clot formation and are based on the analysis of whole blood modifications in viscoelastic forces during the clotting formation. Therefore, the real importance of these assays is related to the possibility to assess the extent of platelet count and function, clotting, and fibrinolytic activation. To date three systems are available: Thromboelastography, performed on TEG (Haemoscope, IL, USA), Thromboelastometry, formerly called Rotational Thromboelastography, performed on ROTEM (TEM Int, Munich, Germany), and Sonoclot analysis performed on Sonoclot Signature (Sienco, CO, USA).

TEG and ROTEM are similar and consist in a rotating system inclusive of a pin suspended by a torsion wire in a cup. In the whole blood sample, owing to the addition of reagents and the shear stress of the rotating system, the forming clot entraps the pin promoting a motion that increases as the clot strengthens and decreases as the clot lyses. The addiction of specific reagents and activator allow to investigate both extrinsic and intrinsic pathways. All these instruments promptly deliver a graphic representation of clot formation and lysis and are currently used as point of care test in particular setting, such as cardiac surgery. More recently, it was developed a TEG platelet mapping system as tool for monitoring of antiplatelet treatment. Although

initial results are encouraging, further large prospective trials are needed to define the possible role of these devices in evaluation of platelet response to therapy and its link with cardiovascular outcomes [71].

2.1.12 Evaluation of Thromboxane metabolites

Serum thromboxane B2 and Urinary 11-dehydro thromboxane B_2 are the two metabolites that could be dose in serum and urine. TXA_2 is the major product of platelet metabolism of AA. When TXA_2 is released from platelets and it is rapidly converted to its stable and inactive metabolite, thromboxane B_2 (TXB_2). The TXB_2 can be dosed in plasma, the 11-dehydro TXB_2 is the stable metabolite released in urine. Both dosages have the advantage to evaluate directly the platelet pathway dependent on aspirin's target, COX-1. The disadvantages of measuring serum TXB_2 or urinary 11-dehydro TXB_2 are that these are indirect measures in the sense that platelets are not directly assayed, and they may not be entirely platelet specific [72]. The potential effects of renal function are obviated by measuring the ratio of urinary 11-dehydro TXB_2 to creatinine [72].

2.2.13 Platelet function test for monitoring antiplatelet therapy

Despite this large possibility of tests, the real problem is how to choose the right assay for each drug or for each clinical setting. In general, for aspirin we can have several options. First able it is possible to use thromboxane as the end point, with an assay of either serum TXB_2 or urinary 11-dehydro TXB_2. The advantage of this approach is that aspirin specifically inhibits platelet COX-1 and, therefore, thromboxane generation. A possible disadvantage is that all patients who are compliant

with taking aspirin are inhibited as judged by serum thromboxane B_2 or urinary 11-dehydro thromboxane B_2 assays, but not necessarily as judged by other assays. Another approach could be the use of AA as stimulus. In this case the AA results in specific signaling through COX-1, but the assumption is that all of the effects of aspirin result from its inhibition of COX-1, which might not be entirely true. Multiple type of assays could be used with AA as agonists impedance platelet aggregometry, the VerifyNow aspirin assay, Plateletworks, platelet surface–activated GP IIb/IIIa, the TEG Platelet Mapping system, and the Impact cone and plate(let) analyzer. Finally, the PFA-100 could be another option even if not COX-1 specific.

There are several assays also for monitoring response to $P2Y_{12}$ inhibitors. VASP phosphorylation assay is specific with regard to signaling through $P2Y_{12}$ and therefore to the platelet inhibitory effects of $P2Y_{12}$ inhibitors. The alternative approach is to add ADP as agonists. However, it is important to consider that ADP binds to its platelet surface $P2Y_1$ receptor as well as to its platelet surface $P2Y_{12}$ receptor and that the $P2Y_{12}$ inhibitors only inhibit $P2Y_{12}$, not $P2Y_1$. Therefore, the effects of ADP on platelet function reflect also the unblocked effect of ADP induced signaling through $P2Y_1$. With ADP as the stimulus, it is possible to choose among several assays: impedance platelet aggregometry, the VerifyNow $P2Y_{12}$ assay, Plateletworks, TEG Platelet Mapping system, and the Impact cone and plate(let) analyzer.

GpIIb/IIIa antagonists can be monitored by 2 categories of assays. GpIIb/IIIa antagonists block the final common pathway of platelet aggregation, in this case available tests are: impedance aggregometry, VerifyNow thrombin receptor–activating peptide assay, or Plateletworks. Moving from the concept that platelet aggregation cannot occur without a conformational change in integrin αIIbβ3 (Gp

IIb/IIIa), flow cytometry can be used to measure a conformational change in platelet surface–activated GpIIb/IIIa reported by monoclonal antibody PAC-1 or a ligand-induced binding site. This latter was no previously reported because out of the interest for this research work [72].

The real issue of monitoring the response to antiplatelet drug is related to the application in clinical practice. If we need to use a test to guide the decision about antiplatelet treatment to administered to a patient, we need of a tool with the subsequent characteristics: easy to use, requiring no special skills, use at or near the patient bedside; no sample processing; rapid readout. In this field, probably VerifyNow System has these characteristics and this will be the assay used in some research projects from this thesis.

2.3 Role of platelet reactivity in prediction of cardiovascular outcome

The issue of clinical significance of variability in response to antiplatelet drugs became of interest in the era of clopidogrel treatment and it is nowadays still debatable, despite the introduction of more potent $P2Y_{12}$ inhibitors [49].

The first step in the evaluation of role of platelet reactivity in prediction of cardiovascular outcome was the definition of low response to antiplatelet drugs. Previous reports have tried to establish a standard definition of low clopidogrel response and have investigated the correlation between results of platelet function tests and clinical outcomes, to demonstrate the prognostic impact of high residual platelet reactivity (HPR) on both short- and long-term outcome. Price et al. have conducted the first clinical study on this issue. They enrolled 380 patients undergoing PCI with

drug eluting stent (DES) with a preprocedural evaluation of response to clopidogrel 600 mg loading dose, using VerifyNow system. A cut-off of PRU value ≥ 235 was identified as optimal cut off value for prediction of 6-month outcome, including cardiovascular death, non-fatal MI or stent thrombosis (ST) [76]. Consistently with this previous evidence in the ARMYDA-PRO (Antiplatelet therapy for Reduction of MYocardial Damage during Angioplasty-Platelet Reactivity Predicts Outcome) study [77], it was prospectively evaluated the relationship between residual platelet reactivity, measured by the VerifyNow $P2Y_{12}$ Assay, and 30-day incidence of MACEs in 160 clopidogrel-treated patients undergoing PCI. In this study, the 30-days incidence of MACEs was greater in patients with periprocedural PRU levels in the highest quartile versus those in the lowest quartile (20% vs 3%, P=0.034). A PRU value ≥ 240 was identified as the optimal cut-off value to predict 30-days outcome, providing a sensitivity of 81% and a specificity of 53% [77]. Other evidences supporting the relation between platelet reactivity and cardiovascular outcome came from the ACS setting. Matetzky et al. demonstrated, in 60 patients undergoing PCI for acute MI, that HPR assessed by LTA and defined as ADP-induced platelet aggregation ≥103±8% of baseline, was associated with an increased incidence of ischemic events (40% vs 6.7%; P for trend 0.007) [78]. Subsequently, two more studies enrolling patients with NSTEMI showed that HPR on clopidogrel measured before PCI was associated with an increased rate of periprocedural MI and higher incidence of ischemic events at 1-month follow up [79,80]. Parodi et al. showed that HPR, defined as an ADP test results ≥70% platelet aggregation, was an independent risk factors for long-term thrombotic events in a population of 1789 ACS patients treated with PCI [81]. In this latter study, patients were treated with a clopidogrel loading dose of 600

mg followed by 75 mg once daily and a loading dose of 325 mg of aspirin. Patients presenting with low responsiveness to clopidogrel were treated with a higher maintance dose (150-300 mg) of clopidogrel and followed with by ADP guidance. The primary composite endpoint (CV death, MI, urgent revascularization, and stroke) at 2-years follow-up was significantly higher in the HPR group compared to patients with normal response (14.6% vs 8.7%, P=0.003) with an absolute risk increase of 5.9%. In the same population, the incidence of ST was higher in HPR compared to normal responders (6.1% vs 2.9%, P=0.01). By multivariate analysis, HPR was independently associated with primary endpoint (P=0.02) and cardiovascular death (P=0.006) [81]. Using VerifyNow System in a population with ACS, Marcucci et al. confirmed the optimal cut-off of PRU≥240 to predict 12-months incidence of CV death and nonfatal MI [82]. Nevertheless, this cutoff distinguishing between patients with and without CV events could be slightly different in patients with unstable CAD compared to those with stable CAD [57]. Indeed, Park et al. reported a strong relationship between HPR and ischemic events in ACS patients, but not in the low risk stable population [83]. These data were confirmed in the large, multicenter registry ADAPT-DES [57], which showed that the probability of ST within after 30 days after DES could be distinguished between patients with and without ACS. In particular, in the multivariate analysis, a PRU> 208 was independently associated with ST risk in ACS patients (P=0.005; HT 3.91), whereas no significant association was identified between platelet reactivity and ischemic events in patients with stable CAD [57]. The message consistently emerging from these evidences is that an adequate degree of platelet inhibition reduces ischemic events in patients undergoing PCI, mostly in patients presenting with ACS. At the same time, other studies have shown that a greater inhibition of platelet reactivity could

expose to higher incidence of bleeding complications, which are also associated with a worse clinical outcome. Ndrepepa et al. in 5684 patients from the ISAR (Intracoronary Stenting and Antithrombotic Regimen) studies demonstrated a relationship between the 30-day frequency of bleeding and 1-year mortality after PCI [83]. Based on these evidences, in several trials it was achieved the inclusion of periprocedural bleeding in a 30-day quadruple endpoint for the assessment of outcome after PCI. Regarding the relationship between platelet reactivity and bleedings, Sibbing et al. showed that the risk of major bleeding in patients treated with 600 mg of clopidogrel undergoing PCI was significantly higher in those with an increased response to treatment (<124 AU/min assessed with the Multiplate analyzer) as compared to those with PR ≥124 AU/min (2.2% vs 0.8%; OR 3.5;95% CI, 1.6-7.3; P=0.001) [85]. Similar results were also reported in the ARMYDA-Bleeding Study, which showed that the 30-day incidence of major bleeding or entry-site complications after PCI occurred more frequently in patients with PRU levels in the lowest quartile (10.1% vs 1.3%, P=0.043). The optimal cut-off for prediction of primary endpoint was a PRU value <189 [86].

Therefore, putting together evidences from these latter studies it was suggested the possibility to identify a therapeutic window for platelet reactivity associated with the low-incidence of ischemic and bleeding events. Indeed, the incidence of thrombotic and bleeding events according to PRU value follows a curvilinear distribution in which below a safety threshold of PRU, thrombotic events are no further reduced at the expense of increased bleeding, and above an efficacy threshold, thrombotic events may be significantly increased and bleeding is not reduce (Figure n. 4). Several studies have tried to define what are the values of a therapeutic window, but using different

methods. Sibbing et al., using the Multiplate analyzer, found that patients with pre-PCI platelet reactivity range of 189-467 AU/min had the lowest rate of risk for occurrence of both bleeding and ischemic events, classified as in-hospital bleeding and 30-day ST [87]. Subsequently, Campo et al. identified a therapeutic window for platelet reactivity between 86 and 238 PRU as assessed by VerifyNow P2Y12 assay. Nevertheless, in this study platelet reactivity was measured 30 days after PCI and, adverse events that occurred within the first month were excluded from the analysis [88]. The ARMYDA-PROVE (Antiplatelet therapy for Reduction of MYocardial Damage during Angioplasty- Platelet Reactivity for Outcome Validation Effort) Study [89] identified in a pre-PCI PRU value ≥239 the optimal cut-off to discriminate the risk of ischemic events and in PRU value ≤178 the cut-off for prediction of bleeding events. In this latter study when population was divided in three groups based on PRU values [low platelet reactivity (LPR) for PRU ≤178; normal platelet reactivity (NPR) for PRU between 178 and 239; high platelet reactivity (HPR) for PRU ≥ 239] the incidence of net adverse clinical events (both thrombotic and bleeding events) was significantly lower in the intermediate level of platelet reactivity (7.8% in NPR, 14.8% in LPR and HPR, P=0.007) [89]. As it was shown from these latter studies before to definitively identify a therapeutic window and its clinical value as guidance for adjusting treatment, it important to underline that these data came from different types of population, with different timing of test and different types of assays used. In this regard, it is also important to consider that not all platelet function tests are equal. In the POPULAR study, which is a registry with the largest head-to-head comparison between platelet function assay, 1069 patients undergoing PCI were evaluated for platelet reactivity in order to determine the prognostic utility of the test. From the five

assays used in the study, only LTA, VerifyNow and Plateletworks were associated with the occurrence of thrombotic events, while IMPACT-R and PFA-100 were not associated with MACE [90]. Multiplate analyzer and VASP were not tested in this registry, but in other studies they were able to predict clinical outcome for both bleeding and ischemic events [63,74]. The differences in measurement principles between assays may have serious consequences on the measured level of PR inhibition, leading to a poor correlation between results of different assays. So, results from studies with a specific device could be not be applicable to all types of assays. Another important issue to consider is the type of agonist used in the test. In the ADAPT-DES among 8500 PCI treated patients, the VerifyNow P2Y12 kit with ADP (and PGE1) stimulation showed a significant association with one-year occurrence of stent thrombosis, mortality and major/clinically relevant bleeding. While results from the VerifyNow Aspirin test (with AA as agonist) in the same population were not correlated with clinical outcome [57]. To date, probably the most robust data to evaluate the role of platelet function test for prediction of clinical events and for stratification of patients in risk categories, come from a collaborative analysis of 17 studies including 20839 patients [91]. In this analysis, only VerifyNow, Mutliplate and VASP were included and patients were divided in three groups according the level of platelet reactivity: LPR, OPR (optimal platelet reactivity), and HPR. Uniform cut-off values were used for the selected platelet function assays (VerifyNow: 95 and 208 PRU, VASP: 16 and 50% PRI, Multiplate: 19 and 46 U). Results showed that patients in the HPR group had a 2.7-fold higher risk for definite or probable ST, while those with LPR had a 1.7-fold higher risk for bleeding compared to the OPR group. These results confirm that in PCI-treated patients, PR on clopidogrel, measured by

VerifyNow, Multiplate or VASP, predicts both thrombotic and bleeding events; therefore, platelet reactivity may be a valuable biomarker for risk stratification. Then, it is possible to conclude that this great amount of data derived from a large number of studies robustly supports the role of PR as predictor of clinical outcome in patients undergoing PCI, especially in the setting of ACS.

2.4 Role of platelet reactivity as guide for a tailored antiplatelet therapy

Once established the role of platelet reactivity as predictor of ischemic and bleeding events, the next step was to investigate whether platelet reactivity could have been used in clinical practice to guide a tailored antiplatelet therapy for a specific patient. Several randomized trials and registries have addressed this issue and results have been matter of debate.

The concept of a tailored therapy based on PR consist in the use of results of periprocedural PR to guide the choice of treatment. In particular, in patients with a low response to clopidogrel and higher ischemic risk, more aggressive antiplatelet regimen might be useful in obtaining platelet reactivity values within the therapeutic range. These strategies include the use of higher clopidogrel doses, newer and more potent $P2Y_{12}$ inhibitors (prasugrel and ticagrelor), administration of GpIIb/IIIa inhibitors. A first laboratory study evaluating platelet reactivity by LTA in ACS patients treated with PCI and receiving three different clopidogrel doses (300 mg loading and 75mg/die maintance dose, 600 mg loading and 75 mg/die maintance dose; 600 mg loading and 150 mg/die maintance dose) showed that a greater overall inhibition can be achieved with higher clopidogrel doses also in this setting of acute patients, even if

in a proportion of patients (8-11%) persisted a low response to treatment [92]. Two pilot studies, that tailored therapy on the base of platelet reactivity, showed a reduction in ST and MACEs, but subsequent results from randomized trials have not been so encouraging [93,94].

The first multicenter, randomized double-blind, active-control trial comparing platelet function-guided antiplatelet treatment in patients undergoing PCI was the GRAVITAS (Gauging Responsiveness with A VerifyNow assay—Impact on Thrombosis And Safety) study [95]. The aim of the study was to compare elevated (150 mg) and standard (75 mg) maintenance doses of clopidogrel in patients with HPR defined as a VerifyNow PRU result greater than 230 PRU after PCI. Altogether, 2214 patients were enrolled into the study: the majority (60%) was low-risk elective patients undergoing PCI, 40% had ACS on admission (10% with acute MI but patients with STEMI were excluded). The primary endpoint was the 6-month incidence of composite endpoint (CV death, nonfatal MI or ST). It is necessary to underline that the study was empowered to a primary endpoint rate of 5% in patients with HPR on standard 75 mg clopidogrel, and anticipated a 50% reduction in the primary endpoint. At six months of follow-up, there was no difference in the incidence of the primary endpoint in the two groups: 150 mg clopidogrel did not reduce the risk of CV death, MI or stent thrombosis (2.3% vs 2.3%, P=0.97). Another point of interest is the absence of a lack of safety: administration of an elevated dose of clopidogrel did not increase the risk of bleeding events. Furthermore, as well as highlighted by Aradi et al. [96], the study presented some important limitations: 1) low-risk patients subset excluding those with STEMI; 2) 150 mg clopidogrel had only a modest impact on HPR; 3) the cutoff chosen (230 PRU) for VerifyNow may have been too high,

currently a lower cutoff (208 PRU) is recommended; 4) lack of power due to inappropriately estimated primary event rate (anticipated: 5% vs. observed: 2.3%) decreases the statistical validity of results. Therefore, a conclusion from the study could be that in low-risk patients undergoing PCI, the increase of clopidogrel dose to 150 mg based on PRU values did not reduce the risk of 6-month MACEs, but is not harmful and did not expose patients to a higher risk for bleeding. These results can be interpreted as a failure of the strategies to increase dose of clopidogrel to 150 mg, but not as a failure of the entire strategies of tailoring antiplatelet therapy following PR.

The ARCTIC (Assessment by a Double Randomization of a Conventional Antiplatelet Strategy versus a Monitoring-guided Strategy for Drug-Eluting Stent Implantation and of Treatment Interruption versus Continuation One Year after Stenting) trial was the second great randomized trial on this issue [97]. Here 2440 patients, scheduled for coronary stenting at 38 centers in France, were randomized to a strategy of platelet-function monitoring, with drug adjustment in patients who had a poor response to antiplatelet therapy, or to a conventional strategy without monitoring and drug adjustment. The primary endpoint was incidence of the composite of death, MI, ST, stroke, or urgent revascularization at 1-year follow up. For patients in the monitoring group, the VerifyNow $P2Y_{12}$ and aspirin point-of-care assays were used in the catheterization laboratory before PCI and in the outpatient clinic 2 to 4 weeks later. In this study patients requiring adjusted treatment comprised a heterogeneous group of patients with HPR on clopidogrel (>235 PRU on VerifyNow $P2Y_{12}$ test), inappropriate platelet inhibition to clopidogrel (<15% inhibition o VerifyNow $P2Y_{12}$ test) and HPR to aspirin (defined as > 550 ARU on VerifyNow Aspirin test). Also, strategies to overcome low responsiveness were quite different: administering GpIIbIIIa inhibitors

during PCI, increasing the dose of clopidogrel and increasing the dose of aspirin after PCI, switching to prasugrel (even if was rarely used in the study, 9%). Also in this trial, the incidence of primary endpoint did not significantly differ between two groups (34.6% vs 31.1% in patients with conventional treatment, P=0.1). Some limitations need to be highlighted: 1) exclusion of high-risk STEMI patients; 2) use of heterogeneous definitions for low responsiveness, 3) inappropriate pharmacological interventions including mostly high-dose clopidogrel and high-dose aspirin, 4) open-label design possibly interfering with data reporting and patient follow-up [96, 97]. The TRIGGER-PCI (Testing platelet Reactivity In patients undergoing elective stent placement on clopidogrel to Guide alternative thErapy with pRasugrel) trial investigated the effectiveness of prasugrel versus clopidogrel in patients with high platelet reactivity after DES implantation [98]. The primary endpoint was a composite of CV death and MI at 6 months. The trial was prematurely stopped for futility due to the lower-than anticipated event rate in study groups. As in the GRAVITAS [95], also in the TRIGGER-PCI there was the limitation that the treatment was tailored post-PCI, whereas pharmacodynamic assessment of platelet inhibition was before the procedure. Even if without a clinical improvement, the study clearly demonstrated that HPR can be corrected by switching from clopidogrel to prasugrel [98]. Similarly the RESPOND (Response to Ticagrelor in Clopidogrel Nonresponders and Responders and the Effect of Switching Therapy) trial [99] showed that also treatment with ticagrelor, regardless of clopidogrel response, can induce a reduction of platelet reactivity below the cut off point for ischemic events (defined as >59% 20 μmol/L ADP-induced maximal platelet aggregation, ≥235 PRU based on the VerifyNow $P2Y_{12}$ assay, and >50% Platelet Reactivity Index based on the VASP phosphorylation assay). But the achievement of

a good response to $P2Y_{12}$ inhibitors was not translated in a clinical benefit [99]. At the same time, some interesting data on this issue came from registries.

Aradi et al. studied the value of treatment intensification based on the Multiplate assay in a single-center, all-comer, high-risk ACS registry [100]. In this analysis including 741 patients, with almost 50% STEMI, subjects with HPR (defined as a Multiplate ADP test >46 U) were treated with either high-dose clopidogrel or were switched over to prasugrel. The primary endpoint was the 1-year incidence of all-cause death, MI, ST or stroke between patients with high-dose clopidogrel or switched to prasugrel due to HPR and those without HPR treated with standard 75 mg clopidogrel. Patients with HPR receiving high-dose clopidogrel had a 2.3-fold higher risk for the primary endpoint compared with those without HPR, with no clinical benefit for high-dose clopidogrel among patients with HPR. However, patients with HPR switched to prasugrel showed a significantly lower risk of MACEs reducing the risk of events to the level of NPR. These results suggested that high risk patients with HPR could benefit from switching to prasugrel, but not from a dose-elevation of clopidogrel [100].

