



Advantages and limitations of clopidogrel response testing methods

Prednosti i ograničenja metoda za testiranje odgovora na klopidogetrel

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Introduction

Clopidogrel is a thienopyridine that irreversibly inhibits platelet P2Y₁₂ receptors and adenosine diphosphate (ADP) mediated platelet aggregation. It is a prodrug that requires activation in the liver by cytochrome P450 enzymes (CYP3A4, CYP3A5, CYP1A2, CYP2C9, CYP2C19, and/or CYP2B6)¹.

Dual antiplatelet therapy with clopidogrel and aspirin has become the mainstay of treatment of patients with acute coronary syndromes undergoing percutaneous coronary intervention^{2–4}.

Clopidogrel response

Despite significant benefits of the combined antiplatelet treatment in large clinical trials, the occurrence of adverse ischemic events remains a serious clinical problem^{5–7}. Clinical trials have shown that 8%–10% of patients experience a recurrent cardiovascular event during the first year after acute coronary syndromes and 1%–3% an acute or subacute stent thrombosis after percutaneous coronary intervention^{8,9}.

A possible reason for these adverse events might be the fact that clopidogrel's antiplatelet effect is not uniform in all patients. Many studies have shown that individual response variability to this thienopyridine derivative and the prevalence of individuals, who are deemed to have an inadequate response to clopidogrel therapy, varies between 4% and 30%^{10–12}. Unfortunately, clopidogrel resistance itself is not yet clearly understood and there is no apparent consensus on the definition of clopidogrel resistance. Also,

there are significant differences between the platelet function tests used, agonist concentrations, and cut-off points. Nevertheless, the term clopidogrel resistance should generally be limited to those who fail to achieve a desired pharmacological response to drug therapy, rather than patients who experience recurrent ischemic events while on anti-thrombotic therapy¹³.

Monitoring of clopidogrel action

Until the early 1990's bleeding time was still considered as the most useful test for the detection of platelet function. Recently, many better tests have become available that may be used to assess the influence of antithrombotic drugs on platelet. Despite that, methodological variability within each technique makes it difficult to compare results, and it is associated with unclear role of platelet function testing in clinical practice.

In this paper we discuss about the most used tests in practice which examine platelet function and aggregation, which is indirect way to evaluate response to clopidogrel.

Light transmission aggregometry (LTA) has been regarded as the gold standard for assessing platelet function for more than two decades. Aggregation of platelets is traditionally measured in platelet rich plasma (PRP) using an optical aggregometer. Aggregation response is simulated by adding of an agonist (ADP for clopidogrel). Transmission amplifies when an agonist is added, platelets aggregate and light transmission increases. Results are presented in percentage, between 0 and 100, according to the degree of light transmission^{14,15}. Also, the rate of aggregation is measured.

LTA test is still widely used, but there are many disadvantages of that method including sample preparation. Methodology for preparation of PRP vary between different laboratories including various types of anticoagulants used (citrate or hirudin), different centrifugation speeds and times reported. Some authors argue that whole blood tests of platelet function are more reliable than PRP assays. In the latter case, isolated platelets are analyzed, which is not their physiological milieu. Also, PRP usually do not include all the platelets; the most active and larger platelets may be lost during centrifugation. In addition, other blood cells are present in the whole blood – erythrocytes and leukocytes, which also interfere with platelet aggregation. This technique is not standardized yet and different concentrations of ADP (5–20 μM) are used. LTA test takes too long, it is laboratory-based, blood samples should be sent as soon as possible to a laboratory, and it requires trained technicians. Also, LTA assay is not suitable to test on large number of samples, which is not convenient for routine clinical practice (Table 1) ^{15–17}.

ceptors. When platelets are activated, microbead aggregation is more rapid and reproducible; therefore, platelet activation is induced when the reagent ADP/prostaglandin E_1 (ADP/PGE1) is incorporated into the assay channel. The reagent is formulated to specifically measure P2Y_{12} -mediated platelet aggregation. Light transmittance increases as activated platelets bind and aggregate fibrinogen-coated beads. The instrument measures this change in optical signal and expresses results in P2Y_{12} Reaction Units (PRU) and a higher PRU reflects greater ADP mediated platelet reactivity. ADP is used to activate platelets by binding to the P2Y_{12} and P2Y_1 receptors, while PGE_1 is used to reduce the ADP-induced P2Y_1 activation which is contributed with increase of the assay sensitivity. This assay is the most commonly used method for monitoring of clopidogrel response in USA.

