Am J Transl Res 2019;11(9):5332-5337
www.ajtr.org /ISSN:1943-8141/AJTR0101207

Review Article

Monitoring of biological response to clopidogrel after treatment for non-cardioembolic ischemic stroke or transient ischemic attack

Jérôme Varvat[1,2], Aurélie Montmartin[2], Magali Epinat[1,2], Sandrine Accassat[3,4], Arnauld Garcin[5], Guorong Li[2], Pierre Garnier[1,2], Claude Lambert[6], Patrick Mismetti[1,2,3,5], Nora Mallouk[2,3,7]

[1]Neurovascular Unit, Saint-Etienne University Hospital Center, North Hospital, Saint-Etienne F-42055, France; [2]University of Lyon, UJM - Saint-Etienne, Inserm, Sainbiose U1089, Saint-Etienne F-42023, France; [3]Department of Vascular and Therapeutic Medicine, Saint-Etienne University Hospital Center, North Hospital, Saint-Etienne F-42055, France; [4]Inserm, CIC1408, Saint-Etienne F-42055, France; [5]Clinical Research, Innovation and Pharmacology Unit, Saint-Etienne University Hospital Center, North Hospital, Saint-Etienne F-42055, France; [6]Immunology Department, Saint-Etienne University Hospital Center, North Hospital, Saint-Etienne F-42055, France; [7]University of Lyon, UJM-Saint-Etienne, CMES, Saint-Etienne F-42023, France

Received August 20, 2019; Accepted August 30, 2019; Epub September 15, 2019; Published September 30, 2019

Abstract: Background and purpose: Biological response to clopidogrel prescribed after a non-cardioembolic ischemic stroke or transient ischemic attack (TIA) has been little studied. The aim of our study (AAPIX) was to assess this response and investigate the agreement between different biological assays in revealing poor responders. Methods: Patients hospitalized following a non-cardioembolic ischemic stroke or transient ischemic attack (TIA) and prescribed clopidogrel were consecutively included from September 2013 to November 2015 in the Stroke Center of Saint-Etienne Hospital. Blood was drawn after 5 to 8 days of standard-dose clopidogrel. Light transmission aggregometry (LTA) and flow cytometric assays, using vasodilator-stimulated phosphoprotein [VASP] and CD62P, were accomplished for all patients. Transmission electron microscopy (TEM) was performed for a poor clopidogrel-responder and for a patient with discordant platelet assay results (platelet reactivity index (PRI) >50% and maximum platelet aggregation <70%), after activation with adenosine diphosphate (ADP) 10 µM. Results: 72 patients were included. According to LTA, VASP assay and CD62P test results, 65%, 71% and 0% of patients, respectively, had a low response to clopidogrel, indicating poor agreement between these assays. Images of ADP-activated platelet samples from a patient manifesting a low response to clopidogrel and from a patient with discordant platelet assay results showed an ultrastructural pattern typical of activation and a state of slight activation, respectively. Conclusions: Platelet function results obtained using different assays for patients having experienced a non-cardioembolic ischemic stroke or TIA were discordant. Transmission electron microscopy could be useful in certain clinical contexts when platelet function assay results disagree.

Keywords: Clopidogrel, VASP assay, light transmission aggregometry, CD62P test, ischemic stroke

Introduction

Poor biological response to clopidogrel has been extensively studied in cardiovascular patients [1], but the results of the ARCTIC and ANTARCTIC clinical trials recently called into question the therapeutic value of personalized antiplatelet therapy monitored by platelet function assays [1, 2].

The choice of therapeutic strategy for patients hospitalized after a non-cardioembolic ischemic stroke or a transient ischemic attack (TIA) is limited and based solely on the prescription of an antiplatelet agent. Aspirin is the first-line treatment following a non-cardioembolic ischemic stroke but if its use is contraindicated, clopidogrel [3, 4] can be prescribed instead. In this specific clinical setting, monitoring of platelet response to clopidogrel remains pertinent. Publications concerning poor biological response to clopidogrel after ischemic stroke are scarce [5-14] and the optimal biological assay has not yet been found. Platelet ultrastructure

has been studied in acute ischemic stroke patients [15] using transmission electron microscopy (TEM) and gives a reliable estimation of the state of platelet activation based on platelet shape changes and aggregation.