In RECLOSE-3 ACS (REsponsiveness to CLOpidogrel and StEnt Thrombosis) study, 550 patients with HPR were compared after treatment with high-dose clopidogrel (historical control, n = 248) or switching to prasugrel (n = 302). According to the results, switching to prasugrel significantly reduced the risk of MACE, including CV mortality (9.7% vs 4.0%, P=0.007). [101]. These data are consistently with those presented by Janssen at the EuroPCR 2015: in the POPULAR Risk Score Registry patients were followed after PCI and treated with a guided antiplatelet intervention based on a risk score integrating PR and clinical features. Patients with high risk score were switched to prasugrel and showed a significant reduction in ischemic events

compared to an historical cohort (8.6% vs 4.1%, P<0.001). Therefore, data from more recent registries support the strategy of switching to prasugrel in high risk patients with HPR [96].

In recent years, with the advent of more potent $P2Y_{12}$ inhibitors (prasugrel and ticagrelor), which are in first line of indication for treatment in ACS [49], also randomized trials have changed their focus moving from the research for superiority to non-inferiority studies in high-risk patients with prasugrel and ticagrelor as reference groups. In the ANTARCTIC study [102], an ACS population of elderly patients (75 years or older) treated with PCI was enrolled. A total of 877 patients were randomized to receive oral prasugrel 5 mg daily with dose or drug adjustment in case of inadequate response (monitoring group) or oral prasugrel 5 mg daily with no monitoring or treatment adjustment (conventional group). The primary end point was a composite of CV death, MI, stroke, ST, urgent revascularization and BARC (Bleeding Academic Research Consortium) bleeding complications [103] at 12-months' follow up. No differences were registered in incidence of primary endpoint between two groups (28% vs 28%; HR 1.003, 05% CI 0.78-1.29; P=0.98). In the TROPICAL-ACS (Testing Responsiveness To Platelet Inhibition On Chronic Antiplatelet Treatment For Acute Coronary Syndromes) study [104], 2610 patients with ACS with successful PCI and a planned duration of DAPT of 12 months, were randomized to standard treatment with prasugrel for 12 months (control group) or a stepdown regimen (1 week prasugrel followed by 1 week clopidogrel and PFT-guided maintenance therapy with clopidogrel or prasugrel from day 14 after hospital discharge; guided de-escalation group). The composite primary endpoint was the 1-year net clinical benefit (CV death, MI, stroke or bleeding grade 2 o higher according

BARC criteria). The study was designed to address the non-inferiority of the de-escalation strategy. The platelet function assay used is the Multiplate analyzer. The primary endpoint occurred in 7% of patients (n=95) in the guided de-escalation group and in 9% of patients (n=118) in the control group ($P_{non\text{-}inferiority}$=0.0004; HR 0·81; 95% CI 0·62–1·06, $P_{superiority}$=0.12). There was no increase in the combined risk of cardiovascular death, myocardial infarction, or stroke in the de-escalation group (3%) versus in the control group (3%; $P_{non\text{-}inferiority}$=0.0115). Incidence of BARC 2 or higher bleeding events was 5% in the de-escalation group vs 6% in the control group (HR 0·82;95% CI 0·59–1·13; P=0.23). The trial substantially demonstrated the non-inferiority of an early guided de-escalation of antiplatelet treatment, suggesting this latter as an alternative approach in patient with ACS treated with PCI [104].

Finally, to date no definitive conclusions are available in relevance of platelet reactivity as guide for treatment in clinical practice. Nevertheless, further studies are needed to better define this role.

2.5 Figures

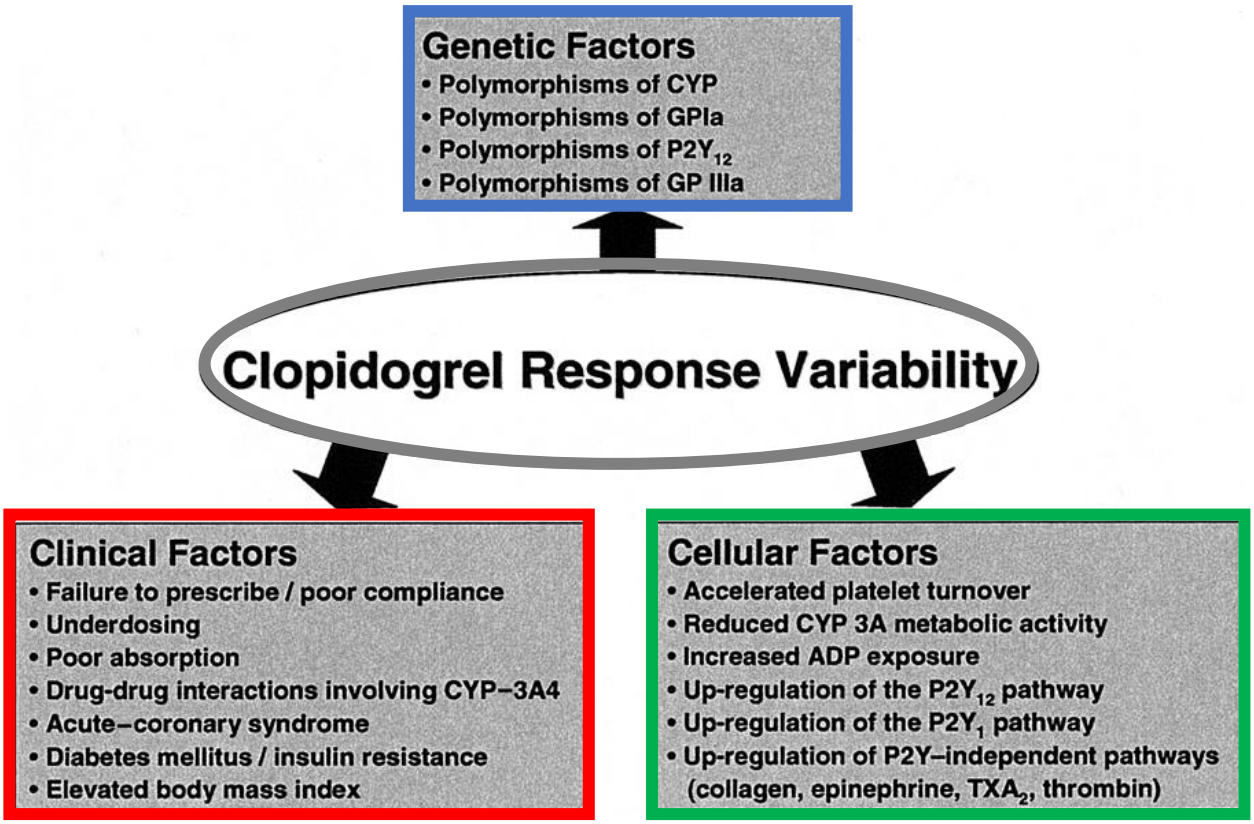

Figure 1. Factors involve in clopidogrel response variability.

Adapted from Angiolillo DJ. Variability in Responsiveness to Oral Antiplatelet Therapy. Am J Cardiol 2009;103[suppl];27A-34A.

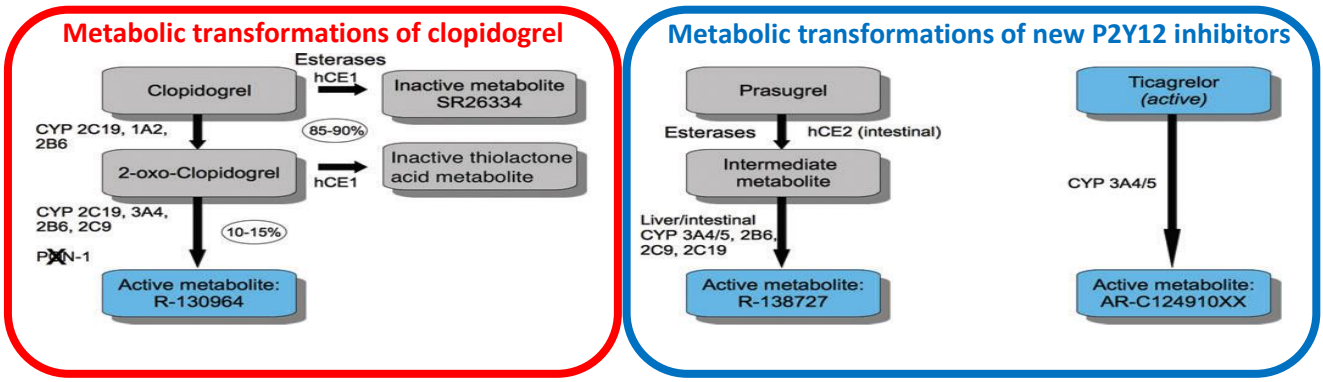

Figure 2. Comparison between the metabolic transformations of clopidogrel, prasugrel and ticagrelor.

Adapted from Aradi D et al. Expert position paper on the role of platelet function testing in patients undergoing percutaneous coronary intervention. Eur Heart J. 2014;35:209-15.

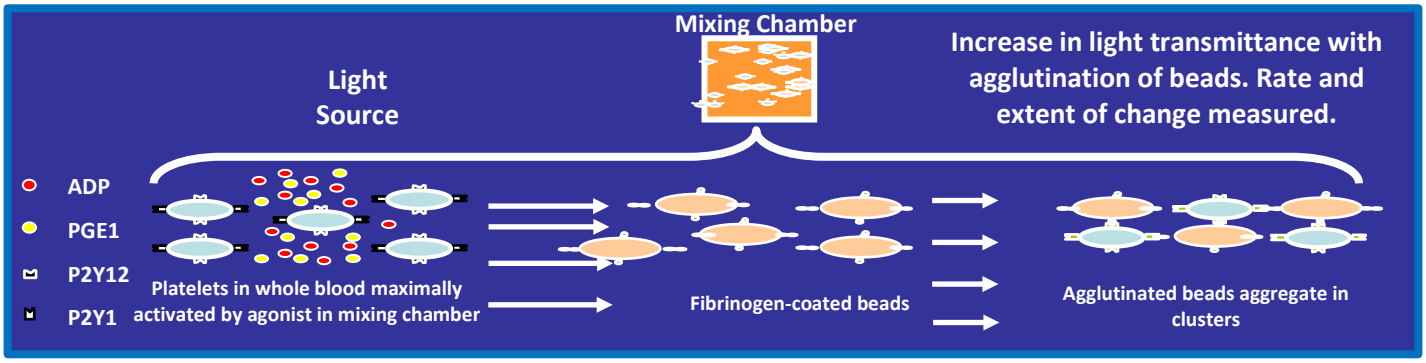

Figure 3. Determination of platelet function with the VerifyNow system (Accriva Diagnostics, San Diego, CA, USA).

Adapted from Michelson AD. Methods for measurment of Platelet Function. Am J Cardiol 2009;103[suppl]:20A–26A.

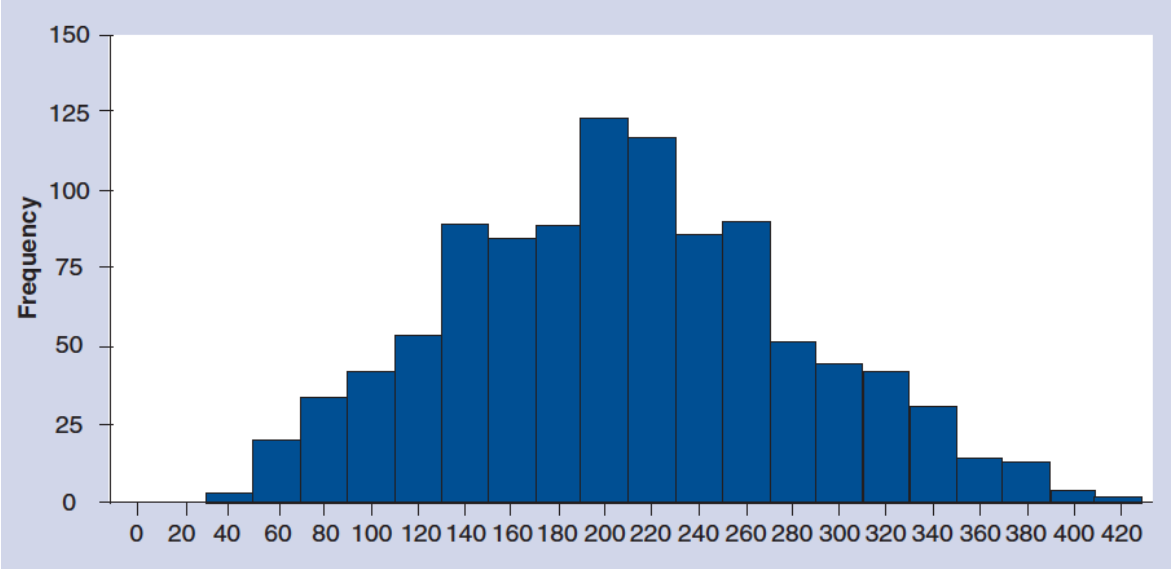

Figure 4. Normal distribution of P2Y12 reaction units (PRU).

PRU was measured by VerifyNow P2Y12 assay, in 1033 patients after 600 mg loading dose of clopidogrel.
Courtesy of F. Mangiacapra, unpublished data.

CHAPTER 3

Role of leptin in atherogenesis and thrombosis

Leptin is a circulating 167-amino acid protein produced by adipocytes, which regulates body weight and food intake through a direct effect on the hypothalamus [105]. In 1950, Ingalls et al. described for the first time a new variant of obese mice (*ob/ob*), which showed a severe obesity with hyperphagia and reduced energetic consume [106]. In 1973, Coleman et al. achieved the normalization of body weight of mice *ob/ob* by connecting circulation of these latter with wild/type mice: these results suggested the existence of a circulating factor involved in energetic balance regulation [107]. Only several years later, Zhang et al. isolated the gene of obesity (gene *ob*), which encode this circulating factor named "leptin" from the greek "λεπτός", which means "thin" [108].

3.1 Leptin, an adipokine with multiple targets

Leptin acts on target cells by interacting with specific membrane receptor, of which at least six different isoforms (from *Ob-Ra to Ob-Rf*) have been identified. The same gene codes all the isoforms and the heterogeneity is the result of alternative splicing processes of a single RNA messenger molecule [109]. Therefore, the different receptor isoforms share the extracellular and trans-membrane domains (except the *Ob-Re* variant), but differ in the length of intracellular domain [110]. The *Ob-Rb* isoform, also

known as the "long" receptor variant, is strongly expressed in the hypothalamus and mediates the anorexing effect of leptin by activating tyrosine kinases belonging to the Janus kinase family (JAK). Once activated, JAKs phosphorylate proteins associated with the leptin's receptor that function as signal transducers and transcriptional activators (STAT proteins). Then the phosphorylated STAT proteins move within the nucleus where they regulate the expression of target genes [111]. Other receptor variants, also known as "short" isoforms (*Ob-Ra, Ob-Rc, Ob-Rd, Ob-Rf*), are not able to activate the JAK-STAT intracellular pathway, but may allow signal transduction through various mechanisms such as, for example, mitogen-activated protein kinases (MAPK) or phosphatidylinositol 3-kinase (PI3K) [112]. *Ob-Ra* receptor isoform is also highly expressed at the level of the choroid plasmas, where its function would seem to transport leptin through the blood-brain barrier [113]. Instead, the *Ob-Re* receptor lacks of intracellular and trans-membrane domains and circulates freely in the blood, where it acts as a leptin-binding protein thereby prolonging its half-life [114].

The main factor influencing serum leptin concentration in patients with normal caloric intake is the amount of fat tissue that is present in the body; indeed, hyperleptinemia of obese subjects is the result of the increase of both fat mass and of adipocytes' size [115]. In addition, changes in the amount of body fat are able to vary the levels of circulating leptin: weight loss usually results in a reduction of leptin levels, while weight gain is associated with hyperleptinemia [116].

Leptin serum levels are also significantly higher in women than men [117], because in women it is more represented subcutaneous adipose tissue that is mainly involved in leptin production compared to the omental adipose tissue, which is normally more

expressed in men. Furthermore, also sex hormones affect the secretion of this adipokine, which is inhibited by testosterone and stimulated by estrogens [118].

Moreover, caloric intake regulates leptinemia regardless of changes in the mass of adipose tissue: a regular diet consisting of three meals per day is able to keep leptin levels constant, with a daily variation of <30% [119]. Even macronutrients in the diet may affect the concentration of this hormone that is inhibited by fat-rich and poor carbohydrate meals [120]. Finally, the sympathetic nervous system regulates the metabolism of adipose tissue through direct innervation or by the release of catecholamines that inhibit leptin synthesis. Nevertheless, mechanisms trough, which catecholamines act on leptin synthesis, have not yet been fully elucidated [121].

In addition to the action in body weight homeostasis, the regulatory role of leptin has been widely recognized in a variety of physiological processes. Many of these functions are mediated by the autonomic nervous system, but the presence of receptors in cells other than nervous system cells indicates that this hormone may have a direct effect on different tissues, including on the cardiovascular system [115]. For example, leptin improves directly the insulin sensitivity [122]. It also induces lipolysis in the cells of the white adipose tissue both in vivo and in vitro experiments [123]: this effect is mediated both by the activation of the central nervous system and by direct action on adipocytes, where oxidation of fatty acids is stimulated [124]. Furthermore, observations derived from several studies suggest that leptin acts also within the reproductive process, signaling to the central nervous system if the stored energies are sufficient to satisfy the high-energy demand of reproduction. In addition, leptin plays a role in ovarian steroidogenesis, placenta development and fetal growth [125].

Leptin is also involved in hematopoiesis and immune system functions by stimulating the proliferation of hematopoietic progenitors in the bone marrow and inducing the formation of in vitro macrophage's and lymphocytes' colonies [126]. Recent studies have suggested that hyperleptinemia may play a key role in the pathogenesis of obesity-related cardiovascular disease, including atherosclerosis. Indeed, leptin can exert many potentially atherogenic effects, such as induction of endothelial dysfunction, promoting oxidative stress, stimulation of inflammatory reactions, platelet aggregation, proliferation and migration of vascular smooth muscle cells [127]. The mechanisms through which hyperleptinemia contributes to cardiovascular complications have not yet been fully clarified, but many of these effects are based on the theory of "selective resistance to leptin" [105].

3.2 Leptin resistance and atherogenesis

The term "resistance to leptin" refers to a particularly complex physio-pathological phenomenon [128], which can be manifested as: 1) a result of genetic mutations affecting the protein or the receptor [129], 2) a receptor downregulation [130] or 3) a reduced access to target tissues through rapid saturation of the transport mechanisms (*Ob-Ra*) [131]. Resistance may also be due to the inhibition of signal transduction mechanism JAK / STAT by intracellular (SOCS 3) [132] or extracellular (SLIPs) factors that binding leptin to alter its bioavailability [133]. Leptin resistance, typical of obese subjects, is characterized by hyperleptinemia, indicating a reduced sensitivity to this adipokine, which seems to be limited to action on the central nervous system [128]. From these evidences the concept of "selective resistance" is born. According to this concept, in obese and hyperleptinemic subjects, only anorexic action is affected, while

the other effects are maintained. Since many of them are potentially atherogenic, hyperleptinemia may contribute to the development of atherosclerosis in obese patients [134]. First, leptin may directly induce atherogenesis, indeed *ob/ob* mice are protected from atherosclerosis [135] and leptin receptors have been identified in human atherosclerotic plaques [136]. In addition, atherosclerosis is now considered an inflammatory disease and leptin and some inflammatory pathways show a reciprocal modulation, as demonstrated by the evidence that many immune system cells, including T lymphocytes, monocytes, macrophages, have receptors for leptin and are activated by this hormone [137]. Indeed, leptin stimulates the central production of T lymphocytes and their peripheral shift in favor of T1 (pro-inflammatory), promotes recruitment of monocytes at intima [138] level, and induces secretion of atherogenic cytokines [137]. In this context, leptin itself can be considered a pro-inflammatory cytokine. Moreover, many data suggest that leptin may contribute to the inflammatory state associated with obesity: the levels of this adipokine are related to those of some acute phase proteins such as the C Reactive Protein (CRP) and the type A amyloid [139]. Finally, recent evidences have shown that leptin is able to induce the expression of CRP in endothelial cells of coronary arteries in humans [140].

Functional receptors for leptin were found on endothelial cells, however the effects of this adipokine on endothelial function remain controversial. In vitro studies have revealed that hyperleptinemia may favor the nitric oxide (NO) mediated vasodilatation [141] and additionally, leptin at pharmacological doses increases NO plasma concentrations [142]. Conversely, in vitro and in vivo studies have shown that leptin at pathologically elevated concentrations, such as those found in obese subjects, alters NO-dependent vasodilatation mediated by acetylcholine [143]. Multiple evidences

suggest that this hormone can contribute to endothelial damage or dysfunction in humans. In fact, leptinemia correlates inversely with NO-mediated coronary vasodilatation in male obese patients [144]. On the other side, a positive correlation of leptin levels was found with the plasma levels of thrombomodulin and vascular cell adhesion molecule (VCAM-1), two endothelial damage markers [145]. Moreover, leptin concentrations correlate negatively with NO production in patients with ischemic heart disease, who develop restenosis after PCI [146].

Leptin promotes oxidative stress: Bouloumie et al. showed that hyperleptinemia induces the formation of reactive oxygen species (ROS) [147] and Porreca et al. observed a significant correlation between plasma levels of leptin and LDL oxidized in 60 healthy women [148]. Furthermore, this adipokine reduces the plasma activity of paroxanase 1, which plays an important anti-atherosclerotic role by preventing lipoprotein oxidation [149].
Regarding lipid metabolism, leptin has been shown to induce secretion of lipoprotein lipase from macrophages by stimulating lipoprotein deposition in subendothelial space [150]; it also favors the formation of foam cells especially in the presence of hyperglycemia [151] and it is inversely correlated with HDL plasma levels [152].

Oda et al. were the first to demonstrate the presence of leptin receptors on vascular smooth muscle cells, which not only stimulated proliferation and migration [153], but also induced the production of type 2 metalloproteinase (MMP-2), that plays a critical role in the rupture of the plaque [154]. Additionally, leptin may favor vascular remodeling through proliferative and prophylactic cytokine production, such as TGF-β [155], angiotensinogen, angiotensin II [156], and endothelin-1 in the obese subjects [157].

Limited data are available about the association between leptin levels and subclinical atherosclerotic forms in humans. Reilly et al. reported in a population of 200 diabetic subjects without clinical evidence of ischemic heart disease, an association between leptin plasma levels and coronary calcification [158]. More recently, similar correlation was also found in 860 healthy, non-diabetic adults, regardless of the presence of the classic cardiovascular risk factors [159]. In 126 normal and obese individuals without diabetes mellitus, familial dyslipidemia, arterial hypertension and clinical manifestations of atherosclerosis, a positive correlation was found between leptin levels and mean-intimal thickness measured at the common carotid artery and considered a marker of subclinical atherosclerosis [160]. Similar results were also reported in children and adolescents with type 1 DM [161]. Conversely, there was no association between leptinemia and mean-intimal thickness in 403 elderly male subjects [162], obese healthy women [163] and in obese or diabetic adolescents [164]. From these results, it is possible to conclude that the correlation between leptin and markers of subclinical atherosclerosis is still controversial.

