Annals of West University of Timisoara

Series of Chemistry **20 (1)** (2011) 47-58

LABORATORY TESTING OF PLATELET FUNCTION BY AGGREGOMETRY: WHAT TEST SHOULD BE USED?

Daniela Maximov[a], Alina Lupu[a], Cristiana Bujor[b], S. I. Dragulescu[b]

[a] Timisoara Institute of Cardiovascular Diseases, Research Department, 13A Gh. Adam St., Timisoara, 300310, ROMANIA, danielamaximov@yahoo.com

[b] Victor Babes University of Medicine and Pharmacy Timisoara, 2 Eftimie Murgu Sq., Nr. 2, Timisoara, 300041, ROMANIA

Received: 18 January 2011 Modified 24 February 2011 Accepted 30 March 2011

SUMMARY

In the last decades, the issue of "aspirin and clopidogrel resistance" was a provocative theme in the medical literature [1-4]. So far, there are a lot of studies published in this area, but its definition, diagnosis, etiology and clinical implications remain uncertain [1, 5].

Keywords: aspirin resistance; clopidogrel resistance; agreggometry.

INTRODUCTION

DEFINITION OF ANTIPLATELET RESPONSE VARIABILITY OR "RESISTANCE". The term of "resistance" to a drug should be used when the drug is unable to hit its pharmacological target, due to inability to reach it (consequence of different factors: reduced bioavailability, negative interaction with other drugs, *in vivo* inactivation) or to alteration of the target [1, 5].

Based on this definition, the term of resistance to aspirin should be limited to situations in which aspirin is unable to inhibit COX-1-dependent thromboxane A_2 (Tx A_2) production and, consequently, TxA_2-dependent platelet functions [5].

Multiple signaling pathways mediate platelet activation and the occurrence of thrombotic events. In consequence, a treatment strategy directed against a single pathway

cannot be expected to prevent the occurrence of all events [6]. But thrombosis results from multiple signaling pathways, and therefore treatment failure alone is not sufficient evidence of drug "resistance". The optimal definition of "resistance" or nonresponsiveness to an antiplatelet agent might be evidence of persistent activity of the specific target of the antiplatelet drug [6, 7].

ASPIRIN

Aspirin acetylates a serine moiety present in cyclooxygenase-1 (COX-1). It irreversibly inhibits the COX-1- dependent synthesis of thromboxane A_2(TxA_2), which is essential for the full aggregation response of platelets [8].

CLOPIDOGREL

The active metabolite of Clopidogrel is responsive of irreversibly inhibition of $P2Y_{12}$ receptor, because of the new covalent disulfide bond; in cases of non-responder patients there are evidence of posttreatment $P2Y_{12}$ reactivity. For aspirin, the diagnosis of resistance would use a laboratory technique that detects residual activity of cyclooxigenase COX-1. The proposed mechanisms of antiplatelet response variability /resistance were described in a large number of publications [1-5, 7].

The status of "clopidogrel non-responder" was mainly detected using LTA in a large number of studies and using ADP as an agonist [6, 9-23, 24]. Also different methods were used to identify the clopidogrel nonresponsiveness: point-of-care assays, VASP phosphorylation, flow-cytometric measurement of activation-dependent receptor expression after ADP stimulation [15, 16-19, 20-23].

Laboratory assessment of platelet responsiveness to aspirin can be divided in COX-1 –specific and COX-1-nonspecific. Arachidonic acid (AA) stimulation of platelet aggregation depends directly on COX-1 activity. *In vivo* production of TxA2 is assessed by measurement of stable metabolites via enzyme-linked immunoassays. Adenosine diphosphate (ADP)- and collagen- stimulated aggregation are COX-1- nonspecific methods. Aggregation occurs through COX-1-independent and-dependent pathways after stimulation using latter agonists (Table I) [6].