The aim of our study was to assess poor biological response to clopidogrel in non-cardioembolic ischemic stroke or TIA patients using three different platelet function assays and to evaluate the agreement among these assays. We also evaluated platelet activation using TEM for a patient responding poorly to clopidogrel and for a patient with discordant results according to light transmittance aggregometry (LTA) and the VASP assay.

The study was registered at ClinicalTrials.gov (no. NCT01955642) and approved by the French health authorities and the local ethics committee. A signed consent form was provided by each patient included.

Materials and methods

Patients

Consecutive patients hospitalized in the Neurovascular Unit of Saint-Etienne University Hospital Center following a non-cardioembolic ischemic stroke or TIA and prescribed treatment with clopidogrel alone were prospectively included between September 2013 and November 2015 in the Stroke Center of Saint-Etienne Hospital.

Clopidogrel (Plavix 75 mg; Sanofi Pharma Bristol-Myers Squibb SNC, Paris, France) was prescribed at the standard dose of 75 mg per day.

A full description of the study population is provided in a previous publication [16].

Evaluation of the biological response to clopidogrel using LTA and flow cytometric assays

The LTA and VASP assay methodologies have been previously described [16, 17].

For the CD62P assay, platelets from platelet-rich plasma (PRP) were activated with ADP 10 µM for 10 minutes, then fixed with Thrombofix Platelet Stabilizer (BD, Galway, Ireland). Platelets were identified by means of a monoclonal antibody directed against CD61 and conjugated with PECy7 (CD61-PECy7, clone SZ21, Beckman Coulter). Activated platelets were labeled with an anti-human monoclonal antibody against CD62P and conjugated with phycoerythrin (CD62P-PE, clone CLB-Thromb/6, Beckman Coulter). Aliquots (10 µl) of fixed activated PRP were incubated in polyethylene tubes (Falcon, BD Bioscience, Le Pont de Claix, France) for 15 minutes with 5 µl of CD62P-PE and 10 µl of CD61-PC7 in a dark room at room temperature. The reaction was stopped by adding 500 µL of phosphate-buffered saline (PBS) and the samples were immediately analyzed using a Navios flow cytometer (Beckman Coulter). The percentage of CD62P-positive platelets was determined after activation with ADP and expressed as the geometric mean value using the Excel Geomean function.

Definitions of low biological response to clopidogrel according to the biological assay

LTA: maximum percentage platelet aggregation >70% following activation by 10 µM ADP.

VASP assay: PRI >50%.

CD62P assay: percentage of CD62P-positive platelets >90%.

For LTA and the VASP assay, published threshold values were used [1]. The threshold value for the CD62P test corresponded to the median value determined for 10 healthy donors from the regional branch of the French Blood Transfusion Service (EFS Auvergne-Loire, Saint-Etienne, France).

Transmission electron microscopy (TEM)

Platelet-rich plasma samples from a poor responder to clopidogrel and from a patient with discordant clopidogrel response values in LTA and the VASP assay were fixed at rest and after 10-minute activation with 10 µM ADP, treated with OsO4, dehydrated in alcohol baths of increasing concentration and embedded in epoxy resin. Ultra-thin sections were cut and treated with uranyl acetate and lead citrate. Sections were examined at high magnification using a transmission electron microscope (H-800, Hitachi) at 100 KV.

Statistical analysis

The linear regression between two variables was assessed using Pearson's test.