Many clinical studies have correlated leptin levels with cardiovascular events. Soderberg et al. were the first to demonstrate a positive association between levels of this adipokine and MI, regardless of the presence of classic risk factors for ischemic heart disease [165]. In addition, leptin was an independent risk factor for myocardial infarction in a group of patients with hypertension [166]. The most convincing evidence for a pro-atherogenic role of this hormone in humans comes from a substudy of the WOSCOPS (West of Scotland Coronary Prevention Study) study. This study showed that hyperleptinemia predicts acute cardiovascular events (death, MI, and new revascularization) during 5-years follow-up period in > 1000 patients. When the

population was subdivided into quintiles based on plasma leptin levels, it was seen a ≈2 fold increase in the risk of cardiovascular events when comparing the highest 2 quintiles with the lowest quintile. Leptin has been confirmed as a positive predictor, regardless of the Body Mass Index, cholesterol, glucose and CRP levels [167].

Wolk et al. examined the relationship between leptin and cardiovascular events in 382 non-diabetic patients with angiographic evidence of significant coronary heart disease during a 4-years follow-up. It has been shown that baseline hyperleptinemia is associated with an increased incidence of the combined end-point (CV death, new MI, cerebrovascular events and need for new revascularization). At the multivariate analysis only the hyperleptinemia and the number of vessel disease were independent predictors of cardiovascular events [168]. In addition, plasma leptin levels were significantly higher in subjects who developed restenosis after coronary angioplasty compared to patients who did not present this complication [146].

Conversely, in the "Quebec Cardiovascular Study" no correlation was established between leptinemia and ischemic heart disease. It is necessary take in account that this latter is a minor trial (86 cases and 95 controls) including different endpoints: stress angina, heart failure and non-fatal MI [169]. Therefore, it is possible that leptin is a major predictor of "hard" endpoints (such as acute coronary syndromes) rather than stable angina. Indeed, Dubey and coll. not only showed that patients with acute coronary syndrome had plasma levels higher than that of stable patients [170], but had also shown that leptin concentrations were significantly higher in patients with complex CAD [171]. Furthermore, a positive correlation was seen between leptin and occurrence of hemorrhagic stroke [172] and ischemic stroke [173]: leptin levels were

significantly higher in patients hospitalized for an acute stroke than healthy controls [174].

3.3 Leptin and platelet reactivity

Nakata et al. were the first to discover by in vitro experiments using Western Blotting, that human platelets express on their surface the long isoform of the receptor for leptin (*Ob-Rb*). Proving that this hormone is able to amplify the ADP-mediated platelet aggregation, they also attributed a functional meaning to this presence. Indeed, the Japanese group initially observed that neither leptin alone (at concentrations ≤ 100 ng/ml) or ADP alone (at concentrations of ≤ 2 μmol/L) are able to exert a prothrombotic effect in vitro. However, the combination of leptin and ADP was effective: in fact, pretreatment for five minutes with leptin at a concentration of 100 ng/ml made platelets responsive to the stimulation of only 2 μmol/L of ADP causing their aggregation. In this way, synergic action between leptin and ADP was also demonstrated (even at sub-threshold concentrations), and it was observed that such interaction was dose-dependent. Leptin at concentrations of 10 ng/ml had a minimum proaggregating effect, instead at the dose of 30 ng/ml showed a significant effect. After pretreatment at doses of 50 ng/ml, the platelet aggregation was markedly increased and a further increase was observed with doses equal to 100 ng/ml. The same authors have also shown how the administration of genisteine, which is tyrosine kinase inhibitors, can block the beneficial activity of leptin on ADP-mediated platelet aggregation. These data suggest that the interaction of this hormone with the *Ob-Platelet Rb* determines the phosphorylation of tyrosine residues of some membrane proteins with the consequent activation of intracellular pathways [175]. Several studies

on animals have subsequently confirmed the prothrombotic effect of leptin in vivo. Indeed, it was found that both *ob/ob* mice (leptin deficiency) and wild type mice treated with an antibody neutralizing this hormone showed a reduced platelet activation, with the formation of an unstable thrombus and with an attenuated thrombotic response to arterial damage. In *ob/ob* animals, the intraperitoneal administration of a single dose of leptin at a dose of 0.06 mg/Kg before inducing arterial damage was able to stabilize thrombus formation; moreover, with the increase of the dose to 0.6 mg/kg, there was evidence of an increase in arterial thrombosis not only in poor leptin but also wild-type animals [176]. Corsonello et al. subsequently confirmed the results of Nakata in a study in which no platelet aggregation was obtained from 14 healthy male controls with BMI<25 kg/m^2, if the stimulus was represented only by the ADP or only by leptin. Instead, the prothrombotic effect of 2 μmol/L of ADP appeared after leptin pretreatment; the percentages of ADP-mediated aggregation were significantly higher than baseline: 40.2 ± 3.7% (P = 0.05), 51.3 ± 3.8% (P = 0.01), 55.9 ± 4.7% (P = 0.01), 69.2 ± 4.1 P = 0.001) for leptin doses respectively of 5, 10, 50 and 100 ng / ml. Again, in this case, it has been seen that such synergy is dose-dependent and it is inhibited by using *anti-ObRb* antibodies [177].

The same authors have subsequently shown that leptin is able to increase, in a dose-dependent manner, intracellular concentrations of free calcium, an important factor in determining the functional changes that lead to platelet activation. Given these findings, it was descripted a possible mechanism through which leptin-induced platelet aggregation could occur: the hormone binds its receptor, causing the activation of the intracellular pathway JAK-STAT and phospholipase C, resulting in hydrolysis of phosphatidyl inositol 4-5-diphosphate and formation of phosphatidyl inositol

triphosphate (IP3) and diacylglycerol (DAG). The DAG activates protein kinase C, while IP3 mobilizes intraplatelets calcium deposits. Protein kinase C, phospholipase A2 and IP3 act synergistically to induce platelet activation, by secretion of alpha and electron granule contents, thromboxane A2 formation and expression on the platelet surface of receptors mediate aggregation (Figure n.1) [178]. It has also been found that the ability of leptin to increase free intracellular calcium is maintained even in the absence of contemporaneous co-stimulation with ADP, although intracellular calcium concentrations presented a greater increase in the presence of both agonists [179]. In the same study, the authors compared the effect of leptin on platelet aggregation in healthy, overweight and obese subjects, demonstrating that the aggregating effect was maintained in healthy controls but it was significantly reduced in overweight patients, where only particularly high leptin concentrations (between 50 and 200 ng/ml) were able to increase ADP-induced platelet aggregation. In obese subjects, the prothrombotic activity of leptin was further decreased and concentrations of this hormone equal to 100-200 ng/ml were needed to produce a significant increase in aggregation. At the same time, while in normal patients there was a substantial increase in leptin-induced intracellular calcium concentrations; this effect was markedly weakened in overweight and obese subjects, where only high leptin concentrations were able to significantly increase free intracytoplasmic calcium. Therefore, these observations suggested that platelets could become resistant to adipokine effects in obese subjects [178].

In this regard, it must be considered that initially the literature data relating to the prothrombotic effect of leptin were contrasting. Ozata et al. hypothesized that this hormone had no ability to stimulate platelet aggregation in a study that compared 4

leptin-deficient subjects (as they carry genetic mutations) with 18 obese subjects and 20 healthy controls. It was first observed that leptin-deficiency patients showed significantly higher aggregation than healthy controls after stimulation with several agonists (ADP, collagen and epinephrine), but substantially similar to those of obese patients. In addition, when platelets are incubated with high concentrations of leptin (100-500 ng/ml), no ADP-mediated aggregation was detected in any subgroup of patients [180].

Nevertheless, the demonstration of aggregating properties of this hormone is mainly derived from a study on healthy volunteers with normal BMI. Here, after leptin incubation, the stimulation of platelets with ADP showed that in some subjects prothrombotic effect were maintained, while in other cases, no enhancement of ADP-mediated aggregation was noted. This result allowed to identify two populations: the leptin-responders and the leptin-non-responders, regardless of BMI. Donors were considered "responders" if the weak and reversible platelet aggregation induced by ADP at low concentrations became, in the presence of leptin, dose-dependent irreversible. In contrast, donors were considered "no responders" if leptin did not show the ability to boost aggregation, even at high doses. Preliminary analysis of platelets derived from 56 different donors indicated that approximately 40% of subjects may be considered "responders", while 60% "no responders". The effects of leptin on the ADP-mediated platelet aggregation were then confirmed by the same authors via experiments conducted on the electronic microscope. In fact, it has been found that if stimulated with low doses of ADP, the "responders" platelets were minimally aggregated and still contained many electron granules inside them. Instead, when the same platelets were pretreated with leptin, low doses of ADP were also able to

determine the formation of platelet aggregates and their almost complete degranulation. Conversely, these changes were not observed when platelets from "no responders" were used. In the same study, other experiments were carried out to clarify the mechanisms underlying responsiveness. In fact, platelet-rich plasma from donor "responders" was mixed with an equal amount of poor platelet plasma from "no-responders" and vice versa. In this way it was seen that leptin was able to maintain its own prothrombotic effect in responders' platelets, suggesting that platelet responsiveness to this hormone was not dependent on plasma factors, but was probably caused by something intrinsic to platelets themselves. Since for leptin-mediated action was critical to interaction with its receptor, it has been hypothesized that responsive platelets express long receptor isoforms, while non-responsive platelets present a short receptor isoform, not able to activate intracellular signal transduction pathways. Indeed, by Western blotting, it has been shown that both populations expressed long isoform. The difference was in the quantity of receptor expressed: no-responder platelets expressed a quantity of receptor less than half compared with responder platelets. Therefore, the decreased responsiveness of platelets to leptin could derived, at least in part, from reduced expression of *Ob-Rb* receptor on their surface, and partly by a reduced affinity for binding to adipokine. The responders' platelets bound 2.5 times more leptin than no-responders; such a phenomenon, according to the authors, could derive from receptor N-glycosylation in a key region for binding to the hormone. Moreover, in the same study, Giandomenico et al. have shown that leptin is capable of enhancing platelet aggregation even in the presence of agonists other than ADP such as collagen and epinephrine, obviously only in responder subjects [181].

The definitive demonstration that platelets from obese subjects are not resistant to leptin capacity to enhance ADP-mediated platelet aggregation was provided by Dellas et al. in a study that included 40 obese subjects (BMI=41.6 ± 1.1 kg / m2) and 36 patients with normal weight (BMI=23.3 ± 0.4 kg / m2). As expected, obese individuals showed significantly higher levels of leptin (index of central resistance) and exhibited more clinical features suggestive of metabolic syndrome (lower HDL cholesterol, diabetes and hypertension). Using ADP increasing doses, platelets of obese subjects have been shown to need of significantly lower doses of this agonist compared to healthy controls to reach a certain grade of aggregation: for example, in obese subjects, 2.6 ± 0.3 μM ADPs are needed to reach at least 10% platelet aggregation versus 3.7 ± 0.4 μM ADP in controls (P = 0.021). Significant differences between the two groups were observed for higher platelet aggregation rates (≥20%, P = 0.010, ≥30%, P = 0.010, ≥40%, P = 0.013 and ≥50%, P = 0.027). In addition, the aggregation level after stimulation with increasing doses of ADP (2, 3, 4, 5 μM) was significantly higher in platelet-rich plasma of obese subjects compared to normal-weight. Therefore, all of these data indicated that obese donor platelets show, in response to ADP, a more pronounced aggregation than normal weight donors. The authors also evaluated whether there was a correlation between leptin plasma levels and the degree of platelet aggregation in response to ADP. In the whole population subjects were distinguished, depending on the degree of activation of platelets in response to increasing ADP doses, in "strong responders" and "weak responders". Strong responders had significantly higher circulating leptin levels compared to the weak responders at all concentrations of ADP tested. Interestingly, BMI did not differ between the two groups at 2, 3, 4 μM ADP; only with 5 μM stimulation, the strong responders group showed BMI levels

significantly higher than the group of weak responders. When the general population was divided based on BMI, leptin levels were found to be very high (about 50 ng/ml) in obese subjects but did not differ significantly when the population was divided into strong and weak responders. In contrast, leptin levels were significantly lower (between 4 and 8 ng/ml) in the subgroup of normal patients. When this latter group was distinguished in strong responders and weak responders, in the first group plasma levels of leptin were significantly higher at all ADP concentrations (2 and 3 μM: $P = 0.056$, 4 μM: $P = 0.049$, 5 μM: $P = 0.014$). BMI did not differ significantly between the responder's groups and this shows that the correlation between platelet aggregation and leptin plasma levels is something more than a simple indicator of increased body weight. Finally, the authors wanted to test the prothrombotic effect of leptin at a dose of 500 ng/ml on the entire population of the study. It has been seen that in obese ($P <0.001$) and in normal weight ($P = 0.002$), this adipokine is able to significantly enhance ADP-mediated aggregation, with an average aggregation increase of 25%. Although individual differences in platelet responses were found in both groups, the effect of leptin on platelet aggregation was similar between obese and normal weight subjects. These latter evidences suggest that the platelet responsiveness to leptin is independent of BMI and of the presence of comorbidities [182].

Regarding the role of comorbidities, Sugiyama et al. examined the correlation between platelet aggregation and leptinemia in the presence of diabetes mellitus, by dividing diabetic patients into two groups according to their platelet aggregation degree in response to different agonists (ADP, collagen and thrombin). It was seen that in the group with aggregation greater than 50%, plasma leptin levels were significantly higher than in those of the aggregate group below 50% ($P<0.05$), suggesting that this

molecule may play a role in determining the prothrombotic tendency of diabetic subjects. These patients were also less responsive to the treatment with antiaggregating drugs. There were no differences between obese subjects and healthy controls in the activation of leptin-pretreated platelets, not only in response to ADP, but also in response to thrombin. After incubation with 100 ng/ml of leptin, 1 U/ml of thrombin was able to determine the same degree of platelet aggregation in both patient groups [183].

The prothrombotic effect of leptin may be explained also by other mechanisms. Nakata and coll. have identified the action of this hormone on the expression of thrombomodulin, a glycoprotein expressed on the surface of endothelial cells that transforms thrombin from pro-coagulant protease into anticoagulant factor. In vitro experiments suggested that leptin, at concentrations between 50 and 100 ng/ml, was able to reduce the expression of thrombomodulin by about 10% and that this effect was enhanced by the simultaneous stimulation from Oxidized- LDL (ox-LDL) and from Advanced Glycoprotein Endo Products (AGE) [184].

The role of leptin in regulating human hemostasis in vivo is not yet fully investigated. In 44 obese women, without the conventional cardiovascular risk factors, plasma leptinemia significantly correlated with urinary excretion of 11-dehydro-TXB_2, a stable metabolite of TXA_2 considered an in vivo marker of platelet activation [185]. In addition, calories restriction, which reduces leptin levels, was associated with a reduced P-selectin plasma expression, in fact, a recent study has shown that the administration of leptin at physiological doses prevented in obese subjects the reduction in P- selectin induced by fasting [186]. In the Health Professionals Follow-up Study, leptinemia significantly correlated with levels of fibrinogen and of von

Willebrand factor in 268 healthy male adults without cardiovascular disease [187]. There was also a reverse correlation between leptinemia and the presence of two coagulation inhibitors: C protein and tissue factor inhibitor [188]. An association between leptin and plasminogen inhibitor (PAI-1), the largest endogenous fibrinolysis inhibitor, has been demonstrated in male subjects with ischemic heart disease [189] and in fertile women [190]. Leptin has been shown not only to correlate positively with PAI-1, but also inversely with the tissue plasminogen activator in 74 overweight and moderately hypertensive subjects [191].

In patients with CAD there are some evidences supporting a correlation of leptin plasma levels and the occurrence of cardiovascular events and with thrombosis mechanisms. Levels of this adipokine were correlated with the efficacy of thrombolytic treatment in patients with myocardial infarction with ST segment elevation (STEMI). Already the results of a sub-study of the GUSTO-1 [192] trial suggested that in patients with body weight >85 kg there was an incidence of ineffective thrombolysis significantly higher than those with lower body weight. Amasyali et al. evaluated whether leptin plasma concentrations could affect the efficacy, in terms of reperfusion and reinfarction, of streptochinase thrombolytic treatment undertaken within six hours of STEMI. 41 patients were enrolled and divided in two groups according to plasma leptin concentrations: 28 patients with leptinemia <14 ng/ml and 13 patients with leptinemia ≥14 ng / ml. The two groups did not differ among themselves for the main clinical features (age, sex, BMI, location of the infarction and cardiovascular risk factors). The time between the onset of symptoms and the beginning of thrombolysis was also similar between the two groups. In the hyperleptinemic group, the left ventricular ejection fraction was significantly

lower (P = 0.031). The mean leptin concentration at the intake was 5.6 ng/ml in a group and 28.8 ng / ml in the other (P <0.001). The failure of thrombolytic therapy, identified by reinfarction or absence of early reperfusion signs, occurred in 11 patients (39%) of the hypoleptinemic group and in 10 patients (77%) of the hyperleptinemic group (P = 0.025). There were no significant differences between the two groups regarding the occurrence of supraventricular and ventricular arrhythmias and the incidence of post-thrombolysis bleeding complications. Hyperleptinemic patients also showed a higher incidence of cardiac failure in Killip II class (21% versus 62%, with P <0.012). In addition, 3 patients in Group 1 (11%) and 5 patients in Group 2 (38%) underwent to emergency coronary angiogram due to a severe impairment of the hemodynamic conditions caused by reperfusion failure, reinfarction or post-infarction angina (P = 0.037). This latter study is very important not only because it confirmed the prothrombotic role of leptin, but also because highlighted the clinical relevance of this phenomenon, that may contribute to the failure of thrombolytic therapy. Probably this hormone can induce the inhibitor of the activators of plasminogen, responsible for the development of resistance to thrombolytic agents. Moreover, these results also suggested that leptin levels can interfere in vivo with platelet aggregation. Indeed, experimental studies have shown that leptin concentrations around 10 ng/ml are able to enhance platelet aggregation in mice, but not in humans, whereas the effect on human platelets is observed at higher concentrations. However, these are in vitro studies, while this latter is an in vivo study and it must be considered that STEMI is a complex event, generally characterized by a prominent tendency to thrombosis, which can occur even in the presence of reduced agonist levels [193].

3.4 Figures

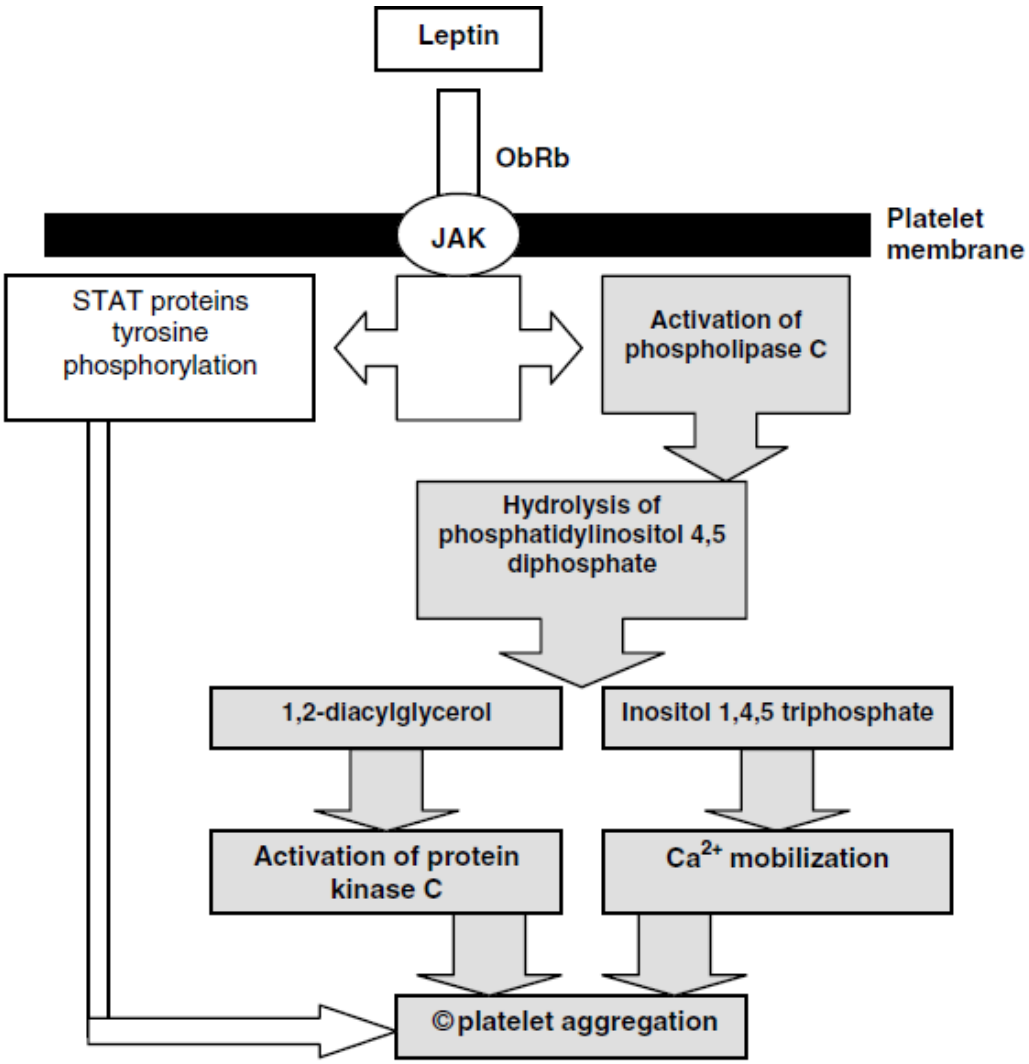

Figure 1. Proposed model of leptin-induced platelet activation.

Corsonello A, Perticone F, Malara A, De Domenico D, Loddo S, Buemi M, Ientile R, Corica F. Leptin-dependent platelet aggregation in healthy, overweight and obese subjects. *Int J Obes Relat Metab Disord.* 2003;27:566-573.