Table I. Laboratory assessment of platelet responsiveness to aspirin

COX-1 specific methods	COX-1non specific methods
1. AA- Induced Platelet Aggregation -LTA (PRP,whole blood) -TEG (whole blood) -VerifyNow (whole blood) - Multiplate	1. ADP and Collagen-Induced Platelet Aggregation -LTA
2. Thromboxane Metabolite - Serum - Urine	2. Shear, Collagen/epinephrine-induced Platelet Aggregation -PFA-100

Laboratory assessment of Clopidogrel responsiveness comprises different methods. ADP stimulates distinct receptors ($P2Y_1$ and $P2Y_{12}$) that are linked to specific signaling pathways. Response can be measured by:

1. Receptor reactivity; intracellular signaling downstream from the $P2Y_{12}$ receptor is measured by flow cytometry that assesses phosphorylation of vasodilator- stimulated phosphoprotein (VASP) with monoclonal antibodies; the P2Y12 is coupled by a Gi protein to adenylyl cyclase which activates protein kinase A (PKA).

2. Activation –dependent receptor expression (active glycoprotein GP IIb/IIIa and P-selectin) identified by monoclonal antibodies (Y) with flow cytometry.

3. Aggregation determined by light transmittance aggregometry (LTA), multiple electrodes aggregometry (MEA- whole blood platelet aggregation assessed with the Multiplate analyzer), aggregation of platelets with fibrinogen-coated beads (VerifiNow), or measuring the contribution of platelet aggregation to total platelet fibrin clot strength by thrombelastography (Table II).

Table II. Laboratory assessment of Clopidogrel responsiveness

Receptor reactivity- intracellular signaling downstream from the $P2Y_{12}$	-Flow cytometry -VASP
Receptor expression	-Flow cytometry -P-selectin
Aggregation	-LTA -MEA -VerifyNow -Thrombelastography

Methods used to measure platelet function during antiplatelet treatment

In vitro platelet function was measured in aspirin- treated patients using a number of functional assays, which were criticized because they do not reproduce the physiological conditions that determine the platelet aggregates development *in vivo*. None of these tests displays sufficient specificity for measuring the effects of aspirin on platelet function. In case of light- transmission aggregometry (LTA), despite of using the arachidonic acid, the precursor of TxA_2, as agonist of platelet aggregation, the results obtained with this technique may overestimate the prevalence of aspirin resistance [1].

Platelet-released factors

Methods that measure directly the capacity of platelets to synthesize TxA_2 are preferable. The urinary levels of the TxB_2 metabolite, 11-dehydrothromboxane B_2, represent a time-integrated index of TxA_2 biosynthesis *in vivo* [5]. The urinary level of this metabolite reflects systemic TxA2 formation, which largely occurs in the platelets. It has been

calculated that about 30% of the urinary metabolite derives from extra-platelets sources and this percent may be increased in particular cases (inflammatory diseases) [6].

In contrast, tromboxane B2 (TxB_2) reflects the total capacity of platelet to synthesize TxA_2, of which it is a stable metabolite; therefore serum TxB_2 is the most specific test to measure the aspirin effect on platelets [10]. Soluble CD40 ligand and P-selectin has been also observed in ACS in elevated levels [25]. The soluble markers are assessed using immunoassays; limitations in their utility include their presence in extraplatelet sources.

Light transmittance aggregometry (turbidimetric) (LTA) is the historical "gold standard" test, which is based on the stimulation of platelet-platelet aggregation in platelet-rich plasma after stimulation with various agonists. LTA has been the most widely used technique to monitor the effect of antiplatelet drugs, including aspirin, clopidogrel, other P2Y12 inhibitors, and platelet glycoprotein (GP)IIb/IIIa inhibitors [8].

Potential disadvantages include the immediate processing, variable reproducibility, large required sample volumes, lengthy processing time, and expenses of the aggregometer and trained operators. LTA has also been the most widely investigated method to predict clinical outcomes [26].

Platelet function analysis using Multiple Electrode Aggregometry (Multiplate®)-Dynabyte, Munich. Impedance aggregometry is conceptually similar to LTA, but it uses whole blood instead of platelet-rich plasma and platelet aggregation is measured by impedance, not light transmittance [27]. This recent method allows an easy and fast assessment of platelet function, with the possibility to decide on treatment regimens when the patient is still in the CathLab (results in 10 minutes). In present, Multiplate is used in many expert centers and pharmaceutical companies throughout Europe.

