CLINICAL RESEARCH

Clopidogrel response: Head-to-head comparison of different platelet assays to identify clopidogrel non-responder patients after coronary stenting

Réponse au clopidogrel : comparaison de différents tests plaquettaires dans l’identification des patients non répondeurs après angioplastie coronaire

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Summary

Objectives. — We investigated the agreement between different platelet tests to identify clopidogrel non-response.

Background. — Biological definition of clopidogrel non-response remains controversial. Different platelet tests have been linked with recurrent ischemic events and proposed for daily practice.

Methods. — We prospectively investigated the agreement of platelet tests to isolate clopidogrel non-response in patients receiving high 150 mg clopidogrel maintenance dose after coronary stenting. Clopidogrel response was assessed with ADP-induced aggregation (ADP-Ag) (non-response if > 70%), Platelet reactivity index VASP (PRI VASP) (non-response if > 50%) and Verify Now Point-of-care assay (VN) (non-response if PRU > 240 AU).

Results. — Seventy consecutive patients were included. The rates of non-responders were respectively: 13% (n = 9) with the ADP-Ag, 39% (n = 27) with the PRI VASP and 33% (n = 23) with the VN. We observed significant correlation between different platelet tests assessing clopidogrel response: r = 0.55 (p < 0.0001) for ADP-Ag and PRI VASP, r = 0.64 (p < 0.0001) for ADP-Ag and
Introduction

Several studies have reported interindividual variability in platelet response to clopidogrel [1] with clinical relevance [2–9]. However, no method of quantification of platelet function inhibition by clopidogrel has consensually been recommended. There is a clinical need to have a reliable platelet assay for measuring platelet function after antiplatelet therapy for monitoring and potentially tailoring antiplatelet dosing regimens to individual patients. Light transmittance platelet aggregometry remains the gold standard for platelet function assessment, and elevated platelet activation in the context of impaired response to clopidogrel using this technique has been associated with recurrent ischemic events [2–4]. For response to clopidogrel, classical aggregometry has been criticized for poor reproducibility and lack of specificity for the P2Y12 pathway. A new flow cytometric vasodilator-stimulated phosphoprotein (VASP) phosphorylation assay has been introduced to measure specific inhibition of clopidogrel’s biochemical target via the P2Y12 receptor. The platelet reactivity index VASP has been also associated with recurrent ischemic events after PCI [5,6]. These laboratory tests demand specific knowledge and skills, requires specialized equipments, and is labor-intensive. Recently, point-of-care assay as Verify Now system was introduced to assess clopidogrel response with clinical relevance [7–9]. However, there is not yet a consensus, neither about the “gold standard” test, nor about the definition of non-response. We therefore designed a prospective study to assess the agreement between the most common threshold of ADP-Ag, PRI VASP and Verify Now point-of-care test to detect clopidogrel non-responders among clopidogrel-treated patients undergoing coronary stenting for Non ST Elevation Acute coronary Syndrome (NSTE ACS).

Methods

Study protocol

Consecutive patients admitted for NSTE ACS in our institution were eligible for this prospective study after successful coronary stenting. NSTE ACS was defined as clinical symptoms compatible with acute myocardial ischemia within 12 h before admission and at least one of the following: a new finding of ST changes at least two leads, elevated levels of cardiac markers or coronary artery disease as documented by a history of revascularization or myocardial infarction. The exclusion criteria were a history of bleeding diathesis, persistent ST elevation ACS, NYHA class IV, PCI or coronary bypass grafting (CABG) < 3 months, contraindications to antiplatelet therapy, platelet count < 100 G/L, and creatinin clearance < 25 mL/min. Patients received oral loading doses
of 250 mg aspirin and 600 mg clopidogrel at least 12 hours before stenting. At discharge, all patients received aspirin 75 mg and clopidogrel 150 mg and assessment of clopidogrel response was performed at 1 month. The study protocol was approved by the ethics committee of our institution, and patients gave written informed consent for participation.

**Blood samples and platelet parameters**

Blood samples for testing platelet reactivity were drawn one month after discharge from a peripheral blood sample.

**ADP-Induced platelet aggregation (ADP-Ag)**

The blood-citrate mixture was centrifuged at 120 g for 5 min. The resulting platelet rich plasma (PRP) was kept at room temperature for use within 1 h. The platelet count was determined in the PRP sample and adjusted to 2.5 × 10^9 mL^-1 with homologous platelet-poor plasma (PPP). Platelets were stimulated with ADP (10 μmol/L) and aggregation was assessed with a PAP4 Aggregometer (Biodata Corporation, Wellcome, Paris, France). Aggregation was expressed as the percentage change in light transmittance from baseline with PPP as reference. Here, we report data on maximal intensity of platelet aggregation. We performed one measurement for each sample. The coefficient of variation of maximal intensity of platelet aggregation with ADP was measured at 6.5%. Non-response to clopidogrel was defined as ADP-Ag > 70%, as previously proposed [2,4].