CHAPTER 4

RESEARCH PROJECT N.1

Correlation of Platelet Reactivity and C-Reactive Protein levels to Occurrence of Peri-Procedural Myocardial Infarction in Patients Undergoing Percutaneous Coronary Intervention (from the ARMYDA-CRP Study) *

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4.1 Background

The degree of platelet inhibition at the time of the procedure influences clinical outcome in patients who undergo percutaneous coronary intervention (PCI) [76,77,86,194]. Given the wide intersubject variability in response to clopidogrel, various studies have explored the issue of whether high on-treatment platelet reactivity (HPR) is associated with a poorer prognosis after coronary stent implantation [76,77, 194]; in particular, impaired response to clopidogrel at platelet function testing was demonstrated to have an independent predictive role for the incidence of periprocedural ischemic events [77] and major adverse cardiac events during follow-up [76,194,195]. Previous data have demonstrated that inflammatory status at the time

of PCI may also influence subsequent clinical outcomes, with higher occurrence of clinical events in patients with increased C reactive protein (CRP) levels [196,197]; however, no study has specifically assessed the correlation between inflammatory status and the incidence of periprocedural myocardial infarction (PMI) in the setting of PCI. Thus, the Antiplatelet Therapy for Reduction of Myocardial Damage During Angioplasty (ARMYDA) study group designed a prospective study to investigate whether (1) elevated baseline CRP level is independently associated with increased risk for PMI after coronary stenting and (2) the combination of pre-PCI high CRP level and HPR may have an incremental predictive value for the occurrence of PMI.

4.2 Methods

ARMYDA-CRP is a prospective investigation of patients who underwent PCI for a variety of coronary ischemic syndromes at Campus Bio-Medico University of Rome from June 2010 to November 2011. All patients were receiving clopidogrel therapy at the time of intervention (75 mg/day for > 7 days or 600-mg loading dose given > 6 hours before PCI). A total of 589 patients were initially screened, and 89 were excluded for the presence of ≥ 1 of the following criteria: primary PCI for ST-segment elevation myocardial infarction, upstream use of glycoprotein IIb/IIIa inhibitors (to avoid interference with pre-PCI platelet reactivity measurement), platelet count <70x109/L, use of prasugrel or ticagrelor, need for oral anticoagulant therapy, chronic renal failure with serum creatinine >2 mg/dl, cardiogenic shock, and concomitant systemic inflammatory conditions.Thus, a total of 500 patients were included in this study.

All interventions were performed using standard techniques. All patients received aspirin before PCI. A blood sample was drawn from the arterial sheath, and platelet reactivity was immediately measured in the catheterization laboratory before PCI using the VerifyNow P2Y12 assay (Accriva Diagnostics, San Diego, CA, USA), which is a rapid cartridge-based assay specifically measuring effects of clopidogrel on the platelet $P2Y_{12}$ receptor. Technical details of the assay have been previously described [198]. Results are expressed as $P2Y_{12}$ reactivity units (PRU); the lower the PRU value, the greater the degree of $P2Y_{12}$ receptor inhibition by clopidogrel, and vice versa. Creatine kinase-MB (mass) and troponin I (mass) levels were evaluated before PCI and at 8 and 24 hours in all patients; further measurements were done if clinically indicated. Measurements were obtained using the Access 2 immunochemiluminometric assay (Beckman Coulter, Brea, California), with normal limits of ≤4 ng/ml for creatine kinase-MB and ≤ 0.08 ng/ml for troponin I. High-sensitivity CRP levels were also measured before the procedure using the Kryptor ultrasensitive immunofluorescent assay (BRAHMS GmbH, Hennigsdorf, Germany), with a detection limit of 0.06 mg/L. The interventional cardiologist performing the procedure was blinded to the PRU and CRP results. Each patient gave informed consent to participate in the study.

The primary end point was the incidence of PMI in relation to baseline platelet reactivity (by PRU) and/or inflammatory status (by CRP level); this end point was prospectively determined. PMI was defined in patients with normal baseline levels of these markers as a postintervention increase in creatine kinase-MB or troponin I increase >3 times the 99th percentile of the upper reference limit [199]; in patients with acute coronary syndromes and increased baseline cardiac marker levels, the

definition of a subsequent elevation ≥50% of the baseline value was applied [200]. The cutoff for defining an elevated inflammatory status was a CRP level >3 mg/L. According to the ARMYDA-Platelet Reactivity Predicts Outcome (ARMYDA-PRO) study [77], which identified a clinically driven threshold of platelet reactivity to identify patients at higher risk for PMI, HPR was defined as PRU _240. The secondary end point was the correlation of platelet reactivity and CRP level with post-PCI peak levels of cardiac markers.

We assumed a 6% overall incidence of PMI in patients without HPR and as an effect size for the power analysis a 2.5-fold increased risk for PMI in those with HPR [77]; we hypothesized a similar increased risk in patients with high CRP. Thus, a study population of ≥495 patients would be needed to verify this hypothesis with an α level of 0.05 (2 tailed, after Sidak correction for multiple comparisons) and a β value of 0.8. Categorical variables are expressed as percentages and continuous variables as mean ± SD, unless otherwise specified. Proportions were compared using Fisher's exact test when the expected frequency was <5; otherwise, the chi-square test (with Yates' correction) was applied, with Bonferroni's correction in case of multiple comparisons. Continuous variables were compared using Student's t tests for normally distributed values (as assessed using the Kolmogorov-Smirnov test); otherwise, the Mann-Whitney U test was used. Correlations were determined using Spearman's rank test. Odds ratios (OR) and 95% confidence intervals (CIs) investigating the independent predictive role of HPR and/or high CRP level on the occurrence of the primary end point were assessed using logistic regression. The following parameters were first evaluated in a univariate model: platelet reactivity, CRP values, and each of the clinical and procedural variables listed in Tables 1 and 2. Variables with P values <0.15 were

then entered into the final model of multivariate logistic regression analysis. We also assessed the incremental value of incorporating HPR and/or high CRP levels into a model of clinical and procedural variables in predicting the primary end point. The area under the curve and its 95% CI were calculated for each logistic regression model, and differences between areas under the curve for different models were assessed using the jackknife method, as previously described [201]. All calculations were performed using SPSS version 15.0 (SPSS, Inc., Chicago, Illinois), and 2-sided P values <0.05 were considered significant.

4.3 Results

Clinical and procedural characteristics according to CRP and PRU status are listed in Tables 1 and 2. Procedural success, defined as a reduction of stenosis to <20% residual narrowing, was obtained in 97% of patients; in patients in whom procedures were unsuccessful, chronic total occlusions were not crossed with the wire. Two patients had stent thrombosis before discharge, with subsequent repeat target vessel angioplasty; no patient died during the hospital stay.

A total of 38 of 500 patients had PMI (7.6%); in the overall population, the prevalence rates of HPR and high CRP level were 38% and 48%, respectively; the latter patients were older and more frequently had histories of myocardial infarction and acute coronary syndromes as clinical presentation; in patients with HPR, the mean age and body mass index were higher. Patients with CRP levels >3 mg/L showed a significantly higher incidence of PMI (26 of 238 patients [10.9%]) compared with those with normal levels (12 of 262 patients [4.6%]) (P= 0.012); average pre-PCI levels of CRP were 27.3±46.3 mg/L in patients with PMI and 10.4±25.4 mg/L in those

without (P<0.001). Similarly, HPR (PRU≥240) was associated with increased rates of PMI: 11% (21 of 188 patients) versus 5.5% (17 of 312) in patients without HPR (P=0.03); baseline PRU values were 239±73 in patients with PMI and 218±79 in those without (P=0.07). The occurrence of PMI was lowest in the subgroup of 194 patients with normal CRP levels and without HPR (3.6%), and it was highest in the 72 patients with high inflammatory status and HPR at baseline (16.6%) (P=0.005; Figure 1); rates of the primary end point were similar in patients with CRP levels ≤ 3 mg/L and PRU≥240 and in those with CRP levels >3 mg/L and PRU <240 (7.8% and 8.5%, respectively). On multivariate analysis (Figure 2), the risk for PMI was 2.4-fold higher in patients with CRP levels >3 mg/L (OR 2.4, 95% CI 1.2 to 4.5, P=0.015) and 2.2-fold higher in those with PRU ≥240 (OR 2.2, 95% CI 1.2 to 4.4, P=0.018); the combination of high inflammatory status and HPR before PCI was associated with the highest predictive value for the occurrence of PMI (OR 4.3, 95% CI 1.5 to 12.6, P=0.008). Other predictors of increased risk for PMI were PCI for non- ST-segment elevation acute coronary syndromes and high-risk PCI requiring the use of glycoprotein IIb/IIIa inhibitors (Figure 2). The combination of pre-PCI CRP level ≤3 mg/L and PRU <240 had a negative predictive value of 96% for excluding the outcome measure of PMI. Table 3 lists the discriminatory power of a model including clinical and procedural variables (age >65 years, diabetes mellitus, previous myocardial infarction, left ventricular ejection fraction <40%, multivessel PCI, number of stents implanted, chronic renal failure, use of glycoprotein IIb/IIIa inhibitors, and therapy with statins) for the prediction of the primary end point, alone and after adding high CRP level, HPR, and their combination. Only the addition of elevated CRP level in association with HPR resulted in a significant increase in the discriminatory power of

the model, with an area under the curve as high as 0.811. Compared with the remainder population, patients with high CRP levels and HPR had increased risk for PMI irrespective of the clinical syndrome (acute coronary syndromes: 26.5% vs 7.9%, P=0.002; stable angina: 13.2% vs 4%, P=0.007; Table 4). No correlation was found between baseline CRP level and PRU in the overall population (R=-0.029, P=0.52); the prevalence of HPR was 38% in patients with CRP>3 mg/L and 36% in those with CRP ≤3 mg/L (P=0.84). In the overall population, average postprocedural peak levels of cardiac markers were significantly more elevated in patients with high inflammatory status and HPR compared with those with CRP levels ≤ 3 mg/L and PRU <240 (troponin I 1.18±4.31 vs 0.29±1.18 ng/ml, P=0.011, creatine kinase-MB 2.94±4.96 vs 1.83±3.40 ng/mL, P=0.037).

4.4 Discussion

This prospective study indicates that enhanced baseline inflammatory status is associated with a significant increase in the risk for PMI in patients who undergo PCI, and this is highest when elevated CRP level is combined with HPR while receiving clopidogrel therapy. Various studies have found significant associations between HPR on clopidogrel treatment and the incidence of early or late follow-up adverse events after PCI [76,77,194]; in particular, a recent patient-level meta-analysis of 6 studies and 3,059 patients demonstrated a higher event rate (hazard ratio 2.62) of a composite end point including death, myocardial infarction, and stent thrombosis for increasing levels of on-treatment platelet reactivity through 2 years [195]. Studies from our institution [77,202] were focused on the correlation between pre-PCI HPR, measured using a point-of care assay, and the incidence of PMI: a threshold of PRU value ≥240

significantly discriminated between patients with and without this outcome measure. ARMYDA-CRP confirms and expands such data; in fact, using the same assay and the same definition for HPR, patients with baseline PRU ≥240 had a 2.2-fold higher incidence of PMI.

Furthermore, previous data identified a significant relation between preprocedural inflammatory status and clinical outcome after PCI; elevated CRP level was able to predict a higher risk for adverse events, including stent thrombosis or in-stent restenosis, during follow-up [197, 203,204]. Of note, normalization of CRP levels early after PCI was associated with very low adverse events rates after intervention [205]. Park et al. [196] recently showed that patients with HPR and high CRP levels before drug-eluting stent implantation had the highest rate of adverse events at 2 years. However, no study has specifically correlated CRP with the acute outcome of PMI in patients who undergo PCI or investigated the predictive value of the association of high inflammatory status and HPR for this complication. Goldberg et al. [206] demonstrated that elevated baseline CRP level is associated with a higher risk for periprocedural myocardial injury, defined as any postprocedural troponin I elevation; moreover, Buffon et al. [204] found a correlation between CRP and periprocedural angiographic end points. However, in the Chimeric c7E3 Antiplatelet Therapy in Unstable Angina Refractory to Standard Treatment (CAPTURE) trial, a very high (and less sensitive) cutoff of CRP (>10 mg/L) before PCI failed to predict mortality and myocardial infarction at 72 hours [207]. In patients with baseline CRP levels >3 mg/L in this study, we observed a 2.4-fold increase in the rate of PMI, and this was independent of possible confounding factors; of note, this cut-off point of 3 mg/L is consistent with that observed in previous investigations as the CRP threshold for

discriminating patients at higher risk for clinical events during follow-up [196]. In the present study, baseline CRP levels were significantly higher in patients with PMI compared with those without. CRP is a soft marker of distal embolization during PCI; however, patients with high inflammatory status might have a more pronounced myocardial damage if distal embolization occurs during intervention. Moreover, there is a close correlation between inflammation and thrombosis, with inflammation enhancing local thrombosis and vice versa [208]; in fact, CRP transgenic mice showed higher rates of thrombotic occlusion [209], and in humans, the increase of CRP levels after the infusion of recombinant CRP produced a concomitant elevation of the 2 markers of inflammation and coagulation [210]. Interestingly, previous data demonstrated that CRP may have direct proinflammatory effect on human endothelial cells [211]. In our study, compared with patients with normal CRP levels and normal responses to clopidogrel, those with the combination of CRP level >3 mg/L and PRU ≥240 before PCI had a 4.3-fold higher incidence of PMI; in such patients with the highest risk profile, PMIs were characterized by more elevated peak levels of cardiac markers. Thus, in patients with enhanced inflammatory status and HPR, the mutual amplification of inflammation and thrombosis may explain the worse periprocedural outcomes. Moreover, when incorporated into a model of clinical and procedural variables, addition of the combination of HPR plus high CRP provided a significant increase in the discriminatory power of the model for predicting PMI. In the acute coronary syndromes and stable angina subgroups, the association of HPR and elevated CRP level resulted in a significant increase in PMI rates, further corroborating the synergistic detrimental effect of elevated platelet reactivity and inflammation in determining myocardial damage irrespective of the clinical syndrome. In our study,

we found no correlation between high platelet reactivity and inflammatory status; however, this apparent lack of a relation was also found in other previous investigations, and it may be explained by the multifactorial pathogenesis of high platelet reactivity and high inflammatory status [196, 212].

Because there could have been a gap of hours between baseline and initial post-PCI marker evaluation, there may have been some challenges in distinguishing a PCI-related event and the normal courses of cardiac markers on the basis of an index-non-ST-segment elevation myocardial infarction. The use of a unique test for platelet reactivity assessment and the enrollment of patients with different clinical syndromes (unstable angina, stable angina, and non-STsegment elevation myocardial infarction) may represent other study limitations.

Although the specific relevance of PMI for late events might be controversial, studies have indicated that PMI by cardiac marker elevation, even if clinically silent, may influence cardiovascular outcomes after PCI [213,214]; of note, the relative increase in mortality at 6 months associated with each increase of creatine kinase-MB was similar for spontaneous and PCI-related myocardial necrosis [213] Thus, the prevention of PMI may translate into an improvement in prognosis during follow-up. We believe that our findings further support the optimization of drug therapy (i.e., interventional pharmacology) to reduce PMI in patients who undergo PCI, considering that antithrombotic treatment may also limit inflammation and that drugs with anti-inflammatory properties (also including statins) may help decrease thrombosis [27; 215-219].

4.5 Tables and Figures

Table 1. Main clinical and procedural features according to baseline C-reactive protein status.

Variable	CRP ≤3 mg/L (n = 262)	CRP >3 mg/L (n = 238)	p Value
Age (yrs)	66 ± 9	68 ± 10	0.019
Men	210 (80%)	184 (77%)	0.50
Systemic hypertension	207 (79%)	200 (84%)	0.18
Diabetes mellitus	107 (41%)	96 (40%)	0.98
Hypercholesterolemia*	190 (71%)	165 (75%)	0.49
Cigarette smoking	48 (18%)	53 (22%)	0.32
Body mass index (kg/m^2)	28 ± 4	28 ± 4	1
Previous myocardial infarction	91 (35%)	96 (40%)	0.11
Previous coronary angioplasty	125 (48%)	85 (35%)	0.009
Previous coronary bypass surgery	20 (8%)	9 (4%)	0.10
Clinical presentation			
Stable angina pectoris	188 (72%)	139 (58%)	0.002
Unstable angina/ non−ST-segment elevation myocardial infarction	74 (28%)	99 (42%)	0.002
Left ventricular ejection fraction (%)	56 ± 5	55 ± 9	0.12
Serum creatinine (mg/dl)	0.96 ± 0.26	1.01 ± 0.28	0.039
Treated coronary artery			
Left main	5 (2%)	5 (2%)	0.84
Left anterior descending	132 (43%)	111 (39%)	0.37
Left circumflex	85 (27%)	81 (28%)	0.90
Right	85 (27%)	87 (30%)	0.49
Saphenous vein graft	3 (1%)	3 (1%)	0.75
Type B2/C lesions	133 (51%)	121 (51%)	0.94
Bifurcating lesions	27 (10%)	18 (8%)	0.36
Restenotic lesions	23 (9%)	29 (12%)	0.27
Multivessel intervention	48 (18%)	49 (21%)	0.60
Use of stents	237 (90%)	222 (93%)	0.32
Number of stents/patient	1.42 ± 0.94	1.38 ± 0.88	0.62
Use of drug-eluting stents	82 (31%)	77 (32%)	0.87
Direct stenting	116 (44%)	105 (44%)	0.95
Medical therapy			
β blockers	99 (38%)	95 (40%)	0.69
Statins	209 (80%)	200 (84%)	0.26
Glycoprotein IIb/IIIa inhibitors	21 (8%)	11 (5%)	0.17
Unfractionated heparin	238 (91%)	210 (88%)	0.42
Bivalirudin	24 (9%)	28 (12%)	0.42

Data are expressed as mean ± SD or as number (percentage).
* Total cholesterol >200 mg/dl.

Table 2. Main clinical and procedural features according to baseline $P2Y_{12}$ reaction unit status.

Variable	PRU <240 (n = 312)	PRU ≥240 (n = 188)	p Value
Age (yrs)	66 ± 10	69 ± 10	0.001
Men	260 (83%)	134 (71%)	0.002
Systemic hypertension	257 (82%)	150 (80%)	0.55
Diabetes mellitus	129 (41%)	74 (39%)	0.73
Hypercholesterolemia*	225 (72%)	130 (69%)	0.54
Cigarette smoking	75 (24%)	26 (14%)	0.008
Body mass index (kg/m^2)	27 ± 4	28 ± 4	0.007
Previous myocardial infarction	119 (38%)	68 (36%)	0.73
Previous coronary angioplasty	129 (41%)	81 (43%)	0.77
Previous coronary bypass surgery	19 (6%)	10 (5%)	0.87
Clinical presentation			
Stable angina pectoris	205 (66%)	122 (65%)	0.93
Unstable angina/ non–ST-segment elevation myocardial infarction	107 (34%)	66 (35%)	0.93
Left ventricular ejection fraction (%)	55 ± 8	56 ± 8	0.18
Serum creatinine (mg/dl)	0.99 ± 0.26	0.98 ± 0.29	0.69
Treated coronary artery			
Left main	6 (2%)	4 (2%)	0.86
Left anterior descending	141 (38%)	102 (45%)	0.09
Left circumflex	112 (30%)	54 (24%)	0.13
Right	110 (29%)	62 (28%)	0.66
Saphenous vein graft	3 (1%)	3 (1%)	0.84
Type B2/C lesions	154 (49%)	100 (53%)	0.46
Bifurcating lesions	30 (10%)	15 (8%)	0.64
Restenotic lesions	30 (10%)	22 (12%)	0.56
Multivessel intervention	60 (19%)	37 (20%)	0.99
Use of stents	288 (92%)	171 (91%)	0.71
Number of stents/patient	1.37 ± 0.88	1.46 ± 0.96	0.28
Use of drug-eluting stents	99 (32%)	60 (32%)	0.95
Direct stenting	134 (43%)	87 (46%)	0.53
Medical therapy			
β blockers	121 (39%)	73 (39%)	0.87
Statins	259 (83%)	150 (80%)	0.43
Glycoprotein IIb/IIIa inhibitors	21 (7%)	11 (6%)	0.84
Unfractionated heparin	285 (91%)	163 (87%)	0.13
Bivalirudin	27 (9%)	25 (13%)	0.13

Data are expressed as mean ± SD or as number (percentage).
* Total cholesterol >200 mg/dl.

Table 3. Discriminatory power of logistic regression models for the primary endpoint.

Model	Area Under the Curve (95% CI)	p Value vs Model 1
Model 1: clinical/procedural variables*	0.725 (0.615–0.835)	—
Model 2: clinical/procedural variables* plus HPR	0.779 (0.675–0.882)	0.147
Model 3: clinical/procedural variables* plus high CRP	0.755 (0.654–0.856)	0.349
Model 4: clinical/procedural variables* plus HPR and high CRP	0.811 (0.713–0.910)	0.041

* Age >65 yrs, diabetes mellitus, previous infarction, ejection fraction <40%, multivessel intervention, number of stents implanted, chronic renal failure, procedural use of glycoprotein IIb/IIIa inhibitors, and statin therapy.

Table 4. Incidence of PMI in different clinical subsets according platelet reactivity and inflammatory status.

Clinical Subset	ACS (n = 173)	p Value	No ACS (n = 327)	p Value
HPR	15.2%	0.25	9.2%	0.025
No HPR	9.3%		3.4%	
High CRP	16%	0.049	6.7%	0.44
No high CRP	6.3%		4.7%	
HPR + high CRP	26.5%	0.002	13.2%	0.007
No HPR + high CRP	7.9%		4%	

ACS = acute coronary syndromes.

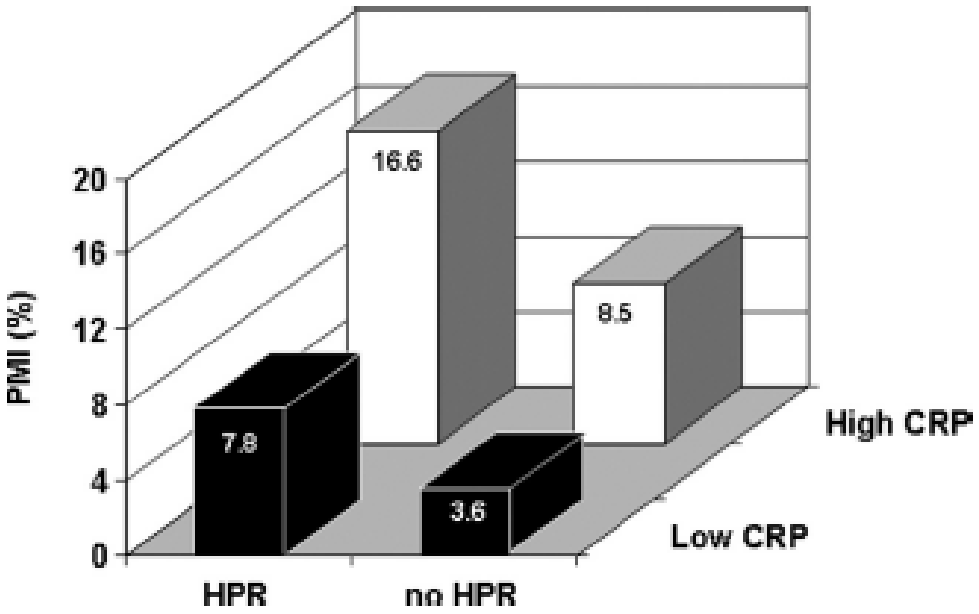

Figure 1. Incidence of primary endpoint (PMI) according to CRP and platelet reactivity status.