Receptor Expression

The resting and stimulated expression of activation-dependent receptors can be assessed by flow cytometry with monoclonal antibodies [8]. This technique was useful to assess pharmacologic effects. The most widely studied receptor includes P-selectin and GPIIb/IIIa. Platelet–leucocytes aggregates also have been measured as a marker of platelet activation, and they have been proposed as a more stable measure of acute coronary syndromes compared with P-selectin [27]. Major disadvantages are the complexity of the technique, which requires a high experienced laboratory staff and high costs.

Intracellular Signaling

The coupling of $P2Y_{12}$ to the inhibition of adenylate cyclase by an inhibitory G protein has been used to measure reactivity of the receptor in the presence of $P2Y_{12}$ inhibitors [31]. Vasodilator-stimulated phosphoprotein (VASP) is phosphorylated by protein kinases that are activated by cyclic adenosine monophosphate. With flow cytometry and methods to make the platelet membrane permeable, it is possible to quantify the amount of phosphorylated VASP by monoclonal antibodies as a measure of unblocked $P2Y_{12}$ [20].

Advantages include the specificity for the $P2Y_{12}$ signaling pathway and the stability of the method in comparison with aggregometry.

Point-of-care Assays

The VerifyNow method (Accumetrics, San Diego, California) uses arachidonic acid, adenosine diphosphate (ADP), or thrombin receptor-activating peptide (TRAP) to assess platelet responsiveness to aspirin, $P2Y_{12}$ inhibitors, or GPIIb/IIIa inhibitors, respectively [21]. The techique measures platelet aggregation with fibrinogen-coated beads and has been used to predict outcomes in patients undergoing percutaneous coronary intervention (PCI). Advantages of the VerfyNow include its ease of use and correlation with turbidimetric aggregometry.

In the thromboelastogram (TEG) Platelet Mapping technique (Haemoscope Corporation, Niles, Illinois), the contribution of arachidonic acid-induced platelet aggregation and ADP-induced aggregation to the overall tensile strength of a platelet-fibrin clot can be quantified and correlated with turbidimetric aggregometry [22]. The preparation of samples for thromboelastography is more complex than that for VerifyNow, but thrombelastography can provide coagulation measurements not possible with VerifyNow.

Rotational thromboelastometry ROTEM is available as point-of-care coagulation monitoring in an increasing number of European operating theatres and emergency rooms. The Platelet Mapping Assay has been described as a platelet aggregation assay for thromboelastography TEG. The aim of this experimental trial was to evaluate feasibility of the Platelet Mapping Assay on the ROTEM test system. Whole blood was drawn from 22 adult volunteers and patients with and without antiplatelet medication. Platelet aggregability was determined in three whole blood assays: the Platelet Mapping Assay using both activators arachidonic acid (AA) and adenosine diphosphate (ADP) on TEG, its adapted version on ROTEM, and the multiple electrode impedance aggregometer Multiplate.

MATERIALS AND METHODS

Several methods are used to analyse platelet function in whole blood. Recently, a new method for measuring whole blood impedance aggregometry was introduced based on a single-use test cell, with a total of four silver-coated electrodes that form two independent sensor units [28].

A new device to measure whole blood platelet aggregation has been developed, called multiple electrode platelet aggregometry (MEA).

Our aim was to evaluate MEA method for the measurement of platelet aggregation and platelet inhibition by aspirin in whole blood. Platelet aggregation induced by different concentrations of ADP, collagen and TRAP-6 and platelet inhibition by aspirin was

determined in hirudin-anticoagulated blood by MEA. MEA indicated that spontaneous platelet aggregation was lower, and stimulated platelet aggregation was higher in hirudin- than citrate-anticoagulated blood.

During a brief period of equilibration, as an alternating current is applied across the electrodes,a monolayer of platelets forms on the exposed portions of the exposed of the electrodes, resulting in a stable impedance value. An antiaggregating agent is added to the cuvette and stimulated platelets aggregate to the platelet monolayer on the immersed electrodes.The accumulation of platelet results in an increase in electrical resistance within the circuit (i.e. as platelet aggregate, the impedance increases). In impedance aggregometry, the extent and rate of aggregation are measured and quantified in ohms and ohms per minute (the measurement of electrical resistance). In MEA analysis the results are expressed in arbitrary “aggregation unit” (AU). The change in impedance is displayed as a function of time on a strip chart recorder or monitor using computer software. Formation of aggregates in the sample has no effect on the measured impedance unless they adhere to the electrodes. There are several factors that may affect the impedance: physical integrity of the electrode, sample temperature, stirring speed.