The Veritas study on 147 patients has shown that VerifyNow P2Y_{12} Assay is a fast and sensitive test for monitoring platelet inhibition during clopidogrel therapy ¹⁸. The main limitations of the test are well-known: it is a closed

Table 1

Available methods for monitoring of clopidogrel response (modified from ^{14–16})

Method	Advantages	Disadvantages
Light transmission aggregometry	• Gold standard • Predictive of outcomes	• Not standardized test (different reagents used, different reagents concentration, different instruments) • Sample preparation • Platelet Rich Plasma test • Time consuming (1h–3h)
VerifyNow P2Y_{12} Assay	• Point of-care (POC) assay • Whole blood test • Simple, fast, small sample volume • Three test cartridges • Widely used in USA • Predictive of outcomes	• Rigidity • Cartridge can only be used for single analysis • Closed system • One canal
Flow cytometry	• Whole blood test • Small blood volumes • Preparation methods flexible	• Specialized laboratories • Expensive instrument • Complex for routine monitoring Experienced operator
Multiple electrode aggregometry	• Point of-care (POC) assay • Whole blood test • Simple, fast, easy to learn • Five channels for parallel tests • Open system • Predictive of outcomes	• Relative new method • Lack of the corresponding number of studies • Comparison with other methods uncertain • No ready-made reagents

The *VerifyNow System* is a turbidimetric based optical detection system, which measures platelet-induced aggregation. The VerifyNow P2Y_{12} assay is a whole blood, fast and standardized point of care analyzer used to measure the level of platelet P2Y_{12} receptor blockade. The system consists of an instrument, a disposable assay device and quality control materials. The assay device contains a lyophilized preparation of human fibrinogen-coated beads, platelet activators, and buffer ¹⁴. A patient's sample is anticoagulated whole blood, which is automatically dispensed from a blood collection tube into an assay device by the instrument. The assay is based upon the ability of activated platelets to bind fibrinogen. Agglutination takes place once the activated platelets are exposed to the fibrinogen coated micro particles and in proportion to the number of the available platelet re-

system without the possibility of assay modification; cartridge can only be used for single analysis; delays in testing or difficulties regarding specimen collection may produce spurious results ¹⁹.

Since clopidogrel irreversibly inhibits ADP binding to the platelet P2Y_{12} receptor and prevents subsequent phosphorylation of vasodilator-stimulated phosphoprotein (VASP), the increase in VASP phosphorylation could be a useful marker of clopidogrel resistance ¹⁷. Standardized *flow cytometric VASP assay* is used for determination the VASP phosphorylation state of whole blood ²⁰. Flow cytometry is a powerful technique that simultaneously measures and then analyzes multiple physical characteristics of single particles, as they flow in a fluid stream through a beam of light. Parameters analyzed with such an assay include particle's rela-

tive size, relative granularity or internal complexity, and relative fluorescence intensity. These characteristics are determined using an optical-to-electronic coupling system that records how the cell or particle scatters incident laser light and emits fluorescence. The flow cytometric assay, like Verify Now System, uses combination of ADP and PGE¹ for analysis of the clopidogrel response. Blood samples were collected in 0.129 M sodium citrate vacutainer tubes and incubated with PGE1 alone or PGE¹ and ADP, before fixation with paraformaldehyde. After this procedure, platelets were permeabilized with non-ionic detergent and labeled with a monoclonal antibody 16C2, specifically directed against serine 239 phosphorylated VASP, followed by a staining reagent, polyclonal anti-mouse antibody IgG-FITC (fluorescein isothiocyanate). Platelet population was identified on its forward and side scatter distribution and 10,000 platelet events were gated. ADP receptor reactivity was calculated using mean fluorescence intensities (MFI) in the presence of PGE1 or PGE1+ADP according the corresponding formula. There is an inverse correlation between clopidogrel treatment efficacy and the ADP receptor reactivity ratio.

