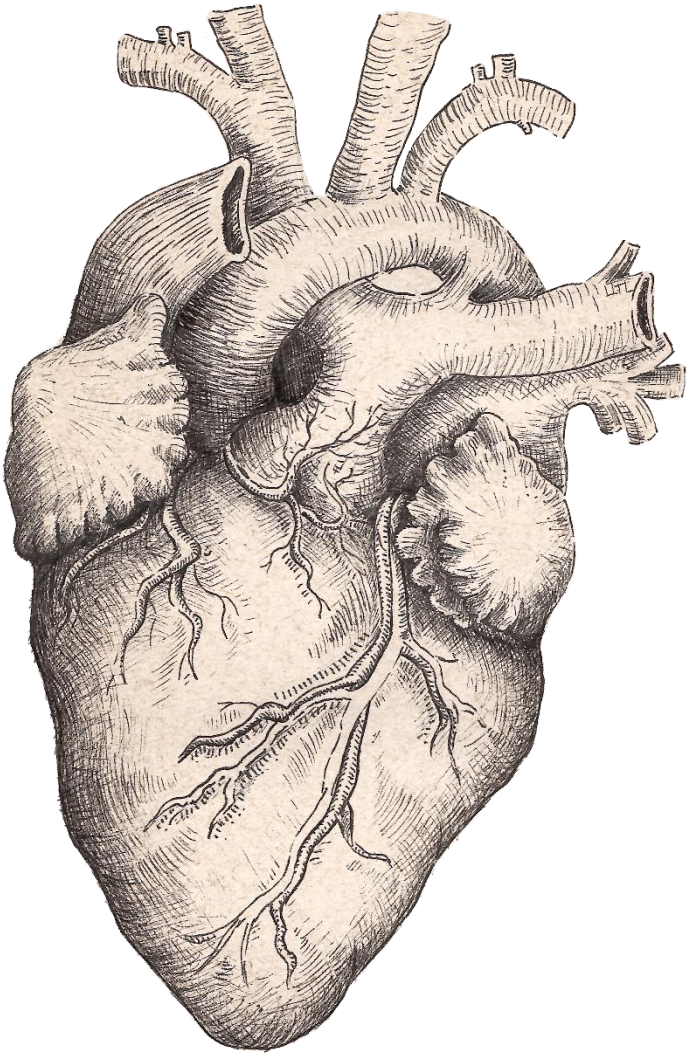
Implications of genetics and major risk factors on platelet morphology, platelet aggregation and their relationship with coronary atherosclerosis



“*Anatomia del Cuore*”, created by Giulia Fontana, MD

Implications of genetics and major risk factors on platelet morphology, platelet aggregation and their relationship with coronary atherosclerosis

DISSERTATION

to obtain the degree of Doctor at the Maastricht University,
on the authority of the Rector Magnificus,
Prof. dr. Pamela Habibović
in accordance with the decision of the Board of Deans,
to be defended in public
on Friday 15 September 2023, at 13:00 hours

by

Matteo Nardin

Supervisors:

Prof. dr. A.W.J. van ’t Hof

Prof. dr. J.M. ten Berg

Co-supervisor:

Prof. G. De Luca, University of Messina, Italy

Members of the Assessment Committee:

Prof. dr. H.J.G.M. Crijns (Chair)

Prof. dr. E. Kedhi, Isala Zwolle

Prof. dr. H. ten Cate

Prof. dr. R.J.M. van Geuns, Radboud University Medical Center Nijmegen

“Speak little, listen a lot,

And look at the goal of what you do”

Chapterhouse, Chiaravalle Abbey, Fiastra, Italy

Contents

Introduction

Several improvements have been achieved from the introduction of percutaneous coronary intervention (PCI) in 1977 to date, where innovations in materials and technologies, together with ameliorations in the new generation of drug eluting stents, have contributed to making PCI an important cornerstone among the options for coronary artery disease treatment (1,2).

Alongside the advances in percutaneous treatment that have allowed to include more complex patients among candidates to PCI, the crucial function exercised by platelets in atherothrombotic disease cannot be prevented. These subcellular fragments are held responsible for the majority of hemostatic processes and for the pathogenesis of acute coronary syndromes (ACS) and thrombotic events after coronary stent implantation (3).

A better understanding of the pathophysiology involved platelet homeostasis would potentially lead to the recognition of markers and targets suitable for pharmacological strategies, which, in turn, would allow adequate platelet inhibition and, thus, a reduction in ischemic events (4).

Moreover, the identification of humoral and genetic factors enhancing platelet reactivity in order to optimize antiplatelet therapies has required growing attention.

Platelet atherosclerosis

Platelets are essential in the hemostatic processes and in the repair of the endothelium after injuries.

However, they have been recognized as playing a decisive role in the development of acute coronary syndrome and in the promotion and extension of atherosclerotic disease. Actually, atherosclerosis is a chronic inflammatory process (5), and inflammation is the common element in endothelial dysfunction and platelet activation.

On the one hand, platelets adhere to the vessel walls characterized by impaired endothelial homeostasis and contribute to chronic atherosclerotic lesions. The acceleration of atherogenesis is mediated by activated platelets releasing adhesive ligands, including P-selectin, which favor the platelet-endothelium interactions. The following actors coming on stage are immunity cells, recruited as a result of the release of chemotaxis factors (**figure 1**).

On the other hand, acute rupture of the luminal surface of vulnerable plaque leads to an unbalanced ratio between the thrombotic and the hemostatic process, where conspicuous platelet and coagulative cascade activation determines the formation of an intraluminal thrombus.

Since the key role of platelets in atherosclerosis has been established, the opportunity of inhibiting platelet activation to reduce clinical sequelae is one of the most desirable therapeutic options. The availability of elements to properly identify patients exposed to increased thrombotic risk will help to select the best antithrombotic strategies, in terms of drug components and duration.

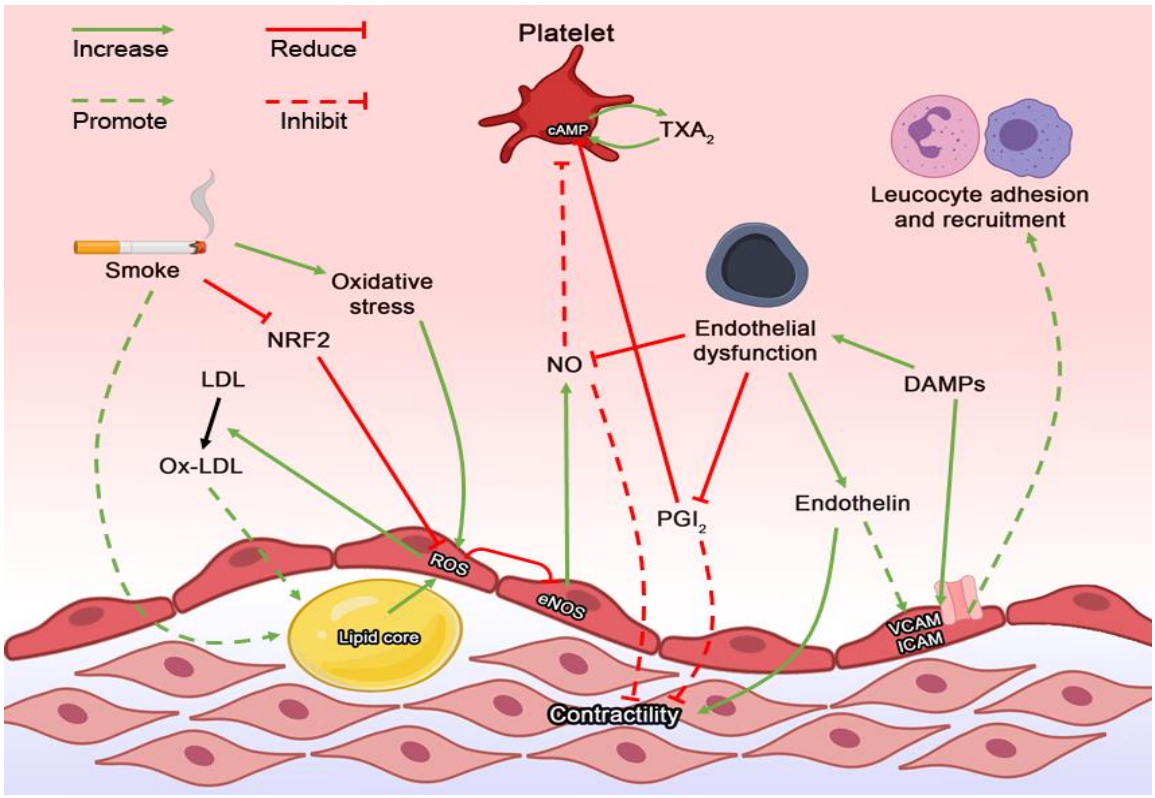


Figure 1. Endothelium dysfunction. The figure reports the key mechanisms involved in endothelium homeostasis and their impairment in stress conditions, mainly driven by cigarette smoke. LDL= low-density lipoprotein cholesterol; Ox-LDL= oxidized low-density lipoprotein cholesterol; NRF2= nuclear factor-erythroid 2-releated factor 2; NO= nitric oxide; cAMP= cyclic adenosine monophosphate; TXA_2= thromboxane A2; DAMPs= damage-associated molecular patterns; PGI_2= prostacyclin; ROS= reactive oxygen species; eNOS= endothelial nitric oxide synthase; V-CAM= Vascular adhesion molecule; I-CAM= Intercellular adhesion molecule.

Platelet morphologic parameters linked to atherosclerosis

One of the first markers of platelet activation investigated was the mean platelet volume (MPV), which is obtained by a routine full blood count(6). Higher MPV has been suggested to reflect an increased platelet production rate and more activation: larger platelets were shown to contain more α-granules, which release amplification substances for platelet aggregation (7,8).

Conflicting results have been reported on the role of MPV in the development of CAD: Murat et. al found MPV to be an independent predictor of the severity of CAD among 395

patients with acute coronary syndrome (ACS) (9), while Halbmayer showed no difference in terms of MPV between healthy subjects and patients with CAD (10). A potential link to a pro-inflammatory and pro-thrombotic clinical condition such as diabetes mellitus and metabolic syndrome has been described for MPV, although conclusive findings were not achieved.

Similarly, the fraction of immature platelets (IPF) has been investigated as a marker of platelet activation and cardiovascular risk: the higher RNA content and number of granules of immature platelets, also called reticulated platelets, have been related to enhanced aggregability and reduced response to antiplatelet agents (11). However, a direct role of IPF as marker of atherosclerotic disease and extension was not confirmed, suggesting IPF as marker of platelet turnover promoted by an inflammatory environment rather than CAD progression (12).

Definition of "High on Treatment Platelet Reactivity"

Increasing accessibility to platelet function tests has led to the investigation of platelet reactivity in PCI patients, aiming to promptly recognize subjects more prone to experience recurrent ischemic and thrombotic events, despite dual antiplatelet therapy (DAPT): they are referred to as poor responders and were found to present enhanced platelet reactivity; this condition was called "High on Treatment Residual Platelet Reactivity" (HTPR or HRPR) (13).

Besides the clinical definition of HTPR, in case of thrombotic events, several platelet assays have been developed to identify patients with enhanced platelet reactivity. However, while all the different whole blood "bedside" tests were in agreement with the prognostic impact of HTPR (14), the use of different cut-off values for the definition of

antiplatelet agents' resistance have led to much uncertainty in adequately identifying the prevalence and the predictors of enhanced platelet reactivity (15).

Indeed, the first attempts in of tailoring DAPT, according to the results of aggregation assays, have failed to provide any clinical benefits. In the GRAVITAS trial (16), the use of high-dose clopidogrel compared with standard-dose clopidogrel among PCI patients displaying HTPR, did not reduce the occurrence of cardiovascular death, myocardial infarction or stent thrombosis, although it slightly increased the risk of bleeding. Similar results were obtained by Montalescot et. al in the ARCTIC trial (17).

One of the most recent platelet function tests using the impendence whole aggregometry, named "Multiplate Electrode Aggregometry", has emerged as the aggregation test best predicting the risk of MACE in a large cohort of more than seven thousand consecutive patients undergoing PCI (18). Hopeful findings come from the MADONNA trial which revealed that adopting a strategy that tailored antiplatelet therapy, according to the HTPR assessed through the multiplate aggregometry, resulted in lower ischemic events whose benefits tended to increase over time (19), and the TROPICAL-ACS trial found the platelet function test tailoring therapy non-inferior to the conventional approach in more than 2500 patients presenting with an ACS (20).

Clinical and genetic determinants of platelet reactivity

Despite promising results, overall evidence does not support routine assessment of platelet reactivity to guide the management of DAPT in PCI patients, limiting that strategy to selected high risk of patients (21). In fact, suboptimal results have been described in the higher-risk population, including patients with diabetes mellitus, obesity and hyperuricemia (22–24).

Diabetic patients display a higher pro-thrombotic status, as the inflammatory background sustained by hyperglycemia favors the production of oxygen radicals and other cytokines that promote endothelial dysfunction and platelet aggregation (25,26). Moreover, the metabolic impairment observed among obese patients alters the pharmacokinetic and pharmacodynamic parameters of drugs, leading to the need for potential dose adjustment or therapy modifications (27).

On the metabolic side, awareness has been raised as to the increase of serum uric acid observed in patients on DAPT composed by ticagrelor. High levels of uricemia are known to determine a pro-oxidant setting in the blood with detrimental consequences on endothelial homeostasis and, hypothetically, on platelet aggregation (28).

Increasing attention has emerged in relation to the impact of chronic drug therapy on platelet aggregation: the proven benefits of some drug classes as renin angiotensin system inhibitors and statin might be partially counterbalanced by increased platelet reactivity due to pharmacodynamic side-effects or specific drug-drug interaction (29,30).

Moreover, the impact of genetics has been explored in the last years, attempting to recognize patients with impaired platelet inhibition despite the optimization of modifiable risk factors. Consistent evidence exists in relation to cytochrome polymorphisms and their impact on platelet reactivity on DAPT, although potential new genes are under investigation for their potential involvement in HTPR (31,32).

This thesis

This thesis addresses several different aspects of the daily clinical practice in the management and decision-making process involving antiplatelet therapy and patients with stable coronary artery disease or acute coronary syndromes, treated with percutaneous coronary intervention, and the identification of factors contributing to the increased atherothrombotic risk profile.

Part 1 is focused on platelet homeostasis and on the main mechanisms sustaining the thrombotic process. The large number of scientific reports of the last years reflect the attention deserved by platelets and the efforts made to understand and identify markers of enhanced platelet reactivity.

Part 2 is centered on morphological parameters that have been addressed as indirect markers of platelet activation and their relationship with coronary artery disease. **Chapter 2** investigated the relationship between the immature platelet fraction levels and the prevalence and extent of coronary artery disease in a large a cohort of consecutive patients undergoing coronary angiography. **Chapter 3** explored the potential impact of active cigarette smoking on immature platelet fraction with potential repercussions on atherosclerosis progression. **Chapter 4** addressed the role played by the metabolic syndrome on mean platelet volume and the impact with angiographically defined coronary artery disease,

Part 3 is dedicated to the assessment of platelet reactivity and the identification of factors affecting it. **Chapter 5** provides insights into the role of genetics in the determination of platelet reactivity in ticagrelor treated patients. **Chapter 6** reports variations of serum uric acid and the impact of platelet aggregation in patients under dual antiplatelet therapy. Remaining on the metabolic field, **Chapter 7** shows the impact of Body Mass Index on platelet reactivity in patients undergoing percutaneous coronary intervention. **Chapter 8**

presents the debated relationship between renin angiotensin system inhibitors and homocysteine concentrations, and the effects on platelet aggregation. **Chapter 9** explores the impact of atorvastatin or rosuvastatin co-administration on platelet reactivity among patients with dual antiplatelet therapy.

References

1. Sheth SD, Giugliano RP. Coronary artery stents: advances in technology. Hosp Pract (1995). 2014 Oct;42(4):83–91.
2. Collet J-P, Thiele H, Barbato E, Barthélémy O, Bauersachs J, Bhatt DL, et al. 2020 ESC Guidelines for the management of acute coronary syndromes in patients presenting without persistent ST-segment elevation. Eur Heart J. 2021 Apr 7;42(14):1289–367.
3. Davì G, Patrono C. Platelet activation and atherothrombosis. N Engl J Med. 2007 Dec 13;357(24):2482–94.
4. Franchi F, Angiolillo DJ. Novel antiplatelet agents in acute coronary syndrome. Nat Rev Cardiol. 2015 Jan;12(1):30–47.
5. Ross R. Atherosclerosis--an inflammatory disease. N Engl J Med. 1999 Jan 14;340(2):115–26.
6. Badimon L, Vilahur G. Thrombosis formation on atherosclerotic lesions and plaque rupture. J Intern Med. 2014 Dec;276(6):618–32.
7. Hekimsoy Z, Payzin B, Ornek T, Kandoğan G. Mean platelet volume in Type 2 diabetic patients. J Diabetes Complications. 18(3):173–6.
8. Briggs C. Quality counts: new parameters in blood cell counting. Int J Lab Hematol. 2009 Jun;31(3):277–97.
9. Murat SN, Duran M, Kalay N, Gunebakmaz O, Akpek M, Doger C, et al. Relation between mean platelet volume and severity of atherosclerosis in patients with acute coronary syndromes. Angiology. 2013 Feb;64(2):131–6.
10. Halbmayer WM, Haushofer A, Radek J, Schön R, Deutsch M, Fischer M. Platelet size, fibrinogen and lipoprotein(a) in coronary heart disease. Coron Artery Dis. 1995 May;6(5):397–402.
11. Hoffmann JJML. Reticulated platelets: analytical aspects and clinical utility. Clin Chem Lab Med. 2014 Jan 1;52(8).
12. Hung J, Lam JY, Lacoste L, Letchacovski G. Cigarette smoking acutely increases platelet thrombus formation in patients with coronary artery disease taking aspirin. Circulation. 1995 Nov 1;92(9):2432–6.
13. Sambu N, Radhakrishnan A, Dent H, Calver AL, Corbett S, Gray H, et al. Personalised antiplatelet therapy in stent thrombosis: observations from the Clopidogrel Resistance in Stent Thrombosis (CREST) registry. Heart. 2012

May;98(9):706–11.

14. Stone GW, Witzenbichler B, Weisz G, Rinaldi MJ, Neumann F-J, Metzger DC, et al. Platelet reactivity and clinical outcomes after coronary artery implantation of drug-eluting stents (ADAPT-DES): a prospective multicentre registry study. Lancet. 2013 Aug 17;382(9892):614–23.
15. Gurbel PA, Becker RC, Mann KG, Steinhubl SR, Michelson AD. Platelet function monitoring in patients with coronary artery disease. J Am Coll Cardiol. 2007 Nov 6;50(19):1822–34.
16. Price MJ, Berger PB, Angiolillo DJ, Teirstein PS, Tanguay J-F, Kandzari DE, et al. Evaluation of individualized clopidogrel therapy after drug-eluting stent implantation in patients with high residual platelet reactivity: design and rationale of the GRAVITAS trial. Am Heart J. 2009 May;157(5):818–24, 824.e1.
17. Montalescot G, Rangé G, Silvain J, Bonnet J-L, Boueri Z, Barthélémy O, et al. High on-treatment platelet reactivity as a risk factor for secondary prevention after coronary stent revascularization: A landmark analysis of the ARCTIC study. Circulation. 2014 May 27;129(21):2136–43.
18. Mayer K, Bernlochner I, Braun S, Schulz S, Orban M, Morath T, et al. Aspirin treatment and outcomes after percutaneous coronary intervention: results of the ISAR-ASPI registry. J Am Coll Cardiol. 2014 Sep 2;64(9):863–71.
19. Siller-Matula JM, Gruber C, Francesconi M, Dechant C, Jilma B, Delle-Karth G, et al. The net clinical benefit of personalized antiplatelet therapy in patients undergoing percutaneous coronary intervention. Clin Sci (Lond). 2015 Jan 2;128(2):121–30.
20. Sibbing D, Aradi D, Jacobshagen C, Gross L, Trenk D, Geisler T, et al. Guided de-escalation of antiplatelet treatment in patients with acute coronary syndrome undergoing percutaneous coronary intervention (TROPICAL-ACS): a randomised, open-label, multicentre trial. Lancet. 2017 Oct 14;390(10104):1747–57.
21. Sibbing D, Aradi D, Alexopoulos D, Ten Berg J, Bhatt DL, Bonello L, et al. Updated Expert Consensus Statement on Platelet Function and Genetic Testing for Guiding P2Y12 Receptor Inhibitor Treatment in Percutaneous Coronary Intervention. JACC Cardiovasc Interv. 2019;12(16):1521–37.
22. De Luca G, Ernst N, van 't Hof AWJ, Ottervanger JP, Hoorntje JCA, Gosselink ATM, et al. Predictors and clinical implications of early reinfarction after primary

angioplasty for ST-segment elevation myocardial infarction. Am Heart J. 2006 Jun;151(6):1256–9.

23. Ohtsubo T, Rovira II, Starost MF, Liu C, Finkel T. Xanthine Oxidoreductase Is an Endogenous Regulator of Cyclooxygenase-2. Circ Res. 2004 Nov 26;95(11):1118–24
24. Cuisset T, Frere C, Quilici J, Morange P-E, Camoin L, Bali L, et al. Relationship between aspirin and clopidogrel responses in acute coronary syndrome and clinical predictors of non response. Thromb Res. 2009 Feb;123(4):597–603.
25. Fukushima Y, Daida H, Morimoto T, Kasai T, Miyauchi K, Yamagishi S, et al. Relationship between advanced glycation end products and plaque progression in patients with acute coronary syndrome: the JAPAN-ACS sub-study. Cardiovasc Diabetol. 2013 Jan 7;12:5.
26. Colwell JA, Nesto RW. The platelet in diabetes: focus on prevention of ischemic events. Diabetes Care. 2003 Jul;26(7):2181–8.
27. Mathijssen RHJ, Sparreboom A, Verweij J. Determining the optimal dose in the development of anticancer agents. Nat Rev Clin Oncol. 2014 May;11(5):272–81.
28. Barbieri L, Verdoia M, Pergolini P, Nardin M, Rolla R, Marino P, et al. Uric acid and high-residual platelet reactivity in patients treated with clopidogrel or ticagrelor. Nutr Metab Cardiovasc Dis. 2016 Jan 6;
29. Pinheiro LF, França CN, Izar MC, Barbosa SP, Bianco HT, Kasmas SH, et al. Pharmacokinetic interactions between clopidogrel and rosuvastatin: Effects on vascular protection in subjects with coronary heart disease. Int J Cardiol. 2012 Jun;158(1):125–9.
30. Jiang S, Pan M, Wu S, Venners SA, Zhong G, Hsu Y-H, et al. Elevation in Total Homocysteine Levels in Chinese Patients With Essential Hypertension Treated With Antihypertensive Benazepril. Clin Appl Thromb. 2016 Mar 30;22(2):191–8.
31. Sibbing D, Koch W, Gebhard D, Schuster T, Braun S, Stegherr J, et al. Cytochrome 2C19*17 allelic variant, platelet aggregation, bleeding events, and stent thrombosis in clopidogrel-treated patients with coronary stent placement. Circulation. 2010 Feb 2;121(4):512–8.
32. Varenhorst C, Eriksson N, Johansson Å, Barratt BJ, Hagström E, Åkerblom A, et al. Effect of genetic variations on ticagrelor plasma levels and clinical outcomes. Eur Heart J. 2015 Aug 1;36(29):1901–12.

Part 1

Platelets and Atherosclerosis

Chapter 1

Platelets and the atherosclerotic process: an overview of new markers of platelet activation and reactivity, and their implications in primary and secondary prevention

Matteo Nardin [a,b]; Monica Verdoia [c,d]; Davide Cao [a]; Simone Nardin [e]; Elvin Kedhi [f]; Gennaro Galasso [g]; Arnoud W J van 't Hof [h]; Gianluigi Condorelli [a,i]; Giuseppe De Luca [j]

a) Humanitas University, Department of Biomedical Sciences, Milan, Italy; b) Third Medicine Division, Department of Medicine ASST Spedali Civili, Brescia, Italy; c) Division of Cardiology, Ospedale degli Infermi, ASL Biella, Biella, Italy; d) Department of Translational Medicine, Eastern Piedmont University, Novara, Italy; e) Department of Internal Medicine and Medical Specialties (DiMI), School of Medicine, University of Genova, Genova, Italy; f) Division of Cardiology, Hopital Erasmus, Universitè Libre de Bruxelles, Bruxelles, Belgium; g) Division of Cardiology, Ospedale Ruggi D'Aragona, Università di Salerno, Salerno, Italy; h) Department of Cardiology, Maastricht University Medical, Maastricht, The Netherlands; i) Department of Cardiovascular Medicine, IRCCS-Humanitas Research Hospital, Rozzano, Italy; j) Division of Clinical and Experimental Cardiology, University Hospital of Sassari, Sassari, Italy

Submitted

Abstract

The key role played by platelets in the atherosclerosis physiopathology, especially in the acute setting is ascertained: they are the main actors during thrombus formation and, thus, one of the major investigated elements related to the atherothrombotic process involving coronary arteries. Platelet have been studied from different perspectives, based on advances in technology and the improvements knowledge on hemostasis achieved recently. Morphology and reactivity constitute the first aspects investigated related to platelets with a significant body of evidence published linking a number of their values and markers to coronary artery disease and cardiovascular events. Recently, the impact of genetics on platelet activation has been explored with promising findings as additional instrument for patients' risk stratification, deserving however further confirmations. Moreover, the interplay between immune system and platelet has been partially elucidated in the last years, providing intriguing elements that will be basic components for the future research to better understand platelet regulation and improve cardiovascular outcome of patients.

Introduction

Platelets enter the circulation after the fragmentation of bone marrow megakaryocytes, as anucleate cells (2 μm in diameter) that lack genomic DNA(1). Because of that, platelets have a limited capacity to synthesize proteins, available by the presence of megakaryocyte-derived messenger RNA (mRNA) together with the equipment required for protein synthesis(2). Thrombopoietin is a glycoprotein synthesized mostly in the liver but also in the kidneys, and regulates megakaryocytic proliferation and maturation, as well as platelet production(3). Once they enter the circulation, platelets have a life span of 7 to 10 days in quiescent discoid state in the presence of healthy vessels without endothelium impaired homeostasis. Platelets' primary function is to stop hemorrhage after tissue trauma and vascular damage.

Injury to the intimal layer of the vessel exposes the underlying subendothelial matrix. Platelets move to sites of vascular disruption and adhere to the exposed matrix protein(4). Adherent platelets undergo activation and subsequently release substances that recruit additional platelets to the site of injury. Additionally, they promote thrombin generation and subsequent fibrin formation. The potent platelet agonist thrombin, together with other amplifying factors, promotes platelet recruitment and activation. Activated platelets then aggregate to form a plug that seals the injury in the vasculature. The understanding of this highly integrated processes is essential to optimize the treatment in patients suffering of the atherothrombotic disease(5–7); therefore the identification of makers of platelet regulation, whether inhibiting or promoting, has deserved increasing attention in the last decades.

Platelet adhesion, activation, and aggregation

The first step of platelets at sites of vascular injury consists in the adhesion and it is mediated by the glycoprotein Ib/V/IX complex, which allows platelets adhering to exposed collagen and von Willebrand factor (vWF). The vWF is synthesized by endothelial cells and megakaryocytes assembles into multimers with a size ranging from 550 kDa to more than 10,000 kDa(8); the prevention of large multimer accumulation is mediated by the metalloprotease ADAMTS13, whose deficiency results in the thrombotic thrombocytopenia purpura.

When released from the α-granules of platelets or the Weibel- Palade bodies of endothelial cells, the majority of the vWF compounds enters the circulation, except for the vWF released from the abluminal surface of the endothelial cells: it accumulates in the subendothelial matrix and binds collagen via its A3 domain. The vWF anchored to the vessel subendothelial matrix changes its conformation, exposes A1 domain that binds platelets.

The adhesion to collagen and vWF endorses the pathways that lead to platelet activation. During this process platelets modify their morphology and stimulate the release of their granule content rich soluble agonists, including adenosine diphosphate (ADP), adhesion molecules, and coagulation factors almost all of which amplify the thrombotic response(9). Among these autocrine and paracrine mediators, platelet activation favors thromboxane A2 (TXA2) generation through cyclooxygenase1. Next step involves the conversion of prothrombin into thrombin through activated factor X(10). Thrombin is the most potent known agonist for platelet activation, binding protease-activated receptor types 1 and 4 (PAR- 1 and PAR-4), but it also plays a critical role in early thrombus formation, converting fibrinogen to fibrin which effectively anchors the growing thrombus.

The final step in formation of the platelet plug is represented by the aggregation, that links platelets to each other to form clumps. Linkages are mediated by glycoprotein IIb/IIIa (GP IIb/IIIa), that has undergone conformational change after platelet activation to increase the affinity for his ligand, fibrinogen (**Figure 1**)(11).

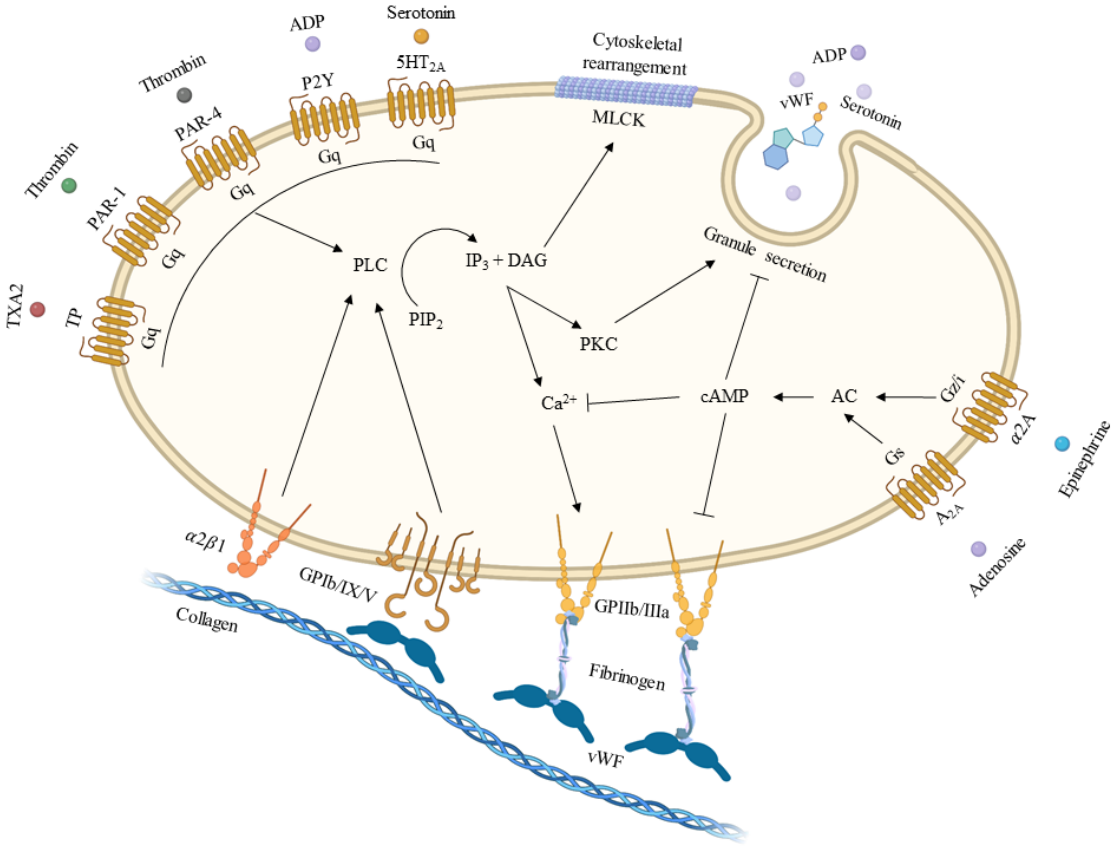


Figure 1. Platelet activation pathways. The figure displays the intricate relationship between different stimuli and platelet activation. AC= adenylate cyclase; ADP= adenosine diphosphate; cAMP= cyclic adenosine monophosphate; DAG= diacylglycerol; IP3= inositol triphosphate; MLKC= myosin light chain kinase; PAR-1= protease activated receptor-1; PAR-4= protease activated receptor-4; PIP2= phosphatidylinositol bisphosphate; PKC= protein kinase C; PLC= phospholipase C; TP= thromboxane receptor; TxA2= thromboxane A2; vWF= von Willebrand factor.

Morphologic and structural

Among first proposed parameters linked to platelet aggregation, there is the mean platelet volume (MPV). MPV is a marker of platelet size, and it is usually provided by the majority of laboratory as a part of the full blood count(10). It constitutes one of most used indirect markers of platelet function. In fact, it has been shown that larger platelets are more active, from a metabolic point of view, leading thus to greater prothrombotic risk(12,13). In their denser granules, larger platelets contain more β-thromboglobulin, serotonin and TXA2 than smaller ones(14–16). Moreover, it has argued that MPV may reflect the platelet production rate(17), stimulation(18) and activation(19–21): in particular larger platelets result more adhesive and reactive(22,23), showing higher expression of glycoprotein Ib and GP IIb/IIIa receptors at the surface(24). For these reasons, MPV has been variously associated to coronary artery disease (CAD), although with contrasting results(25),23). In about 400 patients with ST elevation myocardial infarction undergoing primary percutaneous coronary intervention (PCI), Huczek et al found MPV a strong, independent predictor of impaired angiographic reperfusion and six-month mortality(27). Similarly, Murat et colleagues showed MPV as an independent predictor of the severity of CAD among acute coronary syndrome (ACS) patients(28).

However, the positive relationship between MPV and CAD has not been confirmed in other studies(29,30). Halbmayer et al, in fact, found no differences of MPV between healthy persons and patients with CAD as well as no significant variations of MPV values have been reported between patients without prior myocardial infarction (MI) and MI survivors(31). In a large cohort of more than 1400 patients undergoing PCI no relationship was found between MPV and the extent of CAD(32), neither with platelet aggregation(33). Whether MPV can represent an independent predictor of cardiovascular events or on the contrary the consequence of other cardiovascular risk factors, such as hypertension,

diabetes or smoking is still debated, even if a recent meta-analysis suggests that MPV may be a useful prognostic marker in patients with CAD(34).

Another morphologic parameter of platelet inquired for a potential relationship with CAD is represented by platelet distribution width (PDW): it directly measures the variability in platelet size and has been used to differentiate disorders of platelets such as essential thrombocythemia from reactive thrombocytosis(35), therefore providing more information than MPV. Some studies have described a potential relationship between PDW and chronic total occlusion of coronary artery disease(36) and occluded saphenous vein graft in patients with CABG surgery(37). Opposite results were reported about PDW role to predict CAD and its severity, suggesting the need of further investigations to clarify its contribution in the patients management(38,39).

Also platelet-large cell ratio (P-LCR), an index representing the percentage of platelets larger than 12 fL has deserved attention as marker of platelet activation(40). Despite some promising results showing a relationship between P-LCR and inflammation in CAD patient(41,42), no significant contribution about severity of CAD and platelet reactivity has been found for P-LCR(43,44).

Emerging interest has been denoted about the fraction of reticulated platelets, that represent the proportion of younger platelets, lastly released from the bone marrow, with a higher content in α-granules and RNA: these features have been hypothesized leading to enhanced capability of proteins synthesis and then to a potentially increased overall reactivity(45,46). McBane et al reported that younger reticulated platelets appear to have a greater propensity for thrombus participation in presence of atherosclerotic stenosis and shear conditions compared to older ones. Among suggested mechanisms an increased receptor density of integrin-β3 in younger platelets may contribute to higher predisposition to thrombosis(47).

Immature platelet fraction (IPF) and the absolute immature platelet count (IPC) represent parameters for the identification of the reticulated platelet with a sample for a routine blood count(48). A certain number of studies have reported higher IPF in patients with CAD, especially in the subgroups presenting with ACS(49,50), with potential role of IPF >6.2 to predict mortality(49). In the other hand, Berny-Lan et al found no difference of IPF in patients with and without ACS(51) and Verdoia et al showed no role of IPF to predict CAD and the severity of CAD in patients undergoing PCI, discussing IPF as marker of platelet turnover rather than being directly involved in the pathogenesis of CAD. In patients on antiplatelet drugs IPF has been linked with ineffective therapy, both mono- and dual-antiplatelet therapy(52,53), despite also opposite evidences has been provided(54,55).

Function and reactivity

A direct assessment of platelet activation and aggregation could be performed though a platelet function test (PFT). The identification of impairment in the clot formation for hemorrhagic disorders was the rationale of the first test described by Duke, the evaluation of bleeding time(56). To better assess congenital and acquired platelet disorders the first platelet aggregation test was developed, adopting the light transmission aggregometry (LTA) with platelet rich plasma (PRP)(57,58). After them, several tests with different methods have been proposed to estimate platelet function. Apart of LTA, that required elaborate management of blood sample before proceeding, impedance whole aggregometry allows to assess platelet aggregation using anticoagulated whole blood. It allows to measure platelet aggregation after several stimuli inquiring different activation signaling pathways with two main advantages: small quantity of whole blood and no manipulation before testing, preserving as much as possible physiological condition(59).

Another aggregometry method uses a turbidimetric-based optical detection through a system cartridge containing fibrinogen-coated beads and platelet agonists(60); specific assays for patients under antiplatelet therapy are commercially available(61). A certain number of commercial assays are instead based on the evaluation of platelet adhesion under shear stress, or on viscoelastic methodologies(62–65).

Besides different technologies used, the rationale of assessment platelet function in patients with CAD is for monitoring the response to antiplatelet drugs in order to identify subjects deserving modification in the composition and/or treatment duration(66). The main reason of assessment is to recognize patients poor responders to antiplatelet therapy, both aspirin and $P2Y_{12}$ inhibitors: since these drugs represents the cornerstone in patients with CAD, undergoing PCI, their effectiveness is crucial to prevent adverse events(67). However, the adoption of the expression "drug resistance" is not appropriate, considering that the baseline assessment of platelet function is unavailable in the vast majority of cases, especially in the acute setting: therefore it is recognized to refer to inappropriate platelet reactivity during antiplatelet therapy with the expression of "high on-treatment residual platelet reactivity" (abbreviated as HTPR or HRPR)(68). Even if HTPR is a well-known independent predictor of several adverse events (69), strategies to tailor antiplatelet therapy according to platelet function provided contrasting results in demonstrating clinical benefits(66,70–72), suggesting other factors involved in the determinism of elevated platelet reactivity on top of antiplatelet treatment.

Platelet reactivity

The detection of patients displaying enhanced platelet reactivity despite antiplatelet therapy and deserving more clinical attention and stricter follow-up could improve the overall outcome of those subjects at higher risk for cardiovascular adverse events.

One of the first described element affecting atherosclerosis and platelet is cigarette smoking. The impact on cardiovascular system is recognized, while less detailed are the smoking mechanisms involved in the promotion of platelet reactivity. The increased smoking-induced lipid oxidation products bind platelet scavenger receptor CD36, which in turn results in increased platelet aggregation response(73). Amplifying factors, including ADP and thrombin, have been shown to increase after cigarette smoking exposure, with consequent higher platelet aggregation response to these pathways(74,75), and nicotine displayed a direct role in increasing platelet aggregability(76). However some recent studies have found an opposite effect of smoking, especially on $P2Y_{12}$ antagonists, whose platelet inhibition is resulted enhanced after smoking exposure(77), even if concerns on a basal lower platelet aggregation(78) and smoking effect on platelet morphology(79).

Higher platelet reactivity has been reported among diabetic patients, due to continuous environmental inflammation that characterizes diabetes mellitus. However, diabetes is not only per se a marker of higher risk(80), but also the impaired glycemic control has been linked to HTPR among clopidogrel treated subjects(81) or under more potent P2Y12 inhibitors(82). Conversely, Vivas et al. documented a significant reduction of aggregation in post-ACS patients receiving intensive glucose control treatment with insulin(83). Indeed, hyperglycemia can impact on platelet function both directly and by modulating the release of pro-oxidant and inflammatory substances or by the glycation platelet surface proteins, with consequent amplification of platelet adhesion(83).

Similar pathophysiological elements are shared by the excess of uric acid: it has been addressed as a main determinant of atherosclerosis and metabolic syndrome. In fact, hyperuricemia is a condition characterized by impaired nitric oxygen release and enhanced

pro-inflammatory cytokines(84,85). However, no direct impact has been demonstrated on platelet reactivity under antiplatelet therapy(86).

Increasing interest has grown about the potential cardiovascular impact of vitamins D (87). Alongside the impact on endothelial dysfunction(88), vitamin D receptor was detected on platelet surface, displaying antithrombotic effects(89). Pivotal role in the platelet aggregation and thrombus formation has been described by Aihara et al, who demonstrated in murine models knock-out for the vitamin D receptor gene an enhanced thrombogenicity(90). An indirect antiplatelet therapy has been previously reported by Lòpez-Farré et colleagues: the addition of vitamin D binding protein to whole blood in healthy subjects hampered the antiplatelet inhibitory effect of aspirin(91) and lower levels of vitamin D have been significantly associated to HTPR under ticagrelor treatment(92). Genetic of vitamin D related genes constitutes an interesting ongoing field of investigation(93), despite more studies are needed(94).

Genetics and miRNA

In relation to the antiplatelet therapy with $P2Y_{12}$ inhibitors, several reports have addressed concerns on reduced effectiveness and platelet inhibition especially during clopidogrel treatment. Because clopidogrel is a pro-drug, it is first metabolized by CYP2C19 before it becomes effective. However, in the about 5% of Caucasians and 15 to 20% of Asians display low or absent CYP2C19 activity, leading to a smaller or no clopidogrel effect on platelet function(95). Several studies have reported an increase in adverse cardiovascular events in patients who carry at least one of non-functional copies of the CYP2C19 gene compared with patients wild type for CYP2C19 gene(96,97), portraying these patients to be treated with higher clopidogrel dosage or with an alternative drug(98,99).

Guidelines to describe scenarios deserving modification in antiplatelet therapy have been proposed by Clinical Pharmacogenetics Implementation Consortium (CPIC) and by Dutch Pharmacogenetics Working Group (DPWG) of the Royal Dutch Association for the Advancement of Pharmacy (100,101). However, the recent TAILOR-PCI trial showed that in CYP2C19 loss of function carriers with ACS and stable CAD undergoing PCI, genotype-guided selection of an oral $P2Y_{12}$ inhibitor, compared with conventional clopidogrel therapy without genotyping, resulted in non-statistically significant difference in a composite end point of cardiovascular death, myocardial infarction, stroke, stent thrombosis, and severe recurrent ischemia at 1 year(102), while a meta-analysis suggests the potential usefulness of genetic test to guide de-escalation of antiplatelet therapy after a PCI for ACS(103).

Also more potent and expensive $P2Y_{12}$ inhibitors were inquired for a potential impact of genetics on their effects: prasugrel, a pro-drug like clopidogrel, was found being influenced by some cytochrome P450 polymorphisms(104), even if the Food and Drug Administration stated that there were no relevant effect of genetic variation on

pharmacokinetics of prasugrel's active metabolite and, thus, on its antiplatelet effect(104). On the other hand, ticagrelor, that is direct $P2Y_{12}$ inhibitor not requiring activation, has no specific warning about potential genetic impact: Varenhorst et al found some genetic loci influencing ticagrelor pharmacokinetics through a genome-wide association study, but that did not translate into any clinical detectable effect on ticagrelor efficacy and safety(105), even if genetics of adenosine signaling pathways may affect platelet reactivity under ticagrelor treatment (**Figure 2**)(106).

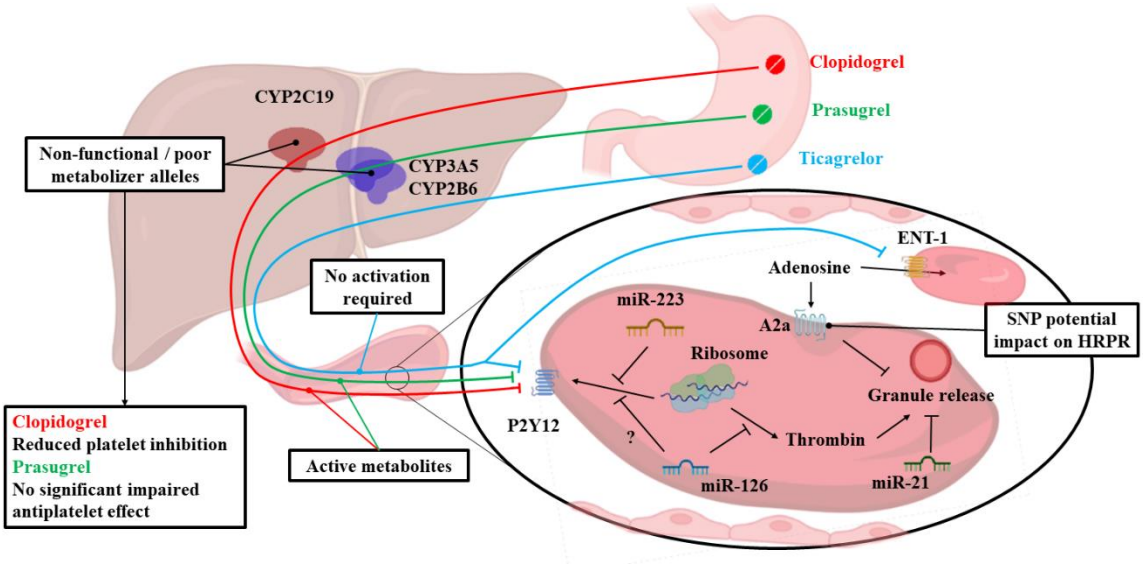


Figure 2. Genetic and miRNA. The figure depicts essential but crucial steps in pharmacokinetic of routinely used P2Y12 inhibitors, coupled with the potential target of the most studied miRNAs. ENT-1= equilibrative nucleoside transporter-1; SNP= single nucleotide polymorphism; HRPR= high residual platelet reactivity.

In the last years increasing interest has been dedicated to RNA carried by platelets, with specific regards to microRNA (miRNAs). Considering that platelets are enriched with miRNAs and represent the second most abundant blood cell type, platelets are the major source of miRNAs in plasma and serum(107,108). Moreover, the levels of both intraplatelet and circulating platelet-derived miRNAs are shown correlating with platelet reactivity(109,110).

Of about 750 intraplatelet miRNAs identified, the most extensively investigated are miR-223, miR-21 and miR-126. The miR-223 results the most representative in platelet, with respect of the overall amount, and it has been linked to $P2Y_{12}$ receptor regulation, despite the exact mechanism need to be clarified(111). However, some suggestions come from murine model without miR-223 expression which displayed increased thrombus size compared to normal miR-223 expression(112) and among diabetic patients a lower level of miR-223 was detected with a concomitant increased platelet reactivity(113). On the other hand, miR-21 together with other miRNA were found upregulated in ACS patient with an enhanced response to clopidogrel(114), deserving however more studies to define its role in regulation of α-granules and platelet-derived pro-fibrotic factors, including transforming growth factor beta-1 (TGF-β1)(115). The role of miR-126 has been shown involving the regulation of $P2Y_{12}$ receptor and, potentially, platelet-derived thrombin generation(116,117).

The role of circulating miRNA in platelet activation is still under investigation, even if a progressive number of evidence have been published, underscoring their role in aggregation homeostasis(118,119). Major shadows are related to the general mechanisms used by circulating miRNA to act on their target, especially considering the abundance of RNases in the circulation that quickly degrade free RNA(120): it has been proposed that circulating miRNAs might regulate the cell surface receptor through physical conformational interaction, but an adversative issue is that miRNAs are contained in vesicles or protein complexes to protect them by degradation(121). Jansen et al have described in 181 patients with stable CAD that miRNAs contained in microvesicles but not circulating miRNAs predict the occurrence of cardiovascular events(122). Also Zampetaki et al described the association of miRNA expression patterns and the incidence of MI in a cohort of 820 patients(123), while other miRNAs levels were significantly

associated with the risk of death in univariate and age- and sex-adjusted analyses in patients with ACS(124). Further investigations will provide us a more detailed understanding of miRNAs pathways to design accurate studies for the definition of their functions in platelet aggregation and atherosclerosis progression.

Interplay immune system

The immune system has a central key role in the atherothrombotic process(125,126). Platelets have been involved in modulation of innate and adaptive immune responses, even if most mechanisms are still an enigma (**Figure 3**). Platelets α-granules contain many molecules with several effects not limited to platelet aggregation amplification and coagulation: a couple of molecule with a key role in several signaling pathways are CD40 and CD154, also known as CD40 ligand (CD40L)(127). In particular, CD154 can interact both with endothelial and immune cells. The stimulation of endothelial cells leads to increased expression of adhesion molecule, mainly intercellular (I-CAM-1) and vascular (V-CAM-1) adhesion molecules(128), and consequent increased leukocytes recruitment and atherosclerotic plaque instability. Platelet derived CD154 interacts with different type of immune cells: it has been described stimulating the B cell differentiation(129) and macrophages activity(130). In murine model of atherosclerosis, CD154 has been shown increasing plaque fragility and reducing its stability by inhibition of regulatory T cells(131). Platelets have been found expressing different toll like receptor (TLR) subtypes, enforcing the concept of inflammation modulatory action by platelets(132).

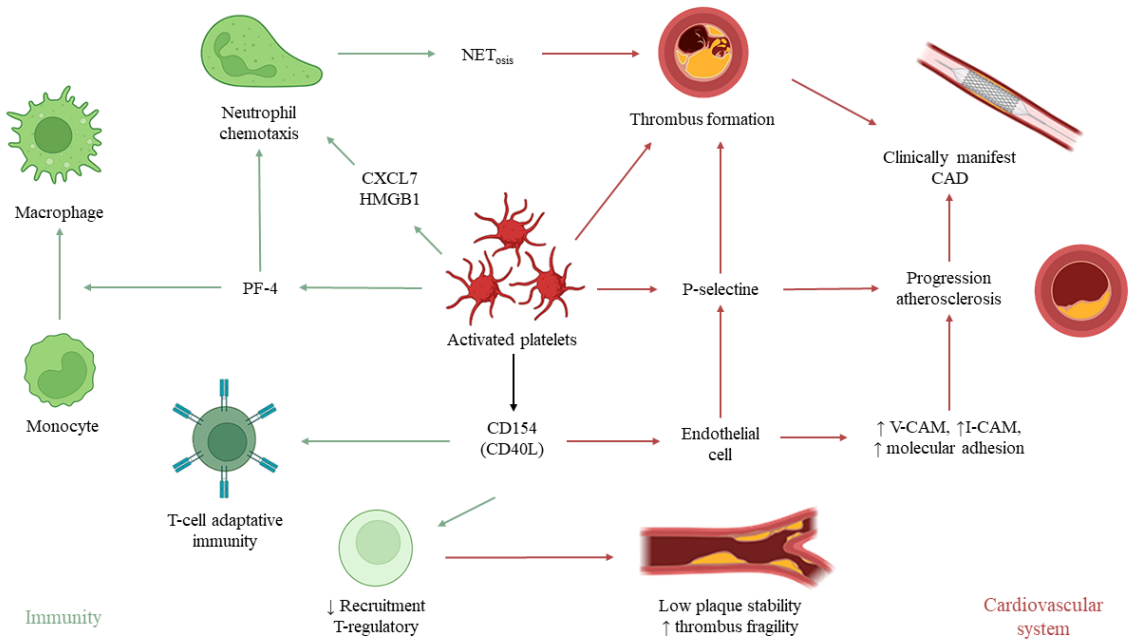


Figure 3. Activated platelet interplay between immune and cardiovascular systems. The figure shows complex interplays involving activated platelets between immune and cardiovascular system: on the left side, crosstalk between platelet and immune system is shown, reporting principal signaling pathways. On the right side it is reported main cardiovascular actions played by activated platelets. CAD= coronary artery disease; CXCL7= chemokine (C-X-C motif) ligand 7; HMGB1= high mobility group box 1; I-CAM= Intercellular adhesion molecule; NET= neutrophil extracellular trap; PF-4= platelet factor-4; V-CAM= Vascular adhesion molecule.

Moreover, platelets have described to have the opportunity to recruit neutrophils and worse thrombo-inflammation(133). The main crosstalk element is constituted by the P-selectin that binds P-selectin glycoprotein ligand-1 expressed by neutrophils(134), but also chemokines CXCL4 (also named platelet factor-4, PF-4) and CXCL7, and high mobility group box 1 (HMGB1) are released by platelet and contribute to neutrophil chemotaxis(135). Final step of neutrophil-platelet interactions is the promotion of neutrophil extracellular traps (NETs)(136,137). They are demonstrated potent inducers of thrombus formation, serving as scaffold for both platelet binding and activation(138), through several pathways including H3 histones and C3b attached to NETs(139).

From the clinical side, Hally et al have investigated the potential utility of a composite biomarker score of NET activation and release, or NETosis, for predicting MACE post-MI: authors demonstrated the importance of these combining biomarkers as risk predictors of MACE at 1 year(140). Furthermore Riegger et al evaluated about two hundred and fifty thrombus specimens in patients with stent thrombosis, demonstrating the recruitment leukocytes, particularly neutrophils and the presence of NETosis in 23% of samples(141). Also in the thrombus of culprit artery from patients with acute myocardial infarction has been demonstrated the presence of NETs formation(142). Promising therapeutics have been investigated, inclacumab and crizanlizumab, that block interaction of P-selectin and neutrophils. In the SELECT-ACS study inclacumab was reported significantly reducing myocardial damage assessed through the CK-MB after PCI in patients with non ST elevation-ACS(143,144). However these findings were not confirmed in patients suffering of bypass graft failure after coronary bypass(145). Therefore, further studies are needed to better assess the potential of monoclonal antibodies therapy against NETs in patients with CAD(146).

Future perspective and conclusions

This overview aimed to show the different approach and methods adopted to study platelets aggregation that appropriately warranted attention by the scientific research: not only because they are an essential component of the physiological hemostasis and pathological atherothrombotic process, but also since the intervention on them have demonstrated dramatic improvements in patients with CAD, including both stable disease and acute presentation. Antiplatelet therapy represents a mandatory approach for subjects with clinically significant atherosclerosis, but the availability of effective parameters to assess platelet function is indispensable to select the best option for each patient. From the first instrument developed and of limited routinary usage, several morphological parameters and methods to assess platelet reactivity have shown significant contribution to daily clinical practice. Auspicious findings are from the genetic investigations, in particular miRNAs, and from the progressive better understanding of the role of immune system cells in platelet thrombus formation too. A single, definite biomarker of platelet aggregation would be probably a chimeric though, whilst the merge of different aspects assessments, including the most recent advances, will help if not to close, at least to move near the circle on a comprehensive valuation of the cardiovascular risk for patients with CAD.

References

1. Italiano JE, Shivdasani RA. Megakaryocytes and beyond: the birth of platelets. J Thromb Haemost. 2003 Jun;1(6):1174–82.
2. Healy AM, Pickard MD, Pradhan AD, Wang Y, Chen Z, Croce K, et al. Platelet expression profiling and clinical validation of myeloid-related protein-14 as a novel determinant of cardiovascular events. Circulation. 2006 May 16;113(19):2278–84.
3. Baigger A, Blasczyk R, Figueiredo C. Towards the Manufacture of Megakaryocytes and Platelets for Clinical Application. Transfus Med Hemother. 2017 Jun;44(3):165–73.
4. Becker RC, Sexton T, Smyth SS. Translational Implications of Platelets as Vascular First Responders. Circ Res. 2018;122(3):506–22.
5. De Luca G, Suryapranata H, Stone GW, Antoniucci D, Tcheng JE, Neumann F-J, et al. Relationship between patient's risk profile and benefits in mortality from adjunctive abciximab to mechanical revascularization for ST-segment elevation myocardial infarction: a meta-regression analysis of randomized trials. J Am Coll Cardiol. 2006 Feb 7;47(3):685–6.
6. De Luca G, Navarese EP, Cassetti E, Verdoia M, Suryapranata H. Meta-analysis of randomized trials of glycoprotein IIb/IIIa inhibitors in high-risk acute coronary syndromes patients undergoing invasive strategy. Am J Cardiol. 2011 Jan 15;107(2):198–203.
7. Verdoia M, Schaffer A, Barbieri L, Cassetti E, Piccolo R, Galasso G, et al. Benefits from new ADP antagonists as compared with clopidogrel in patients with stable angina or acute coronary syndrome undergoing invasive management: a meta-analysis of randomized trials. J Cardiovasc Pharmacol. 2014 Apr;63(4):339–50.
8. Grover SP, Mackman N. Intrinsic Pathway of Coagulation and Thrombosis. Arterioscler Thromb Vasc Biol. 2019;39(3):331–8.
9. Gurbel PA, Kuliopulos A, Tantry US. G-protein-coupled receptors signaling pathways in new antiplatelet drug development. Arterioscler Thromb Vasc Biol. 2015 Mar;35(3):500–12.
10. Badimon L, Vilahur G. Thrombosis formation on atherosclerotic lesions and plaque rupture. J Intern Med. 2014 Dec;276(6):618–32.
11. Davì G, Patrono C. Platelet activation and atherothrombosis. N Engl J Med. 2007 Dec 13;357(24):2482–94.

12. Chu SG, Becker RC, Berger PB, Bhatt DL, Eikelboom JW, Konkle B, et al. Mean platelet volume as a predictor of cardiovascular risk: a systematic review and meta-analysis. J Thromb Haemost. 2010 Jan;8(1):148–56.
13. Jakubowski JA, Thompson CB, Vaillancourt R, Valeri CR, Deykin D. Arachidonic acid metabolism by platelets of differing size. Br J Haematol. 1983 Mar;53(3):503–11.
14. Kodiatte TA, Manikyam UK, Rao SB, Jagadish TM, Reddy M, Lingaiah HKM, et al. Mean platelet volume in Type 2 diabetes mellitus. J Lab Physicians. 2012 Jan;4(1):5–9.
15. Hekimsoy Z, Payzin B, Ornek T, Kandoğan G. Mean platelet volume in Type 2 diabetic patients. J Diabetes Complications. 18(3):173–6.
16. Colwell JA, Nesto RW. The platelet in diabetes: focus on prevention of ischemic events. Diabetes Care. 2003 Jul;26(7):2181–8.
17. Briggs C. Quality counts: new parameters in blood cell counting. Int J Lab Hematol. 2009 Jun;31(3):277–97.
18. Thompson CB, Eaton KA, Princiotta SM, Rushin CA, Valeri CR. Size dependent platelet subpopulations: relationship of platelet volume to ultrastructure, enzymatic activity, and function. Br J Haematol. 1982 Mar;50(3):509–19.
19. Tsiara S, Elisaf M, Jagroop IA, Mikhailidis DP. Platelets as predictors of vascular risk: is there a practical index of platelet activity? Clin Appl Thromb Hemost. 2003 Jul;9(3):177–90.
20. Jagroop IA, Clatworthy I, Lewin J, Mikhailidis DP. Shape change in human platelets: measurement with a channelyzer and visualisation by electron microscopy. Platelets. 2000 Feb;11(1):28–32.
21. Park Y, Schoene N, Harris W. Mean platelet volume as an indicator of platelet activation: methodological issues. Platelets. 13(5–6):301–6.
22. Schoene NW. Design criteria: tests used to assess platelet function. Am J Clin Nutr. 1997 May;65(5 Suppl):1665S-1668S.
23. Martin JF, Trowbridge EA, Salmon G, Plumb J. The biological significance of platelet volume: its relationship to bleeding time, platelet thromboxane B2 production and megakaryocyte nuclear DNA concentration. Thromb Res. 1983 Dec 1;32(5):443–60.
24. Giles H, Smith RE, Martin JF. Platelet glycoprotein IIb-IIIa and size are increased

in acute myocardial infarction. Eur J Clin Invest. 1994 Jan;24(1):69–72.

25. Pizzulli L, Yang A, Martin JF, Lüderitz B. Changes in platelet size and count in unstable angina compared to stable angina or non-cardiac chest pain. Eur Heart J. 1998 Jan;19(1):80–4.
26. Damodar S, Ganesh KV, Murthy S. Mean platelet volume does not predict risk of myocardial infarction or coronary artery disease in Indian patients. Platelets. 2008 Feb;19(1):80–1.
27. Huczek Z, Kochman J, Filipiak KJ, Horszczaruk GJ, Grabowski M, Piatkowski R, et al. Mean platelet volume on admission predicts impaired reperfusion and long-term mortality in acute myocardial infarction treated with primary percutaneous coronary intervention. J Am Coll Cardiol. 2005 Jul 19;46(2):284–90.
28. Murat SN, Duran M, Kalay N, Gunebakmaz O, Akpek M, Doger C, et al. Relation between mean platelet volume and severity of atherosclerosis in patients with acute coronary syndromes. Angiology. 2013 Feb;64(2):131–6.
29. De Luca G, Verdoia M, Cassetti E, Schaffer A, Di Giovine G, Bertoni A, et al. Mean platelet volume is not associated with platelet reactivity and the extent of coronary artery disease in diabetic patients. Blood Coagul Fibrinolysis. 2013 Sep;24(6):619–24.
30. Verdoia M, Camaro C, Barbieri L, Schaffer A, Marino P, Bellomo G, et al. Mean platelet volume and the risk of periprocedural myocardial infarction in patients undergoing coronary angioplasty. Atherosclerosis. 2013 May;228(1):136–41.
31. Halbmayer WM, Haushofer A, Radek J, Schön R, Deutsch M, Fischer M. Platelet size, fibrinogen and lipoprotein(a) in coronary heart disease. Coron Artery Dis. 1995 May;6(5):397–402.
32. De Luca G, Santagostino M, Secco GG, Cassetti E, Giuliani L, Franchi E, et al. Mean platelet volume and the extent of coronary artery disease: results from a large prospective study. Atherosclerosis. 2009 Sep;206(1):292–7.
33. Verdoia M, Pergolini P, Rolla R, Nardin M, Barbieri L, Schaffer A, et al. Mean platelet volume and high-residual platelet reactivity in patients receiving dual antiplatelet therapy with clopidogrel or ticagrelor. Expert Opin Pharmacother. 2015;16(12):1739–47.
34. Sansanayudh N, Numthavaj P, Muntham D, Yamwong S, McEvoy M, Attia J, et al. Prognostic effect of mean platelet volume in patients with coronary artery disease.

A systematic review and meta-analysis. Thromb Haemost. 2015 Nov 25;114(6):1299–309.

35. Vagdatli E, Gounari E, Lazaridou E, Katsibourlia E, Tsikopoulou F, Labrianou I. Platelet distribution width: a simple, practical and specific marker of activation of coagulation. Hippokratia. 2010 Jan;14(1):28–32.
36. Vatankulu MA, Sonmez O, Ertas G, Bacaksiz A, Turfan M, Erdogan E, et al. A New Parameter Predicting Chronic Total Occlusion of Coronary Arteries. Angiology. 2014 Jan 1;65(1):60–4.
37. Ege MR, Guray U, Guray Y, Acıkgoz S, Demirkan B. Platelet distribution width and saphenous vein disease in patients after CABG. Association with graft occlusion. Herz. 2013 Mar;38(2):197–201.
38. Ihara A, Kawamoto T, Matsumoto K, Shouno S, Hirahara C, Morimoto T, et al. Relationship between Platelet Indexes and Coronary Angiographic Findings in Patients with Ischemic Heart Disease. Pathophysiol Haemost Thromb. 2006;35(5):376–9.
39. De Luca G, Venegoni L, Iorio S, Secco GG, Cassetti E, Verdoia M, et al. Platelet distribution width and the extent of coronary artery disease: Results from a large prospective study. Platelets. 2010 Nov 28;21(7):508–14.
40. Bessman JD, Williams LJ, Gilmer PR. Mean platelet volume. The inverse relation of platelet size and count in normal subjects, and an artifact of other particles. Am J Clin Pathol. 1981 Sep;76(3):289–93.
41. Lv H-C, Wu H-Y, Yin J-S, Ge J-B. Thrombin induced platelet-fibrin clot strength in relation to platelet volume indices and inflammatory markers in patients with coronary artery disease. Oncotarget. 2017 Sep 8;8(38):64217–23.
42. Larsen SB, Grove EL, Hvas A-M, Kristensen SD. Platelet turnover in stable coronary artery disease - influence of thrombopoietin and low-grade inflammation. PLoS One. 2014;9(1):e85566.
43. De Luca G, Santagostino M, Secco GG, Cassetti E, Giuliani L, Coppo L, et al. Platelet-large cell ratio and the extent of coronary artery disease: results from a large prospective study. J Thromb Thrombolysis. 2010 Nov;30(4):426–33.
44. Verdoia M, Pergolini P, Rolla R, Nardin M, Barbieri L, Schaffer A, et al. Platelet Larger Cell Ratio and High-on Treatment Platelet Reactivity During Dual Antiplatelet Therapy. Cardiovasc drugs Ther. 2015;29(5):443–50.

45. Ault KA, Rinder HM, Mitchell J, Carmody MB, Vary CPH, Hillman RS. The Significance of Platelets with Increased RNA Content (Reticulated Platelets) A Measure of the Rate of Thrombopoiesis. Am J Clin Pathol. 1992 Dec 1;98(6):637–46.
46. Dusse LMS, Freitas LG. Clinical applicability of reticulated platelets. Clin Chim Acta. 2015 Jan;439:143–7.
47. McBane RD, Gonzalez C, Hodge DO, Wysokinski WE. Propensity for young reticulated platelet recruitment into arterial thrombi. J Thromb Thrombolysis. 2014 Feb 5;37(2):148–54.
48. Hoffmann JJML. Reticulated platelets: analytical aspects and clinical utility. Clin Chem Lab Med. 2014 Jan 1;52(8).
49. López-Jiménez RA, Martín-Herrero F, González-Porras JR, Sánchez-Barba M, Martín-Luengo C, Pabón-Osuna P. Fracción de plaquetas inmaduras, un nuevo marcador pronóstico en el síndrome coronario agudo. Rev Española Cardiol. 2013 Feb;66(2):147–8.
50. Cesari F, Marcucci R, Gori A, Caporale R, Fanelli A, Casola G, et al. Reticulated platelets predict cardiovascular death in acute coronary syndrome patients. Thromb Haemost. 2013 Nov 22;109(05):846–53.
51. Berny-Lang MA, Darling CE, Frelinger AL, Barnard MR, Smith CS, Michelson AD. Do immature platelet levels in chest pain patients presenting to the emergency department aid in the diagnosis of acute coronary syndrome? Int J Lab Hematol. 2015 Feb;37(1):112–9.
52. Bernlochner I, Goedel A, Plischke C, Schüpke S, Haller B, Schulz C, et al. Impact of immature platelets on platelet response to ticagrelor and prasugrel in patients with acute coronary syndrome. Eur Heart J. 2015 Dec 1;36(45):3202–10.
53. Perl L, Lerman-Shivek H, Rechavia E, Vaduganathan M, Leshem-Lev D, Zemer-Wassercug N, et al. Response to Prasugrel and Levels of Circulating Reticulated Platelets in Patients With ST-Segment Elevation Myocardial Infarction. J Am Coll Cardiol. 2014 Feb 1;63(6):513–7.
54. Verdoia M, Sartori C, Pergolini P, Nardin M, Rolla R, Barbieri L, et al. Immature platelet fraction and high-on treatment platelet reactivity with ticagrelor in patients with acute coronary syndromes. J Thromb Thrombolysis. 2016 May 14;41(4):663–70.