We used the Multiplate® analyser, manufacturer Dynabyte, Munich, Germany. The device has 5 channels for parallel tests, an easy to use Windows XP based software, with duplicate sensor for internal quality control, electronic pipetting and automatic analysis and documentation (Figure 1).

Figure 1. Multiplate Analyser

We followed the next steps in performing the measurement: we put the test cell into the measuring position, we attached the sensor cable, we added into the cuvette with the electronic pipette 300 µl of saline + 300 µl of blood (added usually hirudin), we allowed 3 minutes for warming and equilibration, we added the activator (ASPItest, ADP, TRAPtest-

reagents provided by manufacturer), and finally after 6 minutes we printed the results and discarded the test cell. The MEA parameters obtained by Multiplate are: aggregation (AU), the velocity, and the area under curve AUC (AU*min or U).

The result of the determination is displayed on the screen as a curve representing aggregation (AU) function of time (minutes). The 2 curves (tests 1, respective test 2) are corresponding on each electrode. The MEA parameters obtained by Multiplate are: aggregation (AU), the velocity, and the area under curve AUC (AU*min or U).

RESULTS

The group of study consisted of twenty-four adult volunteers who were enrolled in the study after signing the informed consent. The Institutional Ethics Committee of Timisoara Institute of Cardiovascular Diseases approved the protocol for this pilot study. Whole blood MEA was performed after stimulation with thrombin receptor activating peptide (TRAPtest, 32 muM) and arachidonic acid (ASPItest, 0.5 mM). Repeated measurement analysis of variance with a Bonferroni correction for multiple comparisons was performed to detect differences between time points. Assay imprecision was determined by calculating the coefficient of variation. The level of statistical significance was set to $P < 0.05$.

After blood drawing at baseline, 500 mg aspirin was administered to all volunteers. Blood samples were taken at 4, 24, 72 h after aspirin ingestion. At each time point, measurements were performed immediately at 30 and 60 min after drawing blood.

Our aim was to evaluate MEA method for the measurement of platelet aggregation and platelet inhibition by aspirin in whole blood. Platelet aggregation induced by different concentrations of arachidonic acid, ADP, collagen and TRAP-6 and platelet inhibition by aspirin were determined in hirudin-anticoagulated blood by MEA. Whole blood MEA was performed after stimulation with arachidonic acid (ASPItest, 0.5 Mm - Figure 2), thrombin receptor activating peptide (TRAPtest, 32 muM - Figure 3) and ADP test (Figure 4). Repeated measurement analysis of variance with a Bonferroni correction for multiple comparisons was performed to detect differences between time points. Assay imprecision was determined by calculating the coefficient of variation. The level of statistical significance was set to $P < 0.05$.

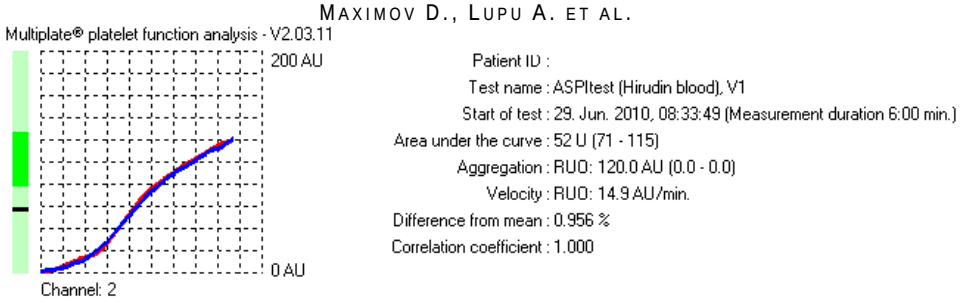

Figure 2. Sample of ASPItest result. Patient with normal aggregation response to Aspirin.