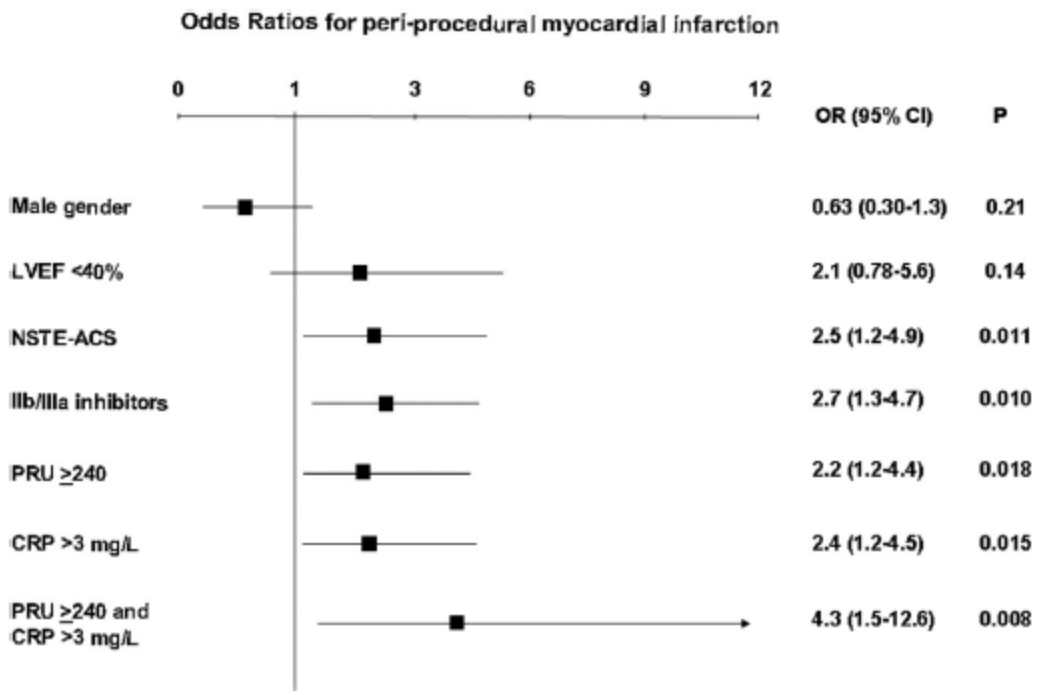

Figure 2. Multivariate analysis for PMI.

LVEF: Left Ventricular Ejection fraction. NSTE-ACS: No ST-segment Elevation Acute Coronary Syndrome

CHAPTER 5

RESEARCH PROJECT N.2

Incremental Value of Platelet Reactivity Over a Risk Score of Clinical and Procedural Variables in Predicting Bleeding After Percutaneous Coronary Intervention via the Femoral Approach. Development and Validation of a New Bleeding Risk Score*

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5.1 Background

Advances in antithrombotic strategies as support to percutaneous coronary intervention (PCI) have improved periprocedural and long-term outcomes, but thrombotic and bleeding events are still frequent complications. Data from recent studies have shown that bleeding complications after PCI are associated with an increased rate of death at both short- and long-term follow-up [84, 220-228]. In particular, it has been shown that both access and non-access site bleeding events after PCI are independently associated with mortality, although this association was considerably stronger with bleeding complications not occurring at access site [227-228]. To timely identify patients at higher risk of bleeding after PCI, various scores have been developed and validated [229-233]. These scores allow estimating the

individual patient the probability to develop bleeding complications simply based on the presence of clinical and procedural risk factors.

Platelet reactivity (PR) assessed by point of care assays seems to be effective in predicting ischemic and bleeding events after PCI [234, 57, 77, 85, 86]; in particular, initial evidences are now available of a therapeutic window for PR after administration of $P2Y_{12}$ inhibitors [87, 89]. Specific thresholds of PR have been identified as associated with a significant increase in the incidence of bleeding complications, helping to identify patients at higher hemorrhagic risk. In particular, we have recently proposed a threshold of 178 $P2Y_{12}$ reaction units (PRU) measured with the VerifyNow $P2Y_{12}$ assay to predict bleeding events after PCI [89]. However, at present, none of the risk scores for bleeding consider PR among variables for risk assessment.

Aim of the present study was to investigate the potential incremental value of PR in predicting bleeding events in patients undergoing PCI via the femoral approach over a validated bleeding risk score (BRS) of clinical and procedural variables.

5.2 Methods

Study Population and Design

This is a prospective study enrolling consecutive patients with stable angina or non–ST-elevation acute coronary syndrome (ACS) undergoing elective PCI via the femoral approach at the Department of Cardiovascular Sciences, Campus Bio-Medico University, Rome, Italy, and at the Cardiovascular Center Aalst, Aalst, Belgium, from June 2011 to May 2012.

Pre-PCI antiplatelet treatment consisted of clopidogrel 600-mg loading dose at least 6 hours before the procedure or 75 mg/d for at least 5 days. Administration of further

antiplatelet drugs to patients already on chronic treatment was left to the operator's discretion and based on clinical presentation. Procedural anticoagulation consisted of unfractionated heparin administrated to achieve an activated clotting time of 250 to 300s. Procedural success was defined as a reduction in percent diameter stenosis to <30% in the presence of thrombolysis in myocardial infarction (TIMI) flow grade 3 in the main vessel and all side branches >2 mm in diameter. After PCI, patients receiving baremetal stents received clopidogrel 75 mg for at least 4 weeks, whereas those with non-ST elevation ACS or undergoing drug-eluting stent implantation received clopidogrel 75 mg for 12 months. Low-dose aspirin (80–100 mg) was administered to all patients before PCI and continued indefinitely. Access site hemostasis after sheath removal was achieved in all patients with manual compression.

Exclusion criteria were use of radial approach, upstream use of glycoprotein IIb/IIIa inhibitors, treatment with oral anticoagulant drugs, platelet count <70×109/L, high bleeding risk (active internal bleeding, history of hemorrhagic stroke, intracranial neoplasm, arteriovenous malformation or aneurysm, and ischemic stroke in the previous 3 months), and coronary artery bypass surgery in the previous 3 months.

Clinical follow-up at 30 days was obtained in all patients by office visit, telephone interview, or chart review. All events were classified and adjudicated by a physician not involved in the follow-up process. This study complied with the Declaration of Helsinki and was approved by the local ethics committees, with all patients giving written informed consent.

BRS Calculation

Bleeding risk score was calculated as previously described by Nikolsky et al. [233]. Briefly, risk score models were created by identifying independent predictors of major

bleeding in databases from the Randomized Evaluation in PCI Linking Angiomax to Reduced Clinical Events (REPLACE)-2 [235] and REPLACE-1 trials [236]. The chosen variables were assigned a weighted integer, the sum of the integers representing the total risk score for each patient. Calculation of the BRS included the following variables: age, sex, intra-aortic balloon pump, glycoprotein IIb/IIIa inhibitors, chronic kidney disease, anemia, and low-molecular-weight heparin within 48-hour pre-PCI.

Blood Sampling and Platelet Function Analysis

Blood samples for PR assessment were collected in the catheterization laboratory immediately before PCI. After discarding the first 5 mL of blood, a sample drawn from the femoral artery immediately after sheath insertion and collected into a 2-mL tube containing 3.2% sodium citrate. PR was assessed using the VerifyNow $P2Y_{12}$ assay. This is an optical turbidimetric point-of-care assay specifically assessing the effects of $P2Y_{12}$ receptor blockers. Results are expressed as PRU: the lower the PRU value, the higher the platelet aggregation inhibition, and vice versa. In all cases, the operators were blinded to platelet function test results.

Bleeding Definitions

Primary end point of this study was the 30-day incidence of bleeding events assessed with the following criteria: TIMI [237], REPLACE-2 [235] and Bleeding Academic Research Consortium (BARC) criteria [103]. For this analysis, TIMI major and minor bleedings, REPLACE-2 major bleedings, and BARC classes 2 to 5 bleedings were considered. Bleedings according to TIMI and REPLACE-2 criteria were prospectively evaluated in all study patients, whereas bleedings according to BARC criteria were retrospectively analyzed. Event adjudication was blinded to platelet function test results.

Statistics

Continuous variables are reported as mean±SD or median with lower and upper quartiles, as appropriate. Categorical variables are reported as frequencies and percentages. Comparisons between continuous variables were performed using the Student t test or Mann–Whitney U test. Comparisons between categorical variables were evaluated using the Fisher exact test or the Pearson χ^2 test, as appropriate. Receiver operating characteristic curve analysis was used to test the ability of BRS and PR values to discriminate between patients with and without bleeding events at 30-day follow-up. We assessed the incremental value of combining BRS and PR together in predicting the primary end point. Area under the curve (AUC) was calculated for the logistic regression model including both BRS and PR; differences between AUCs for different models were assessed using the jackknife method, as described by DeLong et al. [201]. Furthermore, net reclassification improvement (based on 3 risk categories: <2%, 2%–5%, and >5%) and integrated discrimination improvement were used to compare the performance and predictive value of BRS alone or in combination with PR [238]. A bleeding risk score including PR (BRS-PR) was then developed in the study population and validated in the cohort of patients enrolled in the Antiplatelet Therapy for Reduction of Myocardial Damage During Angioplasty-Bleeding Study (ARMYDA-BLEEDS) study [86]. Besides clinical and procedural variables composing the BRS, low PR (LPR), defined as a PRU value ≤178 [89], was also included in BRS-PR. Both BRS and LPR were entered in a multivariable logistic regression model to identify independent predictors of TIMI bleeding. Based on the z-score (model coefficient divided by SE), a weighted integer was assigned to LPR. BRS-PR was calculated as the sum of the integers of each variable included in

the original BRS [233] plus that of LPR. Based on BRS and BRS-PR values, patients were categorized in 4 risk groups: very low risk (0 points), low risk (2–6 points), intermediate risk (7–9 points), and high risk (≥10 points). No a priori sample size calculation was performed. No adjustment for multiple comparisons was performed. Statistical analysis was performed using Stata/IC version 10.0 (STATA Corp, College Station, TX), and P values <0.05 (2-tailed) were considered significant.

5.3 Results

Patient Population

A total of 800 patients were recruited in this study. Main clinical and procedural characteristics are listed in Table 1. Clinical presentation was non–ST-elevation ACS in 226 patients (28%); however, only 87 patients (11%) received glycoprotein IIb/IIIa inhibitors, whereas no patients were treated with low molecular weight heparin or intra-aortic balloon pump. Sheath size was 6 French in 713 patients (89%) and 7 French in the remaining 87 (11%). BRS ranged from 0 to 19, with a mean (±SD) of 7.0±3.7. Pre-PCI PRU levels ranged from 34 to 425, with a mean (±SD) of 207±73 (median 204, lower quartile <155, and upper quartile >256). A total of 272 patients (34%) showed PRU values ≤178 and were classified as having LPR.

Bleeding Events

At 30-day follow-up, a total of 28 (3.5%) TIMI (7 [0.9%] major and 21 [2.6%] minor), 44 (5.5%) BARC class ≥2, 32 (4.0%) BARC class ≥3, and 32 (4.0%) REPLACE-2 bleedings occurred. The source of bleeding was genitourinary in 5 patients, gastrointestinal in 4 patients, and cerebral in 1 patient, related to the entry site in 32 patients (including 2 retroperitoneal hemorrhages), and unknown in 1 patient

with a 5 g/dL hemoglobin loss without an overt source of bleeding. The distribution of bleeding events according to BRS risk groups and to the presence of LPR is shown in Table 2. LPR remained independently associated with a higher risk of bleeding events even after adjustment for diabetes mellitus, serum creatinine, and multivessel disease.

Discrimination Analysis

At receiver operating characteristic curve analysis, BRS could significantly discriminate between patients with and without bleeding according to all definitions (AUC, 0.717; 95% CI, 0.639–0.795 for TIMI bleeding; AUC, 0.733, 95% CI, 0.666–0.800 for BARC class ≥2 bleeding; AUC, 0.629; 95% CI, 0.533–0.726 for BARC class ≥3 bleeding; and AUC, 0.719; 95% CI, 0.646–0.792 for REPLACE-2 bleeding). Similarly, PR could significantly discriminate between patients with and without bleeding according to all definitions (AUC, 0.729; 95% CI, 0.649–0.809 for TIMI bleeding; AUC, 0.736; 95% CI, 0.669–0.802 for BARC class ≥2 bleeding; AUC, 0.708; 95% CI, 0.622–0.793 for BARC class ≥3 bleeding; and AUC, 0.722; 95% CI, 0.645–0.798 for REPLACE-2 bleeding). When BRS and PR were combined in the same logistic regression model for prediction of bleeding, the AUC of the model was 0.818 for TIMI (Hosmer–Lemeshow P=0.989; P=0.002 versus BRS alone), 0.822 for BARC class ≥2 (Hosmer–Lemeshow P=0.678; P<0.001 versus BRS alone), 0.745 for BARC class ≥3 (Hosmer– Lemeshow P=0.591; P=0.003 versus BRS alone), and 0.813 for REPLACE-2 bleeding (Hosmer–Lemeshow P=0.974; P=0.001 versus BRS alone; Figure 1).

The net reclassification improvement was estimated at 0.387 (P=0.006) for TIMI bleeding, at 0.332 (P<0.001) for BARC class ≥2 bleeding, at 0.480 (P=0.001) for BARC class ≥3 bleeding, and at 0.339 (P=0.013) for REPLACE-2 bleeding.

The integrated discrimination improvement was estimated at 0.047 (P<0.001) for TIMI bleeding, at 0.061 (P<0.001) for BARC class ≥2 bleeding, at 0.024 (P<0.001) for BARC class ≥3 bleeding, and at 0.051 (P<0.001) for REPLACE-2 bleeding.

New Bleeding Risk Score

Using this study population as a development cohort, we built a new risk score including LPR in addition to the variables used for the determination of BRS. Henceforth, we used a multivariable model of predictors of TIMI bleeding including LPR and BRS, and, on the basis of the z score, a weighted integer score of 4 was assigned to LPR. BRS-PR ranged from 0 to 23 with a median value of 8 (lower quartile <6, upper quartile >10). The discrimination ability of this new bleeding risk score including PR (BRS-PR) was tested in the development cohort for all bleeding definitions, and in a validation cohort represented by the population of patients enrolled in the ARMYDA-BLEEDS study [86] for TIMI bleeding only.

In the development set, BRS-PR could significantly discriminate between patients with and without bleeding according to all definitions (AUC, 0.809; 95% CI [0.74–0.87] for TIMI bleeding; AUC, 0.814; 95% CI [0.76–0.87] for BARC class ≥2 bleeding; AUC, 0.813; 95% CI [0.75–0.87] for REPLACE-2 bleeding). Discriminatory power was significantly better than that of BRS alone (P<0.001 for all bleeding definitions). The distributions of patients with and without bleeding events in the different risk groups of BRS and BRS-PR are shown in Figure 2. In the validation set (n=310), BRS-PR could significantly discriminate between patients with and

without TIMI bleeding with an AUC of 0.788 (Hosmer–Lemeshow P=0.399). The discriminatory power of BRS-PR was significantly better than that of BRS alone (AUC, 0.709; P=0.036). The rates of TIMI bleedings in the 4 risk score groups are shown in Figure 3 for both the development cohort and the validation cohort.

5.4 Discussion

Major findings of the present study are that (1) PR has incremental value over a validated risk score of clinical and procedural characteristics in predicting bleeding events after PCI via the femoral approach; (2) a new risk score including PR yields significantly better prognostic performance compared with the original BRS, as also confirmed in an independent cohort of patients.

Increasing concern has been raised in the past few years over the bleeding complications associated with aggressive antiplatelet therapy required in patients treated with coronary stenting. There is compelling evidence that hemorrhagic events after PCI are associated with worse clinical outcomes at long-term follow-up [84, 220-228]. Ndrepepa et al. [84] have shown in a study of 5384 patients undergoing PCI that the occurrence of bleeding events within 30 days from stenting was associated with a ≈3-fold increase in 1-year mortality. Similar results have been recently shown in a large registry (>3.3 million PCI procedures), where bleeding after PCI was associated with a significant increase in inhospital mortality, and 12.1% of deaths were related to periprocedural bleeding [228]. Moreover, both access site and non–access site bleeding were associated with increased inhospital mortality, although this association was stronger for nonaccess bleeding [228].The fact that even access-site complications carry important prognostic consequences has been confirmed in a recent analysis of

14180 patients treated with coronary stenting where both access and non–access site bleeding events occurring within 30 days after PCI were independently associated with an increased risk of 1-year mortality [227].

With these premises, an effort to timely identify patients at high bleeding risk seems mandatory to apply appropriate treatment strategies to reduce hemorrhagic complications. In recent years, several risk scores for post-PCI bleeding have been proposed [229-233]. Although most of these were based on ACS patients undergoing urgent revascularization [229,230,232], Nikolsky et al. [233] have developed a risk score model based on the REPLACE-2 trial [235] and validated it in the REPLACE-1 trial [236] population. Similar to the present, the latter studies were mainly composed by stable coronary artery disease patients. This bleeding risk score was based on both clinical and procedural characteristics and demonstrated good prognostic accuracy for major bleeding, significantly discriminating between patients at different levels of risk for major bleeding.

Platelets play a key role in pathogenesis of both thrombotic and bleeding complications. Interindividual variability in response to antiplatelet agents, and clopidogrel in particular, exposes a large proportion of patients to either too high or too low residual PR. Hyporesponders to antiplatelet therapy have high PR and therefore increased risk of thrombotic complications [57,77,202]; on the other extreme of the spectrum, hyperresponders present low residual PR and therefore increased risk of hemorrhagic complications [85,86,88]. Using the VerifyNow $P2Y_{12}$ platelet function test, we have recently proposed a threshold of 178 PRU for the definition of LPR [89], which was associated with a significant increase in 30-day bleeding in patients undergoing elective PCI. Moving from the evidence that both clinical risk

scores and PR could predict post-PCI bleeding, we have investigated whether the combination of these parameters could improve their prognostic performance. In the present study, both PR and BRS alone were able to discriminate between patients with and without bleeding; however, the combination of the two factors together led to a significant increase in discriminatory power. Noteworthy, the superiority of the combination of BRS and PR together over the 2 factors alone was confirmed for all 3 definitions of bleeding used in the present study (TIMI, BARC, and REPLACE-2 definitions). Moreover, we have developed a new risk score including PR in addition to the clinical and procedural variables of BRS. Interestingly, BRS-PR has shown significantly better discriminatory performance compared with the original BRS, showing an AUC >0.8 for all bleeding definition in the development cohort and an AUC of 0.788 for TIMI bleeding in the validation set.

Timely identification of patients at risk of bleeding is key for the adoption of preventive measures. Access-site selection (ie, femoral versus radial approach) is one essential variable, especially when one considers the prognostic implications of often under-rated entry-site complications [227,228]. Identifying a patient at high risk for bleeding after PCI via the femoral approach could lead to the decision to choose the radial approach for coronary angiography and intervention. Growing evidence suggests that the use of radial approach improves patients' outcomes, especially in the setting of ACS, by significantly reducing bleeding complications [239-241]. A pharmacological management tailored on the basis of patients' risk profile is another potential modality of bleeding prevention. This is particularly true in an era in which we have at our disposal a great variety of antithrombotic drugs, with different efficacy and safety profile. Preferring bivalirudin to unfractioned heparin [242], or clopidogrel

instead of prasugrel [28] and ticagrelor [30], and limiting the use of glycoprotein IIb/IIIa inhibitors are all possible pharmacological strategies to reduce the incidence of bleeding complications. However, medical management of patients with CAD should always be a tradeoff between thrombotic and bleeding events prevention, and a thorough evaluation of risk profile on an individual basis is mandatory. In this view, an additional tool such as BRS-PR could be of use in the selection of the appropriate pharmacological and procedural strategies for patients undergoing PCI.

This study has some limitations that are worth mentioning. The assessment of bleeding according to BARC criteria was analyzed retrospectively. By study protocol, only patients undergoing elective PCI via the femoral approach were included; thus, these results might not be applicable to patients undergoing urgent revascularization and radial procedures. However, this allowed to remove a potential confounder related to the vascular access. Although this could be partially responsible for the higher discriminatory power yielded by PR in this compared with other studies [85,89], no specific factors have been identified for these discrepancies. In the light of the composition of our study population, we elected the REPLACE bleeding risk score, which was developed in a cohort of predominantly stable patients, and therefore, the observations of this study cannot be extrapolated for patients with ACS undergoing urgent PCI; however, we think that the specificity of BRS-PR is crucial in determining its high prognostic value. No patient was treated with bivalirudin or $P2Y_{12}$ receptor inhibitor different from clopidogrel, and therefore the observations of the present study cannot be extended to patients receiving those medications. Although the 178 PRU cutoff was derived from our previous work [89] this might not be ideal for the definition of LPR. No adjustment for multiple comparisons was carried out when

analyzing the primary end points; however, given the strong correlation between bleeding events resulting from the wide overlap between different definitions, we think that this would not affect the interpretation of the results. Finally, long-term follow-up data were only available for a minority of patients and no specific analysis was attempted.

In conclusion our study suggests that a new risk score (BRS-PR), taking in account also PR, could help to better stratify patients undergoing elective PCI according to bleeding risk profile. This is of particular importance as bleeding events heavily affect prognosis of patients undergoing PCI via the femoral approach, contributing to increase mortality rate on the long term. A more accurate stratification of patients could lead to a better selection of treatment options to improve their prognosis, avoiding both bleeding complications and thrombotic events. Further investigations are needed to confirm the efficacy of BRS-PR and to evaluate the potential role of PR as an adjunctive parameter to risk scores dedicated to specific clinical settings, such as urgent PCI for ACS.