55. Verdoia M, Nardin M, Negro F, Rolla R, Carriero A, De Luca G. Impact of long-term therapy with acetylsalicylic acid on immature platelet count. J Cardiovasc Med. 2019 May;20(5):306–12.
56. Duke WW. The relation of blood platelets to hemorrhagic disease. J Am Med Assoc. 1910 Oct 1;55(14):1185.
57. BORN G V. Aggregation of blood platelets by adenosine diphosphate and its reversal. Nature. 1962 Jun 9;194:927–9.
58. O'Brien J. Platelet aggregation: Part II Some results from a new method of study. J Clin Pathol. 1962 Sep;15(5):452–5.
59. Tóth O, Calatzis A, Penz S, Losonczy H, Siess W. Multiple electrode aggregometry: a new device to measure platelet aggregation in whole blood. Thromb Haemost. 2006 Dec;96(6):781–8.
60. Smith JW, Steinhubl SR, Lincoff AM, Coleman JC, Lee TT, Hillman RS, et al. Rapid platelet-function assay: an automated and quantitative cartridge-based method. Circulation. 1999 Feb 9;99(5):620–5.
61. Paniccia R, Antonucci E, Gori AM, Marcucci R, Giglioli C, Antoniucci D, et al. Different methodologies for evaluating the effect of clopidogrel on platelet function in high-risk coronary artery disease patients. J Thromb Haemost. 2007 Sep;5(9):1839–47.
62. Kundu SK, Heilmann EJ, Sio R, Garcia C, Davidson RM, Ostgaard RA. Description of an in vitro platelet function analyzer--PFA-100. Semin Thromb Hemost. 1995;21 Suppl 2:106–12.
63. Varon D, Dardik R, Shenkman B, Kotev-Emeth S, Farzame N, Tamarin I, et al. A new method for quantitative analysis of whole blood platelet interaction with extracellular matrix under flow conditions. Thromb Res. 1997 Feb 15;85(4):283–94.
64. Luddington RJ. Thrombelastography/thromboelastometry. Clin Lab Haematol. 2005 Apr;27(2):81–90.
65. Spurgeon BEJ, Aburima A, Oberprieler NG, Taskén K, Naseem KM. Multiplexed phosphospecific flow cytometry enables large-scale signaling profiling and drug screening in blood platelets. J Thromb Haemost. 2014 Oct;12(10):1733–43.
66. Siller-Matula JM, Francesconi M, Dechant C, Jilma B, Maurer G, Delle-Karth G, et al. Personalized antiplatelet treatment after percutaneous coronary intervention:

the MADONNA study. Int J Cardiol. 2013 Sep 1;167(5):2018–23.

67. Collet J-P, Thiele H, Barbato E, Barthélémy O, Bauersachs J, Bhatt DL, et al. 2020 ESC Guidelines for the management of acute coronary syndromes in patients presenting without persistent ST-segment elevation. Eur Heart J. 2021 Apr 7;42(14):1289–367.
68. Cattaneo M. High on-treatment platelet reactivity – definition and measurement. Thromb Haemost. 2013 Nov 22;109(05):792–8.
69. Stone GW, Witzenbichler B, Weisz G, Rinaldi MJ, Neumann F-J, Metzger DC, et al. Platelet reactivity and clinical outcomes after coronary artery implantation of drug-eluting stents (ADAPT-DES): a prospective multicentre registry study. Lancet. 2013 Aug 17;382(9892):614–23.
70. Montalescot G, Rangé G, Silvain J, Bonnet J-L, Boueri Z, Barthélémy O, et al. High on-treatment platelet reactivity as a risk factor for secondary prevention after coronary stent revascularization: A landmark analysis of the ARCTIC study. Circulation. 2014 May 27;129(21):2136–43.
71. Verdoia M, Pergolini P, Nardin M, Rolla R, Suryapranata H, Kedhi E, et al. Ticagrelor and prasugrel in acute coronary syndrome: a single-arm crossover platelet reactivity study. J Cardiovasc Med (Hagerstown). 2021;22(9):686–92.
72. Price MJ, Berger PB, Angiolillo DJ, Teirstein PS, Tanguay J-F, Kandzari DE, et al. Evaluation of individualized clopidogrel therapy after drug-eluting stent implantation in patients with high residual platelet reactivity: design and rationale of the GRAVITAS trial. Am Heart J. 2009 May;157(5):818–24, 824.e1.
73. Podrez EA, Byzova T V, Febbraio M, Salomon RG, Ma Y, Valiyaveettil M, et al. Platelet CD36 links hyperlipidemia, oxidant stress and a prothrombotic phenotype. Nat Med. 2007 Sep;13(9):1086–95.
74. Togna AR, Latina V, Orlando R, Togna GI. Cigarette smoke inhibits adenine nucleotide hydrolysis by human platelets. Platelets. 2008 Nov;19(7):537–42.
75. Hioki H, Aoki N, Kawano K, Homori M, Hasumura Y, Yasumura T, et al. Acute effects of cigarette smoking on platelet-dependent thrombin generation. Eur Heart J. 2001 Jan;22(1):56–61.
76. Renaud S, Blache D, Dumont E, Thevenon C, Wissendanger T. Platelet function after cigarette smoking in relation to nicotine and carbon monoxide. Clin Pharmacol Ther. 1984 Sep;36(3):389–95.

77. Gremmel T, Steiner S, Seidinger D, Koppensteiner R, Panzer S, Kopp CW. Smoking promotes clopidogrel-mediated platelet inhibition in patients receiving dual antiplatelet therapy. Thromb Res. 2009 Nov;124(5):588–91.
78. Crimi G, Somaschini A, Cattaneo M, Angiolillo DJ, Piscione F, Palmerini T, et al. Cigarette smoking reduces platelet reactivity independently of clopidogrel treatment in patients with non-ST elevation acute coronary syndromes. Platelets. 2018 May;29(3):309–11.
79. Nardin M, Verdoia M, Negro F, Rolla R, Tonon F, De Luca G. Impact of active smoking on the immature platelet fraction and its relationship with the extent of coronary artery disease. Eur J Clin Invest. 2020 Feb 18;50(2).
80. De Luca G, Dirksen MT, Spaulding C, Kelbæk H, Schalij M, Thuesen L, et al. Impact of Diabetes on Long-Term Outcome After Primary Angioplasty. Diabetes Care. 2013 Apr 1;36(4):1020–5.
81. Wisman PP, Roest M, Asselbergs FW, de Groot PG, Moll FL, van der Graaf Y, et al. Platelet-reactivity tests identify patients at risk of secondary cardiovascular events: a systematic review and meta-analysis. J Thromb Haemost. 2014 May;12(5):736–47.
82. Nardin M, Verdoia M, Sartori C, Pergolini P, Rolla R, Barbieri L, et al. Diabetes mellitus, glucose control parameters and platelet reactivity in ticagrelor treated patients. Thromb Res. 2016 Jul;143:45–9.
83. Vivas D, Garcia-Rubira JC, Bernardo E, Angiolillo DJ, Martin P, Calle-Pascual A, et al. Effects of intensive glucose control on platelet reactivity in patients with acute coronary syndromes. Results of the CHIPS Study ("Control de Hiperglucemia y Actividad Plaquetaria en Pacientes con Sindrome Coronario Agudo"). Heart. 2011 May 15;97(10):803–9.
84. Filiopoulos V, Hadjiyannakos D, Vlassopoulos D. New Insights into Uric Acid Effects on the Progression and Prognosis of Chronic Kidney Disease. Ren Fail. 2012 May 20;34(4):510–20.
85. Elbasan Z, Şahin DY, Gür M, Kuloğlu O, Kıvrak A, İçen YK, et al. Contrast-Induced Nephropathy in Patients With ST Elevation Myocardial Infarction Treated With Primary Percutaneous Coronary Intervention. Angiology. 2014 Jan 29;65(1):37–42.

86. Barbieri L, Verdoia M, Pergolini P, Nardin M, Rolla R, Marino P, et al. Uric acid and high-residual platelet reactivity in patients treated with clopidogrel or ticagrelor. Nutr Metab Cardiovasc Dis. 2016 Apr;26(4):352–8.
87. Giovannucci E, Liu Y, Hollis BW, Rimm EB. 25-hydroxyvitamin D and risk of myocardial infarction in men: a prospective study. Arch Intern Med. 2008 Jun 9;168(11):1174–80.
88. Tarcin O, Yavuz DG, Ozben B, Telli A, Ogunc AV, Yuksel M, et al. Effect of vitamin D deficiency and replacement on endothelial function in asymptomatic subjects. J Clin Endocrinol Metab. 2009 Oct;94(10):4023–30.
89. Silvagno F, De Vivo E, Attanasio A, Gallo V, Mazzucco G, Pescarmona G. Mitochondrial localization of vitamin D receptor in human platelets and differentiated megakaryocytes. PLoS One. 2010 Jan 13;5(1):e8670.
90. Aihara K, Azuma H, Akaike M, Ikeda Y, Yamashita M, Sudo T, et al. Disruption of nuclear vitamin D receptor gene causes enhanced thrombogenicity in mice. J Biol Chem. 2004 Aug 20;279(34):35798–802.
91. López-Farré AJ, Mateos-Cáceres PJ, Sacristán D, Azcona L, Bernardo E, de Prada TP, et al. Relationship between vitamin D binding protein and aspirin resistance in coronary ischemic patients: a proteomic study. J Proteome Res. 2007 Jul;6(7):2481–7.
92. Verdoia M, Pergolini P, Rolla R, Sartori C, Nardin M, Schaffer A, et al. Vitamin D levels and high-residual platelet reactivity in patients receiving dual antiplatelet therapy with clopidogrel or ticagrelor. Platelets. 2016 Aug 17;27(6):576–82.
93. Verdoia M, Daffara V, Pergolini P, Rolla R, Marino P, Bellomo G, et al. Vitamin D Binding Protein rs7041 polymorphism and high-residual platelet reactivity in patients receiving dual antiplatelet therapy with clopidogrel or ticagrelor. Vascul Pharmacol. 2017;93–95:42–7.
94. Michos ED, Misialek JR, Selvin E, Folsom AR, Pankow JS, Post WS, et al. 25-hydroxyvitamin D levels, vitamin D binding protein gene polymorphisms and incident coronary heart disease among whites and blacks: The ARIC study. Atherosclerosis. 2015 Jul;241(1):12–7.
95. Sibbing D, Koch W, Gebhard D, Schuster T, Braun S, Stegherr J, et al. Cytochrome 2C19*17 allelic variant, platelet aggregation, bleeding events, and stent thrombosis in clopidogrel-treated patients with coronary stent placement. Circulation. 2010 Feb

2;121(4):512–8.

96. Mega JL, Simon T, Collet J-P, Anderson JL, Antman EM, Bliden K, et al. Reduced-function CYP2C19 genotype and risk of adverse clinical outcomes among patients treated with clopidogrel predominantly for PCI: a meta-analysis. JAMA. 2010 Oct 27;304(16):1821–30.
97. Simon T, Verstuyft C, Mary-Krause M, Quteineh L, Drouet E, Méneveau N, et al. Genetic determinants of response to clopidogrel and cardiovascular events. N Engl J Med. 2009 Jan 22;360(4):363–75.
98. Mega JL, Hochholzer W, Frelinger AL, Kluk MJ, Angiolillo DJ, Kereiakes DJ, et al. Dosing clopidogrel based on CYP2C19 genotype and the effect on platelet reactivity in patients with stable cardiovascular disease. JAMA. 2011 Nov 23;306(20):2221–8.
99. Carreras ET, Hochholzer W, Frelinger AL, Nordio F, O'Donoghue ML, Wiviott SD, et al. Diabetes mellitus, CYP2C19 genotype, and response to escalating doses of clopidogrel. Insights from the ELEVATE-TIMI 56 Trial. Thromb Haemost. 2016;116(1):69–77.
100. Scott SA, Sangkuhl K, Stein CM, Hulot J-S, Mega JL, Roden DM, et al. Clinical Pharmacogenetics Implementation Consortium guidelines for CYP2C19 genotype and clopidogrel therapy: 2013 update. Clin Pharmacol Ther. 2013 Sep;94(3):317–23.
101. Royal Dutch Pharmacists Association (KNMP). Dutch Pharmacogenetics Working Group (DPWG). Pharmacogenetic Guidelines [Internet]. Netherlands. Clopidogrel – CYP2C19 [Internet]. Available from: http://kennisbank.knmp.nl
102. Pereira NL, Farkouh ME, So D, Lennon R, Geller N, Mathew V, et al. Effect of Genotype-Guided Oral P2Y12 Inhibitor Selection vs Conventional Clopidogrel Therapy on Ischemic Outcomes After Percutaneous Coronary Intervention. JAMA. 2020 Aug 25;324(8):761.
103. Tavenier AH, Mehran R, Chiarito M, Cao D, Pivato CA, Nicolas J, et al. Guided and unguided de-escalation from potent P2Y12 inhibitors among patients with acute coronary syndrome: a meta-analysis. Eur Hear J - Cardiovasc Pharmacother. 2022 Aug 11;8(5):492–502.
104. Mega JL, Close SL, Wiviott SD, Shen L, Hockett RD, Brandt JT, et al. Cytochrome P450 genetic polymorphisms and the response to prasugrel: relationship to

pharmacokinetic, pharmacodynamic, and clinical outcomes. Circulation. 2009 May 19;119(19):2553–60.

105. Varenhorst C, Eriksson N, Johansson Å, Barratt BJ, Hagström E, Åkerblom A, et al. Effect of genetic variations on ticagrelor plasma levels and clinical outcomes. Eur Heart J. 2015 Aug 1;36(29):1901–12.
106. Nardin M, Verdoia M, Pergolini P, Rolla R, Barbieri L, Marino P, et al. Impact of adenosine A2a receptor polymorphism rs5751876 on platelet reactivity in ticagrelor treated patients. Pharmacol Res. 2018 Mar;129:27–33.
107. Laffont B, Corduan A, Plé H, Duchez A-C, Cloutier N, Boilard E, et al. Activated platelets can deliver mRNA regulatory Ago2•microRNA complexes to endothelial cells via microparticles. Blood. 2013 Jul 11;122(2):253–61.
108. Kaudewitz D, Skroblin P, Bender LH, Barwari T, Willeit P, Pechlaner R, et al. Association of MicroRNAs and YRNAs With Platelet Function. Circ Res. 2016 Feb 5;118(3):420–32.
109. Willeit P, Zampetaki A, Dudek K, Kaudewitz D, King A, Kirkby NS, et al. Circulating MicroRNAs as Novel Biomarkers for Platelet Activation. Circ Res. 2013 Feb 15;112(4):595–600.
110. Nagalla S, Shaw C, Kong X, Kondkar AA, Edelstein LC, Ma L, et al. Platelet microRNA-mRNA coexpression profiles correlate with platelet reactivity. Blood. 2011 May 12;117(19):5189–97.
111. Landry P, Plante I, Ouellet DL, Perron MP, Rousseau G, Provost P. Existence of a microRNA pathway in anucleate platelets. Nat Struct Mol Biol. 2009 Sep 9;16(9):961–6.
112. Elgheznawy A, Shi L, Hu J, Wittig I, Laban H, Pircher J, et al. Dicer Cleavage by Calpain Determines Platelet microRNA Levels and Function in Diabetes. Circ Res. 2015 Jul 3;117(2):157–65.
113. Fejes Z, Póliska S, Czimmerer Z, Káplár M, Penyige A, Gál Szabó G, et al. Hyperglycaemia suppresses microRNA expression in platelets to increase P2RY12 and SELP levels in type 2 diabetes mellitus. Thromb Haemost. 2017 Nov 22;117(03):529–42.
114. Peng L, Liu J, Qin L, Liu J, Xi S, Lu C, et al. Interaction between platelet-derived microRNAs and CYP2C19*2 genotype on clopidogrel antiplatelet responsiveness in patients with ACS. Thromb Res. 2017 Sep;157:97–102.

115. Barwari T, Eminaga S, Mayr U, Lu R, Armstrong PC, Chan M V., et al. Inhibition of profibrotic microRNA-21 affects platelets and their releasate. JCI Insight. 2018 Nov 2;3(21).
116. Wendel Garcia PD, Fumeaux T, Guerci P, Heuberger DM, Montomoli J, Roche-Campo F, et al. Prognostic factors associated with mortality risk and disease progression in 639 critically ill patients with COVID-19 in Europe: Initial report of the international RISC-19-ICU prospective observational cohort. EClinicalMedicine. 2020 Aug;25:100449.
117. Zapilko V, Fish RJ, Garcia A, Reny J-L, Dunoyer-Geindre S, Lecompte T, et al. MicroRNA-126 is a regulator of platelet-supported thrombin generation. Platelets. 2020 Aug 17;31(6):746–55.
118. Becker KC, Kwee LC, Neely ML, Grass E, Jakubowski JA, Fox KAA, et al. Circulating MicroRNA Profiling in Non-ST Elevated Coronary Artery Syndrome Highlights Genomic Associations with Serial Platelet Reactivity Measurements. Sci Rep. 2020 Dec 10;10(1):6169.
119. Braza-Boïls A, Barwari T, Gutmann C, Thomas MR, Judge HM, Joshi A, et al. Circulating MicroRNA Levels Indicate Platelet and Leukocyte Activation in Endotoxemia Despite Platelet P2Y12 Inhibition. Int J Mol Sci. 2020 Apr 21;21(8):2897.
120. Tsui NBY, Ng EKO, Lo YMD. Stability of endogenous and added RNA in blood specimens, serum, and plasma. Clin Chem. 2002 Oct;48(10):1647–53.
121. Gutmann C, Mayr M. Circulating microRNAs as biomarkers and mediators of platelet activation. Platelets. 2022 May 19;33(4):512–9.
122. Jansen F, Yang X, Proebsting S, Hoelscher M, Przybilla D, Baumann K, et al. MicroRNA Expression in Circulating Microvesicles Predicts Cardiovascular Events in Patients With Coronary Artery Disease. J Am Heart Assoc. 2014 Dec 17;3(6).
123. Zampetaki A, Willeit P, Tilling L, Drozdov I, Prokopi M, Renard J-M, et al. Prospective study on circulating MicroRNAs and risk of myocardial infarction. J Am Coll Cardiol. 2012 Jul 24;60(4):290–9.
124. Widera C, Gupta SK, Lorenzen JM, Bang C, Bauersachs J, Bethmann K, et al. Diagnostic and prognostic impact of six circulating microRNAs in acute coronary syndrome. J Mol Cell Cardiol. 2011 Nov;51(5):872–5.

125. De Luca G, Verdoia M, Cassetti E, Schaffer A, Cavallino C, Bolzani V, et al. High fibrinogen level is an independent predictor of presence and extent of coronary artery disease among Italian population. J Thromb Thrombolysis. 2011 May;31(4):458–63.
126. Verdoia M, Schaffer A, Barbieri L, Aimaretti G, Marino P, Sinigaglia F, et al. Impact of diabetes on neutrophil-to-lymphocyte ratio and its relationship to coronary artery disease. Diabetes Metab. 2015 Sep;41(4):304–11.
127. Henn V, Slupsky JR, Gräfe M, Anagnostopoulos I, Förster R, Müller-Berghaus G, et al. CD40 ligand on activated platelets triggers an inflammatory reaction of endothelial cells. Nature. 1998 Feb 5;391(6667):591–4.
128. André P, Nannizzi-Alaimo L, Prasad SK, Phillips DR. Platelet-derived CD40L: the switch-hitting player of cardiovascular disease. Circulation. 2002 Aug 20;106(8):896–9.
129. von Hundelshausen P, Weber C. Platelets as immune cells: bridging inflammation and cardiovascular disease. Circ Res. 2007 Jan 5;100(1):27–40.
130. Semple JW, Italiano JE, Freedman J. Platelets and the immune continuum. Nat Rev Immunol. 2011 Apr;11(4):264–74.
131. Lievens D, Zernecke A, Seijkens T, Soehnlein O, Beckers L, Munnix ICA, et al. Platelet CD40L mediates thrombotic and inflammatory processes in atherosclerosis. Blood. 2010 Nov 18;116(20):4317–27.
132. Shiraki R, Inoue N, Kawasaki S, Takei A, Kadotani M, Ohnishi Y, et al. Expression of Toll-like receptors on human platelets. Thromb Res. 2004;113(6):379–85.
133. Blair P, Flaumenhaft R. Platelet α-granules: Basic biology and clinical correlates. Blood Rev. 2009 Jul;23(4):177–89.
134. Futosi K, Fodor S, Mócsai A. Neutrophil cell surface receptors and their intracellular signal transduction pathways. Int Immunopharmacol. 2013 Nov;17(3):638–50.
135. Bdeir K, Gollomp K, Stasiak M, Mei J, Papiewska-Pajak I, Zhao G, et al. Platelet-Specific Chemokines Contribute to the Pathogenesis of Acute Lung Injury. Am J Respir Cell Mol Biol. 2017 Feb;56(2):261–70.
136. Stark K, Philippi V, Stockhausen S, Busse J, Antonelli A, Miller M, et al. Disulfide HMGB1 derived from platelets coordinates venous thrombosis in mice. Blood. 2016 Nov 17;128(20):2435–49.

137. Maugeri N, Campana L, Gavina M, Covino C, De Metrio M, Panciroli C, et al. Activated platelets present high mobility group box 1 to neutrophils, inducing autophagy and promoting the extrusion of neutrophil extracellular traps. J Thromb Haemost. 2014 Dec;12(12):2074–88.
138. Bonow RO, Fonarow GC, O'Gara PT, Yancy CW. Association of Coronavirus Disease 2019 (COVID-19) With Myocardial Injury and Mortality. JAMA Cardiol. 2020 Jul 1;5(7):751.
139. Middleton EA, He X-Y, Denorme F, Campbell RA, Ng D, Salvatore SP, et al. Neutrophil extracellular traps contribute to immunothrombosis in COVID-19 acute respiratory distress syndrome. Blood. 2020 Sep 3;136(10):1169–79.
140. Hally KE, Parker OM, Brunton-O'Sullivan MM, Harding SA, Larsen PD. Linking Neutrophil Extracellular Traps and Platelet Activation: A Composite Biomarker Score for Predicting Outcomes after Acute Myocardial Infarction. Thromb Haemost. 2021 Dec;121(12):1637–49.
141. Riegger J, Byrne RA, Joner M, Chandraratne S, Gershlick AH, ten Berg JM, et al. Histopathological evaluation of thrombus in patients presenting with stent thrombosis. A multicenter European study: a report of the prevention of late stent thrombosis by an interdisciplinary global European effort consortium. Eur Heart J. 2016 May 14;37(19):1538.1-1549.
142. Stakos DA, Kambas K, Konstantinidis T, Mitroulis I, Apostolidou E, Arelaki S, et al. Expression of functional tissue factor by neutrophil extracellular traps in culprit artery of acute myocardial infarction. Eur Heart J. 2015 Jun 7;36(22):1405–14.
143. Stähli BE, Gebhard C, Duchatelle V, Cournoyer D, Petroni T, Tanguay J, et al. Effects of the P-Selectin Antagonist Inclacumab on Myocardial Damage After Percutaneous Coronary Intervention According to Timing of Infusion: Insights From the SELECT-ACS Trial. J Am Heart Assoc. 2016 Oct 26;5(11).
144. Tardif J-C, Tanguay J-F, Wright SR, Duchatelle V, Petroni T, Grégoire JC, et al. Effects of the P-Selectin Antagonist Inclacumab on Myocardial Damage After Percutaneous Coronary Intervention for Non–ST-Segment Elevation Myocardial Infarction. J Am Coll Cardiol. 2013 May;61(20):2048–55.
145. Stähli BE, Tardif J-C, Carrier M, Gallo R, Emery RW, Robb S, et al. Effects of P-Selectin Antagonist Inclacumab in Patients Undergoing Coronary Artery Bypass Graft Surgery. J Am Coll Cardiol. 2016 Jan;67(3):344–6.

146. Kaiser R, Escaig R, Erber J, Nicolai L. Neutrophil-Platelet Interactions as Novel Treatment Targets in Cardiovascular Disease. Front Cardiovasc Med. 2022 Jan 31;8.

Part 2

Platelets Morphology and Atherosclerosis

Chapter 2

Immature platelet fraction and the extent of coronary artery disease: a single center study

Monica Verdoia [a]; Matteo Nardin [a, b]; Roberta Rolla [c]; Paolo Marino [a]; Giorgio Bellomo [c]; Harry Suryapranata [d]; Giuseppe De Luca [a]; on behalf of the Novara Atherosclerosis Study Group (NAS)

a) Department of Cardiology, Ospedale "Maggiore della Carità", Università del Piemonte Orientale, Novara, Italy; b) Department of Medicine, ASST "Spedali Civili", University of Brescia, Italy; c) Department of Clinical Chemistry, Ospedale "Maggiore della Carità", Università del Piemonte Orientale, Novara, Italy; d) Department of Cardiology, UMC St Radboud, Nijmegen, The Netherlands

Atherosclerosis 260 (2017): 110-115

Abstract

Background and aims: Immature platelet fraction (IPF) represents the quote of younger and larger sized circulating platelets, a potential marker of platelet reactivity and major cardiovascular events. We aimed to assess the relationship between IPF levels and the prevalence and extent of coronary artery disease (CAD) in patients undergoing coronary angiography.

Methods: A cohort of consecutive patients undergoing coronary angiography in a single centre were included. Significant CAD was defined as at least 1 vessel stenosis >50%, while severe CAD was defined as left main and/or three-vessel disease. IPF levels were measured at admission by routine blood cells count (A Sysmex XE-2100).

Results: We included 1789 patients, divided according to quartiles values of IPF. IPF levels were directly related to active smoke ($p = 0.02$), and non-acute coronary syndrome as indication to angiography ($p < 0.001$), higher levels of haemoglobin and uric acid ($p < 0.001$, respectively) and lower platelet count ($p = 0.003$). Angiographic features did not significantly differ according to quartiles values of IPF, but for a lower degree of TIMI flow in patients with a higher percentage of reticulated platelets ($p = 0.01$) and a higher rate of lesions involving bifurcations ($p = 0.05$). IPF levels did not affect the prevalence of CAD (77% vs. 82.2% vs. 79.1% vs. 75.6%, $p = 0.34$, adjusted OR [95% CI] = 0.93 [0.82 – 1.05], $p = 0.22$), nor of severe left main/three-vessel CAD (28.5% vs. 34.4% vs. 32.2% vs. 33.1%, $p = 0.27$; adjusted OR [95% CI] = 0.99 [0.90 – 1.1], $p = 0.88$).

Conclusions: The present study shows that among patients undergoing coronary angiography, the immature platelet fraction (IPF) is not associated with the prevalence and extent of coronary artery disease, and, therefore, should not be overlooked as a marker of coronary atherosclerosis.

Introduction

Coronary artery disease (CAD) still represents the leading cause of mortality worldwide (1), despite the improvements in the management of patients with acute myocardial infarction and the efforts being accomplished to spread preventive measures in the general population (2e5). In fact, the diagnosis of CAD is generally accomplished after a first acute cardiovascular event (6), therefore, raising the attention towards the identification of those early markers of atherosclerosis that could allow a better stratification of cardiovascular risk (7e9). In particular, platelets represent a key player in the pathogenesis of CAD, being involved in endothelial dysfunction, the development of atherosclerotic lesions and its thrombotic complications (10).

Growing attention has been recently addressed to reticulated platelets, the fraction of younger platelets lastly released from the bone marrow, that have been suggested to display a greater aggregating potential, in view of their larger size and protein synthesis capability (11,12). Immature platelets fraction (IPF) is a parameter similarly addressing the turnover of circulating platelets that displays a good correlation with the rate of reticulated platelets, but allows a more reproducible, cheaper and precise measurement (13,14). However, contrasting data has been reported so

far on the association of IPF with ischemic cardiovascular events and platelet reactivity (15e17), with no study specifically addressing the angiographic identification of coronary atherosclerosis.

Therefore, we aimed at assessing the impact of IPF levels on the prevalence and extent of CAD in a cohort of consecutive patients undergoing coronary angiography.

Materials and methods

Patients, undergoing coronary angiography between September 2012 and June 2016 at the Ospedale "Maggiore della Carità", Novara, Italy, were consecutively enrolled to participate in a crosssectional study. The only required inclusion criterium was the signature of a written informed consent. The study was approved by our local Ethical Committee. All demographic and clinical data were prospectively recorded in a dedicated database. Hypertension was defined as systolic pressure >140 mmHg and/or diastolic pressure >90 mmHg or if the individual was taking antihypertensive medications. Diabetes mellitus was defined as previous diagnosis, specific treatment administration (oral drug or insulin), fasting glycaemia >126 mg/dL or HbA1c > 6.5%. Chronic renal failure was defined for history of renal failure or an admission glomerular filtration rate (GFR) < 60 mol/min/1.73 m2 by MDRD (Modification of Diet in renal Disease) formula.

Biochemical measurements

Blood samples were drawn at admission, in elective patients, a fasting period of 12 h was required. Glucose, creatinine, glycosylated haemoglobin and lipid profile were determined by standard methods (18). Blood cells count was performed in a blood sample collected in tripotassium EDTA (7.2 mg) tubes and analyzed within 2 h from drawing by an automatic blood cells counter (A Sysmex XE-2100). The percentage of reticulated plateletswas defined as the percentage of immature platelets within the total platelet count or immature platelet fraction (IPF), determined by a fully automated sorting system (forward light scatter versus fluorescence scatterplot) of the Sysmex XE-2100 instrument, as previously described (17). The expected Coefficient of Variation (CV)was 20% according to the manufacturer.

Coronary angiography

Coronary angiography was routinely performed by a Siemens AXIOM ARTIS dTC instrument (Erlangen, Germany), with a preferential transradial access, by the Judkins technique, using 6-French right and left heart catheters. Quantitative parameters for coronary lesions were derived by an off-line analysis with an automatic edge-detection system for Quantitative Coronary Angiography (Siemens Acom Quantcor QCA, Erlangen, Germany). Minimal luminal diameter, reference diameter, percent diameter stenosis, and length of the lesion were measured. Significant CAD was defined as the presence of at least 1 coronary stenosis more than 50%. Severe CADwas defined as the presence of three-vessel and/or left main disease. In case of patients who had previously undergone percutaneous coronary interventions, the treated lesion was considered in the count of significantly diseased vessels. In carriers of coronary bypass grafts, native arteries and grafts were included in the evaluation of extension of coronary artery disease (number of diseased vessels).

Statistical analysis

Statistical analysis was performed using SPSS 22.0 statistical package. Categorical data were provided as percentage, whereas continuous data were expressed as mean ± SD. Analysis of variance and the Chi-square test were performed for continuous and categorical variables, respectively. Patients were grouped according to quartiles values of IPF. Multiple logistic regression analysis was used to evaluate the relationship between the percentage of reticulated platelets and CAD, after correction for baseline confounding factors (all variables displaying a significant association with IPF at univariate analysis (p value < 0.05) that were entered in the model in block. A p value 0.05 was considered statistically significant.

Results

We included in our study a total of 1789 patients, who were divided according to quartiles values of IPF (<1.8; 1.8-2.89; 2.9-4.39; ≥4.4%). **Table 1** displays the main clinical and demographic features of the study population. As shown, the rate of active smokers was significantly higher in patients with higher reticulated platelets (p = 0.02), as much as the rate of patients undergoing coronary angiography for a non-acute coronary syndrome indication (p < 0.001). We observed a direct relationship between IPF and higher levels of haemoglobin and uric acid (p < 0.001, respectively) and lower platelet count (p = 0.003).

Table 1. Clinical characteristics according to immature platelets fraction quartiles.

Baseline clinical characteristics	**Iquartile (<1.8) n=409**	**IIquartile (1.8-2.89) n=466**	**IIIquartile (2.9-4.39) n=460**	**IVquartile (≥4.4) n=454**	**p-value**
Age (mean±SD)	67.5±11.2	68.4±11	68.6±10.8	68.7±11.2	0.38
Male sex(%)	66.5	76.4	71.7	70	0.64
BMI (mean±SD)	26.9±4.7	27.2±4.4	27.4±4.6	27.91±5	0.36
Hypercholesterolemia(%)	54.8	55.4	58.3	52.9	0.78
Diabetes mellitus(%)	39	38.7	38.3	36.6	0.46
Renal failure(%)	26.8	26.1	29.2	25.5	0.93
Active smokers(%)	15.2	20.2	23	26	0.02
Hypertension(%)	72.9	76.8	78.5	73.6	0.72
History of MI(%)	18.3	22.5	26.7	20.7	0.23
Previous PCI(%)	39.3	39.4	41.3	35.3	0.32
Previous CABG(%)	8.6	12.2	11.5	10.4	0.51
Indication to angiography					<0.001
Stable angina/ silent ischemia(%)	37.7	39.4	39.4	27.9	
ACS(%)	45.3	46.4	42.6	43.5	

Cardiomyopathy/ valvulardisease/ arrhythmias(%)	17	14.2	18	26.8	
Concomitant medications					
ACE inhibitors(%)	32.8	35.3	37.5	37.6	0.11
ARB(%)	27.6	24.5	25.7	24.2	0.36
Beta-blockers(%)	59.9	59.4	64.1	62.7	0.20
Nitrates(%)	36.5	33.8	37.4	33.6	0.62
Statins(%)	53.4	56	57.8	54	0.78
ASA(%)	60.1	62.2	67.6	62.4	0.24
Clopidogrel(%)	16.7	19.8	17.8	18.4	0.74
Calcium antagonists(%)	23.6	20	22.6	23.1	0.86
Diuretics(%)	33.5	31.9	36.5	37.3	0.11
Biochemistry parameters					
Platelets(10^6/ml;mean±SD)	258.6±72.4	238.3±69.9	222±62.5	200.6±68.5	0.003
Haemoglobin(g/dl)	13±1.8	13.3±1.7	13.4±1.7	13.4±2	<0.001
WBC(10^3/ml;mean±SD)	7.8±2.6	8.5±3.3	8.1±2.5	8.3±2.9	0.19
HDL cholesterol(mg/dl)	43.3±13	43.3±12.4	43.2±12.8	44.5±15.2	0.39
LDL cholesterol(mg/dl)	92.9±36.8	95.4±35.9	90.7±33.3	95.6±36.1	0.18
Glycaemia(mg/dl)	121.1±47	122.6±47.3	120.6±43.6	120.8±43.1	0.90
Glycosylated haemoglobin(%)	6.2±1.1	6.2±1.4	6.3±1.3	6.3±1.5	0.52
Creatinine(mg/dl)	0.99±0.55	1.07±0.82	1.06±0.7	1.01±0.58	0.36
C-reactive protein(mg/dl)	0.89±1.8	0.99±2.1	1.1±2.6	1.2±2.9	0.14
Uric acid(mg/dl)	5.6±1.7	5.9±1.9	6.1±1.9	6.2±2	<0.001

Angiographic features did not significantly differ according to quartiles values of IPF, as shown in **Table 2**. but for a lower degree of TIMI flow in patients with higher percentage of reticulated platelets ($p = 0.01$) and a larger prevalence of lesions involving bifurcations ($p = 0.05$).

Table 2. Angiographic features according to immature platelets fraction quartiles.

Angiographic features	**Iquartile (<1.8) n=759**	**IIquartile (1.8-2.89) n=930**	**IIIquartile (2.9-4.39) n=84**	**IVquartile (≥4.4) n=869**	**p-value**
Left main disease(%)	7.6	8.4	7.9	8.4	0.78
LAD(%)	57.2	65.4	59.9	56.3	0.36
CX(%)	48.2	50.1	48.1	45.7	0.36
RCA(%)	46.2	51.1	49.9	52.5	0.08
Type C lesion(%)	18.9	20.3	20.8	18.8	0.24
Lesion length(mm)	24±15.3	22.9±14.5	24.3±15.4	23.8±15.2	0.29
Percent stenosis(%)	85±11.9	86.3±10.7	86.2±11.7	86.2±11.7	0.08
Reference diameter(mm)	2.95±0.5	2.95±0.5	2.92±0.5	2.92±0.5	0.25
Bifurcations(%)	20.7	20.4	22.3	24.2	0.05
Calcifications(%)	12.5	16.2	14.6	13.4	0.95
Chronic occlusion(%)	14.1	13.4	16.6	15.5	0.18
Restenosis(%)	5.8	7	8.2	6.8	0.55
Thrombus(%)	2	3.7	3.4	3.2	0.24
TIMIflow					0.01
3	79.3	76.7	72.4	74.1	
2	3.3	5.7	5.2	5.4	
1	3.2	3.2	3.5	3.6	
0	14.2	14.4	18.9	16.2	

The prevalence of coronary artery disease did not significantly differ according to the levels of IPF (77% vs. 82.2% vs. 79.1% vs. 75.6%, p=0.34, OR [95% CI] = 0.95 [0.86-1.05], p = 0.34), as showed in **Figure 1**.

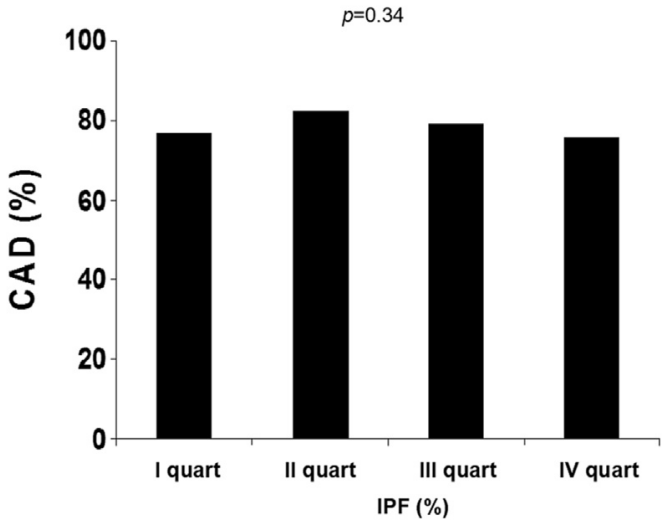


Figure 1. Bar graphs showing the prevalence of coronary artery disease (CAD) according to immature platelet fraction (IPF) quartiles.

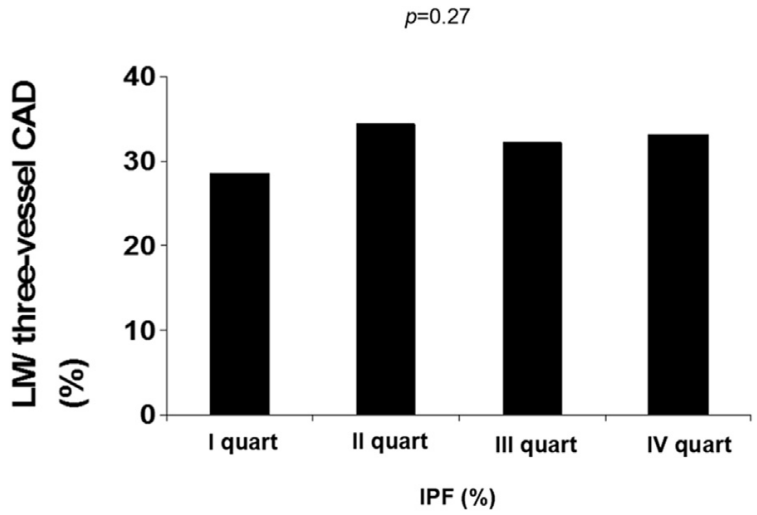


Figure 2. Bar graphs showing the prevalence of severe left main/three-vessel coronary artery disease (CAD) according to immature platelet fraction (IPF) quartiles.

Similar results were obtained when considering the prevalence of severe left main/three-vessel CAD (28.5% vs. 34.4% vs. 32.2% vs. 33.1%, p = 0.27; OR [95% CI] = 1.05 [0.96-1.15], p = 0.27), as shown in **Figure 2**. No impact of reticulated platelets (across quartiles) was confirmed after correction for baseline confounders both for CAD (adjusted OR [95% CI] = 0.93 [0.82-1.05], p = 0.22) and severe CAD (adjusted OR [95% CI] = 0.99 [0.90-1.1], p = 0.88).

Discussion

The present study represents the largest single center cohort study attempting to define a role of immature platelet fraction on the prevalence and extent of coronary disease as detected at angiography. We found no impact of this platelet parameter on the rate of CAD or severe left main/three-vessel disease. Recent advances in pharmacological therapies and percutaneous coronary interventions have dramatically improved the survival of patients with cardiovascular disease, especially after an acute myocardial infarction (19-22). In particular, raising attention has been addressed to the crucial role of platelets in the pathogenesis of acute ischemic events, with emerging evidence in favor of a greater protection from acute thrombotic events with a more prolonged antiplatelet therapy (23,24). Nevertheless, the preventive strategies developed so far have failed to reduce the burden of cardiovascular disease, with CAD still representing the leading cause of mortality worldwide. Therefore, efforts have been made to predict in advance the cardiovascular risk, by attempting to identify early markers of coronary atherosclerosis (25,26). Due to their pivotal role, several parameters of platelet size have been analyzed as potential indicators of CAD, with contrasting results. In particular, previous studies have suggested that larger platelets could display a greater aggregating potential, presenting a larger aggregating surface and a higher content of receptors and granules, that promote their activation (27,28). However, we previously documented no impact of mean platelet volume or platelet size variability on angiographic findings and neither on platelet reactivity in large cohorts of patients (29-31), suggesting that these parameters could be more related to thrombopoiesis or clinical conditions, such as age or diabetes, rather than markers of thrombotic risk. Emerging interest has been addressed to the fraction of reticulated platelets that represent the quote of younger platelets, lastly released from bone marrow, displaying a larger size, higher granules and RNA content and increased

capability of proteins synthesis and then a potentially enhanced reactivity (12,32). Immature platelet fraction (IPF) is a parameter allowing the identification of the fraction of reticulated platelets with a cheap, reliable and easy to obtain analysis, using traditional cells counters (11). Previous reports have linked IPF levels to cardiovascular disease. In fact, Grove et al. have detected higher levels of IPF in patients with acute coronary syndromes and several studies have documented, in small cohorts of patients, that IPF can predict the antiplatelet response to thienopyridines (33-35). However, despite promising results relating reticulated platelets to an impaired response to antiplatelet agents (36,37), other studies, including a cohort of patients from our center, have failed to identify such an association (38,39). In addition, no study has so far addressed the impact of IPF on angiographic findings and the extent of coronary artery disease. In effect, Larsen et al. (40) have demonstrated that the levels of RP relate to low grade inflammation and biomarkers as CRP that are known indicators of the progression of atherosclerosis.

The present study represents the first attempt to define a role of IPF in CAD among consecutive patients undergoing coronary angiography. Our main findings were the absence of an association between reticulated platelets and the prevalence or the extent of CAD in a large cohort of patients, even after correction for baseline differences. According to previous literature, we found an association of IPF levels with active smoke. Consistently with our results, Lupia et al. demonstrated significantly increased thrombopoietin levels in smokers as compared with non-smokers in healthy individuals (41), an association that has been reported to potentially explain a pro-thrombotic state in smokers (42). On the contrary, we identified an association between lower levels of IPF in patients with acute coronary syndrome. Indeed, it must be underlined that the rate of IPF observed in our population was lower than the reference range recently reported (43) for this dosing, and also inferior to the values detected in previous studies (33,44). Moreover,

only half of our population was represented by acute patients, while including a large proportion of patients with stable CAD, with a history of previous PCI and already treated with antiplatelet agents, thus potentially interfering with platelet function and turnover. In addition, we included patients undergoing coronary angiography for valvular disease or cardiomyopathies that were often admitted for heart failure, a condition that has been associated with platelet activation (45). Therefore, it might be inferred that the highest rate of reticulated platelets in these patients might be a consequence of the peripheral consumption of smaller, fully mature platelets, induced by the pro-thrombotic and pro-inflammatory milieu, promoting the release in the bloodstream of immature platelet precursors (40,46). IPF, then, would represent a marker of platelet turnover rather than being directly involved in the pathogenesis of cardiovascular disease. Thus, until new data would become available, according to present findings, reticulated platelets should not be overlooked as an indicator for the assessment of the risk CAD in patients undergoing coronary angiography.

Limitations

A first limitation to our study should be considered the study design, enrolling a heterogeneous population of consecutive patients undergoing coronary angiography for both acute and non-ACS indications. Multicenter patients' recruitment would have further confirmed the applicability of our findings on a large scale. Therefore, additional larger studies are certainly needed to further confirm our results. Moreover, we did not include a control healthy group in our study, since this strategy would have raised some issues: as coronary angiography still represents the gold standard technique to evaluate the presence and extent of CAD, such exam could not have been performed in healthy subjects. In fact, the absence of symptoms would not have excluded with certainty the absence of coronary

atherosclerosis, especially among elderly and diabetic patients representing the majority of our population. By the inclusion of a prospective consecutive cohort of patients undergoing coronary angiography, we could overcome a bias due to a potential patients' selection, when they are retrospectively identified. However, this strategy leads to an unbalance between the cohort of patients with and without CAD, thus not allowing us to perform a proper case-control analysis of the data. The use of intravascular imaging, such as intravascular ultrasound (IVUS) or optical coherence tomography (OCT), would certainly have provided a more precise characterization of coronary plaques, contributing to improve our findings. Nevertheless, such techniques cannot be applied on a very large scale. The isolated association of IPF with single lesions' parameters, such as bifurcation lesions, can be considered only an occasional observation. We did not collect follow-up data, especially in patients undergoing coronary angioplasty, and thus cannot exclude an impact of IPF on the progression of CAD or on the occurrence of acute thrombotic events. Moreover, the study design and the inclusion of a population with a very high cardiovascular risk do not allow us to evaluate the role of reticulated platelets in the early stage of coronary artery disease and its progression and to address the underlying pathophysiological mechanisms either. In addition, no formal sample size calculation was performed. However, based on an expected prevalence of CAD of 75%, and considering as clinically relevant an increase in the relative risk of significant atherosclerosis of 10% associated with IPF above the median value, our sample size would have guaranteed a statistical power of 91%. Finally, the use of the automated measurement of IPF instead of a direct assessment of reticulated platelets, might have conditioned our results, as the instrumental sorting of platelets can lead to the identification of more immature fractions and exclude certain small but young and fully active platelets. However, a good correlation has been previously reported between reticulated platelets and IPF and moreover, flow

cytometric technique, based on RNA staining by thiazole orange, represents a complex and much less reproducible method for analyzing the reticulated platelets (47), especially when aiming at the identification of a marker for a large-scale assessment of cardiovascular risk.

Conclusions

The present study shows that among patients undergoing coronary angiography, the immature platelet fraction (IPF) is not associated with the prevalence and extent of coronary artery disease, and, therefore, should not be overlooked as a marker of coronary atherosclerosis.

References

1. J.A. Finegold, P. Asaria, D.P. Francis, Mortality from ischaemic heart disease by country, region, and age: statistics from world health organization and united, Int. J. Cardiol. 168 (2) (2013) 934e945.
2. G. De Luca, E. Cassetti, P. Marino, et al., Percutaneous coronary intervention related time delay, patient's risk profile, and survival benefits of primary angioplasty vs. lytic therapy in ST-segment elevation myocardial infarction, Am. J. Emerg. Med. 27 (6) (2009) 712e719.
3. E.P. Navarese, G. De Luca, F. Castriota, M. Kozinski, P.A. Gurbel, C.M. Gibson, F. Andreotti, A. Buffon, J.M. Siller-Matula, A. Sukiennik, S. De Servi, J. Kubica, Low-molecular-weight heparins vs. unfractionated heparin in the setting of percutaneous coronary intervention for ST-elevation myocardial infarction: a meta-analysis, J. Thromb. Haemost. 9 (2011) 1902e1915.
4. L. Pace, P. Perrone-Filardi, P. Mainenti, A. Cuocolo, P. Vezzuto, M. Prastaro, A. Varrone, G. De Luca, A. Soricelli, S. Betocchi, M. Chiariello, M. Salvatore, Identification of viable myocardium in patients with chronic coronary artery disease using rest-redistribution thallium-201 tomography: optimal image analysis, J. Nucl. Med. 39 (1998) 1869e1874.
5. M. Verdoia, A. Schaffer, H. Suryapranata, G. De Luca, Effects of HDL-modifiers on cardiovascular outcomes: a meta-analysis of randomized trials, Nutr. Metab. Cardiovasc Dis. 25 (2015) 9e23.
6. D.A. Morrow, Cardiovascular risk prediction in patients with stable and unstable coronary heart disease, Circulation 121 (2010) 2681e2691.
7. L. Barbieri, M. Verdoia, A. Schaffer, P. Marino, H. Suryapranata, G. De Luca, Novara Atherosclerosis Study Group (NAS). Impact of sex on uric acid levels and its relationship with the extent of coronary artery disease: a single-centre study, Atherosclerosis 241 (2015) 241e248.
8. G. De Luca, M. Verdoia, E. Cassetti, A. Schaffer, C. Cavallino, V. Bolzani, P. Marino, Novara Atherosclerosis Study Group (NAS). High fibrinogen level is an independent predictor of presence and extent of coronary artery disease among Italian population, J. Thromb. Thrombolysis 31 (2011) 458e463.

9. M. Verdoia, A. Schaffer, C. Sartori, L. Barbieri, E. Cassetti, P. Marino, G. Galasso, G. De Luca, Vitamin D deficiency is independently associated with the extent of coronary artery disease, Eur. J. Clin. Invest. 44 (7) (2014 Jul) 634e642.
10. G. De Luca, S. Savonitto, A.W. van't Hof, H. Suryapranata, Platelet GP IIb-IIIa receptor antagonists in primary angioplasty: back to the future, Drugs 75 (2015) 1229e1253.
11. J.J. Hoffmann, Reticulated platelets: analytical aspects and clinical utility, Clin. Chem. Lab. Med. 52 (2014) 1107e1117.
12. K.A. Ault, H.M. Rinder, J. Mitchell, M.B. Carmody, C.P. Vary, R.S. Hillman, The significance of platelets with increased RNA content (reticulated platelets). A measure of the rate of thrombopoiesis, Am. J. Clin. Pathol. 98 (1992) 637e646.
13. S.J. Machin, Development of a consensus standard reference method for immature platelets, Int. J. Lab. Hematol. 35 (2013) 26.
14. I. Pons, M. Monteagudo, G. Lucchetti, L. Mu~noz, G. Perea, I. Colomina, J. Guiu, J. Obiols, Correlation between immature platelet fraction and reticulated platelets. Usefulness in the etiology diagnosis of thrombocytopenia, Eur. J. Haematol. 85 (2) (2010 Aug) 158e163.
15. F. Cesari, R. Marcucci, A.M. Gori, R. Caporale, A. Fanelli, G. Casola, D. Balzi, A. Barchielli, S. Valente, C. Giglioli, G.F. Gensini, R. Abbate, Reticulated platelets predict cardiovascular death in acute coronary syndrome patients: insights from the AMI Florence 2 study, Thromb. Haemost. 109 (2013) 846e853.
16. N. Lakkis, H. Dokainish, M. Abuzahra, V. Tsyboulev, J. Jorgensen, A.P. De Leon, A. Saleem, Reticulated platelets in acute coronary syndrome: a marker of platelet activity, J. Am. Coll. Cardiol. 44 (2004) 2091e2093.
17. M. Verdoia, P. Pergolini, M. Nardin, R. Rolla, L. Barbieri, A. Schaffer, P. Marino, G. Bellomo, H. Suryapranata, G. De Luca, Impact of diabetes on immature platelets fraction and its relationship with platelet reactivity in patients receiving dual antiplatelet therapy, J. Thromb. Thrombolysis 42 (2) (2016) 245e253.
18. G. De Luca, G.G. Secco, M. Santagostino, L. Venegoni, S. Iorio, E. Cassetti, M. Verdoia, L. Coppo, C. Di Mario, G. Bellomo, P. Marino, Novara Atherosclerosis Study Group (NAS). Uric acid does not affect the prevalence and extent of coronary artery disease. Results from a prospective study, Nutr. Metab. Cardiovasc Dis. 22 (5) (2012 May) 426e433.

19. G. De Luca, A. Schaffer, J. Wirianta, H. Suryapranata, Comprehensive metaanalysis of radial vs. femoral approach in primary angioplasty for STEMI, Int. J. Cardiol. 168 (2013) 2070e2081.
20. E. Di Lorenzo, R. Sauro, M. Capasso, F. Lanni, T. Lanzillo, G. Carbone, F. Manganelli, V. Palmieri, V. Serino, M.R. Pagliuca, G. Rosato, H. Suryapranata, G. De Luca, Long-term results of the randomized comparison of everolimuseluting stents and sirolimus-eluting stent in patients with ST elevation myocardial infarction (RACES-MI trial), Int. J. Cardiol. 202 (2016) 177e182.
21. G. De Luca, H. Suryapranata, G.W. Stone, D. Antoniucci, J.E. Tcheng, F.J. Neumann, E. Bonizzoni, E.J. Topol, M. Chiariello, Relationship between patient's risk profile and benefits in mortality from adjunctive abciximab to mechanical revascularization for ST-segment elevation myocardial infarction: a meta-regression analysis of randomized trials, J. Am. Coll. Cardiol. 47 (2006) 685e686.
22. G. De Luca, M.T. Dirksen, C. Spaulding, H. Kelbaek, M. Schalij, L. Thuesen, B. van der Hoeven, M.A. Vink, C. Kaiser, C. Musto, T. Chechi, G. Spaziani, L.S. Díaz de la Llera, V. Pasceri, E. Di Lorenzo, R. Violini, G. Cortese, H. Suryapranata, G.W. Stone, Drug-Eluting Stent in Primary Angioplasty (DESERT) Cooperation. Drug-eluting vs. bare-metal stents in primary angioplasty: a pooled patient-level meta-analysis of randomized trials, Arch. Intern Med. 172 (8) (2012) 611e621.
23. L. Mauri, S.C. Smith Jr., Focused update on duration of dual antiplatelet therapy for patients with coronary artery disease, JAMA Cardiol. 1 (6) (2016) 733e734.
24. D.L. Bhatt, M.P. Bonaca, S. Bansilal, D.J. Angiolillo, M. Cohen, R.F. Storey, K. Im, S.A. Murphy, P. Held, E. Braunwald, M.S. Sabatine, P.G. Steg, Reduction in ischemic events with ticagrelor in diabetic patients with prior myocardial infarction in PEGASUS-TIMI 54, J. Am. Coll. Cardiol. 67 (23) (2016) 2732e2740.
25. M.T. Cooney, A.L. Dudina, I.M. Graham, Value and limitations of existing scores for the assessment of cardiovascular risk: a review for clinicians, J. Am. Coll. Cardiol. 54 (14) (2009) 1209e1227.
26. C. Vlachopoulos, P. Xaplanteris, V. Aboyans, M. Brodmann, R. Cífkova, F. Cosentino, M. De Carlo, A. Gallino, U. Landmesser, S. Laurent, J. Lekakis, D.P. Mikhailidis, K.K. Naka, A.D. Protogerou, D. Rizzoni, A. Schmidt-Trucksass, L. Van Bortel, T. Weber, A. Yamashina, R. Zimlichman, P. Boutouyrie, J. Cockcroft, M. O'Rourke, J.B. Park, G. Schillaci, H. Sillesen, R.R. Townsend, The role of vascular

biomarkers for primary and secondary prevention. A position paper from the european society of cardiology working group on peripheral circulation: endorsed by the association for research into arterial structure and physiology (ARTERY) society, Atherosclerosis 241 (2) (2015) 507e532.

27. S.G. Khaspekova, I.T. Ziuriaev, V.V. Iakushkin, IaA. Naĭmushin, et al., Mean platelet volume: interactions with platelet aggregation activity and glycoprotein IIb-IIIa and Ib expression levels, Biomed. Khim 60 (2014) 94e108.
28. A. Ihara, T. Kawamoto, K. Matsumoto, S. Shouno, C. Hirahara, T. Morimoto, Y. Noma, Relationship between platelet indexes and coronary angiographic findings in patients with ischemic heart disease, Pathophysiol. Haemost. Thromb. 35 (5) (2006) 376e379.
29. G. De Luca, M. Verdoia, E. Cassetti, A. Schaffer, G. Di Giovine, A. Bertoni, C. Di Vito, S. Sampietro, G. Aimaretti, G. Bellomo, P. Marino, F. Sinigaglia, Novara Atherosclerosis Study (NAS) group. Mean platelet volume is not associated with platelet reactivity and the extent of coronary artery disease in diabetic patients, Blood Coagul. Fibrinolysis 24 (2013) 619e624.
30. M. Verdoia, P. Pergolini, R. Rolla, M. Nardin, L. Barbieri, A. Schaffer, G. Bellomo, P. Marino, H. Suryapranata, G. De Luca, Novara atherosclerosis study group (NAS).Platelet larger cell ratio and high-on treatment platelet reactivity during dual antiplatelet therapy, Cardiovasc. Drugs Ther. 29 (5) (2015) 443e450.
31. M. Verdoia, L. Barbieri, A. Schaffer, E. Cassetti, G. Di Giovine, G. Bellomo, P. Marino, F. Sinigaglia, G. De Luca, Platelet distribution width and the risk of periprocedural myocardial infarction in patients undergoing percutaneous coronary intervention, J. Thromb. Thrombolysis 37 (3) (2014) 345e352.
32. L.M. Dusse, L.G. Freitas, Clinical applicability of reticulated platelets, Clin. Chim. Acta 439 (2015) 143e147.
33. E.L. Grove, A.-M. Hvas, S.D. Kristensen, Immature platelets in patients with acute coronary syndromes, Thromb. Haemost. 101 (2009) 151e156.
34. H. Ibrahim, S. Nadipalli, T. Delao, J. Paranilam, C. Barker, N. Kleiman, Immature platelets for prediction of clinical events: a possible future target for antiplatelet therapy, Cathet. Cardiovasc. Interv. 81 (2013) S108eS109.
35. I. Bernlochner, A. Goedel, C. Plischke, S. Schüpke, B. Haller, C. Schulz, K. Mayer, T. Morath, S. Braun, H. Schunkert, W. Siess, A. Kastrati, K.L. Laugwitz, Impact of

immature platelets on platelet response to ticagrelor and prasugrel in patients with acute coronary syndrome, Eur. Heart J. 36 (45) (2015) 3202e3210.

36. M. Vaduganathan, N. Zemer-Wassercug, E. Rechavia, H. Lerman-Shivek, L. Perl, D. Leshem-Lev, K. Orvin, R. Kornowski, E.I. Lev, Relation between ticagrelor response and levels of circulating reticulated platelets in patients with non-ST elevation acute coronary syndromes, J. Thromb. Thrombolysis 40 (2) (2015) 211e217.
37. S. Guthikonda, C.L. Alviar, M. Vaduganathan, M. Arikan, A. Tellez, T. DeLao, J.F. Granada, J.F. Dong, N.S. Kleiman, E.I. Lev, Role of reticulated platelets and platelet size heterogeneity on platelet activity after dual antiplatelet therapy with aspirin and clopidogrel in patients with stable coronary artery disease, J. Am. Coll. Cardiol. 52 (2008) 743e749.
38. C. Stratz, T. B€omicke, I. Younas, A. Kittel, M. Amann, C.M. Valina, T. Nührenberg, D. Trenk, F.J. Neumann, W. Hochholzer, Comparison of immature platelet count to established predictors of platelet reactivity during thienopyridine therapy, J. Am. Coll. Cardiol. 68 (3) (2016) 286e293.
39. M. Verdoia, C. Sartori, P. Pergolini, M. Nardin, R. Rolla, L. Barbieri, A. Schaffer, P. Marino, G. Bellomo, H. Suryapranata, G. De Luca, Immature platelet fraction and high-on treatment platelet reactivity with ticagrelor in patients with acute coronary syndromes, J. Thromb. Thrombolysis 41 (2016) 663e670.
40. S.B. Larsen, E.L. Grove, A.M. Hvas, S.D. Kristensen, Platelet turnover in stable coronary artery disease - influence of thrombopoietin and low-grade inflammation, PLoS One 9 (2014) e85566.
41. E. Lupia, O. Bosco, S. Bergerone, A.E. Dondi, A. Goffi, et al., Thrombopoietin contributes to enhanced platelet activation in patients with unstable angina, J. Am. Coll. Cardiol. 48 (2006) 2195e2203.
42. Y. Takajo, H. Ikeda, N. Haramaki, T. Murohara, T. Imaizumi, Augmented oxidative stress of platelets in chronic smokers. Mechanisms of impaired plateletderived nitric oxide bioactivity and augmented platelet aggregability, J. Am. Coll. Cardiol. 38 (2001) 1320e1327.
43. L. Meintker, M. Haimerl, J. Ringwald, S.W. Krause, Measurement of immature platelets with Abbott CD-Sapphire and Sysmex XE-5000 in haematology and oncology patients, Clin. Chem. Lab. Med. 51 (11) (2013) 2125e2131.

44. R.A. Lopez-Jimenez, F. Martín-Herrero, J.R. Gonzalez-Porras, M. Sanchez- Barba, C. Martín-Luengo, P. Pabon-Osuna, Immature platelet fraction: a new prognostic marker in acute coronary syndrome, Rev. Esp. Cardiol. Engl. Ed. 66 (2) (2013) 147e148.
45. I. Chung, A. Choudhury, G.Y. Lip, Platelet activation in acute, decompensated congestive heart failure, Thromb. Res. 120 (5) (2007) 709e713.
46. N.M. Smith, R. Pathansali, P.M. Bath, Altered megakaryocyte-platelethaemostatic axis in patients with acute stroke, Platelets 13 (2) (2002) 113e120.
47. J.J. Hoffmann, Reticulated platelets: analytical aspects and clinical utility, Clin. Chem. Lab. Med. 52 (2014) 1107e1117.

Chapter 3

Impact of active smoking on the immature platelet fraction and its relationship with the extent of coronary artery disease

Matteo Nardin [a,b]; Monica Verdoia [a,c]; Federica Negro[a]; Roberta Rolla[d]; Francesco Tonon[a]; Giuseppe De Luca[a,]

a) Division of Cardiology, Azienda Ospedaliera-Universitaria "Maggiore della Carità", EasternPiedmontUniversity, Novara, Italy; b) Department of Medicine, ASST "Spedali Civili", University of Brescia, Italy; c) Division of Cardiology, "Ospedale degli Infermi", Biella, Italy; d) Division of Clinical Chemistry, Azienda Ospedaliera-Universitaria "Maggiore della Carità", Eastern Piedmont University, Novara, Italy

Eur J Clin Invest (2020) 50: e13181

Abstract

Introduction. Smoking represents a major cardiovascular risk factor, due to the induction of oxidative stress and low-grade, continuous, inflammation that contribute to promote atherothrombosis. However, the mechanisms leading to increased platelet aggregability associated with smoking are only partially defined. A potential role has been hypothesized for immature platelets, a younger and potentially more reactive fraction, previously associated to the main determinants of coronary artery disease (CAD). Therefore, the aim of our study was to define the impact of smoking on the immature platelet fraction (IPF) and its relationship with prevalence and extent of coronary artery disease.

Methods. We enrolled a cohort of consecutive patients undergoing coronary angiography in a single center. Significant CAD was defined as at least 1 vessel stenosis >50%, while severe CAD was defined as left main and/or three-vessel disease. IPF was measured at admission by routine blood cells count (A Sysmex XE-2100).

Results. We included in our study 2553 patients, that were divided according to smoking status (active smokers: 512; non-active smokers: 2041). Smokers were younger, more frequent males, with lower rate of diabetes mellitus, previous PCI, previous CABG ($p<0.001$, respectively) and were in treatment less often with ARB, BB, nitrates, statins, ASA, Clopidogrel, CCB, diuretics ($p<0.001$, respectively) as compared to non-active smokers. Higher percentage of smokers was observed in patients with higher IPF values, and at multivariate analysis active smoking resulted an independent predictor of higher IPF (adjusted OR [95% CI]=1.59[1.03 – 2.45] p=0.035). Among smokers, higher IPF were associated to lower ejection fraction (p=0.034), percentage of acute coronary syndrome (p=0.002) and platelet count ($p<0.001$) compared to ones with lower IPF. However, the IPF (according to quartiles values) were not associated to the prevalence and extent of CAD (82.5%, 80.4%, 86.1%, 80.9%, from 1^{st} to 4^{th} quartile, respectively, adjusted OR

[95% CI] = 0.98 [0.79 – 1.23] p=0.89) and severe CAD (31%, 31.1%, 39.1%, 35.2%,from 1st to 4th quartile, respectively, adjusted OR[95% CI]=1.03 [0.86 – 1.23] p=0.76).

Conclusion. The present study shows an independent association between active smoking and the levels of immature platelet fraction in patients undergoing coronary angiography. However, among active smokers, IPF did not result an independent predictor of CAD or severe CAD

Introduction

Active smoking represents one of main cardiovascular risk factor, enhancing the development of both atherosclerosis and its thrombotic complications (1). In fact, smoking has been established to enhance oxidative stress and low-grade inflammation, increasing radical oxygen production (ROS) and triggering the response of the immune system and platelets, the major actors in the development of atherothrombotic events (2).

Great interest has been recently focused on platelet subpopulations, and in particular on the role of reticulated platelets, the younger platelets lastly released from the bone marrow, that have been hypothesized as having an increased aggregating potential and, then, a larger impact on the atherothrombotic processes. However, contrasting data have been reported on the association between reticulated platelets and coronary artery disease (CAD)(3–5). A similar role has been also hypothesized for the Immature platelet fraction (IPF), that constitutes a parameter addressing the turnover of circulating platelets. Being a cheaper, more reproducible and precise measurement, and displaying a good correlation with the reticulated platelets, the assessment of IPF has been preferred to the direct evaluation of reticulated thrombocytes, in daily clinical practice (6,7).

Previous studies have reported a potential increase in the thrombotic risk due to the effect of smoking, mediated by an enhanced platelet activation and turnover (8,9).

In fact, previous reports have documented that the systemic inflammation sustained by smoking could raise acute phase proteins and proinflammatory cytokines, stimulating the hematopoietic system and resulting in the release of leukocytes and platelets into bloodstream(10).

However, no study has so far evaluated the relationship between smoking and reticulated plateletsand their impact on CAD, that was therefore the aim of our investigation.

Methods

Our study population is represented by consecutive patients undergoing coronary angiography between September 2012 and September 2018 at the Ospedale "Maggiore dellaCarità", Novara, Italy. We excluded patients who refused to sign the informed consent before angiography. The study was approved by our local Ethical Committee. All demographic and clinical data were prospectively collected in a dedicated database. Active smokers were defined for smoking of any number of cigarettes per day at admission, while patients were considered non-active smoking if they never smoked or were former smokers.

Biochemical measurements

Blood samples were drawn at admission in patients undergoing elective (following a fasting period of 12 h) or urgent coronary angiography. Glucose, creatinine, glycosylated haemoglobin and lipid profile were determined by standard methods. Blood cells count was performed in a blood sample collected in tripotassium EDTA (7.2 mg) tubes and analysed within 2 h from drawing by an automatic blood cells counter (A Sysmex XE-2100). The percentage of reticulated platelets was defined as the percentage of immature platelets within the total platelet count or immature platelet fraction (IPF), determined by a fully automated sorting system (forward light scatter versus fluorescence scatterplot) of the Sysmex XE-2100 instrument, as previously described(11). The expected Coefficient of Variation (CV) was ≤ 20% according to the manufacturer. Immature platelet count (IPC) was defined as the absolute value of IPF according to the total platelet count.

Coronary angiography

Coronary angiography (carried out by Siemens AXIOM ARTIS dTC, Erlangen, Germany) was routinely performed by the Judkins technique using 6-French right and left heart catheters. Quantitative coronary angiography was performed by two experienced interventional cardiologists, by an automatic edge-detection system for Quantitative Coronary Angiography (Siemens AcomQuantcor QCA, Erlangen, Germany). After a visual inspection of the coronary artery, the frame of optimal clarity was selected, showing lesion at maximal narrowing and arterial silhouette in sharpest focus. After the calibration of guiding catheter, analysed arterial segment with coronary lesion was defined by moving the cursor from the proximal to the distal part of coronary artery to ensure adequate determination of reference diameter. Minimal luminal diameter, reference diameter, percent diameter stenosis, and length of the lesion were measured. Significant CAD was defined as the presence of at least 1 coronary stenosis more than 50%. Severe CAD was defined as the presence of three-vessel disease and/or left main disease. For patients who had previously undergone percutaneous coronary interventions, the treated lesion was considered as significantly diseased vessel. In previously bypassed patients, both native arteries and grafts were taken into account in the evaluation of extension of coronary artery disease (number of diseased vessels).

Statistical analysis

Statistical analysis was performed using SPSS 22.0 statistical package. Continuous data were expressed as mean + SD and categorical data as percentage. Analysis of variance and the chi-square test were used for continuous and categorical variables, respectively.

Patients were grouped according to smoking status. Multiple logistic regression analysis was performed to evaluate the relationship between smoking status and IPF values, after

correction for baseline confounding factors, that were entered in the model in block. In active smokers, multiple logistic regression analysis was performed to evaluate the relationship between IPF values and CAD and severe CAD. A p value < 0.05 was considered statistically significant.