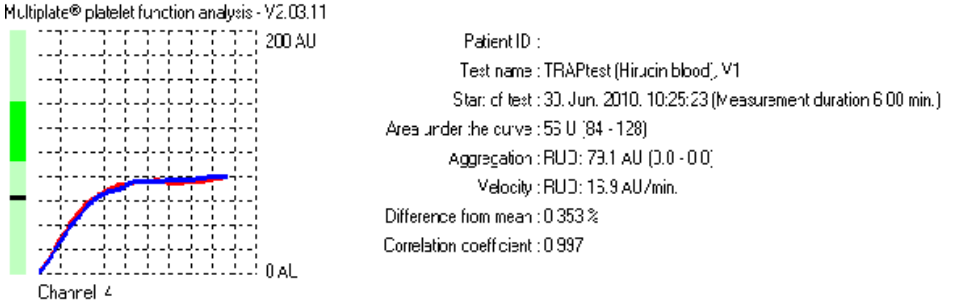

Figure 3. Sample of TRAP test result in a patient with normal aggregation response.

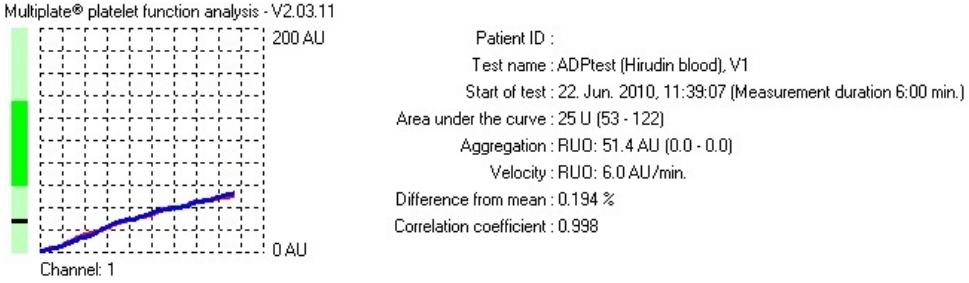

Figure 4. Sample of ADP test result

Analysis of ASPItest and TRAPtest in Aspirin treated patients was performed at 30 and 60 minutes after blood sampling. The result of regression analysis of the two determinations is showed in the Figure 5; a good reproducibility is shown.

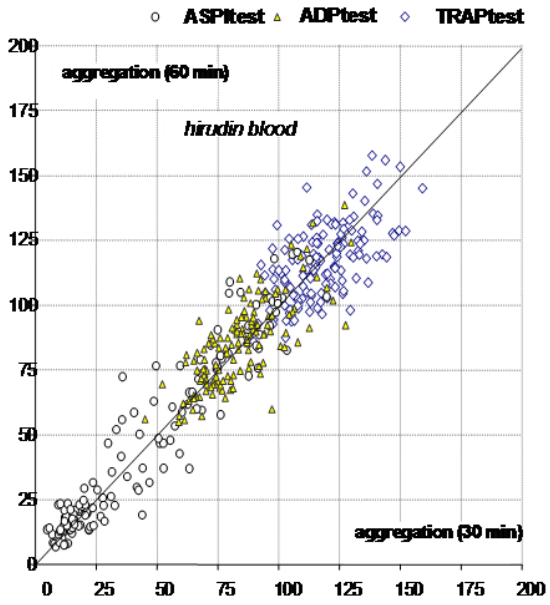

Figure 5. Regression analysis of the ASPI test, ADP test and TRAP test in the study group

DISCUSSION

ASPIRIN

Aspirin is one of the most commonly ingested over-the-counter drugs. In addition to its analgesic and antiinflammatory actions, it also potently inhibits platelet aggregation. Evaluation of aspirin-induced platelet dysfunction is relevant in various clinical situations, including during complex surgeries with high bleeding risk in individuals who have ingested aspirin. In this study, we examined the suitability of multiple electrode aggregometry (MEA) for time course assessment of the antiplatelet effects of a single oral dose of 500 mg aspirin. We also determined the applicability of this method in the point-of-care (POC) setting by comparing the results of the test after different time intervals after blood sampling.

Aspirin resistance is infrequent among patients undergoing elective PCI who are treated with 325 mg daily as assessed by arachidonic acid-induced platelet aggregation with LTA [9, 10]. The incidence of aspirin resistance seems to be highly assay-dependent and is rare when determined by methods that directly indicate the activity of COX-1 [11, 12].

