Comparison of the VASP assay and platelet aggregometry in the evaluation of platelet P2Y_{12} receptor blockade

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INTRODUCTION It has been shown that incomplete blockade of platelet reactivity is a risk factor for future ischemic events in patients with cardiovascular diseases. Despite these findings, there is yet no gold standard of platelet reactivity estimation. The 2 most commonly used methods in platelet testing are platelet aggregometry and vasodilator-stimulated phosphoprotein phosphorylation (VASP) assay. They both showed the predictive value for future adverse events in cardiac patients; however, there are few data that compare these 2 methods.

OBJECTIVES The aim of the study was to compare the results of aggregometry (multi-electrode aggregometer [MEA]) and flow cytometric VASP assay used to determine platelet reactivity after the administration of P2Y_{12} receptor blockers.

PATIENTS AND METHODS The study included 17 healthy volunteers (12 men, 5 women; aged 41 ± 10 years) and 12 patients (men, aged 62 ± 12 years) with stable coronary artery disease treated with elective percutaneous coronary intervention with stent implantation. In volunteers, the blood was collected and tests were performed before and after 10-minute incubation with 5 nmol/l of cangrelor. In patients, the blood was collected for measurements before and after ingestion of 300 mg of clopidogrel. Aggregometry measurements included adenosine-diphosphate (ADP)-induced maximal aggregation (A_{max}) and ADP-induced area under the aggregation curve (AUC). The platelet reactivity index (PRI) was determined using the VASP assay.

RESULTS The use of cangrelor and clopidogrel was associated with a significant inhibition of platelet reactivity measured using the above methods. In both groups, the degree of inhibition was significantly greater when measured with the aggregation method compared with the VASP assay. The only significant coefficient of correlation between the VASP assay and aggregation results was observed in volunteers after platelet incubation with cangrelor (r = 0.81 between PRI and A_{max}, r = 0.68 between PRI and AUC).

CONCLUSIONS Compared with the VASP assay, ADP-induced platelet aggregation shows a greater ability to detect a decrease in platelet aggregation after P2Y_{12} antagonists. These tests are not interchangeable because they measure different aspects of the P2Y_{12} receptor blockade.

INTRODUCTION Antiplatelet therapy monitoring is currently extensively investigated. It has been shown that incomplete blockade of platelet reactivity is a risk factor for future ischemic events in patients with cardiovascular diseases.³ Despite these findings, there is yet no gold standard for platelet reactivity estimation. Numerous methods and devices have been developed to date, but there is still no consensus as to which is the most appropriate.⁴
There are tests that measure the overall platelet reactivity as well as tests that can specifically show the action of antiplatelet drugs. Both types showed some predictive value for future ischemic events. The most commonly used method is aggregometry modified with newer devices such as VerifyNow (Accumetrics, United States) or Multiplate (Dynabyte, Germany). There are also flow cytometry and biochemical methods with determination of serum thromboxane B₂ levels. These methods have 2 weaknesses. First, most of them lack standardization (such as for example the international normalized ratio), so the results can vary from one laboratory to another. Second, the positive predictive value of these methods is quite low. On the other hand, if there would be an idea to individualize the antiplatelet therapy with the drug dose change, one should consider using more specific tests that evaluate the specific pathway of platelet activation blocked by a given drug. It should also be mentioned that there is currently no recommendation to assess the antplatelet drug action on a routine basis, but numerous studies are underway. Researchers are particularly interested in the monitoring of antiplatelet actions of clopidogrel. This drug is used in patients after myocardial infarction for secondary prevention of future ischemic events and after coronary artery angioplasty as the prevention of stent thrombosis. Its nonoptimal antiplatelet action has been shown to increase risk for subsequent stent thrombosis after percutaneous coronary intervention (PCI).