Results

We included in our study 2553 patients, that were divided according to smoking status (active smokers: 512; non-active smokers: 2041).

Main clinical and demographic features are shown in **Table 1**. Smokers were younger (p<0.001), more frequently males (p<0.001), displayed a lower rate of renal failure (p=0.04), diabetes mellitus, hypertension, previous PCI, previous CABG (p<0.001 respectively) as compared to non-active smokers.

Table 1. Clinical and demographic characteristics according to smoking status

Baseline clinical characteristics	**Active smokers (n=512)**	**Non-active smokers (n=2041)**	**p-value**
Age (mean□SD)	62.01 (±10.86)	70.05 (±10.55)	<0.001
Age ≥75 years (%)	15.3	38.4	<0.001
Male sex (%)	78.5	70.3	<0.001
BMI (mean±SD)	27.23 (±5.14)	27.16 (±4.89)	0.77
Hypercholesterolemia (%)	54.4	57.6	0.19
Renal failure (%)	13.7	19.1	0.04
Hypertension (%)	63.9	78.9	<0.001
Diabetes mellitus (%)	30.5	40.2	<0.001
History of MI (%)	22.1	22.3	0.95
Previous PCI (%)	32.8	42.4	<0.001
Previous CABG (%)	6.3	11.6	<0.001
EF (%)	50.57 (±11.79)	50 (±10.01)	0.26
Indication to angiography			0.16
Stable Angina (%)	28.2	35.6	
ACS (%)	54.2	44.7	
CMD / Valvulopathy (%)	17.6	19.7	
Concomitant medications			
ACE inhibitors(%)	32.5	37.1	0.057
ARB (%)	19	27.7	<0.001
Beta blockers (%)	51.5	65.1	<0.001
Nitrates (%)	21.4	35.5	<0.001
Statins (%)	47.9	57.7	<0.001

ASA (%)	55	66.1	<0.001
Clopidogrel (%)	14.7	20.9	0.001
Calcium channel blockers (%)	16.2	25.3	<0.001
Diuretics (%)	22.7	39.8	<0.001
Vitamin D (%)	2.9	4.9	0.051
Biochemistry parameters (mean± SD)			
Platelets (10^3/µl)	236.9 (±70.93)	230.29 (±72.07)	0.063
Haemoglobin g/dl)	13.82 (±1.79)	13.17 (±1.76)	<0.001
WBC (10^3/µl)	9.3 (±2.7)	7.93 (±4.2)	0.03
Glycaemia (mg/dL)	120.06 (±50.56)	121.54 (±55.99)	0.59
HbA1c (%)	6.2 (±1.2)	6.3 (±1.3)	0.05
Creatinine (mg/dL)	0.99 (±0.79)	1.04 (±0.61)	0.11
HDL cholesterol (mg/dl)	41.56 (±11.98)	44.27 (±13.53)	<0.001
Total cholesterol (mg/dL)	172.8 (±43.79)	159.22 (±41.6)	<0.001
LDL cholesterol (mg/dL)	105.62 (±48.34)	90.9 (±35.85)	<0.001
Triglycerides (mg/dl)	138.64 (±84.79)	119.93 (±69.06)	<0.001
C-reactive protein (mg/dl)	1.2 (±2.61)	1.05 (±2.51)	0.23
Uric acid (mg/dL)	5.77 (±2.8)	6.09 (±4.66)	0.15
IPF (%)	4.09 (±2.77)	3.61 (±2.55)	<0.001
IPC (10^3/µl)	908.27 (±592.94)	776.96 (±524.16)	<0.001
Angiographic features			
CAD yes (%)§	82.4	79.4	0.15
Multivessel (%)§	57	55.7	0.28
Left main/three-vessel disease (%)§	34	35	0.71

BMI=Body Mass Index; MI = Myocardial Infarction; PCI = Percutaneous Coronary Interventions; CABG = Coronary Artery Bypass Grafting; ACS = Acute Coronary Syndrome; CMD =Dilated Cardiomyopathy; EF = Ejection Fraction; ACE = Angiotensin Converting Enzyme; ARB = Angiotensin Receptor Blockers; ASA = Acetylsalicylic Acid; WBC= White Blood Cells; LDL = Low-Density Lipoproteins; HDL= High-Density Lipoproteins; CAD = Coronary Artery Disease; LAD= Left anterior descending; CX= circumflex coronary artery; RCA= Right coronary artery;

Smokers were less often in treatment with ARB, beta-blockers, nitrates, statins, ASA, Clopidogrel, calcium channel blockers, diuretics ($p<0.001$, respectively) than non-smokers. In relation to biochemistry parameters, active smoking associated to higher levels of haemoglobin ($p<0.001$), total cholesterol, LDL cholesterol, triglycerides ($p<0.001$,

respectively) and lower values of HDL cholesterol (p<0.001). No differences were found in angiographic features.

The mean levels of IPF and immature platelet count (IPC) were higher in smokers than in non-smokers (4.09±2.77 vs 3.61±2.55, p<0.001 and 908.27±592.94 vs 776.96±524.16, p<0.001, respectively). A significant higher percentage of patients with IPF values in 4th quartile (≥4.7 %) were observed among active smokers (24.5% vs 29.5%, p=0.02, as in **Figure 1**).

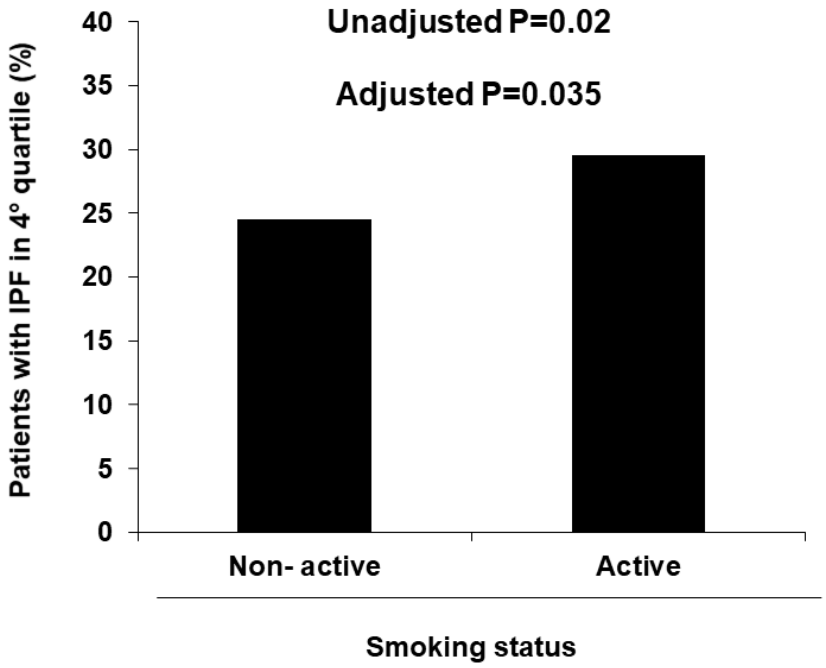


Figure 1. Bar graphs showing the percentage of patients with IPF in 4th quartile according to smoking status.

At multivariate analysis, after correction for main baseline differences (age, male sex, diabetes, hypertension, chronic renal failure, previous MI, total cholesterol, LDL cholesterol, haemoglobin, white blood cell count, indication to angiography, therapy with ARB, BB, ASA, clopidogrel, CCB, diuretics, statins), active smoking resulted an

independent predictor of higher IPF (as above 4th quartile, adjusted OR[95%CI] = 1.59[1.03-2.45] P=0.035).

IPF and CAD in active smokers

Active smoking patients (n= 512) were divided according to the quartiles values of IPF (<2.2 %; 2.2-3.2 %; 3.3-5.1 %; ≥ 5.2 %). **Table 2** shows main clinical and demographic features according to IPF values.

Table 2. Clinical and demographic characteristics according to IPF quartiles in active smokers

Baseline clinical characteristics	**I quart <2.2 n=118**	**II quart 2.2-3.2 n=138**	**III quart 3.3-5.1 n=124**	**IV quart ≥5.2 n=132**	**p-value**
Age (mean±SD)	61.84 (±10.59)	61.73 (±10.91)	62.89 (±10.98)	61.61 (±10.01)	0.78
Male sex (%)	78.8	79	78.2	78	0.85
BMI (mean±SD)	27.4 (±4.29)	27.23 (±4.58)	27.34 (±5.1)	26.98 (±6.36)	0.93
Hypercholesterolemia (%)	53	55.8	57.3	51.5	0.85
Renal failure (%)	13.8	14.1	17.9	9.3	0.56
Hypertension (%)	61	71	62.1	56.8	0.22
Diabetes mellitus (%)	34.7	25.4	30.6	32.1	0.94
History of MI (%)	22.9	14.5	29	22.7	0.37
Previous PCI (%)	36.4	21.7	37.1	37.1	0.29
Previous CABG (%)	5.1	3.6	8.1	8.3	0.13
Previous CVA (%)	5.1	2.2	2.4	3.0	0.43
Ejection fraction (mean±SD)	52.16 (±9.68)	52.23 (±10.05)	49.54 (±12.86)	48.79 (±13.69)	0.034
Indication to angiography					0.002
Stable Angina (%)	29	33.6	27.3	22.3	
ACS (%)	61	53.9	51.8	50.9	
CMD / Valvulopathy (%)	10	12.5	20.9	26.8	
Concomitant Medications					
ACE inhibitors(%)	32.5	29.7	39.5	28.8	0.93
ARB (%)	20.5	23.9	14.5	16.7	0.17

Beta blockers (%)	51.3	45.7	58.1	51.5	0.5
Nitrates (%)	20.7	15.9	27.4	22	0.34
Statins (%)	46.2	42.8	52.4	50.8	0.23
ASA (%)	58.1	50.7	55.6	56.1	0.99
Clopidogrel (%)	17.9	13	16.9	11.4	0.27
Calcium antagonists (%)	14.5	13	21	16.7	0.33
Diuretics (%)	23.1	23.9	22.6	21.2	0.67
Vitamin D (%)	2.7	3.8	2.5	2.4	0.73
Biochemistry parameters (mean± SD)					
Platelets (10^3/µl)	269.25 (±61.38)	244.82 (±73.53)	226.07 (±65.24)	209.89 (±68.97)	<0.001
Hemoglobin (g/dl)	13.71 (±1.79)	13.73 (±1.77)	13.87 (±1.62)	13.99 (±1.95)	0.57
WBC (10^3/µl)	9.3 (±2.8)	9.4 (±2.9)	9.3 (±2.7)	9.2 (±2.7)	0.36
Total cholesterol (md/dl)	171.58 (±42.02)	179.63 (±43.51)	161.4 (±38.67)	177.47 (±48.21)	0.004
HDL cholesterol (mg/dl)	42.25 (±11.52)	42.08 (±12.24)	40.45 (±11.97)	41.45 (±12.17)	0.63
LDL cholesterol (mg/dL)	101.67 (±36.82)	112.21 (±49.58)	100.27 (±62.92)	107.59 (±38.26)	0.18
Tryglicerides (mg/dl)	140.36 (±80.33)	139.18 (±85.15)	123.56 (±61.88)	150.81 (±77.52)	0.083
Glycaemia (mg/dL)	124.36 (±58.87)	119.57 (±50.69)	116.66 (±46.5)	119.93 (±46.08)	0.7
HbA1c (%)	6.18 (±1.09)	6.21 (±1.56)	6.19 (±1.26)	6.26 (±1.39)	0.97
Creatinine (mg/dL)	0.94 (±0.31)	1.04 (±1.13)	1.05 (±0.98)	0.92 (±0.33)	0.44
C-reactive protein (mg/dl)	1.16 (±2.71)	1.14 (±2.5)	1.33 (±2.74)	1.18 (±2.52)	0.94
Uric acid (mg/dL)	5.5 (±2.11)	5.93 (±3.49)	5.63 (±2.75)	5.99 (±2.59)	0.46

BMI=Body Mass Index; MI = Myocardial Infarction; PCI = Percutaneous Coronary Interventions; CABG = Coronary Artery Bypass Grafting; ACS = Acute Coronary Syndrome; CMD =Dilated Cardiomyopathy; EF = Ejection Fraction; ACE = Angiotensin Converting Enzyme; ARB = Angiotensin Receptor Blockers; ASA = Acetylsalicylic Acid; WBC= White Blood Cells; LDL = Low-Density Lipoproteins; HDL= High-Density Lipoproteins.

Smokers with higher IPF values displayed lower ejection fraction (p=0.034), lower percentage of stable angina and acute coronary syndrome as indication to angiography (p=0.002), lower platelet count (p<0.001) and slightly lower total cholesterol (p=0.004), Among angiographic features, we observed a higher percentage of chronic occlusions (p=0.002) and impaired TIMI flow (p=0.017) in smokers with higher IPF as compared to those with lower IPF (**Table 3**).

Table 3. Angiographic characteristics according to IPF quartiles in active smokers

Angiographic features		**I quart <2.2 n=118**	**II quart 2.2-3.2 n=138**	**III quart 3.3-5.1 n=124**	**IV quart ≥5.2 n=132**	**p-value**
CAD yes (%)§		82.5	80.4	86.1	80.9	0.95
Multivessel (%)§		60.2	51.1	62.6	55.5	0.94
Left main/three-vessel disease (%)§		31	31.1	39.1	35.2	0.29
Left main disease (%)§		10.5	10.2	9.8	6.9	0.32
LAD (%)§		62.3	51.4	60.7	59.5	0.88
Intermediate (%)§		4.6	8.1	6.1	4.1	0.68
CX (%)§		45.6	49.6	56.6	48.1	0.52
RCA (%)§		53.5	51.4	56.6	50.4	0.82
Type C Lesion (%)		37.4	35.7	44.6	37.9	0.45
Lesion length (mm±SD)		24.1 (±15.4)	22.8 (±14.71)	23.77 (±15.18)	22.67 (±16.65)	0.69
Percent stenosis (%)		84.99 (±11.01)	85.36 (±11.72)	86.13 (±11.58)	86.79 (±11.05)	0.31
Reference Diameter (mm)		3.02 (±0.53)	3 (±0.5)	3.05 (±0.54)	2.96 (±0.54)	0.24
Calcifications (%)		7.9	6	13.7	7.4	0.42
Chronic occlusion (%)		8.6	11.1	16.4	16.9	0.002
Restenosis (%)		7.4	6.1	7.7	8.3	0.57
Thrombus (%)		3.5	6.4	6	4.1	0.85
Bifurcation lesion (%)		23.9	19.9	19.4	30.9	0.087
TIMI Flow						0.017
	3	80.9	78.2	71.9	73.7	
	2	3.9	6	5.2	5.3	

	1	4.3	1.5	3.2	3.7
	0	10.9	14.3	19.7	17.3

§ Per patient definition
CAD = Coronary Artery Disease; LAD= Left anterior descending; CX= circumflex coronary artery; RCA= Right coronary artery;

The prevalence of CAD was not different between groups based on IPF quartiles (82.5%, 80.4%, 86.1%, 80.9%, from 1st to 4th IPF quartile, respectively, p=0.95) (**Figure 2**). Similar results were observed for severe CAD (31%, 31.1%, 39.1%, 35.2%, from 1st to 4th IPF quartile, respectively, p=0.29) (**Figure 3**). At multivariate analysis, after correction for main baseline differences (ejection fraction, platelets, total cholesterol) IPF quartiles did not result an independent predictor of CAD (adjusted OR [95% CI] = 0.98[0.79 – 1.23] p=0.89) and severe CAD (adjusted OR [95% CI] = 1.03 [0.86 – 1.23] p=0.76).

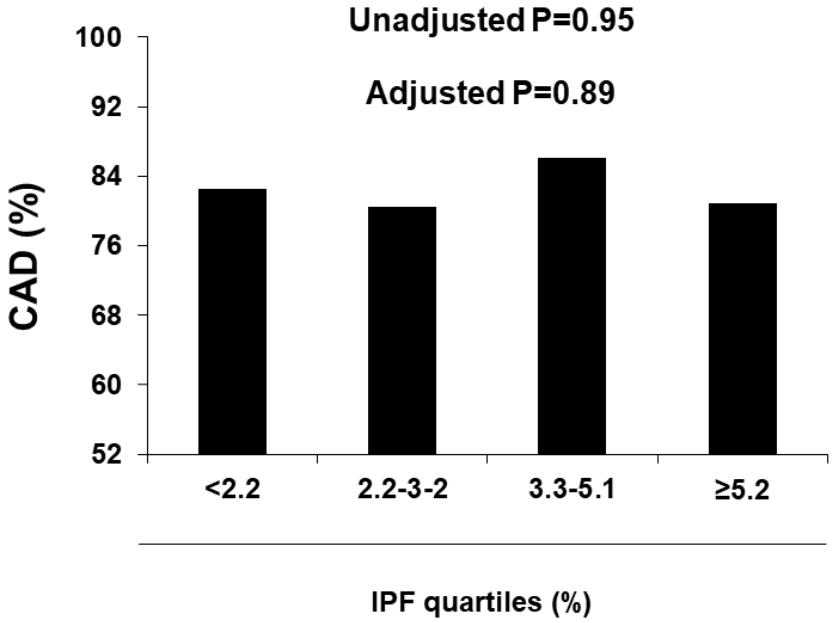


Figure 2. Bar graphs showing the prevalence of CAD according to IPF quartiles in smoking patients

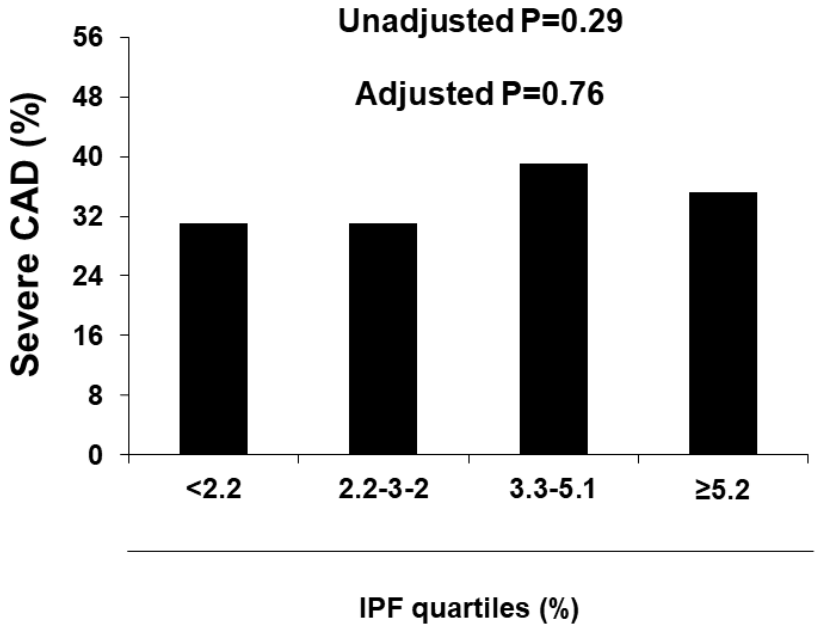


Figure 3. Bar graphs showing the prevalence of severe CAD according to IPF quartiles in smoking patients

Discussion

The present study is the largest so far conduced to attempt to define the impact of active smoking on reticulated platelets. Our main findings are consistent with a significant, independent role of active smoking to predict higher immature platelet fraction (IPF) among patients undergoing percutaneous coronary intervention. However, among active smokers, higher IPF did not result an independent predictor of CAD neither severe CAD.

Undoubtedly in last years many improvements have been obtained in relation to cardiovascular diseases, especially in the treatment of coronary artery disease(12–16). Despite recent advances, still consistent percentage of patients displays higher mortality and morbidity due to CAD, especially in acute coronary syndromes(17–19). Understanding the underlying processes involved in pathogenesis of CAD is essential to detect potential site of intervention to improve patients' outcome.

Active smoking is a well-known risk factor of CAD (20). Strong evidence exists on the increased risk of death among smokers, independently from other cardiovascular risk factors (21). The 10-year fatal CVD risk is approximately doubled in smokers and the relative risk in smokers 50 years of age is five-fold higher than in non-smokers(22). On the other hand smoking cessation is potentially the most effective of all preventive measures, being associated with a reduction in mortality of 36% after myocardial infarction(23).

However, the underlying mechanisms of enhanced cardiovascular risk among smokers is not completely enlightened, also considering the high number of chemicals (a few thousands) contained in cigarette smoke(24).

Besides nicotine and carbon monoxide, the oxidant action of a cigarette smoke constitutes a main stimulus for the formation of atherosclerotic plaques and thrombi, inducing oxidative stress in various cell types, including endothelial cells, leading to endothelial

injury and dysfunction. In addition, reactive oxygen species (ROS) can trigger the activation of NF-kB and, therefore, the transcription and expression of proinflammatory cytokines and adhesion molecules (25), resulting in a stronger haemostatic disequilibrium(26,27), with the exposure of subendothelial compounds that facilitates platelets activation and aggregation and the interaction between endothelial cells, leucocytes and platelets. (28).

Thus, in active smokers, platelets are prone to higher reactivity than non-smokers. Yarlioglues et al (29) have shown that after 1 hour of acutely exposition to smoking platelets displayed marked increased activation in healthy volunteers, and Fusegawa et colleagues(30) have reported increased stimulated and spontaneous aggregation of platelets isolated from smokers. Several mechanisms have been hypothesized to impact on platelets aggregability in smokers, including lipid oxidation products (31), reduced release of platelet-derived nitric oxide (32), higher sensitivity of GPIIB/IIIA to fibrinogen(30) impaired balance of thromboxane A2 and prostaglandin I2(33).

On the clinical side the need to early identifying more reactive platelets has led to focus growing interest to younger thrombocytes, that are lastly released from bone marrow, and named “reticulated platelet”, containing still a small amount of RNA, in analogy with the reticulocytes for erythropoiesis (34). Reticulated platelets display a larger size, higher alpha-granules and RNA content and increased capability of proteins synthesis and then a potentially enhanced reactivity(34,35).

Immature platelet fraction (IPF) is a parameter allowing the identification of the fraction of reticulated platelets(7) with a cheap, reliable and easy to obtain analysis, using traditional cells counters (36). Some authors have reported that high levels of circulating immature platelet are associated to a pro-thrombotic condition (4,37,38). Furthermore, previous studies have shown the potential relationship between immature platelet and

cardiovascular disease. In fact, Grove at al. have found higher IPF values in patients with acute coronary syndromes (39) and increased levels of immature platelets have been linked to a worse clinical outcome (40,41).

Influence of smoking on platelet turnover and volume has been already described(42), but few and contrast data are available on impact of smoking on IPF. In a previous study including 35 large Spanish families, Pujol-Moix et colleagues have evaluated the quote of genetic and environmental determinants on following parameters: platelet volume, platelet count and platelet function indexes, including IPF. Their results were consistent with an impact of smoking in a covariance model on platelet volume and IPF(43), while Butkiewicz et al have found a significant higher percentage of reticulated platelet among smoking women compared to non-smokers (44). On the other side, Joergensena et al have reported no impact of smoking status on IPF assessment in general population of Denmark(45).

In our study, conducted among patients undergoing coronary angiography, we found higher IPF among active smokers as compared to the non-active ones, that were considered comparable to a "normal" control population, since comprising a heterogeneous and largely sized cohort of patients, including patients with and without CAD. In fact, despite we included also patients admitted for an acute coronary syndrome, the IPF% of our cohort was similar to the values those reported by other studies on European cohorts(45,46). Platelet count was found slightly higher among smokers, even if that difference was not statistically significant, but immature platelet count was statistical significant higher in smokers. Moreover, smokers showed lower prevalence and diabetes,hypertension and renal failure and less pharmacological therapies. This difference in pharmacological therapy and cardiovascular risk factor could be explained by different of mean age between smokers and non-smokers (62.01 vs 70.05 years, respectively, $p<0.001$). However, the

association between active smoking and high IPF was confirmed at multivariate analysis after correction for all baseline confounding factors.

Partially in agreement with our results are the findings of Butkiewicz et al(44) in a cohort of 125 healthy blood donors they reported significant higher number of reticulated platelets among smoking women compared to non-smoking ones. They found no differences among men according to smoking status. In our cohort sex has been included in multivariate analysis and active smoking was confirmed independently associated with higher IPF.

Immature platelets are larger and enzymatically and metabolically more active, and have a higher thrombotic potential than smaller platelets (47). Larsen et al. (48) have demonstrated that the levels of reticulated platelet relate to low grade inflammation and biomarkers as C-reactive protein. In active smokers nitric oxide insufficiency combined with increased reactive oxygen species cause an increase in intracellular calcium levels in platelets, promoting platelet activation (49). Moreover, platelet α-granule constituents, such as platelet factor-4, β-thromboglobulin, and platelet activating factor, are increased in the plasma of smokers(9,50,51), and Lupia et al have reported demonstrated significantly increased thrombopoietin levels in smokers as compared with non-smokers (52).

Indeed, immature platelets have been previously hypothesized playing a role in the prediction of atherosclerosis (39), but its role especially in acute settings is not clear whether enhanced platelet turnover is the cause or the consequence of the acute atherothrombotic event(53).

However, we have found no relationship between IPF and CAD among active smokers. In fact, no significant impact of higher IPF values has been detected on prediction the presence of CAD at angiography, after correction for main baselines differences.

Similarly, we previously reported no association of IPF with prevalence and extension of CAD as evaluated by coronary angiography(54).

Raising attention has been focused on potential impact of immature platelet on response to antiplatelet drugs. However contrasting results have been found relating reticulated platelets to an impaired response to antiplatelet agents (38,55–57), implying further studies to assess the clinical significance of immature platelet on clinical settings. IPF, then, would represent a marker of platelet turnover rather than being directly involved in the pathogenesis of cardiovascular disease.

Active smoking, thus, represents an independent predictor of reticulated platelet in patients undergoing coronary angiography, however IPF does not represent an indicator for the assessment of the risk of CAD among smokers, and further studies are needed to define the clinical significance and of immature platelet.

Limitations

A limitation could be the use of the automated measurement of immature platelets instead of a direct assessment of RPs, although flow cytometric technique, based on RNA staining by thiazole orange, represents a complex and much less reproducible method for analyzing RPs (36). However previous reports have shown an appreciable precision of measurement of IPF values through Sysmex XE-series haematology analysers and moreover, the coefficient of variation on IPF% values, representing only <20%, could have played only a minor role in affecting our conclusions (58,59). Also measurements of thromboglobulin and thrombopoietin could have allowed a better evaluation of platelet turnover.

We have not assessed the oxidative stress in our patients, one of essential mechanisms of action of cigarette smoking, neither the number of cigarettes smoked per day. However,

impact of active smoking on prediction of higher IPF values was independent from other baseline differences.

Indeed, biochemical assessment of smoking status through the measurement of cotinine on blood, salivary or urine samples or CO on exhaled air could have prevented a potential misclassification of smoking status based on self-reporting, that has been documented anyhow in less that 10% of the patients in literature(60). Nevertheless, considering the large cohort of patients included in our study we expect only a minor impact of such misclassification on our results.

Indeed, the inclusion in our study of a cohort of patients undergoing coronary angiography, could have lead to a possible selection bias. However only coronary angiography can provide certainty about the absence or presence of significant CAD, still representing the gold standard technique to evaluate the presence and extension of CAD. In addition, we included also an important percentage (almost 18%) of patients undergoing coronary angiography for arrhythmias or dilated cardiomiopathy or valvular disease, and thus with a lower pre-test probability of CAD, therefore quite similar to a “normal” control population.

Conclusions

The present study shows an independent association between active smoking and the levels of immature platelet fraction in patients undergoing coronary angiography. However, among active smokers, IPF did not result an independent predictor of CAD or severe CAD.

References

1. Piepoli MF, Hoes AW, Agewall S, Albus C, Brotons C, Catapano AL, et al. 2016 European Guidelines on cardiovascular disease prevention in clinical practice. Eur Heart J. 2016 Aug 1;37(29):2315–81.
2. De Luca G, Savonitto S, van't Hof AWJ, Suryapranata H. Platelet GP IIb-IIIa Receptor Antagonists in Primary Angioplasty: Back to the Future. Drugs. 2015 Jul;75(11):1229–53.
3. Trovati M, Anfossi G, Cavalot F, Massucco P, Mularoni E, Emanuelli G. Insulin directly reduces platelet sensitivity to aggregating agents. Studies in vitro and in vivo. Diabetes. 1988 Jun;37(6):780–6.
4. Cesari F, Marcucci R, Caporale R, Paniccia R, Romano E, Gensini GF, et al. Relationship between high platelet turnover and platelet function in high-risk patients with coronary artery disease on dual antiplatelet therapy. Thromb Haemost. 2008 May;99(5):930–5.
5. Lakkis N, Dokainish H, Abuzahra M, Tsyboulev V, Jorgensen J, De Leon AP, et al. Reticulated platelets in acute coronary syndrome: a marker of platelet activity. J Am Coll Cardiol. 2004 Nov 16;44(10):2091–3.
6. Ko YJ, Kim H, Hur M, Choi SG, Moon H-W, Yun Y-M, et al. Establishment of reference interval for immature platelet fraction. Int J Lab Hematol. 2013 Oct;35(5):528–33.
7. Pons I, Monteagudo M, Lucchetti G, Muñoz L, Perea G, Colomina I, et al. Correlation between immature platelet fraction and reticulated platelets. Usefulness in the etiology diagnosis of thrombocytopenia. Eur J Haematol. 2010 Aug;85(2):158–63.
8. Hunter KA, Garlick PJ, Broom I, Anderson SE, McNurlan MA. Effects of smoking and abstention from smoking on fibrinogen synthesis in humans. Clin Sci (Lond). 2001 Apr;100(4):459–65.
9. Hung J, Lam JY, Lacoste L, Letchacovski G. Cigarette smoking acutely increases platelet thrombus formation in patients with coronary artery disease taking aspirin. Circulation. 1995 Nov 1;92(9):2432–6.
10. Csordas A, Bernhard D. The biology behind the atherothrombotic effects of cigarette smoke. Nat Rev Cardiol. 2013 Apr 5;10(4):219–30.
11. Verdoia M, Pergolini P, Nardin M, Rolla R, Barbieri L, Schaffer A, et al. Impact of

diabetes on immature platelets fraction and its relationship with platelet reactivity in patients receiving dual antiplatelet therapy. J Thromb Thrombolysis. 2016 Aug;42(2):245–53.

12. De Luca G, Schaffer A, Wirianta J, Suryapranata H. Comprehensive meta-analysis of radial vs femoral approach in primary angioplasty for STEMI. Int J Cardiol. 2013 Oct 3;168(3):2070–81.
13. De Luca G, Navarese EP, Cassetti E, Verdoia M, Suryapranata H. Meta-analysis of randomized trials of glycoprotein IIb/IIIa inhibitors in high-risk acute coronary syndromes patients undergoing invasive strategy. Am J Cardiol. 2011 Jan 15;107(2):198–203.
14. De Luca G, Navarese EP, Suryapranata H. A meta-analytic overview of thrombectomy during primary angioplasty. Int J Cardiol. 2013 Jul 1;166(3):606–12.
15. De Luca G, Dirksen MT, Spaulding C, Kelbaek H, Schalij M, Thuesen L, et al. Drug-eluting vs bare-metal stents in primary angioplasty: a pooled patient-level meta-analysis of randomized trials. Arch Intern Med. 2012 Apr 23;172(8):611–21; discussion 621-2.
16. De Luca G, Smits P, Hofma SH, Di Lorenzo E, Vlachojannis GJ, Van't Hof AWJ, et al. Everolimus eluting stent vs first generation drug-eluting stent in primary angioplasty: A pooled patient-level meta-analysis of randomized trials. Int J Cardiol. 2017 Oct 1;244:121–7.
17. De Luca G, Suryapranata H, Ottervanger JP, van 't Hof AWJ, Hoorntje JCA, Gosselink ATM, et al. Circadian variation in myocardial perfusion and mortality in patients with ST-segment elevation myocardial infarction treated by primary angioplasty. Am Heart J. 2005 Dec;150(6):1185–9.
18. De Luca G, Suryapranata H, Dambrink J-H, Ottervanger JP, van 't Hof AWJ, Zijlstra F, et al. Sex-related differences in outcome after ST-segment elevation myocardial infarction treated by primary angioplasty: data from the Zwolle Myocardial Infarction study. Am Heart J. 2004 Nov;148(5):852–6.
19. De Luca G, Dirksen MT, Spaulding C, Kelbæk H, Schalij M, Thuesen L, et al. Impact of diabetes on long-term outcome after primary angioplasty: insights from the DESERT cooperation. Diabetes Care. 2013 Apr;36(4):1020–5.
20. Nicholls SJ, Hsu A, Wolski K, Hu B, Bayturan O, Lavoie A, et al. Intravascular

ultrasound-derived measures of coronary atherosclerotic plaque burden and clinical outcome. J Am Coll Cardiol. 2010 May 25;55(21):2399–407.

21. Meyers DG, Neuberger JS, He J. Cardiovascular effect of bans on smoking in public places: a systematic review and meta-analysis. J Am Coll Cardiol. 2009 Sep 29;54(14):1249–55.
22. Prescott E, Hippe M, Schnohr P, Hein HO, Vestbo J. Smoking and risk of myocardial infarction in women and men: longitudinal population study. BMJ. 1998 Apr 4;316(7137):1043–7.
23. Critchley J, Capewell S. Smoking cessation for the secondary prevention of coronary heart disease. Cochrane database Syst Rev. 2003;(4):CD003041.
24. Messner B, Bernhard D. Smoking and Cardiovascular Disease. Arterioscler Thromb Vasc Biol. 2014 Mar;34(3):509–15.
25. Collins T. Endothelial nuclear factor-kappa B and the initiation of the atherosclerotic lesion. Lab Invest. 1993 May;68(5):499–508.
26. Bernhard D, Csordas A, Henderson B, Rossmann A, Kind M, Wick G. Cigarette smoke metal-catalyzed protein oxidation leads to vascular endothelial cell contraction by depolymerization of microtubules. FASEB J. 2005 Jul;19(9):1096–107.
27. Csordas A, Kreutmayer S, Ploner C, Braun PR, Karlas A, Backovic A, et al. Cigarette smoke extract induces prolonged endoplasmic reticulum stress and autophagic cell death in human umbilical vein endothelial cells. Cardiovasc Res. 2011 Oct 1;92(1):141–8.
28. Lippi G, Franchini M, Targher G. Arterial thrombus formation in cardiovascular disease. Nat Rev Cardiol. 2011 Jul 5;8(9):502–12.
29. Yarlioglues M, Ardic I, Dogdu O, Akpek M, Zencir C, Kasapkara HA, et al. The acute effects of passive smoking on mean platelet volume in healthy volunteers. Angiology. 2012 Jul;63(5):353–7.
30. Fusegawa Y, Goto S, Handa S, Kawada T, Ando Y. Platelet spontaneous aggregation in platelet-rich plasma is increased in habitual smokers. Thromb Res. 1999 Mar 15;93(6):271–8.
31. Podrez EA, Byzova T V, Febbraio M, Salomon RG, Ma Y, Valiyaveettil M, et al. Platelet CD36 links hyperlipidemia, oxidant stress and a prothrombotic phenotype. Nat Med. 2007 Sep;13(9):1086–95.

32. Ichiki K, Ikeda H, Haramaki N, Ueno T, Imaizumi T. Long-term smoking impairs platelet-derived nitric oxide release. Circulation. 1996 Dec 15;94(12):3109–14.
33. Reinders JH, Brinkman HJ, van Mourik JA, de Groot PG. Cigarette smoke impairs endothelial cell prostacyclin production. Arteriosclerosis. 6(1):15–23.
34. Ault KA, Rinder HM, Mitchell J, Carmody MB, Vary CP, Hillman RS. The significance of platelets with increased RNA content (reticulated platelets). A measure of the rate of thrombopoiesis. Am J Clin Pathol. 1992 Dec;98(6):637–46.
35. Dusse LMS, Freitas LG. Clinical applicability of reticulated platelets. Clin Chim Acta. 2015 Jan;439:143–7.
36. Hoffmann JJML. Reticulated platelets: analytical aspects and clinical utility. Clin Chem Lab Med. 2014 Aug;52(8):1107–17.
37. Richards EM, Baglin TP. Quantitation of reticulated platelets: methodology and clinical application. Br J Haematol. 1995 Oct;91(2):445–51.
38. Guthikonda S, Alviar CL, Vaduganathan M, Arikan M, Tellez A, DeLao T, et al. Role of reticulated platelets and platelet size heterogeneity on platelet activity after dual antiplatelet therapy with aspirin and clopidogrel in patients with stable coronary artery disease. J Am Coll Cardiol. 2008 Aug 26;52(9):743–9.
39. Grove EL, Hvas A-M, Kristensen SD. Immature platelets in patients with acute coronary syndromes. Thromb Haemost. 2009 Jan;101(1):151–6.
40. Cesari F, Marcucci R, Gori AM, Caporale R, Fanelli A, Casola G, et al. Reticulated platelets predict cardiovascular death in acute coronary syndrome patients. Insights from the AMI-Florence 2 Study. Thromb Haemost. 2013 May;109(5):846–53.
41. Ibrahim H, Schutt RC, Hannawi B, DeLao T, Barker CM, Kleiman NS. Association of immature platelets with adverse cardiovascular outcomes. J Am Coll Cardiol. 2014;64(20):2122–9.
42. Kario K, Matsuo T, Nakao K. Cigarette smoking increases the mean platelet volume in elderly patients with risk factors for atherosclerosis. Clin Lab Haematol. 1992;14(4):281–7.
43. Pujol-Moix N, Vázquez-Santiago M, Morera A, Ziyatdinov A, Remacha A, Nomdedeu JF, et al. Genetic determinants of Platelet Large-Cell Ratio, Immature Platelet Fraction, and other platelet-related phenotypes. Thromb Res. 2015 Aug;136(2):361–6.
44. Butkiewicz AM, Kemona-Chetnik I, Dymicka-Piekarska V, Matowicka-Karna J,

Kemona H, Radziwon P. Does smoking affect thrombocytopoiesis and platelet activation in women and men? Adv Med Sci. 2006;51:123–6.

45. Joergensen MK, Bathum L. Reference intervals for mean platelet volume and immature platelet fraction determined on a sysmex XE5000 hematology analyzer. Scand J Clin Lab Invest. 2016 Feb 17;76(2):172–6.
46. Ali U, Knight G, Gibbs R, Tsitsikas DA. Reference intervals for absolute and percentage immature platelet fraction using the Sysmex XN-10 automated haematology analyser in a UK population. Scand J Clin Lab Invest. 2017 Nov 17;77(8):658–64.
47. Brown AS, Martin JF. The megakaryocyte platelet system and vascular disease. Eur J Clin Invest. 1994 Feb;24 Suppl 1:9–15.
48. Larsen SB, Grove EL, Hvas A-M, Kristensen SD. Platelet Turnover in Stable Coronary Artery Disease – Influence of Thrombopoietin and Low-Grade Inflammation. Schulz C, editor. PLoS One. 2014 Jan 21;9(1):e85566.
49. Barua RS, Ambrose JA. Mechanisms of Coronary Thrombosis in Cigarette Smoke Exposure. Arterioscler Thromb Vasc Biol. 2013 Jul;33(7):1460–7.
50. Benowitz NL, Fitzgerald GA, Wilson M, Zhang Q. Nicotine effects on eicosanoid formation and hemostatic function: comparison of transdermal nicotine and cigarette smoking. J Am Coll Cardiol. 1993 Oct;22(4):1159–67.
51. Blache D. Involvement of hydrogen and lipid peroxides in acute tobacco smoking-induced platelet hyperactivity. Am J Physiol. 1995 Feb;268(2 Pt 2):H679-85.
52. Lupia E, Bosco O, Bergerone S, Dondi AE, Goffi A, Oliaro E, et al. Thrombopoietin Contributes to Enhanced Platelet Activation in Patients With Unstable Angina. J Am Coll Cardiol. 2006 Dec;48(11):2195–203.
53. Lev EI. Immature Platelets: Clinical Relevance and Research Perspectives. Circulation. 2016;134(14):987–8.
54. Verdoia M, Nardin M, Rolla R, Marino P, Bellomo G, Suryapranata H, et al. Immature platelet fraction and the extent of coronary artery disease: A single centre study. Atherosclerosis. 2017 May;260:110–5.
55. Verdoia M, Sartori C, Pergolini P, Nardin M, Rolla R, Barbieri L, et al. Immature platelet fraction and high-on treatment platelet reactivity with ticagrelor in patients with acute coronary syndromes. J Thromb Thrombolysis. 2016 May 14;41(4):663–70.

56. Stratz C, Bömicke T, Younas I, Kittel A, Amann M, Valina CM, et al. Comparison of Immature Platelet Count to Established Predictors of Platelet Reactivity During Thienopyridine Therapy. J Am Coll Cardiol. 2016 Jul;68(3):286–93.
57. Vaduganathan M, Zemer-Wassercug N, Rechavia E, Lerman-Shivek H, Perl L, Leshem-Lev D, et al. Relation between ticagrelor response and levels of circulating reticulated platelets in patients with non-ST elevation acute coronary syndromes. J Thromb Thrombolysis. 2015 Aug 29;40(2):211–7.
58. Briggs C, Kunka S, Hart D, Oguni S, Machin SJ. Assessment of an immature platelet fraction (IPF) in peripheral thrombocytopenia. Br J Haematol. 2004 Jul;126(1):93–9.
59. Ruisi MM, Psaila B, Ward MJ, Villarica G, Bussel JB. Stability of measurement of the immature platelet fraction. Am J Hematol. 2010 Apr 27;85(8):622–4.
60. From Attebring M, Herlitz J, Berndt A-K, Karlsson T, Hjalmarson A. Are patients truthful about their smoking habits? A validation of self-report about smoking cessation with biochemical markers of smoking activity amongst patients with ischaemic heart disease. J Intern Med. 2001 Feb;249(2):145–51.

Chapter 4

Impact of metabolic syndrome on mean platelet volume and its relationship with coronary artery disease

Matteo Nardin [a,b]; Monica Verdoia [a,c]; Lucia Barbieri[a]; Giuseppe De Luca[a,]

a) Division of Cardiology, Azienda Ospedaliera-Universitaria "Maggiore della Carità", EasternPiedmontUniversity, Novara, Italy; b) Department of Medicine, ASST "Spedali Civili", University of Brescia, Italy;

Platelets (2019) 30: 615-623

Abstract

Platelets represent one of the main actors involved in pathogenesis of coronary artery disease (CAD). Mean platelet volume (MPV) has been proposed as marker of platelet reactivity and thrombotic risk. However, still debated is whether higher MPV constitutes an independent determinant of CAD or just the consequence of an association with several cardiovascular risk factors. Therefore, the aim of the present study was to assess the impact of metabolic syndrome (MetS), on MPV and its relationship with angiographically defined CAD.

Consecutive patients undergoing coronary angiography were included. Admission samples were collected for MPV and chemistry assessment. MetS was defined according to IDF-criteria. Significant CAD was defined as at least 1 vessel stenosis >50%, while severe CAD as left main and/or 3-vessel disease, as evaluated by quantitative coronary angiography

We included 4730 patients, among them 2167 (45.8%) had MetS. Patients with MetS were older ($p<0.001$),more often females ($p<0.001$), and displayed higher BMI, higher prevalence of hypercholesterolemia, renal failure, hypertension, diabetes mellitus, history of myocardial infarction (MI), previous PCI ($p<0.001$, respectively), previous CABG ($p=0.002$),treatment with ACE inhibitors, ARB, beta-blockers, nitrates, statins, ASA, calcium channel blockers, diuretics ($p<0.001$, respectively),higher values of glycaemia,HbA1c, fibrinogen ($p<0.001$, respectively), creatinine ($p=0.01$),uric acid ($p=0.02$) and lower values of haemoglobin ($p=0.001$),total-cholesterol, LDL-cholesterol, HDL-cholesterol ($p<0.001$, respectively). MetS patients showed a higher prevalence of CAD ($p=0.002$) and severe CAD ($p=0.01$).

MPV values were slightly higher in patients with MetS (10.91±1.01 vs 10.84±1.03 fL, p=0.02), although MetS did not emerge as an independent predictor of higher MPV values (above 4th quartile; adjusted OR [95%CI]=1.01[0.84-1.22],p=0.93).

When metabolic syndrome patients were analyzed according to MPV quartiles, higher MPV values did not result as an independent predictor of CAD (adjusted OR[95%CI]=0.79[0.61-1.03], p=0.08) and severe CAD (adjusted OR[95%CI]=0.82 [0.65-1.03],p=0.084). Results did not change when applying the new harmonized definition of MetS.

This study shows that among patients undergoing coronary angiography MetS is not an independent predictor of higher MPV. Moreover, among MetS patients, larger sized platelets are not associated to the prevalence and extent of CAD.

Introduction

Coronary artery disease still represents the leading cause of mortality in western countries. In fact, despite the great achievements in the treatment of coronary atherosclerosis and acute coronary syndromes (ACS) (1–4), the outcome is still unsatisfactory in high-risk subsets of patients(5,6). Therefore, large investigations have been conducted in the last decades in order to identify and evaluate new factors involved in the atherothrombotic process, with a special focus on platelets.

A large number of studies has underlined the central role of platelets in coronary artery disease (CAD)(7,8), suggesting platelets activation being one of the main factors involved in atherothrombotic processes (9),

Therefore, in the last years scientific research has largely investigated to detect an effective and world-wide feasible parameter to measure platelet activity, in order to better assess patients' thrombotic and cardiovascular risk(10). Previous investigations (11,12) have shown that larger platelets are more active, from a metabolic point of view, leading thus to greater prothrombotic risk than smaller platelets. For these reasons, mean platelet volume (MPV), a marker of platelet size, has been variously associated to CAD, although with contrasting results (13)(14). In fact, still debated is whether MPV can represent an independent predictor of cardiovascular events or on the contrary an increased platelet volume simply represents the consequence of other cardiovascular risk factors, such as hypertension, diabetes or smoking, that have been linked to a raise in MPV.

Metabolic syndrome (MetS) represents a cluster of several cardiovascular risk factors that has shown a positive correlation with CAD (15)(16), with insulin-resistance playing a key-role in its pathogenesis(17). Moreover insulin-resistance has been related with an enhanced platelet reactivity, due to higher thromboxane biosynthesis and calcium mobilization(18).

Few studies have investigated the impact of MetS on MPV and its relationship with CAD, providing inconclusive results. (19), (20).

Therefore, the aim of the present study was to assess the relationship between MetS and MPV in a large cohort of patients undergoing coronary angiography, and its impact on CAD.

Methods

Our study population is represented by consecutive patients undergoing coronary angiography between September 2009 and June 2015 at the Ospedale "Maggiore della Carità", Eastern Piedmont University, Novara, Italy. Indication to coronary angiography was either elective or an acute coronary syndrome, defined as the presence of chest pain or cardiac biomarkers elevation > ULN (respectively 0,04 µg/l for Troponin I and 5,00 µg/l for CK-MB), with or without ECG changes (21,22). Established CAD was defined for a history of previous coronary revascularization (with either PCI or CABG). We excluded patients who refused to sign informed consent before angiography. The study was approved by our local Ethical Committee. All demographic and clinical data were prospectively collected in a dedicated database.

Biochemical measurement.

Blood samples were drawn at admission in patients undergoing elective (following a fasting period of 12 h) or urgent coronary angiography. Glucose, creatinine, glycosylated haemoglobin and lipid profile were determined by standard methods. MPV values were measured in a blood sample collected in tripotassium EDTA (7.2 mg) tubes and analysed within 2 hours from collection.

Metabolic syndrome definition

Metabolic syndrome was defined according to modified IDF criteria (23) for Europoids: 1) waist circumference ≥ 94 cm and ≥ 80 cm respectively in men and women; 2) two between following four elements: a) hypertriglyceridemia (> 150 mg/dl) or specific treatment, b) reduced HDL-cholesterol (<40 mg/dl and <50 mg/dl respectively in men and women) or specific treatment, c) systolic blood pressure ≥130 and/or diastolic blood

pressure ≥85 mmHg or treatment of previously diagnosed hypertension, d) fasting plasma glucose ≥126 mg/dL or previously diagnosed type 2 diabetes mellitus. In addition, the most recent Adult Treatment Panel III (ATPIII) criteria were also applied, considering any three among the previous five elements (24).

Coronary angiography

Coronary angiography (carried out by Siemens AXIOM ARTIS *d*TC, Erlangen, Germany) was routinely performed by the Judkins technique using 6-French right and left heart catheters. Quantitative coronary angiography was performed by two experienced interventional cardiologists, by an automatic edge-detection system for Quantitative Coronary Angiography (Siemens Acom Quantcor QCA, Erlangen, Germany) (25). After a visual inspection of the coronary artery, the frame of optimal clarity was selected, showing lesion at maximal narrowing and arterial silhouette in sharpest focus. After the calibration of guiding catheter, analysed arterial segment with coronary lesion was defined by moving the cursor from the proximal to the distal part of coronary artery to ensure adequate determination of reference diameter. Minimal luminal diameter, reference diameter, percent diameter stenosis, and length of the lesion were measured. Significant CAD was defined as the presence of at least 1 coronary stenosis more than 50%. Severe CAD was defined as the presence of left main and /or 3-vessel disease. For patients who had previously undergone percutaneous coronary interventions, the treated lesion was considered as significantly diseased vessel. In previously coronary artery bypassed (CABG) patients, both native arteries and grafts were taken into account in the evaluation of the extension of coronary artery disease (number of diseased vessels).

Statistical analysis

Statistical analysis was performed using SPSS 17.0 statistical package. Continuous data were expressed as mean $\pm$ SD and categorical data as percentage. Analysis of variance and the chi-square test were used for continuous and categorical variables, respectively.

Patients were grouped according to metabolic syndrome status and MPV quartiles. Multiple logistic regression analysis was performed to evaluate the relationship between MetS and MPV or between MPV and CAD, after correction for baseline confounding factors, that were entered in the model in block. A p value $\leq$ 0.05 was considered statistically significant.

Results

Our population included 4730 patients; among them among them 2167 (45.8%) were affected by metabolic syndrome.

Table 1 shows main baseline differences according to diagnosis of metabolic syndrome in overall population. As shown in **Table 1**, patients with MetS were older (p<0.001), more often females (p<0.001), and displayed a higher cardiovascular risk profile as compared to patients without MetS.

Table 1. Clinical characteristics according to metabolic syndrome diagnosis.

Baseline clinical characteristics	**MetS (n=2167)**	**non MetS (n=2563)**	**p-value**
Age (mean±SD)	68.48 (±10.42)	67.07 (±11.94)	<0.001
Age ≥75 years (%)	32.1	30.7	0.31
Male sex (%)	62.7	75.6	<0.001
BMI (mean±SD)	28.89 (±4.7)	25.3 (±3.75)	<0.001
Hypercholesterolemia (%)	65.4	46.1	<0.001
Renal failure (%)	30.1	22.5	<0.001
Smokers (%)			0.92
Active smokers	22.9	28.7	
Previous smoker	21.2	18.4	
Hypertension (%)	88.2	57.6	<0.001
Diabetes mellitus (%)	50.7	22.5	<0.001
History of MI (%)	26.8	20.6	<0.001
Previous PCI (%)	30.7	22.7	<0.001
Previous CABG (%)	12.8	9.9	0.002
Indication to angiography			0.08
Stable Angina (%)	27	23.5	
ACS (%)	55.1	58.5	
CMD / Valvulopathy (%)	17.9	17.9	
ACS type (n=2685)			<0.001
STEMI (%)	18.6	25.7	
NSTEMI/UA (%)	81.4	74.3	
Concomitant medications			

ACE inhibitors (%)	40.3	34.2	<0.001
ARB (%)	28.4	15.4	<0.001
Beta blockers (%)	59.6	45.9	<0.001
Nitrates (%)	39.8	30.8	<0.001
Statins (%)	58	40.6	<0.001
ASA (%)	63.4	53.6	<0.001
Clopidogrel (%)	22.6	20.8	0.16
Calcium channel blockers (%)	25.3	15.9	<0.001
Diuretics (%)	39.1	23.4	<0.001
Biochemistry parameters (mean± SD)			
Platelets (10^3/µl)	219.8 (±64.85)	217.95 (±68.59)	0.35
Haemoglobin g/dl)	13.27 (±1.8)	13.45 (±1.74)	0.001
WBC (10^3/µl)	7.94 (±2.52)	8.45 (±2.83)	0.15
Glycaemia (mg/dL)	133.76 (±58.72)	117.5 (±44.46)	<0.001
HbA1c (%)	6.59 (±1.39)	5.93 (±1.07)	<0.001
Creatinine (mg/dL)	1.087 (±0.545)	1.047 (±0.517)	0.01
HDL cholesterol (mg/dl)	39 (±11.30)	43.5 (±13.23)	<0.001
Total cholesterol (mg/dL)	159.38 (±41.16)	166.75 (±42.05)	<0.001
LDL cholesterol (mg/dL)	89.49 (±33.89)	98.48 (±35.88)	<0.001
Triglycerides (mg/dl)	152.95 (±84.56)	122.97 (±70.08)	<0.001
C-reactive protein (mg/dl)	1.29 (±2.78)	1.36 (±3.18)	0.4
Uric acid (mg/dL)	6.29 (±2.35)	6.07 (±3.87)	0.02
Fibronogen (mg/dl)	443.41 (±144.34)	415.02 (±143.88)	<0.001
CAD (%)§	79.3	75.5	0.002
Multivessel CAD (%)§	51.8	45.3	<0.001
Left main-/3-vessel (%)§	31.1	27.8	0.01

§ Per patient definition
BMI=Body Mass Index; MI = Myocardial Infarction; PCI = Percutaneous Coronary Interventions; CABG = Coronary Artery Bypass Grafting; ACS = Acute Coronary Syndrome; CMD =Dilated Cardiomyopathy; EF = Ejection Fraction; ACE = Angiotensin Converting Enzyme; ARB = Angiotensin Receptor Blockers; ASA = Acetylsalicylic Acid; WBC= White Blood Cells; LDL = Low-Density Lipoproteins; HDL= High-Density Lipoproteins.

In terms of biochemistry parameters, patients with MetS displayed higher values of glycaemia ($p<0.001$), HbA1c ($p<0.001$), creatinine ($p=0.01$), uric acid ($p=0.02$), fibrinogen ($p<0.001$), triglycerides ($p < 0.001$) and lower values of haemoglobin

(p=0.001), total cholesterol (p<0.001), LDL cholesterol (p<0.001), HDL cholesterol (p<0.001) than patients without MetS.

MPV values were significant higher in patients with MetS than in ones without (10.91 ± 1.01 vs 10.84 ±1.03 fL, p=0.02) (**Figure 1**).

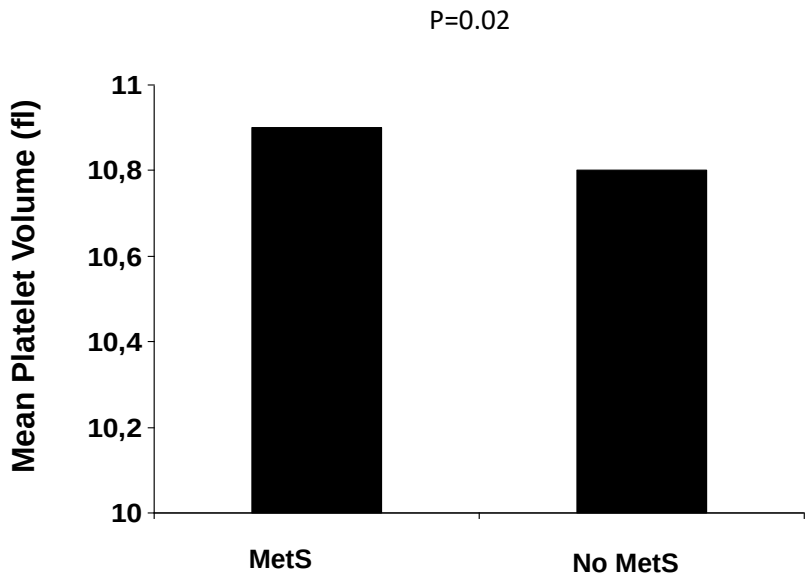


Figure 1. Bar graphs showing the mean values of mean platelet volume (MPV) according to metabolic syndrome (MetS) diagnosis.

Moreover, when dividing our patient according to the quartiles values of MPV (<10.2 fL; 10.2-10.7 fL; 10.8-11.4 fL; ≥11.5 fL), the prevalence of patients with MPV in IV quartile was larger among MetS patients (26.6% vs 24.9%, p=0.02). At multivariate analysis, however, after correction for main baseline differences (age, male sex, BMI, renal failure, previous MI, ACE inhibitors, ARB, statins, beta blockers, ASA, CCB, diuretics, creatinine, haemoglobin), metabolic syndrome did not result as an independent predictor of higher MPV values (as ≥IV quartile; adjusted OR[95%CI]= 1.01[0.84-1.22], p=0.93).

Similar results were achieved when applying the new 2008 definition criteria, leading to the classification of 2749 patients (58%) as having MetS. Mean MPV values were more

elevated in patients with MetS (10.9 ± 1 vs 10.82 ±1.05 fL, p=0.01), with a higher prevalence of patients displaying MPV values ≥ IV quartile (26.1% vs 24.7%, p=0.02; adjusted OR[95%CI]= 1.09[0.94-1.27], p=0.26).

MPV values and coronary artery disease in patients with metabolic syndrome.

We divided 2167 patients with metabolic syndrome according to quartiles of MPV values. As shown in **Table 2**, higher MPV values were associated to higher age (p<0.001), percentage of ≥75 years old patients (p=0.002), hypercholesterolemia (p=0.02), renal failure (p=0.003), treatment with ASA (p=0.038), higher levels of haemoglobin (p<0.001), creatinine (0.049), uric acid (p=0.01) and to lower platelets count (p<0.001).

In terms of angiographic characteristics, patients with higher MPV values displayed a slightly lower rate of CAD (81.8% vs 78.7% vs 80.7% vs 76.7%, p=0.10; **Figure 2A**), but showed a lower prevalence of severe CAD (35.4% vs 30.4% vs 31.3% vs 28.4%, p=0.03; **Figure 2B**).

Table 2. Clinical characteristics according to MPV quartiles in patients with metabolic syndrome

Baseline clinical characteristics	**I quartile <10.2 fl n=486**	**II quartile 10.2-10.7 fl n=543**	**III quartile 10.8-11.4 fl n=563**	**IV quartile ≥11.5 fl n=575**	**p-value**
Age (mean±SD)	67.34 (±10.76)	67.48 (±11.17)	69.32 (±9.51)	69.33 (±10.26)	<0.001
Age ≥75 years (%)	26.8	31	33.9	35.6	0.002
Male sex (%)	56.2	68.4	63.4	62.7	0.19
BMI (mean±SD)	28.39 (±4.61)	29.06 (±4.69)	28.81 (±4.59)	29.25 (±4.93)	0.02
Hypercholesterolemia (%)	66.4	67.5	64.4	64.1	0.27
Renal failure (%)	27.8	25.5	32.4	34.2	0.003
Smokers (%)					0.42
Active smokers	21.1	24.9	21.6	24.4	
Previous smoker	20.9	21.3	20.5	22.1	
Hypertension (%)	87.3	88.7	88.7	87.4	0.99
Diabetes mellitus (%)	47.3	51.5	51.6	51.7	0.19
History of MI (%)	23.2	27.6	28	28.5	0.07
Previous PCI (%)	28.5	31.8	33	29.6	0.69
Previous CABG (%)	12.9	11.5	12	14.3	0.41
Indication to angiography					0.08
Stable Angina (%)	25.1	28.8	28.7	25	
ACS (%)	60.6	53.7	55	52.8	
CMD / Valvulopathy (%)	14.3	17.5	16.3	22.2	
ACS type (n=1201)					0.85
STEMI (%)	17.3	18.4	21.9	16.8	
NSTEMI/UA (%)	82.7	81.6	78.1	83.2	
Concomitant medications					
ACE inhibitors (%)	40	39	42	40.1	0.74
ARB (%)	27.5	27.7	25.7	32.6	0.12
Beta blockers (%)	56.2	62.9	57.8	61.1	0.37
Nitrates (%)	35.8	40.7	41.1	41.8	0.065
Statins (%)	55.4	58.4	59	59.1	0.24
ASA (%)	59.1	63.5	64.5	65.5	0.038
Clopidogrel (%)	21	20.2	26.8	22.1	0.24
Calcium channel blockers (%)	26.1	25	23.2	27.6	0.71
Diuretics (%)	37.2	37.7	38.4	42.1	0.09

Biochemistry parameters (mean± SD)

Platelets (10^3/µl)	245.31 (±72.51)	229.07 (±62.01)	215.19 (±51.3)	196.9 (±61.58)	<0.001
Haemoglobin g/dl)	12.96 (±1.88)	13.26 (±1.76)	13.43 (±1.71)	13.5 (±1.77)	<0.001
WBC (10^3/µl)	7.87 (±2.45)	7.96 (±2.46)	7.9 (±2.45)	7.96 (±2.47)	0.95
Glycaemia (mg/dL)	132.46 (±53.57)	132.96 (±67.69)	133.15 (±54.56)	136.37 (±58.63)	0.69
HbA1c (%)	6.48 (±1.3)	6.58 (±1.43)	6.57 (±1.38)	6.72 (±1.43)	0.07
Creatinine (mg/dL)	1.039 (±0.414)	1.067 (±0.47)	1.116 (±0.785)	1.119 (±0.415)	0.049
HDL cholesterol (mg/dl)	38.94 (±11.93)	39.66 (±10.97)	38.96 (±10.62)	38.82 (±11.79)	0.62
Total cholesterol (mg/dL)	161.01 (±43.8)	162.1 (±40.12)	157.7 (±40.14)	158.07 (±40.99)	0.22
LDL cholesterol (mg/dL)	90.55 (±36.2)	90.97 (±32.92)	88.98 (±33.83)	88.33 (±32.87)	0.55
Triglycerides (mg/dl)	156.4 (±88.86)	154.64 (±82.89)	150.64 (±82.91)	151.29 (±85.52)	0.66
C-reactive protein (mg/dl)	1.46 (±3.18)	1.12 (±2.32)	1.25 (±2.65)	1.32 (±2.93)	0.28
Uric acid (mg/dL)	6.12 (±1.84)	6.09 (±1.74)	6.43 (±1.82)	6.46 (±1.95)	0.01
Fibronogen (mg/dl)	446.29 (±143.5)	435.43 (±140.43)	451.27 (±151.17)	440.72 (±141.28)	0.32

BMI=Body Mass Index; MI = Myocardial Infarction; PCI = Percutaneous Coronary Interventions; CABG = Coronary Artery Bypass Grafting; ACS = Acute Coronary Syndrome; CMD =Dilated Cardiomyopathy; EF = Ejection Fraction; ACE = Angiotensin Converting Enzyme; ARB = Angiotensin Receptor Blockers; ASA = Acetylsalicylic Acid; WBC= White Blood Cells; LDL = Low-Density Lipoproteins; HDL= High-Density Lipoproteins.

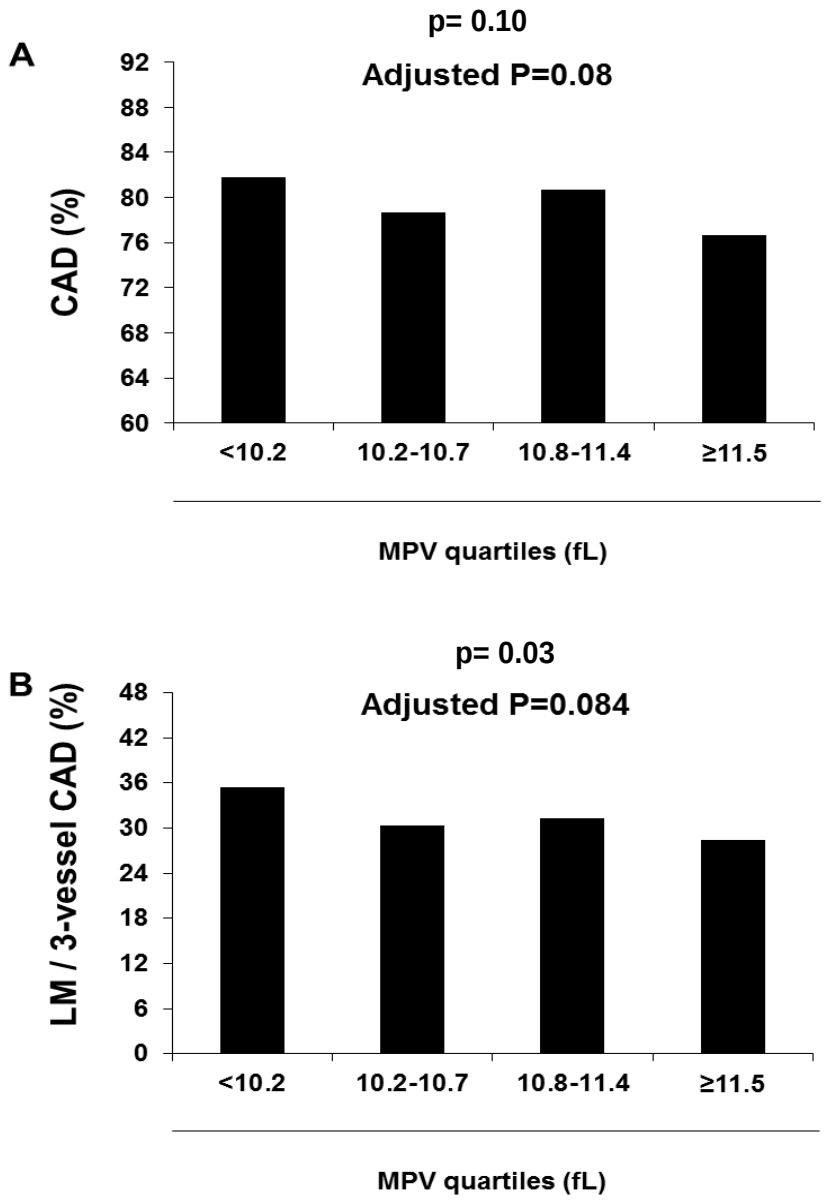


Figure 2. Bar graphs showing the prevalence of coronary artery disease (CAD) (figure A, upper graph) and severe CAD (Figure B, lower graph) according to MPV quartiles in metabolic syndrome.

As displayed in **Table 3**, MPV was inversely associated to multivessel disease (p=0.03), calcifications (p=0.028), chronic occlusion (p=0.004) and related with lower reference diameter (p=0.03).

Table 3. Angiographic characteristics according to MPV quartiles in patients with metabolic syndrome.

Angiographic features		I quartile <10.2 fl n=486	II quartile 10.2-10.7 fl n=543	III quartile 10.8-11.4 fl n=563	IV quartile ≥11.5 fl n=575	p-value
CAD (%)§		81.8	78.7	80.7	76.7	0.1
Multivessel CAD (%)§		56.4	51.2	51.1	49.4	0.03
Left main/3-vessel disease		35.4	30.4	31.3	28.4	0.03
Left main disease (%)§		10.8	8.6	8.0	9.8	0.59
LAD (%)§		57.1	57.8	56.4	52.2	0.09
CX (%)§		47.6	43.5	46.7	43.4	0.37
RCA (%)§		56.9	49.3	50.7	50.3	0.1
Type C Lesion (%)		30.4	33.6	34.8	33.4	0.18
Lesion length (mm±SD)		20.6 (±13.27)	20.82 (±13.89)	20.58 (±13.61)	20.17 (±13.49)	0.83
Percent stenosis (%)		85.73 (±14.89)	85.73 (±14.4)	85.94 (±14.34)	85.25 (±15.51)	0.81
Reference Diameter (mm)		2.88 (±0.59)	2.92 (±0.63)	2.9 (±0.57)	2.97 (±0.61)	0.03
Calcifications (%)		19.2	19.4	27.1	21.4	0.028
Chronic occlusion (%)		14.8	14.9	16.8	19.7	0.004
Restenosis (%)		5	4.7	6.6	4.7	0.73
Thrombus (%)		4.2	3.6	3	3.5	0.36
Bifurcation lesion (%)		23.5	19.3	20.6	19.8	0.13
TIMI Flow						0.1
	3	72.2	72.7	72.5	70.4	
	2	6.1	5.4	3.5	4.8	
	1	3.4	2.9	2.9	2.5	
	0	18.3	19	21.1	22.3	

§ Per patient definition
CAD= Coronary artery disease; LAD= Left anterior descending; CX= circumflex coronary artery; RCA= Right coronary artery;

At multivariate analysis, after correction for main baseline differences (age, male sex, BMI, renal failure, previous MI, diuretics, creatinine, haemoglobin, platelets), higher MPV values (across quartiles) did not result as an independent predictor of CAD (adjusted

OR[95%CI] = 0.79[0.61-1.03], p=0.08) and severe CAD (adjusted OR[95%CI]= 0.82 [0.65-1.03], p=0.084).