**Platelet Reactivity Index VASP (PRI VASP)**

To determine the VASP phosphorylation state of whole blood, we used a standardized flow cytometric assay (Platelet VASP®; Diagnostica Stago [Biocytex], Asnières, France), which is an adaptation of the method of Schwarz et al. [11]. A platelet reactivity index (PRI VASP) was calculated from the median fluorescence intensity (MFI) of samples incubated with PGE1 or PGE1 and ADP according to the formula: PRI VASP = [MFI (PGE1) − MFI (PGE1 + ADP)/MFI(PGE1)] × 100. Non-response to clopidogrel was defined as PRI VASP > 50%, as previously proposed [5].

**Point of care Verify Now Assay**

We used the VerifyNow P2Y12 (Accumetrics, San Diego, CA, USA) point-of-care system, a rapid platelet-function cartridge-based assay with specific cartridges for the P2Y12 pathway. The Verify Now P2Y12 is designed to directly measure the effects of drugs on the P2Y12 receptor. VerifyNow-P2Y12 assay uses prostaglandin E1 in addition to ADP to increase intraplatelet cAMP. VerifyNow-P2Y12 assay results are expressed in P2Y12 Reaction Units (PRU) and % inhibition P2Y12 from baseline activation via TRAP (% inhibition P2Y12). The Verify Now analyser is designed to measure this agglutination as an increase in light transmittance. We used the PRU to define non-response to clopidogrel with a PRU > 240 AU [8,9].

**Statistical analysis**

Statistical analysis was performed with the SAS Software (v 8.01; SPSS Inc., Chicago, IL, USA). Continuous variables are expressed as mean ± S.D. or median and interquartile range. Categorical variables are expressed as frequencies and percentages. The Wilcoxon rank-sum test was used to compare continuous variables. Comparison between categorical variables were performed using the v2-test or the Fisher’s exact test when frequencies were below five. The agreements between ADP-Ag, PRI VASP and PRU were determined by linear regression (Pearson’s correlation coefficient) and Bland-Altman analysis. For each comparison, agreement between the two tests was calculated using the kappa statistic. A kappa statistic value of < 0.40 represents poor-to-fair agreement, a value of 0.41–0.60 reflects moderate agreement, a value of 0.61–0.80 is considered substantial agreement, and a kappa value of 0.81–1.00 is considered excellent agreement. P < 0.05 was considered significant.

**Results**

A total of 70 consecutive patients who fulfilled the enrolment criteria were prospectively included. Demographic data of the studied population are summarized in Tables 1. Platelet testing was performed in all the patients after one month, receiving 150 mg clopidogrel.

**Clopidogrel response with different tests**

The mean values of platelet parameters were: 53 ± 17% for ADP-Ag, 42 ± 19% for PRI VASP and 199 ± 104 for the PRU with the Verify Now assay. The rates of non-responders with different tests were respectively: 13% (n = 9) with the ADP-Ag (ADP-Ag > 70%), 39% (n = 27) with the PRI VASP (PRI VASP > 50%) and 33% (n = 23) with the Verify Now Assay (PRU > 240). The demographic, clinical and therapeutic parameters were similar between responders and non-responders whatever test is chosen (data not shown).

**Agreements between different tests (Fig. 1)**

Linear regression revealed a good correlation between different platelet tests assessing clopidogrel response:

- ADP-Ag and PRI VASP (Y = 9.1923 + 0.6257X, r = 0.55, p < 0.0001);
- ADP-Ag and PRU (Y = −11.6388 + 3.9888X, r = 0.64, p < 0.0001);
- PRI VASP and PRU (Y = 64.4765 + 3.1868X, r = 0.59, p < 0.0001).

<table>
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<th>Table 1 Baseline characteristics of the population.</th>
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<td>Mean age, years (mean ± SD)</td>
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<td>Men</td>
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<td>Hypertension</td>
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<td>Diabetes mellitus</td>
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<td>Use of statin</td>
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Figure 1. Correlation and agreement between different platelet parameters with ADP-induced aggregation (ADP), Platelet Reactivity Index VASP (PRI VASP) and PRU assessed with Verify Now assay (PRU).

The Bland-Altman plot showed a mean difference of 10.6 for ADP-Ag and PRI VASP, −146 for ADP-Ag and PRI VASP and −157 for PRI VASP and PRU (Fig. 2).