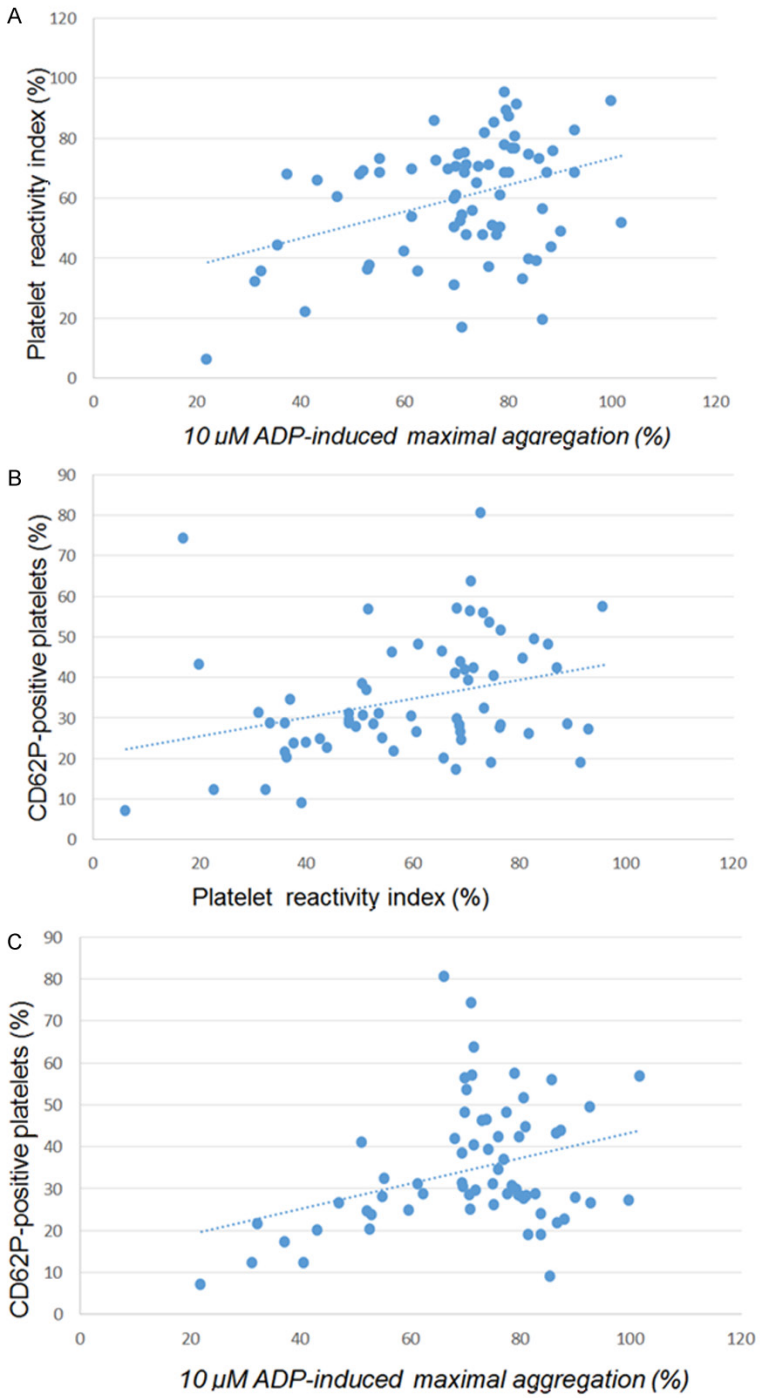

Figure 1. Linear regression coefficients determined for comparisons between light transmittance aggregometry, VASP assay and CD62P assay results: R2 values of 0.14, 0.09 and 0.1, respectively, were obtained for VASP assay vs. LTA results (A), for CD62P assay vs. VASP assay results (B) and for CD62P assay vs. LTA results (C) with statistically significant (<0.05) *P* values of 0.001, 0.001 and 0.006, respectively.

Correlation coefficients (R2) and *P*-values were calculated with a significance threshold of <0.05 using Microsoft Excel.

Results

Seventy-two patients were included in the study. Using LTA, 47 (65%) patients were classified as poor responders to clopidogrel and 25 (35%) as good responders.

The VASP assay discriminated 51 (71%) patients as poor responders to clopidogrel and 21 (29%) as good responders. 15 patients showed discordant platelet assay results, with a PRI >50% in the VASP assay, but a maximal aggregation of <70% using LTA. No patient was identified as a poor responder to clopidogrel with the CD62P assay.

Overall, there was poor agreement between the results of LTA, the VASP assay and the CD62P (CD62P) assay (**Figure 1**). Linear regression analyses yielded R2 values of 0.14, 0.09 and 0.1 respectively, for the VASP assay vs. LTA (**Figure 1A**), the CD62P assay vs. the VASP assay (**Figure 1B**) and the CD62P assay vs. LTA (**Figure 1C**) with statistically significant *P* values (<0.05) of 0.001, 0.001 and 0.006, respectively.