A similar association was observed when analyzing our 2749 patients with the new MetS definition. Higher MPV values were not associated with higher prevalence of CAD (82.9% vs 80.1% vs 80.6% vs 87.9%, p=0.049; adjusted OR[95%CI]= 0.78 [0.35-1.75], p=0.55) and severe CAD (34.7% vs 32.2% vs 32.1% vs 31.1%, p=0.20¸ adjusted OR[95%CI]= 0.96 [0.82-1.05], p=0.24).

Similar results were confirmed in special subgroups of patients, with or without established CAD, ACS at presentation and use of ASA, with no significant interaction with MPV (defined as above the median, ≥ 10.8 fL), except for the risk of severe CAD in patients with or without established CAD (p-interaction =0.002) as displayed in **Figure 3**.

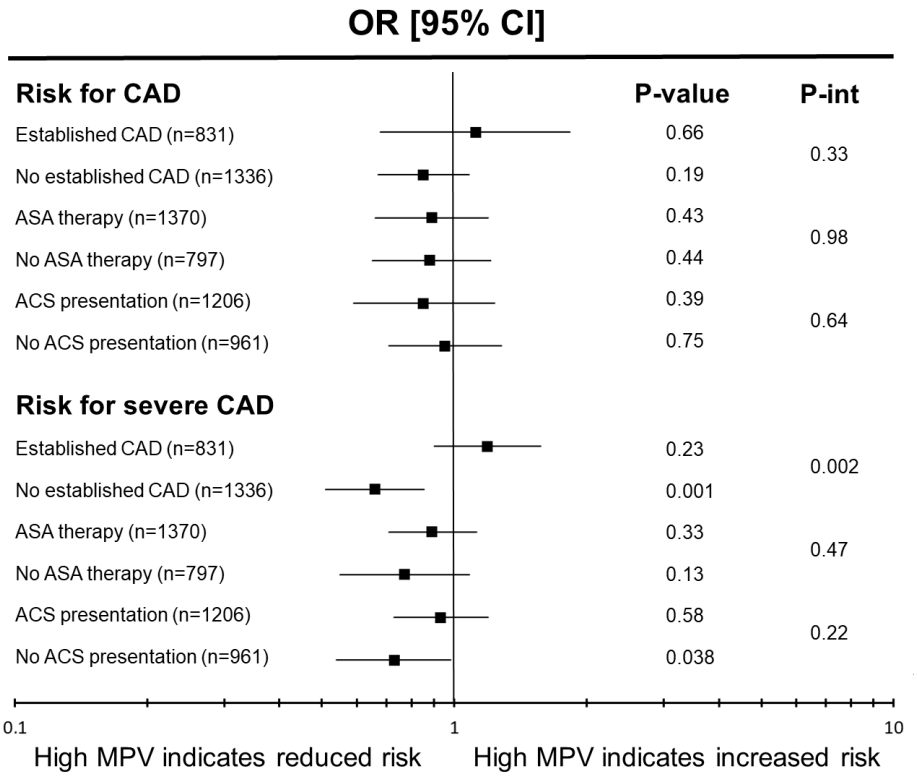


Figure 3. Forest plot showing the risk for coronary artery disease (CAD) and severe CAD according to mean platelet volume (MPV) values in specific subgroups of patients with metabolic syndrome. (ASA: acetylsalicylic acid; ACS: acute coronary syndrome; P-int: p-interaction).

Discussion

The present study represents to the best of our knowledge the largest study so far conducted on metabolic syndrome and mean platelet volume (MPV) in patients undergoing coronary angiography.

We found no independent association between metabolic syndrome (MetS) and higher MPV values, that was confirmed after correction for main baseline differences. Moreover, in the subgroup of patients with MetS, MPV values did not show any independent association with CAD.

Despite the significant improvements in the treatment of coronary artery disease, especially in the acute setting, CAD still remains one of the main causes of mortality in developed countries. Therefore, large efforts have been done in the identification of new risk factors in order to better assess the risk of CAD and optimally prevent its occurrence and complications. (26,27).

Among factors involved in the pathophysiology of CAD, platelets play a key-role in the atherothrombotic process, as their activation and aggregation is closely related to cardiovascular complications (28). Therefore, the assessment of platelet reactivity and thrombotic risk could potentially lead to improvements in patients' outcome(29).

It has been observed that larger platelets are metabolically and enzymatically more active as compared to smaller ones: in particular larger platelets display a larger content in granules, higher levels of thromboxane A2 and increased expression of adhesion proteins as glycoproteins Ib and IIB//IIIA(12,30–32). Therefore, mean platelet volume has been addressed for its potential role as a marker of platelet activity and CAD. In two hundred patients undergoing coronary angiography Kiliçli-Camur et al have found that MPV was an independent risk factor for CAD and MI, suggesting the importance of considering MPV together with conventional risk factors(33). Similarly, Murat et colleagues have

investigated among 395 patients with acute coronary syndrome (ACS), where MPV emerged as an independent predictor of the severity of CAD(34).

However, the positive relationship between MPV and CAD has not been confirmed in other studies(35,36). Halbmayer et al, in fact, have compared about four hundred patients with coronary heart disease and 125 healthy individuals. They found no difference of MPV between healthy persons and patients with CAD; additionally no significant variations of MPV values have been reported between patients without myocardial infarction (MI) and MI survivors (37). We have previously reported similar results(38): in a cohort of more than 1400 patients undergoing coronary angiography no relationship was found between MPV and the extent of CAD and platelet aggregation (39).

Different findings, however, could be expected in patients with metabolic disorders (40). In fact, MPV has been previously linked to diabetes mellitus, with a positive relationship between higher MPV values and diabetes mellitus diagnosis (41)(42). Moreover, MPV has been investigated also in patients with Metabolic syndrome (MetS)(43), that represents a cluster of cardiovascular risk factors including hypertension, impaired glucose metabolism, hypertriglyceridemia, hypercholesterolemia, abdominal obesity(24). The main common mechanisms involved in MetS determinants is constitute by insulin resistance and hyperinsulinemia(17). It has been demonstrated that insulin stimulates the production of bigger platelets during megakaryocytopoiesis(44), potentially affecting platelet reactivity and thrombosis.

Positive relationship between MPV and MetS has been reported by Furman-Niedziejko et al, who found higher MPV values in patients with metabolic syndrome compared with controls in a cohort of 382 persons(45). Similar results have been observed by Tavil et colleagues, with a positive correlation between MPV and MetS (19).

However, opposite results have been reported by other authors(46). In a large cohort of patients Shah et al have shown no significant difference in MPV values between subjects with and without MetS(47). Similarly Lee et al have found no correlation in MPV values and MetS diagnosis in their cohort after correction for main baseline differences of patients(20).

Our main findings are consistent with higher MPV values in MetS patients as compared with patients without MetS. However, at multivariate analysis after correction for main baseline characteristics we found no significant relationship between metabolic syndrome and MPV values. Analogous findings have been documented in a recent meta-analysis (40) pooling thirty-nine studies where MPV and other platelet parameters were investigated in relation to diabetes mellitus, impaired fasting glucose and metabolic syndrome. Their results were consistent with no differences in MPV values according to metabolic syndrome diagnosis.

We also investigated the role of MPV in relation to CAD in a cohort of patients with MetS. In particular, we found no association between MPV and the prevalence and extension of CAD, that was confirmed after adjustment for main baseline differences. However, we observed a positive interaction between established CAD and MPV for the risk of severe CAD. Indeed, it might be argued that patients without established CAD were younger and displayed, anyhow, a better control of metabolic profile and less frequent comorbidities, with a lower degree of inflammation, parameters that are known to increase platelet turnover and therefore the MPV(48). On the contrary, among patients with established CAD, the longer exposure to MetS, the increased severity of metabolic profile and the interference of drugs, and especially antiplatelet agents, could have interfered with higher MPV values and with a larger extent of coronary disease. In fact, we previously reported a tendency to increase in the immature platelet count among patients receiving a more

aggressive antiplatelet regimen, that were also, probably, those with a more relevant coronary disease(49).

Few studies have investigated the role of MPV to predict CAD in high-risk population such as metabolic syndrome patients. In their study, Tavil et al, have reported that higher MPV values correlated to severity of CAD among MetS patients, suggesting a potential role of MPV values in atherosclerosis process(19). However this evidence has not been confirmed by Arican Ozluk et al who in a large cohort of patients (more than 1300) have found no significant changes in MPV values according to angiographically defined peripheral artery disease (50).

Speculative hypothesis about MPV could be formulated taking into account two main points. First, in agreement with previous studies, we have found an inverse correlation between MPV values and platelet count, suggesting the peripheral consumption of small platelets with consequent compensatory production of larger reticulated platelets (51); secondly, a positive correlation has been shown between MPV and thrombopoietin levels, which represents a key hormone in thrombopoietic process(52). These evidences may suggest a mainly haemopoietic dysregulation marker role of MPV respect of predictor role of platelet activation. Additionally, it should be mentioned that previous reports showed significantly higher values of MPV in elderly patients (53)(54): in fact, impaired ability to replace blood cells due to stimuli like blood loss has been documented especially above 75 years of age (55).

Therefore, the general agreement is far from being achieved and additional studies are certainly needed to better define the true role of MPV as marker of elevated atherothrombotic risk, especially at long-term follow-up, with a special focus on higher risk subgroups of patients, such as individuals with metabolic syndrome.

Limitations

The inclusion in our study of a cohort of patients undergoing coronary angiography, could have led to a higher prevalence of CAD among our patients, therefore potentially affecting the possibility of observing any impact of MPV. However, such kind of selection was made on purpose, as only coronary angiography can provide certainty about the presence or absence of significant CAD, therefore preventing us from identifying a proper control group. In fact, the absence of symptoms could not have excluded the presence of CAD, especially among high-risk patients, as the large proportion of elderly and diabetics enrolled in our study. Nevertheless, we included also an important percentage (about 18%) of patients undergoing coronary angiography for arrhythmias or dilated cardiomiopathy or valvular disease, and thus with a lower pre-test probability of CAD.

In addition, we did not exclude patients with previous PCI or CABG, since atherosclerosis is a progressive disease. Patients with already established CAD are not free from the disease, that does persist and can progress. Therefore, considering these subjects as "normal" would have led to a selection bias, for the loss of a certain number of patients with CAD and a spuriously lower CAD prevalence. On the contrary, the inclusion of a higher risk population, such as carriers of coronary stents that can favor the thrombotic processes, would have, instead, potentially resulted in an even stronger association between MPV and CAD, should have been one. However, we did not find any significant interaction between MPV and major cardiovascular risk conditions at subgroup analysis.

Moreover, in patients with previous coronary revascularization, both treated and untreated vessels (and also CABG disease) were taken into account for the count of the diseased vessels, therefore allowing to consider both previous treatment and the progression of the disease**.**

In addition, the use of intravascular imaging, such as intravascular ultrasound (IVUS) or optical coherence tomography (OCT) would certainly have provided a more precise characterization of coronary plaques, contributing to improve our findings. However, such techniques cannot still be applied on a very large scale.

Moreover, in our definition of metabolic syndrome, according to guidelines, we did not exclude diabetic patients, since it would have led to an underestimation of the burden of metabolic syndrome. Indeed, in our study we did not include patients with mild impairment of glucose metabolism (fasting glucose 110-125 mg/dl), since modest variations in glucose homeostasis have been reported among patients with acute coronary syndromes (56), and therefore we preferred to apply a more stringent definition, and exclude a potential bias to the over-inclusion of patients with normal baseline glucose metabolism.

Finally, we did not collect follow-up data, especially in patients undergoing coronary angioplasty, and thus cannot exclude an impact of MPV and metabolic syndrome on the progression of CAD or on the occurrence of acute thrombotic events at distance.

Conclusions

The present study shows that among patients undergoing coronary angiography MetS is not an independent predictor of higher MPV. Furthermore, among patients with MetS, MPV does not predict the prevalence and the severity of CAD.

References

1. De Luca G, Suryapranata H, Marino P. Reperfusion strategies in acute ST-elevation myocardial infarction: an overview of current status. Prog Cardiovasc Dis. 50(5):352–82.
2. Verdoia M, Schaffer A, Barbieri L, Suryapranata H, De Luca G. Bivalirudin Versus Unfractionated Heparin in Acute Coronary Syndromes: An Updated Meta-analysis of Randomized Trials. Rev Esp Cardiol (Engl Ed). 2016 Aug;69(8):732–45.
3. De Luca G, Navarese EP, Cassetti E, Verdoia M, Suryapranata H. Meta-analysis of randomized trials of glycoprotein IIb/IIIa inhibitors in high-risk acute coronary syndromes patients undergoing invasive strategy. Am J Cardiol. 2011 Jan 15;107(2):198–203.
4. De Luca G, Schaffer A, Wirianta J, Suryapranata H. Comprehensive meta-analysis of radial vs femoral approach in primary angioplasty for STEMI. Int J Cardiol. 2013 Oct 3;168(3):2070–81.
5. De Luca G, Dirksen MT, Spaulding C, Kelbæk H, Schalij M, Thuesen L, et al. Impact of diabetes on long-term outcome after primary angioplasty: insights from the DESERT cooperation. Diabetes Care. 2013 Apr;36(4):1020–5.
6. De Luca G, Dirksen MT, Spaulding C, Kelbæk H, Schalij M, Thuesen L, et al. Time course, predictors and clinical implications of stent thrombosis following primary angioplasty. Insights from the DESERT cooperation. Thromb Haemost. 2013 Oct;110(4):826–33.
7. Sandercock PA, van den Belt AG, Lindley RI, Slattery J. Antithrombotic therapy in acute ischaemic stroke: an overview of the completed randomised trials. J Neurol Neurosurg Psychiatry. 1993 Jan;56(1):17–25.
8. Rasmanis G, Vesterqvist O, Gréen K, Edhag O, Henriksson P. Effects of intermittent treatment with aspirin on thromboxane and prostacyclin formation in patients with acute myocardial infarction. Lancet. 1988 Jul 30;2(8605):245–7.
9. Davì G, Patrono C. Platelet activation and atherothrombosis. N Engl J Med. 2007 Dec 13;357(24):2482–94.
10. Collaborative meta-analysis of randomised trials of antiplatelet therapy for prevention of death, myocardial infarction, and stroke in high risk patients. BMJ. 2002 Jan 12;324(7329):71–86.

11. Chu SG, Becker RC, Berger PB, Bhatt DL, Eikelboom JW, Konkle B, et al. Mean platelet volume as a predictor of cardiovascular risk: a systematic review and meta-analysis. J Thromb Haemost. 2010 Jan;8(1):148–56.
12. Jakubowski JA, Thompson CB, Vaillancourt R, Valeri CR, Deykin D. Arachidonic acid metabolism by platelets of differing size. Br J Haematol. 1983 Mar;53(3):503–11.
13. Pizzulli L, Yang A, Martin JF, Lüderitz B. Changes in platelet size and count in unstable angina compared to stable angina or non-cardiac chest pain. Eur Heart J. 1998 Jan;19(1):80–4.
14. Damodar S, Ganesh KV, Murthy S. Mean platelet volume does not predict risk of myocardial infarction or coronary artery disease in Indian patients. Platelets. 2008 Feb;19(1):80–1.
15. Isomaa B, Almgren P, Tuomi T, Forsén B, Lahti K, Nissén M, et al. Cardiovascular morbidity and mortality associated with the metabolic syndrome. Diabetes Care. 2001 Apr;24(4):683–9.
16. Kahn R, Buse J, Ferrannini E, Stern M. The metabolic syndrome: time for a critical appraisal: joint statement from the American Diabetes Association and the European Association for the Study of Diabetes. Diabetes Care. 2005 Sep;28(9):2289–304.
17. Reaven GM. Banting lecture 1988. Role of insulin resistance in human disease. Diabetes. 1988 Dec;37(12):1595–607.
18. Gami AS, Witt BJ, Howard DE, Erwin PJ, Gami LA, Somers VK, et al. Metabolic syndrome and risk of incident cardiovascular events and death: a systematic review and meta-analysis of longitudinal studies. J Am Coll Cardiol. 2007 Jan 30;49(4):403–14.
19. Tavil Y, Sen N, Yazici HU, Hizal F, Abaci A, Cengel A. Mean platelet volume in patients with metabolic syndrome and its relationship with coronary artery disease. Thromb Res. 2007 Jan;120(2):245–50.
20. Lee EY, Kim SJ, Song YJ, Choi SJ, Song J. Immature platelet fraction in diabetes mellitus and metabolic syndrome. Thromb Res. 2013 Jan;132(6):692–5.
21. Roffi M, Patrono C, Collet J-P, Mueller C, Valgimigli M, Andreotti F, et al. 2015 ESC Guidelines for the management of acute coronary syndromes in patients presenting without persistent ST-segment elevation. Eur Heart J. 2016 Jan 14;37(3):267–315.

22. Ibanez B, James S, Agewall S, Antunes MJ, Bucciarelli-Ducci C, Bueno H, et al. 2017 ESC Guidelines for the management of acute myocardial infarction in patients presenting with ST-segment elevation. Eur Heart J. 2017 Aug 26;
23. Alberti KGMM, Zimmet P, Shaw J. The metabolic syndrome--a new worldwide definition. Lancet. 366(9491):1059–62.
24. Alberti KGMM, Eckel RH, Grundy SM, Zimmet PZ, Cleeman JI, Donato KA, et al. Harmonizing the Metabolic Syndrome: A Joint Interim Statement of the International Diabetes Federation Task Force on Epidemiology and Prevention; National Heart, Lung, and Blood Institute; American Heart Association; World Heart Federation; International. Circulation. 2009 Oct 5;120(16):1640–5.
25. De Luca G, Secco GG, Santagostino M, Venegoni L, Iorio S, Cassetti E, et al. Uric acid does not affect the prevalence and extent of coronary artery disease. Results from a prospective study. Nutr Metab Cardiovasc Dis. 2012 May;22(5):426–33.
26. Pitsilos S, Hunt J, Mohler ER, Prabhakar AM, Poncz M, Dawicki J, et al. Platelet factor 4 localization in carotid atherosclerotic plaques: correlation with clinical parameters. Thromb Haemost. 2003 Dec;90(6):1112–20.
27. Coppinger JA, Cagney G, Toomey S, Kislinger T, Belton O, McRedmond JP, et al. Characterization of the proteins released from activated platelets leads to localization of novel platelet proteins in human atherosclerotic lesions. Blood. 2004 Mar 15;103(6):2096–104.
28. Tsiara S, Elisaf M, Jagroop IA, Mikhailidis DP. Platelets as predictors of vascular risk: is there a practical index of platelet activity? Clin Appl Thromb Hemost. 2003 Jul;9(3):177–90.
29. De Luca G, Secco GG, Iorio S, Verdoia M, Bellomo G, Marino P. Short-term effects of aspirin and clopidogrel on mean platelet volume among patients with acute coronary syndromes. A single-center prospective study. Blood Coagul Fibrinolysis. 2012 Dec;23(8):756–9.
30. Martin JF, Trowbridge EA, Salmon G, Plumb J. The biological significance of platelet volume: its relationship to bleeding time, platelet thromboxane B2 production and megakaryocyte nuclear DNA concentration. Thromb Res. 1983 Dec 1;32(5):443–60.
31. Giles H, Smith RE, Martin JF. Platelet glycoprotein IIb-IIIa and size are increased in acute myocardial infarction. Eur J Clin Invest. 1994 Jan;24(1):69–72.

32. Kamath S, Blann AD, Lip GY. Platelet activation: assessment and quantification. Eur Heart J. 2001 Sep;22(17):1561–71.
33. Kiliçli-Camur N, Demirtunç R, Konuralp C, Eskiser A, Başaran Y. Could mean platelet volume be a predictive marker for acute myocardial infarction? Med Sci Monit. 2005 Aug;11(8):CR387-92.
34. Murat SN, Duran M, Kalay N, Gunebakmaz O, Akpek M, Doger C, et al. Relation between mean platelet volume and severity of atherosclerosis in patients with acute coronary syndromes. Angiology. 2013 Feb;64(2):131–6.
35. De Luca G, Verdoia M, Cassetti E, Schaffer A, Di Giovine G, Bertoni A, et al. Mean platelet volume is not associated with platelet reactivity and the extent of coronary artery disease in diabetic patients. Blood Coagul Fibrinolysis. 2013 Sep;24(6):619–24.
36. Verdoia M, Camaro C, Barbieri L, Schaffer A, Marino P, Bellomo G, et al. Mean platelet volume and the risk of periprocedural myocardial infarction in patients undergoing coronary angioplasty. Atherosclerosis. 2013 May;228(1):136–41.
37. Halbmayer WM, Haushofer A, Radek J, Schön R, Deutsch M, Fischer M. Platelet size, fibrinogen and lipoprotein(a) in coronary heart disease. Coron Artery Dis. 1995 May;6(5):397–402.
38. Verdoia M, Pergolini P, Rolla R, Nardin M, Barbieri L, Schaffer A, et al. Mean platelet volume and high-residual platelet reactivity in patients receiving dual antiplatelet therapy with clopidogrel or ticagrelor. Expert Opin Pharmacother. 2015;16(12):1739–47.
39. De Luca G, Santagostino M, Secco GG, Cassetti E, Giuliani L, Franchi E, et al. Mean platelet volume and the extent of coronary artery disease: results from a large prospective study. Atherosclerosis. 2009 Sep;206(1):292–7.
40. Zaccardi F, Rocca B, Pitocco D, Tanese L, Rizzi A, Ghirlanda G. Platelet mean volume, distribution width, and count in type 2 diabetes, impaired fasting glucose, and metabolic syndrome: a meta-analysis. Diabetes Metab Res Rev. 2015 May 29;31(4):402–10.
41. Sahin DY, Gür M, Elbasan Z, Özdoğru I, Uysal OK, Kivrak A, et al. Mean platelet volume and extent and complexity of coronary artery disease in diabetic and nondiabetic patients with ST elevation myocardial infarction. Angiology. 2013 Oct;64(7):505–11.

42. Han J-Y, Choi D-H, Choi S-W, Kim B-B, Ki Y-J, Chung J-W, et al. Stroke or coronary artery disease prediction from mean platelet volume in patients with type 2 diabetes mellitus. Platelets. 2013;24(5):401–6.
43. Kutlucan A, Bulur S, Kr S, Bulur S, Önder E, Aslantaş Y, et al. The relationship between mean platelet volume with metabolic syndrome in obese individuals. Blood Coagul Fibrinolysis. 2012 Jul;23(5):388–90.
44. Watanabe Y, Kawada M, Kobayashi B. Effect of insulin on murine megakaryocytopoiesis in a liquid culture system. Cell Struct Funct. 1987 Jun;12(3):311–6.
45. Furman-Niedziejko A, Rostoff P, Rychlak R, Golinska-Grzybala K, Wilczynska-Golonka M, Golonka M, et al. Relationship between abdominal obesity, platelet blood count and mean platelet volume in patients with metabolic syndrome. Folia Med Cracov. 2014 Jan;54(2):55–64.
46. Jesri A, Okonofua EC, Egan BM. Platelet and white blood cell counts are elevated in patients with the metabolic syndrome. J Clin Hypertens (Greenwich). 2005 Dec;7(12):705-11-3.
47. Shah B, Sha D, Xie D, Mohler ER, Berger JS. The relationship between diabetes, metabolic syndrome, and platelet activity as measured by mean platelet volume: the National Health And Nutrition Examination Survey, 1999-2004. Diabetes Care. 2012 May;35(5):1074–8.
48. Santilli F, Vazzana N, Liani R, Guagnano MT, Davì G. Platelet activation in obesity and metabolic syndrome. Obes Rev. 2012 Jan;13(1):27–42.
49. Verdoia M, Pergolini P, Rolla R, Barbieri L, Schaffer A, Marino P, et al. Impact of Long-Term Dual Antiplatelet Therapy on Immature Platelet Count and Platelet Reactivity. Angiology. 2017 Jan 1;3319717736407.
50. Arican Ozluk O, Ber I, Peker T, Yilmaz M, Tenekecioglu E, Karaagac K, et al. Mean platelet volume levels in the presence of angiographically documented peripheral artery disease. Int J Clin Exp Med. 2015;8(2):2899–904.
51. McCabe DJH, Harrison P, Sidhu PS, Brown MM, Machin SJ. Circulating reticulated platelets in the early and late phases after ischaemic stroke and transient ischaemic attack. Br J Haematol. 2004 Sep;126(6):861–9.

52. ŞENARAN H, Ileri M, AltinbaŞ A, KoŞar A, Yetkin E, ÖZTÜRK M, et al. Thrombopoietin and mean platelet volume in coronary artery disease. Clin Cardiol. 2001 May;24(5):405–8.
53. Verdoia M, Schaffer A, Barbieri L, Bellomo G, Marino P, Sinigaglia F, et al. Impact of age on mean platelet volume and its relationship with coronary artery disease: a single-centre cohort study. Exp Gerontol. 2015 Feb;62:32–6.
54. Verdoia M, Schaffer A, Barbieri L, Bellomo G, Marino P, Sinigaglia F, et al. Impact of age on mean platelet volume and its relationship with coronary artery disease: A single-centre cohort study. Exp Gerontol. 2015 Feb;62:32–6.
55. Henry CJ, Marusyk A, DeGregori J. Aging-associated changes in hematopoiesis and leukemogenesis: what's the connection? Aging (Albany NY). 2011 Jun;3(6):643–56.
56. Ito S, Nagoshi T, Minai K, Kashiwagi Y, Sekiyama H, Yoshii A, et al. Possible increase in insulin resistance and concealed glucose-coupled potassium-lowering mechanisms during acute coronary syndrome documented by covariance structure analysis. PLoS One. 2017;12(4):e0176435.

Part 3

Platelets Reactivity and Atherosclerosis

Chapter 5

Impact of adenosine A2a receptor polymorphism rs5751876 on platelet reactivity in ticagrelor treated patients

Matteo Nardin[a, b]; Monica Verdoia[a]; Patrizia Pergolini[c]; Roberta Rolla[c]: Lucia Barbieri[a]; Gennaro Galasso[d]; Paolo Marino[a]; Giorgio Bellomo[c]; Giuseppe De Luca[a]; on behalf of the Novara Atherosclerosis Study Group (NAS)

a) Division of Cardiology, Azienda Ospedaliera-Universitaria "Maggiore della Carità", Eastern Piedmont University, Novara, Italy; b) Department of Medicine, ASST "Spedali Civili", University of Brescia, Italy; c) Clinical Chemistry, Azienda Ospedaliera-Universitaria "Maggiore della Carità", Eastern Piedmont University, Novara, Italy; d) Division of Cardiology, Department of Medicine and Surgery, University of Salerno, Salerno, Italy.

Pharmacological Research (2018) 129: 27–33

Abstract

Dual antiplatelet therapy constitutes a key point in the management of patients with acute coronary syndromes. In particular, ticagrelor, an ADP-antagonist, can provide a more potent and predictable platelet inhibition as compared to clopidogrel, and adenosine-mediated pathways have been involved in its beneficial effects on mortality and myocardial perfusion. However, a quote of patients still displays a suboptimal platelet inhibition on ticagrelor, and, while the role of genetics in conditioning clopidogrel resistance is well established, few data have been reported for ticagrelor. We investigated the impact of rs5751876 C>T polymorphism of adenosine A2a receptor (ADORA2a) on platelet reactivity in patients during chronic treatment with ticagrelor.

We included patients treated with ASA and ticagrelor for a recent ACS or elective coronary revascularization. Platelet reactivity was assessed at 30-90 days post-discharge by multiple-electrode aggregometry. HRPR for ticagrelor was defined as ADP-test results >417 AU*min. Genetic analysis was performed to assess the presence of rs5751876 C>T polymorphism of ADORA2a receptor. We included 244 patients in our study, 174 (71.3%) patients carried the polymorphism (T allele), 51 (20.9%) of them in homozygosis (T/T). C-allele carriers (homozygotes C/C and heterozygotes C/T) showed no difference in baseline characteristics but for lower HDL-cholesterol (p=0.01). An absolute lower rate of HRPR on ticagrelor was observed in homozygotes T/T (p=0.03). At multivariate analysis, C allele carriage was independently associated with the rate of HRPR on ticagrelor (adjusted OR[95%CI]= 4.63[1.02-21.01], p=0.048).

Our study results showed a significant independent association between rs5751876 allele C carriage and a higher rate of high residual platelet reactivity in patients on ticagrelor after a recent ACS or PCI

Introduction

Alongside with the amelioration of technical issues leading to an increase in the number and complexity of percutaneous coronary interventions (PCI), optimal antithrombotic therapies, with the achievement of an adequate platelet inhibition, have become one of the main targets for the management of these patients (1-3). Therefore, pharmacological therapy has been enriched by new antiplatelet agents with a more potent and predictable antiplatelet effect, which have demonstrated to lower mortality in combination with acetylsalicylic acid (ASA) (4-8).

In fact, the most recently introduced ticagrelor is a direct acting antiplatelet drug, not requiring any metabolic activation, thus providing a faster and more predictable onset of action as compared to clopidogrel, where a persistent high residual platelet reactivity (HRPR) has been observed in almost 30% of patients (9), with an increased risk of stent thrombosis and recurrent cardiovascular events (10-11).

However, recent studies have shown a modest occurrence of HRPR and a more delayed effect also in ticagrelor treated patients, especially in certain higher risk subsets, such as diabetics (12-13) or the elderly(14-15), suggesting the existence of factors potentially modulating the response to ticagrelor. In addition, it has been demonstrated that the mechanism of platelet inhibition due to ticagrelor can involve the adenosine pathway (16), by binding the adenosine receptor (ADORA) and modulating cellular uptake of adenosine (16–18), whose increased plasma levels could determine platelet inhibition through the A2a adenosine receptor (ADORA2a) on platelets' surface. Such additional mechanism has been deemed respinsible for the potential side effects of ticagrelor, but also for its additional benefits, as compared to other antiplatelet agents, on mortality and myocardial perfusion (8,16). However, a genetic single nucleotide polymorphism (rs5751876) has been identified for adenosine A2a receptor consisting in a C > T substitution in position

13772 on the exon 3, potentially modulating the response to different receptor agonists/antagonists as adenosine or caffeine (19,20). However, no study has so far addressed the role of A2a receptor rs5751876 polymorphism on platelet reactivity in patients treated with ticagrelor, that was therefore the aim of the present study.

Methods

We included consecutive patients admitted for acute coronary syndromes to the Division of Cardiology, "Maggiore della Carità" Hospital, Eastern Piedmont University in Novara, Italy, from September 2013 to August 2015. Invasive treatment with coronary angiography and eventual coronary stenting was not a required inclusion criterion. All patients receiving at discharge dual antiplatelet therapy with ASA (100 to 160 mg daily) and ticagrelor (90 mg every 12 hours) were scheduled for chemistry and platelet function tests evaluation at 30-90 days from discharge. Blood samples were drawn in the early morning, following a fasting period of 12 h. The study was approved by our local Ethical Committee and informed consent was obtained from all patients.

Main demographic, clinical and angiographic data, together with the indication to dual antiplatelet therapy were recorded at discharge and included in a dedicated database, protected by password. Main cardiovascular risk factors were identified. Hypertension was defined as systolic pressure > 140 mm Hg and/or diastolic pressure was >90 mmHg or if the individual was taking antihypertensive medications. The diagnosis of diabetes was based on previous history of diabetes treated with or without drug therapies, fasting glucose >126 g/dl or HbA1c > 6.5% at the moment of admission (21). Compliance to therapy was assessed by medical interview.

Platelet aggregation

Platelet aggregation was determined by Multiplate electrical impedance aggregometry (MEA), within about 2 hours from the morning dose of ticagrelor. The aggregation tests were performed from 30 minutes to 2 hours from blood collection(22). Platelets aggregation was assessed after stimulation with arachidonic acid (0.5 mM) (ASPI test), collagen (3.2 µg/ml) (COL test), ADP (6.4 µM) with prostaglandin E1 and thrombin

receptor activating peptide, (TRAP-6; 30 µM). Results were expressed as arbitrary Aggregation Units (AU) and plotted against time, defining platelet function as the area under curve (AUC or AU*min). HRPR for Aspirin was defined for ASPI-test above 862 AU*min, (normal range: [862-1344]), whereas for ticagrelor was defined for ADP test above 417 AU*min; (normal range: [417-1030])(23). The test was repeated in patients with HRPR to confirm the findings.

Genetic analysis

We obtained an informed consent from all patients, and we performed a genetic analysis to define the presence of 1976 C > T ADORA2a polymorphism. Genomic DNA was obtained from 200 ml of whole blood through a dedicated kit (GenElute Blood Genomic DNA, Sigma Aldrich). The target region of ADORA2a gene was amplified by polymerase chain reaction (PCR) using following primers: 5'-TCC CCA CCA TGA GCG GAG GCC CAA TGG CGA-3 ' and 5'-CAA GCC AAC CAG AAA GAT AAA G-3'. A negative control containing no genomic DNA was added for every PCR reaction. PCR product of 235 base pairs was then digested by restriction enzyme Hin1I, producing a reduced fragment of 180 base pairs in absence of the C > T substitution.

Statistical analysis

Statistical analysis was performed using SPSS 23.0 statistical package. Continuous data were expressed as mean $\pm$ SD and categorical data as percentage. Analysis of variance and the chi-square test were used for continuous and categorical variables, respectively. Patients were grouped according to genotype results, considering T allele homozygotes vs C allele carriers (wild-type allele). Multiple logistic regression analysis was performed to evaluate the relationship between ADORA2a polymorphism and HRPR after correction

for main baseline differences, that were entered in the model in block. A p value < 0.05 was considered statistically significant.

Results

Our population is represented by a total of 244 patients receiving dual antiplatelet therapy with ASA and ticagrelor. Among them 174 (71.3%) patients carried the ADORA2a-T allele, 51 (20.9%) of them in homozygosis.

In **Table 1** are displayed the main clinical and demographic features according to ADORA2a polymorphism genotype. No significant differences were found in clinical and demographic characteristics. Ongoing medications did not differ between two groups, whereas for major biochemistry parameters no differences were shown except for HDL-cholesterol levels, that resulted lower in T-allele carriers as compared to other genotypes (37.59 mg/dl vs 42.27 mg/dl, p=0.01).

Table 1. Clinical and demographic characteristics according to ADORA2a polymorphism.

Baseline clinical characteristics	**ADORA2a-C allele carriers n= 193**	**ADORA2a-TT n=51**	**p-value**
Age (mean±SD)	66.6 (±11.1)	65.9 (±12.1)	0.68
Age ≥75 years	27.5	31.4	0.6
Male sex (%)	78.2	76.5	0.85
BMI (mean±SD)	27 (± 4.7)	27.6 (±4.8)	0.49
Hypercholesterolemia(%)	53.9	66.7	0.11
Renal failure (%)	16.1	11.8	0.52
Active Smokers (%)	32.1	31.4	0.94
Hypertension (%)	71.5	76.5	0.6
Diabetes mellitus (%)	35.2	39.2	0.63
History of MI (%)	21.4	11.8	0.16
Previous PCI (%)	29	17.6	0.11
Previous CABG (%)	8.8	7.8	1
Ejection fraction % (mean±SD)	51 (±10)	52.6 (±8.9)	0.28
Indication to angiography			0.51
Unstable angina (%)	20.2	21.6	
NSTEMI (%)	74.6	78.4	
STEMI (%)	4.7	0	
Multivessel disease	66.3	75.6	0.27

Left Main/ Three-Vessel CAD (%)	36.7	39	0.86
Concomitant medications			
ACE inhibitors(%)	57	70.6	0.11
ARB (%)	18.1	13.7	0.54
Beta blockers (%)	88.6	96.1	0.18
Nitrates (%)	38.3	45.1	0.42
Statins (%)	88.6	98	0.06
Calcium antagonists (%)	20.2	15.7	0.55
Diuretics (%)	30.1	29.4	1
Biochemistry parameters (mean± SD)			
Haemoglobin (g/dl)	13.49 (±1.64)	13.7 (±1.9)	0.51
Platelets (10^3/µl)	236.74 (±70.04)	233.49 (±74.6)	0.77
WBC (10^3/µl)	7.95 (±2.33)	8.31 (±2.2)	0.32
Total cholesterol (mg/dl)	136.7 (±33.8)	128.35 (±32.6)	0.12
HDL cholesterol (mg/dl)	42.27 (±12.28)	37.59 (±10.6)	0.01
LDL cholesterol (mg/dL)	71.85 (±29.13)	66.8 (±22.5)	0.256
Triglycerides (mg/dl)	117.29 (±63.53)	123.24 (±77.6)	0.57
Glycaemia (mg/dL)	120.7 (± 42.3)	125.47 (45.5)	0.49
HbA1c (%)	6.3 (±1.1)	6.51 (±1.4)	0.38
Creatinine (mg/dL)	1.03 (±0.8)	0.95 (±0.3)	0.42
Uric acid (mg/dl)	5.83 (±1.94)	6.1 (±2.2)	0.39
C-reactive protein (mg/dl)	0.51 (±0.94)	0.45 (±0.6)	0.72
COL test (AU*min; mean±SD)	439.03 (±147.09)	420.31 (±131)	0.41
ASPI test (AU*min; mean±SD)	358.65 (±187.14)	353.77 (±167.9)	0.87
TRAP test (AU*min; mean±SD)	1155.07 (±296.68)	1167.14 (±296)	0.8
ADP test (AU*min; mean±SD)	286.86 (±135.58)	271.26 (±113.8)	0.45

CAD = Coronary Artery Disease; MI = Myocardial Infarction; PCI = Percutaneous Coronary Interventions; CABG = Coronary Artery Bypass Graft; ACE = Angiotensin Converting Enzyme; ARB = Angiotensin Receptor Blockers; ASA = Acetylsalicylic Acid; LDL = Low-Density Lipoproteins;

The rate of ASA resistance was low, and not affected by the presence of the polymorphism (2.1 % for C-allele carriers vs 2 % for T/T homozygotes, p=0.99, adjusted OR[95%CI]=0.73[0.08-6.84], p=0.78) (**Figure 1**).

Moreover, as shown in **Figure 2**, mean values of platelet reactivity with different activating stimuli did not significantly differ between the two groups. At **Figure 3** we showed ADP-test values of patients according to rs5751876 genotype.

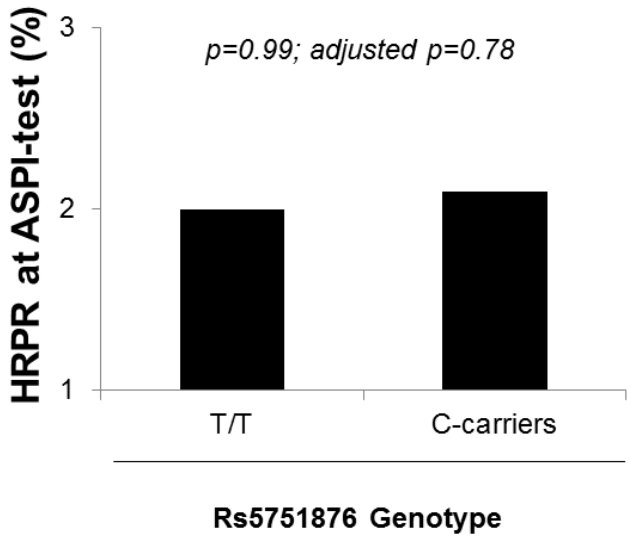


Figure 1. Bar graphs showing the prevalence of HRPR with aspirin at ASPI test according to rs5751876 genotype.

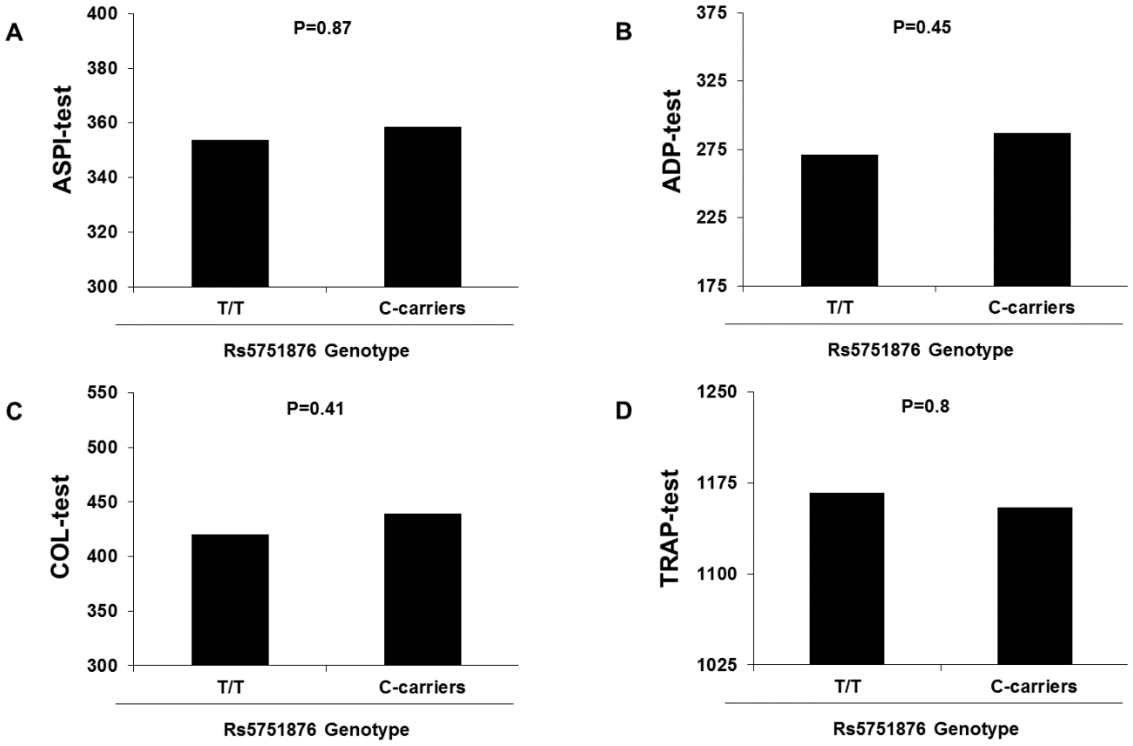


Figure 2. Bar graphs showing results of multiplate aggregometry at ASPI-test (panel A), ADP-test (panel B), COL-test (panel C) and TRAP-test (panel D) according to rs5751876 genotype

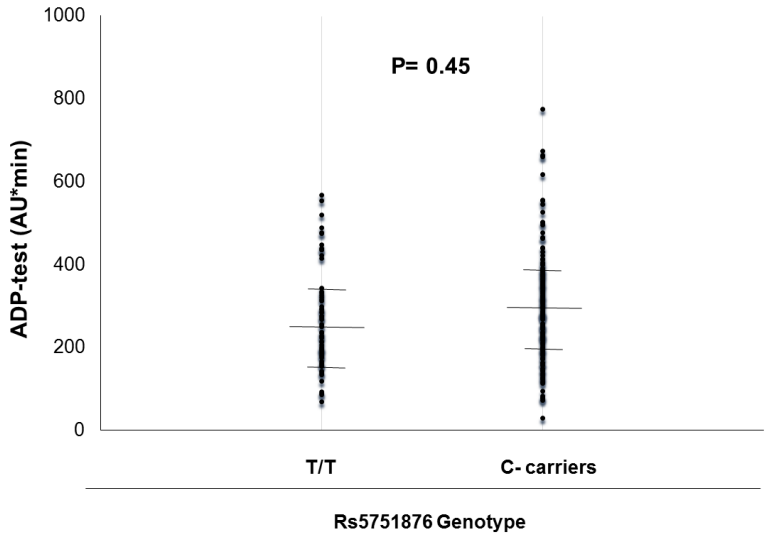


Figure 3. Graph showing the distribution of ADP-test values according to rs5751876 genotype.

HRPR with ticagrelor was observed in a total of 31 patients. As shown in **Figure 4**, we observed a significantly lower percentage of HRPR for ticagrelor in subjects homozygous for the T-allele (15.1% for C-carriers vs 3.9% for T/T, p=0.03). At multivariate analysis after correction for main baselines differences (HDL-cholesterol), we confirmed C allele carriage as the only independent predictor of HRPR with ticagrelor (adjusted OR[95%CI]= 4.63[1.02-21.01], p=0.048).

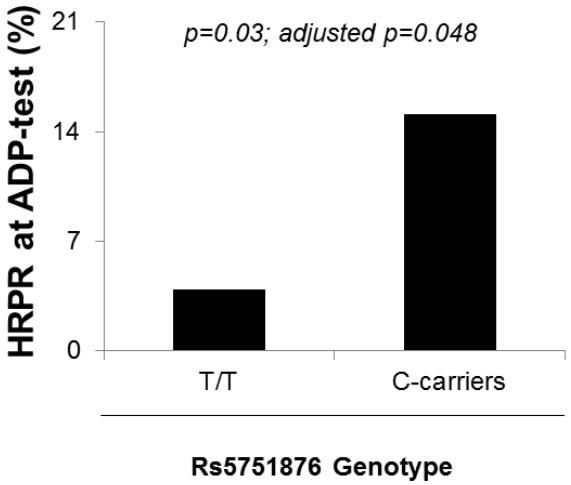


Figure 4. Bar graphs showing the prevalence of HRPR with ticagrelor at ADP test according to rs5751876 genotype

Discussion

The present study represents the first attempt to define a relationship between ADORA2a rs5751876 polymorphism and HRPR with ticagrelor. Our main results are consistent with a statistically significant association of the C-allele carriage and a suboptimal effectiveness of this drug.

Recent advances in the management of acute coronary syndromes have provided a significant improvement in patients' outcome, reducing overall mortality and morbidity(24–27).

Platelet aggregation plays a major role in the pathogenesis of acute cardiovascular events, consequently representing the key therapeutic target in the management of patients with coronary artery disease(28,29), especially in acute coronary syndrome and after drug-eluting stent implantation, where a dual antiplatelet therapy (DAPT) is indicated for 6 to 12 months (30-31) in order to prevent thrombotic complications (32).

In particular, the combination of ASA and ticagrelor has demonstrated a significant reduction in mortality and recurrent ischemic events as compared to clopidogrel. In fact, a significant percentage of patients does not achieve adequate levels of platelet inhibition with clopidogrel, maintaining a high residual platelet reactivity (HRPR), that has been clearly demonstrated to enhance the risk of major cardiovascular ischemic events (33,34). The complex metabolic pathway required for its transformation into an active drug, as much as inter-individual variability in the drug absorption and in the processes of hepatic activation, have been claimed for the occurrence of a suboptimal response to clopidogrel that is observed in about 30% of treated patients. Genetic variants of genes encoding specific proteins involved in clopidogrel transportation and metabolism have been shown to play certainly a crucial role in such occurrence (35,36). A meta-analysis including

23035 subjects has confirmed an increased risk of myocardial infarction, stent thrombosis and repeat revascularization in patients carriers of mutant variation of CYP2C19 (37).

Ticagrelor has greatly overcome these problems of HRPR, being a direct ADP P2Y12 receptor antagonist that does not require metabolic activation for exerting its antiplatelet activity(38), therefore avoiding the problems showed by clopidogrel with metabolic activation. However, about 10% of ticagrelor treated patients still display HRPR, and the underlying mechanisms are yet largely undefined (14,30,39,40).

Nevertheless, a role of genetics could be hypothesized also for explaining the suboptimal effectiveness of ticagrelor. In a recent genome-wide association study (GWAS), in fact, point mutations of the cytochrome 3A4 enzyme were associated to significant variations in the plasmatic levels of the drug, although not conditioning any clinical impact (41). Similar results were found in relation to CYP2C19 and ABCB1 polymorphisms in PLATO trial population, with ticagrelor resulting more effective than clopidogrel in acute coronary syndrome management, irrespective of these polymorphisms (42).

In addition, recent studies (16–18) have reported that a certain quote of platelet inhibition induced by ticagrelor, could be dependent from a parallel pathway mediated by adenosine. In fact, ticagrelor has been shown to inhibit the sodium-independent equilibrative nucleoside transporter (ENT1) expressed by human erythrocytes, leading to decreased cellular adenosine uptake and increased plasmatic levels of adenosine (18). Such adenosine release could then inhibit platelet aggregation by binding A2a receptor expressed on platelets' surface. The evidence of adenosine mediated platelet inhibition during ticagrelor treatment has been shown by Nylander et al (16), who documented in 50 healthy subjects that part of the antiplatelet effect of ticagrelor is mediated by a drug-induced increase in extracellular adenosine levels and by a direct adenosine-mediated platelet inhibition via A2a receptor. In fact, ENT blockage is not the only possible

explanation of enhanced adenosine mediated effects during ticagrelor treatment(40,41), as Armstrong and colleagues have found that ticagrelor could act as an A2a agonist role in recombinant cells (17). This evidence was confirmed by the inhibition of ticagrelor-induced A2a receptor stimulation obtained with a selective A2a antagonist(17).

A single nucleotide polymorphisms (SNP) has been identified on the gene coding for the adenosine A2a receptor (ADORA2a), the rs5751876, that is responsible for a C >T substitution in position 13772 of the exon 3. This SNP represents a synonym mutation Tyr361Tyr and thus, in theory, without consequences on the receptor transcription and function. However this genetic variant has been shown to display a significant clinical impact, possiblly mediated by quantitative changes in the number of exposed receptors or by variations in the folding of the protein, potentually modulating its binding activity(45). In fact, homozygotic T/T patients have been reported to display higher anxiogenic responses to caffeine administration(46) and Domschke et al have found higher startle magnitudes in T/T carriers after caffeine administration(47). In addition, Deckert and colleagues have investigated the relationship between A2a polymorphisms and panic disorder, finding a significant association with allele T carriage (48).

Therefore, while an impact of this polymorphism has clearly emerged in conditioning the response to different antagonists, data on the cardiovascular impact of rs5751876 are still contrasting. In a previous study, arterial vasodilatation of the brachial artery after adenosine infusion was not modulated by the ADORA2a polymorphism in a cohort of healthy subjects(49), whereas in a subsequent study(50) T allele carriers with dilated cardiomyopathy displayed an enhanced vasodilatation in response to adenosine. No previous studies, in fact, have identified a genetic determinant of ticagrelor antiplatelet effect. Investigations on PLATO- trial patients have found no genetic variants significantly

influencing ticagrelor efficacy(41,42,51).However, no study has so far addressed the impact of rs5751876 on platelet reactivity.

Our main findings suggest a relationship between ADORA2a rs5751876 polymorphism and platelet reactivity. In particular, we observed a higher rate of poor ticagrelor responders among C allele carriers, and at multivariate analysis patients with C/T and C/C genotypes resulted associated with an enhanced risk of HRPR for ticagrelor compared to homozygotes T/T. Thus, it might be argued that the T allele mutation could translate into an elevation of the number of adenosine receptors on platelets' surface, translating into an increased sensibility to adenosine and a higher rate of binding of ticagrelor, favouring its antiplatelet effect. In fact, adenosine A2a receptor activation leads to increase cAMP levels, inhibiting glycoprotein IIb/IIIa ability to bind fibrinogen and, thus, platelet aggregation(52). However, such pathophysiological hypothesis certainly needs molecular confirmation.

In addition, we found a significant correlation between HDL-cholesterol levels and ADORA2a polymorphism. Previous reports have investigated and suggested a potential involvment of adenosine pathway through A2a receptor in cholesterol transcellular transport (53). Bingham et al have shown that adenosine A2a receptor stimulation can reduce foam cell formation by stimulating increased reverse cholesterol transport via ABCA1(54) and therefore the formation of HDL particles. Contrastingly, our findings seem to suggest the presence of lower HDL-cholesterol in T allele homozygotes, although a potential interaction due to statins use, that was not standardized among our patients, can certainly have affected the results, raising the need of further studies to better the role of adenosine receptor in cholesterol metabolism.

Indeed, the present data endorse a potential interest for the ADORA2a rs5751876 in cardiovascular disease, and especially among higher-risk patients as those experiencing an

acute coronary syndrome and undergoing percutaneous coronary interventions, that are the patients that can most benefit from aggressive antiplatelet treatment with ticagrelor(55). However, the influence on ADORA2a genotype remains of no univocal explanation, especially considering that not only increased plasmatic adenosine levels, by ENT blocking, could influence A2a receptor activity but also ticagrelor itself could directly bind A2a receptor (17).

Therefore, future investigations are needed to enlighten the exact pharmacodynamics of ticagrelor and adenosine induced platelet inhibition, and their potential clinical impact.

Limitations

A first limitation can be considered the relatively small size of our population, although present study already represents the first ever conducted to evaluate the relationship between ADORA2a rs5751876 SNP and HRPR during ticagrelor treatment. Furthermore, the we did not performed pharmacokinetics test, however as ticagrelor is a direct and reversible blocker of the ADP-receptor, we expect that baseline platelet reactivity, rather that pharmacokinetics, could have influenced our results, as suggested from previous studies (56). Compliance to ongoing treatment was not confirmed by plasmatic ticagrelor measurements, however the very low percentage of aspirin resistance among our cohort, without statistically significant differences according to genotype, suggests the almost complete and compliance to therapy.

In addition, our results and patients with HRPR were not confirmed by the use of light transmission aggregometry that still represents the gold standard for platelet aggregation. However, a good correlation between ADP-mediated IPA and ADP-LTA has already been reported (57).

The discrepancy between significant clinical differences according to ADORA2a genotype and the absence of changes in the encoding amino acid sequence, induced by rs5751876 SNP could be explained by the presence of a linkage disequilibrium (LD) between rs5751876 and other SNP responsible of clinical different ADORA2a related manifestation(20,58). In fact, a strong LD has been reported for example between rs5751876 and rs35320474, a SNP determining transcriptional block, and rs2298383 SNP, causing an intron 1 substitution(58-60).

Finally, we did not perform a systematic follow-up of our patients. Therefore, we could not definitely evaluate the impact of genetic polymorphism and HRPR with ticagrelor on clinical outcomes. However, a significant benefit in the prevention of ischemic events has been recently reported in the MADONNA trial, when antiplatelet therapy was modified according to platelet reactivity as evaluated by Multiplate(61).

Conclusions

The present study represents the first evaluation of the impact of rs5751876 polymorphism of adenosine A2a receptor on platelet aggregation during ticagrelor treatment in post ACS patients. Our study results showed a significant independent association between rs5751876 allele C carriage and a higher rate of high-on treatment platelet reactivity on ticagrelor. Future additional studies are certainly needed to further investigate on the prognostic impact of this polymorphism in the context of ACS patients treated with ticagrelor.

References

1. De Luca G, Navarese EP, Suryapranata H. A meta-analytic overview of thrombectomy during primary angioplasty. Int J Cardiol. 2013 Jul 1;166(3):606-12.
2. Di Lorenzo E, Sauro R, Varricchio A, Capasso M, Lanzillo T, Manganelli F, et al. Randomized comparison of everolimus-eluting stents and sirolimus-eluting stents in patients with ST elevation myocardial infarction: RACES-MI trial. JACC Cardiovasc Interv. 2014 Aug;7(8):849–56.
3. Navarese EP, De Luca G, Castriota F, Kozinski M, Gurbel PA, Gibson CM, Andreotti F, Buffon A, Siller-Matula JM, Sukiennik A, De Servi S, Kubica J. Low-molecular-weight heparins vs. unfractionated heparin in the setting of percutaneous coronary intervention for ST-elevation myocardial infarction: a meta-analysis. J Thromb Haemost. 2011 Oct;9(10):1902-15.
4. De Luca G, Suryapranata H, Stone GW, Antoniucci D, Tcheng JE, Neumann FJ, Van de Werf F, Antman EM, Topol EJ. Abciximab as adjunctive therapy to reperfusion in acute ST-segment elevation myocardial infarction: a meta-analysis of randomized trials. JAMA. 2005; 293: 1759-65.
5. Verdoia M, Schaffer A, Barbieri L, Cassetti E, Piccolo R, Galasso G, Marino P, Sinigaglia F, De Luca G. Benefits from new ADP antagonists as compared with clopidogrel in patients with stable angina or acute coronary syndrome undergoing invasive management: a meta-analysis of randomized trials. J Cardiovasc Pharmacol 2014; 63: 339-50.
6. Yusuf S, Zhao F, Mehta SR, Chrolavicius S, Tognoni G, Fox KK. Effects of clopidogrel in addition to aspirin in patients with acute coronary syndromes without ST-segment elevation. N Engl J Med 2001; 345: 494–502.
7. Wiviott SD, Braunwald E, McCabe CH, Montalescot G, Ruzyllo W, Gottlieb S, et al. Prasugrel versus clopidogrel in patients with acute coronary syndromes. N Engl J Med 2007; 357: 2001–15.
8. Wallentin L, Becker RC, Budaj A, Cannon CP, Emanuelsson H, Held C, et al. Ticagrelor versus clopidogrel in patients with acute coronary syndromes. N Engl J Med. 2009 Sep 10;361(11):1045–57.
9. Müller I, Besta F, Schulz C, Massberg S, Schönig A, Gawaz M. Prevalence of clopidogrel non-responders among patients with stable angina pectoris scheduled for elective coronary stent placement. Thromb Haemost 2003; 89: 783–7.

10. Stone GW, Witzenbichler B, Weisz G, Rinaldi MJ, Neumann F-J, Metzger DC, et al. Platelet reactivity and clinical outcomes after coronary artery implantation of drug-eluting stents (ADAPT-DES): a prospective multicentre registry study. Lancet. 2013 Aug 17;382(9892):614–23.
11. Wisman PP, Roest M, Asselbergs FW, de Groot PG, Moll FL, van der Graaf Y, et al. Platelet-reactivity tests identify patients at risk of secondary cardiovascular events: a systematic review and meta-analysis. J Thromb Haemost. 2014 May;12(5):736–47.
12. De Luca G, Dirksen MT, Spaulding C, Kelbæk H, Schalij M, Thuesen L, van der Hoeven B, Vink MA, Kaiser C, Musto C, Chechi T, Spaziani G, Diaz de la Llera LS, Pasceri V, Di Lorenzo E, Violini R, Suryapranata H, Stone GW; DESERT cooperation. Impact of diabetes on long-term outcome after primary angioplasty: insights from the DESERT cooperation. Diabetes Care 2013; 36: 1020-5.
13. Nardin M, Verdoia M, Sartori C, Pergolini P, Rolla R, Barbieri L, et al. Diabetes mellitus, glucose control parameters and platelet reactivity in ticagrelor treated patients. Thromb Res. 2016 Apr 27;143:45–9.
14. De Luca G, Dirksen MT, Spaulding C, Kelbæk H, Schalij M, Thuesen L, van der Hoeven B, Vink MA, Kaiser C, Musto C, Chechi T, Spaziani G, Diaz de la Llera LS, Pasceri V, Di Lorenzo E, Violini R, Suryapranata H, Stone GW; DESERT cooperation. Impact of age on long-term outcome after primary angioplasty with bare-metal or drug-eluting stent (from the DESERT cooperation). Am J Cardiol. 2013 Jul 15;112(2):181-6.
15. Verdoia M, Pergolini P, Rolla R, Nardin M, Schaffer A, Barbieri L, et al. Advanced age and high-residual platelet reactivity in patients receiving dual antiplatelet therapy with clopidogrel or ticagrelor. J Thromb Haemost. 2016 Jan;14(1):57–64.
16. Nylander S, Femia EA, Scavone M, Berntsson P, Asztély A-K, Nelander K, et al. Ticagrelor inhibits human platelet aggregation via adenosine in addition to P2Y 12 antagonism. J Thromb Haemost. 2013 Oct;11(10):1867-76
17. Armstrong D, Summers C, Ewart L, Nylander S, Sidaway JE, van Giezen JJJ. Characterization of the Adenosine Pharmacology of Ticagrelor Reveals Therapeutically Relevant Inhibition of Equilibrative Nucleoside Transporter 1. J Cardiovasc Pharmacol Ther. 2014 Mar 1;19(2):209–19.
18. van Giezen JJJ, Sidaway J, Glaves P, Kirk I, Björkman J-A. Ticagrelor inhibits

adenosine uptake in vitro and enhances adenosine-mediated hyperemia responses in a canine model. J Cardiovasc Pharmacol Ther. 2012 Jun;17(2):164–72.

19. Hohoff C, McDonald JM, Baune BT, Cook EH, Deckert J, de Wit H. Interindividual variation in anxiety response to amphetamine: possible role for adenosine A2A receptor gene variants. Am J Med Genet B Neuropsychiatr Genet. 2005 Nov 5;139B(1):42–4.
20. Chen X, Xue A, Chen W, Ding Y, Yan D, Peng J, et al. Assessment of exonic single nucleotide polymorphisms in the adenosine A2A receptor gene to high myopia susceptibility in Chinese subjects. Mol Vis. 2011 Jan;17:486–91.
21. De Luca G, Secco GG, Santagostino M, Venegoni L, Iorio S, Cassetti E, et al. Uric acid does not affect the prevalence and extent of coronary artery disease. Results from a prospective study. Nutr Metab Cardiovasc Dis. 2012 May;22(5):426–33.
22. Verdoia M, Pergolini P, Camaro C, Restifo M, Rolla R, Schaffer A, et al. PlA(1)/PlA(2) polymorphism does not influence response to Gp IIb-IIIa inhibitors in patients undergoing coronary angioplasty. Blood Coagul Fibrinolysis. 2013 Jun;24(4):411–8.
23. Vidali M, Rolla R, Parrella M, Cassani C, Manzini M, Portalupi MR, et al. Role of the laboratory in monitoring patients receiving dual antiplatelet therapy. Int J Lab Hematol. 2012 Oct;34(5):484–94.
24. Navarese EP, Kubica J, Castriota F, Gibson CM, De Luca G, Buffon A, et al. Safety and efficacy of biodegradable vs. durable polymer drug-eluting stents: evidence from a meta-analysis of randomised trials. EuroIntervention. 2011 Dec;7(8):985–94.
25. Capozzolo C, Piscione F, De Luca G, Cioppa A, Mazzarotto P, Leosco D, et al. Direct coronary stenting: effect on coronary blood flow, immediate and late clinical results. Catheter Cardiovasc Interv. 2001 Aug;53(4):464–73.
26. De Luca G, Dirksen MT, Spaulding C, Kelbæk H, Schalij M, Thuesen L, et al. Meta-analysis comparing efficacy and safety of first generation drug-eluting stents to bare-metal stents in patients with diabetes mellitus undergoing primary percutaneous coronary intervention. Am J Cardiol. 2013 May 1;111(9):1295–304.
27. De Luca G, Dirksen MT, Spaulding C, Kelbaek H, Schalij M, Thuesen L, et al. Drug-eluting vs bare-metal stents in primary angioplasty: a pooled patient-level meta-analysis of randomized trials. Arch Intern Med. 2012 Apr 23;172(8):611–21;

discussion 621–2.

28. De Luca G, Bellandi F, Huber K, Noc M, Petronio AS, Arntz H-R, et al. Early glycoprotein IIb-IIIa inhibitors in primary angioplasty-abciximab long-term results (EGYPT-ALT) cooperation: individual patient's data meta-analysis. J Thromb Haemost. 2011 Dec;9(12):2361–70.
29. De Luca G, Verdoia M, Suryapranata H. Benefits from intracoronary as compared to intravenous abciximab administration for STEMI patients undergoing primary angioplasty: a meta-analysis of 8 randomized trials. Atherosclerosis. 2012 Jun;222(2):426–33.
30. Roffi M, Patrono C, Collet J-P, Mueller C, Valgimigli M, Andreotti F, et al. 2015 ESC Guidelines for the management of acute coronary syndromes in patients presenting without persistent ST-segment elevation. Eur Heart J. 2016 Jan 14;37(3):267–315.
31. 2014 ESC/EACTS Guidelines on myocardial revascularization. Eur Heart J. 2014 Oct 1;35(37):2541–619.
32. De Luca G, Dirksen MT, Spaulding C, Kelbæk H, Schalij M, Thuesen L, van der Hoeven B, Vink MA, Kaiser C, Musto C, Chechi T, Spaziani G, Diaz de la Llera LS, Pasceri V, Di Lorenzo E, Violini R, Suryapranata H, Stone GW; DESERT cooperation. Time course, predictors and clinical implications of stent thrombosis following primary angioplasty. Insights from the DESERT cooperation. Thromb Haemost 2013; 110: 826-33
33. Mayer K, Bernlochner I, Braun S, Schulz S, Orban M, Morath T, et al. Aspirin treatment and outcomes after percutaneous coronary intervention: results of the ISAR-ASPI registry. J Am Coll Cardiol. 2014 Sep 2;64(9):863–71.
34. Buonamici P, Marcucci R, Migliorini A, Gensini GF, Santini A, Paniccia R, et al. Impact of platelet reactivity after clopidogrel administration on drug-eluting stent thrombosis. J Am Coll Cardiol. 2007 Jun 19;49(24):2312–7.
35. Giusti B, Gori AM, Marcucci R, Saracini C, Sestini I, Paniccia R, et al. Relation of cytochrome P450 2C19 loss-of-function polymorphism to occurrence of drug-eluting coronary stent thrombosis. Am J Cardiol. 2009 Mar 15;103(6):806–11.
36. Sibbing D, Stegherr J, Latz W, Koch W, Mehilli J, Dörrler K, et al. Cytochrome P450 2C19 loss-of-function polymorphism and stent thrombosis following percutaneous coronary intervention. Eur Heart J. 2009 Apr;30(8):916–22.

37. Mao L, Jian C, Changzhi L, Dan H, Suihua H, Wenyi T, et al. Cytochrome CYP2C19 polymorphism and risk of adverse clinical events in clopidogrel-treated patients: a meta-analysis based on 23,035 subjects. Arch Cardiovasc Dis. 2013 Oct;106(10):517–27.
38. VAN Giezen JJJ, Nilsson L, Berntsson P, Wissing B-M, Giordanetto F, Tomlinson W, et al. Ticagrelor binds to human P2Y(12) independently from ADP but antagonizes ADP-induced receptor signaling and platelet aggregation. J Thromb Haemost. 2009 Sep;7(9):1556–65.
39. Nardin M, Verdoia M, Pergolini P, Rolla R, Barbieri L, Schaffer A, et al. Serum uric acid levels during dual antiplatelet therapy with ticagrelor or clopidogrel: Results from a single-centre study. Nutr Metab Cardiovasc Dis. 2016 Mar 15;
40. Verdoia M, Pergolini P, Rolla R, Nardin M, Barbieri L, Daffara V, et al. Gender Differences in Platelet Reactivity in Patients Receiving Dual Antiplatelet Therapy. Cardiovasc Drugs Ther. 2016 Apr;30(2):143–50.
41. Varenhorst C, Eriksson N, Johansson Å, Barratt BJ, Hagström E, Åkerblom A, et al. Effect of genetic variations on ticagrelor plasma levels and clinical outcomes. Eur Heart J. 2015 Aug 1;36(29):1901–12.
42. Wallentin L, James S, Storey RF, Armstrong M, Barratt BJ, Horrow J, et al. Effect of CYP2C19 and ABCB1 single nucleotide polymorphisms on outcomes of treatment with ticagrelor versus clopidogrel for acute coronary syndromes: a genetic substudy of the PLATO trial. Lancet. 2010 Oct 16;376(9749):1320–8.
43. Wittfeldt A, Emanuelsson H, Brandrup-Wognsen G, van Giezen JJJ, Jonasson J, Nylander S, et al. Ticagrelor Enhances Adenosine-Induced Coronary Vasodilatory Responses in Humans. J Am Coll Cardiol. 2013 Feb;61(7):723–7.
44. Nanhwan MK, Ling S, Kodakandla M, Nylander S, Ye Y, Birnbaum Y. Chronic Treatment With Ticagrelor Limits Myocardial Infarct Size: An Adenosine and Cyclooxygenase-2-Dependent Effect. Arterioscler Thromb Vasc Biol. 2014 Sep 1;34(9):2078–85.
45. Kimchi-Sarfaty C, Oh JM, Kim I-W, Sauna ZE, Calcagno AM, Ambudkar S V, et al. A “silent” polymorphism in the MDR1 gene changes substrate specificity. Science. 2007 Jan 26;315(5811):525–8.
46. Alsene K, Deckert J, Sand P, de Wit H. Association between A2a receptor gene polymorphisms and caffeine-induced anxiety. Neuropsychopharmacology. 2003

Sep;28(9):1694–702.