5.5 Tables and Figures

Table 1. Clinical and Procedural Characteristics

	Overall (n=800)	LPR (n=272)	No LPR (n=528)	*P* Value
Age, y	67±10	66±10	67±10	0.129
Male	590 (74)	195 (72)	395 (75)	0.342
Body mass index	26.1±3.3	25.9±3.0	26.2±3.4	0.219
Diabetes mellitus	236 (30)	77 (28)	206 (39)	0.003
Hypertension	630 (79)	213 (78)	417 (79)	0.827
Current smoking	162 (20)	63 (23)	99 (19)	0.141
Previous myocardial infarction	199 (25)	89 (33)	149 (28)	0.187
Previous percutaneous coronary intervention	274 (34)	94 (35)	180 (34)	0.895
Previous coronary artery bypass graft	61 (8)	17 (6)	44 (8)	0.293
Previous cerebrovascular accident	25 (3)	7 (3)	18 (3)	0.520
Non–ST-segment–elevation acute coronary syndrome	226 (28)	82 (30)	144 (27)	0.392
Left ventricle ejection fraction, %	55±7	55±8	56±7	0.695
Left ventricle ejection fraction <40%	75 (9)	31 (11)	44 (8)	0.159
Hematocrit, %	42.0±4.5	41.7±4.6	42.1±4.6	0.244
Hemoglobin, g/dL	13.9±1.8	13.7±1.9	14.0±1.9	0.145
Anemia	231 (29)	87 (34)	144 (26)	0.164
Platelet count, 10^9/L	231±69	229±71	232±68	0.561
Serum creatinine, mg/dL	1.04±0.29	1.01±0.31	1.05±0.28	0.066
Estimated glomerular filtration rate, mL/min per 1.73 m^2	76.9±24.2	78.1±25.2	76.1±23.9	0.271
Estimated glomerular filtration rate <60 mL/min per 1.73 m^2	173 (22)	57 (21)	116 (22)	0.741
Multivessel disease	336 (42)	102 (38)	234 (44)	0.064
Target vessel				0.788
Left main stem	6 (1)	2 (1)	4 (1)	
Left anterior descending	429 (54)	139 (51)	290 (55)	
Left circumflex	139 (17)	49 (18)	90 (17)	
Right coronary artery	221 (27)	81 (30)	140 (27)	
Saphenous vein graft	5 (1)	1 (1)	4 (1)	
No. of stents implanted	1.4±0.9	1.3±0.8	1.4±1.0	0.153
Intraortic balloon pump	…	…	…	…
Glycoprotein IIb/IIa inhibitors	87 (11)	36 (13)	51 (10)	0.124
Sheath size				0.734
6 French	713 (89)	241 (89)	472 (89)	
7 French	87 (11)	31 (11)	56 (11)	

Values are mean±SD or n (%). LPR indicates low platelet reactivity.

Table 2. Bleeding Events at 30-Day Follow-Up According to Bleeding Risk Score Groups and to the Presence Low Platelet Reactivity

	Bleeding Definition					
	Any TIMI	TIMI Major	TIMI Minor	BARC ≥2	BARC ≥3	REPLACE-2
Very low risk (n=50)	0 (0.0)	0 (0.0)	0 (0.0)	1 (2.0)	1 (2.0)	0 (0.0)
Low risk (n=366)	5 (1.4)	1 (0.3)	4 (1.1)	5 (1.4)	3 (0.8)	5 (1.4)
Intermediate risk (n=175)	7 (4.0)	2 (1.1)	5 (2.9)	13 (7.4)	9 (5.1)	9 (5.1)
High risk (n=209)	16 (7.7)	4 (1.9)	12 (5.7)	25 (12.0)	19 (9.1)	18 (8.6)
P value	<0.001	0.032	<0.001	<0.001	<0.001	<0.001
No LPR (n=528)	6 (1.1)	1 (0.2)	5 (0.9)	11 (2.1)	7 (1.3)	7 (1.3)
LPR (n=272)	22 (8.1)	6 (2.2)	16 (5.9)	33 (12.1)	25 (9.9)	25 (9.2)
P value	<0.001	0.004	<0.001	<0.001	<0.001	<0.001

BARC indicates Bleeding Academic Research Consortium; LPR, low-platelet reactivity; REPLACE, Randomized Evaluation in PCI Linking Angiomax to Reduced Clinical Events; and TIMI, thrombolysis in myocardial infarction.

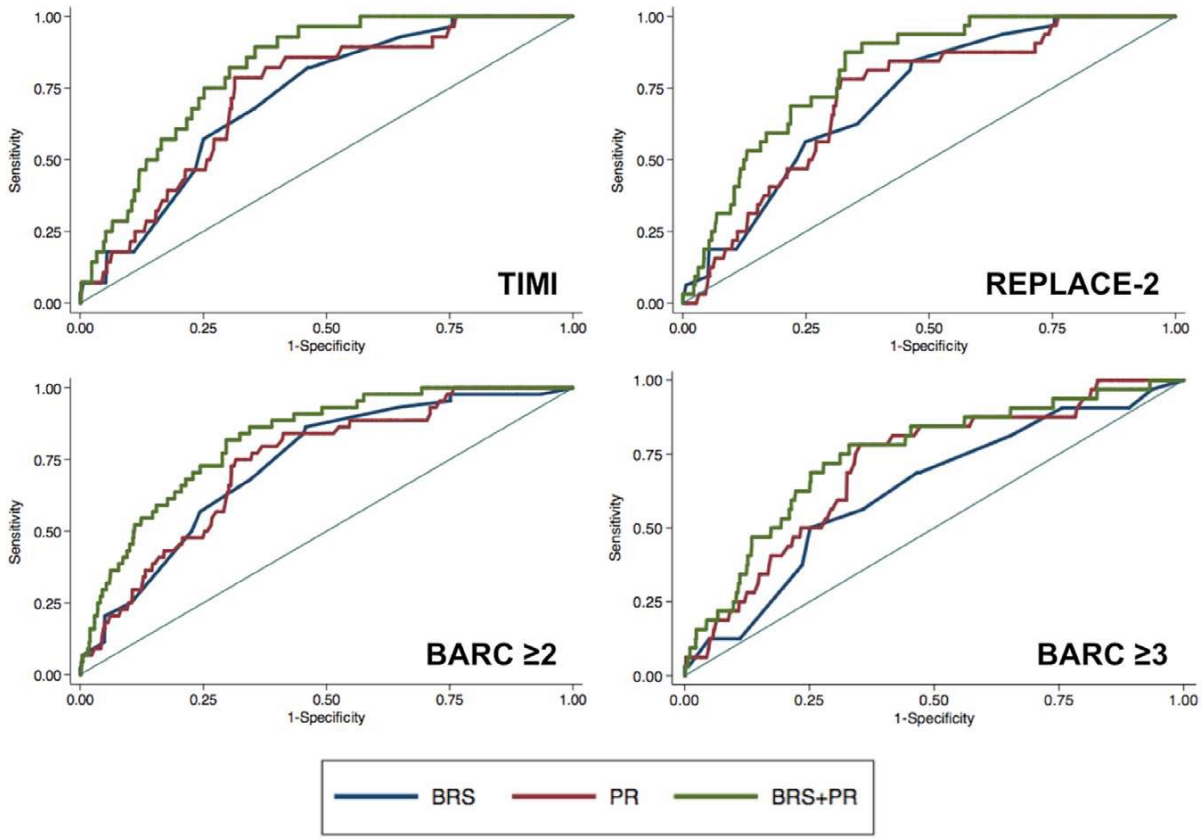

Figure 1. Receiver operating characteristic curves for bleeding events.

Bleeding was defined according to thrombolysis in myocardial infarction (TIMI), Bleeding Academic Research Consortium (BARC) ≥2, BARC ≥3, and Randomized Evaluation in PCI Linking Angiomax to Reduced Clinical Events (REPLACE)-2 definitions. BRS indicates bleeding risk score; and PR, platelet reactivity.

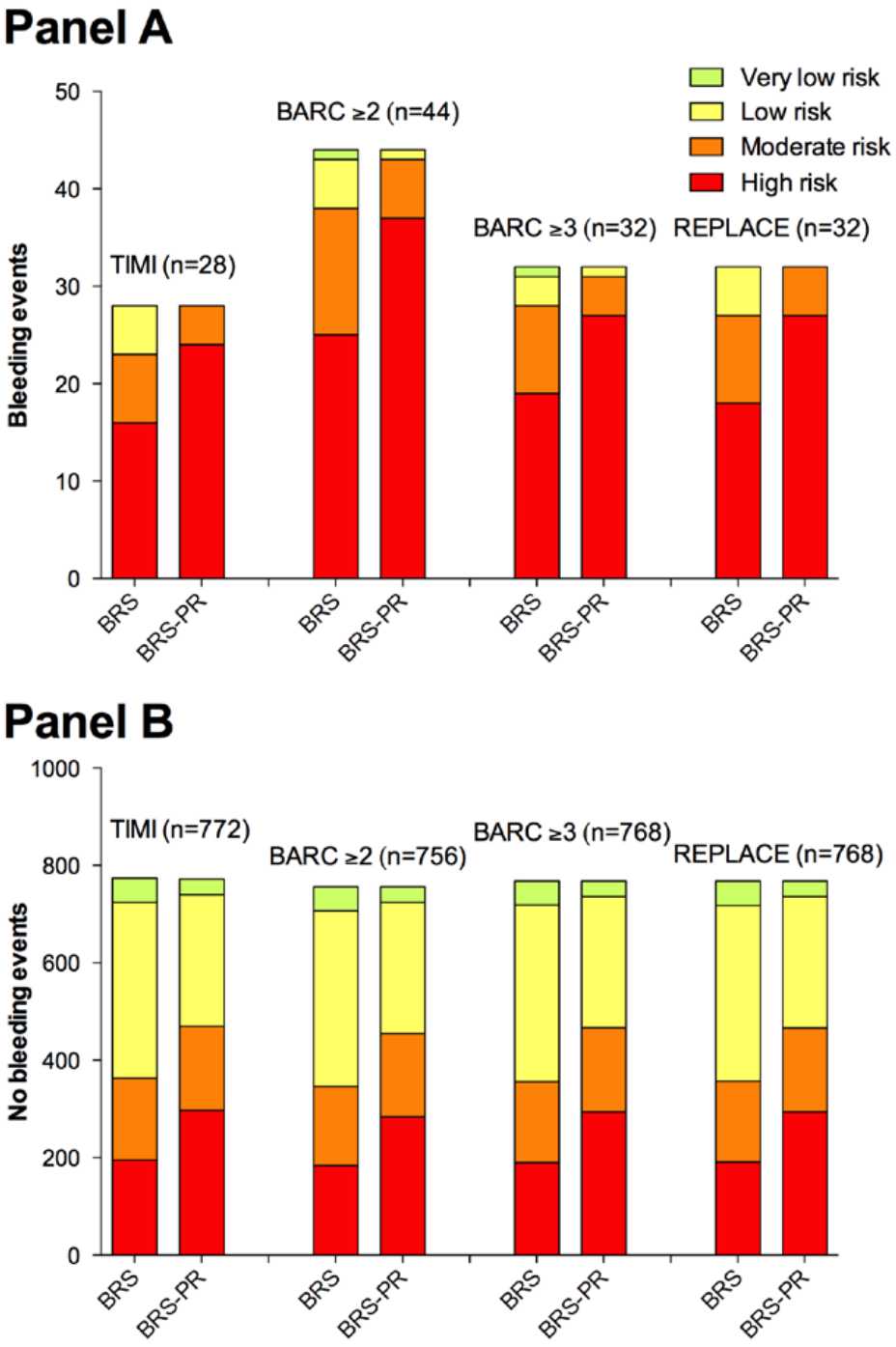

Figure 2. Distribution of patients with (A) and without (B) of bleeding events according to bleeding risk score (BRS) and BRS platelet reactivity platelet reactivity (PR) risk score groups.

Risk was categorized as: very low (0 points), low (2–6 points), intermediate (7–9 points), and high (≥10 points). BARC indicates Bleeding Academic Research Consortium; REPLACE, Randomized Evaluation in PCI Linking Angiomax to Reduced Clinical Events; and TIMI, thrombolysis in myocardial infarction.

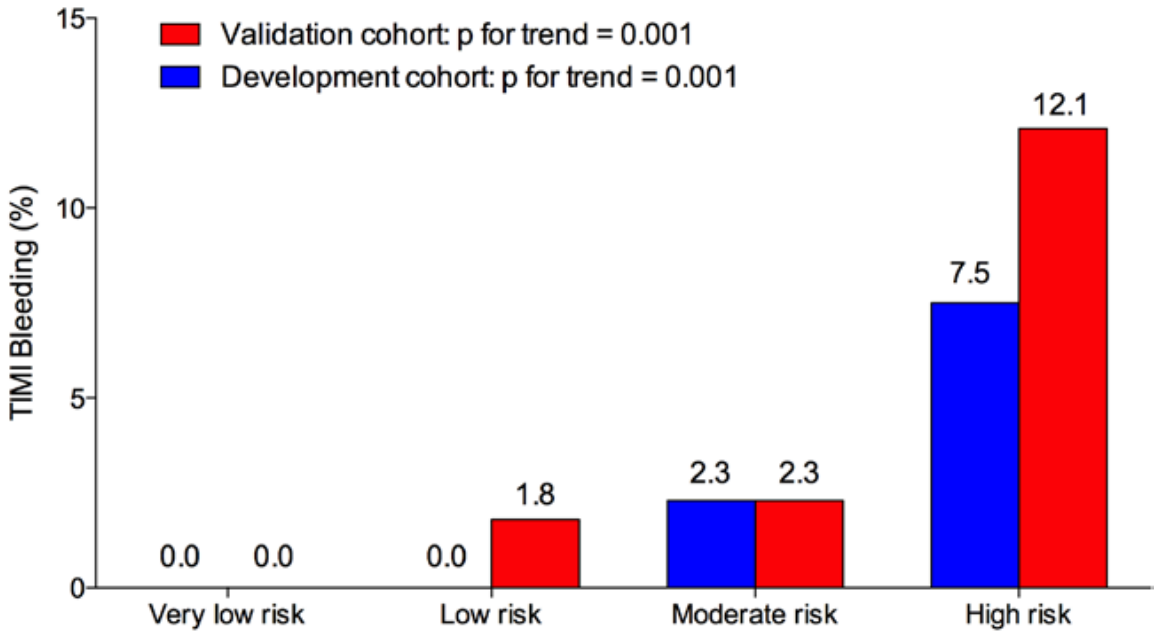

Figure 3. Incidence of any thrombolysis in myocardial infarction (TIMI) bleeding according to bleeding risk score (BRS) and BRS-platelet reactivity (PR) risk score groups in the development cohort and the validation cohort.

Risk was categorized as: very low (0 points), low (2–6 points), intermediate (7–9 points), and high (≥10 points).

CHAPTER 6

RESEARCH PROJECT N.3

Relationship of platelet indices with platelet reactivity and periprocedural myocardial infarction in patients undergoing percutaneous coronary angioplasty *

**This article has been submitted to the American Journal of Cardiology.*

6.1 Background

Percutaneous coronary intervention (PCI) has become in the last two decades one of the treatments of choice for patients with coronary artery disease (CAD) [243, 244]. Despite technological and pharmacological advances, cardiovascular events following PCI still occur in a clinically significant proportion of patients [51,52]. Among these, periprocedural myocardial infarction (PMI) still represents a frequent complication with an incidence varying from 2.6% to 23% based on the definition applied [59, 245]. Dual antiplatelet therapy with aspirin and $P2Y_{12}$ inhibitors is the cornerstone of the pharmacological treatment of patients undergoing PCI. Nevertheless, a wide interindividual variability in the response to antiplatelet drugs has been reported [145], and it has been shown that the degree of platelet inhibition at the time of the procedure has a significant impact on clinical outcomes of PCI,

including the occurrence of PMI [76,77,247]. Numerous platelet function tests are nowadays available, most of which are expensive or require dedicated training and time-consuming laboratory procedures [72].

Platelet indices such as platelet count, mean platelet volume (MPV), platelet distribution width (PDW) and MPV/P ratio have been proposed as reliable markers of platelet activation and are related to several traditional risk factors and to long-term outcomes of patients with coronary artery disease (CAD) [248-256]. These indices are routinely reported in a complete blood count (CBC), and therefore readily available in patients undergoing PCI. However, no comprehensive data are available on the relationship of platelet indices with on-treatment platelet reactivity and periprocedural outcomes in patients undergoing PCI. Therefore, we aimed to investigate the correlation of platelet indices with platelet reactivity and with the occurrence of PMI in patients undergoing PCI.

6.2 Methods

This is a retrospective study enrolling patients with stable angina or non-ST segment elevation acute coronary syndrome (NSTE-ACS) undergoing percutaneous coronary intervention at Campus Bio-Medico University. All patients were given aspirin (100 mg/day) and received either a 600 mg clopidogrel loading dose (at least 6 hours before PCI) or continued clopidogrel treatment if they were on clopidogrel 75 mg/day therapy for at least 5 days. Exclusion criteria were: primary PCI for ST-segment elevation myocardial infarction, upstream use of glycoprotein IIb/IIIa inhibitors, platelet count <70 $x10^3$/µl, use of prasugrel or ticagrelor, need for oral

anticoagulant therapy, left ventricular ejection fraction <30%, renal failure with creatinine serum level >2 mg/dL, history of systemic inflammatory or autoimmune disease, hemoproliferative disorders and neoplasms.

Interventions were performed using a standard technique. Procedural success was defined as postprocedure Thrombolysis In Myocardial Infarction grade 3 flow, with decrease of stenosis to <30% residual narrowing by quantitative coronary angiographic analysis. A non-ionic, low-osmolarity (915 mOsm/kg) iodinated contrast agent (iobitridol, Xenetix, Guerbet, RoissyCdGCedex, France) was used. PCI procedures were performed using the femoral approach after administration of weight-adjusted intravenous unfractionated heparin (70 IU/kg body weight) or bivalirudin (bolus 0.75 mg/kg, followed by intra-procedural infusion adjusted according to renal function). Periprocedural use of glycoprotein IIb/IIIa inhibitors was only in bailout following operator discretion. After the procedure, aspirin (100 mg/day) was continued indefinitely, whereas clopidogrel (75 mg/day) was administered for at least 1 month (12 months in patients treated for NSTE-ACS or receiving a drug eluting stent). This study complied with the Declaration of Helsinki and was approved by the local ethics committee, with all patients giving written informed consent.

Blood sample for assessment of platelet indices (platelet count, MPV, PDW and MPV/P ratio) were collected before PCI in tripotassium EDTA tubes and were analyzed within 2 hours from venipuncture by automatic blood counter (Sysmex XT-400), as per standard clinical practice in our institution. Normal range for platelet count were set at 150-400 $x10^3$/µl for platelet count, 9-13 fL for MPV and 10-16 fL for PDW. Platelet reactivity was assessed on blood samples drawn from the arterial sheath, and platelet reactivity was immediately measured in the catheterization laboratory before

PCI using the VerifyNow $P2Y_{12}$ assay (Accriva Diagnostics, San Diego, CA, USA), which is a rapidcartridge-based assay specifically measuring effects of clopidogrel on the platelet $P2Y_{12}$ receptor. Technical details of the assay have been previously described [198]. Results are expressed as $P2Y_{12}$ reactivity units (PRU). Blood samples were also drawn in all patients before PCI and at 8 and 24 h after PCI for measurement of creatine kinase-MB (CK-MB) and troponin I (TnI) levels. Additional samples were obtained to measure myocardial necrosis markers if patients after PCI developed symptoms suggestive of myocardial ischemia, as per standard clinical practice in our institution. Measurements were performed using the Access 2 immunochemiluminometric assay (Beckman Coulter, Fullerton, California). Normal limits were set at 3.6 ng/mL for CK-MB and at 0.06 ng/mL for TnI.

Primary endpoint was the incidence of PMI according to tertiles of different platelet indices and platelet reactivity. PMI was defined according to the Joint ESC/ACCF/AHA/WHF task force consensus statement on the redefinition of MI for clinical trials on coronary intervention [257]. Secondary endpoint was evaluation of platelet indices in high platelet reactivity (HPR) patient compared to those with normal responsiveness to clopidogrel. HPR was defined as a value of PRU ≥240 (10).

Categorical variables are reported as frequencies and percentages. Continuous variables are reported as mean±SD. Normal distribution was tested by the Kolmogorov-Smirnov test. Continuous variables were compared by two tailed t test for normally distributed values; otherwise the Mann–Whitney U test was used. Proportions were compared by Fisher's exact test when the expected frequency was <5, or the Yates-corrected Chi-square test otherwise. A P value <0.05 was considered

statistically significant. Statistical analysis was performed using the SPSS 16.0 release software (SPSS, Inc., Chicago, Illinois).

6.3 Results

A total of 502 patients fulfilling inclusion criteria were included in the study. Main clinical and procedural features are listed in Table 1 and Table 2, respectively. Clinical presentation was NSTE-ACS in 171 patients (34%). No patient died during in-hospital stay.

PMI occurred in 33 patients (6.6%) and its incidence was not significantly different among tertiles of platelet count (P=0.74, Figure 1A). Similarly, no difference in PMI incidence was observed in tertiles of MPV (P=0.86, Figure 1B), tertiles of PDW (I tertile P=0.74, Figure 1C), and tertiles of MPV/P ratio (P=0.91, Figure 1D). A significant difference in the occurrence of PMI was identified among PRU tertiles (P=0.006, Figure 2). Mean absolute PRU levels were significant higher in patients with PMI compared to patients who do not develop PMI (262.4±66.7 vs 216.5±79.7; P=0.001). In the overall population, 186 patients presented HPR (37.1%). The incidence of PMI was 24.4% among patients with HPR and 3.8% among patients without HPR (P=0.003).

No significant difference was observed in platelet count between patients with and without PMI (218.3±65.9 $x10^3/\mu l$ vs. 212.1±59.1$x10^3/\mu l$, P=0.56). Likewise, no significant differences were identified in other platelet indices between patients with and without PMI (MPV: 10.63±0.80 fL vs. 10.77±0.99, P=0.43; PDW: 12.92±1.69 vs. 13.27±2.15fL, P=0.36; MPV/P ratio 52.97±15.90 vs. 54.99±17.46, P=0.52). However,

platelet count and MPV/P were significantly different between patients with and without HPR (platelet count: 221.8±58.6x10^3/µl vs. 207±59.4x10^3/µl, P=0.008, Figure 3A; MPV/P ratio 51.73±15.17 vs. 56.7±18.3, P=0.002, Figure 3B), while no significant differences were identified in MPV (10.69±0.86 fL vs. 10.8±1, P=0.22) and PDW values (13.05±1.83 vs. 13.37±2.27, P=0.10).