Two methods that are most widely used to estimate clopidogrel action are platelet aggregometry and the vasodilator-stimulated phosphorylation (VASP) assay. While adenosine-diphosphate (ADP)-induced aggregation assess the more general aspect of platelet receptor P2Y₁₂ blockade, the VASP assay aims at the specific intraplatelet pathway blocked by clopidogrel. Both methods showed the predictive value for future adverse events in cardiac patients; yet, there are between those 2 methods at the laboratory level.

The aim of the study was to compare the changes in platelet reactivity after the action of P2Y₁₂ receptor blockers as measured by aggregometry or using the flow cytometric VASP detection assay.

**PATIENTS AND METHODS** The study included 17 healthy volunteers (12 men, 5 women; aged 41 ±10 years) and 12 patients (all men, aged 62 ±12 years) with stable coronary artery disease treated with elective PCI with stent implantation. None of the patients or healthy volunteers had used any drugs that could affect the platelet reactivity, including nonsteroidal drugs, for at least 7 days before the study. Individuals receiving acetylsalicylic acid (ASA) in the patient group were eligible.

Blood was collected into a vacuum tube containing 0.105 M buffered sodium citrate (Becton Dickinson, United Kingdom) for the VASP assay and into a vacuum tube containing hirudin (Sarstedt, Germany) for platelet aggregation. Blood was drawn with special caution to avoid undesirable activation of circulating platelets. All platelet reactivity measurements were performed within 1 hour after blood collection.

**Experimental protocol in volunteers** Platelet aggregation and VASP measurement were performed using the same blood sample (baseline), while the remaining blood volume was incubated with 5 nmol/l cangrelor for 10 minutes at 37°C prior to aggregation and VASP measurements (inhibition).

**Experimental protocol in patients** Blood for the study was collected before PCI in patients on 75 mg/d ASA alone (baseline). Coronary angiography was performed according to the current guidelines and stent implantation was performed in the culprit lesion. Patients were given 300 mg of clopidogrel and the second blood specimen was collected 24 hours after PCI (inhibition). Patients were excluded from the study if they received any platelet glycoprotein IIb/IIIa blocker during the PCI or if they were on oral anticoagulants.

**Platelet inhibitors** We used clopidogrel (Plavix, Sanofi, United States) for in vivo study, and cangrelor (formerly AR-C69931MX, kindly provided by AstraZeneca, United Kingdom) for ex vivo study. Both are P2Y₁₂ receptor inhibitors. Clopidogrel requires hepatic metabolism to become an active form, while cangrelor is a short-acting intravenous direct antiplatelet agent.

**Platelet aggregation** Aggregation was determined in whole blood using Multiplate (Dynabyte, Germany), the five-channel aggregometer based on measurements of electric impedance, so called multi-electrode aggregometer (MEA). The measurements were performed according to the manufacturer’s protocol. Shortly, to evaluate effectiveness of cangrelor or clopidogrel, the whole blood sample (0.3 ml) was anticoagulated with hirudin (Sarstet, Germany), diluted 1:1 with 0.9% saline, preincubated for 10 minutes at 37°C, and then supplemented with ADP (with the final concentration of 6.4 μmol/l) (Dynabyte, Germany).

Aggregation curves were registered and analyzed using Dynabyte software enabling the calculation of the total area under the aggregation curve (AUC) and the maximal value of platelet aggregation (Amax) expressed in arbitrary units of aggregation. The aggregation measurements were done in duplicate and the maximal inter-assay variability was 15%.

**VASP measurements** To monitor specific platelet ADP receptor antagonists, we used VASP/P2Y₁₂ flow cytometry kit (BioCytex, France). Under the test conditions, VASP correlates with the P2Y₁₂ receptor inhibition, while its nonphosphorylation
We defined the high on-treatment platelet reactivity when the result after P2Y₁₂ blockade was above the upper quartile separately for the group of patients and volunteers. This general cut-off value was applied in previous studies.¹¹

Ethics  The study was performed according to the guidelines of the Helsinki Declaration for human research and approved by the committee on the Ethics of Research in Human Experimentation at the Medical University of Lodz (No. RNN/13/07/KB).