Concordance of different tests to identify non-responder patient

The correlation was significant as continuous variables, but using the most common threshold, the agreement between the difference tests was weak: kappa = 0.35 for ADP-Ag and PRI VASP, 0.36 for ADP-Ag and Verify Now Assay and 0.46 for PRI VASP and Verify Now Assay. We repeated this analysis with another cut-off value for ADP-Ag of 50%. The analysis with 50% resulted in slightly better concordance between different assays: kappa = 0.42 for ADP-Ag and PRI VASP and 0.52 for ADP-Ag and Verify Now (Fig. 3).

Discussion

This study showed that assessment of platelet function inhibition by clopidogrel is highly test-specific. Indeed, our results demonstrated a poor agreement between different platelet assays to identify clopidogrel non-responders, suggesting a probable need for a "double test" assessment to identify a non-responder patient before tailoring antiplatelet therapy. Moreover, these results showed that the rate of non-responders is very different with the three platelet function tests. This could have a great impact in daily practice for identifying the appropriate candidate for tailored therapy. The PRI VASP identified as high as 39% of non-responder patients despite a high maintenance dose of 150 mg with a threshold of 50%. This cut-off value is probably too sensitive while large randomized trial showed a clear benefit of clopidogrel for ischemic events prevention,
not suggesting that half the patients are "non-responders". Biological studies have demonstrated a broad interindividual variability of platelet response to clopidogrel associated with increased risk of ischemic events [1—9]. Several mechanisms have been proposed to explain this variability of response including genetic factors, metabolic parameters or interaction with other medications [1]. The active metabolite of clopidogrel, which irreversibly blocks platelet ADP P2Y12 receptors, arises from complex biochemical reactions involving several CYP450 isoforms [10]. Accordingly, genetic variations affecting the cytochrome activity will therefore modify clopidogrel response as recently demonstrated [11,12]. In addition, interaction with medications metabolized by the cytochrome have been described, such as atorvastatine [13], omeprazole [14] or calcium-channel blockers [15]. Definition of clopidogrel resistance is not consensual and different platelet tests have been proposed to assess clopidogrel response [1]. It was in the first reports mainly based on the ADP-induced platelet aggregation [1]. However, platelet aggregation could have some limitations: it is a non-specific method to measure the inhibitory effects of clopidogrel, as it is mediated by many factors, including multiple ADP-receptor pathways, while only one of these receptors is inhibited by clopidogrel.
Nonetheless, it is most likely that ADP-induced light transmission aggregometry reflects at least some aspects of platelet in vivo behaviour. Therefore, clopidogrel resistance should be strictly defined from a specific laboratory point of view: as an inability to achieve the expected inhibition of the solitary target P2Y12 receptor. Fortunately, platelet function assays have recently been introduced to measure the P2Y12 pathway. Indeed, a flow cytometric VASP phosphorylation assay has been introduced to measure more specific inhibition of clopidogrel’s biochemical target, the P2Y12 receptor [16]. It has been investigated in both biological and clinical studies [5,6]. The clinical relevance of clopidogrel non-response underlines the necessity to optimize the degree of platelet inhibition at the time of PCI. Several strategies have been proposed to improve clinical prognosis of these 'non-responder' patients including higher clopidogrel loading dose [17,18], additional repeated loading doses [19] or GPIIbIIIa antagonists [20]. However, this tailored approach requires consensual definition of non-response and validation of point-of-care platelet assay usable in daily clinical practice is a prerequisite for such tailored antiplatelet therapy. Accordingly, a recent point-of-care Verify Now has been proposed and associated with clinical prognosis after PCI [7—9]. However, concordance of these different tests has not been well investigated in ACS patients. The level of correlations in our study is quite low as compared with previous studies [21]. This might be explained by different platelet parameters (maximal platelet aggregation rather than residual), chronic therapy evaluation, unstable patients or high maintenance load. Although the use of correlation to report on the association between two measurements is widespread, it is often inappropriate as it does not imply agreement. The present study showed that despite significant correlation, agreement is poor, and many patients identified as non-responders with one test will be good responders with another one. This may suggest that concordance of two tests is probably needed before identifying a patient as a non-responder and modifying his antiplatelet therapy. Indeed, the ideal platelet test must be reliable and valid, with well-defined diagnostic cut-off values, and treatment decisions based on such tests should cost-effectively improve patient outcome. No laboratory or point-of-care platelet function tests currently fulfils all of these criteria. In a near future, platelet function testing could help to determine which patients would derive the most benefit from newer, more powerful P2Y12 inhibitors, such as prasugrel. Whether, genetic testing will have additional value for individualized therapy will also have to be addressed.

Conflicts of interest

None.

Financial disclosure

None.

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