6.4 Discussion

The present study suggests that platelet indices (i.e. platelet count, MPV, PDW, MPV/P ratio) are not associated with the occurrence of PMI in patients undergoing PCI. Moreover, our data suggest that MPV and PDW are not associated with responsiveness to clopidogrel, while higher platelet count and MPV/P ratio were observed among patients with HPR.

Conflicting data have been reported on the potential impact of platelet indices on clinical outcomes of patient undergoing PCI. Higher values of platelet indices, in particular MPV and PDW, were previously associated with a higher incidence of adverse events both in the setting of stable angina and ACS [248-255]. However, recent investigations by Verdoia et al. have found no significant association of MPV and PDW with the occurrence of PMI defined as CK-MB mass release ≥3 times ULN or increased by 50% if already elevated at the time of the procedure [258, 259, 260]. Our results are in line with those of the latter studies, although we have taken into account a more recent definition of PMI, including clinical features besides an increase of cardiac markers [257]. A strong correlation between HPR and the incidence of PMI in patients undergoing PCI has also been widely demonstrated [76,77,247], however

no definitive data are available on the relationship between platelet indices derived from CBC and platelet reactivity. MPV is an expression of platelet size, whereas PDW represent the range of variability in platelet volume. Larger platelets are considered metabolically and enzymatically more active in reason of greater concentration of granules containing prothrombotic materials. In fact, they have a higher expression of molecules like glycoprotein IIbIIIa and P selectin on their surface, furthermore produce an increased amount of platelet factor 4 and thromoboxane A2 and B2 [261-264]. Nevertheless, in our study indices of platelet size, like MPV and PDW were not increased in patients with HPR.

Previous investigations have found conflicting results also regarding the association of platelet reactivity and platelet size. Kim et al. have found that large platelets (with high MPV and PDW) were associated with HPR after treatment with aspirin and clopidogrel in patients undergoing PCI, although no correlation with clinical outcomes was observed [265]. Asher et al. in a series of 276 ACS patients have shown that increased MPV could predict clopidogrel non-responsiveness [266]. Conversely, data from De Luca et al. are in agreement with our findings. In their study of 50 patients not taking any antiplatelet therapy evaluated with the Platelet Function Assay-100 (PFA-100 System; Dade Behring, Miami, Flo), no relationship between MPV and platelet aggregation was found [255]. A lack of correlation between MPV and platelet reactivity was also shown in the same study among 255 patients treated with aspirin and evaluated by the PFA-100 and by the Multiplate analyzer (Dynabyte, Munich, Germany) [255]. Similar data were obtained in a cohort of 50 diabetic patients not receiving antiplatelet drug: there was no significant relationship between MPV and platelet reactivity evaluated by light transmission aggregometry (LTA). In addition,

MPV was found not related with thromoboxane B2 plasma levels and with increase of P-selectin after stimulation with U26619 (synthetic analogue of prostaglandin PGH2) [260]. In our study we included only PCI patients on dual antiplatelet therapy with aspirin and clopidogrel in order to analyze the relationship with periprocedural outcome and with platelet reactivity while on antiplatelet treatment. Identification of higher risk of PMI and/or inadequate response to traditional antiplatelet therapy is an important issue in the management of patients undergoing PCI. In the present study, we have considered all platelet indices and we have found that among patients with HPR values of MPV and PDW are not significantly different compared with patients with good response to clopidogrel. Moreover, platelet count and MPV/P ratio were significantly different in patients with HPR. Previous reports about association of platelet count with platelet reactivity are rare and have enrolled smaller population. In agreement with our study mean platelet count was found to be higher in patients with clopidogrel non-responsiveness tested by Multiplate analyzer [267]. Similarly, platelet count was found to be predictive of aspirin resistance [268]. To our knowledge none of these studies evaluated the association between MPV/P ratio and platelet reactivity. It is known that an inverse relationship between MPV and platelet count exists. MPV/P ratio was proposed to be superior to MPV alone in predicting long-term mortality after NSTEMI [251]. Our data confirm this inverse relationship and show that MPV/P ratio is lower in patients with HPR, reflecting the higher platelet count observed in these patients. In our population platelet count is not associated with PMI but it is higher in HPR patients: the mechanisms linking platelet count and platelet reactivity are still not clear. A possible explanation could be reconducted to a high platelet turnorver [268] or to a correlation with a higher inflammatory state [248]. Iijima et al. have found a

significant increase in C reactive protein (CRP) levels with the increase of tertiles of platelet count [248] and high inflammatory state was associated with worse clinical outcome [269,270]. Our study confirms that HPR is related with increased incidence of PMI, but it is still not clear the reason why MPV and PDW, which are traditionally related to enhanced platelet activity, are not associated in our population with platelet reactivity. Probably, our population of patients with coronary artery disease undergoing PCI may represent a high-risk population in which baseline levels of MPV and PDW tend to be high.

We acknowledge a number of limitations in our study. First limitation is related to the retrospective nature of this study. Furthermore, no data are available on long term follow up, which was beyond the specific aims of the present study, that it is focused on acute phase of PCI. Our study was not designed to address the molecular pathways affecting relationship between platelet indices and platelet reactivity.

In conclusion, the present study shows no significant correlation between platelet indices and the occurrence of PMI and platelet reactivity in patients undergoing PCI, confirming however that HPR is associated with an increased incidence of PMI. Thus, platelet indices alone seem to be not able to identify patients at higher risk of PMI, but use of a bedside assay for monitoring platelet reactivity remains a useful tool for periprocedural risk stratification.

6.5 Tables and Figures

Table 1. Main clinical features.

Main clinical features	Overall patients (N=502)
Age (years)	67±10
Male gender	393 (78)
Hypertension	409 (81)
Diabetes mellitus	208 (41)
Hypercholesterolemia	357 (71)
Body Mass Index	28±4
Previous Myocardial Infarction	188 (37)
Previous coronary angioplasty	209 (42)
Previous CABG	31(6)
ACS/NSTEMI	171 (34)
Left ventricular ejection fraction	55.4±7.9
Serum Creatinine (mg/dl)	0.98±0.29
Platelet count (x10^3/µl)	212.5±59.5
Mean platelet volume (fL)	10.76±0.98
Platelet distribution width (fL)	13.25±2.12
Medical treatment	
Aspirin	502 (100)
Clopidogrel	502 (100)
Statin	400 (80)
ACE/AT antagonists	381 (76)
β-blockers	196 (39)

Value are given as n(%) or mean±SD MI- myocardial infarction; PCI - percutaneous myocardial infarction; CABG – coronary artery bypass surgery; ACS/NSTEMI: acute coronary syndrome/non ST acute myocardial infarction; ACE/AT antagonists – Angiotensin Converting Enzyme inhibitors or Angiotensin-receptor antagonists

Table 2. Main procedural features

Main procedural features	Overall patients (N=502)
Multivessel coronary disease	267 (53)
Coronary vessel treated	
Left main	10 (2)
Left anterior descending artery	244 (40)
Left circumflex	168 (28)
Right coronary artery	173 (29)
Saphenous vein grafts	6 (1)
Lesion B2/C	238 (47)
Restenotic lesion	54 (11)
Multivessel intervention	99 (20)
Use of stents	472 (94)
Use of Drug Eluting Stents	161 (32)
Glycoprotein IIb/IIIa inhibitors	32 (6)
Heparin	450 (90)
Bivalirudin	52 (10)

Value are given as n (%) or mean±SD

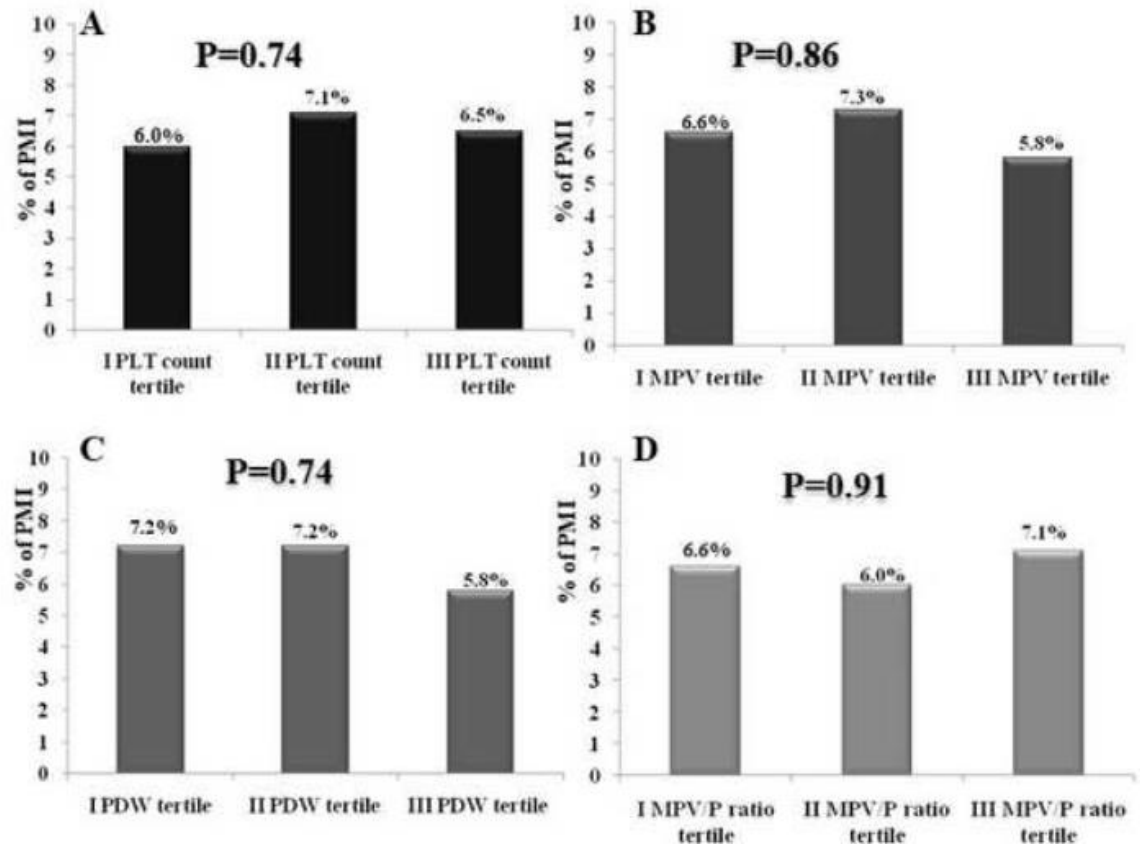

Figure 1. Incidence of periprocedural myocardial infarction (PMI) according tertiles of platelet indices.

Platelet (PLT) count (A: I tertile< 183 x10^3/µl; II tertile ≥ 183 x10^3/µl and < 228 x10^3/µl; III tertile ≥ 228 x10^3/µl), MPV (B: I tertile<10.3 fl; II tertle ≥ 10.3 fL and < 11.1; III terile ≥ 11.1 fL), PDW (C: I tertile<12.2 fl; II tertle ≥ 12.2 fL and < 13.9; III terile ≥ 13.9 fL), and MPV/P ratio (D: I tertile < 46.3; II tertile ≥ 46.3 and <59.3; III tertile ≥ 59.3).

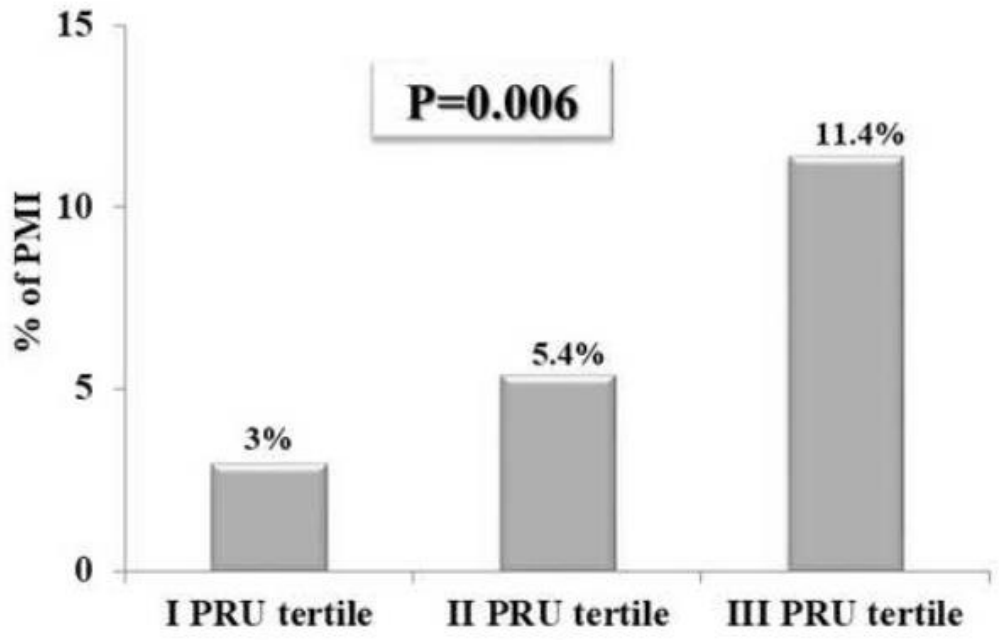

Figure 2. Incidence of periprocedural myocardial infarction (PMI) according tertiles of $P2Y_{12}$ reaction unit (PRU).

I tertile < 187 PRU; II tertile ≥ 87 and < 251 PRU; III tertile ≥ 251 PRU.

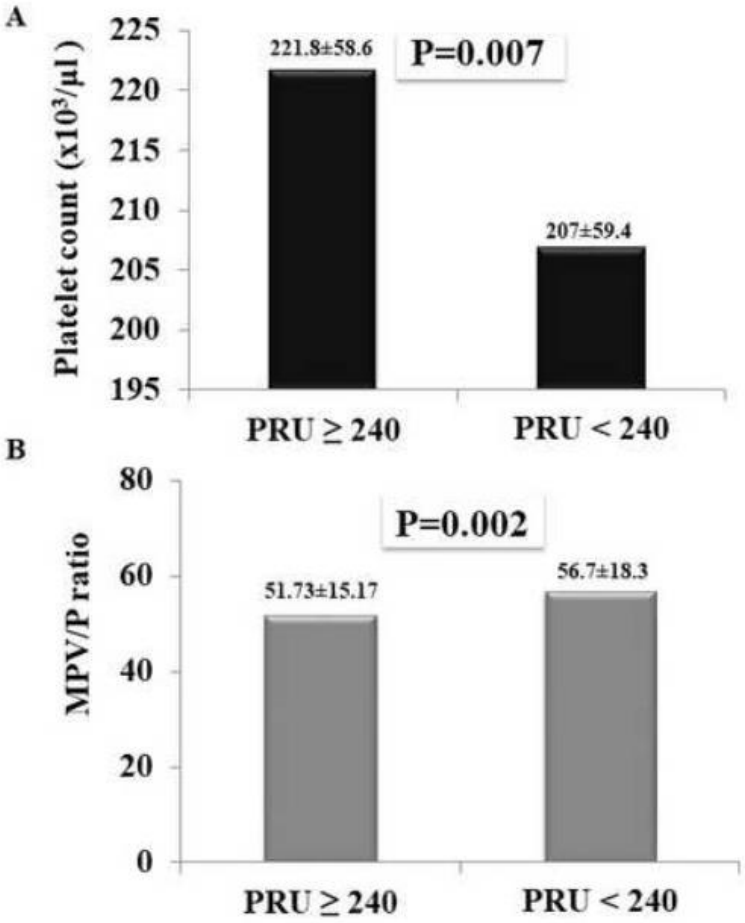

Figure 3. Values of platelet count in patients with PRU ≥ 240 (HPR) and with PRU < 240 (A). Values of MPV/P ratio in patients with PRU ≥ 240 (HPR) and with PRU < 240 (B).

CHAPTER 7

RESEARCH PROJECT N.4

Hyperleptinemia as risk factor for high platelet reactivity and cardiovascular events in patients undergoing percutaneous coronary intervention

7.1 Background

Leptin is a circulating 167-amino acid protein produced by the adipose tissue, which through a direct effect on the hypothalamus is involved in the regulation of food intake and energy balance [108, 178]. Although major role of leptin seems to be related to the balance of body weight and energy metabolism, several studies have suggested that this hormone could exert an important role also in other pathophysiological mechanisms [271]. Leptin receptors are expressed in a variety of tissues and leptin is involved in regulation of immune function, in fertility, bone formation and angiogenesis [110, 126, 137, 272-274]. Leptin was also proposed as a risk factor for development of coronary artery disease (CAD) [167]. Furthermore, the long form of the leptin receptor (*ObRb*) has been identified on platelet membrane and there are evidences suggesting a possible role in favoring platelet aggregation trough activation of this receptor [175].

Platelets activation plays a critical role in thrombosis and in clinical outcome of patient with cardiovascular disease. Antiplatelet therapy with aspirin and $P2Y_{12}$ inhibitors is a cornerstone of treatment for patients affected by stable and unstable CAD and treated with invasive procedure. Nevertheless, a wide interindividual

variability in the response to antiplatelet drugs has been reported [145] and seems to affect clinical outcome. In recent years, several evidences have suggested that platelet reactivity (PR), evaluated by point of care assays, is effective in predicting ischemic and bleeding events in patient undergoing percutaneous coronary intervention (PCI) [77,86, 91, 195] and it is possible to identify a therapeutic window of PR after $P2Y_{12}$ inhibitors administration [89].

No previous investigations have explored the possible association of leptin plasma levels and platelet reactivity in the setting of PCI. Therefore, the aim of present study was to investigate the role of leptin plasma levels as risk factor for high platelet reactivity (HPR) and for occurrence of cardiovascular events in patients undergoing PCI.

7.2 Methods

This is a prospective study enrolling consecutive patients with stable angina or non-ST segment elevation acute coronary syndrome (NSTE-ACS) undergoing PCI at Campus Bio-Medico University from March 2009 to August 2009. The design of the study is illustrated in Figure 1. All patients were given aspirin (100 mg/day) and received either a 600 mg clopidogrel loading dose (at least 6 h before PCI) or continued clopidogrel treatment if they were on clopidogrel 75 mg/day therapy for at least 5 days. Exclusion criteria were: primary PCI for ST-segment elevation myocardial infarction, upstream use of glycoprotein IIb/IIIa inhibitors, platelet count <70 $x10^3$/µl, use of prasugrel or ticagrelor, need for oral anticoagulant therapy, left ventricular ejection fraction <30%, renal failure with creatinine glomerular filtration rate <30

ml/min/1.73m^2, history of systemic inflammatory or autoimmune disease, hemoproliferative disorders and neoplasms.

Interventions were performed using a standard technique. Procedural success was defined as postprocedure Thrombolysis In Myocardial Infarction grade 3 flow, with decrease of stenosis to <30% residual narrowing by quantitative coronary angiographic analysis. A non-ionic, low-osmolarity (915 mOsm/kg) iodinated contrast agent (iobitridol, Xenetix, Guerbet, RoissyCdGCedex, France) was used. PCI procedures were performed using the femoral approach after administration of weight-adjusted intravenous unfractionated heparin (70 IU/kg body weight) or bivalirudin (bolus 0.75 mg/kg, followed by intra-procedural infusion adjusted according to renal function). Periprocedural use of glycoprotein IIb/IIIa inhibitors was only in bailout following operator discretion. After the procedure, aspirin (100 mg/day) was continued indefinitely, whereas clopidogrel (75 mg/day) was administered for at least 1 month (12 months in patients treated for NSTE-ACS or receiving a drug eluting stent). This study complied with the Declaration of Helsinki and was approved by the local ethics committee, with all patients giving written informed consent.

Blood samples for assessment of leptin plasma levels were collected before PCI in tripotassium EDTA tubes. Blood samples were immediately refrigerated at 4°C until processing within 2 hours of collection. The quantitative analysis was performed by ELISA (Leptina Sandwich - ELISA Standard Curve). A value of leptin plasma levels ≥ 14 ng/ml was considered as cut-off to define hyperleptinemic patients [193].

Platelet reactivity was assessed on blood samples drawn from the arterial sheath, and platelet reactivity was immediately measured in the catheterization

laboratory before PCI using the VerifyNow $P2Y_{12}$ assay (Accriva Diagnostics, San Diego, CA, USA), which is a rapid cartridge-based assay specifically measuring effects of clopidogrel on the platelet $P2Y_{12}$ receptor. Technical details of the assay have been previously described [198]. Results are expressed as $P2Y_{12}$ reactivity units (PRU). Patients were divided in three groups based on PRU values: low platelet reactivity (LPR) for PRU $\leq$178; normal platelet reactivity (NPR) for PRU between 178 and 239; high platelet reactivity (HPR) for PRU $\geq$ 239 [89].

Blood samples were also drawn in all patients before PCI and at 8 and 24 h after PCI for measurement of creatine kinase-MB (CK-MB) and troponin I (TnI) levels. Additional samples were obtained to measure myocardial necrosis markers if patients after PCI developed symptoms suggestive of myocardial ischemia, as per standard clinical practice in our institution. Measurements were performed using the Access 2 immunochemiluminometric assay (Beckman Coulter, Fullerton, California). Normal limits were set at 3.6 ng/mL for CK-MB and at 0.06 ng/mL for TnI.

All patients were followed with office visit or telephone interview every 12 months for up 8 years.

Primary endpoint was evaluation of leptin plasma levels according group of platelet reactivity. Secondary endpoints were incidence of periprocedural myocardial infarction (PMI) and incidence of MACE (cardiovascular death, MI and target lesion revascularization) at long-term follow up.

PMI was defined according to the Joint ESC/ACCF/AHA/WHF task force consensus statement on the redefinition of MI for clinical trials on coronary intervention [257]. Myocardial injury was defined as a TnI level elevation >5 x 99th

percentile URL within 48 hours of PCI (in patients with normal baseline values) or an increase in TnI levels of >20% in patients with elevated but stable or falling baseline levels.