47. Domschke K, Gajewska A, Winter B, Herrmann MJ, Warrings B, Mühlberger A, et al. ADORA2A Gene variation, caffeine, and emotional processing: a multi-level interaction on startle reflex. Neuropsychopharmacology. 2012 Feb;37(3):759–69.
48. Deckert J, Nöthen MM, Franke P, Delmo C, Fritze J, Knapp M, et al. Systematic mutation screening and association study of the A1 and A2a adenosine receptor genes in panic disorder suggest a contribution of the A2a gene to the development of disease. Mol Psychiatry. 1998 Jan;3(1):81–5.
49. Riksen NP, Franke B, van den Broek P, Smits P, Rongen GA. The 1976C>T polymorphism in the adenosine A2A receptor gene does not affect the vasodilator response to adenosine in humans in vivo. Pharmacogenet Genomics. 2007 Jul;17(7):551–4.
50. Andreassi MG, Laghi Pasini F, Picano E, Capecchi PL, Pompella G, Foffa I, et al. Adenosine A2(A) receptor gene polymorphism (1976C>T) affects coronary flow reserve response during vasodilator stress testing in patients with non ischemic-dilated cardiomyopathy. Pharmacogenet Genomics. 2011 Aug;21(8):469–75.
51. Akerblom A, Eriksson N, Wallentin L, Siegbahn A, Barratt BJ, Becker RC, et al. Polymorphism of the cystatin C gene in patients with acute coronary syndromes: Results from the PLATelet inhibition and patient Outcomes study. Am Heart J. 2014 Jul;168(1):96–102.e2.
52. Davì G, Patrono C. Platelet activation and atherothrombosis. N Engl J Med. 2007 Dec 13;357(24):2482–94.
53. Voloshyna I, Carsons S, Littlefield MJ, Rieger JM, Figler R, Reiss AB. Adenosine A(2A) receptor activation supports an atheroprotective cholesterol balance in human macrophages and endothelial cells. Biochim Biophys Acta. 2013 Feb;1831(2):407–16.
54. Bingham TC, Fisher EA, Parathath S, Reiss AB, Chan ES, Cronstein BN. A2A adenosine receptor stimulation decreases foam cell formation by enhancing ABCA1-dependent cholesterol efflux. J Leukoc Biol. 2010 Apr;87(4):683–90.
55. Bonaca MP, Bhatt DL, Cohen M, Steg PG, Storey RF, Jensen EC, et al. Long-term use of ticagrelor in patients with prior myocardial infarction. N Engl J Med. 2015 May 7;372(19):1791–800.
56. Price MJ, Clavijo L, Angiolillo DJ, Carlson G, Caplan R, Teng R, et al. A

randomised trial of the pharmacodynamic and pharmacokinetic effects of ticagrelor compared with clopidogrel in Hispanic patients with stable coronary artery disease. J Thromb Thrombolysis. 2015 Jan;39(1):8–14.

57. Paniccia R, Antonucci E, Maggini N, Romano E, Gori AM, Marcucci R, et al. Assessment of platelet function on whole blood by multiple electrode aggregometry in high-risk patients with coronary artery disease receiving antiplatelet therapy. Am J Clin Pathol. 2009 Jun;131(6):834–42.
58. Beste C, Stock A-K, Ness V, Epplen JT, Arning L. Differential effects of ADORA2A gene variations in pre-attentive visual sensory memory subprocesses. Eur Neuropsychopharmacol. 2012 Aug;22(8):555–61.
59. Kobayashi H, Ujike H, Iwata N, Inada T, Yamada M, Sekine Y, et al. The adenosine A2A receptor is associated with methamphetamine dependence/psychosis in the Japanese population. Behav Brain Funct. 2010 Jan;6:50.
60. Childs E, Hohoff C, Deckert J, Xu K, Badner J, de Wit H. Association between ADORA2A and DRD2 polymorphisms and caffeine-induced anxiety. Neuropsychopharmacology. 2008 Dec;33(12):2791–800.
61. Siller-Matula JM, Gruber C, Francesconi M, Dechant C, Jilma B, Delle-Karth G, et al. The net clinical benefit of personalized antiplatelet therapy in patients undergoing percutaneous coronary intervention. Clin Sci (Lond). 2015 Jan;128(2):121–30.

Chapter 6

Serum uric acid levels during dual antiplatelet therapy with ticagrelor or clopidogrel: results from a single center study

Matteo Nardin[a]; Monica Verdoia[a]; Patrizia Pergolini[b]; Roberta Rolla[b]: Lucia Barbieri[a]; Alon Schaffer[a]; Paolo Marino[a]; Giorgio Bellomo[b]; Harry Suryapranata[c]; Giuseppe De Luca[a]; on behalf of the Novara Atherosclerosis Study Group (NAS)

a) Division of Cardiology, Azienda Ospedaliera-Universitaria "Maggiore della Carità", Eastern Piedmont University, Novara, Italy; b) Clinical Chemistry, Azienda Ospedaliera-Universitaria "Maggiore della Carità", Eastern Piedmont University, Novara, Italy; c) Department of Cardiology, UMC St Radboud, Nijmegen, The Netherlands.

Nutrition, Metabolism & Cardiovascular Diseases (2016) 26: 567-574

Abstract

Background. New antithrombotic therapies have significantly improved the outcomes of patients with Acute Coronary Syndromes (ACS), where the introduction of ticagrelor has provided the greatest mortality benefits. However, ticagrelor treatment has been associated with a potential raise in serum uric acid (SUA), whose contribution to endothelial dysfunction and pro-thrombotic status may affect the risk of acute cardiovascular events in patients requiring dual antiplatelet therapy (DAPT). The aim of the present study was to compare the impact of the antiplatelet agents ticagrelor or clopidogrel on SUA levels and their effect on platelet reactivity.

Methods. We included patients admitted for ACS or elective percutaneous coronary intervention and discharged with ASA (100-160 mg) and clopidogrel (75 mg) or ticagrelor (90 mg twice a day). Chemistry was assessed at admission (baseline) and after a 30-90 days period of DAPT (together with platelet reactivity). Absolute and percentage variations of SUA after DAPT instruction were considered. Multiple-electrode aggregometry was used to assess platelet function.

Results. A total of 378 patients were enrolled, 145 in aspirin and clopidogrel (AC) treatment, 233 in aspirin and ticagrelor (AT). AC patients displayed higher age (p=0.003), more often elective PCI as indication to DAPT (<0.001), chronic therapy with ARB (p=0.001), nitrates (p=0.044), CCB (p=0.005), diuretics (p=0.044). AT patients displayed higher percentage of ACS diagnosis (p<0.001), chronic therapy with ACE- inhibitors (p=0.001), beta blockers (p=0.001), statins (p=0.013). AC patients displayed higher platelet reactivity at COL test, ASPI test and ADP test (p=0.03, 0.001 and <0.001, respectively) and higher percentage of HRPR at ADP-test (p=0.001).

No difference was found in baseline uric acid and creatinine levels between AC and AT patients. At 30-90 days a significant absolute and percentage increase in SUA levels was

found in AT as compared to AC patients (0.204 mg/dl vs. -0.165 mg/dl, p=0.034; 6,26% vs. -0.005%, p=0.018, respectively). Results were not influenced by variations in renal function. At multivariate analysis, in fact, ticagrelor therapy emerged as an independent predictor of increase of uric acid levels (OR[95%CI]= 2.79 [1.66-4.67], p<0.001). However, SUA levels variation did not affect platelet reactivity or HRPR in both AC and AT patients.

Conclusion. Among patients receiving chronic dual antiplatelet therapy ticagrelor but not clopidogrel treatment is associated with an increase in serum uric acid levels at 30-90 days. However, the SUA changes do not impact on platelet aggregation.

Introduction

The significant innovations achieved in interventional cardiology and medical therapy, have not decreased the burden of coronary artery disease (CAD), still representing a leading cause of mortality (1). Nevertheless, the introduction of more potent antithrombotic treatments and early reperfusion strategies have dramatically improved the prognosis of patients after an acute coronary event (2–4).

In particular, ticagrelor, a direct inhibitor of platelet P2Y12 receptor, has provided a significant reduction in mortality and ischemic events as compared to clopidogrel, in patients experiencing an acute cardiovascular events, due to its more potent and predictable antiplatelet effect, whose benefits have been suggested to increase over time (5,6).

However, a potential warning has been raised since the first clinical studies with ticagrelor, being associated with an increase of serum uric acid levels than was not observed in clopidogrel treated patients (7). In fact, elevated serum uric acid (SUA) has been associated with the development and progression of CAD (8), by modulating endothelial dysfunction, oxidative stress and inflammation (9–11). Even though controversies still exist on its independent role in the pathogenesis of CAD (12), its potential impact on thrombosis, platelet hyperreactivity and plaque instabilization could display an even more harmful effects among those high-risk patients requiring a dual antiplatelet therapy (DAPT) for an acute coronary event.

However, no study, with the exception of PLATO trial, has so far investigated the dynamic changes of serum uric acid levels during DAPT administration and their impact on platelet reactivity among patients with CAD, that was, therefore, the aim of the present study.

Methods

We included patients admitted Division of Cardiology, "Maggiore della Carità" Hospital, Eastern Piedmont University in Novara, Italy, from September 2011 to April 2015 requiring dual antiplatelet therapy for acute coronary syndromes (ACS) or undergoing elective PCI. Invasive treatment with coronary angiography and eventual coronary stenting was not a required inclusion criterion. All patients receiving at discharge dual antiplatelet therapy with ASA (100 to 160 mg daily) an ADP-antagonist (clopidogrel 75 mg daily or ticagrelor 90 mg b.i.d) were scheduled for chemistry and platelet function tests evaluation within a period ranging from 30 to 90 days from hospital discharge.

The study was approved by our local Ethical Committee and informed consent was obtained by all patients. Main demographic, clinical and angiographic data, together with the indication to dual antiplatelet therapy were recorded at discharge and included in a dedicated database, protected by password. As previously described (13) hypertension was defined as systolic pressure > 140 mm Hg and/or diastolic pressure > 90 mm Hg or if the individual was taking antihypertensive medications. Diabetes mellitus was defined as previous diagnosis, specific treatment administration (oral drug or insulin), fasting glycaemia > 126 mg/dL or HbA1c > 6.5% (14). Chronic renal failure was considered for history of renal failure or an admission glomerular filtrate (GFR) < 60 mol/min/1.73m^2 by MDRD (Modification of Diet in renal Disease) formula. Exclusion criteria were patients' refusal or if the patient had given up DAPT.

Biochemical measurements

Blood samples were drawn in the early morning, following a fasting period of 12 h. Glucose, creatinine, glycosylated haemoglobin and lipid profile were determined as previously described (13). Blood cells count was performed in a blood sample collected in

tripotassium EDTA (7.2 mg) tubes. These blood samples were analysed within 2 h of venepuncture by automatic blood cells counter (A Sysmex XE-2100).

Platelet aggregation

Platelet aggregation was determined by Multiplate electrical impedance aggregometry (MEA). The aggregation tests were performed after more than 30 minutes and within 2 hours from blood collection, as suggested by the manufacturers (15). Platelets aggregation was assessed in a multi-channel system, where in separate cuvettes platelets could be stimulated either with ADP (6.4 µM + 20 µl prostaglandin E1), collagen (3.2 µg/ml) (COL test), arachidonic acid (AA) (0.5 mM), or thrombin receptor activating peptide (TRAP-6; 30 µM). Results were expressed as arbitrary Aggregation Units (AU) and plotted against time, defining platelet function as the area under curve (AUC or AU*min). High residual platelet reactivity (HRPR) for clopidogrel/ ticagrelor was defined for ADP test above 417 AU*min, (normal range: [417-1030]), while HRPR for Aspirin was defined for ASPI-test above 862 AU*min, (normal range: [862-1344])(16). The test was repeated in patients with HRPR to confirm the findings.

Statistical analysis

Statistical analysis was performed using SPSS 17.0 statistical package. Continuous data were expressed as mean $\pm$ SD and categorical data as percentage. Analysis of variance and the chi-square test were used for continuous and categorical variables, respectively.

Differences in serum uric acid and creatinine levels from baseline (at admission) to the planned re-assessment were expressed as absolute change ([final serum concentration-baseline value]) and as percentage ([final serum concentration- baseline value]/[baseline value]). Patients were grouped according to the type of antiplatelet therapy used

(ASA+clopidogrel; “AC” or ASA+ticagrelor; “AT”). Multiple logistic regression analysis was performed to evaluate the relationship between serum uric acid, defined as any increase in SUA, and P2Y12 antagonist drug after correction for baseline differences, that were entered in the model in block. A p-value < 0.05 was considered statistically significant.

Results.

We included in our study a population of 378 patients. Among them, 145 patients (38.4%) were treated with clopidogrel (AC) and 233 (61.6%) with ticagrelor (AT). Mean time from baseline to re-assessment of serum uric acid and creatinine was similar in the two groups (39.2±18.5 vs 40.3±20.1 days in AC and AT patients respectively, p=0.68).

Table 1 shows main clinical and demographic features according to the type of dual antiplatelet therapy received. Clopidogrel treated patients displayed higher age (p=0.003), higher prevalence of previous PCI (p<0.001), higher percentage of stable angina diagnosis (<0.001), chronic therapy with ARB (p=0.001), nitrates (p=0.044), CCB (p=0.005), diuretics (p=0.044). Ticagrelor treated patients displayed higher percentage of ACS diagnosis (p<0.001) and were receiving a concomitant therapy with ACE inhibitors (p=0.001), beta blockers (p=0.001), statins (p=0.013). Results for main biochemistry parameters as displayed in **Table 2**. AC patients displayed higher levels of total and LDL cholesterol (p=0.01 and p=0.04, respectively), lower platelet count (p=0.04) and fibrinogen (p=0.044), and higher platelet reactivity at COL test, ASPI test and ADP test (p=0.03, 0.001 and <0.001, respectively), with a more elevated percentage of HRPR at ADP-test (p=0.001). No difference was found in baseline uric acid and creatinine levels between AC and AT patients (**Table 2**).

Table 1 Clinical and demographic characteristics according to dual antiplatelet treatment.

Baseline clinical characteristics	ASA-clopidogrel n=145	ASA-ticagrelor n= 233	p-value
Age (mean±SD)	69.3 (±9.3)	65.9 (±11.3)	0.003
Male sex (%)	82.8	79.4	0.5
BMI (mean±SD)	27.73 (±4.6)	27.08 (±4.5)	0.19
Hypercholesterolemia (%)	58.6	58	0.94
Renal failure (%)	16	17.7	0.68
Smokers (%)	18.6	31.9	0.35
Hypertension (%)	78.6	72.4	0.18
Diabetes mellitus (%)	46.2	38.2	0.13
Previous MI (%)	25.5	20.3	0.25
Previous PCI (%)	51.7	28.4	<0.001
Previous CABG (%)	13.8	9.1	0.17
Ejection fraction (mean±SD)	52.3 (±11.8)	51.06 (±9.9)	0.28
Indication to angiography			<0.001
Stable angina/ Silent ischemia (%)	53.1	21	
Acute coronary syndrome (%)	35.9	75.1	
Dilated CMP/ Arrythmias (%)	11	3.9	
Concomitant medications			
ACE inhibitors(%)	41.4	58.4	0.001
ARB (%)	33.1	18	0.001
Beta blockers (%)	76.6	90.1	0.001
Nitrates (%)	52.4	416	0.044
Statins (%)	80.7	90.1	0.013
Calcium antagonists (%)	34.5	21	0.005
Diuretics (%)	40	29.6	0.044

CAD = Coronary Artery Disease; MI = Myocardial Infarction; PCI = Percutaneous Coronary Interventions; CABG = Coronary Artery Bypass Graft ;ACE = Angiotensin Converting Enzyme; ARB = Angiotensin Receptor Blockers;

Table 2. Chemistry parameters according to dual antiplatelet treatment

Baseline clinical characteristics	ASA-clopidogrel n=145	ASA-ticagrelor n= 233	p-value
Biochemistry parameters (mean± SD)			
Platelets (10^3/µl)	222.5 (±68.4)	237.84 (±72.2)	0.04
Haemoglobin (g/dL)	13.5 (±1.8)	13.67 (±1.7)	0.23
WBC (10^3/µl)	8.2 (±2.8)	7.95 (±2.2)	0.73
Glycaemia (mg/dL)	129.3 (±72.6)	121.71 (±43.2)	0.21
HbA1c (%)	6.5 (±1.2)	6.44 (±1.2)	0.76
Total cholesterol (mg/dL)	145 (±36.5)	135.38 (±34)	0.01
HDL cholesterol (mg/dL)	41.9 (±13.3)	41.06 (±11.2)	0.49
LDL cholesterol (mg/dL)	76.8 (±29.2)	70.62 (±28.3)	0.04
C-reactive protein (mg/dl)	0.6 (±1.5)	0.5 (±0.9)	0.21
Fibrinogen (mg/dL)	388.3 (±111.5)	414.51 (±125.6)	0.044
Creatinine baseline (mg/dL)	1 (±0.6)	1 (±0.8)	0.92
Creatinine re-assessment(mg/dL)	1 (±0.6)	1 (±0.7)	0.98
DELTA Creatinine (mg/dl)	0 (±0.2)	0.2 (±0.3)	0.76
% variation Creatinine*	3.4 (±1.8)	4.7(±2.1)	0.52
Uric acid baseline (mg/dL)	5.9 (±1.8)	5.7 (±1.6)	0.42
Uric acid re-assessment (mg/dL)	5.7 (±1.8)	5.92 (±1.9)	0.26
Δ Uric Acid (mg/dL)	-0.2 (±1.6)	0.2 (±1.7)	0.034
% variation Uric Acid*	-0.05 (±2.5)	6.4 (±2.7)	0.018
HRPR for ASA (%)	3.5	1.7	0.31
HRPR for ADP antagonists (%)	36.6	12.4	<0.001
COL test (AUC; mean±SD)	467.1 (±154.9)	434.2 (±132.7)	0.03
ASPI test (AUC; mean±SD)	411.5 (±207.9)	343.6 (±170.6)	0.001
TRAP test (AUC; mean±SD)	1099.8 (±298.7)	1153.9 (±275.2)	0.075
ADP test (AUC; mean±SD)	458.3 (±202.6)	281.9 (±122.6)	<0.001

WBC: White Blood cells; HDL: High-Density Lipoprotein; LDL: Low-Density Lipoprotein; HRPR: High Residual Platelet Reactivity; ASA: Acetylsalicylic Acid; AUC: Area Under Curve;

In ticagrelor treated patients we found a significantly higher increase of uric acid levels, expressed both in absolute and percentage variations than clopidogrel treated patients (0.204 VS -0.165, P=0.034 and 6.26% VS -0.005%, P=0.018, respectively), (**Table 2**, **Figure 1**); whereas no differences were found in creatinine levels absolute and percentage variation between the two groups (0.019 VS 0.028, P=0.76 and 4.7% vs 3.4%, p=0.52 respectively) (**Table 2; Figure 2**).

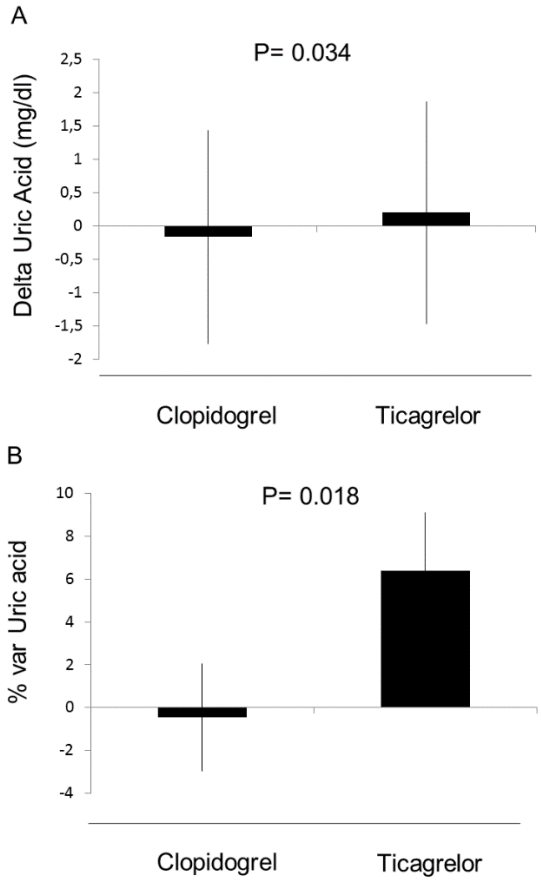


Figure 1. Bar graphs showing the mean absolute (panel A) and percentage (panel B) values with standard deviations of serum uric acid changes according to ADP-antagonist treatment.

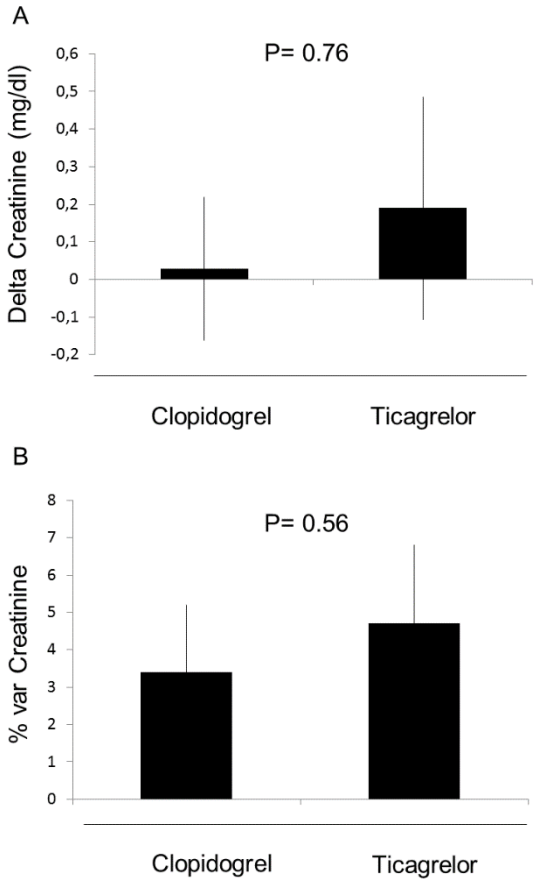


Figure 2. Bar graphs showing the mean absolute (panel A) and percentage (panel B) values with standard deviations of serum creatinine changes according to ADP-antagonist treatment.

In fact, at multivariate analysis, after correction for potential confounders (clinical presentation, chronic treatment with ACE inhibitor, ARB, Beta blockers, nitrates, statins, calcium antagonists, diuretics, previous PCI, age, platelet count, total and LDL cholesterol and fibrinogen), therapy with ticagrelor resulted as an independent predictor of a positive increase of uric acid levels (adjustedOR [95%CI] = 2.79 [1.66-4.67], p<0.001).

However, as displayed in **Table 3**, the variations in uric acid levels did not affect mean values of ADP-mediated aggregation in both AC and AT treated patients, when comparing increase vs decrease in SUA levels. Similar results were obtained when considering the percentage of patients displaying HRPR for ASA (1.5% vs 3.4%, p=0.31), clopidogrel (60.9% vs 52.7%, p=0.40) or ticagrelor (12% vs 13%, p=0.84), as reported in **Figure 3**.

Table 3. Platelet reactivity assessment according to uric acid levels variations and dual antiplatelet treatment

Platelet reactivity parameters	**ASA-clopidogrel**			**ASA-ticagrelor**		
	SUA +	SUA -	p-value	SUA +	SUA -	p-value
COL test (AUC; mean±SD)	492.8 (±162.4)	453.1 (±149.8)	0.14	442.5 (±132.7)	424.7 (±132.8)	0.31
ASPI test (AUC; mean±SD)	412.9 (±215.3)	410.8 (±204.9)	0.95	334.7 (±144.3)	354 (±196.9)	0.39
TRAP test (AUC; mean±SD)	1098.2 (±317.7)	1100.6 (±289.9)	0.96	1177.8 (±280)	1126.3 (±268.3)	0.16
ADP test (AUC; mean±SD)	450.8 (±201.1)	462.4 (±204.4)	0.75	283.5 (±117.5)	280.1 (±126.8)	0.84

SUA+ : positive variation of serum uric acid. SUA - : negative variation of serum uric acid.

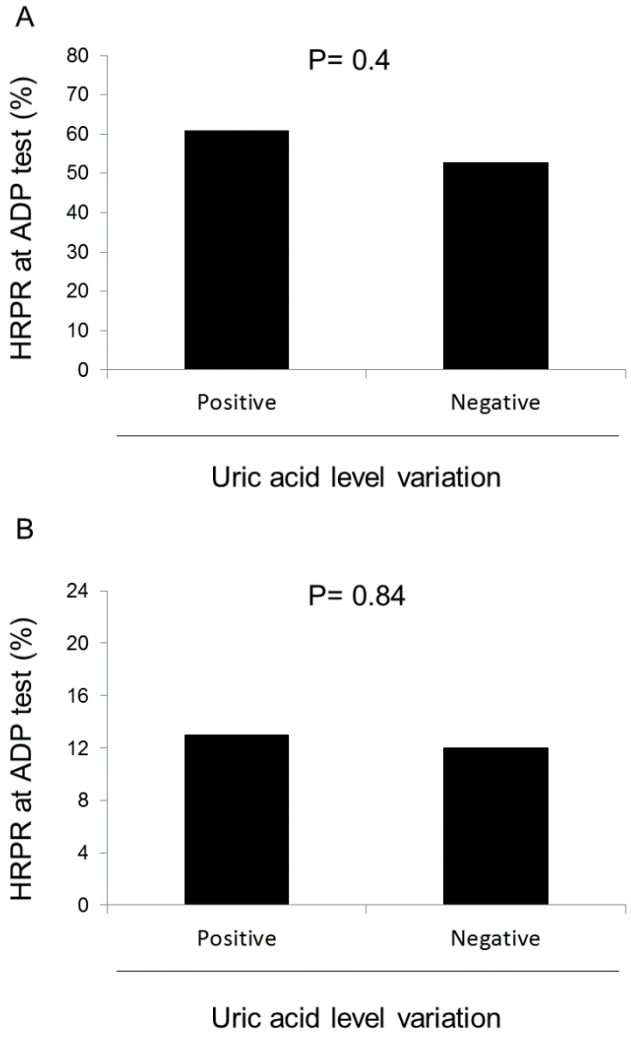


Figure 3. Bar graphs showing the prevalence of HRPR with clopidogrel at ADP test according to positive/negative uric acid variations (panel A), and the prevalence of HRPR with ticagrelor at ADP test according to positive/negative uric acid variations (panel B).

Discussion

The present study represents one of the largest cohorts of patients on DAPT where we assessed the impact of chronic therapy with ticagrelor or clopidogrel on uric acid levels and their effect on platelet aggregation.

Our main results are consistent with an increase of serum uric acid (SUA) values during chronic therapy with ticagrelor but not with clopidogrel chronic treatment, although not influencing platelet aggregation.

During the last years many developments have concerned treatment of CAD improving patients' outcome. Multifactorial pathogenesis of atherothrombotic disease has yet to be completely understood, in order to identify involved risk factors and reduce mortality.

High serum uric acid levels have frequently been associated with the progression of cardiovascular disease; however, large cohort studies have reported so far contrasting results on the role of SUA. In the Framingham study Culleton et al. (17). have not demonstrated any independent association between SUA levels and cardiovascular mortality or coronary heart disease. On the other side, Fang et al in the NHANES I have shown the independent role of SUA on cardiovascular mortality(18).

However, the importance of serum uric acid levels has been underlined in cohort studies of patients with elevated cardiovascular risk, most of them suggesting an independent association between SUA and adverse cardiovascular events. Alderman et al (18) have demonstrated in hypertensive patients that annual measurements of SUA were an effective independent predictor of cardiovascular risk and Lehto et al (19) have found that diabetic patients with higher SUA levels had a significantly increased risk of stroke when compared with lower SUA levels. In addition, a recent meta-analysis of Kin et al have reported that uric acid modestly correlated with coronary artery disease and clinical outcome, especially in women (20).

From a pathogenic point of view, uric acid has been reported increasing oxidative stress, endothelial dysfunction and smooth muscle cells proliferation (21,22). Moreover, Chitalia et al (23) showed the importance of uric acid level in thrombosis development, as they identified uric acid upregulation of the production of tissue factor in vascular smooth muscle cells, increasing thrombogenic risk. Furthermore, among STEMI patients Akpek et al (24) have reported the independent role of uric acid in predicting poor coronary blood flow and in-hospital MACE, included stent thrombosis.

In the Clopidogrel in Unstable Angina to Prevent Recurrent Events (CURE) trial (25) the use of the P2Y12 ADP-receptor antagonist clopidogrel in association with aspirin has demonstrated a reduction of cardiovascular events and mortality as compared with aspirin alone. Therefore, dual antiplatelet therapy has become an essential element for the management of patients with coronary artery disease undergoing percutaneous coronary revascularization or acute coronary syndromes, allowing to prevent stent thrombosis and major adverse cardiovascular events (26).

However, recent evidence has emerged of a worst outcome in patients with clopidogrel resistance and high residual platelet reactivity (27,28), a problem that has been only partially overcome from the introduction of new, more potent ADP antagonists, as ticagrelor. In fact, ticagrelor is a highly selective inhibitor of P2Y12 receptor, not requiring metabolic conversion to an active form (29), thus achieving a more predictable antiplatelet effect and significant clinical benefits in the reduction of ischemic events as compared to clopidogrel (5).

However, warnings have been raised on a potential role of ticagrelor in elevating serum uric acid levels after its administration, risking then to vanish its antithrombotic benefits. The DISPERSE-2, a phase II study, has reported a statistically significant increase of SUA levels in ticagrelor treated groups compared with clopidogrel. Anyhow, during the planned

follow up of 12 weeks, there was a non-statistically significant increase of gout attacks or joint-related adverse events occurrence in ticagrelor treated patients (30).

In the same PLATO trial this side effect has been underlined, with SUA increased values being identified in ticagrelor groups more than clopidogrel group after both 1 and 12 months of dual antiplatelet therapy. Moreover, they also reported that 1 month after end of treatment the SUA increase was no more different between two groups, suggesting that this side effect could be reversible (5).

After PLATO trial, the effects of ticagrelor on SUA were investigated by Bulter et al (31). They performed a double blind, placebo controlled, randomized study in 24 healthy male volunteers to evaluate the magnitude of the effect of ticagrelor on serum and urinary levels of uric acid during 5 days of treatment. Findings of this study were consistent with hyperuricemia associated with ticagrelor intake. The authors found that the ticagrelor treated group showed elevated serum uric acid levels at all time-points evaluated, while the uric acid returned to baseline values by 72 hours after the last ticagrelor dose.

Such metabolic interaction could play a relevant role in conditioning platelet reactivity and therefore cardiovascular risk in patients requiring DAPT for a recent ischemic event. In fact,

SUA levels above 6.45 mg/dl have been shown to independently predict aspirin resistance with 79% sensitivity and 65% specificity(32), although opposite findings have been described by Barbieri et al. in a large cohort of patients treated with DAPT (33). Nevertheless, different results could be expected in patients treated with Ticagrelor, displaying an even more enhanced raise in SUA values.

This is the first study evaluating the impact of ticagrelor on uric acid in an unselected cohort of patients with ACS, therefore representing a more realistic picture of daily practice. Our results are in agreement with previous studies, having found a statistically

significant relationship between ticagrelor chronic treatment and SUA increase compared with clopidogrel chronic treatment. Multivariate analysis has confirmed these results identifying ticagrelor intake as independent predictor of serum uric acid increase.

Possible explanation of ticagrelor action on SUA levels has been reported by Butler and Tend(31). In fact, they suggested that the main reason of SUA increase with ticagrelor could be represented by its blockage of adenosine uptake by erythrocytes, through the erythrocyte nucleoside transporters, without directly influencing creatinine levels and kidney function. Consequently, the increase of extracellular and blood levels of adenosine would lead to an increased uric acid synthesis by tissues with high levels of xanthine oxidase, like liver and kidney.

In agreement with Butler et al(31) we have not found any significant variations of creatinine levels both in clopidogrel and ticagrelor treated population, suggesting no direct interaction on renal function of ticagrelor.

In addition, in order to better assess the impact of SUA increase on thrombosis development risk we have evaluated the relationship of SUA variations on platelet reactivity (34). Our findings have shown no significant impact on aggregometry tests results in patients displaying a positive serum uric acid variation, suggesting no relationship between uric acid increase and platelet reactivity. Also, the prevalence of high residual platelet reactivity at ADP-test was not different between patients with increased uric acid levels than with decreased levels. These results have been confirmed both in clopidogrel treated population and ticagrelor treated patients. Similar results, in effect had previously been reported by Verdoia et al.(35) suggesting no role of SUA levels on platelet aggregation among diabetic patients.

Present study represents the first work, to our best knowledge, that has evaluated the relationship between SUA variations and platelet reactivity during ticagrelor chronic

treatment. Despite our short-term results did not underline any negative effect of the SUA increase produced by ticagrelor, these data certainly deserve long term confirmations, for their potential negative interaction on the progression of atherosclerosis, recurrent ischemic events or even with renal function.

Limitations

A first limitation can be considered the relatively small sample of our patients, although representing one of largest cohort so far analyzed. Certainly, the lack of randomization could be an important limitation to our study, in fact even though main baseline clinical characteristics were similar in both groups, an acute cardiovascular event as clinical presentation was more often observed in Ticagrelor treated patients. However, having already postponed our measurements of platelet function after more than 30 days from discharge, we do not expect that initial presentation could have played a role in our findings.

Moreover, the differences in concomitant medications could have influenced uric acid metabolism, as certain drugs have been shown different effects on SUA concentration and excretion (36). However, despite the differential use of angiotensin-receptor blockers or diuretics between AT and AC patients, at logistic regression the independent association between Ticagrelor and SUA raise was confirmed after correction for baseline differences, including concomitant medications use.

Furthermore, the absence of any impact of change in uric acid on platelet reactivity may have been influenced by the relatively small change in uric acid observed in our study.

However, despite the negative findings of our study, the role of uric acid in cardiovascular disease may be independent from the strict impact on platelet aggregation. Therefore, the

evaluation of its prognostic relevance, especially in case of prolonged administration of ticagrelor as secondary prevention(37), certainly deserve further investigation.

In addition, we have not evaluated urinary uric acid levels and serum hypoxanthine and xanthine concentration, that could have added most complete information in relation to ticagrelor influence on uric acid metabolism enlightening its pathophysiological mechanism. However, previous studies have already suggested a relationship between circulating adenosine and urate metabolites in plasma and urines (38,39).

The high prevalence of HRPR with clopidogrel observed in our study is in line with data largely reported in the literature (up to 10-30% of patients)(28). The occurrence of inadequate platelet inhibition was confirmed in 100% of patients by a second repeated platelet function test. Therefore, we do not believe that compliance may have played a relevant role in our study.

Finally, we did not perform a systematic follow-up of our patients leaving unanswered the question whether the SUA increase induced by ticagrelor could be associated with any impact on clinical outcomes.

Conclusions

The present study showed that among patients receiving chronic dual antiplatelet therapy ticagrelor but not clopidogrel treatment was associated with an increase in serum uric acid levels at 30-90 days. However, the SUA changes did not impact on platelet aggregation.

References

1. Bortnick AE, Epps KC, Selzer F, Anwaruddin S, Marroquin OC, Srinivas V, et al. Five-year follow-up of patients treated for coronary artery disease in the face of an increasing burden of co-morbidity and disease complexity (from the NHLBI Dynamic Registry). Am J Cardiol. 2014 Feb 15;113(4):573–9.
2. De Luca G, Suryapranata H, Marino P. Reperfusion strategies in acute ST-elevation myocardial infarction: an overview of current status. Prog Cardiovasc Dis. 50(5):352–82.
3. De Luca G, Smit JJ, Ernst N, Suryapranata H, Ottervanger JP, Hoorntje JCA, et al. Impact of adjunctive tirofiban administration on myocardial perfusion and mortality in patients undergoing primary angioplasty for ST-segment elevation myocardial infarction. Thromb Haemost. 2005 May;93(5):820–3.
4. De Luca G, Verdoia M, Suryapranata H. Benefits from intracoronary as compared to intravenous abciximab administration for STEMI patients undergoing primary angioplasty: a meta-analysis of 8 randomized trials. Atherosclerosis. 2012 Jun;222(2):426–33.
5. Wallentin L, Becker RC, Budaj A, Cannon CP, Emanuelsson H, Held C, et al. Ticagrelor versus clopidogrel in patients with acute coronary syndromes. N Engl J Med. 2009 Sep 10;361(11):1045–57.
6. Bonaca MP, Bhatt DL, Cohen M, Steg PG, Storey RF, Jensen EC, et al. Long-Term Use of Ticagrelor in Patients with Prior Myocardial Infarction. N Engl J Med. 2015 Mar 14;
7. Abergel E, Nikolsky E. Ticagrelor: an investigational oral antiplatelet treatment for reduction of major adverse cardiac events in patients with acute coronary syndrome. Vasc Health Risk Manag. 2010 Jan;6:963–77.
8. Bortnick AE, Epps KC, Selzer F, Anwaruddin S, Marroquin OC, Srinivas V, et al. Five-year follow-up of patients treated for coronary artery disease in the face of an increasing burden of co-morbidity and disease complexity (from the NHLBI Dynamic Registry). Am J Cardiol. 2014 Feb;113(4):573–9.
9. Ohtsubo T, Rovira II, Starost MF, Liu C, Finkel T. Xanthine oxidoreductase is an endogenous regulator of cyclooxygenase-2. Circ Res. 2004 Nov 26;95(11):1118–24.
10. Cattaneo M. Resistance to antiplatelet drugs: molecular mechanisms and laboratory detection. J Thromb Haemost. 2007 Jul;5 Suppl 1:230–7.

11. Aroor AR, McKarns S, Demarco VG, Jia G, Sowers JR. Maladaptive immune and inflammatory pathways lead to cardiovascular insulin resistance. Metabolism. 2013 Nov;62(11):1543–52.
12. De Luca G, Secco GG, Santagostino M, Venegoni L, Iorio S, Cassetti E, et al. Uric acid does not affect the prevalence and extent of coronary artery disease. Results from a prospective study. Nutr Metab Cardiovasc Dis. 2012 May;22(5):426–33.
13. Verdoia M, Schaffer A, Sartori C, Barbieri L, Cassetti E, Marino P, et al. Vitamin D deficiency is independently associated with the extent of coronary artery disease. Eur J Clin Invest. 2014 Jul;44(7):634–42.
14. De Luca G, Santagostino M, Secco GG, Cassetti E, Giuliani L, Franchi E, et al. Mean platelet volume and the extent of coronary artery disease: results from a large prospective study. Atherosclerosis. 2009 Sep;206(1):292–7.
15. Verdoia M, Pergolini P, Camaro C, Restifo M, Rolla R, Schaffer A, et al. PlA(1)/PlA(2) polymorphism does not influence response to Gp IIb-IIIa inhibitors in patients undergoing coronary angioplasty. Blood Coagul Fibrinolysis. 2013 Jun;24(4):411–8.
16. Vidali M, Rolla R, Parrella M, Cassani C, Manzini M, Portalupi MR, et al. Role of the laboratory in monitoring patients receiving dual antiplatelet therapy. Int J Lab Hematol. 2012 Oct;34(5):484–94.
17. Culleton BF, Larson MG, Kannel WB, Levy D. Serum uric acid and risk for cardiovascular disease and death: the Framingham Heart Study. Ann Intern Med. 1999 Jul 6;131(1):7–13.
18. Fang J, Alderman MH. Serum uric acid and cardiovascular mortality the NHANES I epidemiologic follow-up study, 1971-1992. National Health and Nutrition Examination Survey. JAMA. 2000 May 10;283(18):2404–10.
19. Lehto S, Niskanen L, Rönnemaa T, Laakso M. Serum uric acid is a strong predictor of stroke in patients with non-insulin-dependent diabetes mellitus. Stroke. 1998 Mar;29(3):635–9.
20. Kim SY, Guevara JP, Kim KM, Choi HK, Heitjan DF, Albert DA. Hyperuricemia and coronary heart disease: a systematic review and meta-analysis. Arthritis Care Res (Hoboken). 2010 Feb;62(2):170–80.
21. Butler R, Morris AD, Belch JJ, Hill A, Struthers AD. Allopurinol normalizes endothelial dysfunction in type 2 diabetics with mild hypertension. Hypertension.

2000 Mar;35(3):746–51.

22. Rao GN, Corson MA, Berk BC. Uric acid stimulates vascular smooth muscle cell proliferation by increasing platelet-derived growth factor A-chain expression. J Biol Chem. 1991 May 5;266(13):8604–8.
23. Chitalia VC, Shivanna S, Martorell J, Balcells M, Bosch I, Kolandaivelu K, et al. Uremic serum and solutes increase post-vascular interventional thrombotic risk through altered stability of smooth muscle cell tissue factor. Circulation. 2013 Jan 22;127(3):365–76.
24. Akpek M, Kaya MG, Uyarel H, Yarlioglues M, Kalay N, Gunebakmaz O, et al. The association of serum uric acid levels on coronary flow in patients with STEMI undergoing primary PCI. Atherosclerosis. 2011 Nov;219(1):334–41.
25. Yusuf S, Zhao F, Mehta SR, Chrolavicius S, Tognoni G, Fox KK. Effects of clopidogrel in addition to aspirin in patients with acute coronary syndromes without ST-segment elevation. N Engl J Med. 2001 Aug 16;345(7):494–502.
26. Steg PG, James SK, Atar D, Badano LP, Blömstrom-Lundqvist C, Borger MA, et al. ESC Guidelines for the management of acute myocardial infarction in patients presenting with ST-segment elevation. Eur Heart J. 2012 Oct;33(20):2569–619.
27. Matetzky S, Shenkman B, Guetta V, Shechter M, Beinart R, Bienart R, et al. Clopidogrel resistance is associated with increased risk of recurrent atherothrombotic events in patients with acute myocardial infarction. Circulation. 2004 Jun 29;109(25):3171–5.
28. Müller I, Besta F, Schulz C, Massberg S, Schönig A, Gawaz M. Prevalence of clopidogrel non-responders among patients with stable angina pectoris scheduled for elective coronary stent placement. Thromb Haemost. 2003 May;89(5):783–7.
29. Siller-Matula JM, Jilma B. Ticagrelor: from discovery to Phase III clinical trial. Future Cardiol. 2010 Nov;6(6):753–64.
30. Cannon CP, Husted S, Harrington RA, Scirica BM, Emanuelsson H, Peters G, et al. Safety, tolerability, and initial efficacy of AZD6140, the first reversible oral adenosine diphosphate receptor antagonist, compared with clopidogrel, in patients with non-ST-segment elevation acute coronary syndrome: primary results of the DISPERSE-2 tri. J Am Coll Cardiol. 2007 Nov 6;50(19):1844–51.
31. Butler K, Teng R. Evaluation and characterization of the effects of ticagrelor on serum and urinary uric acid in healthy volunteers. Clin Pharmacol Ther. 2012

Feb;91(2):264–71.

32. Yildiz BS, Ozkan E, Esin F, Alihanoglu YI, Ozkan H, Bilgin M, et al. Does high serum uric acid level cause aspirin resistance? Blood Coagul Fibrinolysis. 2015 Dec 11;
33. Barbieri L, Verdoia M, Pergolini P, Nardin M, Rolla R, Marino P, et al. Uric acid and high-residual platelet reactivity in patients treated with clopidogrel or ticagrelor. Nutr Metab Cardiovasc Dis. 2016 Jan 6;
34. Wisman PP, Roest M, Asselbergs FW, de Groot PG, Moll FL, van der Graaf Y, et al. Platelet-reactivity tests identify patients at risk of secondary cardiovascular events: a systematic review and meta-analysis. J Thromb Haemost. 2014 May;12(5):736–47.
35. Verdoia M, Sartori C, Pergolini P, Nardin M, Rolla R, Barbieri L, et al. Prevalence and predictors of high-on treatment platelet reactivity with ticagrelor in ACS patients undergoing stent implantation. Vascul Pharmacol. 2015 May 6;
36. Matsumura K, Arima H, Tominaga M, Ohtsubo T, Sasaguri T, Fujii K, et al. Effect of losartan on serum uric acid in hypertension treated with a diuretic: the COMFORT study. Clin Exp Hypertens. 2015 Jan;37(3):192–6.
37. Bonaca MP, Bhatt DL, Cohen M, Steg PG, Storey RF, Jensen EC, et al. Long-term use of ticagrelor in patients with prior myocardial infarction. N Engl J Med. 2015 May 7;372(19):1791–800.
38. Suzuki S, Yoneyama Y, Sawa R, Otsubo Y, Takeuchi T, Araki T. Relation between serum uric acid and plasma adenosine levels in women with preeclampsia. Gynecol Obstet Invest. 2001;51(3):169–72.
39. Fromonot J, Deharo P, Bruzzese L, Cuisset T, Quilici J, Bonatti S, et al. Adenosine plasma level correlates with homocysteine and uric acid concentrations in patients with coronary artery disease. Can J Physiol Pharmacol. 2015 Aug 28;1–6.

Chapter 7

Body Mass Index and platelet reactivity during dual antiplatelet therapy with clopidogrel or ticagrelor

Matteo Nardin[a]; Monica Verdoia[a]; Chiara Sartori[a]; Patrizia Pergolini[b]; Roberta Rolla[b]: Lucia Barbieri[a]; Alon Schaffer[a]; Paolo Marino[a]; Giorgio Bellomo[b]; Harry Suryapranata[c]; Giuseppe De Luca[a]; on behalf of the Novara Atherosclerosis Study Group (NAS)

a) Division of Cardiology, Azienda Ospedaliera-Universitaria "Maggiore della Carità", Eastern Piedmont University, Novara, Italy; b) Clinical Chemistry, Azienda Ospedaliera-Universitaria "Maggiore della Carità", Eastern Piedmont University, Novara, Italy; c) Department of Cardiology, UMC St Radboud, Nijmegen, The Netherlands.

J Cardiovasc Pharmacol (2015) 66: 364-370

Abstract

Introduction. Dual antiplatelet therapy (DAPT) is considered essential in clinical management of patients undergoing percutaneous coronary revascularization (PCI) or Acute Coronary Syndromes (ACS). However, the optimal platelet inhibition is not always obtained, with high residual platelet reactivity (HRPR) increasing stent thrombosis and recurrent ischemic events. Aim of the present study was to investigate the impact of body mass index (BMI) on platelet reactivity in patients on DAPT.

Methods. We included patients treated with ASA (100-160 mg) and clopidogrel (75 mg) or ticagrelor (90 mg twice a day) for ACS or Drug-Eluting Stent (DES) implantation. Platelet reactivity was assessed at 30-90 days post-discharge by multiple-electrode aggregometry. HRPR for ADP-antagonists was defined as ADP-test results >417 AU*min. HRPR for ASA was considered for ASPI-test >862 AU*min.

Results. Our population is represented by 498 patients, 308 (61.8%) were treated with clopidogrel and 190 (38.2%) with ticagrelor. Overall, higher BMI was related with younger age (p=0.003), higher prevalence of diabetes mellitus (p<0.001), hypercholesterolemia (p=0.017), hypertension (p<0.001), chronic therapy with ARB (p=0.019), CCB (p=0.003). Higher values of BMI directly related with haemoglobin (p=0.02), triglycerides (p<0.001), glycaemia (p=0.035), HbA1c (p<0.001), and inversely related with HDL cholesterol (p=0.01). BMI did not influence the effectiveness of ASA, while it was associated to a non-significant trend for higher platelet reactivity (r=0.08, p=0.08) for ADP-antagonists. In fact, 111 patients (22.3%) displayed HRPR at ADP test (> 417 AU*min), with no statistically significant difference according to BMI (20.3% vs 27.1% vs 25.7%, p=0.28; adjusted OR[95%CI]= 1.19[0.86-1.64], p=0.30). However, results were different when considering separately patients receiving clopidogrel or ticagrelor.

In the clopidogrel-treated subgroup significantly higher ADP-mediated aggregation values were found in patients with higher BMI (r=0.14, p=0.023), that emerged as an independent predictor of HRPR with clopidogrel (OR[95%CI]= 1.45 [1.01-2.12], p=0.049).

On the contrary, no impact of BMI was observed in the ticagrelor-treated subgroup for platelet reactivity (r=-0.036, p=0.62) or the prevalence of HRPR (adjusted OR[95%CI]= 0.73[0.39-1.36], p=0.32).

Conclusion-Present study shows that among patients treated with DAPT for coronary artery disease, higher BMI is related to an increased platelet reactivity and a higher prevalence of HRPR in clopidogrel treated patients, while not significantly influencing the effectiveness of ticagrelor or ASA.

Introduction

Coronary artery disease (CAD) still represents the leading cause of mortality and morbidity in Western countries (1). Great improvements in the field of percutaneous coronary revascularization (PCI)(2,3) have indeed, provided significant outcomes benefits in patients with CAD, and especially in the settings of acute myocardial infarction, although increasing the need for more potent antithrombotic strategies (4).

Dual antiplatelet therapy (DAPT) is a cornerstone of the clinical management in post-PCI patients, in order to prevent recurrent ischemic events and stent thrombosis(5,6). For several years the only partner of aspirin in DAPT has been clopidogrel (7), that has however, recently been superseded in acute coronary syndromes by new, more potent ADP-antagonists, as prasugrel or ticagrelor, due to the evidence of a high residual platelet reactivity (HRPR) in almost 30% of patients during treatment with clopidogrel. However, suboptimal platelet inhibition has not completely disappeared, even with these new antiplatelet drugs (8). In fact, HRPR is not only a pure laboratory parameter, but a clinically relevant problem, associated to 2 to 9 fold increased risk of stent thrombosis and major cardiovascular events (9–11), and therefore large efforts have been addressed to understand the mechanism of antiplatelet resistance.

Previous studies (12,13) have shown that obesity could intervene negatively in clopidogrel responsiveness, and in particular elevated Body Mass Index (BMI) has emerged as an independent predictor of suboptimal platelet response after 300 mg of loading dose of clopidogrel (14). On the other hand few data have been reported so far on clopidogrel maintenance therapy and also with new ADP-antagonists. Indeed ticagrelor provides a more powerful and predictable antiplatelet effect and in the PLATO trial (15), subgroups according to BMI or weight didn’t show any significant differences compared to overall

trial results. Nevertheless a recent meta-analysis suggested an association between BMI and platelet reactivity during ticagrelor maintenance therapy (16).

Therefore, aim of present study was to evaluate the impact of BMI on platelet aggregation during maintenance DAPT with clopidogrel or ticagrelor.

Methods

We included patients admitted Division of Cardiology, “Maggiore della Carità” Hospital, Eastern Piedmont University in Novara, Italy, from September 2011 to October 2014 requiring dual antiplatelet therapy for acute coronary syndromes (ACS) or after PCI with DES implantation. Invasive treatment with coronary angiography and eventual coronary stenting was not a required inclusion criterion. All patients receiving at discharge dual antiplatelet therapy with ASA (100 to 160 mg daily) an ADP-antagonist (clopidogrel 75 mg daily or ticagrelor 90 mg b.i.d) were scheduled for chemistry and platelet function tests evaluation within a period ranging from 30 to 90 days from hospital discharge. The study was approved by our local Ethical Committee and informed consent was obtained by all patients. Main demographic, clinical and angiographic data, including BMI, together with the indication to dual antiplatelet therapy were recorded at discharge and included in a dedicated database, protected by password. As previously described (18) hypertension was defined as systolic pressure > 140 mm Hg and/or diastolic pressure > 90 mm Hg or if the individual was taking antihypertensive medications. Diabetes mellitus was defined as previous diagnosis, specific treatment administration (oral drug or insulin), fasting glycaemia > 126 mg/dL or HbA1c > 6.5% (17). Chronic renal failure was considered for history of renal failure or an admission glomerular filtrate (GFR) < 60 mol/min/1.73m2 by MDRD (Modification of Diet in renal Disease) formula. Exclusion criteria were patients’ refusal or if the patient had given up DAPT.

Biochemical measurements

Blood samples were drawn in the early morning, following a fasting period of 12 h. Glucose, creatinine, glycosylated haemoglobin and lipid profile were determined as previously described (18). Blood cells count was performed in a blood sample collected in

tripotassium EDTA (7.2 mg) tubes. These blood samples were analyzed within 2 h of venipuncture by automatic blood cells counter (A Sysmex XE-2100) .

Platelet aggregation

Platelet aggregation was determined by Multiplate electrical impedance aggregometry (MEA). The aggregation tests were performed from 30 minutes to 2 hours from blood collection(19). Platelets aggregation was assessed after stimulation with arachidonic acid (0.5 mM) (ASPI test), collagen (3.2 µg/ml) (COL test), ADP (6.4 µM) with prostaglandin E1 and thrombin receptor activating peptide, (TRAP-6; 30 µM). Results were expressed as arbitrary Aggregation Units (AU) and plotted against time, defining platelet function as the area under curve (AUC or AU*min). HRPR for clopidogrel/ ticagrelor was defined for ADP test above 417 AU*min; (normal range: [417-1030])(20). The test was repeated in patients with HRPR to confirm the findings.

Statistical analysis

Statistical analysis was performed using SPSS 17.0 statistical package. Continuous data were expressed as mean $\pm$ SD and categorical data as percentage. Analysis of variance and the chi-square test were used for continuous and categorical variables, respectively.

Patients were grouped according to tertiles values of BMI. Linear regression analysis was performed to compare BMI values and platelet reactivity at ADP-test. Multiple logistic regression analysis was performed to evaluate the relationship between BMI and HRPR after correction for baseline differences, that were entered in the model in block. A p-value < 0.05 was considered statistically significant.

Results

We included in our study a population of 498 patients. Among them 308 patients (61.8%) were treated with clopidogrel and 190 (38.2%) with ticagrelor. **Table 1** shows main clinical and demographic features according to tertiles values of BMI (<24.9 kg/m^2, 24.9-28.29 kg/m^2, ≥28.3 kg/m^2). Higher values of BMI were related with younger age (p=0.003), higher prevalence of diabetes mellitus (p<0.001), hypercholesterolemia (p=0.017), hypertension (p<0.001), chronic therapy with ARB (p=0.019), Calcium Channel Blockers (p=0.003). Higher values of BMI directly related with haemoglobin (p=0.02), triglycerides (p<0.001), glycaemia (p=0.035), HbA1c (p<0.001), and inversely related with HDL cholesterol (p=0.01).

Mean timing of platelet function assessment from hospital discharge did not differ across BMI tertiles (42.9±20.9 vs 47.5±19.9 vs 39.9±21.2, p=0.20).

A non-significant trend for higher platelet reactivity in upper BMI tertiles was observed for COL test (p=0.11) and ADP test (p=0.08), but not for ASPI or TRAP test (**Table 1**).

Table 1 Clinical and demographic characteristics according to BMI values tertiles.

Parameters	**I tert < 24.9 BMI n=164**	**II tert 24.9-28.29 BMI n=164**	**III tert ≥28.3 BMI n=170**	**p-value**
Age (years)	68.7 (±11.28)	68.72 (±10.24)	65.22 (±11.07)	0.003
Age ≥75 years old	34.1	30.7	24.1	0.045
Male sex (%)	73.2	86.6	72.9	0.93
Diabetes mellitus (%)	31.1	42.7	50	<0.001
Hypercholesterolemia (%)	50.9	65	63.9	0.017
Renal failure (%)	19.5	19.6	19.4	0.98
Active smokers (%)	23.9	17.2	25.9	0.61
Hypertension (%)	62.6	75.5	80	<0.001
History of MI (%)	23.3	21.5	24.1	0.86
Previous PCI (%)	31.1	35	31.8	0.9
Previous CABG (%)	14	9.8	11.2	0.42

Ejection Fraction (%)	50.11 (±11.9)	52.61 (±10.97)	51.34 (±10.61)	0.13
Indication to angiography				0.89
Stable angina/ silent ischemia (%)	26.8	36.6	21.8	
Acute coronary syndrome (%)	65.2	57.3	74.7	
Dilated cardiomyopathy/ Arrythmias/ Valvular disease (%)	7.9	6.1	3.5	
Concomitant Medications				
ACE inhibitors(%)	49.4	42.7	45.9	0.53
ARB (%)	14.6	25.6	25.3	0.019
Beta blockers (%)	67.7	69.5	69.4	0.74
Nitrates (%)	43.9	44.5	42.9	0.86
Statins (%)	68.3	77.4	68.8	0.93
Calcium antagonists (%)	15.9	23.3	29.4	0.003
Diuretics (%)	25.6	31.7	31.2	0.27
ADP receptor antagonist				0.55
Clopidogrel (%)	57.3	67.7	60.6	
Ticagrelor (%)	42.7	32.3	39.4	
Biochemistry parameters (mean± SD)				
Platelets (10^3/µl)	226.65 (±69.53)	219.47 (±63.67)	229.44 (±68.98)	0.38
Haemoglobin (g/dl)	13.21 (±1.76)	13.63 (±1.5)	13.66 (±1.73)	0.02
WBC (10^3/µl)	9.16 (±2.63)	7.78 (±2.42)	8.41 (±2.52)	0.14
Total cholesterol (mg/dl)	145.84 (±36.77)	150.13 (±38.31)	153.64 (±42.13)	0.2
HDL cholesterol (mg/dl)	43.87 (±16.41)	42.25 (±14.37)	39.22 (±12.12)	0.01
LDL cholesterol (mg/dL)	80.84 (±31.45)	84.41 (±38.6)	84.45 (±35.33)	0.58
Glycaemia (mg/dL)	121.24 (±59.67)	124.87 (±45.43)	136.02 (±55.96)	0.035
HbA1c (%)	6.1 (±1.12)	6.45 (±1.39)	6.72 (±1.54)	<0.001
Creatinine (mg/dL)	1.013 (±0.454)	1.077 (±0.515)	1.082 (±0.703)	0.47
C-reactive protein (mg/dl)	1.006 (±2.329)	0.664 (±1.404)	1.142 (±2.234)	0.09
Fibronogen (mg/dl)	439.04 (±142.97)	419.85 (±119.91)	445.64 (±144.58)	0.21
Platelet aggregation				
ASPI-test (AU*min)	294.98 (±198.28)	300.66 (±179.6)	302.45 (±185.85)	0.92
COL-test (AU*min)	412.56 (±158.83)	450.36 (±178.94)	422.98 (±167.84)	0.11
TRAP-test (AU*min)	976.84 (±350.94)	1012.2 (±327.7)	1022.64 (±310.8)	0.44
ADP-test (AU*min)	293.49 (±165.55)	336.77 (±180.64)	326.89 (±181.98)	0.08

BMI=Body Mass Index; MI = Myocardial Infarction; PCI = Percutaneous Coronary Interventions; CABG = Coronary Artery Bypass Grafting; ACE = Angiotensin Converting Enzyme; ARB = Angiotensin Receptor Blockers; WBC= White Blood Cells; LDL = Low-Density Lipoproteins; HDL= High-Density Lipoproteins.

A similar trend was observed, at linear regression analysis, as displayed in **Figure 1**, between platelet reactivity at ADP test and BMI values (r=0.08, p=0.08).

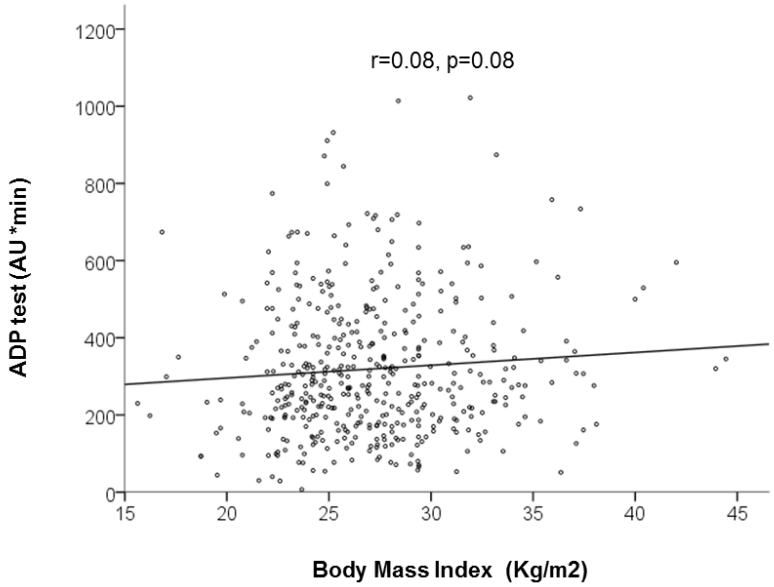


Figure 1. Linear regressions showing the relationship between platelet reactivity at ADP test and BMI values.

In fact, 8 (1.6%) patients displayed HRPR for ASA, with no impact of BMI (1.8% vs 1.8% vs 1.2%, p=0.85), **Figure 2A**. A total of 111 patients (22.3%) displayed HRPR at ADP test (≥ 417 AU*min), with no statistically significant difference according to BMI (20.3% vs 27.1% vs 25.7%, p=0.28), **Figure 2B**.

Results were confirmed at multivariate analysis, after correction for potential confounders, for both ASA (adjusted OR [95%CI] = 0.90 [0.36-2.24], p = 0.81) and ADP-antagonists (adjusted OR [95%CI] = 1.19 [0.86-1.64], p = 0.30).

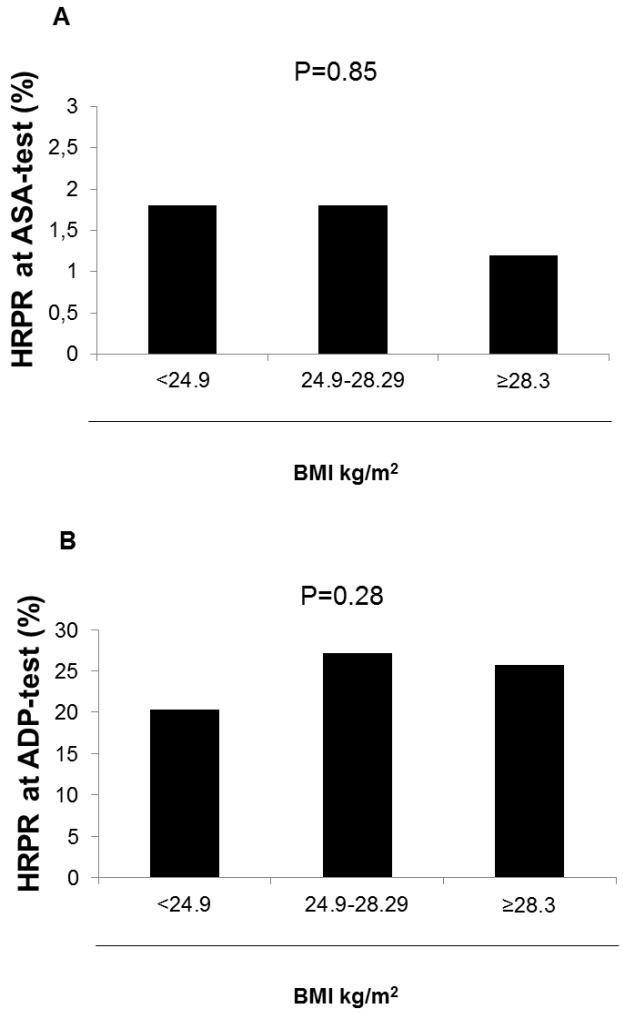


Figure 2. Bar graphs showing the overall prevalence of HRPR at ASP-test according to BMI values tertiles (figure A) and the overall prevalence of HRPR at ADP-test according to BMI values tertiles (figure B).

However, different results were observed when considering separately clopidogrel and ticagrelor treated patients.

In the clopidogrel-treated subgroup, we found significantly higher values of COL-test, TRAP-test and ADP-test in patients with higher BMI (p=0.03, P= 0.02, P=0.02, respectively) (**Table 2)**, with a linear relationship between ADP-test values and BMI. At linear regression analysis, as displayed in **Figure 3A**, a direct statistically significant relationship was identified between platelet reactivity at ADP test in clopidogrel treatment and BMI values (r=0.14, p=0.023).

In fact, among the 88 HRPR patients with clopidogrel, the occurrence was higher in patient with elevated BMI (24.1% vs 36.3% vs 37.6%, p=0.06), **Figure 4A**, and at multivariate analysis, after correction for baseline differences, BMI was confirmed as an independent predictor of HRPR with clopidogrel (OR [95%CI] = 1.45 [1.01-2.12], p = 0.049).

Table 2 Platelet aggregation assessment in clopidogrel-treated subgroup.

Parameter	**I tert < 24.9 BMI n=94**	**II tert 24.9-28.29 BMI n=111**	**III tert ≥28.3 BMI n=103**	**p-value**
Platelet aggregation				
ASPI-test (AU*min)	271.6 (±223.17)	294.55 (±183.84)	286.13 (±190.37)	0.71
COL-test (AU*min)	394.58 (±176.35)	463.02 (±192.08)	431.97 (±184.88)	0.03
TRAP-test (AU*min)	835.62 (±325.46)	961.78 (±320.28)	931.15 (±307.28)	0.02
ADP-test (AU*min)	298.95 (±186.5)	371.49 (±197.12)	371.48 (±198.54)	0.02

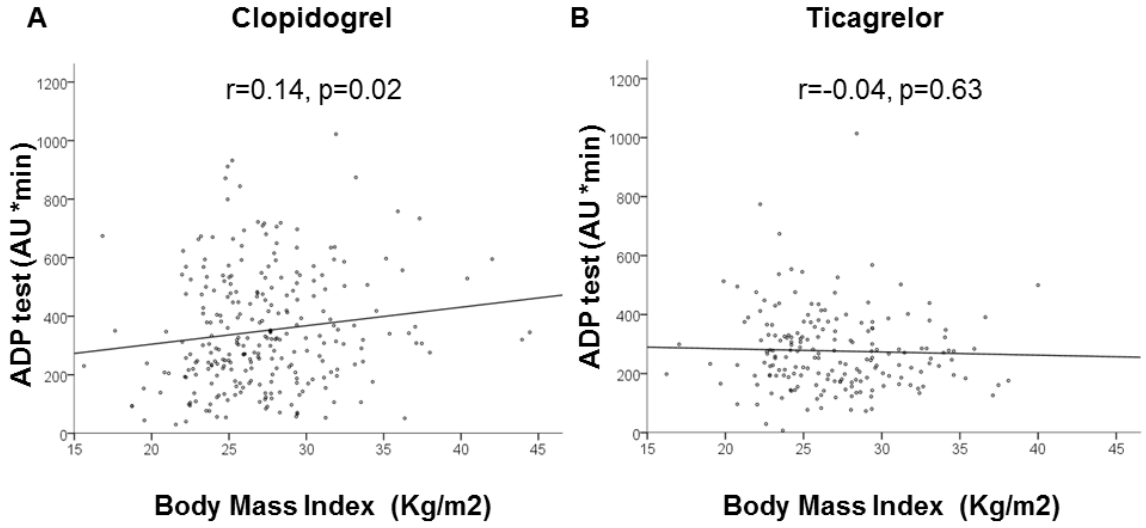


Figure 3. Linear regressions showing the relationship between platelet reactivity at ADP test and BMI values in clopidogrel subgroup (figure A), and between platelet reactivity at ADP test and BMI values in ticagrelor subgroup (figure B).