Statistical analysis  The Shapiro-Wilk’s test was used to verify normal distribution of the data. For platelet aggregation and for the VASP assay, the data were analyzed using a nonparametric analysis of variance (Kruskal-Wallis test) and the all-pairwise comparison Connover-Inman test (data presented as median and interquartile range: from 25% quartile, lower quartile, to 75% quartile, upper quartile). The calculations were performed using the StatsDirect statistical software.

RESULTS  The clinical characteristics of the study groups are presented in TABLE 1. The use of cangrelor and clopidogrel was associated with a significant inhibition of platelet reactivity, measured with the VASP assay and ADP-induced aggregation (TABLE 2, FIGURES 1–4). In both groups, the degree of inhibition was significantly greater when measured with the aggregation method (TABLE 3, TABLE 4, FIGURE 5). There were no differences in baseline platelet reactivity or inhibited platelet reactivity between the groups. The only significant (P <0.05) coefficient of correlation between the VASP assay and aggregation results was observed in volunteers after in vitro cangrelor use: r = 0.81 between PRI and A max and r = 0.68 between PRI and AUC.

We defined the high on-treatment platelet reactivity when the result after P2Y₁₂ blockade was above the upper quartile separately for the group of patients and volunteers. Considering this definition, there were 3 patients and 3 volunteers with MEA AUC and Amax and 3 patients and 5 volunteers with the VASP assay. The only significant

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**TABLE 1**  Clinical characteristics of the study group (n = 12)

<table>
<thead>
<tr>
<th>Age, y, mean ± SD</th>
<th>62 ±12</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sex, n men</td>
<td>12</td>
</tr>
<tr>
<td>Diabetes, n (%)</td>
<td>3 (25)</td>
</tr>
<tr>
<td>Arterial hypertension, n (%)</td>
<td>8 (66)</td>
</tr>
<tr>
<td>Chronic renal diseases, n (%)</td>
<td>2 (16)</td>
</tr>
<tr>
<td>History of myocardial infarction, n (%)</td>
<td>1 (8)</td>
</tr>
<tr>
<td>History of PCI or CABG, n (%)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Active smoking, n (%)</td>
<td>5 (41)</td>
</tr>
<tr>
<td>Single/double vessel disease</td>
<td>10/2</td>
</tr>
<tr>
<td>Implanted stent, BMS/DES*</td>
<td>13/4</td>
</tr>
<tr>
<td>Implanted stent, median (min–max)</td>
<td>1 (1–2)</td>
</tr>
<tr>
<td>Periprocedural complications, n (%)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Statins, n (%)</td>
<td>12 (100)</td>
</tr>
<tr>
<td>β-blockers, n (%)</td>
<td>12 (100)</td>
</tr>
<tr>
<td>ACEIs, n (%)</td>
<td>12 (100)</td>
</tr>
<tr>
<td>Oral antidiabetics, n (%)</td>
<td>3 (25)</td>
</tr>
<tr>
<td>ARBs, n (%)</td>
<td>2 (17)</td>
</tr>
<tr>
<td>Proton-pump inhibitors, n (%)</td>
<td>12 (100)</td>
</tr>
</tbody>
</table>

a 3 patients received 2 BMSs and 2 patients received 2 DESs, the rest received 1 stent


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**TABLE 2**  Results of aggregation and VASP measurements

<table>
<thead>
<tr>
<th></th>
<th>Before P2Y₁₂ antagonist</th>
<th>After P2Y₁₂ antagonist</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Volunteers, AUC</td>
<td>950 ±130</td>
<td>184 ±171</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Volunteers, A max</td>
<td>87.5 ±11.5</td>
<td>26.2 ±16.2</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Volunteers, VASP, PRI</td>
<td>83.4 ±6.7</td>
<td>49.5 ±14.1</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Patients, AUC</td>
<td>874 ±134</td>
<td>213 ±276</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Patients, A max</td>
<td>84.9 ±2.4</td>
<td>24.9 ±14.8</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Patients, VASP, PRI</td>
<td>84.0 ±2.3</td>
<td>44.6 ±23.0</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