TEM images from patients treated with clopidogrel are displayed in **Figure 2**. Platelet samples were examined before activation (in the resting state) and after activation by 10-minute exposure to 10 µM ADP. Ultrastructural analysis of a resting platelet sample from a patient presenting discordant assay results (a PRI >50% in the VASP assay, but a maximal aggregation of <70% using LTA) showed a discoid shape, the presence of alpha and delta granules and a dense tubular system (**Figure 2A**). TEM images of a platelet sample from this

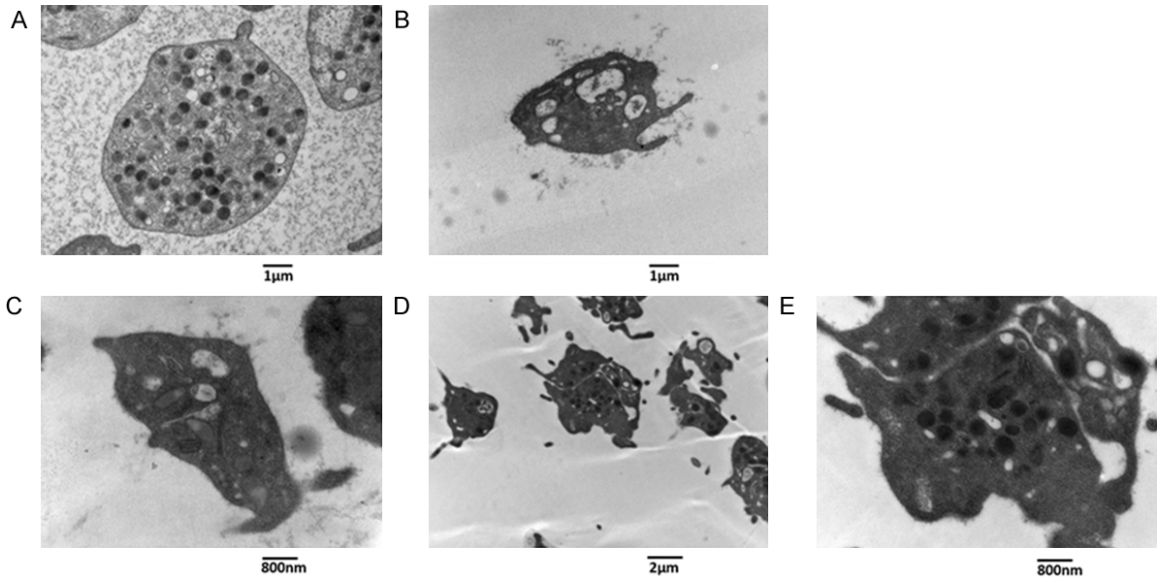

Figure 2. Transmission electron microscopy images of citrated platelet samples from patients treated with clopidogrel. An accelerating voltage of 100 kV was chosen. Ultrastructural patterns of platelet samples at rest (A and C), platelet samples activated by exposure to 10 µM ADP for 10 minutes (B, D and E), comprising platelet samples from a poor responder to clopidogrel (C-E), and platelet samples from a patient with discordant assay results (A, B). Magnification: (A, B) × 20,000, (C, E) × 30,000, (D) × 10,000.

patient after exposure to ADP (**Figure 2B**) revealed a state of slight activation manifested by the presence of pseudopodia and a dilated open canalicular system (OCS).

TEM images from a resting platelet sample from a poor biological responder to clopidogrel showed moderate activation, with reorganization of the OCS and granule centralization (**Figure 2C**). Images obtained after platelet exposure to ADP showed strong platelet activation, with the formation of platelet aggregates and the presence of cell projections visible at both low and high magnification (**Figure 2D** and **2E**).

Discussion

Surprisingly, numerous patients were classified as poor responders to clopidogrel according to both LTA and VASP assays. This could be explained by the prescription of a single antiplatelet agent rather than dual antiplatelet therapy in view of the bleeding risk associated with ischemic stroke. Furthermore, in the acute phase of non-cardioembolic ischemic stroke, platelets are highly activated and substantial percentages of poor responders to clopidogrel have been reported [18-20]. The cut-off value defining poor response is probably too low for patients having experienced ischemic stroke or TIA.

Interestingly, no patient was classified as a poor responder to clopidogrel according to the CD62P assay. One explanation for this result could be the choice of an inappropriate cut-off value for poor response in the context of ischemic stroke. The cut-off value in the CD62P assay was calculated from values obtained in healthy volunteers under the same experimental conditions as those used for our patients. Several cut-off values have been published [18-20] but we did not choose any of these because the experimental conditions employed were different from ours, involving retreatment of the platelets with PGE1 and use of a different flow cytometer.

The poor correlation between the different assays in our study could be explained by their assessment of different mechanisms of platelet activation. LTA reflects platelet aggregation due to conformational changes in platelet membrane GPIIb/IIIa glycoproteins leading to increased affinity for fibrinogen. The VASP assay evaluates P2Y12 receptor activity, while the CD62P assay measures the externalization of CD62P from alpha granules to the platelet membrane during platelet activation. The use

of multiple assays to identify a truly poor responder is recommended [21].