The main disadvantage of flow cytometry assay is that it needs suspension of single cells or other particles, with minimum clumps and debris. This means that tissue architecture and any information about the spatial relationship among different cells are lost when single cells or nuclei are prepared. Also, this test is too complex for routine monitoring of clopidogrel and requires experienced person who will perform analysis. Other limitations are total cost of the procedure, and interactions of drugs with VASP assay (e.g. drugs affecting intracellular cyclic adenosine monophosphate and/or nitric oxide (NO)/cyclic guanosine monophosphate (cGMP) signals may influence VASP phosphorylation)²¹.

Multiple electrode aggregometry (MEA) is a new technique for detection of platelet function. This method is performed to analyze platelet function in whole blood based on impedance aggregometry. In Europe, the multiplate assay is slowly but surely winning over other methods for monitoring of clopidogrel resistance. The system registers the electrical impedance change due to the aggregation of platelets on two independent electrode set surfaces in the test cuvette and the analysis are measured simultaneously on two sensor units-internal QC. The obtained impedance is transformed to arbitrary aggregation units (AU) that are plotted against time (Aumin)²². Like VerifyNow P2Y₁₂ and flow cytometry assay, MEA uses ADP and PGE1 as agonists. Main advantages of such a test involve the use of heparin and hirudin, because

these anticoagulants do not interfere with serum calcium, an important second messenger of platelet activation and aggregation. Also, MEA is rapid test; smaller samples of blood are needed; up to five parallel samples could be analyzed at the same time; electronic pipetting reduces operator errors and it is easy to learn. However, MEA has not been sufficiently tested in clinical settings as a relatively new method.

In general, there is a correlation between light transmission aggregometry ("gold standard") and other tests of platelet function, e.g. VerifyNow P2Y₁₂ assay, VASP assay and MEA²³⁻²⁶. In addition, Varenhorst et al.²⁷ reported that the VerifyNowP2Y₁₂ correlated strongly with inhibition of P2Y₁₂ receptors, as measured with either VASP or LTA. The only exception is the Platelet Function Analyzer PFA-100, a reliable test for monitoring of aspirin but not clopidogrel response. Gremmel et al.²⁸ investigated correlation between LTA and other tests in the same time. These authors evaluated clopidogrel response in 80 patients on combined antiplatelet therapy after coronary stent implantation – throw LTA, VerifyNowP2Y₁₂, VASP assay, MEA and Impact R measure of platelet inhibition. The results showed that all of these methods correlated significantly with LTA, where VerifyNowP2Y₁₂ had the strongest correlation. Despite significant correlation between LTA and VerifyNowP2Y₁₂, VASP assay, MEA and Impact R, registered sensitivities and specificities ranged from 55% to 35% and from 85% to 78.3%, respectively²⁸. Unfortunately, it was not emphasized in which patients this disagreement was detected. Similarly, Panicia et al.²⁹ showed that MEA significantly correlated with VerifyNowP2Y₁₂ with moderate agreement and 81.5% of concordant values. Also, a significant correlation was shown between MEA and LTA with good agreement and 88.8% of concordant values.

When Bland-Altman analysis was used instead of correlation, only a low agreement was found between light transmission aggregometry, whole-blood aggregometry, PFA-100 and VerifyNow P2Y₁₂ Assay (randomized, double-blind trial on 116 patients with stable coronary artery disease treated with clopidogrel)³⁰.