Values are presented as mean ± SD

Abbreviations: A max – maximal aggregation, AUC – area under the aggregation curve, PRI – platelet reactivity index, VASP – vasodilator-stimulated phosphoprotein phosphorylation, others – see TABLE 1
There is still no consensus as to which platelet reactivity test is the most suitable for the measurement of clopidogrel effect. Two distinct questions that should be addressed are: which test is more appropriate for the evaluation of pharmacodynamic effects and which test could be more useful in the prediction of patient clinical outcomes. Bauman et al.\(^\text{13}\) showed that the VASP method and the VerifyNow ADP test correlate most with active clopidogrel metabolite levels, while the correlation of classic light transmittance with this metabolite aggregometry is weaker. On the other hand, Siller-Matula et al.\(^\text{14}\) demonstrated that MEA high-sensitivity ADP (with addition of prostaglandin) was more predictive for future stent thrombosis in patients after coronary interventions than the VASP method.

**DISCUSSION** Our study showed that the use of P2Y\(_{12}\) agonist (both clopidogrel in patients and cangrelor ex vivo in volunteers) results in a greater change in the MEA ADP-induced aggregometry parameters compared with the VASP method. This finding is in line with the results by Siller-Matula et al.,\(^\text{15}\) who showed that clopidogrel response in a group of patients on clopidogrel was strongest when measured with MEA ADP-induced aggregometry. We confirmed this finding and additionally showed that this is also true for direct intravenous P2Y\(_{12}\), receptor agonist – cangrelor.

(P < 0.05) Spearman coefficient of correlation was \(r = 0.78\) for the comparison between MEA AUC and \(A_{max}\) in patients and \(r = 0.59\) for the same comparison in volunteers. There were no significant correlations between the VASP assay and MEA with regard to the detection of high on-treatment platelet reactivity (\(r = 0.56\) for VASP and \(A_{max}\) and \(r = 0.26\) for VASP and AUC).

There is still no consensus as to which platelet reactivity test is the most suitable for the measurement of clopidogrel effect. Two distinct questions that should be addressed are: which test is more appropriate for the evaluation of pharmacodynamic effects and which test could be more useful in the prediction of patient clinical outcomes. Bauman et al.\(^\text{13}\) showed that the VASP method and the VerifyNow ADP test correlate most with active clopidogrel metabolite levels, while the correlation of classic light transmittance with this metabolite aggregometry is weaker. On the other hand, Siller-Matula et al.\(^\text{14}\) demonstrated that MEA high-sensitivity ADP (with addition of prostaglandin) was more predictive for future stent thrombosis in patients after coronary interventions than the VASP method.

There still remains the problem of test interchangeability. Lordkipanidze et al.\(^\text{15}\) showed that there is hardly any significant correlation between 6 different tests used with regard to...
In our study, there was a fairly good association of the results of various tests depends on the laboratory methodology. In our study, there was a fairly good association between PRI and $A_{\text{max}}$ and between PRI and AUC, but only after ex vivo cangrelor use and not in patients treated with clopidogrel.

In other studies, there was a weak but significant correlation between VASP and aggregation in patients treated with clopidogrel. However, it rather confirms our results that while testing the effects of clopidogrel, these tests are hardly interchangeable.

The MEA method is fairly new and uses the ADP concentration of 6.4 μmol/l. We thus decided to use the recommended concentration, but it shows that the platelet reactivity tests are hardly interchangeable even within the aggregation methods.