The TEM images of ADP-treated platelets from a poor responder to clopidogrel and from a patient with discordant clopidogrel response values in the LTA and VASP assay provide valuable information, as platelet ultrastructure reflects the state of platelet activation. A low response to clopidogrel is clearly indicated by the pattern of platelet activation during clopidogrel treatment revealed by this technique. TEM could therefore be used when biological assays are not concordant and not conclusive for specific clinical cases. This approach could be very helpful for patient care. These preliminary results warrant more extensive studies in a new clinical trial.

Conclusions

TEM could be particularly useful in specific clinical contexts when the results of different platelet function assays are discordant.

This transverse study involved a clinical and a research team. The manuscript was written by NM, with revision of English by GL. AM and NM were responsible for assay analysis. AG was in charge of clinical research management. JV, SA and ME took care of patient recruitment. PG and PM supervised the clinical and pharmacological aspects of the study. The clinical team was also involved in interpretation of the results.

Acknowledgements

This work was supported by Saint-Etienne University Hospital grants (1208094, AOL 2012).

Disclosure of conflict of interest

None.

Address correspondence to: Dr. Nora Mallouk, Faculty of Medicine, Centre de Microscopie Electronique Stéphanois (CMES), Campus Santé Innovations, 10 rue de la Marandière, Saint-Priest-En-Jarez 42270, France. Tel: +33 4 77 42 14 34; E-mail: nora.mallouk@univ-st-etienne.fr

References

[1] Mallouk N, Labruyère C, Reny JL, Chapelle C, Piot M, Fontana P, Gris JC, Delavenne X, Mismetti P, Laporte S. Prevalence of poor biological response to Clopidogrel: a systematic review. Thromb Haemost 2012; 107: 494-506.

[2] Collet JP, Cuisset T, Rangé G, Cayla G, Elhadad S, Pouillot C, Henry P, Motreff P, Carrié D, Boueri Z, Belle L, Van Belle E, Rousseau H, Aubry P, Monségu J, Sabouret P, O'Connor SA, Abtan J, Kerneis M, Saint-Etienne C, Barthélémy O, Beygui F, Silvain J, Vicaut E, Montalescot G; ARCTIC Investigators. Bedside monitoring to adjust antiplatelet therapy for coronary stenting. N Engl J Med 2012; 367: 2100-2109.

[3] Cayla G, Cuisset T, Silvain J, Leclercq F, Manzo-Silberman S, Saint-Etienne C, Delarche N, Bellemain-Appaix A, Range G, El Mahmoud R, Carrié D, Belle L, Souteyrand G, Aubry P, Sabouret P, du Fretay XH, Beygui F, Bonnet JL, Lattuca B, Pouillot C, Varenne O, Boueri Z, Van Belle E, Henry P, Motreff P, Elhadad S, Salem JE, Abtan J, Rousseau H, Collet JP, Vicaut E, Montalescot G; ANTARCTIC investigators. Platelet function monitoring to adjust antiplatelet therapy in elderly patients stented for an acute coronary syndrome (ANTARCTIC): an open-label, blinded-endpoint, randomised controlled superiority trial. Lancet 2016; 388: 2015-2022.

[4] Cattaneo M. The platelet P2Y12 receptor for adenosine diphosphate: congenital and drug-induced defects. Blood 2011; 117: 2102-2112.

[5] Fong J, Cheng-Ching E, Hussain MS, Katzan I, Gupta R. Predictors of biochemical aspirin and clopidogrel resistance in patients with ischemic stroke. J Stroke Cerebrovasc Dis 2011; 20: 227-230.

[6] Fukuoka T, Furuya D, Takeda H, Dembo T, Nagoya H, Kato Y, Deguchi I, Maruyama H, Horiuchi Y, Tanahashi N. Evaluation of clopidogrel resistance in ischemic stroke patients. Intern Med 2011; 50: 31-35.

[7] Depta JP, Fowler J, Novak E, Katzan I, Bakdash S, Kottke-Marchant K, Bhatt DL. Clinical outcomes using a platelet function-guided approach for secondary prevention in patients with ischemic stroke or transient ischemic attack. Stroke 2012; 43: 2376-2381.