In the ticagrelor-treated subgroup no statistically significant relationship was found between BMI values and platelet reactivity, as in **Table 3**. Moreover, at linear regression analysis, as displayed in **Figure 3B**, no relationship was identified between platelet reactivity at ADP test and BMI values (r=-0.036, p=0.62). In fact, in the 23 patients with HRPR during ticagrelor, the prevalence was not conditioned by BMI (15.9% vs 9.4% vs 10.4%, p=0.33; adjusted OR [95%CI] = 0.73 [0.39-1.36], p=0.32), **Figure 4B**.

Table 3 Platelet aggregation assessment in ticagrelor-treated subgroup.

Parameter	**I tert < 24.9 BMI n=70**	**II tert 24.9-28.29 BMI n=53**	**III tert ≥28.3 BMI n=67**	**p-value**
Platelet aggregation				
ASPI-test (AU*min)	325.65 (±154.39)	313.47 (±171.39)	327.54 (±177.16)	0.89
COL-test (AU*min)	436.78 (±128.91)	423.85 (±145.83)	409.15 (±137.81)	0.5
TRAP-test (AU*min)	1152.86 (±299.76)	1111,15 (±322.36)	1142.81 (±273.94)	0.73
ADP-test (AU*min)	287.23 (±138.85)	269.94 (±119.37)	270.31 (±140.68)	0.7

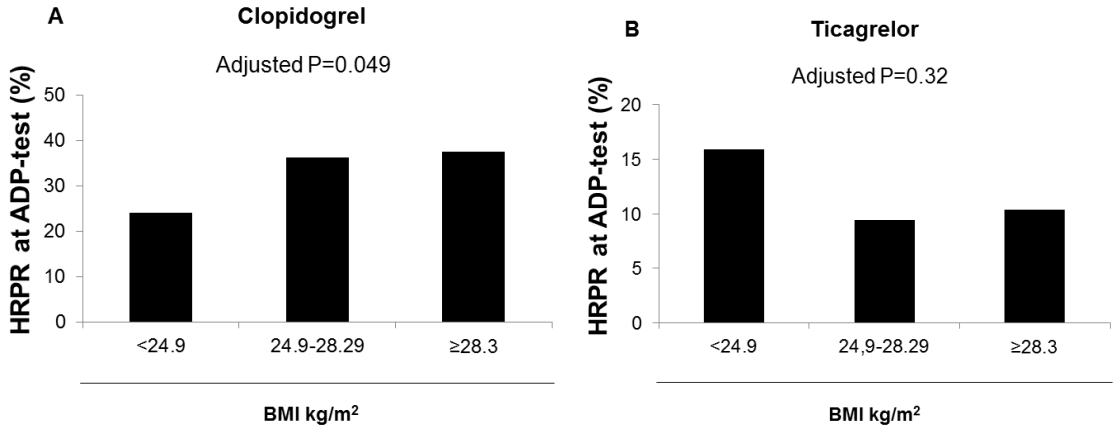


Figure 4. Bar graphs showing the prevalence of HRPR with clopidogrel at ADP test according to BMI values tertiles (figure A), and the prevalence of HRPR with ticagrelor at ADP test according to BMI values tertiles (figure B).

Discussion

Present study represents one of the largest cohorts of patients on DAPT where the impact of BMI on platelet reactivity was assessed.

Our main results are consistent with an increase of platelet reactivity and the prevalence of HRPR in patients with higher BMI treated with clopidogrel. On the contrary, BMI did not influence the response to ASA or ticagrelor.

Platelet aggregation plays a dominant role in the pathogenesis of acute cardiovascular events, consequently representing the key therapeutic targets in the management of patients with coronary artery disease, especially after an acute coronary syndrome (ACS)(21–24).

Actual Guidelines recommend at least 12 months dual antiplatelet therapy after percutaneous coronary revascularization with drug eluting stents or after ACS, that represent categories at higher thrombotic risk, irrespectively from their invasive management or coronary stenting (25). In fact, the Clopidogrel in Unstable Angina to Prevent Recurrent Events (CURE) trial (5) has clearly demonstrated in ACS the effectiveness of a DAPT with clopidogrel in addiction to aspirin, as compared to ASA alone, to prevent MACEs.

However, some patients were demonstrated having a suboptimal antiplatelet response to clopidogrel, showing an increased risk of recurrent cardiac events (26). Several authors identified a relationship between high residual platelet reactivity (HRPR) during clopidogrel treatment, as assessed by platelet function tests, and recurrent ischemic events. Buonamici at al in a prospective cohort study have shown that non-responsiveness to clopidogrel is a strong independent predictor of stent thrombosis in DES-implanted patients (27). In the ISAR-ASPI trial, impaired ASA response was associated with a higher

risk for death or stent thrombosis at 1 year follow-up in a large cohort of consecutive 7090 patients undergoing PCI (28).

Therefore, several attempts have been accomplished, to identify and prevent the causes of HRPR, focusing on clinical conditions associated with a higher pro-thrombotic status, as diabetes mellitus, or on genetic factors (29).

Previous studies, in particular, have suggested that high body mass index (BMI) could be associated with higher platelet reactivity.

Indeed, obesity has been demonstrated involving endothelium dysfunction and platelet activation by an enhanced oxidative stress (30). The consequent pro-inflammatory status could determine an acceleration of atherothrombotic processes, due to an intrinsic higher platelet reactivity (31). In addition, many authors, not only from cardiovascular medicine, have remarked that several pharmacokinetic and pharmacodynamic parameters and metabolic regulation are altered in obesity, implying the need to determining the optimal dose of drugs in overweight patients (32–34).

This metabolic deregulation, therefore, could play a significant impact on the effectiveness of clopidogrel in obese patients, as clopidogrel is a pro-drug, whose transformation into active metabolites is strictly dependent on the activity of liver cytochromes. In fact, carriers of a polymorphic variant of CYP2C19, causing its loss of function, have been clearly associated to a higher risk of clopidogrel resistance (35).

In effects, in a cohort of 73 patients Bonello-Palot et al have found that BMI was the only independent predictor of failed clopidogrel dose adjustment in patients with HRPR after 600 mg loading dose of clopidogrel (36), and moreover, Angiolillo et al have found in 48 patients that platelet aggregation was higher after 300 mg loading dose in overweight patients (> 25 kg/m^2) than normal weight patients (37).

However, few data have been reported so far on the impact of BMI during clopidogrel maintenance therapy, where the prevalence of HRPR is significantly lower, but with potentially far more relevant clinical consequences (38).

In addition, even fewer studies are available with the new, more powerful, antiplatelet agent ticagrelor, that being independent from a metabolic activation was expected to overcome the problem of clopidogrel resistance. In PLATO trial the subgroups analysis did not show any difference according to patients weight or BMI and the HRs were consistent with overall trial results(15).

However in a recent meta-analysis including a total of 445 patients (29.4±7.1 of BMI mean value), Alexopoulos et al (16) concluded that age, BMI, and current smoking status were independent related with enhanced platelet reactivity during ticagrelor maintenance therapy.

This study represents one of the largest studies addressing the relationship between BMI and platelet reactivity during dual antiplatelet therapy with clopidogrel or ticagrelor.

We identified a direct association between BMI values and platelet reactivity assessed by ADP-test at Multiplate electrical impedance aggregometry (MEA) only in clopidogrel treated patients. Moreover, higher BMI tertiles independently predicted, at multivariate analysis, the risk of HRPR during clopidogrel maintenance therapy. On the contrary, BMI did not affect overall platelet aggregation with different activating stimuli or the response to ASA or ticagrelor.

Our findings are in accordance with the results of Pankert et al, that in a large cohort study have identified BMI having a strong impact on effectiveness of clopidogrel maintenance therapy, with higher incidence of HRPR(39). Similar findings were reported by Wagner et at, showing higher platelet reactivity in higher body weight patients during clopidogrel maintenance treatment(40).

In ticagrelor-treated subgroup we found no evidence of relationship between ticagrelor maintenance treatment and BMI values; this result was confirmed ad multivariate analysis. The difference between our results and those by Alexopoulos et al (16) could be partially explained by BMI values in the two populations: in fact, their BMI mean value was 29.4, instead in our population 65% of patients had BMI <28.3. However, our results are completely in accordance with PLATO trial, showing no impact of BMI on the benefit of ticagrelor(15). In fact, the more potent antiplatelet effect of ticagrelor, together with its directly effective mechanism of action can prevent the large quote of HRPR observed with clopidogrel in overweight patients.

According to present findings, patients with higher BMI values should be considered a category at higher thrombotic risk, especially when treated with clopidogrel. Therefore, platelet function testing or the use of more potent antiplatelet drugs should be recommended in patients with higher BMI (37), in order to improve outcome and prevent major cardiovascular events.

Limitations

A first limitation can be considered the relatively small size of our population, although present study already represents one of the largest ever conducted to evaluate the relationship between BMI and platelet aggregation.

Indeed, we did not consider the metabolic status or the concomitant presence of a metabolic syndrome in our population. In fact, impaired glucose homeostasis, even beyond diabetic threshold, can affect aggregation, and in fact Pankert et al have shown the possibility that metabolic status might be a better predictor of platelet reactivity(39). However, our results were confirmed also after correction for baseline differences, including diabetic status and lipids parameters.

In addition, our results and patients with HRPR were not confirmed by the use of light transmission aggregometry that still represents the gold standard for platelet aggregation. However, a good correlation between ADP-mediated IPA and ADP-LTA has already been reported (41). Platelet reactivity was assessed after at least 30 days of dual antiplatelet therapy and within 90 days. Even if the range can appear wide, the greatest changes in the responsiveness to antiplatelet therapy have been reported within the first month of treatment (42), while no difference in the rate of HRPR could therefore be hypothesized after that period.

Finally, we did not perform a systematic follow-up of our patients, also because the sample size would not have probably allowed raising significant clinical outcome results; therefore, we could not definitely evaluate the impact of platelet reactivity assessment and HRPR on clinical outcomes. However, a significant benefit in the prevention of ischemic events has been recently reported in the MADONNA trial, when antiplatelet therapy was modified according to platelet reactivity as evaluated by multiplate (43).

Conclusions

Present study shows that among patients treated with DAPT for coronary artery disease, higher BMI is related to an increased platelet reactivity and a higher prevalence of HRPR in clopidogrel treated patients, while not significantly influencing the effectiveness of ticagrelor or ASA.

References

1. Amsterdam EA, Wenger NK, Brindis RG, Casey DE, Ganiats TG, Holmes DR, et al. 2014 AHA/ACC Guideline for the Management of Patients With Non-ST-Elevation Acute Coronary Syndromes: A Report of the American College of Cardiology/American Heart Association Task Force on Practice Guidelines. J Am Coll Cardiol. 2014 Dec 23;64(24):e139–228.
2. Di Lorenzo E, Sauro R, Varricchio A, Capasso M, Lanzillo T, Manganelli F, et al. Benefits of drug-eluting stents as compared to bare metal stent in ST-segment elevation myocardial infarction: four year results of the PaclitAxel or Sirolimus-Eluting stent vs bare metal stent in primary angiOplasty (PASEO) randomized trial. Am Heart J. 2009 Oct;158(4):e43–50.
3. De Luca G, Schaffer A, Wirianta J, Suryapranata H. Comprehensive meta-analysis of radial vs femoral approach in primary angioplasty for STEMI. Int J Cardiol. 2013 Oct 3;168(3):2070–81.
4. De Luca G, Suryapranata H. Recent Advances in Optimal Adjunctive Antithrombotic Therapy in STEMI Patients Undergoing Primary Angioplasty: an Overview. Curr Vasc Pharmacol. 2014 Dec 28;
5. Yusuf S, Zhao F, Mehta SR, Chrolavicius S, Tognoni G, Fox KK. Effects of clopidogrel in addition to aspirin in patients with acute coronary syndromes without ST-segment elevation. N Engl J Med. 2001 Aug 16;345(7):494–502.
6. De Luca G, Dirksen MT, Spaulding C, Kelbæk H, Schalij M, Thuesen L, et al. Time course, predictors and clinical implications of stent thrombosis following primary angioplasty. Insights from the DESERT cooperation. Thromb Haemost. 2013 Oct;110(4):826–33.
7. Deharo P, Pankert M, Bonnet G, Quilici J, Bassez C, Morange P, et al. Body mass index has no impact on platelet inhibition induced by ticagrelor after acute coronary syndrome, conversely to prasugrel. Int J Cardiol. 2014 Oct 20;176(3):1200–2.
8. Parodi G, Bellandi B, Xanthopoulou I, Capranzano P, Capodanno D, Valenti R, et al. Morphine Is Associated With a Delayed Activity of Oral Antiplatelet Agents in Patients With ST-Elevation Acute Myocardial Infarction Undergoing Primary Percutaneous Coronary Intervention. Circ Cardiovasc Interv. 2015 Jan 30;8(1).

9. Müller I, Besta F, Schulz C, Massberg S, Schönig A, Gawaz M. Prevalence of clopidogrel non-responders among patients with stable angina pectoris scheduled for elective coronary stent placement. Thromb Haemost. 2003 May;89(5):783–7.
10. Matetzky S, Shenkman B, Guetta V, Shechter M, Beinart R, Bienart R, et al. Clopidogrel resistance is associated with increased risk of recurrent atherothrombotic events in patients with acute myocardial infarction. Circulation. 2004 Jun 29;109(25):3171–5.
11. Bonello L, Tantry US, Marcucci R, Blindt R, Angiolillo DJ, Becker R, et al. Consensus and future directions on the definition of high on-treatment platelet reactivity to adenosine diphosphate. J Am Coll Cardiol. 2010 Sep 14;56(12):919–33.
12. Sibbing D, von Beckerath O, Schömig A, Kastrati A, von Beckerath N. Impact of body mass index on platelet aggregation after administration of a high loading dose of 600 mg of clopidogrel before percutaneous coronary intervention. Am J Cardiol. 2007 Jul 15;100(2):203–5.
13. Cuisset T, Frere C, Quilici J, Morange P-E, Camoin L, Bali L, et al. Relationship between aspirin and clopidogrel responses in acute coronary syndrome and clinical predictors of non response. Thromb Res. 2009 Feb;123(4):597–603.
14. Angiolillo DJ, Fernández-Ortiz A, Bernardo E, Barrera Ramírez C, Sabaté M, Fernandez C, et al. Platelet aggregation according to body mass index in patients undergoing coronary stenting: should clopidogrel loading-dose be weight adjusted? J Invasive Cardiol. 2004 Apr;16(4):169–74.
15. Wallentin L, Becker RC, Budaj A, Cannon CP, Emanuelsson H, Held C, et al. Ticagrelor versus clopidogrel in patients with acute coronary syndromes. N Engl J Med. 2009 Sep 10;361(11):1045–57.
16. Alexopoulos D, Xanthopoulou I, Storey RF, Bliden KP, Tantry US, Angiolillo DJ, et al. Platelet reactivity during ticagrelor maintenance therapy: a patient-level data meta-analysis. Am Heart J. 2014 Oct;168(4):530–6.
17. De Luca G, Secco GG, Santagostino M, Venegoni L, Iorio S, Cassetti E, et al. Uric acid does not affect the prevalence and extent of coronary artery disease. Results from a prospective study. Nutr Metab Cardiovasc Dis. 2012 May;22(5):426–33.
18. Verdoia M, Schaffer A, Sartori C, Barbieri L, Cassetti E, Marino P, et al. Vitamin D deficiency is independently associated with the extent of coronary artery disease. Eur J Clin Invest. 2014 Jul;44(7):634–42.

19. Verdoia M, Pergolini P, Camaro C, Restifo M, Rolla R, Schaffer A, et al. PlA(1)/PlA(2) polymorphism does not influence response to Gp IIb-IIIa inhibitors in patients undergoing coronary angioplasty. Blood Coagul Fibrinolysis. 2013 Jun;24(4):411–8.
20. Vidali M, Rolla R, Parrella M, Cassani C, Manzini M, Portalupi MR, et al. Role of the laboratory in monitoring patients receiving dual antiplatelet therapy. Int J Lab Hematol. 2012 Oct;34(5):484–94.
21. De Luca G, Suryapranata H, Marino P. Reperfusion strategies in acute ST-elevation myocardial infarction: an overview of current status. Prog Cardiovasc Dis. 50(5):352–82.
22. De Luca G, Verdoia M, Suryapranata H. Benefits from intracoronary as compared to intravenous abciximab administration for STEMI patients undergoing primary angioplasty: a meta-analysis of 8 randomized trials. Atherosclerosis. 2012 Jun;222(2):426–33.
23. DE Luca G, Bellandi F, Huber K, Noc M, Petronio AS, Arntz H-R, et al. Early glycoprotein IIb-IIIa inhibitors in primary angioplasty-abciximab long-term results (EGYPT-ALT) cooperation: individual patient's data meta-analysis. J Thromb Haemost. 2011 Dec;9(12):2361–70.
24. De Luca G, Navarese EP, Cassetti E, Verdoia M, Suryapranata H. Meta-analysis of randomized trials of glycoprotein IIb/IIIa inhibitors in high-risk acute coronary syndromes patients undergoing invasive strategy. Am J Cardiol. 2011 Jan 15;107(2):198–203.
25. Hamm CW, Bassand J-P, Agewall S, Bax J, Boersma E, Bueno H, et al. ESC Guidelines for the management of acute coronary syndromes in patients presenting without persistent ST-segment elevation: The Task Force for the management of acute coronary syndromes (ACS) in patients presenting without persistent ST-segment elevatio. Eur Heart J. 2011 Dec;32(23):2999–3054.
26. Patti G, Nusca A, Mangiacapra F, Gatto L, D'Ambrosio A, Di Sciascio G. Point-of-care measurement of clopidogrel responsiveness predicts clinical outcome in patients undergoing percutaneous coronary intervention results of the ARMYDA-PRO (Antiplatelet therapy for Reduction of MYocardial Damage during Angioplasty-Platelet Reac. J Am Coll Cardiol. 2008 Sep 30;52(14):1128–33.

27. Buonamici P, Marcucci R, Migliorini A, Gensini GF, Santini A, Paniccia R, et al. Impact of platelet reactivity after clopidogrel administration on drug-eluting stent thrombosis. J Am Coll Cardiol. 2007 Jun 19;49(24):2312–7.
28. Mayer K, Bernlochner I, Braun S, Schulz S, Orban M, Morath T, et al. Aspirin treatment and outcomes after percutaneous coronary intervention: results of the ISAR-ASPI registry. J Am Coll Cardiol. 2014 Sep 2;64(9):863–71.
29. Varenhorst C, James S, Erlinge D, Brandt JT, Braun OO, Man M, et al. Genetic variation of CYP2C19 affects both pharmacokinetic and pharmacodynamic responses to clopidogrel but not prasugrel in aspirin-treated patients with coronary artery disease. Eur Heart J. 2009 Jul;30(14):1744–52.
30. Freedman JE. Oxidative stress and platelets. Arterioscler Thromb Vasc Biol. 2008 Mar;28(3):s11–6.
31. Davì G, Guagnano MT, Ciabattoni G, Basili S, Falco A, Marinopiccoli M, et al. Platelet activation in obese women: role of inflammation and oxidant stress. JAMA. 288(16):2008–14.
32. Badimon L, Hernández Vera R, Padró T, Vilahur G. Antithrombotic therapy in obesity. Thromb Haemost. 2013 Oct;110(4):681–8.
33. Janson B, Thursky K. Dosing of antibiotics in obesity. Curr Opin Infect Dis. 2012 Dec;25(6):634–49.
34. Mathijssen RHJ, Sparreboom A, Verweij J. Determining the optimal dose in the development of anticancer agents. Nat Rev Clin Oncol. 2014 May;11(5):272–81.
35. Collet J-P, Hulot J-S, Pena A, Villard E, Esteve J-B, Silvain J, et al. Cytochrome P450 2C19 polymorphism in young patients treated with clopidogrel after myocardial infarction: a cohort study. Lancet. 2009 Jan 24;373(9660):309–17.
36. Bonello-Palot N, Armero S, Paganelli F, Mancini J, De Labriolle A, Bonello C, et al. Relation of body mass index to high on-treatment platelet reactivity and of failed clopidogrel dose adjustment according to platelet reactivity monitoring in patients undergoing percutaneous coronary intervention. Am J Cardiol. 2009 Dec 1;104(11):1511–5.
37. Angiolillo DJ, Fernández-Ortiz A, Bernardo E, Barrera Ramírez C, Sabaté M, Fernandez C, et al. Platelet aggregation according to body mass index in patients undergoing coronary stenting: should clopidogrel loading-dose be weight adjusted? J Invasive Cardiol. 2004 Apr;16(4):169–74.

38. Gurbel PA, Bliden KP, Guyer K, Cho PW, Zaman KA, Kreutz RP, et al. Platelet reactivity in patients and recurrent events post-stenting: results of the PREPARE POST-STENTING Study. J Am Coll Cardiol. 2005 Nov 15;46(10):1820–6.
39. Pankert M, Quilici J, Loundou AD, Verdier V, Lambert M, Deharo P, et al. Impact of obesity and the metabolic syndrome on response to clopidogrel or prasugrel and bleeding risk in patients treated after coronary stenting. Am J Cardiol. 2014 Jan 1;113(1):54–9.
40. Wagner H, Angiolillo DJ, Ten Berg JM, Bergmeijer TO, Jakubowski JA, Small DS, et al. Higher body weight patients on clopidogrel maintenance therapy have lower active metabolite concentrations, lower levels of platelet inhibition, and higher rates of poor responders than low body weight patients. J Thromb Thrombolysis. 2014 Aug;38(2):127–36.
41. Paniccia R, Antonucci E, Maggini N, Romano E, Gori AM, Marcucci R, et al. Assessment of platelet function on whole blood by multiple electrode aggregometry in high-risk patients with coronary artery disease receiving antiplatelet therapy. Am J Clin Pathol. 2009 Jun;131(6):834–42.
42. Gurbel PA, Bliden KP, Hiatt BL, O'Connor CM. Clopidogrel for coronary stenting: response variability, drug resistance, and the effect of pretreatment platelet reactivity. Circulation. 2003 Jun 17;107(23):2908–13.
43. .Siller-Matula JM, Gruber C, Francesconi M, Dechant C, Jilma B, Delle-Karth G, Grohs K, Podczeck-Schweighofer A, Christ G. The net clinical benefit of personalized antiplatelet therapy in patients undergoing percutaneous coronary intervention. Clin Sci (Lond). 2015;128(2):121-30

Chapter 8

Impact of renin angiotensin system inhibitors on homocysteine levels and platelets reactivity in patients on dual antiplatelet therapy

Matteo Nardin[a, b]; Monica Verdoia[a, c]; Rocco Gioscia[a]; Federica Negro[a]; Giuseppe De Luca[a]; on behalf of the Novara Atherosclerosis Study Group (NAS)

a) Division of Cardiology, Azienda Ospedaliera-Universitaria “Maggiore della Carità”, Eastern Piedmont University, Novara, Italy; b) Department of Medicine, ASST “Spedali Civili”, University of Brescia, Italy; c) Division of Cardiology, "Ospedale degli Infermi", Biella, Italy.

Nutrition, Metabolism & Cardiovascular Diseases (2021) 31: 1276-1285

Abstract

Background and aims. Dual antiplatelet therapy (DAPT)and Renin-angiotensin system inhibitors (RASi) represent the cornerstone in the treatment of patients undergoing percutaneous coronary interventions(PCI),mainly after an acute ischemic event. However, high-on treatment residual platelet reactivity (HRPR),is not infrequent despite optimal medical treatment. Homocysteine (Hcy) is a metabolite of methionine catabolism linked to atherothrombosis. Recently, a potential crosstalk between RAS and Hcy has been suggested, potentially favoring platelet aggregation and cardiovascular disease. Therefore, we aimed to investigate the impact of RASi on Hcy levels and platelet aggregation in patients on DAPT after PCI.

Methods and Results. Patients undergoing PCI on DAPT with ASA plus an ADP-antagonist (clopidogrel, ticagrelor or prasugrel), were included. RASi comprised angiotensin converting enzyme inhibitors (ACEi) and angiotensin receptor blockers (ARB). Aggregation tests were performed by Multiple Electrode Aggregometry. We included 1210 patients, of whom 862(71.2%) were on treatment with RASi. Overall, DAPT composition was ASA+clopidogrel in 566(46.8%)patients, ASA+ticagrelor in 428(35.4%)and ASA+prasugrel in 216(17.9%).Median values of Hcy were higher in RASi patients(p=0.006),who displayed a higher percentage of Hcy above the median value(52.4% vs. 44.8%, p=0.019, adjusted OR [95%CI]=1.40[1.04-1.88],p=0.027).No differences in HRPR rate were found according to RASi use for ASPI test (3.6%vs.3.3 %,p=0.88) and ADP test (25.6%vs.24.3%,p=0.62;adjustedOR[95%CI]=1.23[0.89-1.70],p=0.220)and according to ADP-antagonist type. A direct linear relationship was observed between platelet reactivity and Hcy in both patients receiving RASi and untreated ones, with higher values of platelet aggregation being observed in patients with Hcy above the median, independently from RASi administration and DAPT strategy.

Conclusion. In patients on DAPT after PCI, RASi treatment did not emerge as an independent predictor of HRPR. However, the levels of Hcy were significantly elevated in patients on RASi and related to higher values of platelet reactivity, independently from the DAPT strategy.

Introduction

Platelets activation represents a crucial step in the progression of atherosclerosis and its acute complications. Antiplatelet agents have, in fact, significantly reduced the rate of cardiovascular events and improved the outcome among patients with coronary artery disease (CAD) (1–3). In particular, a dual antiplatelet therapy (DAPT) comprising acetylsalicylic acid (ASA) and an ADP antagonist is currently indicated in patients after an acute coronary syndrome (ACS) or percutaneous coronary intervention (PCI) (4). Nevertheless, several reports have pointed up to the problem of sub-optimal antiplatelet effect in a relevant proportion of patients under DAPT, with a poor prognostic consequence (5–7). Therefore, increasing efforts have focused on the identification of the predictors of impaired platelet inhibition despite DAPT.

Homocysteine (Hcy) is a sulphur amino-acid, metabolite of methionine catabolism and folate cycle. Increased plasma levels of Hcy have been related to a negative impact on the pathophysiology of vascular disease (8–11), including endothelial dysfunction, impairment of methylation, and enhanced oxidative stress (12) and platelet activation (13,14), by raising the production of thromboxane A2 (TxA2) (15) and reducing nitric oxide (NO) (16). In fact, previous reports documented an association between suboptimal inhibition with antiplatelet agents (high-on treatment residual platelet reactivity, HRPR) and elevated Hcy (17).

Inversely, the renin-angiotensin system inhibitors (RASi), both angiotensin converting enzyme inhibitors (ACEi) and angiotensin receptor blockers (ARB) (18,19), have been suggested to reduce platelet reactivity, mainly modulating the NO pathway. In fact, ramipril showed an amelioration of the responsiveness to NO in platelets and (20) ARBs can increase the NO release from platelet and endothelial cells (21).

Moreover, RASi have been hypothesized to restore the normal endothelial functionality after exposure to Hcy (22), to prevent the ventricular hypertrophy induced by elevated levels of Hcy (23) and, in addition, the impact of RAS inhibition on renal filtration and flow could condition the clearance of Hcy, suggesting a potential positive interplay between Hcy and this class of drugs. However, Hcy could also oppositely attenuate the effects of RASi, (24) by inducing a conformational change of the AT-1 receptor (25), whose expression on platelets surface could result in a potential role of Hcy in modulating the positive antithrombotic effects of RASi.

No definite association is, so far, available about the interaction of Hcy and RASi on platelet reactivity in patients on DAPT, that was, then, the aim of the present study.

Methods

We included all consecutive patients admitted at the Division of Cardiology, "Maggiore della Carità" Hospital, Eastern Piedmont University in Novara, Italy, from September 2014 to June 2018 and discharged on DAPT for an acute coronary syndrome (ACS) or elective PCI. All patients were on treatment with ASA (100 to 160 mg daily) and an ADP-antagonist (clopidogrel 75 mg daily or ticagrelor 90 mg b.i.d or prasugrel 10 mg daily). The study was approved by our local Ethical Committee and informed consent was obtained by all patients. Patients were excluded in case of unavailable data on RASi treatment, homocysteine levels or platelet aggregation or if refusing to sign the informed consent.

Main demographic, clinical and angiographic data, together with the domiciliary therapy and indication to DAPT were recorded and included in a dedicated database, protected by password.

Biochemical measurements

Blood samples were drawn in the early morning, following a fasting period of 12 h. Glucose, creatinine, glycosylated haemoglobin, homocysteine and lipid profile were determined as previously described(26). Blood cells count was performed in a blood sample collected in tripotassium EDTA (7.2 mg) tubes. These blood samples were analyzed within 2h of venipuncture by automatic blood cells counter (A Sysmex XE-2100).

Platelet aggregation

Platelet aggregation was determined by Multiplate electrical impedance aggregometry (MEA). The aggregation tests were performed from 30 minutes to 2 hours from blood

collection, made in the morning(27). Platelets aggregation was assessed after stimulation with arachidonic acid (0.5 mM) (ASPI test), collagen (3.2 µg/ml) (COL test), ADP (6.4 µM) with prostaglandin E1 and thrombin receptor activating peptide, (TRAP 6; 30 µM). Results were expressed as arbitrary Aggregation Units (AU) and plotted against time, defining platelet function as the area under curve (AUC or AU*min). high-on treatment residual platelet reactivity (HRPR) for ADP-antagonists was defined for ADP test above 417 AU*min; (normal range: [417-1030])(28). The test was repeated in patients with HRPR to confirm the findings.

Statistical analysis

All statistical analyses were performed by SPSS Statistics Software 22.0 (SPSS Inc, Chicago, IL). Continuous variables were represented as median and interquartile range (IQR), while categorical variables as percentage. Chi-square and analysis of variance (or Mann-Whitney in case of non-normal distribution) were used to compare clinical and chemistry features according to RASi treatment. Multiple logistic regression analysis was performed to evaluate the independent predictors of HRPR, after correction for potential confounders (all variables displaying a significant difference at univariate analysis) which were entered in the model in block. Linear regression analysis was used to evaluate the association between aggregation test results and homocysteine levels in patients with and without RASi therapy. A p-value of 0.05 was considered statistically significant.

Results

Our population is represented by 1210 patients on treatment with DAPT. Among them, a total of 862 were on treatment with RASi. In patients receiving RASi, 374 (43.4%) were on treatment with ASA + clopidogrel, 324 with ASA + ticagrelor (37.6%) and 164 with ASA + prasugrel (19.0%). Patients without RASi were treated with ASA + clopidogrel in 192 cases (55.2%), ticagrelor in 104 (29.9%) and prasugrel in 52 (14.9%).

Main baseline clinical features according to RASi treatment are shown in **Table 1**. Patients with RASi displayed a higher prevalence of cardiovascular risk factors, including BMI and hypertension, and had more frequently a previous PCI. Consequently, they were more often under treatment with beta-blockers, statins and diuretics. The prevalence of chronic kidney disease (CKD) and serum creatinine were lower among patients on RASi.

In relation to platelet aggregation, higher values at TRAP-test were found among patients on RASi ($p<0.001$) compared to the untreated ones (**Table 1**). No differences were found in the prevalence of HRPR at ASPI test according to the use of RASi (3.6% vs. 3.3 %, $p=0.877$) and neither at ADP-tests (25.6 % vs. 24.3 %, $p=0.623$). After adjustment for main baseline significant differences, including chronic kidney disease, BMI, diabetes mellitus, statin, beta-blockers and diuretics intake, RASi did not result an independent predictor of HRPR at ADP-test (adjusted OR [95%CI] = 1.23 [0.89-1.70] $p=0.220$) (**Figure 1**).

Table 1. Baseline clinical characteristics according to RASi treatment.

Baseline clinical characteristics	No RAS inhibitors (n=348)	RAS inhibitors use (n=862)	p-value
Age (median [IQR])	68.0 [59-75]	69 [60-76]	0.363
Male sex (%)	79.0	76.9	0.410
Age > 75 years old (%)	27.9	30.0	0.450
BMI (kg/m^2) (median [IQR])	26.4 [23.9-29.3]	26.7 [24.2-29.4]	0.013
Hypercholesterolemia (%)	60.9	60.1	0.769
Diabetes mellitus (%)	35.8	41.3	0.053
Renal failure (%)	24.5	18.9	0.019
Smokers (%)			0.551
Active smokers	26.8	24.6	
Previous smokers	28.0	27.6	
Hypertension (%)	58.5	79.9	<0.001
History of MI (%)	23.0	23.3	0.946
Previous PCI (%)	30.7	38.0	0.009
Previous CVA (%)	4.5	6.0	0.316
Left Ventricular EF (%)	54 [48-59]	54 [45-59]	0.126
Indication to angiography			0.618
Stable angina/ silent ischemia (%)	30.3	31.5	
Acute coronary syndrome (%)	61.1	59.7	
Dilated cardiomyopathy/Arrhythmias/ Valvular disease (%)	8.5	8.8	
Beta blockers (%)	71.5	82.5	<0.001
Nitrates (%)	38.0	42.7	0.103
Statins (%)	66.7	85.3	<0.001
Calcium antagonists (%)	22.9	25.0	0.423
Diuretics (%)	24.1	40.9	<0.001
ADP-antagonist			<0.001
Clopidogrel (%)	55.17	43.39	
Ticagrelor (%)	29.89	37.59	
Prasugrel (%)	14.94	19.03	
Biochemistry parameters (median [IQR])			
Platelets (10^3/µl)	232 [184-276]	233 [195-279]	0.269
Haemoglobin (g/dl)	13.5 [12.1-14.6]	13.6 [12.3-14.6]	0.586
WBC (10^3/µl)	7.7 [6.1-9.4]	7.5 [6.4-9.1]	0.134
Total cholesterol (mg/dl)	143 [123-174]	136 [116-159]	<0.001
HDL cholesterol (mg/dl)	41 [33-47]	39 [33-47]	0.465
LDL cholesterol (mg/dL)	78 [63-101]	72 [57-90]	<0.001

Triglycerides (mg/dl)	116 [82-156]	106 [79-146]	0.153
Glycaemia (mg/dL)	106 [93-128]	107 [97-133]	0.579
HbA1c (%)	5.9 [5.5-6.6]	6.1 [5.7-6.7]	0.298
Creatinine (mg/dL)	0.92 [0.77-1.13]	0.93 [0.80-1.12]	<0.001
C-reactive protein (mg/dl)	0.28 [0.08-0.97]	0.19 [0.06-0.62]	0.001
Homocysteine (μmol/L)	16.9 [12.8-21.9]	18.2 [14.2-23.4]	0.006
Fibrinogen (mg/dl)	385 [317-479]	376 [314-473]	0.238
MPV (fl)	10.5 [10.0-11.8]	11.0 [10.0-12.0]	0.246
PDW (fl)	12.6 [11.4]	12.6 [11.3-14.1]	0.954
PLCR (fl)	30.6 [26.2-35.8]	30.6 [25.5-36.1]	0.362
IPF (%)	2.8 [1.9-4.0]	2.9 [.8-4.2]	0.993
Platelet aggregation			
ASPI test (AU*min)	297 [204-440]	330 [238-455]	0.065
COL test (AU*min)	411 [314-524]	412 [329-517]	0.283
TRAP test (AU*min)	1067 [865-1298]	1143 [944-1321]	<0.001
ADP test (AU*min)	283 [196-418]	303 [214-428]	0.991
Angiographic features			
Multivessel disease (%)	58.4	68.4	0.001
Severe CAD (%)	38.2	38.3	1.000

RAS=renin angiotensin system; BMI=Body Mass Index; MI = Myocardial Infarction; PCI = Percutaneous Coronary Interventions; CABG = Coronary Artery Bypass Grafting; CVA= Cerebrovascular Accident; EF = Ejection Fraction; WBC= White Blood Cells; LDL = Low-Density Lipoproteins; HDL= High-Density Lipoproteins; MPV= Mean Platelet Volume; PDW= Platelet Distribution Width; PLCR= Platelet Large Cell Ratio; IPF= Immature Platelet Fraction; CAD = Coronary Artery Disease.

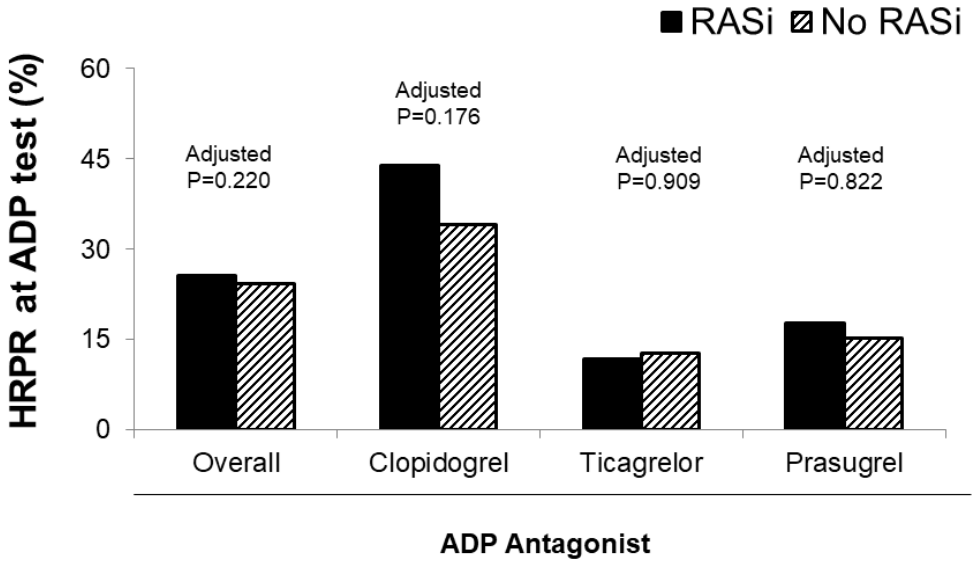


Figure 1. Bar graphs showing the prevalence of HRPR at ADP test according to RASi treatment in overall population and according to ADP antagonist.

Similar results were observed when dividing our patients according to type of ADP-antagonist (ticagrelor: 11.7% vs. 12.7% p=0.739; prasugrel: 17.6% vs. 15.2%, p=0.715) while a significant difference was observed with clopidogrel (43.8% vs. 34.0%, p=0.030). However, at multivariate analysis, in patients with clopidogrel RASi intake did not result an independent predictor of ADP resistance (clopidogrel: adjusted OR [95%CI]= 1.31 [0.89-1.93] p=0.176; ticagrelor: adjusted OR [95%CI]= 0.96 [0.46-1.99] p=0.909; prasugrel: adjusted OR [95%CI]= 0.91 [0.39-2.10] p=0.822).

RASi and Homocysteine and platelet reactivity

We further analysed our population according to homocysteine (Hcy) levels. Treatment with RASi was associated with a higher prevalence of Hcy levels above the median (≥17.7 μmol/L) (52.4% vs. 44.8%, p=0.019) (**Figure 2**).

Figure 2. Bar graphs showing the prevalence of Hcy above the median value according to RASi treatment.

Data were confirmed when considering hyperhomocysteinemia, (as above the upper reference limit; 73.9% vs. 63.8%, p<0.001 in patients with and without RASi, respectively). The elevation of Hcy was also confirmed in the majority of patients on RASi, compared to ones without, when evaluating Hcy as continuous variable (18.2 [14.2-23.4] μmol/L vs. 16.9 [12.8-21.9] μmol/L, respectively, p=0.006) and as differential value (delta Hcy, variation of Hcy from upper reference limit; 13.9 μmol/L) (4.1[0.15 – 9.35] μmol/L vs. 2.7 [-1.2 – 8] μmol/L, respectively, p=0.006)

At multivariate analysis, RASi use was confirmed as an independent predictor of Hcy above the median (adjusted OR[95%CI] = 1.40 [1.04-1.88], p=0.027) and hyperhomocysteinemia (adjusted OR [95%CI] = 1.43 [1.04-1.97], p=0.027).

Mean levels of platelet reactivity were more elevated in patients with Hcy above the median (**Table 2**), with similar results in patients with and without RASi and with the different DAPT strategies (ASA + clopidogrel, ASA + ticagrelor and ASA + prasugrel), as shown in **Table 2**.

Our findings were confirmed at linear regression analysis among patients without RASi (**Figure 3**) and with RASi (**Figure 4**).

Table 2. Multiplate aggregometry tests according to Hcy median values in patients with and without RASi.

	Overall population			NO RASi			RASi use		
Overall	**Hcy <17.7 µmol/L**	**Hcy ≥17.7 µmol/L**	**p- value**	**Hcy <17.7 µmol/L**	**Hcy ≥17.7 µmol/L**	**p-value**	**Hcy <17.7 µmol/L**	**Hcy ≥17.7 µmol/L**	**p-value**
ASPI test (AU*min)	342.5 (±203.2)	398.46 (±226.5)	<0.001	310.2 (±196.6)	382.7 (±197.5)	0.001	346.1 (±191.2)	400.8 (±224.3)	<0.001
COL test (AU*min)	431.6 (±171.6)	455.1 (±172.9)	0.022	405.8 (±166.4)	469.1 (±184.9)	0.002	418.0 (±149.0)	442.1 (±155.3)	0.034
TRAP test (AU*min)	1056.8 (±319.0)	1142.2 (±306.1)	<0.001	993.3 (±334.7)	1133.8 (±312.5)	<0.001	1089.1 (±315.7)	1144.8 (±307.0)	0.010
ADP test (AU*min)	350.1 (±214.1)	375.1 (±205.5)	0.035	313.5 (±210.5)	371.5 (±202.0)	0.012	323.1 (±170.4)	356.7 (±187.7)	0.007
Clopidogrel									
ASPI test (AU*min)	318.8 (±203.6)	399.2 (±238.8)	<0.001	303.6 (±210.7)	386.0 (±234.3)	0.013	327.4 (±198.9)	404.2 (±241.6)	0.001
COL test (AU*min)	422.8 (±179.1)	448.4 (±172.2)	0.116	416.3 (±187.1)	462.5 (±188.3)	0.126	427.1 (±174.0)	442.5 (±165.4)	0.426
TRAP test (AU*min)	976.1 (±332.0)	1070.4 (±333.5)	0.001	956.1 (±348.4)	1041.7 (±342.9)	0.106	986.0 (±321.7)	1079.0 (±331.0)	0.008
ADP test (AU*min)	376.0 (±213.7)	425.0 (±225.8)	0.011	353.6 (±240.6)	424.4 (±246.9)	0.063	388.8 (±194.6)	423.6 (±218.9)	0.119
Ticagrelor									
ASPI test (AU*min)	352.0 (±186.2)	383.0 (±204.8)	0.102	323.1 (±174.5)	359.2 (±144.6)	0.254	361.8 (±191.2)	389.9 (±220.7)	0.224
COL test (AU*min)	403.6 (±111.7)	455.2 (±161.4)	0.001	370.7 (±98.0)	500.3 (±188.5)	<0.001	413.7 (±114.0)	440.4 (±149.3)	0.099
TRAP test (AU*min)	1138.8 (±296.4)	1193.3 (±289.0)	0.054	993.6 (±270.6)	1214.7 (±286.4)	<0.001	1181.0 (±293.5)	1185.0 (±289.6)	0.903
ADP test (AU*min)	263.6 (±118.0)	295.4 (±135.8)	0.010	247.1 (±115.7)	305.5 (±127.4)	0.017	271.9 (±117.7)	292.4 (±138.6)	0.155
Prasugrel									
ASPI test (AU*min)	348.7 (±170.5)	417.6 (±200.2)	0.004	316.9 (±169.4)	420.4 (±181.9)	0.040	358.3 (±170.8)	415.0 (±188.1)	0.046
COL test (AU*min)	410.0 (±150.6)	440.8 (±152.3)	0.162	423.3 (±160.6)	431.3 (±168.0)	0.871	406.2 (±148.6)	444.2 (±147.3)	0.125
TRAP test (AU*min)	1139.1 (±295.9)	1209.8 (±245.7)	0.050	1164.6 (±345.1)	1211.2 (±201.7)	0.553	1131.3 (±281.2)	1208.4 (±258.3)	0.069
ADP test (AU*min)	281.4 (±161.7)	351.6 (±159.0)	0.002	269.3 (±175.4)	373.0 (±163.2)	0.034	285.1 (±158.3)	338.3 (±146.6)	0.027

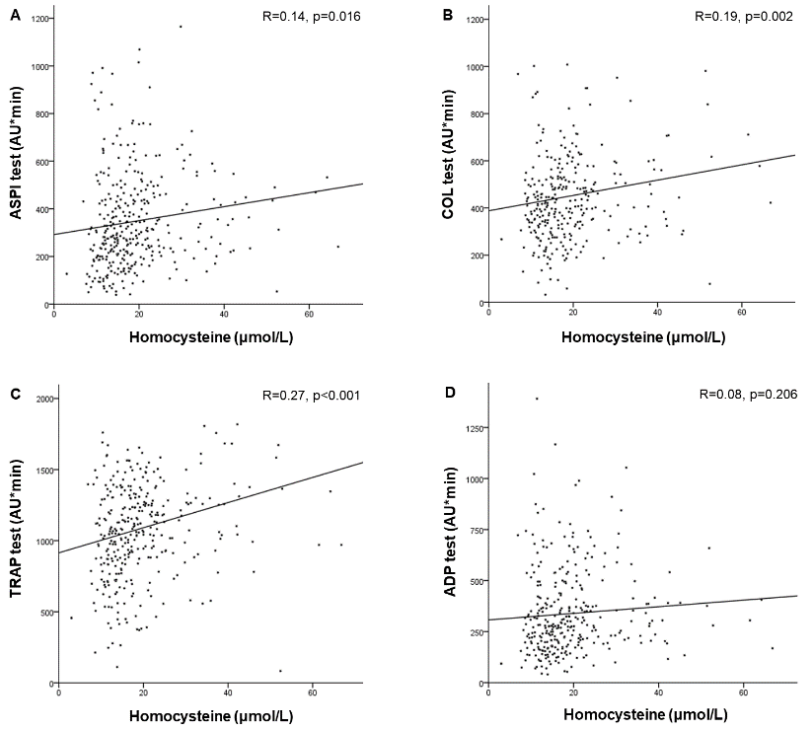


Fig. 3. Linear regressions showing the relationship between platelet Hcy values and platelet reactivity at ASPI test (panel A), COL test (panel B), TRAP test (panel C) and ADP test (panel D) in patients without RASi.

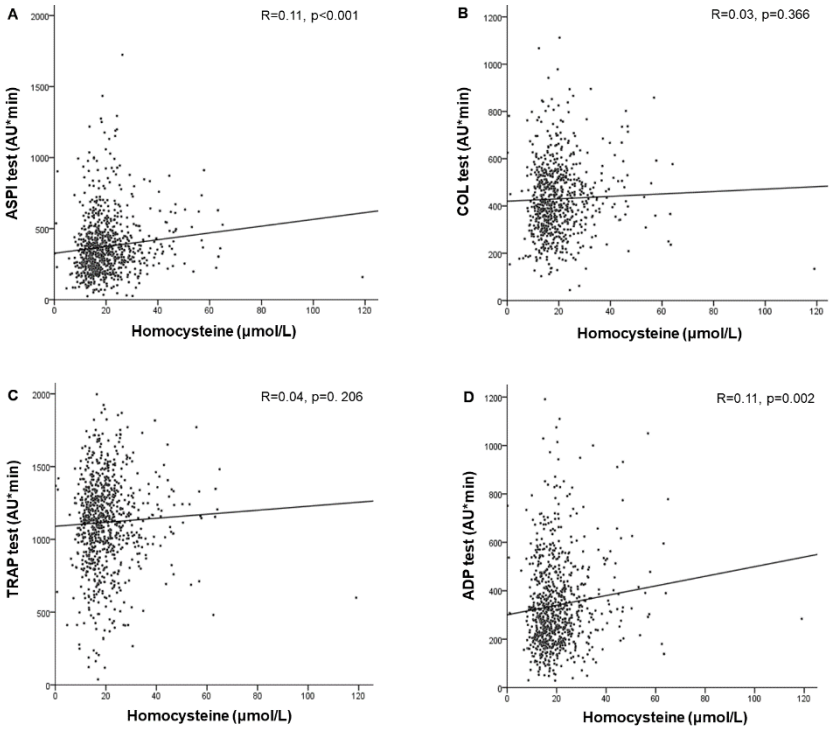


Figure 4. Linear regressions showing the relationship between platelet Hcy values and platelet reactivity at ASPI test (panel A), COL test (panel B), TRAP test (panel C) and ADP test (panel D) in patients with RASi.

Discussion

The present study represents, to our knowledge, the first attempt to assess the impact of renin angiotensin system inhibitors (RASi) and homocysteine (Hcy) on platelet reactivity in patient on DAPT after percutaneous coronary revascularization. Main findings are consistent with no independent role of RASi intake to predict the rate of HRPR in patients on DAPT regimen. However, higher Hcy levels were observed in RASi treated patients, being associated with enhanced platelet reactivity. However, the use of RAS blockers did not affect platelet aggregation in patients on DAPT.

Platelets represent one of main mediators of atherosclerosis, interfering with the plaque formation , the progression of the disease and also plaque rupture (29,30). During the last years, alongside with technical and instrumental improvement (31,32), efforts in the pharmacological field have allowed to reduce platelet activation in patients affected by CAD (33), and especially for those who experience an acute event, where a dual antiplatelet therapy (DAPT) with ASA plus clopidogrel, ticagrelor or prasugrel has provided significant improvements in patients' outcome (3).

Platelets activation and their aggregation are regulated by a fine balance including different signaling pathways, either promoting or inhibiting aggregation. TxA2, collagen, ADP and thrombin are the most well-known platelet activating factors, mediating their effects through G-protein and intracellular calcium concentration (34), while nitric oxide (NO) plays a crucial role among inhibitors of platelet activation. NO stimulates the production of cyclic guanosine monophosphate (cGMP) and regulates cGMP–dependent protein kinases (PKG), causing a secondary decrease in intracellular Ca^{2+} flux. Intracellular calcium levels reduction suppresses the conformational change in glycoprotein IIb/IIIa that is required for binding of the integrin to fibrinogen, thus decreasing the number and affinity of fibrinogen binding sites on the platelets' membrane (35).

Renin-angiotensin system inhibitors, including ACE inhibitors (ACEi) and angiotensin receptor blockers (ARB), have been demonstrated to reduce mortality, myocardial infarction and stroke in patients with high cardiovascular risk profile (36). In fact, the cardiovascular expression of angiotensin II type 1 receptor (AT-1) has been related to increasing severity of atherosclerosis, enhanced inflammation and vascular remodelling (37), while RAS blockage improves vascular homoeostasis and can inhibit the progression of atherosclerosis (38,39).

Cumulative evidence has been provided about the potential modulation of platelet reactivity by RASi. In a cohort of patients with angiographically documented CAD, Bauriedel et al have reported a significant reduction of platelet reactivity in subjects under treatment with ACEi (40). Similar results were achieved by Krämer et al for losartan and its metabolites reducing inflammation and platelet aggregation (19). Main mechanisms of the antiplatelet properties of RASi involve the NO signalling pathway. Willoughby et colleagues found a significant improvement of NO sensitiveness of platelets in patients with high cardiovascular risk profile treated with ramipril compared to placebo(20). The role of NO in mediating the anti-platelet action of ARB has been shown by Kalinowski et al, that documented a relationship between ARBs, and their concentrations, and the activation of NO release (41). In fact, AT-1 is expressed on platelets surface (42), and its activation causes NO synthase (NOS) uncoupling with consequent reduction in NO production and increased platelet aggregation(43).

The majority of previous studies enlightening the advantages of RASi on platelet function were conducted without concomitant treatment of dual antiplatelet drug and with a lower cardiovascular risk profile, whilst we firstly investigated the impact of the therapy with RASi on top of DAPT in patients with established CAD. RASi group displayed a higher percentage of cardiovascular risk factors, including hypertension, obesity and diabetes, but

a lower rate of renal dysfunction. Overall, we have not found a significant difference in platelet reactivity or in the prevalence of HRPR according to RAS inhibition.

In contrast to our results Marinšek and Sinkovic (44) investigated the inflammatory parameters and platelet aggregation in STEMI patients on DAPT according to the intake of ACEi, ARB or neither. They observed a higher magnitude of platelet inhibition in RASi treated patients as compared to controls without a RASi. However, in this study the included population comprised 64 patients and 9 controls (1:16 of the population enrolled in the present analysis). Moreover, the authors did not perform platelet aggregation assays with different stimuli for platelet activation. In addition, Schieffer et al conduced a comparison between ARB and ACEi in a small cohort of patients (n=48 subjects) with stable CAD and under treatment only with ASA. They found an higher platelet inhibition with ARB as compared to ACEi, although no comparison was performed in patients without RASi (45).

Explanations of lack of differences in our cohort could be derived from the study by Montón, et al. They reported that the concentration of ARBs required to inhibit platelet aggregation (5×10^{-7} M) is higher compared with that obtained in humans with such treatment ($\sim 5 \times 10^{-9}$M) (42).

Alongside with impact of drugs, a wide spectrum of elements and substances contributes to the balance and modulation of platelet aggregation. In particular, homocysteine (Hcy) has been hypothesized to increase the cardiovascular risk (46,47) favouring a pro-inflammatory, pro-oxidant and thrombotic milieu. McGarrigle et al have found a direct relationship between plasmatic Hcy levels and platelet surface expression of integrin αIIbβ3, that is crucial in the process of thrombus formation; moreover, Signorello et colleagues demonstrated a reduced NO bioavailability in platelets which were stimulated by increasing Hcy concentrations(16).

Homocysteine is cleared from the body through excretion in the kidneys after filtration, like creatinine (48). A potential relationship between Hcy levels and RASi has been previously pointed, given the role of ACEi and ARB in modulating renal flow and vascular resistances. However, whilst RASi have been suggested to prevent the decline of glomerular filtration rate (GFR), especially in concomitant with proteinuria (49), concerns were reported in relation to a potential worsening of GFR in presence of CKD or heart failure, driven by excessive lowering of blood pressure and renal flow during RASi treatment (50). In essential hypertension, Poduri et al suggested potential ACEi benefits in reduction of Hcy levels (51), whilst other investigators oppositely found in hypertensive patients with normal baseline Hcy levels, that ACEi treatment raised serum Hcy values, potentially vanishing the net benefit of RASi administration, if translating into an increase of Hcy levels (52).

A crosstalk in signaling pathways between Hcy and RASi impacting on cardiovascular system has been previously suggested. Kassab et al. have found that, in murine model of cardiac hypertrophy induced by Hcy, the administration of an ARB (valsartan) can attenuate hypertension and reduce the cardiac collagen, improvement ventricular hypertrophy (23). The exact mechanisms underlying RAS and Hcy interplay has not been completely clarified. Prior investigations have claimed a direct Hcy action on ACE activity, with amelioration of endothelial dysfunction after administration of RASi (53). More recently a direct relationship between Hcy levels and AT-1 receptor expression have been reported by Sen et al, leading to stimulation of matrix metalloproteinase-9 (MMP-9) and, thus, vascular remodeling and detrimental effect on cardiovascular system (54).

A deep investigation on the relation between Hcy on AT-1 receptor has been provided by Li et colleagues: in murine model they found that Hcy can allosterically modulate the AT-1 receptor conformation, and synergistically increase Angiotensin II-induced vascular

injury, suggesting a potential protective effect of RASi against damage mediated by Hcy and AT1 receptor (25)

However, there are no data about any variations in platelet reactivity during DAPT based on homocysteine and RASi, that was the aim of our study. Higher levels of Hcy were found in patients on treatment with RASi as compared to the non-treated ones with RASi resulting as an independent predictor of higher Hcy levels. Indeed, our results were in contrast with the previous reports suggesting a decrease in Hcy levels alongside with the amelioration of renal function among patients with a lower cardiovascular risk profile (51,55). However, our population was represented by patients with cardiovascular disease, where Hcy levels could have been more elevated independently from the use of RASi and moreover reduced ventricular function and latent renal dysfunction could have conditioned the effects of RASi on the kidneys. In fact, similarly to our findings Jiang et al. (52) reported a raise of Hcy with RAS inhibition in hypertensive subjects.

At multiplate aggregometry we found higher values with higher HCY in all the different tests, in both patients with and without RASi and irrespective of ADP-antagonist type in the composition of DAPT. Data were in accordance with our previous observations (56,57), documenting enhanced platelet reactivity with hyperhomocysteinemia only in patients on single antiplatelet therapy and irrespective of RASi intake.

Thus, despite the cardiovascular benefits of Hcy lowering strategies are still debated (58–60) further dedicated studies are needed to clarify the interplay between RASi and Hcy in platelet homeostasis, and the potential benefits of Hcy reduction in patients with cardiovascular disease and RASi use.

Limitations

The accessibility to follow-up data including the assessment of the clinical impact of high Hcy on adverse events and thrombotic episodes according to concomitant RASi intake would have certainly strengthened the conclusions of our study.

Patients with RASi had significantly higher values of Hcy, potentially leading to an impact on final results. Moreover, subjects under RASi displayed a more complex cardiovascular risk profile, including higher percentage of hypertension and obesity, known factors related to higher Hcy. However, oppositely lower rates of renal failure were observed in association to RASi therapy, which is often contraindicated in association to renal dysfunction. Therefore, we do not expect that the impact of renal function on the clearance of Hcy could have affected our results. Also, drug regimens differed between subjects under RASi treatment to ones without, including diuretics, beta-blockers and statins. Thus, a potential attenuation in RASi effect and its underestimation in our results cannot be excluded. In particular, diuretics can increase aldosterone, that is held responsible of inflammation, atherosclerosis, thrombotic diseases and cardiovascular complications, potentially limiting the effect of RASi. Nevertheless, our results were confirmed at multivariate analysis, accounting for all significant potential confounding factors. However, we cannot exclude the role of a third unidentified factor, and in particular for other metabolites, in the modulation of platelet reactivity.

We did not stratify our population according to the type and dosage of ACEi and ARB, leading to lack of estimation of potential differences among the different drugs. However, a pharmacological “class” effect of RASi on platelet activation has been suggested, with studies including a wide spectrum of drug and dosage types, reporting similar positive results.

Conclusions

The present study shows that renin angiotensin system inhibitors intake is not an independent predictor of elevated platelet reactivity in patients on DAPT for coronary artery disease.

However, the levels of Hcy were significantly higher in patients on RASi and related to higher values of platelet reactivity, independently from the DAPT strategy. Future dedicated studies are certainly needed to assess whether the elevated Hcy levels and platelet reactivity associated with the use of RASi could hamper the antiplatelet effects of currently available potent DAPT strategies.

References

1. De Luca G, Smit JJ, Ernst N, Suryapranata H, Ottervanger JP, Hoorntje JCA, et al. Impact of adjunctive tirofiban administration on myocardial perfusion and mortality in patients undergoing primary angioplasty for ST-segment elevation myocardial infarction. Thromb Haemost. 2005 May;93(5):820–3.
2. De Luca G, Suryapranata H, Marino P. Reperfusion strategies in acute ST-elevation myocardial infarction: an overview of current status. Prog Cardiovasc Dis. 50(5):352–82.
3. Roffi M, Patrono C, Collet J-P, Mueller C, Valgimigli M, Andreotti F, et al. 2015 ESC Guidelines for the management of acute coronary syndromes in patients presenting without persistent ST-segment elevation: Task Force for the Management of Acute Coronary Syndromes in Patients Presenting without Persistent ST-Segment Elevation of. Eur Heart J. 2016 Jan 14;37(3):267–315.
4. Brener SJ, Steinhubl SR, Berger PB, Brennan DM, Topol EJ, CREDO Investigators. Prolonged dual antiplatelet therapy after percutaneous coronary intervention reduces ischemic events without affecting the need for repeat revascularization: insights from the CREDO trial. J Invasive Cardiol. 2007 Jul;19(7):287–90.
5. Collet J-P, Cuisset T, Rangé G, Cayla G, Elhadad S, Pouillot C, et al. Bedside monitoring to adjust antiplatelet therapy for coronary stenting. N Engl J Med. 2012 Nov 29;367(22):2100–9.
6. Steinhubl SR, Talley JD, Braden GA, Tcheng JE, Casterella PJ, Moliterno DJ, et al. Point-of-care measured platelet inhibition correlates with a reduced risk of an adverse cardiac event after percutaneous coronary intervention: results of the GOLD (AU-Assessing Ultegra) multicenter study. Circulation. 2001 May 29;103(21):2572–8.
7. Pettersen A-ÅR, Seljeflot I, Abdelnoor M, Arnesen H. High On-Aspirin Platelet Reactivity and Clinical Outcome in Patients With Stable Coronary Artery Disease: Results From ASCET (Aspirin Nonresponsiveness and Clopidogrel Endpoint Trial). J Am Heart Assoc. 2012 Jun;1(3):e000703.
8. De Luca G, Suryapranata H, Gregorio G, Lange H, Chiariello M. Homocysteine and its effects on in-stent restenosis. Circulation. 2005 Nov 8;112(19):e307-11.
9. Schaffer A, Verdoia M, Cassetti E, Marino P, Suryapranata H, De Luca G, et al.

Relationship between homocysteine and coronary artery disease. Results from a large prospective cohort study. Thromb Res. 2014 Aug;134(2):288–93.

10. Verdoia M, Schaffer A, Cassetti E, Barbieri L, Di Giovine G, Marino P, et al. MTHFR polymorphism and risk of periprocedural myocardial infarction after coronary stenting. Nutr Metab Cardiovasc Dis. 2014 May;24(5):532–7.
11. Verdoia M, Schaffer A, Barbieri L, Cassetti E, Di Giovine G, Marino P, et al. Homocysteine and risk of periprocedural myocardial infarction in patients undergoing coronary stenting. J Cardiovasc Med (Hagerstown). 2015 Feb;16(2):100–5.
12. Loscalzo J. Homocysteine trials--clear outcomes for complex reasons. N Engl J Med. 2006 Apr 13;354(15):1629–32.
13. Karolczak K, Pieniazek A, Watala C. Inhibition of glutamate receptors reduces the homocysteine-induced whole blood platelet aggregation but does not affect superoxide anion generation or platelet membrane fluidization. Platelets. 2017 Jan;28(1):90–8.
14. McGarrigle SA, O'Neill S, Walsh GM, Moran N, Graham IM, Cooney M-T, et al. Integrin $\alpha(IIb)\beta_3$ exists in an activated state in subjects with elevated plasma homocysteine levels. Platelets. 2011;22(1):65–73.
15. Signorello MG, Pascale R, Leoncini G. Effect of homocysteine on arachidonic acid release in human platelets. Eur J Clin Invest. 2002 Apr;32(4):279–84.
16. Signorello MG, Segantin A, Passalacqua M, Leoncini G. Homocysteine decreases platelet NO level via protein kinase C activation. Nitric oxide Biol Chem. 2009 Mar;20(2):104–13.
17. Karolczak K, Kamysz W, Karafova A, Drzewoski J, Watala C. Homocysteine is a novel risk factor for suboptimal response of blood platelets to acetylsalicylic acid in coronary artery disease: a randomized multicenter study. Pharmacol Res. 2013 Aug;74:7–22.
18. Skowasch D, Viktor A, Schneider-Schmitt M, Lüderitz B, Nickenig G, Bauriedel G. Differential antiplatelet effects of angiotensin converting enzyme inhibitors: comparison of ex vivo platelet aggregation in cardiovascular patients with ramipril, captopril and enalapril. Clin Res Cardiol. 2006 Apr;95(4):212–6.
19. Krämer C, Sunkomat J, Witte J, Luchtefeld M, Walden M, Schmidt B, et al. Angiotensin II receptor-independent antiinflammatory and antiaggregatory

properties of losartan: role of the active metabolite EXP3179. Circ Res. 2002 Apr 19;90(7):770–6.

20. Willoughby SR, Rajendran S, Chan WP, Procter N, Leslie S, Liberts EA, et al. Ramipril sensitizes platelets to nitric oxide: implications for therapy in high-risk patients. J Am Coll Cardiol. 2012 Sep 4;60(10):887–94.
21. Kawai T, Forrester SJ, O'Brien S, Baggett A, Rizzo V, Eguchi S. AT1 receptor signaling pathways in the cardiovascular system. Pharmacol Res. 2017 Nov;125(Pt A):4–13.
22. Fu Y, Xiong Y, Fu S. Captopril restores endothelium-dependent relaxation of rat aortic rings after exposure to homocysteine. J Cardiovasc Pharmacol. 2003 Oct;42(4):566–72.
23. Kassab S, Garadah T, Abu-Hijleh M, Golbahar J, Senok S, Wazir J, et al. The angiotensin type 1 receptor antagonist valsartan attenuates pathological ventricular hypertrophy induced by hyperhomocysteinemia in rats. J Renin Angiotensin Aldosterone Syst. 2006 Dec;7(4):206–11.
24. Qin X, Li Y, Sun N, Wang H, Zhang Y, Wang J, et al. Elevated Homocysteine Concentrations Decrease the Antihypertensive Effect of Angiotensin-Converting Enzyme Inhibitors in Hypertensive Patients. Arterioscler Thromb Vasc Biol. 2017;37(1):166–72.
25. Li T, Yu B, Liu Z, Li J, Ma M, Wang Y, et al. Homocysteine directly interacts and activates the angiotensin II type I receptor to aggravate vascular injury. Nat Commun. 2018;9(1):11.
26. Verdoia M, Schaffer A, Sartori C, Barbieri L, Cassetti E, Marino P, et al. Vitamin D deficiency is independently associated with the extent of coronary artery disease. Eur J Clin Invest. 2014 Jul;44(7):634–42.
27. Verdoia M, Pergolini P, Camaro C, Restifo M, Rolla R, Schaffer A, et al. PlA(1)/PlA(2) polymorphism does not influence response to Gp IIb-IIIa inhibitors in patients undergoing coronary angioplasty. Blood Coagul Fibrinolysis. 2013 Jun;24(4):411–8.
28. Vidali M, Rolla R, Parrella M, Cassani C, Manzini M, Portalupi MR, et al. Role of the laboratory in monitoring patients receiving dual antiplatelet therapy. Int J Lab Hematol. 2012 Oct;34(5):484–94.
29. Massberg S, Brand K, Grüner S, Page S, Müller E, Müller I, et al. A critical role of

platelet adhesion in the initiation of atherosclerotic lesion formation. J Exp Med. 2002 Oct 7;196(7):887–96.

30. Patko Z, Csaszar A, Acsady G, Peter K, Schwarz M. Roles of Mac-1 and glycoprotein IIb/IIIa integrins in leukocyte-platelet aggregate formation: stabilization by Mac-1 and inhibition by GpIIb/IIIa blockers. Platelets. 2012;23(5):368–75.
31. De Luca G, Navarese EP, Suryapranata H. A meta-analytic overview of thrombectomy during primary angioplasty. Int J Cardiol. 2013 Jul 1;166(3):606–12.
32. De Luca G, Schaffer A, Wirianta J, Suryapranata H. Comprehensive meta-analysis of radial vs femoral approach in primary angioplasty for STEMI. Int J Cardiol. 2013 Oct 3;168(3):2070–81.
33. De Luca G, Navarese EP, Cassetti E, Verdoia M, Suryapranata H. Meta-analysis of randomized trials of glycoprotein IIb/IIIa inhibitors in high-risk acute coronary syndromes patients undergoing invasive strategy. Am J Cardiol. 2011 Jan 15;107(2):198–203.
34. Tomaiuolo M, Brass LF, Stalker TJ. Regulation of Platelet Activation and Coagulation and Its Role in Vascular Injury and Arterial Thrombosis. Interv Cardiol Clin. 2017;6(1):1–12.
35. Moncada S. Adventures in vascular biology: a tale of two mediators. Philos Trans R Soc Lond B Biol Sci. 2006 May 29;361(1469):735–59.
36. Knuuti J, Wijns W, Saraste A, Capodanno D, Barbato E, Funck-Brentano C, et al. 2019 ESC Guidelines for the diagnosis and management of chronic coronary syndromes. Eur Heart J. 2019 Aug 31;
37. Werner C, Baumhäkel M, Teo KK, Schmieder R, Mann J, Unger T, et al. RAS blockade with ARB and ACE inhibitors: current perspective on rationale and patient selection. Clin Res Cardiol. 2008 Jul;97(7):418–31.
38. Warnholtz A, Nickenig G, Schulz E, Macharzina R, Bräsen JH, Skatchkov M, et al. Increased NADH-oxidase-mediated superoxide production in the early stages of atherosclerosis: evidence for involvement of the renin-angiotensin system. Circulation. 1999 Apr 20;99(15):2027–33.
39. Candido R, Jandeleit-Dahm KA, Cao Z, Nesteroff SP, Burns WC, Twigg SM, et al. Prevention of accelerated atherosclerosis by angiotensin-converting enzyme

inhibition in diabetic apolipoprotein E-deficient mice. Circulation. 2002 Jul 9;106(2):246–53.