Recently, the concept of high on-treatment platelet reactivity has been developed. This condition, which is partly responsible for the worse outcome in cardiovascular patients, has not been clearly defined yet due to a number of factors. For this reason, it is generally not recommended to perform routine platelet reactivity measurements, although the results of the relevant studies will be published soon. In our study, we defined high on-treatment platelet reactivity when the result of a given test exceeded the upper quartile for the group. There was no significant correlation between the VASP assay and both MEA tests in identifying patients/volunteers with such platelet reactivity. Subjects with high on-treatment platelet reactivity detected by one test did not show the same when another test was used. This is in line with the study by von Beckerath et al., in which the coefficient of correlation was 0.35 between MEA ADP AUC and the VASP method ($r = 0.26$ in our study). On the other hand, we observed a strong correlation between MEA AUC and MEA $A_{\text{max}}$ for the detection of high on-treatment platelet reactivity, but it is quite understandable and only confirms good adjustment of the MEA method.

One of the limitations of our study is the small number of subjects, but this is rather typical of this type of studies. Another limitation is the use of only 2 tests for comparisons, but according to the current literature, these 2 tests and VerifyNow seem to be the best choice for the estimation of P2Y$_{12}$ receptor functional blockade.

In conclusion, the change in ADP-induced aggregation with the use of MEA in response to P2Y$_{12}$ antagonists is greater than that observed using the VASP assay. The tests are not interchangeable because they measure different aspects of the P2Y$_{12}$ receptor blockade.

**Acknowledgements** This study was supported by a grant from the Polish Ministry of Science and Higher Education (N405 065 034).

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**TABLE 3** The percentage of inhibition of the platelet reactivity measured with aggregation and VASP methods

<table>
<thead>
<tr>
<th></th>
<th>AUC</th>
<th>$A_{\text{max}}$</th>
<th>VASP</th>
<th>$P$</th>
</tr>
</thead>
<tbody>
<tr>
<td>volunteers</td>
<td>81.0 ±16.9</td>
<td>70.4 ±16.6</td>
<td>40.5 ±16.3</td>
<td>&lt;0.001 (AUC vs. VASP and $A_{\text{max}}$ vs. VASP)</td>
</tr>
<tr>
<td>patients</td>
<td>77.4 ±29.3</td>
<td>71.7 ±17.2</td>
<td>46.8 ±27.4</td>
<td>0.001 (AUC vs. VASP); 0.045 ($A_{\text{max}}$ vs. VASP)</td>
</tr>
</tbody>
</table>

**Abbreviations:** see **TABLE 2**

**TABLE 4** Differences between baseline and maximal platelet inhibition for VASP and aggregometry methods; median (min–max)

<table>
<thead>
<tr>
<th></th>
<th>$A_{\text{max}}$</th>
<th>AUC</th>
<th>VASP, PRI</th>
</tr>
</thead>
<tbody>
<tr>
<td>cangrelor</td>
<td>3.4 (1.6–29.0)</td>
<td>7.3 (1.6–61.5)</td>
<td>1.5 (1.2–3.4)</td>
</tr>
<tr>
<td>clopidogrel</td>
<td>4.7 (1.5–10.0)</td>
<td>14.2 (1.1–50.0)</td>
<td>1.7 (1.0–6.1)</td>
</tr>
</tbody>
</table>

**Abbreviations:** see **TABLE 2**

**FIGURE 5** Blood platelet reactivity inhibition monitored by MEA and the VASP assay in the presence of P2Y$_{12}$ platelet inhibitors (A – in vitro studies) and in patients after clopidogrel ingestion (B – ex vivo studies). Data shown as median (interquartile range) of inhibition of blood platelet reactivity (%). Abbreviations: ADP – adenosine diphosphate, MEA – multi-electrode aggregometer, others – see **TABLE 2**

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the aspirin-induced effect. We demonstrated that as for P2Y$_{12}$ receptor blockade, the strength of correlations between the results of various tests depends on the laboratory methodology. In our study, there was a fairly good association between PRI and $A_{\text{max}}$ and between PRI and AUC, but only after ex vivo cangrelor use and not in patients before and after clopidogrel.