Statystical Analysis

Categorical variables are reported as frequencies and percentages. Proportions were compared by Fisher's exact test when the expected frequency was <5, or the Yates-corrected Chi-square test otherwise. Continuous variables are reported as mean±SD. Normal distribution was tested by the Kolmogorov-Smirnov test. Continuous variables were compared by two tailed t test for normally distributed values; otherwise the Mann–Whitney U test was used. In the case of multiple groups, continuous variables were compared by One-way ANOVA. Event analyses were displayed using Kaplan-Meier plot and were compared with log-rank test. A cox model for MACE was performed, correcting for those clinical variables that were significantly different among the groups: sex, body mass index (BMI) and hypertension. A P value <0.05 was considered statistically significant. Statistical analysis was performed using the SPSS 16.0 release software (SPSS, Inc., Chicago, Illinois).

7.3 Results

A total of 212 patients were screened during the study period for enrollment, 57 were excluded for the presence of exclusion criteria. The population of the study was composed of 155 patients. Patients with baseline leptin levels ≥14 ng/ml were 33. In Table 1 are reported principal clinical features of the study population according the

level of leptin. Hyperleptinemic patients were more frequently female, with hypertension and with significantly higher medium value of BMI. No significant differences were identified in principal procedural features as reported in Table 2.

Regarding the primary endpoint, leptin plasma levels were significantly different among groups of PR (P=0.047). In particular leptin levels were significantly higher in patients with HPR (12.61±16.58 ng/ml) compared to LPR (7.83±8.87 ng/ml, P=0.044) and NPR (7.04±7.03 ng/ml, P=0.01) group (Figure n.2). Nonsignificant difference was identified between leptin plasma levels in LPR and NPR group (P=0.66, Figure 2).

Incidence of PMI in general population of the study was 8% (13 patients). Rate of PMI was higher, even not significant, among hyperleptinemic patients compared with the other group (15.1% vs 6.5%, P=0.22, Figure 3). Incidence of myocardial injury was 39% (13 patients) in hyperleptinemic group compared to 26% (32 patients) in group with leptin levels < 14 ng/ml (P=0.20).

Clinical long-term follow-up was complete in 140 patients (90.3%). Incidence of MACE were 40% (12 patients) in hyperleptinemic group and 21% (23 patients) in the normoleptinemic group. A landmark analysis of the Kaplan-Meier estimates of clinical outcomes during 8 year-follow up is provided in Figure 4. Patients with hyperleptinemia experienced a significantly higher rate of MACE compared with those in the normleptinemic group (HR 2.3; CI 95% 1.14-4.6, P=0.02). These results remained substantially unchanged after adjusting for BMI, hypertension and sex (Table 3).

7.4 Discussion

Major findings of the present study are: 1) patients with HPR show higher leptin plasma levels; 2) hyperleptinemia is associated with an increased incidence of periprocedural myocardial damage and with higher rate of cardiovascular events a long-term follow-up after PCI.

Leptin is a hormone produced by fat tissue, whose main task is to maintain homeostasis of body weight by regulating the sense of hunger and increasing energy consumption [105]. However, multiple evidences suggest that hyperleptinemia may play a role in the development of atherosclerosis, thus this adipokine is considered a cardiovascular risk factor [127]. In addition, there is a growing number of data that, also demonstrate that this hormone is able to enhance both in vitro and in vivo ADP-mediated platelet aggregation in a dose-dependent manner, making platelets particularly responsive even at reduced concentrations of such agonist, which is normally inhibited by clopidogrel [176-179, 180]. Nevertheless, to the best of our knowledge this is the first study assessing the association of HPR with leptin levels in vivo and in the context of PCI. In particular in our population, PCI patients with HPR after treatment with clopidogrel showed higher levels of leptin compared with patients with NPR and LPR, while no difference in leptin levels were identified in patients of these latter groups. This is also the first study to evaluate the association of leptin levels and PR measured by a point-of-care assay. The prognostic value of PR was widely demonstrated in previous studies and growing evidences are available on the possibility to identify a therapeutic window of PR after treatment with $P2Y_{12}$ inhibitors [77, 86, 89, 91, 195]. However, none of the available studies has shown that alone the achievement of the therapeutic window is able to improve the clinical outcomes of

patients undergoing PCI [95,97,102]. In addition, variability in the response to antiplatelet drugs (in particular clopidogrel) is the result of numerous factors, most of which are not yet fully recognized [53,59]. In this context mechanisms linking obesity and platelet reactivity are lacking. The results of the present study can be explained in the light of the enhancement of platelet aggregation exerted by leptin, which could act by promoting the binding of $P2Y_{12}$ receptor to ADP, rather than clopidogrel, thus inhibiting its antiplatelet action. Possible mechanism through which leptin can exert its role in platelet activation is proposed in an elegant work by Corsonello et al.: in this model leptin binds its receptor, causing the activation of the intracellular pathway JAK-STAT and phospholipase C, resulting in hydrolysis of phosphatidyl inositol 4-5-diphosphate and formation of phosphatidyl inositol triphosphate (IP3) and diacylglycerol (DAG). The DAG activates protein kinase C, while IP3 mobilizes intra-platelets calcium deposits. Protein kinase C, phospholipase A2 and IP3 act synergistically to induce platelet activation, by secretion of alpha and electron granule contents, thromboxane A2 formation and by expression on the platelet surface of receptors mediate aggregation [178]. The ability of leptin to induce platelet aggregation in the presence of ADP improves with the increase in circulating levels of this hormone [175, 178]. Previous experimental studies have shown that concentrations of this adipokine at 10 ng/ml are able to enhance platelet aggregation in the mice, but not in humans, whereas the effect on human platelets is observed at higher concentrations (including 30 and 100 ng/ml) [176]. Indeed, in our study patients with HPR showed average leptin values around 13 ng/ml. PCI with a stent implantation induces a damage of the endothelium, resulting in the exposure of the atherosclerotic plaque and the connective tissue; arterial wall lesion triggers the coagulation cascade

and favors the adhesion of platelets to the exposed collagen, with subsequent aggregation. Therefore, it is reasonable to speculate that in such pro-thrombotic context, even leptin average levels not so high, as those identified in our population, may strongly increase platelet reactivity. In our population leptin average levels identified in patients with HPR were similar to the cut-off of another in vivo study on humans, in which leptin values ≥ 14 ng/ml were associated with a higher incidence of failure of thrombolytic streptochinase therapy in patients with STEMI [193]. We have applied the cut-off from this substudy of GUSTO-1 for the anlysis of clinical outcome of our population, because we have needed of a cut-off derived from a population of patients with CAD, even if this was derived in the setting of ACS.

Clinical results of our study show that hyperleptinemic patients had an increase in rate of PMI, even this result is not significant probably due to the small size of our population. More interesting are data on long-term follow up, in which hyperleptinemia was identified as predictor of MACE, even after adjustment for BMI, hyperntension and sex. These latter were the variables significantly different in our population, although the differences observed are in line with previous reports. Leptin levels tend to be higher in female patients [118] and obviously in patients with higher BMI [189] and with other cardiovascular risk factors [151]. The association of high leptin levels with a worse clinical outcome was previously assessed in other studies. In the WOSCOPS (West of Scotland Coronary Prevention Study) study, hyperleptinemia predicts acute cardiovascular events (death, myocardial infarction, and new revascularization) during 5-year follow-up [167]. Wolk et al. examined the relationship between leptin and cardiovascular events in 382 non-diabetic patients with angiographic evidence of significant CAD during a 4-year follow-up period. It has

been shown that baseline hyperleptinemia is associated with an increased incidence of the combined end-point (CV death, new MI, cerebrovascular events and need for new revascularization) [168]. In addition, a high plasma leptin level was associated with occurrence of restenosis after PCI [146]. Finally, Dubey and coll. not only have shown that patients with acute coronary syndrome had plasma levels higher than stable patients [170], but have also shown that leptin concentrations were significantly higher in patients with complex CAD [171]. Results of our study are in line with those evidences and confirm the association of hyperleptinemia with an increased rate of cardiovascular events events at a very long-term follow up. Therefore, the presence of high leptin levels could be an adjunctive factor to take in account in order to assess the risk profile of patients undergoing PCI. Risk stratification in the context of PCI, and in general in CAD, represent a fundamental tool to guide the treatment strategies of these patients, from the cath-lab to the secondary prevention's therapy.

We acknowledge a number of limitation of our study. First limitation is related to the prospective nature of the study. The small size of our population is another issue to be considered especially for evaluation of periprocedural outcome. No repeated measures of leptin levels are available, as the aim of our study was to assess the leptin levels at the same time of PR evaluation. Our study was not designed to address the molecular pathways affecting relationship between leptin levels and platelet reactivity.

In conclusion our study suggests that high levels of leptin are associated with HPR and with a worse clinical outcome in patients treated with clopidogrel undergoing PCI. Further studies are needed to better define the pathophysiological pathways underlying this association and to evaluate the possible efficacy of targeting leptin as a measure of effectiveness of secondary prevention treatments in patients with CAD.

7.5 Tables and Figures.

Table 1. Main clinical features.

Main clinical features	Overall patients (N=155)	Leptin <14 ng/ml (N=122)	Leptin ≥ 14 ng/ml (N=33)	P
Age (years)	66±9	66±10	68±9	0.32
Male gender	124 (80)	106 (87)	18 (55)	<0.01
Hypertension	136 (88)	104 (85)	32 (97)	<0.01
Diabetes mellitus	52 (33)	43 (35)	9 (27)	0.51
Hypercholesterolemia	127 (82)	100 (82)	27 (82)	0.81
Current Smokers	41 (26)	33 (27)	8 (24)	0.91
Body Mass Index	28±5	27±4	31±7	0.003
Previous MI	55 (35)	47 (38)	8 (24)	0.18
Previous PCI	62 (40)	52 (43)	10 (30)	0.28
Previous CABG	13 (8)	12 (10)	1 (3)	0.37
ACS/NSTEMI	38 (25)	31 (25)	7 (21)	0.79
LVEF (%)	55.8±7.9	55.6±7.8	56.3±8.4	0.96
Serum Creatinine (mg/dl)	0.94±0.25	0.94±0.21	0.94±0.35	0.68
CRP (ng/ml)	4.53±6.33	4.72±6.7	3.82±4.78	0.47
Medical treatment				
Aspirin	155 (100)	122 (100)	33 (100)	-
Clopidogrel	155 (100)	122 (100)	33 (100)	-
Statin	114 (74)	88 (72)	26 (79)	0.58
ACE/AT antagonists	120 (77)	95 (78)	25 (76)	0.98
β-blockers	66 (43)	49 (40)	17 (52)	0.33
Insulin	10 (6)	7 (6)	3 (9)	0.76
PPI	97 (78)	79 (65)	18 (55)	0.38

Table 2. Main procedural features

Main procedural features	Overall patients (N=155)	Leptin <14 ng/ml (N=122)	Leptin ≥ 14 ng/ml (N=33)	P
Multivessel coronary disease	73 (48)	59 (48)	14 (42)	0.68
Coronary vessel treated				
Left main	2 (1)	2 (1)	0 (0)	0.86
Left anterior descending artery	73 (42)	56 (40)	17 (47)	0.47
Left circumflex	41 (23)	32 (23)	9 (25)	0.89
Right coronary artery	55 (31)	45 (32)	10 (28)	0.84
Saphenous vein grafts	5 (3)	5 (3)	0 (0)	0.57
Lesion B2/C	107 (61)	88 (60)	19 (53)	0.47
Restenotic lesion	29 (19)	22 (18)	7 (21)	0.87
Multivessel intervention	21 (64)	18 (15)	3 (9)	0.58
Use of stents	144 (93)	114 (93)	30 (91)	0.9
Use of Drug Eluting Stents	53 (34)	46 (38)	7 (21)	0.12
Glycoprotein IIb/IIIa inhibitors	4 (3)	2 (2)	2 (6)	0.42
Heparin	136 (88)	108 (89)	28 (85)	0.79
Bivalirudin	19 (12)	14 (11)	5 (15)	0.79

Value are given as n (%) or mean±SD

Table 3. Cox model for incidence of MACE correcting for BMI, Sex and hypertension.

	HR	IC 95%		P
Hyperleptinemia	2.7	1.2	6.3	0.016
BMI	0.9	0.9	1.06	0.63
Hypertension	1.5	0.46	5.1	0.49
Sex (Male)	1.6	0.7	3.9	0.29

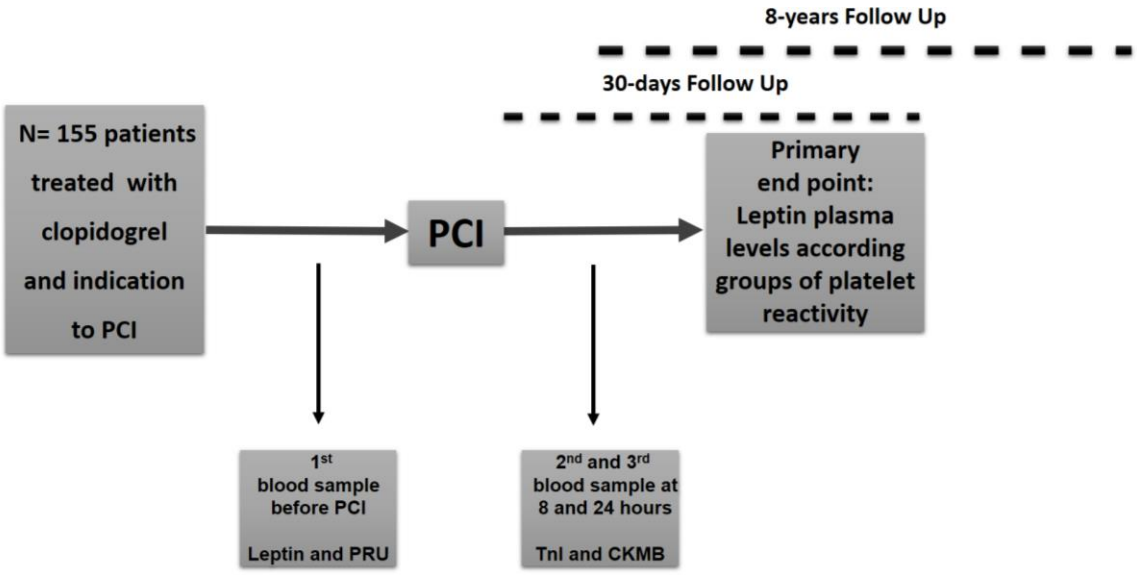

Figure 1. Study Design.

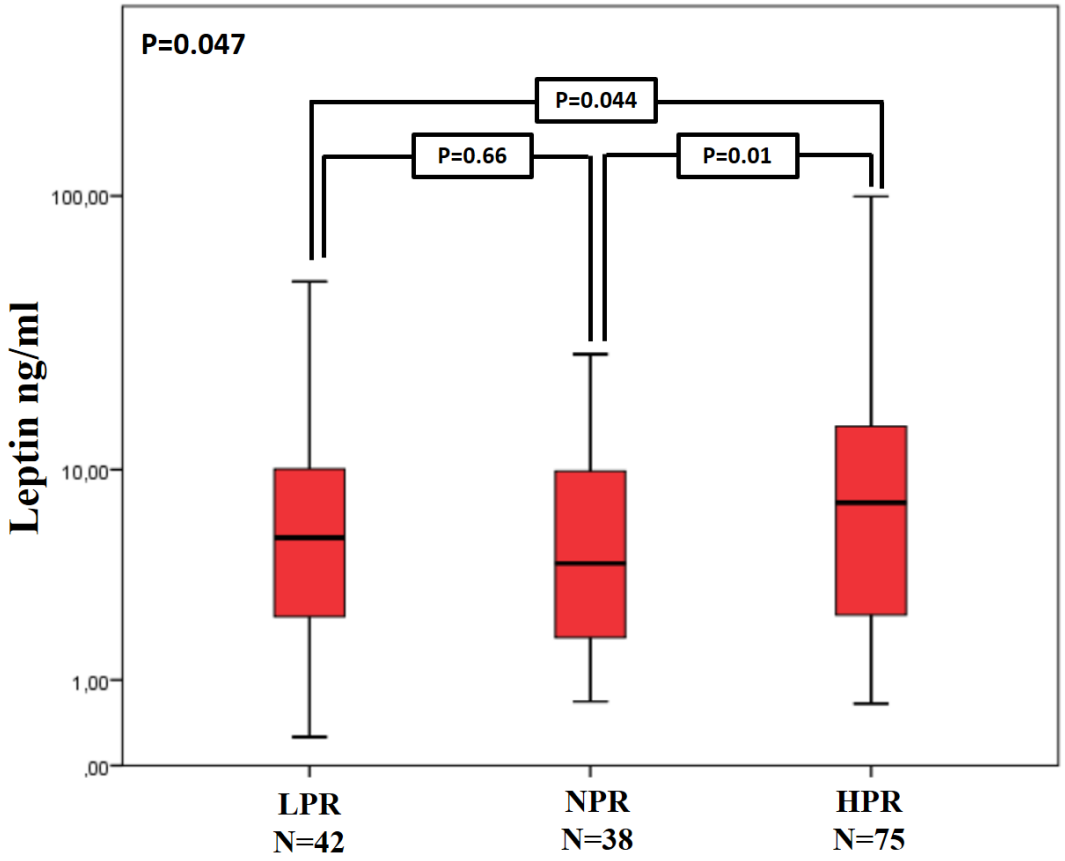

Figure 2. Leptin levels according group of PR.

Low platelet reactivity (LPR) was defined by PRU ≤178; normal platelet reactivity (NPR) for PRU between 178 and 239; high platelet reactivity (HPR) for PRU ≥ 239.

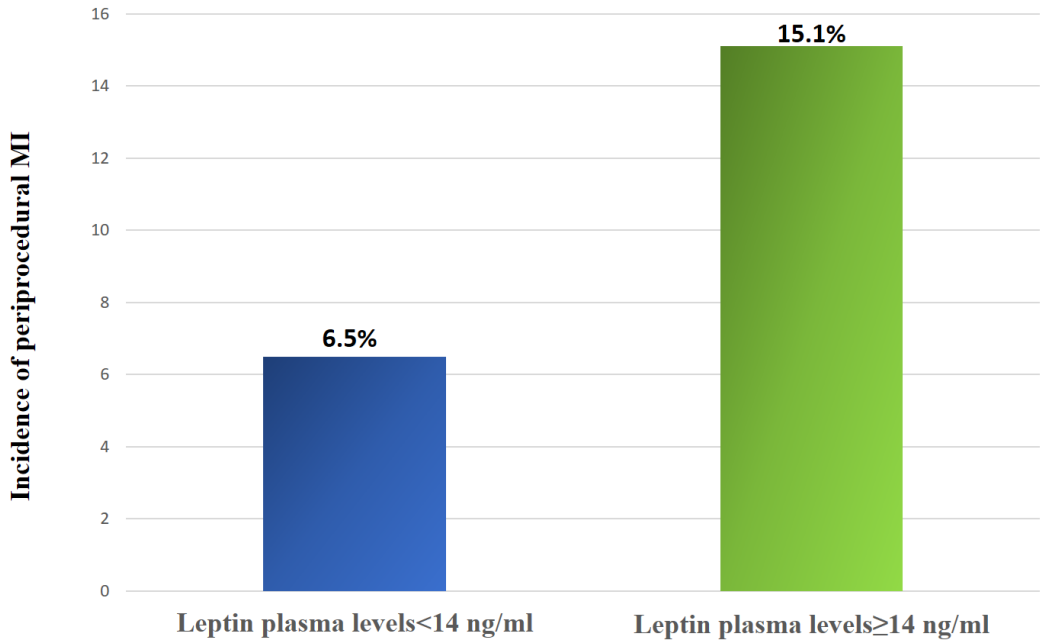

Figure 3. Incidence of PMI according leptin levels.

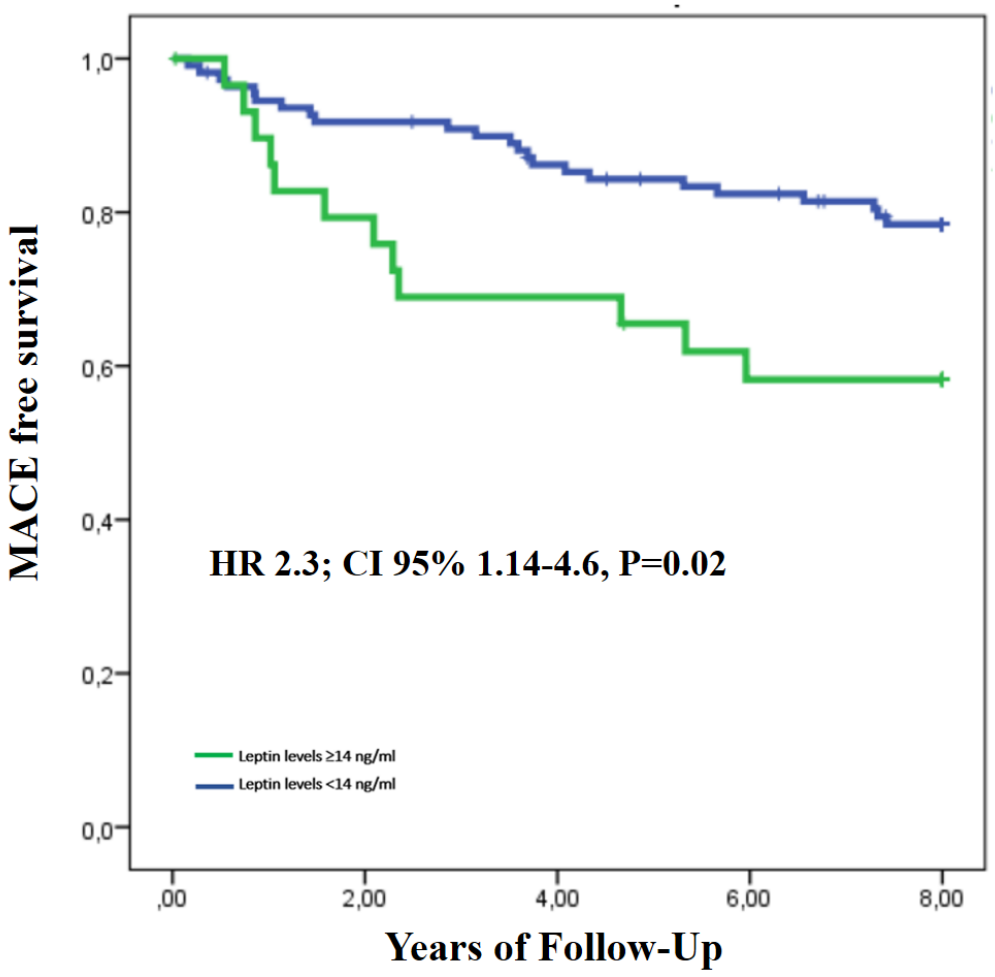

Figure 4. Kaplan-Meier estimates of clinical outcomes during 8-year follow-up.

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