Treatment non-compliance can also affect the identification of aspirin "resistance" [9, 10].

Aspirin resistance also might to be associated with concomitant clopidogrel "resistance" [12, 13]. Patients identified as aspirin-and clopidogrel-resistant have exhibited high platelet reactivity to collagen in addition to ADP and arachidonic acid stimulation [12-14]. Recent studies suggest a generalized high-platelet-reactivity phenotype that might be associated with an increased risk for ischemic events.

CLOPIDOGREL

The therapeutic response to clopidogrel has been most studied in patients undergoing PCI, and numerous studies have reported wide variations in response to therapy and rates of nonresponders of 5% to 44% [6-18]. Determination of VASP phosphorylation has also shown high residual reactivity of the P2Y12 receptor in selected patients treated with clopidogrel [24]. Differences in the prevalence of non-responder status in different studies might be related to differences in definitions (relative versus absolute change in aggregation, maximum versus late aggregation), laboratory methods, different dosages. Clopidogrel response variability has multiple proposed etiologies.

This pilot study on a small group of healthy patients was necessary prior to study of the variability of individual response of aggregation in patients with coronary atherosclerotic disease. This response of aggregation to Aspirin administration in healthy individuals was useful to compare the values with these obtained in other study group patients, i.e. on chronic administration of Aspirin 75-100 mg QD doses (usually), respective Clopidogrel 75 mg QD, in order to classify the patients in "responders" or non-responders" to Aspirin or Clopidogrel treatment according with the values obtained for AUC to ASPItest, ADPtest or TRAPtest using the MEA method by Multiplate analyser.

CONCLUSIONS

In conclusion, MEA is an easy, reproducible and sensitive method for measuring spontaneous and stimulated platelet aggregation, and evaluating antiplatelet drugs in whole blood. The use of hirudin as an anticoagulant is preferable to the use of citrate. MEA is a promising technique for experimental and clinical applications.

REFERENCES

1. Cattaneo M., "Aspirin and Clopidogrel. Efficacy, safety and the issue of drug resistance", *Arterioscler Thromb Vasc Biol*, **24** (2002) 1980-1987.
2. Patrono C., "Aspirin resistance: definition, mechanisms and clinical read-outs", *J Thromb Haemost* **1** (2003) 1710-1713.
3. Campbell C.L., Steinhubl S.R., "Variability in response to aspirin: do we understand the clinical relevance?", *J Thromb Haemost*, **3** (2005) 665-669.