In other studies, there was a weak but significant correlation between VASP and aggregation in patients treated with clopidogrel. However, it rather confirms our results that while testing the effects of clopidogrel, these tests are hardly interchangeable.

The MEA method is fairly new and uses the ADP concentration of 6.4 μmol/l. In the majority of studies, ADP concentrations of 5 and 20 μmol/l were used, but it should be mentioned that the MEA method is based on the principles of impedance aggregometry, while the latter concentrations were used in optical aggregometry. The MEA device allows for the changes of agonist concentration, but the current literature and the reference ranges provided by the manufacturer refer to the ADP concentration of 6.4 μmol/l. We thus decided to use the recommended concentration, but it shows that the platelet reactivity tests are hardly interchangeable even within the aggregation methods.


Porównanie metody VASP i agregacji w ocenie blokady płytkowego receptora P2Y<sub>12</sub>

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STRESZCZENIE

W grupie pacjentów z chorobami układu sercowo-naczyniowego niepełna blokada funkcji płytek krwi ma związek ze zwiększonym ryzykiem incydentów niedokrwiennych w przyszłości. Mimo to nadal nie dysponujemy standardem laboratoryjnym oceny reaktywności płytek krwi. Najczęściej stosowanymi metodami w badaniach płytkowych są agregacja i ocena fosforylacji białka VASP (vasodilator-stimulated phosphoprotein phosphorylation). Obie metody mają wartość rokowniczą dla zdarzeń niedokrwiennych u pacjentów z chorobami układu sercowo-naczyniowego, niewiele natomiast jest danych porównujących obie te metody.

CELE

Celem badania było porównanie wyników oznaczeń agregometrycznych (za pomocą metody multi-electrode aggregometer [MEA]) i oceny cytofluorymetrycznej (ocena VASP) reaktywności płytek krwi po zastosowaniu antagonistów receptora P2Y<sub>12</sub>.

PACJENTI I METODY

Do badania włączono 17 zdrowych ochotników (12 mężczyzn i 5 kobiet w wieku 41 ± 10 lat) oraz 12 chorych (mężczyźni w wieku 62 ± 12 lat) na stabilną chorobę niedokrwienną serca leczonych elektrycznie przezskórą angioplastyką naczyń wieńcowych z implantacją stentu. Od ochotników pobierano krew i wykonywano oznaczenia przed inkubacją i po 10 min. inkubacji z 5 nmol kangrelorem. U pacjentów krew do oznaczeń pobierano przed i po zastosowaniu dawki 300 mg klopidogrelu. Wykonywano agregację płytek krwi indukowaną adenozyndifosforanem (ADP), oceniając maksymalną agregację (A<sub>max</sub>) oraz pole pod krzywą agregacji (area under the curve – AUC). Metodą VASP określano współczynnik reaktywności płytek (platelet reactivity index – PRI).

WYNIKI

Zastosowanie kangrelorem i klopidogrelu wiązało się z istotnym zahamowaniem reaktywności płytek ocenianej zastosowanymi metodami. W obu grupach stopień blokady płytek krwi był większy w pomiarach agregometrycznych w porównaniu z metodą VASP. Zaobserwowano tylko jedną istotną korelacją obu metod w grupie zdrowych ochotników po inkubacji płytek krwi z kangrelorem (r = 0,81 pomiędzy PRI i A<sub>max</sub> oraz r = 0,68 pomiędzy PRI i AUC).

WNIOSKI

W ocenie blokady receptora P2Y<sub>12</sub> większe zahamowanie reaktywności płytek krwi obserwuje się podczas stosowania agregacji wywołanej ADP niż podczas stosowania metody VASP. Testy te nie powinny być stosowane zamiennie, gdyż oceniają odmienne aspekty blokady receptora P2Y<sub>12</sub>.