[8] Kinsella JA, Tobin WO, Cox D, Coughlan T, Collins R, O'Neill DMurphy RP, McCabe DJ. Prevalence of ex vivo high on-treatment platelet reactivity on antiplatelet therapy after transient ischemic attack or ischemic stroke on the PFA-100(®) and VerifyNow(®). J Stroke Cerebrovasc Dis 2013; 22: e84-e92.

[9] Zhou BR, Shi HT, Wang R, Zhang M, Guan HT, Liu ZF, Deng YH. Dynamic changes and associated factors of clopidogrel resistance in patients after cerebral infarction. J Neurol 2013; 260: 2928-2937.

[10] Meves SH, Schröder KD, Endres HG, Krogias C, Krüger JC, Neubauer H. Clopidogrel high-on-treatment platelet reactivity in acute ischemic stroke patients. Thromb Res 2014; 133: 396-401.
[11] Lundström A, Laska AC, Von Arbin M, Jörneskog G, Wallén H. Glucose intolerance and insulin resistance as predictors of low platelet response to clopidogrel in patients with minor ischemic stroke or TIA. Platelets 2014; 25: 102-110.
[12] Qiu LN, Wang L, Li X, Han RF, Xia XS, Liu J. Predictive value of high residual platelet reactivity by flow cytometry for outcomes of ischemic stroke patients on Clopidogrel therapy. J Stroke Cerebrovasc Dis 2015; 24: 1145-1152.
[13] Maruyama H, Fukuoka T, Deguchi I, Ohe Y, Kato Y, Horiuchi Y, Hatashi T, Nagamine Y, Sano H, Tanahashi N. Response to clopidogrel and its association with chronic kidney disease in noncardiogenic ischemic stroke patients. Intern Med 2014; 53: 215-219.
[14] Maruyama H, Fukuoka T, Deguchi I, Ohe Y, Horiuchi Y, Kato Y, Sehara Y, Nagamine Y, Sano H, Hayashi T, Tanahashi N. Relationship between smoking and responsiveness to clopidogrel in non-cardiogenic ischemic stroke patients. Intern Med 2014; 53: 2575-2579.
[15] Joseph R, Riddle JM, Welch KM, D'Andrea G. Platelet ultrastructure and secretion in acute ischemic stroke. Stroke 1989; 20: 1316-1319.
[16] Varvat J, Epinat M, Montmartin A, Accassat S, Boutet C, Garcin A, Li G, Malergue F, Chapelle C, Laporte S, Garnier P, Lambert C, Mallouk N, Mismetti P. Role of platelet α2-adrenoreceptor in biological low response to Clopidogrel for patients with non cardioembolic ischemic stroke or transient ischemic attack. Am J Transl Res 2018; 15; 10: 2712-2721.
[17] Mallouk N, Varvat J, Berger A, Epinat M, Accassat S, Garcin A, Montmartin A, Li G, Garnier P, Mismetti P, Lambert C. Assessment of a flow cytometry technique for studying signaling pathways in platelets: monitoring of VASP phosphorylation in clinical samples. Pract Lab Med 2018; 11: 10-18.
[18] Godino C, Mendolicchio L, Figini F, Latib A, Sharp AS, Cosgrave J, Calori G, Cera M, M, Chieffo A, Castelli A, Maseri A, Ruggeri ZM, Colombo A. Comparison of VerifyNow-P2Y12 test and Flow Cytometry for monitoring individual platelet response to clopidogrel. What is the cut-off value for identifying patients who are low responders to clopidogrel therapy? Thromb J 2009; 7: 4.
[19] Fox SC, May JA, Dovlatova N, Glenn JR, Johnson A, White AE, Radhakrishnan A, Heptinstall S. How does measurement of platelet CD62P compare with other methods of measuring platelet function as a means of determining the effectiveness of antiplatelet therapy? Platelets 2019; 30: 290-295.
[20] Gremmel T, Koppensteiner R, Panzer S. Comparison of aggregometry with flow cytometry for the assessment of agonists'-induced platelet reactivity in patients on dual antiplatelet therapy. PLoS One 2015; 10: e0129666.
[21] Lemesle G, Landel JB, Bauters A, Delhaye C, Bonello L, Sudre A, Susen S, Bauters C, Lablanche JM. Poor agreement between light transmission aggregometry, Verify Now P2Y12 and vasodilatator-stimulated phosphoprotein for clopidogrel low-response assessment: a potential explanation of negative results of recent randomized trials. Platelets 2014; 25: 499-505.