The importance of evaluating the (non)adequate response to clopidogrel could be seen in a possible relation with adverse cardiovascular events. Numerous studies have found that low response to clopidogrel is associated with an increased risk of ischemic events after percutaneous coronary intervention (PCI) (Table 2)^{22, 31-34}. In Excelsior study, which investigated platelet function in 802 patients, throw LTA, was shown that attenuated response to clopidogrel is

Table 2

Clinical implication of inadequate response to clopidogrel^{22, 31-34}

Study	Number of patients	Method	Results	Outcome
Hochholzer et al, 2006 ³¹	802	LTA	↑ Platelet aggregation	30-day MACE
Buonamici et al, 2007 ³²	804	LTA	↑ Platelet aggregation	Stent thrombosis
Patti et al, (ARMYDA PRO Study)2008 ³³	160	VerifyNow P2Y ₁₂	↑ PRU (4 th quartile)	30-day MACE
Sibbing et al, 2009 ²²	1608	MEA	↑ Platelet reactivity	Stent thrombosis
Siller-Matula et al, 2010 ³⁴	416	MEA	↑ Platelet reactivity	Stent thrombosis

MACE – major adverse cardiac events; ARMYDA-PRO – Antiplatelet Therapy for Reduction of Myocardial Damage during Angioplasty-Platelet Reactivity Predicts Outcome; PRU – platelet (P2Y₁₂) reaction units; LTA – light transmission aggregometry; MEA – multiple electrode aggregometry

an independent predictor of major adverse cardiac events (MACE)³¹. Also, by using LTA assay, Buonamici et al.³² found that nonresponsiveness to clopidogrel is a strong and independent predictor of stent thrombosis in patients receiving sirolimus- or paclitaxel-eluting stents. Other studies using other methods have also reported on correlation regarding testing response to clopidogrel and cardiovascular outcomes. In ARMYDA- PRO trial platelet reactivity was evaluated in 160 patients before PCI and at 8 h and 24 h after intervention with the VerifyNow P2Y12 assay. The results have shown that pre-PCI PRU levels in the fourth quartile were associated with 6-fold increase in risk of 30-day MACE³³. Sibbing et al.²² used MEA to detect the response to clopidogrel. The authors found that a low response to this thienopyridine is to a significant level associated with higher risk of stent thrombosis. The predictive value of MEA was also pointed on by Siller-Matula et al.³⁴ who have examined platelet reactivity in their prospective study with 416 patients by the use of MEA and VASP assay. These results have shown that MEA can predict stent thrombosis better, than the VASP assay.

However, a prospective cohort study called Popular involved 1,069 patients subjected to intracoronary stent implantation and treated with clopidogrel. The primary endpoint (composite of all-cause death, nonfatal acute myocardial infarction, stent thrombosis, and ischemic stroke) occurred more frequently in patient with high on-treatment platelet reactivity detected by the LTA, VerifyNow P2Y12, and Plateletworks assay (11.7%, 13.3%, and 12.6%). Despite a significant association between those tests and the primary endpoint, their predictive accuracy was only modest³⁵.

We found no significant difference in one-year mortality between good and bad responders to clopidogrel in our open, prospective, controlled study on 52 participants (VASP assay)¹². Also, myocardial infarction and/or revascularization did not occur in good or bad responders during a follow-up period of one year. However, an insufficient number of participants and open design preclude firm conclusion from those data.

Finally, the role of a reduced platelet response to clopidogrel in patients with certain comorbidities (e.g. type 2 diabetes) as compared with platelet function tests still remains to be clarified³⁶.

Conclusion

Thienopyridine (clopidogrel) resistance still remains to be clarified. More well-designed clinical trials with a sufficient number of participants are needed in order to draw valid conclusions. The very first step in clopidogrel resistance problem solving is to establish a unique platelet aggregation test which is reliable, effective, simple and low cost. Also, it would be useful to establish cut-off values for high on-treatment platelet reactivity that provides accurate prognostic information for high-risk patients subjected to intracoronary stent implantation.

The greatest benefit from the determination of response to clopidogrel should have patients who need a long-term usage of this drug. Based on the response to clopidogrel is possible to decide on further course of treatment, in other words, patients with inadequate response to the drug can receive either a higher dose of clopidogrel or switch to another antiplatelet drug.

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