40. Bauriedel G, Skowasch D, Schneider M, Andrié R, Jabs A, Lüderitz B. Antiplatelet effects of angiotensin-converting enzyme inhibitors compared with aspirin and clopidogrel: a pilot study with whole-blood aggregometry. Am Heart J. 2003 Feb;145(2):343–8.
41. Kalinowski L, Matys T, Chabielska E, Buczko W, Malinski T. Angiotensin II AT1 receptor antagonists inhibit platelet adhesion and aggregation by nitric oxide release. Hypertens (Dallas, Tex 1979). 2002 Oct;40(4):521–7.
42. Montón M, Jiménez A, Núñez A, López-Blaya A, Farré J, Gómez J, et al. Comparative effects of angiotensin II AT-1-type receptor antagonists in vitro on human platelet activation. J Cardiovasc Pharmacol. 2000 Jun;35(6):906–13.
43. Mollnau H, Wendt M, Szöcs K, Lassègue B, Schulz E, Oelze M, et al. Effects of angiotensin II infusion on the expression and function of NAD(P)H oxidase and components of nitric oxide/cGMP signaling. Circ Res. 2002 Mar 8;90(4):E58-65.
44. Marinšek M, Sinkovič A. Ramipril and Losartan Exert a Similar Long-Term Effect upon Markers of Heart Failure, Endogenous Fibrinolysis, and Platelet Aggregation in Survivors of ST-Elevation Myocardial Infarction: A Single Centre Randomized Trial. Biomed Res Int. 2016;2016:9040457.
45. Schieffer B, Bünte C, Witte J, Hoeper K, Böger RH, Schwedhelm E, et al. Comparative effects of AT1-antagonism and angiotensin-converting enzyme inhibition on markers of inflammation and platelet aggregation in patients with coronary artery disease. J Am Coll Cardiol. 2004 Jul 21;44(2):362–8.
46. Homocysteine Studies Collaboration. Homocysteine and risk of ischemic heart disease and stroke: a meta-analysis. JAMA. 288(16):2015–22.
47. Wald DS, Law M, Morris JK. Homocysteine and cardiovascular disease: evidence on causality from a meta-analysis. BMJ. 2002 Nov 23;325(7374):1202.
48. Refsum H, Helland S, Ueland PM. Radioenzymic determination of homocysteine in plasma and urine. Clin Chem. 1985 Apr;31(4):624–8.
49. Jafar TH, Stark PC, Schmid CH, Landa M, Maschio G, de Jong PE, et al. Progression of chronic kidney disease: the role of blood pressure control, proteinuria, and angiotensin-converting enzyme inhibition: a patient-level meta-analysis. Ann Intern Med. 2003 Aug 19;139(4):244–52.

50. Williams B, Mancia G, Spiering W, Agabiti Rosei E, Azizi M, Burnier M, et al. 2018 ESC/ESH Guidelines for the management of arterial hypertension. Eur Heart J. 2018 Sep 1;39(33):3021–104.
51. Poduri A, Kaur J, Thakur JS, Kumari S, Jain S, Khullar M. Effect of ACE inhibitors and beta-blockers on homocysteine levels in essential hypertension. J Hum Hypertens. 2008 Apr;22(4):289–94.
52. Jiang S, Pan M, Wu S, Venners SA, Zhong G, Hsu Y-H, et al. Elevation in Total Homocysteine Levels in Chinese Patients With Essential Hypertension Treated With Antihypertensive Benazepril. Clin Appl Thromb Hemost. 2016 Mar;22(2):191–8.
53. Huang A, Pinto JT, Froogh G, Kandhi S, Qin J, Wolin MS, et al. Role of homocysteinylation of ACE in endothelial dysfunction of arteries. Am J Physiol Heart Circ Physiol. 2015 Jan 15;308(2):H92-100.
54. Sen U, Herrmann M, Herrmann W, Tyagi SC. Synergism between AT1 receptor and hyperhomocysteinemia during vascular remodeling. Clin Chem Lab Med. 2007;45(12):1771–6.
55. Wollesen F, Brattström L, Refsum H, Ueland PM, Berglund L, Berne C. Plasma total homocysteine and cysteine in relation to glomerular filtration rate in diabetes mellitus. Kidney Int. 1999 Mar;55(3):1028–35.
56. Verdoia M, Rolla R, Negro F, Tonon F, Pergolini P, Nardin M, et al. Homocysteine levels and platelet reactivity in coronary artery disease patients treated with ticagrelor. Nutr Metab Cardiovasc Dis. 2020;30(2):292–9.
57. Verdoia M, Schaffer A, Pergolini P, Rolla R, Barbieri L, Bellomo G, et al. Homocysteine Levels Influence Platelet Reactivity in Coronary Artery Disease Patients Treated With Acetylsalicylic Acid. J Cardiovasc Pharmacol. 2015 Jul;66(1):35–40.
58. Lange H, Suryapranata H, De Luca G, Börner C, Dille J, Kallmayer K, et al. Folate therapy and in-stent restenosis after coronary stenting. N Engl J Med. 2004 Jun 24;350(26):2673–81.
59. Schaffer A, Verdoia M, Barbieri L, Cassetti E, Suryapranata H, De Luca G. Impact of Diabetes on Homocysteine Levels and Its Relationship with Coronary Artery Disease: A Single-Centre Cohort Study. Ann Nutr Metab. 2016;68(3):180–8.
60. Singh Y, Samuel VP, Dahiya S, Gupta G, Gillhotra R, Mishra A, et al.

Combinational effect of angiotensin receptor blocker and folic acid therapy on uric acid and creatinine level in hyperhomocysteinemia-associated hypertension. Biotechnol Appl Biochem. 2019 Sep;66(5):715–9.

Chapter 9

Impact of atorvastatin or rosuvastatin co-administration on platelet reactivity in patients treated with dual antiplatelet therapy

Monica Verdoia[a]; Matteo Nardin[a]; Chiara Sartori[a]; Patrizia Pergolini[b]; Roberta Rolla[b]: Lucia Barbieri[a]; Paolo Marino[a]; Giorgio Bellomo[b]; Harry Suryapranata[c]; Giuseppe De Luca[a]; on behalf of the Novara Atherosclerosis Study Group (NAS)

a) Division of Cardiology, Azienda Ospedaliera-Universitaria "Maggiore della Carità", Eastern Piedmont University, Novara, Italy; b) Clinical Chemistry, Azienda Ospedaliera-Universitaria "Maggiore della Carità", Eastern Piedmont University, Novara, Italy; c) Department of Cardiology, UMC St Radboud, Nijmegen, The Netherlands.

Atherosclerosis 243 (2015): 389-394

Abstract

Background: Residual high-on treatment platelet reactivity (HRPR) still represents a challenging matter in patients with coronary artery disease. Drug-to-drug interaction has been suggested between some statin and antiplatelet agents, despite their co-administration is mandatory in patients after an acute cardiovascular event or coronary stenting. Therefore, the aim of the current study was to investigate any impact of rosuvastatin or atorvastatin co-administration on platelet reactivity in patients receiving dual antiplatelet therapy (DAPT).

Methods: Our population is represented by patients on DAPT (ASA and either clopidogrel 75 mg or ticagrelor 90 mg b.i.d) after an ACS or percutaneous revascularization and receiving rosuvastatin or atorvastatin. Platelet function was assessed by Multiplate Impedance Aggregometry (Roche Diagnostics AG).

Results: We included a total of 374 patients, 240 (64.2%) receiving atorvastatin, 134 (35.8%) rosuvastatin. Rosuvastatin treated patients were more often using beta-blockers ($p = 0.05$), diuretics ($p = 0.04$) and displayed higher HDL ($p < 0.001$) and lower LDL cholesterol ($p < 0.001$). The prevalence of HRPR for ASA was low, with no difference according to statin type (0.8% vs 1.5%, $p = 0.62$, adjusted OR[95%CI] = 2 [0.23 -16.6], $p = 0.52$). Concerning ADP-antagonists, in the 163 patients treated with clopidogrel, rosuvastatin co-administration was associated with a significantly increased rate of HRPR (55.6% vs 32%, $p = 0.01$, adjusted OR[95%CI] = 2.69[1.22-5.96], $p = 0.015$) with higher ADP-mediated platelet reactivity ($p = 0.01$) and TRAP-test results ($p = 0.04$). On the contrary, in the 211 ticagrelor treated patients, statin type did not affect mean platelet reactivity or the prevalence of HRPR with ticagrelor (10.5% vs 11.2%, $p = 0.99$, adjusted OR[95%CI] = 0.86[0.34-2.22], $p = 0.76$)

Conclusions: Among patients receiving DAPT, rosuvastatin but not atorvastatin is associated with an increased rate of HRPR for clopidogrel, without any influence on the antiplatelet effect of ASA or ticagrelor. Therefore, cautiousness should be exerted for clopidogrel and rosuvastatin therapeutic association.

Background

Improvements in percutaneous coronary revascularization techniques and pharmacological strategies have positively affected the outcome of patients with coronary artery disease (CAD), especially in acute coronary syndromes (ACS) (1,2), where largest benefits have been obtained from high-intensity statins and more potent dual antiplatelet therapy (DAPT) (3,4). However, suboptimal platelet inhibition or high-residual on treatment platelet reactivity (HRPR) still occurs in a large quote of patients on DAPT, enhancing the risk of recurrent ischemic events and stent thrombosis (5,6). Thus, recently large attention has been paid to the identification of the causes of HRPR, with a special focus on potential drug-todrug interactions, in particular for clopidogrel, whose complex metabolic activation via the hepatic cytochromes CYP2C19 and CYP3A4, has been identified as a major determinant of its interindividual variability of response (7,8).

However, contrasting results have been achieved with high intensity statins (9), such as rosuvastatin and atorvastatin. In fact, their co-administration with clopidogrel often occurs in patients with CAD and, being these statins inactivated by the CYP2C19 and CYP3A4 enzymes, they could interfere with clopidogrel activation. Moreover, even few data have been reported with the new antiplatelet agent ticagrelor that, not requiring hepatic activation, has partially overcome the phenomenon of HRPR observed with clopidogrel (10). Nevertheless, a modest hepatic biotransformation into active metabolites has been documented also for ticagrelor via the CYP3A4 and 3A5, accounting for about 30% of the circulating drug (11) and potentially being exposed to the same risk of interaction with statins as for clopidogrel. Therefore, the aim of present study was to evaluate the impact of different statin types on the antiplatelet effect of clopidogrel or ticagrelor among patients receiving dual antiplatelet therapy for CAD.

Methods

We included patients admitted to the Division of Cardiology, “Maggiore della Carita” Hospital, Eastern Piedmont University in Novara, Italy, from September 2011 to October 2014 and treated with DAPT and statins for either ACS or stable CAD. All patients receiving at discharge atorvastatin or rosuvastatin and DAPT with ASA (100e160mg daily) and either ticagrelor (90 mg every 12 h) or clopidogrel (75 mg daily) were scheduled for chemistry and platelet function tests evaluation at 30e90 days from discharge. Invasive treatment with coronary angiography and eventual coronary stenting was not a required inclusion criteria. The study was approved by our local Ethical Commitee and informed consent was obtained by all patients. Main demographic, clinical and angiographic data, together with the indication to dual antiplatelet therapy were recorded at discharge and included in a dedicated database, protected by password. Main cardiovascular risk factors were identified. Hypertension was defined as systolic pressure >140 mmHg and/or diastolic pressure was >90 mmHg or if the individual was taking antihypertensive medications. The diagnosis of diabetes was based on previous history of diabetes treated with or without drug therapies, fasting glucose >126 g/dL or HbA1c > 6.5% at the moment of admission (12). Exclusion criteria were patients’ refusal or nonadherence to prescribed medications. High-intensity statin therapy was defined as atorvastatin 40 mg daily or rosuvastatin 20 mg daily according to 2013 AHA/ ACC Guidelines (13). Proton pump inhibitors were routinely administered in all patients on DAPT.

Biochemistry analysis

Blood samples were drawn in the early morning, following a fasting period of 12 h. Glucose, creatinine, glycosylated hemoglobin and lipid profile were determined as previously described (14). Blood cells count was performed in a blood sample collected in

tripotassium EDTA (7.2 mg) tubes. These blood samples were analyzed within 2 h of venipuncture by automatic blood cells counter (A Sysmex XE-2100).

Platelet aggregation

Platelet aggregation was assessed by Multiplate electrical impedance aggregometry (MEA), in the early morning (between 8 and 9 a.m.). The aggregation tests were performed from 30 min to 2 h from blood collection (15). Platelets aggregation was assessed after stimulation with arachidonic acid (0.5 mm/L) (ASPI test), collagen (3.2 mg/mL) (COL test), ADP (6.4 mmol/L) with prostaglandin E1 and thrombin receptor activating peptide, (TRAP-6; 30 mmol/L). Results were expressed as arbitrary Aggregation Units (AU) and plotted against time, defining platelet function as the area under curve (AUC or AU*min). HRPR was considered for AU*min values above lower limit normal for ASA (HAPR), (range: 862-1344) or after ADP stimulation (range: 417-1030) (16). In addition, the previously reported cut-off of 468AU*min was also applied (17).

Statistical analysis

Statistical analysis was performed by SPSS Statistics Software 17.0. (SPSS Inc., Chicago, Illinois). Continue variables were represented as mean ± SD, while categorical variables as percentage. Chi-Squared and ANOVA test were appropriately used to compare clinical and laboratory features according to the type of statin treatment. Multiple logistic regression analysis was performed to assess the potential association between the occurrence of HRPR and statin type, after correction for baseline differences that were entered in the model in block. A p-value < 0.05 was considered statistically significant.

Results

Our population is represented by 374 patients, 240 of them (64.2%) were on atorvastatin, 134 (35.8%) on rosuvastatin. **Table 1** displays main demographic and clinical characteristics according to statin type. The percentage of patients receiving a high-dose statin did not differ between the two study groups. Rosuvastatin treated patients were more often using beta-blockers (p = 0.05), diuretics (p = 0.04) and displayed higher HDL (p<0.001) and lower LDL cholesterol (p < 0.001) as compared to patients on atorvastatin. No difference in the timing of platelet function tests from discharge was observed among our patients according to statin treatment (45 ± 14.2 vs 55.8 ± 15.3, p = 0.62).

Table 1. Main clinical and demographic features in study population.

Clinical features	**Atorvastatin (n=240)**	**Rosuvastatin (n=134)**	**p-value**
Age (mean±SD)	66.7±11.1	65±10.6	0.15
Male sex (%)	78.8	79.9	0.99
BMI (mean±SD)	27±4.1	27.5±4.6	0.33
Hypertension (%)	74.2	70.7	0.47
Active Smokers (%)	23.3	30.1	0.10
Diabetes mellitus (%)	40.8	43.3	0.66
Hypercholesterolemia (%)	61.1	61.7	0.99
Previous MI (%)	23.8	25.5	0.99
Previous PCI (%)	32.1	39.8	0.14
Previous CABG (%)	12.1	11.3	0.87
Renal failure (%)	16.3	13.5	0.55
Indication to DAPT (%)			0.35
Elective PCI	33.5	32.6	
Acute coronary syndrome	47.7	44.1	
STEMI	18.8	23.3	
Multivessel CAD	61.9	65.2	0.53
Left main/trivessel CAD*	37.2	29.6	0.18
Therapy at discharge			
ACE inhibitors (%)	55	55.2	0.99
ARBs (%)	17.5	25.4	0.08

Betablockers (%)	78.8	87.3	0.05
Nitrates (%)	44.6	44	0.99
Ca^{2+}-antagonists (%)	25	18.7	0.20
Diuretics (%)	32.5	22.4	0.04
Proton pump inhibitors (%)	100	100	0.99
High-dose statin (%)	63.3	68.5	0.57
Main chemistry parameters			
Glycaemia (mean±SD)	125.2±56.2	125.7±45	0.93
HbA1c (mean±SD)	6.5±1.3	6.5±1.2	0.82
Creatinine (mean±SD)	1±0.5	1±0.6	0.59
Cholesterol HDL (±SD)	39.1±11.7	43.9±13.6	<0.001
Cholesterol LDL (±SD)	77.5±29.8	65.5±28.3	<0.001
ΔLDL (±SD)	29.5±39	31.8±45.9	0.30
Triglycerides (mean±SD)	126.1±81	130.1±71	0.59
C-reactive protein (mg/dL,±SD)	0.78±1.8	0.59±1.8	0.33
Platelets (10^5/mL; mean±SD)	213.5±73.5	223.6±64	0.30
Hemoglobin (mean±SD)	13.5±1.7	13.8±1.5	0.10
WBC (10^3/mL;mean±SD)	8.2±2.7	7.8±1.9	0.58

BMI=Body Mass Index; MI = Myocardial Infarction; PCI = Percutaneous Coronary Interventions; CABG = Coronary Artery Bypass Grafting; DAPT= Dual antiplatelet therapy; STEMI= ST-elevation myocardial infarction; CAD = Coronary artery disease; ACE= Angiotensin converting enzyme; ARB= angiotensin receptor blocker; HDL= High-Density Lipoproteins; LDL = Low-Density Lipoproteins; WBC= White Blood Cells.

No significant difference in mean platelet reactivity was observed between patients on rosuvastatin vs atorvastatin, but for higher values of TRAP-mediated aggregation in rosuvastatin treated patients (p = 0.01), as displayed in **Table 2**. The prevalence of HRPR for ASA was low, with no difference according to statin type (0.8% vs 1.5%, p = 0.62, **Figure 1**). Results were confirmed after correction for baseline differences, with no association between statin type and impaired ASA response (adjusted OR [95%CI] = 2 [0.23 - 16.6], p = 0.52 for rosuvastatin vs atorvastatin). In the 163 patients treated with clopidogrel, higher mean ADP-mediated platelet reactivity (p = 0.01) and TRAP-test

results (p = 0.04) were observed for the concomitant use of rosuvastatin and clopidogrel (**Table 2**).

Table 2. Platelet reactivity at Multiplate impedance aggregometry according to treatment.

Multiplate aggregometry	**Atorvastatin (n=240)**	**Rosuvastatin (n=134)**	**p-value**
Overall dual antiplatelet therapy			
COLtest (mean±SD)	423.1±149.1	446.7±155.2	0.16
TRAPtest (mean±SD)	1030.7±310.7	1110.2±262.7	0.01
ASPItest (mean±SD)	316.2±175.5	351.6±195.3	0.07
ADPtest (mean±SD)	309.5±156	328.9±183	0.29
ASA+Clopidogrel	Atorvastatin (n=115)	Rosuvastatin (n=48)	
COLtest (mean±SD)	432.2±173	468.5±155	0.21
TRAPtest (mean±SD)	924.3±306.3	1029.8±252.2	0.04
ASPItest (mean±SD)	310.4±200.2	339.2±185	0.39
ADPtest (mean±SD)	343.5±184.5	429.9±214	0.01
ASA+Ticagrelor	Atorvastatin (n=125)	Rosuvastatin (n=86)	
COLtest (mean±SD)	416.2±123.3	434.5±154.5	0.34
TRAPtest (mean±SD)	1119.1±286	1154.1±259.2	0.37
ASPItest (mean±SD)	321.6±149.9	358.6±201.5	0.13
ADPtest (mean±SD)	281.5±123.4	276.1±139.6	0.77

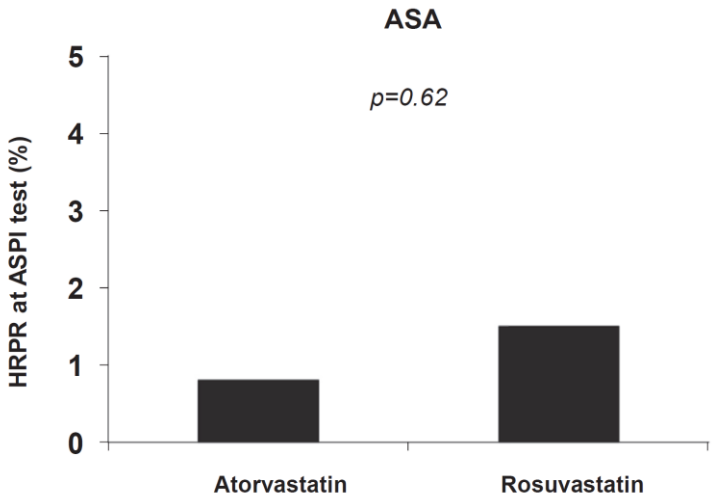


Figure 1. Bar graph showing the prevalence of high-residual on treatment platelet reactivity (HRPR) at ASPI test according to statin type.

Rosuvastatin co-administration was associated with a significant increase of HRPR (55.6% vs 32%, p = 0.01, **Figure 2**), that was confirmed after adjustment for baseline confounders (adjusted OR [95%CI] = 2.69 [1.22-5.96], p = 0.015). On the contrary, in the 211 ticagrelor treated patients, statin type did not affect mean platelet reactivity (**Table 2**) or the prevalence of HRPR with ticagrelor (10.5% vs 11.2%, p = 0.99, **Figure 3**), with results being confirmed at multivariate analysis (adjusted OR [95% CI] = 0.86 [0.34-2.22], p = 0.76 for rosuvastatin vs atorvastatin).

No dose-dependent effect on ADP-mediated aggregation was observed in patients receiving low vs high intensity atorvastatin with clopidogrel (326.3 ± 185.1 vs 344.7 ± 185.4, p = 0.80) or ticagrelor (282.5 ± 200.7 vs 281.4 ± 113.6, p = 0.97) and neither in patients receiving low vs high intensity rosuvastatin with clopidogrel (413.6 ± 207 vs 429.9 ± 214.7, p = 0.55) or ticagrelor (324 ± 147.5 vs 315.5 ± 140.4, p = 0.83).

Results did not significantly change when considering the previously reported cut-off of 468AU*min for the definition of HRPR for both clopidogrel (28.2% vs 42.2%, p = 0.12; adjusted OR [95% CI] = 2.15 [0.95-4.84], p = 0.06), and ticagrelor (8% vs 7%, p = 0.99; adjusted OR [95%CI] = 0.98 [0.32-2.99], p = 0.97).

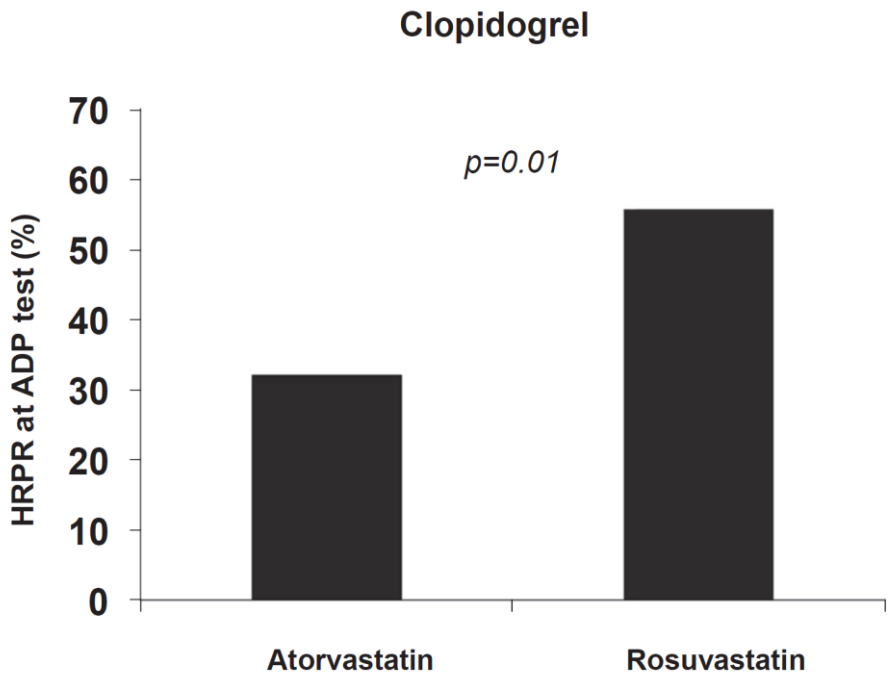


Figure 2. Bar graph showing the prevalence of high-residual on treatment platelet reactivity (HRPR) at ADP test according to statin type in patients receiving clopidogrel.

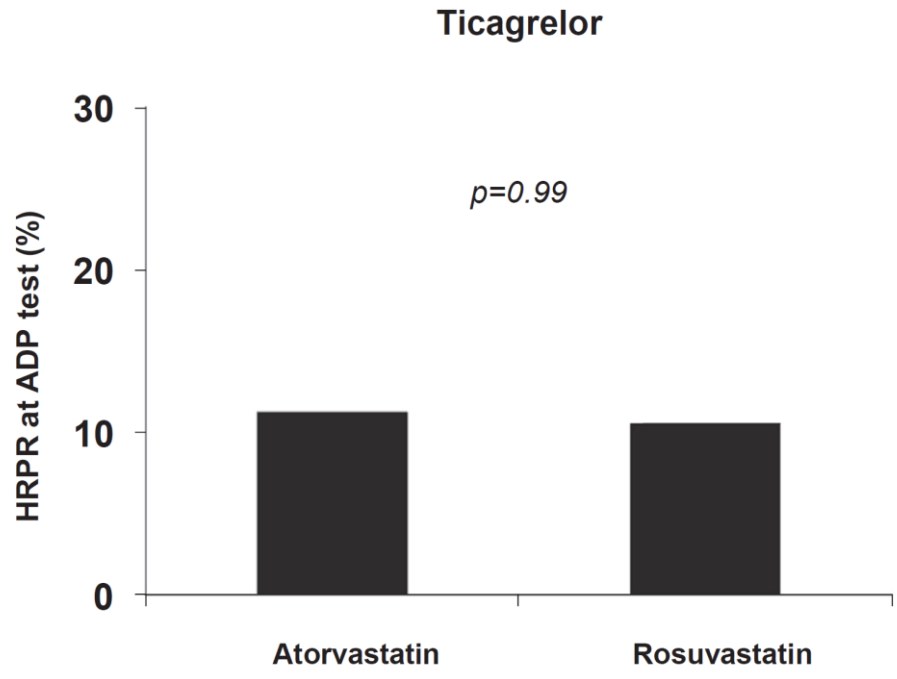


Figure 3. Bar graph showing the prevalence of high-residual on treatment platelet reactivity (HRPR) at ADP test according to statin type in patients receiving ticagrelor.

Discussion

The present study represents one of the largest studies addressing the role of statin type on the platelet reactivity and the response to DAPT with ASA, clopidogrel and ticagrelor among patients with CAD.

Our main finding is that among patients receiving dual antiplatelet therapy for CAD, the concomitant treatment with rosuvastatin is associated with an increased rate of HRPR with clopidogrel. On the contrary, statin type does not influence the antiplatelet effect of ASA or ticagrelor. Relevant improvements have been achieved in the field of CAD, where advances in mechanical reperfusion strategies and innovations in pharmacological treatments have allowed to reduce mortality and the risk of recurrent cardiovascular events, especially in the settings of STEMI (18,19). In particular, a potent platelet inhibition represents the pillar of anti-ischemic treatment, with a DAPT being recommended as soon as possible after an acute cardiovascular event or coronary stent implantation, and indicated for at least 12 months in the majority of patients (20). However, despite aggressive therapeutic strategies still a relevant quote of patients experiences recurrent ischemic events for a suboptimal platelet inhibition, or high HRPR, accounting for a 2e9 fold increased risk of stent thrombosis and major CV events (6). Certainly, suboptimal response to clopidogrel has been documented in up to 30% of patients, and the complex metabolic pathway required for its transformation into active metabolites has been claimed as one of the major determinants of HRPR (21). Growing evidence has associated the occurrence of clopidogrel resistance to a reduced function of the cytochrome P450 (CYP) enzymes, and primarily CYP2C19, the main responsible for clopidogrel activation (22). However, genetic allelic loss of function of the CYP2C19 can explain only the 12% of clopidogrel response variability. Indeed, other genetic variants of CYP enzymes, as CYP3A4, might condition the antiplatelet effect of clopidogrel, although

raising interest has been addressed to non-genetic causes of impaired clopidogrel metabolism, including drug-to-drug interactions (23). In fact, previous studies have documented a negative competition of clopidogrel with other drugs, such as proton pump inhibitors, calcium antagonists, and statins (24), whose coadministration is not infrequent, and especially for statins, that are recommended in current guidelines in all patients with CAD, irrespective from lipid profile. In particular, the high-intensity statins rosuvastatin and atorvastatin are not only the most effective in cardiovascular prevention, being then suggested in higher risk patients and in acute settings, but also those more prone to interfere with clopidogrel metabolism, as rosuvastatin undergoes inactivation via the CYP2C9 and CYP2C19 enzymes, while atorvastatin interacts with CYP3A4 (25). In fact, Lau et al. reported that atorvastatin co-administration attenuated the antiplatelet effect of clopidogrel (26), while on the contrary, the ACHIDO study documented lower platelet reactivity with clopidogrel 150 mg loading dose, if co-administered with atorvastatin (27). More recently, Pelliccia et al. (28) documented in 155 patients with stable CAD that atorvastatin increased the risk of HRPR with clopidogrel only in patients displaying already higher platelet reactivity before statin introduction, suggesting that the blockage of CYP3A4 pathway by atorvastatin could shift the clopidogrel metabolism to CYP2C19, playing a relevant impact only in patients with a genetic hypo-functioning variant of this enzyme (7). Contrasting results, instead, have been reported with rosuvastatin, that can directly interact with the main principal clopidogrel metabolizer. In a previous small study, Malmstrom et al. (29) documented that statin type did not significantly impact on ADP-mediated platelet reactivity, although the percentage of platelet inhibition was 40% in the rosuvastatin arm and 57% in patients on atorvastatin. Furthermore, the CILON-T trial (30) confirmed that among 915 patients with a recent percutaneous coronary intervention, the use of rosuvastatin was a significant predictor of a platelet reactivity in the highest tertile

(OR 1.67, 95% confidence interval 1.05e2.65, p ¼ 0.031). Moreover, Pinheiro et al. clearly demonstrated dynamical changes in rosuvastatin and clopidogrel concentrations when the two drugs were co-administered (31). Opposite findings were suggested by Riondino et al. (32) in patients undergoing PCI and receiving either atorvastatin or rosuvastatin and in the 122 patients of the PEARL study (33), showing no difference in platelet aggregation. Finally, in a recent sub-analysis of the TRITON-TIMI 38 study there was no influence of concomitant use of statin on clinical outcome in patients treated with clopidogrel (34). Indeed, a different scenario can be hypothesized after the introduction of new ADP-antagonists, and especially for ticagrelor a completely reversible and fully active antiplatelet agent, not requiring metabolic activation, thus being less subject to the problems of drug-to-drug interaction of clopidogrel. However, despite ticagrelor can provide a more potent and predictable antiplatelet effect, recent evidence has emerged of the existence of two ticagrelor active metabolites, produced by the CYP3A4 and CYP3A5 enzymes, and accounting for about 30% of the circulating drug (11). In fact, Teng et al. showed that ticagrelor could display a pharmacokinetic interaction at the CYP3A4 level with statin, in heathy volunteers, demonstrating a significant role for simvastatin, but not for atorvastatin (35). However, no study has explored so far the relationship between statin type and the antiplatelet effectiveness of ticagrelor, whereas uncertainty still exists on such statins-clopidogrel interaction. In our study, we evaluated the impact of commonly administered statins, atorvastatin and rosuvastatin, on platelet reactivity in patients receiving DAPT with clopidogrel or ticagrelor for a recent ACS or PCI. We demonstrated that rosuvastatin therapy significantly increased the risk of HRPR for clopidogrel, as compared to atorvastatin, while statin type did not influence the effectiveness of ticagrelor maintenance therapy. Therefore, present findings confirm with a different aggregation test (Multiplate vs VerifyNow) the results obtained by Suh et al. (30), in a large population of

patients treated with clopidogrel for 6 months. On the contrary, the smaller population enrolled and the differences in dosage and duration of statin therapy can provide explanation for the discrepant results of previous studies with clopidogrel, such as in Park et al. (36), where patients on chronic (>6 months) low-dose atorvastatin were randomly assigned to a brief therapy with pravastatin or high-dose rosuvastatin before platelet function re-assessment. In addition, we showed no statin-ticagrelor interaction among CAD patients. Thus, according to present findings, the clopidogrel-rosuvastatin combination should not be advised in secondary cardiovascular prevention, whereas several treatment combinations appear feasible with no evidence of drug-drug interaction, suggesting the option of switching to a different statin or to the new antiplatelet agents in patients requiring DAPT for CAD.

Limitations

A first limitation can be represented by the lack of long-term follow-up of our patients, and thus we could not investigate the impact of HRPR or pharmacologic interactions on clinical outcome, including the risk of thrombotic events or drug adverse reactions. In addition, as our study lacked a randomized allocation of patients, a certain heterogeneity in statin dose could be observed among our patients, although all our population received an intensive statins treatment, while the largest differences have been reported between high-intensity and moderate-low intensity statin therapy (37). We did not confirm our findings with a second aggregation test, but good concordance has been recently reported between Multiplate and light transmission aggregometry, that still represents the gold standard for platelet aggregation (38). Moreover, Multiplate has demonstrated a predictive role on outcomes and in the tailoring of antiplatelet therapy (39). Indeed, different cut-offs have been recently reported for definition of HRPR with clopidogrel. In particular, a value

of 468AU*min has been recently identified by Sibbings et al. (40) as best predictor of the risk of stent thrombosis in a population of patients undergoing PCI. However, as no study has ever validated this cut-off for ticagrelor and aiming to define the prevalence of HRPR with different statin types rather than its clinical impact, we preferred to apply a more strict laboratory value of 417 AU*min to consider inadequate the levels of platelet inhibition. In fact, this cut-off represents not only the upper normal range for our laboratory (validated in a control population) (16), but was also similarly observed as the highest quintile for platelet reactivity in the study of Sibbings et al. (40). We evaluated platelet aggregation between 30 and 90 days after initiation of the therapy. In fact, it has been shown that platelet reactivity tends to be higher in the acute phase of ACS as compared to a later stable phase (at 30 days in the vast majority of studies) (41). Having already postponed the measurement (between 30 and 90 days), we would not certainly expect different results by going even further. This was an exploratory study, and no formal sample size calculation was performed. Therefore, our study may have potentially been underpowered to detect a statistically significant difference in the rate of HRPR between statins in patients treated with ticagrelor. However, based on the observed rate of HRPR (10.5% vs 11.2%) and based on a study power of 80%, 61973 patients would have been required to reach the statistical significance ($p < 0.05$) among ticagrelor treated patients. However, it must be recognized that such a small difference, even if statistically different, would not certainly imply a clinical relevance. Finally, we did not consider the role of genetic variants of hepatic CYP enzymes in modulating the response to pharmacological therapies. However, previous studies with clopidogrel have demonstrated the superiority of direct platelet function assessment as compared with genotyping in terms of prognostic predictivity (42).

Conclusion

Among patients receiving DAPT, rosuvastatin but not atorvastatin concomitant treatment is associated with an increased rate of HRPR for clopidogrel, while not influencing the antiplatelet effect of ASA or ticagrelor. Therefore, cautiousness should be exerted for clopidogrel and rosuvastatin therapeutic association.

References

1. G. De Luca, H. Suryapranata, P. Marino, Reperfusion strategies in acute STelevation myocardial infarction: an overview of current status, Prog. Cardiovasc Dis. 50 (2008) 352-382.
2. G. De Luca, M.T. Dirksen, C. Spaulding, H. Kelbaek, M. Schalij, L. Thuesen, B. van der Hoeven, M.A. Vink, C. Kaiser, C. Musto, T. Chechi, G. Spaziani, L.S. Díaz de la Llera, V. Pasceri, E. Di Lorenzo, R. Violini, G. Cortese, H. Suryapranata, G.W. Stone, Drug-Eluting stent in Primary Angioplasty (DESERT) Cooperation. Drug-eluting vs bare-metal stents in primary angioplasty: a pooled patientlevel meta-analysis of randomized trials, Arch. Intern. Med. 172 (2012) 611-621.
3. M. Verdoia, A. Schaffer, L. Barbieri, E. Cassetti, R. Piccolo, G. Galasso, P. Marino, F. Sinigaglia, G. De Luca, Benefits from new ADP antagonists as compared with clopidogrel in patients with stable angina or acute coronary syndrome undergoing invasive management: a meta-analysis of randomized trials, J. Cardiovasc Pharmacol. 63 (4) (2014) 339-350.
4. T.R. Pedersen, N.B. Cater, O. Faergeman, J.J. Kastelein, A.G. Olsson, M.J. Tikkanen, I. Holme, M.L. Larsen, C. Lindahl, M. Szarek, Comparison of atorvastatin 80 mg/day versus simvastatin 20 to 40 mg/day on frequency of cardiovascular events late (five years) after acute myocardial infarction (from the incremental decrease in end points through aggressive Lipid Lowering IDEAL. trial), Am. J. Cardiol. 106 (3) (2010) 354-359.
5. K. Mayer, I. Bernlochner, S. Braun, S. Schulz, M. Orban, T. Morath, L. Cala, P. Hoppmann, H. Schunkert, K.L. Laugwitz, A. Kastrati, D. Sibbing, Aspirin treatment and outcomes after percutaneous coronary intervention: results of the ISAR-ASPI registry, J. Am. Coll. Cardiol. 64 (9) (2014) 863-871.
6. U.S. Tantry, L. Bonello, D. Aradi, M.J. Price, Y.H. Jeong, D.J. Angiolillo, G.W. Stone, N. Curzen, T. Geisler, J. Ten Berg, A. Kirtane, J. Siller-Matula, E. Mahla, R.C. Becker, D.L. Bhatt, R. Waksman, S.V. Rao, D. Alexopoulos, R. Marcucci, J.L. Reny, D. Trenk, D. Sibbing, P.A. Gurbel, Working Group on ontreatment platelet Reactivity. Consensus and update on the definition of ontreatment platelet reactivity to adenosine diphosphate associated with ischemia and bleeding, J. Am. Coll. Cardiol. 62 (24) (2013) 2261-2273.

7. P.A. Gurbel, U.S. Tantry, Clopidogrel response variability and the advent of personalised antiplatelet therapy. A bench to bedside journey, Thromb. Haemost. 106 (2) (2011) 265-271.
8. X.L. Jiang, S. Samant, L.J. Lesko, S. Schmidt, Clinical pharmacokinetics and pharmacodynamics of clopidogrel, Clin. Pharmacokinet. 54 (2) (2015) 147-166.
9. M. Leoncini, A. Toso, M. Maioli, F. Bellandi, Statin and clopidogrel pharmacological interaction, G. Ital. Cardiol. (Rome) 14 (9) (2013) 574-584.
10. P.P. Dobesh, J.H. Oestreich, Ticagrelor: pharmacokinetics, pharmacodynamics, clinical efficacy, and safety, Pharmacotherapy 34 (10) (2014) 1077-1090.
11. S. Husted, H. Emanuelsson, S. Heptinstall, P.M. Sandset, M. Wickens, G. Peters, Pharmacodynamics, pharmacokinetics, and safety of the oral reversible P2Y12 antagonist AZD6140 with aspirin in patients with atherosclerosis: a double-blind comparison to clopidogrel with aspirin, Eur. Heart J. 27 (9) (2006) 1038-1047.
12. R.T. Ackermann, Y.J. Cheng, D.F. Williamson, E.W. Gregg, Identifying adults at high risk for diabetes and cardiovascular disease using hemoglobin A1c National Health and Nutrition examination survey 2005-2006, Am. J. Prev. Med. 40 (1) (2011) 11-17.
13. N.J. Stone, J.G. Robinson, A.H. Lichtenstein, D.C. Goff Jr., D.M. Lloyd-Jones, S.C. Smith Jr., C. Blum, J.S. Schwartz, 2013 ACC/AHA Cholesterol guideline Panel. Treatment of blood cholesterol to reduce atherosclerotic cardiovascular disease risk in adults: synopsis of the 2013 American College of Cardiology/ American Heart Association cholesterol guideline, Ann. Intern. Med. 160 (5) (2014) 339-343.
14. G. De Luca, G.G. Secco, M. Santagostino, L. Venegoni, S. Iorio, E. Cassetti, M. Verdoia, L. Coppo, C. Di Mario, G. Bellomo, P. Marino, Uric acid does not affect the prevalence and extent of coronary artery disease. Results from a prospective study, Nutr. Metab. Cardiovasc Dis. 22 (2012) 426-433.
15. M. Verdoia, P. Pergolini, C. Camaro, M. Restifo, R. Rolla, A. Schaffer, G. Di Giovine, P. Marino, G. Bellomo, H. Suryapranata, G. De Luca, Novara Atherosclerosis Study Group (NAS). PlA(1)/PlA(2) polymorphism does not influence response to GpIIb-IIIa inhibitors in patients undergoing coronary angioplasty, Blood Coagul. Fibrinolysis 24 (2013) 411-418.

16. M. Vidali, R. Rolla, M. Parrella, C. Cassani, M. Manzini, M.R. Portalupi, R. Serino, M.D. Prando, G. Bellomo, P. Pergolini, Role of the laboratory in monitoring patients receiving dual antiplatelet therapy, Int. J. Lab. Hematol. 34 (2012) 484-494.
17. L. Bonello, U.S. Tantry, R. Marcucci, R. Blindt, D.J. Angiolillo, R. Becker, D.L. Bhatt, M. Cattaneo, J.P. Collet, T. Cuisset, C. Gachet, G. Montalescot, L.K. Jennings, D. Kereiakes, D. Sibbing, D. Trenk, J.W. Van Werkum, F. Paganelli, M.J. Price, R. Waksman, P.A. Gurbel, Working Group on High on-treatment platelet Reactivity. Consensus and future directions on the definition of high on-treatment platelet reactivity to adenosine diphosphate, J. Am. Coll. Cardiol. 56 (2010) 919-933.
18. G. De Luca, E.P. Navarese, H. Suryapranata, A meta-analytic overview of thrombectomy during primary angioplasty, Int. J. Cardiol. 166 (3) (2013) 606-612.
19. G. De Luca, F. Bellandi, K. Huber, M. Noc, A.S. Petronio, H.R. Arntz, M. Maioli, H.M. Gabriel, S. Zorman, M. DE Carlo, T. Rakowski, M. Gyongyosi, D. Dudek, Early glycoprotein IIb-IIIa inhibitors in primary angioplasty-abciximab longterm results (EGYPT-ALT) cooperation: individual patient's data meta-analysis, J. Thromb. Haemost. 9 (12) (2011) 2361-2370.
20. Authors/Task Force members S. Windecker, P. Kolh, F. Alfonso, J.P. Collet, J. Cremer, V. Falk, G. Filippatos, C. Hamm, S.J. Head, P. Jüni, A.P. Kappetein, A. Kastrati, J. Knuuti, U. Landmesser, G. Laufer, F.J. Neumann, D.J. Richter, P. Schauerte, M. Sousa Uva, G.G. Stefanini, D.P. Taggart, L. Torracca, M. Valgimigli, W. Wijns, A. Witkowski, 2014 ESC/EACTS guidelines on myocardial revascularization: the Task Force on myocardial revascularization of the European society of cardiology (ESC) and the European association for Cardio-Thoracic Surgery (EACTS)Developed with the special contribution of the European association of percutaneous cardiovascular interventions (EAPCI), Eur. Heart J. 35 (37) (2014) 2541-2619.
21. N.C. Chan, J.W. Eikelboom, J.S. Ginsberg, M.N. Lauw, T. Vanassche, J.I. Weitz, J. Hirsh, Role of phenotypic and genetic testing in managing clopidogrel therapy, Blood 124 (5) (2014) 689-699.
22. B. Guo, Q. Tan, D. Guo, Z. Shi, C. Zhang, W. Guo, Patients carrying CYP2C19 loss of function alleles have a reduced response to clopidogrel therapy and a greater risk

of in-stent restenosis after endovascular treatment of lower extremity peripheral arterial disease, J. Vasc. Surg. 60 (4) (2014) 993-1001.

23. M.F. Notarangelo, F. Bontardelli, P.A. Merlini, Genetic and nongenetic factors influencing the response to clopidogrel, J. Cardiovasc Med. Hagerst. 14 (Suppl. 1) (2013) S1-S7.
24. I. Urtane, A. Aitullina, K. Pukite, Clopidogrel and the possibility of drug-drug interaction in primary health care, J. Young Pharm. 5 (1) (2013) 18-21.
25. P.J. Neuvonen, Drug interactions with HMG-CoA reductase inhibitors (statins): the importance of CYP enzymes, transporters and pharmacogenetics, Curr. Opin. Investig. Drugs 11 (3) (2010) 323-332.
26. W.C. Lau, L.A. Waskell, P.B. Watkins, C.J. Neer, K. Horowitz, A.S. Hopp, et al., Atorvastatin reduces the ability of clopidogrel to inhibit platelet aggregation: a new drug-drug interaction, Circulation 107 (2003) 32-37.
27. M. Leoncini, A. Toso, M. Maioli, D.J. Angiolillo, B. Giusti, R. Marcucci, et al., High-dose atorvastatin on the pharmacodynamic effects of double-dose clopidogrel in patients undergoing percutaneous coronary interventions: the ACHIDO (Atorvastatin and Clopidogrel High DOse in stable patients with residual high platelet activity) study, JACC Cardiovasc Interv. 6 (2013) 169-179.
28. F. Pelliccia, G. Rosano, G. Marazzi, C. Vitale, I. Spoletini, F. Franzoni, et al., Pharmacodynamic comparison of pitavastatin versus atorvastatin on platelet reactivity in patients with coronary artery disease treated with dual antiplatelet therapy, PORTO trial Circ J 78 (2014) 679-684.
29. R.E. Malmstr€om, J. Ostergren, L. Jørgensen, P. Hjemdahl, CASTOR investigators. Influence of statin treatment on platelet inhibition by clopidogrel e a randomized comparison of rosuvastatin, atorvastatin and statin co-treatment, J. Intern Med. 266 (5) (2009) 457-466.
30. J.W. Suh, M.J. Cha, S.P. Lee, I.H. Chae, J.H. Bae, T.G. Kwon, J.W. Bae, M.C. Cho, S.W. Rha, H.S. Kim, Relationship between statin type and responsiveness to clopidogrel in patients treated with percutaneous coronary intervention: a subgroup analysis of the CILON-T trial, J. Atheroscler. Thromb. 21 (2) (2014) 140-150.
31. L.F. Pinheiro, C.N. França, M.C. Izar, S.P. Barbosa, H.T. Bianco, S.H. Kasmas, G.D. Mendes, R.M. Povoa, F.A. Fonseca, Pharmacokinetic interactions between

clopidogrel and rosuvastatin: effects on vascular protection in subjects with coronary heart disease, Int. J. Cardiol. 158 (1) (2012) 125-129.

32. S. Riondino, N. Petrini, L. Donato, C. Torromeo, G. Tanzilli, F.M. Pulcinelli, F. Barilla, Effects of rosuvastatin on platelet inhibition by clopidogrel in cardiovascular patients, J. Thromb. Thrombolysis 28 (2) (2009) 151-155.
33. F. Pelliccia, G. Rosano, G. Marazzi, C. Vitale, I. Spoletini, F. Franzoni, G. Speziale, M. Polacco, C. Greco, C. Gaudio, Pharmacodynamic effects of atorvastatin versus rosuvastatin in coronary artery disease patients with normal platelet reactivity while on dual antiplatelet therapyethe PEARL randomized crossover study, Eur. J. Pharmacol. 725 (2014) 18-22.
34. O. Ojeifo, S.D. Wiviott, E.M. Antman, S.A. Murphy, J.A. Udell, E.R. Bates, M.S. Mega JL,Sabatine, M.L. O'Donoghue, Concomitant administration of clopidogrel with statins or calcium-channel blockers: insights from the TRITONTIMI 38 (trial to assess improvement in therapeutic outcomes by optimizing platelet inhibition with prasugrel-thrombolysis in myocardial infarction 38), JACC Cardiovasc Interv. 6 (12) (2013) 1275-1281.
35. R. Teng, P.D. Mitchell, K.A. Butler, Pharmacokinetic interaction studies of coadministration of ticagrelor and atorvastatin or simvastatin in healthy volunteers, Eur. J. Clin. Pharmacol. 69 (2013) 477-487.
36. Y. Park, Y.H. Jeong, U.S. Tantry, J.H. Ahn, T.J. Kwon, J.R. Park, et al., Accelerated platelet inhibition by switching from atorvastatin to a non-CYP3A4- metabolized statin in patients with high platelet reactivity (ACCEL-STATIN) study, Eur. Heart J. 33 (2012) 2151-2162.
37. P.L. Thompson, A.G. Thompson, C. Judkins, Optimising pharmacotherapy for secondary prevention of non-invasively managed acute coronary syndrome, Med. J. Aust. 201 (10) (2014) S100-S105
38. L.Z. Guo, M.H. Kim, C.D. Jin, J.Y. Lee, S.J. Yi, M.K. Park, Y.R. Cho, T.H. Park, Comparison of pharmacodynamics between low dose ticagrelor and clopidogrel after loading and maintenance doses in healthy Korean subjects, Platelets 2 (2014) 1-7.
39. J.M. Siller-Matula, C. Gruber, M. Francesconi, C. Dechant, B. Jilma, G. Delle-Karth, K. Grohs, A. Podczeck-Schweighofer, G. Christ, The net clinical benefit of personalized antiplatelet therapy in patients undergoing percutaneous coronary

intervention, Clin. Sci. (Lond) 128 (2) (2015) 121e130; (b) D. Sibbing, S. Schulz, S. Braun, T. Morath, J. Stegherr, J. Mehilli, A. Schomig, N. von Beckerath, A. Kastrati, Antiplatelet effects of clopidogrel and bleeding in patients undergoing coronary stent placement, J. Thromb. Haemost. 8 (2010) 250-256.

40. D. Sibbing, S. Braun, T. Morath, J. Mehilli, W. Vogt, A. Schömig, A. Kastrati, N. von Beckerath, Platelet reactivity after clopidogrel treatment assessed with point-of-care analysis and early drug-eluting stent thrombosis, J. Am. Coll. Cardiol. 53 (10) (2009) 849-856.
41. P.A. Gurbel, K.P. Bliden, B.L. Hiatt, C.M. O'Connor, Clopidogrel for coronary stenting: response variability, drug resistance, and the effect of pretreatment platelet reactivity, Circulation 107 (2003) 2908-2913.
42. J.M. Siller-Matula, G. Delle-Karth, I.M. Lang, T. Neunteufl, M. Kozinski, J. Kubica, G. Maurer, K. Linkowska, T. Grzybowski, K. Huber, B. Jilma, Phenotyping vs. genotyping for prediction of clopidogrel efficacy and safety: the PEGASUS-PCI study, J. Thromb. Haemost. 10 (4) (2012) 529-542.

Summary and conclusions

Summary and conclusions

In this thesis we have addressed the main aspects related to platelet aggregation in patients with coronary artery disease, focusing on markers and predictors of platelet reactivity: morphologic parameters, metabolic and genetic factors have been investigated to better understand the complex role played by platelets in atherosclerotic disease.

Chapter 1 described the principal fields of investigation regarding platelet homeostasis, trying to identify the key elements that might be the future targets of therapeutics in patients with atherothrombotic disease. The overview includes a part on platelet morphology and reactivity assessments, which are the most extensively studied, together with the latest aspects under investigation: immunological patterns and genetics, especially micro-RNA.

In **Part 2** we evaluated the role of platelet morphologic parameters including the immature platelet fraction (IPF) and the mean platelet volume (MPV) on the prevalence and the severity of CAD.

Chapter 2, specifically, reports the relationship between immature platelet fraction levels and the prevalence and extent of CAD in 1789 consecutive patients undergoing coronary angiography. The immature platelet fraction (IPF) was not associated with the prevalence (adjusted OR [95% CI] = 0.93 [0.82 – 1.05], p = 0.22) and extent (adjusted OR [95% CI] = 0.99 [0.90 – 1.1], p = 0.88) of CAD, and, therefore, it should not be considered as a marker of coronary atherosclerosis.

The aim in **Chapter 3** was to define the impact of smoking on the IPF and its relationship with prevalence and extent of CAD. Overall, we included 2553 patients in our study, who were divided according to smoking status (active smokers: 512; non-active smokers: 2041). A higher percentage of patients with higher IPF values was observed in smokers, and active smoking was an independent predictor of higher IPF (adjusted OR [95% CI] = 1.59 [1.03 – 2.45] P=0.035). However, higher IPF (according to quartile values) was not associated with the prevalence and extent of CAD (adjusted OR [95% CI] =0.98[0.79 – 1.23] P=0.89) and severity of CAD (adjusted OR [95% CI] = 1.03 [0.86 – 1.23] P=0.76).

Chapter 4 dealt with the impact of metabolic syndrome on MPV and its relationship with CAD in 4730 consecutive patients undergoing PCI. Subjects affected by metabolic syndrome displayed a more pronounced cardiovascular risk profile and more prior cardiac events, and MPV was slightly higher among them compared to patients without (p=0.02), although metabolic syndrome did not emerge as an independent predictor of higher MPV values (adjusted OR [95% CI] = 1.01 [0.84 – 1.22], p=0.93). In the subgroup of metabolic syndrome patients, higher MPV values did not result as an independent predictor of CAD (adjusted OR [95% CI] = 0.79 [0.61 – 1.03], p=0.08) and severe CAD (adjusted OR [95% CI] = 0.82 [0.65 – 1.03], p=0.084).

In **Part 3** we discussed the challenging and intricate matter of platelet aggregation with specific regard to genetic, metabolic and drug determinants of enhanced reactivity among patients with CAD.

Chapter 5 investigated the potential impact of the single nucleotide polymorphisms rs5751876 (C > T) of the adenosine A2a receptor gene on platelet reactivity in ticagrelor

treated patients. Among the 244 patients included, 174 (71.3%) patients carried polymorphism (T allele), 51 (20.9%) of them in homozygosis (T/T). A lower rate of High on Treatment Residual Platelet Reactivity (HTPR) was observed in homozygotes T/T (p=0.03). Taking into account potential confounders, C allele carriage was independently associated with the rate of HTPR on ticagrelor (adjusted OR [95% CI]= 4.63 [1.02 – 21.01], p=0.048), suggesting a crucial role of adenosine signaling pathway in ticagrelor-mediated platelet inhibition and the potential significant impact of genetic variants.

Chapter 6 addressed the topic of serum uric acid variation in patients under dual antiplatelet therapy (DAPT) with ticagrelor or clopidogrel admitted for acute coronary syndrome or elective PCI. A total of 378 patients were included: 145 (38.4%) subjects with clopidogrel and 233 (61.6%) with ticagrelor, in addition to aspirin. As expected, clopidogrel patients had a higher percentage of HTPR than the ticagrelor ones (p=0.001), while no differences in creatinine levels were detected. At 1-3 months a significant increase in serum uric acid values was found in the ticagrelor treated patients as compared to the clopidogrel (p=0.034 for absolute changes; p=0.018 for percentage changes). Results were not influenced by variations in renal function. Multivariate analysis confirmed ticagrelor as predictor of increase in uric acid levels (adjusted OR [95% CI] = 2.79 [1.66 – 4.67], p<0.001). However, uric acid levels variation did not affect platelet reactivity or HTPR in both clopidogrel and ticagrelor patients.

In **Chapter 7** we investigated the potential impact of Body Mass Index (BMI) on platelet reactivity in 498 patients under DAPT with aspirin, plus either clopidogrel (61.8%) or ticagrelor (38.2%). BMI did not influence the effectiveness of aspirin, while it was associated to a non-significant trend in higher platelet reactivity (r=0.08, p=0.08) for ADP-

antagonists. Overall, BMI tertile values did not predict HTPR at ADP-test (adjusted OR [95%CI]= 1.19[0.86 – 1.64], p=0.30). However, in the clopidogrel-treated subgroup, significantly higher ADP-mediated aggregation values were found in patients with higher BMI (r=0.14, p=0.023), which resulted as an independent predictor of HRPR (adjusted OR[95%CI]= 1.45 [1.01 – 2.12], p=0.049). On the contrary, no impact of BMI was observed in the ticagrelor-treated subgroup for platelet reactivity (r=-0.036, p=0.62) or the prevalence of HRPR (adjusted OR[95%CI]= 0.73[0.39 – 1.36], p=0.32).

In **Chapter 8** we explored the role of Renin Angiotensin System Inhibitors (RASi) on homocysteine levels and their potential influence on platelet reactivity in DAPT patients. The final cohort was composed of 1210 patients, 862 of whom (71.2%) were on treatment with RASi. Overall, DAPT composition was aspirin + clopidogrel in 566 (46.8%) patients, aspirin + ticagrelor in 428 (35.4%) and aspirin + prasugrel in 216 (17.9%). Median values of homocysteine were higher in patients receiving RASi (p=0.006), which resulted as an independent predictor of higher homocysteine (adjusted OR [95% CI] = 1.40[1.04 – 1.88], p=0.027). No differences were detected in the HTPR rate at the ADP aggregation test when RASi was used (adjusted OR [95% CI] = 1.23 [0.89 – 1.70], p=0.220). Levels of homocysteine were directly related to higher values of platelet reactivity, independently of the DAPT strategy.

Chapter 9 assessed the potential effect of high intensity statin, atorvastatin or rosuvastatin, and co-administration on platelet aggregation in patients receiving DAPT with aspirin and either clopidogrel or ticagrelor. The majority of the 374 patients included was taking atorvastatin (64.2%) as opposed to rosuvastatin (35.8%). According to statin type, no difference was found in relation to platelet inhibition mediated by aspirin (adjusted OR

[95% CI] = 2 [0.23 – 16.6], p = 0.52). In the clopidogrel subgroup (163 patients), rosuvastatin co-administration, in comparison with atorvastatin, was associated with a significantly higher rate of HTPR (55.6% vs 32%, respectively; adjusted OR [95% CI] = 2.69 [1.22 – 5.96], p = 0.015). On the contrary, in the 211 ticagrelor treated patients, statin type did not affect the prevalence of HTPR (10.5% vs 11.2%, for rosuvastatin and atorvastatin respectively; adjusted OR [95% CI] = 0.86 [0.34 – 2.22], p = 0.76)

Future directions in the management of platelet reactivity and antiplatelet therapy for patients with coronary artery disease

Substantial advances have been made in understanding the intricate mechanisms of platelet activation and aggregation, although several questions remain unanswered. While a number of signaling amplifying pathways have been identified and characterized, contrasting results are reported in relation to potential morphologic and functional markers of platelet aggregation, that would allow optimization of antiplatelet therapies in patients with atherothrombotic disease. The recent description of the interaction between the immune system and cardiovascular disease has also shown an active role also for platelets. The interplay between platelets and immunity has been described as biunivocal, with both platelets, stimulating granulocytes and lymphocyte recruitment, and immune cells, in particular neutrophils, promoting platelet activation and participating in thrombus formation. Knowledge in this field is rapidly growing, but several issues still need to be clarified, especially in the selection of new targets aimed at inhibiting platelets, and therefore at reducing an adverse outcome in CAD patients.

Of course, the innumerable assays available in daily clinical practice represent a resource in the assessment of platelet function in countless patients. The multitude of methods, however, prevents full agreement on platelet aggregation cut off to be reached in the clinical decision-making process and limits comparison among studies adopting different approaches. The complexity of ~~in~~ reproducing the phenomenon of aggregation in vitro remains a critical challenge. Definite findings on the potential use of platelet function tests to optimize the antiplatelet effect in patients receiving DAPT are not available. Promising results have suggested tailoring antiplatelet therapy with a reduction approach, both

through platelet function tests and genetic guidance, although, so far, general consensus has not been obtained.

Awareness has been raised by evidence of the potential impact of drug co-administration on platelet reactivity, including renin angiotensin system inhibitors and statin, two keystones in the treatment of patients with CAD. The large amount of stimuli and network of signals involved in platelet aggregation result in exposure to potential interference by other drugs, especially in chronic treatment, affecting platelet homeostasis directly or indirectly. In addition to that, drug-drug interactions may also explain impaired platelet inhibition: direct interaction, competitive receptor binding, common cytochrome pathways would affect the eventual platelet inhibition, leading to an increased thrombotic risk. Improvement in reducing cardiovascular mortality in the last decades has globally led to an extended lifespan in patients undergoing PCI, namely polypharmacotherapy.

The conclusive message, from the numerous and occasionally contrasting evidence from platelet research, focusing on the clinical aspects related to patients with CAD receiving antiplatelet treatments is that precise findings may be infeasible. The paradigm to achieve a solution that is valid for the greatest amount of patients should be switched to a treatment whose drug composition is adjusted to the subject, considering not only results from platelet function tests but also metabolic and genetic factors, and polypharmacotherapy. One size does not fit all.

Samenvatting en conclusies

Samenvatting en conclusies

In dit proefschrift hebben we de belangrijkste aspecten met betrekking tot trombocytenaggregatie bij patiënten met coronaire hartziekte besproken, waarbij we ons richten op markers en voorspellers van trombocytenreactiviteit: morfologische parameters, metabolische en genetische factoren zijn onderzocht om de complexe rol van trombocyten bij atherosclerotische ziekte beter te begrijpen.

Hoofdstuk 1 beschreef de belangrijkste onderzoeksgebieden met betrekking tot de trombocytenhomeostase en probeerde de belangrijkste elementen te identificeren die in de toekomst therapeutische doelen zouden kunnen zijn bij patiënten met een atherotrombotische ziekte. Het overzicht omvat een deel over de morfologie en reactiviteitevalusaties van de trombocyten, de meest uitgebreid bestudeerde tot nu toe, samen met de laatste aspecten in onderzoek: immunologische patronen en genetica, met name micro-RNA.

In **Deel 2** evalueerden we de rol van de morfologische parameters voor trombocyten, waaronder de onvolwassen trombocytenfractie (IPF) en het gemiddelde trobocytenvolume (MPV) op CAD.

Hoofdstuk 2, specifiek, rapporteert de relatie tussen onvolgroeide trombocytenfractiespiegels en de prevalentie en omvang van CAD bij 1789 opeenvolgende patiënten die coronaire angiografie ondergaan. De onvolgroeide trombocytenfractie (IPF) werd niet geassocieerd met de prevalentie (adjusted OR[95% CI] = 0.93[0.82-1.05], p=0.22) en de mate (adjusted OR[95%CI]=0.99 [0.90-1.1], p=0.88) van

CAD, en daarom mag deze niet over het hoofd worden gezien als een marker van coronaire atherosclerose.

Het doel van **Hoofdstuk 3** was om de impact van roken op de IPF en de relatie met de prevalentie en omvang van CAD te definiëren. In totaal namen we in ons onderzoek 2553 patiënten op, die waren verdeeld naar rookstatus (actieve rokers: 512; niet-actieve rokers: 2041). Een hoger percentage rokers werd waargenomen bij patiënten met hogere IPF-waarden, en actief roken resulteerde in een onafhankelijke voorspeller van hogere IPF (adjusted OR[95%CI]= 1.59[1.03-2.45], p=0.035). De IPF (volgens kwartielwaarden) werd echter niet geassocieerd met de prevalentie en omvang van CAD (adjusted OR[95% CI] =0.98[0.79 – 1.23], p=0.89) en ernstige CAD (adjusted OR[95% CI] = 1.03[0.86 – 1.23], p=0.76).

Hoofdstuk 4 behandelde de impact van het metabool syndroom op MPV en de relatie met CAD bij 4730 opeenvolgende patiënten die PCI ondergingen. Proefpersonen met metabool syndroom vertoonden een meer uitgesproken cardiovasculair risicoprofiel en eerdere cardiale voorvallen, en MPV werd enigszins onder hen gevonden in vergelijking met patiënten zonder (p=0.02), hoewel het metabool syndroom niet naar voren kwam als een onafhankelijke voorspeller van hogere MPV-waarden (adjusted OR[95% CI] = 1.01 [0.84-1.22], p=0.93). In de subgroep van patiënten met metabool syndroom resulteerden hogere MPV-waarden niet als een onafhankelijke voorspeller van CAD (adjusted OR[95%CI] = 0.79[0.6 - 1.03], p=0.08) en ernstige CAD (adjusted OR[95%CI] = 0.82 [0.65-1.03], p=0.084).

In **Deel 3** bespraken we de uitdagende en ingewikkelde kwestie van trombocytenaggregatie met specifieke aandacht voor genetische, metabole en geneesmiddeldeterminanten van verhoogde reactiviteit bij patiënten met CAD.

Hoofdstuk 5 onderzocht de mogelijke impact van de enkelvoudige nucleotide polymorfismen rs5751876 (C > T) van het adenosine A2a receptorgen op de trombocytenreactiviteit bij met ticagrelor behandelde patiënten. 174 (71.3%) van de 244 patiënten droegen het polymorfisme (T-allel), 51 (20.9%) van hen bij homozygose (T/T). Bij homozygoten T/T werd een lagere mate van hoge residuele trombocytenreactiviteit (HTPR) tijdens de behandeling waargenomen (p=0.03). Rekening houdend met potentiële confounders, werd het C-allelvervoer onafhankelijk geassocieerd met de snelheid van HTPR op ticagrelor (adjusted OR[95% CI]= 4.63 [1.02 -21.01], p=0.048), wat wijst op een cruciale rol van de adenosinesignaleringsmethode bij door ticagrelor gemedieerde trombocytenremming en de potentiële significante impact van genetische varianten.

Hoofdstuk 6 behandelde het onderwerp van variatie in serumurinezuur bij patiënten onder duale trombocytenaggregatietherapie (DAPT) met ticagrelor of clopidogrel die werden opgenomen voor een acuut coronair syndroom of een electieve PCI. In totaal werden 378 patiënten opgenomen, 145 (38.4%) patiënten met clopidogrel en 233 (61.6%) patiënten met ticagrelor, naast aspirine. Zoals verwacht hadden patiënten met clopidogrel een hoger percentage HTPR dan patiënten met ticagrelor (p=0.001), terwijl er geen verschillen in creatininespiegels werden gedetecteerd. Na 1-3 maanden werd een significante stijging in serumurinezuurwaarden gevonden bij met ticagrelor behandelde patiënten in vergelijking met met met clopidogrel behandelde patiënten (p=0.034 voor absolute veranderingen; p=0.018 voor procentuele veranderingen). De resultaten werden niet beïnvloed door

variaties in de nierfunctie. Multivariate analyse bevestigde ticagrelor als voorspeller van verhoging van urinezuurspiegels (adjusted OR[95% CI] = 2.79 [1.66-4.67], p<0.001). De variatie in urinezuurspiegels had echter geen invloed op de trombocytenreactiviteit of HTPR bij zowel clopidogrel- als ticagrelorpatiënten.

In **Hoofdstuk 7** onderzochten we de mogelijke impact van de body mass index (BMI) op de trobocytenreactiviteit bij 498 patiënten onder DAPT met aspirine plus clopidogrel (61.8%) of ticagrelor (38.2%). BMI had geen invloed op de werkzaamheid van aspirine, terwijl het geassocieerd werd met een niet-significante trend voor een hogere trombocytenreactiviteit (r=0.08, p=0.08) voor ADP-antagonisten. Over het algemeen voorspelden de BMI-tertielenwaarden geen HTPR van een ADP-test (adjusted OR[95%CI]= 1.19[0.86-1.64], p=0.30). Echter, in de met clopidogrel behandelde subgroep werden significant hogere ADP-gemedieerde aggregatiewaarden gevonden bij patiënten met een hogere BMI (r=0.14, p=0.023), resulterend in een onafhankelijke voorspeller van HRPR (adjusted OR[95%CI]= 1.45 [1.01 -2.12], p=0.049). Integendeel, er werd geen effect van BMI waargenomen in de met ticagrelor behandelde subgroep voor trombocytenreactiviteit (r= -0.036, p=0.62) of de prevalentie van HRPR (adjusted OR[95%CI]= 0.73[0.39 -1.36], p=0.32).