4. Undas A., Brummel-Ziedins K.E., Mann K.G., "Antithrombotic properties of aspirin and resistance to aspirin: beyond strictly antiplatelet actions" *Blood,* **109** (2007) 2285-2292.
5. Cattaneo M., "Laboratory detection of 'aspirin resistance': what test should be use (if any)?", *Eur Heart J*, **28** (2007) 1673-1675.
6. Gurbel P.A., Becker C.R., Kenneth G.M., Steinhubl S.R., Michelson A.D., "Platelet function monitoring in patients with coronary artery disease", *J Am Coll Cardiol*, **50** (2007) 1901-1920.
7. Gurbel P.A., Tantry U.S., "Clopidogrel resistance?", *Thromb Res*, **120** (2007) 311-321.
8. Michelson A.D., "Platelet function testing in cardiovascular diseases", *Circulation*, **110,** (2004) e489-e493
9. Tantry U.S., Bliden K.P., Gurbel P.A., "Overestimation of platelet aspirin resistance detection by thrombelastograph platelet mapping and validation by conventional aggregometry using arachidonic acid stimulation", *J Am Coll Cardiol*, **46** (2005) 1705-1709.
10. Schwartz K.A., Schwartz D.E., Ghosheh K., Reeves M.J., Barber K., DeFranco A., "Compliance as a critical consideration in patients who appear to be resistant to aspirin after healing of myocardial infarction", *Am J Cardiol*, **95** (2005) 973-975.
11. Gurbel PA, Bliden K.P, Di Chiara J., et al., "Evaluation of dose- related effects of aspirin on platelet function: results from the Aspirin- Induced Platelet Effect (ASPECT) study", *Circulation*, **115** (2007) 3156-3164.
12. Pulcinelli F.M., Riondino S., Celestini A., et al., "Persistent production of platelet thromboxane A2 in patients chronically treated with aspirin", *J Thromb Haemost*, **3** (2007) 2784-2789.
13. Templin C., Schaefer A., Stumme B., Drexler H., von Depka M., "Combined aspirin and clopidogrel resistances associated with recurrent coronary stent thrombosis", *Clin Res Cardiol,* **95** (2006) 122-126.
14. Dichiara J., Bliden K.P., Tantry U.S., et al., "Platelet aspirin responsiveness measured by VerifyNow: correlation with multiple methods and identification of a high platelet reactivity phenotype", *Platelets* **18** (2007) 414-483.
15. Gurbel P.A., Bliden K.P., Hiatt B.L., O'Connor C.M., "Clopidogrel for coronary stenting: response variability, drug resistance, and the effect of pretreatment platelet reactivity", *Circulation* **107** (2003) 2908-2913.
16. Geisler T., Langer H., Wydymus M., et al., "Low response to clopidogrel is associated with cardiovascular outcome after coronary stent implantation", *Eur Heart J,* **27** (2006) 2420-2425.
17. Hochholzer W., Trenk D., Bestehorn H.P., et al., "Impact of the degree of peri-interventional platelet inhibition after loading with clopidogrel on early clinical outcome of elective coronary stent placement", *J Am Coll Cardiol*, **48** (2006) 1742-1750.
18. Buonamici P., Marcucci R., Migliorini A., et al., "Impact of platelet reactivity after clopidogrel administration on drug-eluting stent thrombosis", *J Am Coll Cardiol*, **49** (2007) 2312-2317.
19. Gurbel P.A., Bliden K.P., Guyer K., et al., "Platelet reactivity in patients and recurrent events post-stenting: results of the PREPARE POST-STENTING study", *J Am Coll Cardiol*, **46** (2005) 1820-1826.
20. Barragan P., Bouvier J.L., Roquebert P.O., et al., "Resistance to thienopyridines: clinical detection of coronary stent thrombosis by monitoring of VASP", *Catheter Cardiovasc Interv*, **59** (2003) 295-302.
21. Cuisset T., Frere C., Quilici J., Barbou F., Morange P.E., Hovasse T., Bonnet J.L., Alessi M.C.J., *Thromb Haemost*, **4** (2007) 542-549.
22. Gurbel P.A., Bliden K.P., Samara W., et al., "Clopidogrel effect on platelet reactivity in patients with stent thrombosis: results of the CREST study", *J Am Coll Cardiol*, **46** (2005) 1827-1832.
23. Ajzenberg N., Aubry P., Huisse M.G., et al., "Enhanced shear- induced platelet aggregation in patients who experience subacute stent thrombosis: a case- control study", *J Am Coll Cardiol* **45** (2005) 1753-1756.
24. Aleil B., Ravanat C., Cazenave J.P., Rochoux G., et al., "Flow cytometric analysis of intraplatelet VASP phosphorylation for the detection of clopidogrel resistance in patients with ischemic cardiovascular diseases", *J Thromb Haemost*, **3** (2005) 85-92.
25. Gurbel P.A., Kereiakes D.J., D'Alesandro M.R., Bahr R.D., et al., "Role of soluble and platelet-

bound P-selectin in discriminating cardiac from noncardiac chest pain at presentation in the emergency department", *Am Heart J,* **139** (2006) 320-328.
26. Gum P.A., Kottke-Marchant K., Poggio E.D., et al., "Profile and prevalence of aspirin resistance in patients with cardiovascular disease", *Am J Cardiol* **88** (2001) 230-235.
27. Ingerman –Wojenski C., Smith J.B., Silver M.J., "Evaluation of electrical aggregometry: comparison with optical aggregometry, secretion of ATP, and accumulation of radiolabeled platelets", *J Lab Clin Med* **101** (1983) 44-52
28. Geiger J., Brich J., Honig-Liedl P., et al., "Specific impairment of human platelet P2Y(AC) ADP receptor- mediated signalling by the antiplatelet drug clopidogrel Arterioscler", *Thromb Vasc Biol*, **19** (1999) 2007-2011