In **Hoofdstuk 8** onderzochten we de rol van renine angiotensine systeem remmers (RASi) op homocysteïne niveaus en de mogelijke invloed op de trombocytenreactiviteit bij DAPT-patiënten. Het uiteindelijke cohort bestond uit 1210 patiënten, van wie er 862 (71.2%) behandeld werden met RASi. In totaal was de DAPT-samenstelling aspirine + clopidogrel bij 566 (46.8%) patiënten, aspirine + ticagrelor bij 428 (35.4%) en aspirine + prasugrel bij 216 (17.9%). De mediane waarden van homocysteïne waren hoger bij

patiënten die RASi kregen (p=0.006), wat resulteerde in een onafhankelijke voorspeller van hoger homocysteïne (adjusted OR[95% CI] = 1.40[1.04 – 1.88], p=0.027). Er werden geen verschillen in HTPR-snelheid bij de ADP-aggregatietest gevonden op basis van het gebruik van RASi (adjusted OR[95%CI] = 1.23 [0.89-1.70], p=0.220). Homocysteïnespiegels waren direct gerelateerd aan hogere waarden van de trombocytenreactiviteit, onafhankelijk van de DAPT-strategie.

In **Hoofdstuk 9** werd het potentiële effect van statine, atorvastatine of rosuvastatine met een hoge intensiteit op de trombocytenaggregatie beoordeeld bij patiënten die DAPT met aspirine en clopidogrel of ticagrelor kregen. De meerderheid van de 374 geïncludeerde patiënten ging uit van atorvastatine (64.2%) in vergelijking met rosuvastatine (35.8%). Er werd geen verschil gevonden naargelang van het statinetype in relatie tot de trobocytenremming gemedieerd door aspirine (adjusted OR[95%CI] = 2 [0.23 -16.6], p = 0.52). In de clopidogrel subgroep (163 patiënten) werd gelijktijdige toediening van rosuvastatine in vergelijking met atorvastatine geassocieerd met een significant verhoogd percentage HTPR (respectievelijk 55.6% versus 32%; adjusted OR[95% CI] = 2.69 [1.22-5.96], p = 0.015). Integendeel, bij de 211 met ticagrelor behandelde patiënten had het statinetype geen invloed op de prevalentie van HTPR (10.5% versus 11.2%, voor respectievelijk rosuvastatine en atorvastatine; adjusted OR[95%CI] = 0.86[0.34 – 2.22], p = 0.76).

Toekomstige aanwijzingen voor de behandeling van trombocytenreactiviteit en trombocytenaggregatietherapie bij patiënten met coronaire hartziekte

Er is consistente vooruitgang geboekt bij het begrijpen van de ingewikkelde mechanismen van de activering en aggregatie van trombocyten, zelfs als verschillende vragen nog steeds niet zijn beantwoord. Hoewel een aantal signaalversterkingsroutes zijn geïdentificeerd en gekarakteriseerd, worden contrasterende resultaten gerapporteerd in relatie tot potentiële morfologische en functionele markers van trombocytenaggregatie, die het mogelijk zouden maken om trombocytenaggregatietherapieën bij patiënten met atherotrombotische ziekte te optimaliseren. De recente beschrijving van de interactie tussen het immuunsysteem en hart- en vaatziekten heeft ook voor trombocyten een actieve rol laten zien. Het samenspel tussen trombocyten en immuniteit is beschreven als biunivocal, met zowel trombocyten, stimulerende granulocyten en lymfocytenrekrutering, en immuuncellen, in het bijzonder neutrofielen, het bevorderen van de activering van bloedplaatjes en deelname aan trombusvorming. De kennis op dit gebied groeit snel, maar verschillende kwesties moeten nog worden opgehelderd, speciaal om nieuwe doelen te selecteren om trombocyten te remmen en daardoor de negatieve resultaten bij CAD-patiënten te verminderen.

Het grote aantal tests dat in de dagelijkse klinische praktijk beschikbaar is, is natuurlijk een hulpmiddel om de trombocytenfunctie bij zoveel mogelijk patiënten te beoordelen. Anderzijds bemoeilijkt de veelheid aan methoden een brede overeenstemming te bereiken over trombocytenaggregatie die wordt afgesneden voor klinische besluitvormingsprocessen en de vergelijking tussen studies die verschillende benaderingen hanteren, te beperken. De complexiteit van de reproductie in vitro van het

verschijnsel aggregatie blijft een kritieke uitdaging. Er zijn geen definitieve bevindingen beschikbaar over het mogelijke gebruik van trombocytenfunctietests om het trombocytenaggregatie-remmende effect te optimaliseren bij patiënten die DAPT krijgen. Er zijn veelbelovende resultaten gesuggereerd voor het op maat maken van trombocytenaggregatietherapie in een step-down view, zowel trombocytenfunctietest- als genetisch geleid, zelfs als er tot nu toe geen algemene consensus beschikbaar is.

De waarschuwingen zijn verhoogd door het bewijs van mogelijke effecten van gelijktijdige toediening van geneesmiddelen op de trombocytenreactiviteit, waaronder renine angiotensine systeemremmers en statine, twee sleutelelementen in de behandeling van patiënten met CAD. Het hoge aantal stimuli en het web van signalen die in de trombocytenaggregatie worden geïmpliceerd is gevoelig voor aan potentiële interferentie door andere drugs, vooral in chronische behandeling, en beïnvloedt direct of indirect de homeostase van trombocyten. Daarnaast kunnen ook geneesmiddelinteracties een verstoorde remming van de trombocyten verklaren: directe interactie, competitieve receptorbinding, gemeenschappelijke cytochroomroutes zouden de uiteindelijke remming van de trombocyten beïnvloeden, wat zou leiden tot een verhoogd trombotisch risico. De verbetering in het verminderen van cardiovasculaire mortaliteit in de laatste decennia heeft wereldwijd geleid tot een hogere leeftijd van patiënten die PCI ondergaan en, dus, gekenmerkt door een polyfarmacotherapie.

De sluitende boodschap van het talrijke en soms contrasterende bewijs in trombocytenonderzoek, gericht op klinische aspecten met betrekking tot patiënten met CAD en die trombocytenaggregatieremmers krijgen, is dat definitieve zinnen onwerkbaar kunnen zijn. Het paradigma om de oplossing te bereiken die werkt voor zo mogelijk

patiënten, moet worden verschoven naar een behandeling die gepersonaliseerd is in de samenstelling van het geneesmiddel, rekening houdend met niet alleen de resultaten van trombocytenfunctietests, maar ook metabole en genetische factoren en de polyfarmacotherapie. Een maat past niet iedereen.

Valorisation

Valorization

In this thesis we aimed to explore some crucial aspects related to platelets, that act as main actor in the atherothrombotic process, with specific regard to coronary artery disease. Our investigations have been focused to better characterize the platelet morphology and aggregation in patients referring for coronary angiography, in the search of new, user-friendly and cheap prognostic parameters for daily practice.

Given the central role of platelet in almost all the steps of atherosclerosis, from endothelial dysfunction to plaque promotion and rupture, any intervention on them has potential benefit on detrimental consequences of the disease. Considering the leading position in mortality and morbidity burden of ischemic heart disease, improvements in that field play a crucial role not only from the scientific side of knowledge advances but also could impact on life expectancy.

Laboratory values on platelets are easy to be obtained, during a routine blood test and provide a lot of information contributing to define patients' risk profile. The additional data should be interpreted according to individual features because platelet indexes should be considered as an addendum to traditional risk factors but not as a substitute. Moreover, specific measurement of platelet aggregation must be taken together with the platelet regimen that is ongoing, in order to maximize the info on platelet reactivity in the clinical management of antiplatelet therapy.

More in details, morphologic information on platelets have found related to coronary artery disease, but they do not represent independent predictor of atherosclerotic process

including its severity. Platelet volume and immature fraction remain attractive items of research even if their contribution should be still defined.

We also inquired the reactivity of platelets during dual antiplatelet therapy, the cornerstone of treatment in patients undergoing percutaneous coronary intervention. The selection of optimal antiplatelet regimen is crucial to avoid adverse events and re-intervention and to prevent harmful bleeding. Inappropriate platelet reactivity under antiplatelet treatment is related to detrimental consequences and the evaluation of adequate platelet inhibition is crucial, but contrasting results were reported into the application of platelet test- guided strategies. Therefore, the identification of the determinants of enhanced platelet aggregation may upgrade the expertise to choose the best antiplatelet drug regimen for each specific patient.

The emerging role of genetics results always more important as magnitude in contribution to regulation of atherosclerosis progression and it must be part of all further investigations: both pharmacological and non-pharmacological interventions might be favored or hampered by different patients' genetic profile.

The medicine tailored as much as possible will provide not only the best individualized treatment for every patient, but also will avoid to waste resources from several point of view, including the economic one. An increased ability to finely stratify the risk, that every patient presents, allows to schedule the appropriate treatment during the hospitalization and after the discharge. Moreover, the ability to early recognize factors that precipitate atherosclerosis may provide the opportunity to plan intervention to contrast them.

Of course, the complexity of atherosclerotic disease is a huge obstacle to achieve a comprehensive understanding of the underlying mechanisms, but all studies, even if little, might be a piece of puzzle to solve the picture.

The Constitution of the World Health Organization includes several crucial principles to guide our actions, and the first sentence is represented by the definition of health: "it is a state of complete physical, mental and social well-being and not merely the absence of disease or infirmity" (**Figure 1**). The vast majority of research projects conduced so far in all fields, including the cardiovascular branch, have been focused to reduce and limit the incidence and impact of the diseases. They have been efficacious. Now we must consider in our future investigations the well-being concept, because we do not have to limit our action to reduce the disease burden, but extend it to the health of our patients, who, out of the hospital, are persons we meet every day.

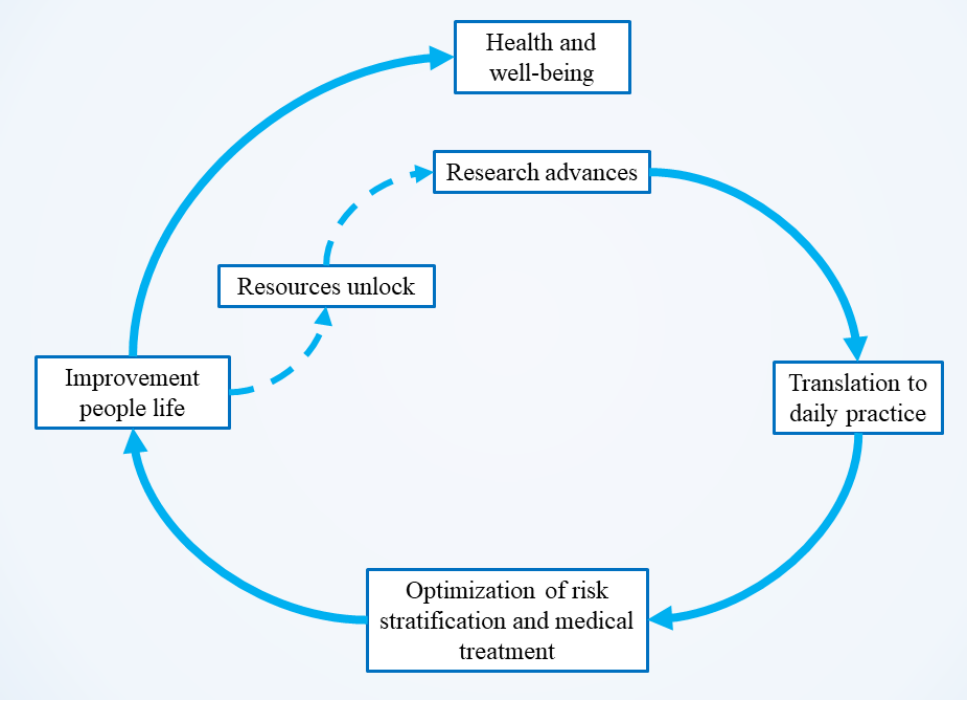


Figure 1. Vicious circle of medical research.

The findings of this thesis have been and will be shared with others in several ways. All except one chapters are published in international indexed scientific journal and are available online, the last has been submitted for publication. This thesis will be published online, to make it readable for everyone interested. Principal findings have been submitted as abstract to congress and general results of our research will be part of communication to scientific meeting.

Curriculum Vitae

Matteo Nardin was born on February 19, 1989, in Gattinara (Italy). He attended the secondary school in Vercelli, at Liceo Classico “Lagrangia”. He was admitted at the School of Medicine of the Eastern Piedmont University “A. Avogadro” in Novara and graduated in 2014 with the notation of 110/110 cum laude under the supervision of Professor Giuseppe De Luca. He obtained the specialization in Internal Medicine at University of Brescia in 2020 with the notation of 50/50 cum laude. During the last months of residency he started a research fellowship at the Icahn School of Medicine at Mount Sinai under the supervision of Professor Roxana Mehran. In 2022 he started the residency in Cardiology at the Humanitas University. Across the years after the medicine degree he was involved in different research group, contributing to several publications in international peer reviewed journals. Cardiovascular and internist arguments were the main topics of the investigations, allowing to grow an international network of research and friends. He has been awarded by the Italian Society of Internal Medicine with the prize for the Best Specialty Thesis in Internal Medicine – 2021, and is Fellow of the European Federation of Internal Medicine (FEFIM) since 2021.

Publications list on international indexed journals

1: Verdoia M, Schaffer A, Barbieri L, Cassetti E, **Nardin M**, Bellomo G, Marino P, Sinigaglia F, De Luca G; Novara Atherosclerosis Study (NAS) group. Diabetes, glucose control and mean platelet volume: a single-centre cohort study. Diabetes Res Clin Pract. 2014 May;104(2):288-94. doi: 10.1016/j.diabres.2013.12.020. Epub2014 Jan 17. PMID: 24530116.

2: Verdoia M, Cassetti E, Schaffer A, Barbieri L, Giovine GD, **Nardin M**, Marino P, Sinigaglia F, Luca GD; Novara Atherosclerosis Study Group (NAS). Relationship between glycoprotein IIIa platelet receptor gene polymorphism and coronary artery disease. Angiology. 2015 Jan;66(1):79-85. doi: 10.1177/0003319714524296. Epub 2014 Feb 28. PMID: 24586027.

3: Verdoia M, Barbieri L, Schaffer A, Cassetti E, **Nardin M**, Bellomo G, Aimaretti G, Marino P, Sinigaglia F, De Luca G; Novara Atherosclerosis Study Group (NAS). Impact of diabetes on uric acid and its relationship with the extent of coronary artery disease and platelet aggregation: a single-centre cohort study. Metabolism. 2014 May;63(5):640-6. doi: 10.1016/j.metabol.2014.01.010. Epub 2014 Jan 29. PMID: 24606806.

4: Barbieri L, Verdoia M, Schaffer A, **Nardin M**, Marino P, De Luca G. The role of statins in the prevention of contrast induced nephropathy: a meta-analysis of 8 randomized trials. J Thromb Thrombolysis. 2014 Nov;38(4):493-502. doi: 10.1007/s11239-014-1076-3. PMID: 24705677.

5: Verdoia M, Barbieri L, Schaffer A, Cassetti E, Di Giovine G, **Nardin M**, Bellomo G, Marino P, De Luca G; Novara Atherosclerosis Study Group (NAS). Effect of diabetes mellitus on periprocedural myocardial infarction in patients undergoing coronary stent implantation. Diabetes Metab Res Rev. 2015 Jan;31(1):85-92. doi: 10.1002/dmrr.2567. PMID: 24898423.

6: Verdoia M, Sartori C, Pergolini P, **Nardin M**, Rolla R, Barbieri L, Schaffer A, Marino P, Bellomo G, Suryapranata H, De Luca G; Novara Atherosclerosis Study Group (NAS). Prevalence and predictors of high-on treatment platelet reactivity with ticagrelor in ACS patients undergoing stent implantation. Vascul Pharmacol. 2016 Feb;77:48-53. doi: 10.1016/j.vph.2015.04.014. Epub 2015 May 6. PMID: 25956733.

7: **Nardin M**, Verdoia M, Sartori C, Pergolini P, Rolla R, Barbieri L, Schaffer A, Marino P, Bellomo G, Suryapranata H, De Luca G; Novara Atherosclerosis Study Group. Body Mass Index and Platelet Reactivity During Dual Antiplatelet Therapy With Clopidogrel or Ticagrelor. J Cardiovasc Pharmacol. 2015 Oct;66(4):364-70. doi: 10.1097/FJC.0000000000000288. PMID: 26065844.

8: Verdoia M, Pergolini P, Rolla R, **Nardin M**, Barbieri L, Schaffer A, Bellomo G, Marino P, Suryapranata H, De Luca G. Mean platelet volume and high-residual platelet reactivity in patients receiving dual

antiplatelet therapy with clopidogrel or ticagrelor. Expert Opin Pharmacother. 2015;16(12):1739-47. doi: 10.1517/14656566.2015.1056151. Epub 2015 Jun 12. PMID: 26067422.

9: Verdoia M, Sartori C, Pergolini P, **Nardin M**, Rolla R, Barbieri L, Schaffer A, Marino P, Bellomo G, Suryapranata H, De Luca G. Immature platelet fraction and high-on treatment platelet reactivity with ticagrelor in patients with acute coronary syndromes. J Thromb Thrombolysis. 2016 May;41(4):663-70. doi: 10.1007/s11239-015-1279-2. PMID: 26370198.

10: Verdoia M, Pergolini P, Rolla R, **Nardin M**, Barbieri L, Schaffer A, Bellomo G, Marino P, Suryapranata H, De Luca G; Novara Atherosclerosis Study Group (NAS). Platelet Larger Cell Ratio and High-on Treatment Platelet Reactivity During Dual Antiplatelet Therapy. Cardiovasc Drugs Ther. 2015;29(5):443-50. doi: 10.1007/s10557-015-6616-3. PMID: 26428927.

11: Verdoia M, Barbieri L, Schaffer A, **Nardin M**, Suryapranata H, De Luca G. Acetylsalicylic acid desensitization in patients with coronary artery disease: A comprehensive overview of currently available protocols. Vascul Pharmacol. 2016 May;80:43-9. doi: 10.1016/j.vph.2015.09.005. Epub 2015 Oct 9. PMID: 26456910.

12: Verdoia M, Pergolini P, Rolla R, **Nardin M**, Schaffer A, Barbieri L, Marino P, Bellomo G, Suryapranata H, De Luca G. Advanced age and high-residual platelet reactivity in patients receiving dual antiplatelet therapy with clopidogrel or ticagrelor. J Thromb Haemost. 2016 Jan;14(1):57-64. doi: 10.1111/jth.13177. Epub 2015 Dec 29. PMID: 26512550.

13: Barbieri L, Pergolini P, Verdoia M, Rolla R, **Nardin M**, Marino P, Bellomo G, Suryapranata H, De Luca G; Novara Atherosclerosis Study Group (NAS). Platelet reactivity in patients with impaired renal function receiving dual antiplatelet therapy with clopidogrel or ticagrelor. Vascul Pharmacol. 2016 Apr;79:11-15. doi: 10.1016/j.vph.2015.10.006. Epub 2015 Oct 28. PMID: 26518440.

14: Verdoia M, **Nardin M**, Sartori C, Pergolini P, Rolla R, Barbieri L, Marino P, Bellomo G, Suryapranata H, De Luca G; Novara Atherosclerosis Study Group (NAS). Impact of atorvastatin or rosuvastatin co-administration on platelet reactivity in patients treated with dual antiplatelet therapy. Atherosclerosis. 2015 Dec;243(2):389-94. doi: 10.1016/j.atherosclerosis.2015.10.005. Epub 2015 Oct 9. PMID: 26520891.

15: Barbieri L, Verdoia M, Pergolini P, **Nardin M**, Rolla R, Marino P, Bellomo G, Suryapranata H, De Luca G; Novara Atherosclerosis Study Group (NAS). Uric acid and high-residual platelet reactivity in patients treated with clopidogrel or ticagrelor. Nutr Metab Cardiovasc Dis. 2016 Apr;26(4):352-8. doi: 10.1016/j.numecd.2015.12.015. Epub 2016 Jan 6. PMID: 26857781.

16: Verdoia M, Pergolini P, Rolla R, **Nardin M**, Barbieri L, Daffara V, Marino P, Bellomo G, Suryapranata H, Luca GD; Novara Atherosclerosis Study Group (NAS). Gender Differences in Platelet Reactivity in Patients Receiving Dual Antiplatelet Therapy. Cardiovasc Drugs Ther. 2016 Apr;30(2):143-50. doi: 10.1007/s10557-016-6646-5. PMID: 26868495.

17: Verdoia M, Pergolini P, **Nardin M**, Rolla R, Barbieri L, Schaffer A, Marino P, Bellomo G, Suryapranata H, De Luca G. Impact of diabetes on immature platelets fraction and its relationship with platelet reactivity in patients receiving dual antiplatelet therapy. J Thromb Thrombolysis. 2016 Aug;42(2):245-53. doi: 10.1007/s11239-016-1348-1. PMID: 26951167.

18: Verdoia M, Pergolini P, Rolla R, **Nardin M**, Barbieri L, Schaffer A, Bellomo G, Marino P, Suryapranata H, De Luca G; Novara Atherosclerosis Study Group (NAS). Parathyroid Hormone Levels and High-Residual Platelet Reactivity in Patients Receiving Dual Antiplatelet Therapy With Acetylsalicylic Acid and Clopidogrel or Ticagrelor. Cardiovasc Ther. 2016 Aug;34(4):209-15. doi: 10.1111/1755-5922.12188. PMID: 27086085.

19: **Nardin M**, Verdoia M, Pergolini P, Rolla R, Barbieri L, Schaffer A, Marino P, Bellomo G, Suryapranata H, De Luca G; Novara Atherosclerosis Study Group (NAS). Serum uric acid levels during dual antiplatelet therapy with ticagrelor or clopidogrel: Results from a single-centre study. Nutr Metab Cardiovasc Dis. 2016 Jul;26(7):567-574. doi: 10.1016/j.numecd.2016.03.001. Epub 2016 Mar 15. PMID: 27134063.

20: **Nardin M**, Verdoia M, Sartori C, Pergolini P, Rolla R, Barbieri L, Schaffer A, Bellomo G, Suryapranata H, De Luca G; Novara Atherosclerosis Study Group (NAS). Diabetes mellitus, glucose control parameters and platelet reactivity in ticagrelor treated patients. Thromb Res. 2016 Jul;143:45-9. doi: 10.1016/j.thromres.2016.04.021. Epub 2016 Apr 27. PMID: 27179132.

21: **Nardin M**, Verdoia M, Schaffer A, Barbieri L, Marino P, De Luca G; Novara Atherosclerosis Study Group (NAS). Vitamin D status, diabetes mellitus and coronary artery disease in patients undergoing coronary angiography. Atherosclerosis. 2016 Jul;250:114-21. doi: 10.1016/j.atherosclerosis.2016.05.019. Epub 2016 May 10. PMID: 27205868.

22: Verdoia M, Pergolini P, Rolla R, Sartori C, **Nardin M**, Schaffer A, Barbieri L, Daffara V, Marino P, Bellomo G, Suryapranata H, De Luca G; Novara Atherosclerosis Study Group (NAS). Vitamin D levels and high-residual platelet reactivity in patients receiving dual antiplatelet therapy with clopidogrel or ticagrelor. Platelets. 2016 Sep;27(6):576-82. doi: 10.3109/09537104.2016.1149159. Epub 2016 Apr 11. PMID: 27540959.

23: Barbieri L, Verdoia M, **Nardin M**, Marino P, Suryapranata H, De Luca G; Novara Atherosclerosis Study Group (NAS). Gender Difference in the Risk of Contrast- Induced Nephropathy in Patients Undergoing

Coronary Angiography or Percutaneous Coronary Intervention. Angiology. 2017 Jul;68(6):542-546. doi: 10.1177/0003319716669429. Epub 2016 Sep 22. PMID: 27662891.

24: Verdoia M, Pergolini P, Rolla R, **Nardin M**, Schaffer A, Barbieri L, Daffara V, Marino P, Bellomo G, Suryapranata H, De Luca G; Novara Atherosclerosis Study Group (NAS). Impact of high-dose statins on vitamin D levels and platelet function in patients with coronary artery disease. Thromb Res. 2017 Feb;150:90-95. doi: 10.1016/j.thromres.2016.12.019. Epub 2016 Dec 28. PMID: 28068529.

25: Verdoia M, **Nardin M**, Rolla R, Marino P, Bellomo G, Suryapranata H, De Luca G; Novara Atherosclerosis Study Group (NAS). Immature platelet fraction and the extent of coronary artery disease: A single centre study. Atherosclerosis. 2017 May;260:110-115. doi: 10.1016/j.atherosclerosis.2017.03.044. Epub 2017 Mar 31. PMID: 28388444.

26: **Nardin M**, Verdoia M, Barbieri L, Schaffer A, Suryapranata H, De Luca G. Radial vs Femoral Approach in Acute Coronary Syndromes: A Meta- Analysis of Randomized Trials. Curr Vasc Pharmacol. 2017;16(1):79-92. doi: 10.2174/1570161115666170504125831. PMID: 28490313.

27: Valsecchi O, **Nardin M**, Vassileva A, Bernelli C. Transulnar recanalization of a chronic radial artery occlusion. Cardiovasc Revasc Med. 2017 Oct- Nov;18(7):504-506. doi: 10.1016/j.carrev.2017.04.011. Epub 2017 Apr 23. PMID: 28600020.

28: Malerba M, **Nardin M**, Radaeli A, Montuschi P, Carpagnano GE, Clini E. The potential role of endothelial dysfunction and platelet activation in the development of thrombotic risk in COPD patients. Expert Rev Hematol. 2017 Sep;10(9):821-832. doi: 10.1080/17474086.2017.1353416. Epub 2017 Jul 12. PMID: 28693343.

29: Verdoia M, Barbieri L, **Nardin M**, Suryapranata H, De Luca G. Dual Versus Single Antiplatelet Regimen With or Without Anticoagulation in Transcatheter Aortic Valve Replacement: Indirect Comparison and Meta-analysis. Rev Esp Cardiol (Engl Ed). 2018 Apr;71(4):257-266. English, Spanish. doi: 10.1016/j.rec.2017.06.012. Epub 2017 Jul 19. PMID: 28734880.

30: Malerba M, Radaeli A, **Nardin M**, Clini E, Carpagnano GE, Sciatti E, Salghetti F, Bonadei I, Platto F, Vizzardi E. Endothelial dysfunction assessment by noninvasive peripheral arterial tonometry in patients with chronic obstructive pulmonary disease compared with healthy subjects. Clin Respir J. 2018 Apr;12(4):1466-1472. doi: 10.1111/crj.12686. Epub 2017 Aug 16. PMID: 28779547.

31: Daffara V, Verdoia M, Rolla R, **Nardin M**, Marino P, Bellomo G, Carriero A, De Luca G; Novara Atherosclerosis Study Group (NAS). Impact of polymorphism rs7041 and rs4588 of Vitamin D Binding

Protein on the extent of coronary artery disease. Nutr Metab Cardiovasc Dis. 2017 Sep;27(9):775-783. doi: 10.1016/j.numecd.2017.06.002. Epub 2017 Jun 24. PMID: 28779988.

32: **Nardin M**, Verdoia M, Pergolini P, Rolla R, Barbieri L, Marino P, Bellomo G, Kedhi E, Suryapranata H, Carriero A, De Luca G; Novara Atherosclerosis Study Group (NAS). Impact of adenosine A2a receptor polymorphism rs5751876 on platelet reactivity in ticagrelor treated patients. Pharmacol Res. 2018 Mar;129:27-33. doi: 10.1016/j.phrs.2017.12.035. Epub 2018 Jan 31. PMID: 29355684.

33: Verdoia M, Pergolini P, Barbieri L, Rolla R, **Nardin M**, Negro F, Suryapranata H, De Luca G; Novara Atherosclerosis Study Group (NAS). Impact of pre-procedural dual antiplatelet therapy on periprocedural myocardial infarction in patients undergoing percutaneous coronary interventions with adjunctive tirofiban. Thromb Res. 2018 Apr;164:17-23. doi: 10.1016/j.thromres.2018.02.008. Epub 2018 Feb 15. PMID: 29475177.

34: Favero G, Paini A, De Ciuceis C, Rodella LF, Moretti E, Porteri E, Rossini C, Ministrini S, Solaini L, Stefano C, Coschignano MA, Brami V, Petelca A, **Nardin M**, Valli I, Tiberio GAM, Bonomini F, Agabiti Rosei C, Portolani N, Rizzoni D, Rezzani R. Changes in extracellular matrix in subcutaneous small resistance arteries of patients with essential hypertension. Blood Press. 2018 Aug;27(4):231-239. doi: 10.1080/08037051.2018.1448256. Epub 2018 Mar 9. PMID: 29523048.

35: Malerba M, **Nardin M**, Santini G, Mores N, Radaeli A, Montuschi P. Single- inhaler triple therapy utilizing the once-daily combination of fluticasone furoate, umeclidinium and vilanterol in the management of COPD: the current evidence base and future prospects. Ther Adv Respir Dis. 2018 Jan-Dec;12:1753466618760779. doi: 10.1177/1753466618760779. PMID: 29537340.

36: Verdoia M, Ceccon C, **Nardin M**, Suryapranata H, De Luca G; Novara Atherosclerosis Study Group (NAS). Vitamin D deficiency and periprocedural myocardial infarction in patients undergoing percutaneous coronary interventions. Cardiovasc Revasc Med. 2018 Oct;19(7 Pt A):744-750. doi: 10.1016/j.carrev.2018.03.002. Epub 2018 Mar 4. PMID: 29656938.

37: Caletti S, Paini A, Coschignano MA, De Ciuceis C, **Nardin M**, Zulli R, Muiesan ML, Salvetti M, Rizzoni D. Management of VEGF-Targeted Therapy-Induced Hypertension. Curr Hypertens Rep. 2018 Jun 29;20(8):68. doi: 10.1007/s11906-018-0871-1. PMID: 29959593.

38: **Nardin M**, Verdoia M, Barbieri L, De Luca G; Novara Atherosclerosis Study Group (NAS). Impact of metabolic syndrome on mean platelet volume and its relationship with coronary artery disease. Platelets. 2019;30(5):615-623. doi: 10.1080/09537104.2018.1499885. Epub 2018 Jul 26. PMID: 30047817.

39: Verdoia M, **Nardin M**, Negro F, Rolla R, De Luca G; Novara Atherosclerosis Study Group (NAS). Impact of statin therapy on the immature platelet count in patients with coronary artery disease: A single centre cohort study. Int J Cardiol. 2018 Dec 1;272:40-44. doi: 10.1016/j.ijcard.2018.08.039. Epub 2018 Aug 11. PMID: 30150123.

40: Verdoia M, Pergolini P, **Nardin M**, Rolla R, Barbieri L, Marino P, Carriero A, Suryapranata H, De Luca G; Novara Atherosclerosis Study Group. Prevalence and predictors of high-on treatment platelet reactivity during prasugrel treatment in patients with acute coronary syndrome undergoing stent implantation. J Cardiol. 2019 Mar;73(3):198-203. doi: 10.1016/j.jjcc.2018.10.005. Epub 2018 Dec 3. PMID: 30522897.

41: **Matteo Nardin**, Maria Antonietta Coschignano, Claudia Rossini, Carolina De Ciuceis, Stefano Caletti, Marco Rizzoni, Franco Docchio, Enzo Porteri, Damiano Rizzoni; Methods of evaluation of microvascular structure: state of the art. Eur J Trans Clin Med. 2018;1(1):7-17. doi:10.31373/ejtcm/95161

42: Rizzoni D, Rizzoni M, **Nardin M**. Vitamin D and Ischaemic Heart Disease: A Casual or A Causal Association? : Commentary on: "Raslan E et al. Association of Vitamin D Deficiency with Chronic Stable Angina: A Case-Control Study". High Blood Press Cardiovasc Prev. 2019 Apr;26(2):151-155. doi: 10.1007/s40292-019-00302-y. Epub 2019 Jan 23. PMID: 30673972.

43: Verdoia M, **Nardin M**, Negro F, Rolla R, Carriero A, De Luca G; Novara Atherosclerosis Study Group (NAS). Impact of long-term therapy with acetylsalicylic acid on immature platelet count: a single-centre cohort study. J Cardiovasc Med (Hagerstown). 2019 May;20(5):306-312. doi: 10.2459/JCM.0000000000000786. PMID: 30865138.

44: Verdoia M, Pergolini P, **Nardin M**, Rolla R, Tonon F, Kedhi E, Suryapranata H, Carriero A, De Luca G; Novara Atherosclerosis Study Group (NAS). Impact of aging on platelet reactivity in diabetic patients receiving dual antiplatelet therapy. J Thromb Thrombolysis. 2019 Oct;48(3):413-421. doi: 10.1007/s11239-019-01873-2. PMID: 31065927.

45: Malerba M, Foci V, Patrucco F, Pochetti P, **Nardin M**, Pelaia C, Radaeli A. Single Inhaler LABA/LAMA for COPD. Front Pharmacol. 2019 Apr 25;10:390. doi: 10.3389/fphar.2019.00390. PMID: 31105560; PMCID: PMC6494943.

46: Rizzoni D, Rizzoni M, **Nardin M**, Chiarini G, Agabiti-Rosei C, Aggiusti C, Paini A, Salvetti M, Muiesan ML. Vascular Aging and Disease of the Small Vessels. High Blood Press Cardiovasc Prev. 2019 Jun;26(3):183-189. doi: 10.1007/s40292-019-00320-w. Epub 2019 May 29. PMID: 31144248.

47: Verdoia M, Pergolini P, **Nardin M**, Rolla R, Negro F, Kedhi E, Suryapranata H, Marcolongo M, Carriero A, De Luca G; Novara Atherosclerosis Study Group (NAS). Vitamin D levels and platelet reactivity in

diabetic patients receiving dual antiplatelet therapy. Vascul Pharmacol. 2019 Sep;120:106564. doi: 10.1016/j.vph.2019.106564. Epub 2019 Jun 7. PMID: 31176855.

48: Campana F, **Nardin M**, Coppini A, Muiesan ML. Case report of a sub-occluding thrombus in thoracic aorta: what is the origin? Eur Heart J Case Rep. 2019 Sep 1;3(3):ytz114. doi: 10.1093/ehjcr/ytz114. PMID: 31367736; PMCID: PMC6764550.

49: **Nardin M**, Verdoia M, Negro F, Rolla R, Tonon F, De Luca G. Impact of active smoking on the immature platelet fraction and its relationship with the extent of coronary artery disease. Eur J Clin Invest. 2020 Feb;50(2):e13181. doi: 10.1111/eci.13181. Epub 2020 Jan 18. PMID: 31659742.

50: Radaeli A, **Nardin M**, Azzolina D, Malerba M. Determinants of Smoking Status in a Sample of Outpatients Afferent to a Tertiary Referral Hospital. Int J Environ Res Public Health. 2019 Oct 27;16(21):4136. doi: 10.3390/ijerph16214136. PMID: 31717846; PMCID: PMC6862272.

51: Verdoia M, Rolla R, Negro F, Tonon F, Pergolini P, **Nardin M**, Marcolongo M, De Luca G; Novara Atherosclerosis Study Group (NAS). Homocysteine levels and platelet reactivity in coronary artery disease patients treated with ticagrelor. Nutr Metab Cardiovasc Dis. 2020 Feb 10;30(2):292-299. doi: 10.1016/j.numecd.2019.09.018. Epub 2019 Sep 30. PMID: 31780401.

52: Verdoia M, **Nardin M**, Rolla R, Pergolini P, Suryapranata H, Kedhi E, Carriero A, De Luca G; Novara Atherosclerosis Study Group (NAS). Impact of diabetes mellitus on immature platelet fraction and its association with coronary artery disease. Diabetes Metab Res Rev. 2020 May;36(4):e3290. doi: 10.1002/dmrr.3290. Epub 2020 Feb 5. PMID: 31943700.

53: Verdoia M, **Nardin M**, Negro F, Tonon F, Gioscia R, Rolla R, De Luca G. Impact of aging on immature platelet count and its relationship with coronary artery disease. Platelets. 2020 Nov 16;31(8):1060-1068. doi: 10.1080/09537104.2020.1714572. Epub 2020 Jan 23. PMID: 31973643.

54: Verdoia M, Pergolini P, **Nardin M**, Rolla R, Negro F, Gioscia R, Fierro N, Saghir Afifeh AM, Viglione F, Suryapranata H, De Luca G; Novara Atherosclerosis Study Group (NAS). Gender Differences in Platelet Reactivity in Diabetic Patients Receiving Dual Antiplatelet Therapy. Cardiovasc Revasc Med. 2020 Sep;21(9):1144-1149. doi: 10.1016/j.carrev.2020.02.007. Epub 2020 Feb 13. PMID: 32085942.

55: Negro F, Verdoia M, Tonon F, **Nardin M**, Kedhi E, De Luca G; Novara Atherosclerosis Study Group (NAS). Impact of gender on immature platelet count and its relationship with coronary artery disease. J Thromb Thrombolysis. 2020 May;49(4):511-521. doi: 10.1007/s11239-020-02080-0. PMID: 32189190.

56: **Nardin M**, Verdoia M, Negro F, Suryapranata H, Khedi E, De Luca G. Relationship between adenosine A2a receptor polymorphism rs5751876 and fractional flow reserve during percutaneous coronary intervention. Heart Vessels. 2020 Oct;35(10):1349-1359. doi: 10.1007/s00380-020-01609-w. Epub 2020 May 4. PMID: 32367186.

57: Verdoia M, Ceccon C, **Nardin M**, Negro F, Marcolongo M, De Luca G; Novara Atherosclerosis Study Group (NAS). Polymorphism rs2762939 of CYP24A1 enzyme and
coronary artery disease: angiographic results from a large prospective cohort of patients. Blood Coagul Fibrinolysis. 2020 Sep;31(6):366-371. doi: 10.1097/MBC.0000000000000922. PMID: 32516167.

58: Verdoia M, **Nardin M**, Gioscia R, Negro F, Marcolongo M, Suryapranata H, Kedhi E, De Luca G; Novara Atherosclerosis Study Group (NAS). Higher neutrophil-to-lymphocyte ratio (NLR) increases the risk of suboptimal platelet inhibition and major cardiovascular ischemic events among ACS patients receiving dual antiplatelet therapy with ticagrelor. Vascul Pharmacol. 2020 Sep;132:106765. doi: 10.1016/j.vph.2020.106765. Epub 2020 Jul 16. PMID: 32681888.

59: Verdoia M, Gioscia R, **Nardin M**, Negro F, Tonon F, Suryapranata H, Khedi E, Marcolongo M, De Luca G. Impact of Age on the Functional Evaluation of Intermediate Coronary Stenoses With Instantaneous Wave-Free Ratio and Fractional Flow Reserve. Angiology. 2021 Jan;72(1):62-69. doi: 10.1177/0003319720947578. Epub 2020 Aug 20. PMID: 32815383.

60: Verdoia M, **Nardin M**, Rolla R, Negro F, Gioscia R, Afifeh AMS, Viglione F, Suryapranata H, Marcolongo M, De Luca G; Novara Atherosclerosis Study Group (NAS). Prognostic impact of Vitamin D deficiency in patients with coronary artery disease undergoing percutaneous coronary intervention. Eur J Intern Med. 2021 Jan;83:62-67. doi: 10.1016/j.ejim.2020.08.016. Epub 2020 Aug 20. PMID: 32830035.

61: Verdoia M, **Nardin M**, Rolla R, Negro F, Gioscia R, Saghir Afifeh AM, Viglione F, Suryapranata H, Marcolongo M, De Luca G; Novara Atherosclerosis Study Group (NAS). Vitamin D levels condition the outcome benefits of renin-angiotensin system inhibitors (RASI) among patients undergoing percutaneous coronary intervention. Pharmacol Res. 2020 Oct;160:105158. doi: 10.1016/j.phrs.2020.105158. Epub 2020 Aug 22. PMID: 32841717.

62: Verdoia M, Tonon F, Gioscia R, **Nardin M**, Fierro N, Sagazio E, Negro F, Pergolini P, Rolla R, De Luca G; Novara Atherosclerosis Study Group (NAS). Impact of the rs73598374 polymorphism of the adenosine deaminase gene on platelet reactivity and long-term outcomes among patients with acute coronary syndrome treated with ticagrelor. Thromb Res. 2020 Dec;196:231-237. doi: 10.1016/j.thromres.2020.09.006. Epub 2020 Sep 3. PMID: 32916566.

63: Verdoia M, **Nardin M**, Rolla R, Negro F, Gioscia R, Afifeh AMS, Viglione F, Suryapranata H, Marcolongo M, De Luca G; Novara Atherosclerosis Study Group (NAS). Association of lower vitamin D levels with inflammation and leucocytes parameters in patients with and without diabetes mellitus undergoing coronary angiography. Eur J Clin Invest. 2021 Apr;51(4):e13439. doi: 10.1111/eci.13439. Epub 2020 Nov 20. PMID: 33112413.

64: Verdoia M, **Nardin M**, Rolla R, Negro F, Gioscia R, Saghir Afifeh AM, Viglione F, Suryapranata H, Marcolongo M, De Luca G; Novara Atherosclerosis Study Group (NAS). Cholecalciferol levels, inflammation and leukocytes parameters: Results from a large single-centre cohort of patients. Clin Nutr. 2021 Apr;40(4):2228-2236. doi: 10.1016/j.clnu.2020.09.054. Epub 2020 Oct 19. PMID: 33121835.

65: Boari GEM, Chiarini G, Bonetti S, Malerba P, Bianco G, Faustini C, Braglia- Orlandini F, Turini D, Guarinoni V, Saottini M, Viola S, Ferrari-Toninelli G, Pasini G, Mascadri C, Bonzi B, Desenzani P, Tusi C, Zanotti E, **Nardin M**, Rizzoni D. Prognostic factors and predictors of outcome in patients with COVID-19 and related pneumonia: a retrospective cohort study. Biosci Rep. 2020 Dec 23;40(12):BSR20203455. doi: 10.1042/BSR20203455. PMID: 33201172; PMCID: PMC7724688.

66: Nicolas J, Claessen BE, Cao D, Chiarito M, Sartori S, Qiu H, Goel R, **Nardin M**, Roumeliotis A, Vogel B, Turfah A, Chandiramani R, Baber U, Barman N, Sweeny J, Krishnan P, Kini A, Sharma SK, Dangas GD, Mehran R. A sex paradox in clinical outcomes following complex percutaneous coronary intervention. Int J Cardiol. 2021 Apr 15;329:67-73. doi: 10.1016/j.ijcard.2020.11.067. Epub 2020 Dec 2. PMID: 33278415.

67: Saghir Afifeh AM, Verdoia M, **Nardin M**, Negro F, Viglione F, Rolla R, De Luca G; Novara Atherosclerosis Study Group (NAS). Determinants of vitamin D activation in patients with acute coronary syndromes and its correlation with inflammatory markers. Nutr Metab Cardiovasc Dis. 2021 Jan 4;31(1):36-43. doi: 10.1016/j.numecd.2020.09.021. Epub 2020 Sep 24. PMID: 33308994.

68: Negro F, Verdoia M, **Nardin M**, Suryapranata H, Kedhi E, Dudek D, De Luca G; Novara Atherosclerosis Study Group (NAS). Impact of the Polymorphism rs5751876 of the Purinergic Receptor ADORA2A on Periprocedural Myocardial Infarction in Patients Undergoing Percutaneous Coronary Intervention. J Atheroscler Thromb. 2021 Feb 1;28(2):137-145. doi: 10.5551/jat.53405. Epub 2020 Dec 19. PMID: 33342966; PMCID: PMC7957027.

69: **Nardin M**, Verdoia M, Negro F, Tonon F, De Luca G. Impact of uric acid on immature platelet fraction in patients undergoing percutaneous coronary intervention. Thromb Res. 2021 Feb;198:171-181. doi: 10.1016/j.thromres.2020.12.006. Epub 2020 Dec 16. PMID: 33348191.

70: Verdoia M, Rolla R, Pergolini P, Gioscia R, **Nardin M**, Negro F, Viglione F, Suryapranata H, Kedhi E, De Luca G; Novara Atherosclerosis Study Group (NAS). Low hemoglobin predicts high-platelet reactivity

and major cardiovascular ischemic events at long-term follow-up among ACS patients receiving dual antiplatelet therapy with ticagrelor. Catheter Cardiovasc Interv. 2021 Feb 1. doi: 10.1002/ccd.29512. Epub ahead of print. PMID: 33527669.

71: Verdoia M, **Nardin M**, Gioscia R, Saghir Afifeh AM, Viglione F, Negro F, Marcolongo M, De Luca G; Novara Atherosclerosis Study Group (NAS). Association between vitamin D deficiency and serum Homocysteine levels and its relationship with coronary artery disease. J Thromb Thrombolysis. 2021 Feb 4:1–9. doi: 10.1007/s11239-021-02391-w. Epub ahead of print. PMID: 33538987; PMCID: PMC7859464.

72: **Nardin M**, Verdoia M, Gioscia R, Negro F, De Luca G. Impact of renin angiotensin system inhibitors on homocysteine levels and platelets reactivity in patients on dual antiplatelet therapy. Nutr Metab Cardiovasc Dis. 2021 Apr 9;31(4):1276-1285. doi: 10.1016/j.numecd.2020.12.004. Epub 2020 Dec 10. PMID: 33549433.

73: Verdoia M, Gioscia R, **Nardin M**, Viola O, Brancati MF, Soldà PL, Marcolongo M, De Luca G. Preprocedural β-Blockers in the Functional Assessment of Intermediate Coronary Lesions by Instantaneous Wave-Free Ratio. Angiology. 2021 Aug;72(7):687-692. doi: 10.1177/0003319721996170. Epub 2021 Feb 25. PMID: 33626884.

74: Coschignano MA, De Ciuceis C, Agabiti-Rosei C, Brami V, Rossini C, Chiarini G, Malerba P, Famà F, Cosentini D, Muiesan ML, Salvetti M, Petelca A, Capellini S, Arnoldi C, **Nardin M**, Grisanti S, Rizzoni D, Berruti A, Paini A. Microvascular Structural Alterations in Cancer Patients Treated With Antiangiogenic Drugs. Front Cardiovasc Med. 2021 Mar 10;8:651594. doi: 10.3389/fcvm.2021.651594. PMID: 33778028; PMCID: PMC7987651.

75: Verdoia M, **Nardin M**, Viola O, Brancati MF, Soldà PL, Marcolongo M, De Luca G. Impact of sex on the functional assessment of intermediate coronary lesions by instantaneous wave-free ratio. Cardiovasc Revasc Med. 2021 Jul 10:S1553-8389(21)00512-1. doi: 10.1016/j.carrev.021.07.007. Epub ahead of print. PMID: 34275742.

76: Verdoia M, Pergolini P, **Nardin M**, Rolla R, Suryapranata H, Kedhi E, De Luca G; Novara Atherosclerosis Study Group (NAS). Ticagrelor and prasugrel in acute coronary syndrome: a single-arm crossover platelet reactivity study. J Cardiovasc Med (Hagerstown). 2021 Jul 16. doi: 10.2459/JCM.0000000000001222. Epub ahead of print. PMID: 34280175.

77: Nicolas J, Beerkens F, Cao D, Sartori S, Pivato CA, Qiu H, Giustino G, Chiarito M, Claessen BE, Zhang Z, **Nardin M**, Razuk V, Jones D, Camaj A, Power D, Banashefski B, Sweeny J, Baber U, Dangas G, Sharma SK, Kini A, Mehran R. Performance of the academic research consortium high-bleeding risk criteria in

patients undergoing PCI for acute myocardial infarction. J Thromb Thrombolysis. 2021 Aug 4. doi: 10.1007/s11239-021-02534-z. Online ahead of print. PMID: 3434720

78: Kini A, Cao D, **Nardin M**, Sartori S, Zhang Z, Pivato CA, Chiarito M, Nicolas J, Vengrenyuk Y, Krishnamoorthy P, Sharma SK, Dangas G, Fuster V, Mehran R. Types of myocardial injury and mid-term outcomes in patients with COVID-19. Eur Heart J Qual Care Clin Outcomes. 2021 Aug 30:qcab053. doi: 10.1093/ehjqcco/qcab053. Epub ahead of print. PMID: 34458912.

79: Tavenier AH, Mehran R, Chiarito M, Cao D, Pivato CA, Nicolas J, Beerkens F, **Nardin M**, Sartori S, Baber U, Angiolillo DJ, Capodanno D, Valgimigli M, Hermanides RS, van 't Hof AWJ, Ten Berg JM, Chang K, Kini AS, Sharma SK, Dangas G. Guided and unguided de-escalation from potent P2Y12 inhibitors among patients with ACS: a meta-analysis. Eur Heart J Cardiovasc Pharmacother. 2021 Aug 30:pvab068. doi: 10.1093/ehjcvp/pvab068. Epub ahead of print. PMID: 34459481.

80: **Nardin M**, Cao D, Mehran R, Chandiramani R, Chiarito M, Sartori S, Qiu H, Zhang Z, Golani E, Nicolas J, Baber U, Dangas G, De Luca G, Stefanini GG, Sweeny J, Sharma SK, Kini A. Sex-Related Differences in the Prevalence and Prognostic Value of the Academic Research Consortium for High Bleeding Risk Criteria. Circ Cardiovasc Interv. 2021 Sep 3. doi: 10.1161/CIRCINTERVENTIONS.120.010392. Epub ahead of print. PMID: 34474588.

81: Beerkens FJ, Singh R, Cao D, Claessen BE, Nicolas J, Sartori S, Snyder C, Camaj A, Giustino G, Power D, Razuk V, Jones D, Tavenier AH, Pivato CA, **Nardin M**, Chiarito M, Krishnan P, Barman N, Baber U, Sweeny J, Dangas G, Sharma SK, Mehran R, Kini A. Impact of target vessel choice on outcomes following percutaneous coronary intervention in patients with a prior coronary artery bypass graft. Catheter Cardiovasc Interv. 2021 Sep 3. doi: 10.1002/ccd.29935. Epub ahead of print. PMID: 34478235.

82: 1: De Luca G, Algowhary M, Uguz B, Oliveira DC, Ganyukov V, Zimbakov Z, Cercek M, Jensen LO, Loh PH, Calmac L, Roura-Ferrer G, Quadros A, Milewski M, Scotto di Uccio F, von Birgelen C, Versaci F, Ten Berg J, Casella G, Wong ASL, Kala P, Diez Gil JL, Carrillo X, Dirksen MT, Becerra-Muñoz VM, Kang-Yin Lee M, Juzar DA, de Moura Joaquim R, Paladino R, Milicic D, Davlouros P, Bakraceski N, Zilio F, Donazzan L, Kraaijeveld AO, Galasso G, Lux A, Marinucci L, Guiducci V, Menichelli M, Scoccia A, Yamac A, Ugur Mert K, Flores Rios X, Kovarnik T, Kidawa M, Moreu J, Flavien V, Fabris E, Lozano Martìnez-Luengas I, Boccalatte M, Bosa Ojeda F, Arellano-Serrano C, Caiazzo G, Cirrincione G, Kao HL, Sanchis Fores J, Vignali L, Pereira H, Manzo-Silberman S, Ordonez S, Özkan AA, Scheller B, Lehtola H, Teles R, Mantis C, Ylitalo A, Brum Silveira JA, Zoni R, Bessonov I, Savonitto S, Kochiadakis G, Alexopoulos D, Uribe C, Kanakakis J, Faurie B, Gabrielli G, Gutiérrez A, Bachini JP, Rocha A, Tam FC, Rodriguez A, Lukito A, Saint-Joy V, Pessah G, Tuccillo B, Cortese G, Parodi G, Bouraghda MA, Kedhi E,
Lamelas P, Suryapranata H, **Nardin M**, Verdoia M; ISACS-STEMI COVID-19; Collaborators. COVID-19 pandemic, mechanical reperfusion and 30-day mortality in ST elevation myocardial infarction. Heart. 2021

Oct 28:heartjnl-2021-319750. doi: 10.1136/heartjnl-2021-319750. Epub ahead of print. PMID: 34711661; PMCID: PMC8561823.

83: Salvotti F, Poiatti F, Bressa S, Montani G, **Nardin M**, Rizzoni D. Platypnoea-Orthodeoxia Syndrome in COVID-19. Eur J Case Rep Intern Med. 2021 Oct 22;8(10):002849. doi: 10.12890/2021_002849. PMID: 34790629; PMCID: PMC8592651.

84: Mehran R, Owen R, Chiarito M, Baber U, Sartori S, Cao D, Nicolas J, Pivato CA, **Nardin M**, Krishnan P, Kini A, Sharma S, Pocock S, Dangas G. A contemporary simple risk score for prediction of contrast-associated acute kidney injury after percutaneous coronary intervention: derivation and validation from an observational registry. Lancet. 2021 Nov 27;398(10315):1974-1983. doi: 10.1016/S0140-6736(21)02326-6. Epub 2021 Nov 15. PMID: 34793743.

85: Chiarito M, Kini A, Roumeliotis A, Cao D, Power D, Sartori S, Reisman A, Zhang Z, Mtisi T, Nicolas J, **Nardin M**, Stefanini G, Baber U, Giustino G, Sweeny J, Mehran R, Sharma S, Dangas G. Prevalence and Impact of High Bleeding Risk in Patients Undergoing Left Main Artery Disease PCI. JACC Cardiovasc Interv. 2021 Nov 22;14(22):2447-2457. doi: 10.1016/j.jcin.2021.08.056. PMID: 34794650.

86: De Luca G, **Nardin M**, Algowhary M, Uguz B, Oliveira DC, Ganyukov V, Zimbakov Z, Cercek M, Jensen LO, Loh PH, Calmac L, Roura Ferrer G, Quadros A, Milewski M, Scotto di Uccio F, von Birgelen C, Versaci F, Ten Berg J, Casella G, Lung AWS, Kala P, Díez Gil JL, Carrillo X, Dirksen M, Becerra-Munoz VM, Lee MK, Juzar DA, de Moura Joaquim R, Paladino R, Milicic D, Davlouros P, Bakraceski N, Zilio F, Donazzan L, Kraaijeveld A, Galasso G, Lux A, Marinucci L, Guiducci V, Menichelli M, Scoccia A, Yamac AH, Mert KU, Flores Rios X, Kovarnik T, Kidawa M, Moreu J, Flavien V, Fabris E, Martínez-Luengas IL, Boccalatte M, Bosa Ojeda F, Arellano-Serrano C, Caiazzo G, Cirrincione G, Kao HL, Sanchis Forés J, Vignali L, Pereira H, Manzo S, Ordoñez S, Arat Özkan A, Scheller B, Lehtola H, Teles R, Mantis C, Antti Y, Brum Silveira JA, Zoni R, Bessonov I, Savonitto S, Kochiadakis G, Alexopulos D, Uribe CE, Kanakakis J, Faurie B, Gabrielli G, Gutierrez Barrios A, Bachini JP, Rocha A, Tam FC, Rodriguez A, Lukito AA, Saint-Joy V, Pessah G, Tuccillo A, Cortese G, Parodi G, Bouraghda MA, Kedhi E, Lamelas P, Suryapranata H, Verdoia M. Renin-angiotensin system inhibitors and mortality among diabetic patients with STEMI undergoing mechanical reperfusion during the COVID-19 pandemic. Diabet Epidemiol Manag. 2021 Oct-Dec;4:100022. doi: 10.1016/j.deman.2021.100022. Epub 2021 Oct 30. PMID: 35072135.

87: Pivato CA, Jones D, Cao D, Sartori S, Chiarito M, Nicolas J, Zhang Z, Beerkens F, **Nardin M**, Qiu H, Razuk V, Feldman D, Kumaraguru V, Stefanini GG, Sweeny J, Baber U, Dangas G, Sharma SK, Kini A, Mehran R. Prognostic Value of Baseline Inflammation in Diabetic and Nondiabetic Patients Undergoing Percutaneous Coronary Intervention. Can J Cardiol. 2022 Jun;38(6):792-800. doi: 10.1016/j.cjca.2022.02.002. Epub 2022 Feb 10. PMID: 35151783.

88: Cao D, Swain JA, Sartori S, **Nardin M**, Zhang Z, Roumeliotis A, Nicolas J, Chiarito M, Chandiramani R, Pivato CA, Spirito A, Giustino G, Stefanini GG, Dangas GD, Baber U, Bhatt DL, Adams DH, Sharma SK, Kini AS, Mehran R. Perioperative Management of P2Y12 Inhibitors in Patients Undergoing Cardiac Surgery within 1 Year of PCI. Eur Heart J Cardiovasc Pharmacother. 2022 Apr 7:pvac024. doi: 10.1093/ehjcvp/pvac024. Epub ahead of print. PMID: 35389474.

89: Pivato CA, Cao D, Spirito A, Sartori S, Nicolas J, Chiarito M, Snyder C, Mehilli J, Lefèvre T, Stefanini GG, Presbitero P, Capranzano P, Tchetche D, Iadanza A, Vogel B, Sardella G, Van Mieghem NM, **Nardin M**, Tavenier AH, Meliga E, Dumonteil N, Fraccaro C, Trabattoni D, Mikhail G, Sharma S, Ferrer MC, Naber C, Kievit P, Baber U, Petronio AS, Morice MC, Chieffo A, Dangas G, Mehran R. Impact of Small Valve Size on 1-Year Outcomes After Transcatheter Aortic Valve
Implantation in Women (from the WIN-TAVI Registry). Am J Cardiol. 2022 Jun 1;172:73-80. doi: 10.1016/j.amjcard.2022.02.025. Epub 2022 Apr 20. PMID: 35461697.

90: Verdoia M, Gioscia R, **Nardin M**, Rognoni A, De Luca G. Low levels of vitamin D and coronary artery disease: Is it time for therapy? Kardiol Pol. 2022;80(4):409-416. doi: 10.33963/KP.a2022.0079. PMID: 35546270.

91: De Luca G, **Nardin M**, Algowhary M, Uguz B, Oliveira DC, Ganyukov V, Zimbakov Z, Cercek M, Okkels Jensen L, Loh PH, Calmac L, Roura Ferrer G, Quadros A, Milewski M, Scotto di Uccio F, von Birgelen C, Versaci F, Ten Berg J, Casella G, Wong Sung Lung A, Kala P, Díez Gil JL, Carrillo X, Dirksen M, Becerra-Munoz VM, Lee MK, Arifa Juzar D, de Moura Joaquim R, Paladino R, Milicic D, Davlouros P, Bakraceski N, Zilio F, Donazzan L, Kraaijeveld A, Galasso G, Lux A, Marinucci L, Guiducci V, Menichelli M, Scoccia A, Yamac AH, Ugur Mert K, Flores Rios X, Kovarnik T, Kidawa M, Moreu J, Flavien V, Fabris E, Martínez-Luengas IL, Boccalatte M, Bosa Ojeda F, Arellano-Serrano C, Caiazzo G, Cirrincione G, Kao HL, Sanchis Forés J, Vignali L, Pereira H, Manzo S, Ordoñez S, Özkan AA, Scheller B, Lehtola H, Teles R, Mantis C, Antti Y, Brum Silveira JA, Zoni R, Bessonov I, Savonitto S, Kochiadakis G, Alexopoulos D, Uribe CE, Kanakakis J, Faurie B, Gabrielli G, Gutierrez Barrios A, Bachini JP, Rocha A, Tam FC, Rodriguez A, Lukito AA, Saint-Joy V, Pessah G, Tuccillo A, Cortese G, Parodi G, Bouraghda MA, Kedhi E, Lamelas P, Suryapranata H, Verdoia M. Impact of chronic obstructive pulmonary disease on short-term outcome in patients with ST- elevation myocardial infarction during COVID-19 pandemic: insights from the international multicenter ISACS-STEMI registry. Respir Res. 2022 Aug 15;23(1):207. doi: 10.1186/s12931-022-02128-0. PMID: 35971173;

92: Rosei CA, Gaggero A, Famà F, Malerba P, Chiarini G, **Nardin M**, Brami V, Rossini C, Coschignano MA, Porteri E, Salvetti M, Muiesan ML, Rizzoni D, De Ciuceis C. Skin capillary alterations in patients with acute SarsCoV2 infection. J Hypertens. 2022 Aug 19. doi: 10.1097/HJH.0000000000003271. Epub ahead ofprint. PMID: 35983856.

93: De Luca G, Silverio A, Verdoia M, Siudak Z, Tokarek T, Kite TA, Gershlick AH, Rodriguez-Leor O, Cid-Alvarez B, Jones DA, Rathod KS, Montero-Cabezas JM, Jurado-Roman A, **Nardin M**, Galasso G. Angiographic and clinical outcome of SARS- CoV-2 positive patients with ST-segment elevation myocardial infarction undergoing primary angioplasty: A collaborative, individual patient data meta-analysis of six registry-based studies. Eur J Intern Med. 2022 Aug 18:S0953-6205(22)00298-9. doi: 10.1016/j.ejim.2022.08.021. Epub ahead of print. PMID: 35999094; PMCID: PMC9385833.

94: De Luca G, Algowhary M, Uguz B, Oliveira DC, Ganyukov V, Zimbakov Z, Cercek M, Okkels Jensen L, Loh PH, Calmac L, Roura I Ferrer G, Quadros A, Milewski M, Scotto Di Uccio F, von Birgelen C, Versaci F, Ten Berg J, Casella G, Wong Sung Lung A, Kala P, Díez Gil JL, Carrillo X, Dirksen M, Becerra-Munoz VM, Kang-Yin Lee M, Juzar DA, de Moura Joaquim R, De Simone C, Milicic D, Davlouros P, Bakraceski N, Zilio F, Donazzan L, Kraaijeveld A, Galasso G, Arpad L, Marinucci L, Guiducci V, Menichelli M, Scoccia A, Yamac AH, Ugur Mert K, Flores Rios X, Kovarnik T, Kidawa M, Moreu J, Flavien V, Fabris E, Lozano Martínez-Luengas I, Boccalatte M, Bosa Ojeda F, Arellano-Serrano C, Caiazzo G, Cirrincione G, Kao HL, Sanchis Forés J, Vignali L, Pereira H, Manzo-Silbermann S, Ordoñez S, Arat Özkan A, Scheller B, Lehtola H, Teles R, Mantis C, Antti Y, Brum Silveira JA, Bessonov I, Zoni R, Savonitto S, Kochiadakis G, Alexopoulos D, Uribe CE, Kanakakis J, Faurie B, Gabrielli G, Gutierrez Barrios A, Bachini JP, Rocha A, Tam FC, Rodriguez A, Lukito AA, Bellemain-Appaix A, Pessah G, Cortese G, Parodi G, Burgadha MA, Kedhi E, Lamelas P, Suryapranata H, **Nardin M**, Verdoia M. SARS-CoV-2 Positivity, Stent Thrombosis, and 30-day Mortality in STEMI PatientsUndergoing Mechanical Reperfusion. Angiology. 2022 Oct 12:33197221129351. doi: 10.1177/00033197221129351. Epub ahead of print. PMID: 36222189.

95: Verdoia M, **Nardin M**, Gioscia R, Suryapranata H, Kedhi E, Rognoni A, DE Luca G; Novara Atherosclerosis Study Group (NAS). Impact of renal failure and high-platelet reactivity on major cardiovascular ischemic events among patients with acute coronary syndrome receiving dual antiplatelet therapy with ticagrelor. Minerva Cardiol Angiol. 2022 Oct 12. doi: 10.23736/S2724-5683.22.06001-X. Epub ahead of print. PMID: 36222601.

96: **Nardin M**, Pivato CA, Cao D, Sartori S, Zhang Z, Vogel B, Nicolas J, Chiarito M, Qiu H, Chandrasekhar J, Spirito A, Abizaid A, Christiansen EH, Colombo A, de Winter RJ, Haude M, Jakobsen L, Jensen LO, Krucoff MW, Landmesser U, Saito S, Suryapranata H, De Luca G, Dangas G, Mehran R. The mega COMBO collaboration: An individual patient data pooled analysis of patients undergoing PCI with COMBO stent. Int J Cardiol. 2023 Jan 1;370:149-155. doi: 10.1016/j.ijcard.2022.10.133. Epub 2022 Oct 19. PMID: 36270496.

97: De Luca G, Algowhary M, Uguz B, Oliveira DC, Ganyukov V, Zimbakov Z, Cercek M, Jensen LO, Loh PH, Calmac L, Ferrer GRI, Quadros A, Milewski M, Scotto D'Uccio F, von Birgelen C, Versaci F, Ten Berg J, Casella G, Wong Sung Lung A, Kala P, Díez Gil JL, Carrillo X, Dirksen M, Becerra-Munoz VM, Lee MK, Juzar DA, Joaquim RM, Paladino R, Milicic D, Davlouros P, Bakraceski N, Zilio F, Donazzan L, Kraaijeveld A, Galasso G, Arpad L, Lucia M, Vincenzo G, Menichelli M, Scoccia A, Yamac AH, Ugur Mert

K, Flores Rios X, Kovarnik T, Kidawa M, Moreu J, Vincent F, Fabris E, Martínez-Luengas IL, Boccalatte M, Bosa Ojeda F, Arellano-Serrano C, Caiazzo G, Cirrincione G, Kao HL, Sanchis Forés J, Vignali L, Pereira H, Manzo S, Ordoñez S, Özkan AA, Scheller B, Lehtola H, Teles R, Mantis C, Antti Y, Silveira JAB, Zoni R, Bessonov I, Savonitto S, Kochiadakis G, Alexopulos D, Uribe CE, Kanakakis J, Faurie B, Gabrielli G, Barrios AG, Bachini JP, Rocha A, Tam FC, Rodriguez A, Lukito AA, Saint-Joy V, Pessah G, Tuccillo A, Cortese G, Parodi G, Bouraghda MA, Kedhi E, Lamelas P, Suryapranata H, **Nardin M**, Verdoia M. Impact of Smoking Status on Mortality in STEMI Patients Undergoing Mechanical Reperfusion for STEMI: Insights from the ISACS-STEMI COVID-19 Registry. J Clin Med. 2022 Nov 13;11(22):6722. doi: 10.3390/jcm11226722. PMID: 36431198; PMCID: PMC9698021.

Acknowledgements

It is often considered the most difficult section to approach in writing a thesis: the acknowledgements section. The risk to forget someone is elevated, out of any scoring. I apologize in advance for the accidental forgetfulness.

The most important, huge thank is due to Professor De Luca: about nine years ago I strongly wanted to reach and ask you for my medicine degree thesis. After a number of attempts I have met you and a lot of what you have made and given me is reported in the above curriculum vitae. But not everything. What is not written is the opportunity, the time, the chances, the teachings to grow and ameliorate as physician and as researcher.

I could not know which fortune I had when, dear Monica, you supervised me for the MD thesis. You did not teach ex cathedra, you showed me which is the meaning of "passion", "dedication". I always say to colleagues, students, friends, what treasure I received being a student of yours. The number of thanks I should tell you never ends. Without you and your mentorship, never of this would have been possible.

Thank you, Professor van 't Hof for having given me the special, unique chance to reach a goal in my medical career, the highest that a researcher could hope.

Dear Carolina, thank you for having been a guide during the specialization period. You have taught me so many things, ranging from the medicine to the wine with a common element: there is no limit to enjoy the knowledge.

Dear Enzo, I have recently talked about the best features that a teacher should promote to be effective with own students. All time you appear in my mind.

Dear Davide, we met in the middle of the first coronavirus pandemic wave, far from our country, on the opposite side, in New York. Despite the situation, you welcomed me, and showed the level of research that could be achieved with rigors and precision.

Dear Mauro, the opportunity to talk to someone with a so deep knowledge made me happy. It has been like discovering a hidden part of the scientific world, funnier, where only the best players, like you and Davide, take the field.

A global thanks to all colleagues, mates, friends, that I met during these years, in all working and non-working contexts. A personalized thought is for each of you: everyone is the puzzle of own experience and memories.

A special, unique, thank to my parents and my brother, because, like for the medicine degree and the specialization, also in this occasion, without you, I would not have succeeded.