# **Republished Article**

# **Evaluating the Utility of a Novel Research Use Index in Platelet Aggregation Analysis Featured in an Automated Blood Coagulation Analyzer to Confirm the Effect of Antiplatelet Drugs**

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The main purpose of measuring platelet aggregation is to confirm the effect of antiplatelet drugs. Although the automated blood coagulation analyzers (CS-5100, CS-2500, CS-2400, CS-2100i and CS-2000i) measure platelet aggregation, they are not equipped to confirm the effect of antiplatelet drugs, unlike the semiautomated analyzers currently used in Japan. We have developed platelet aggregation level (PAL), a research use only (RUO) scoring system to use the current platelet aggregation testing method to confirm the effect of antiplatelet drugs.

The values of ADP-induced PAL (APAL), obtained when ADP was used as an agonist, and collagen-induced PAL (CPAL), obtained when collagen was used as an agonist, decreased in a concentration-dependent manner in cangrelor (P2Y12 inhibitor) and aspirin-treated samples, respectively. In addition, the values significantly decreased before and after the addition. APAL and CPAL showed a significantly close relationship, with a correlation coefficient of  $\geq 0.90$ , with the existing natural standard range-II (NSR-II) system.

We suggested that the automated blood coagulation analyzer can test the effect of antiplatelet drugs as a dedicated semi-automated analyzer. Thus, our innovation is expected to assist institutions that cannot afford the new instrument due to cost and space limitations by enabling easy testing using an automated blood coagulation analyzer.

Key Words

Platelet Aggregation, Light Transmission Aggregometry, Antiplatelet Drug, PAL (Platelet Aggregation Level)

### INTRODUCTION

Light transmission aggregometry (LTA), developed as a method of *in vitro* platelet function testing in 1962,<sup>1)</sup> has been widely used for the diagnosis of platelet dysfunction, including thrombasthenia and Von Willebrand disease, and was termed the gold standard method by the Scientific and Standardization Committee of the International Society of Thrombosis and Haemostasis in 2013.<sup>2)</sup> Previously the platelet aggregation test was performed using the dedicated semi-automated analyzer. In 2015, however, the platelet aggregation test function was installed on the automated blood coagulation analyzers (CS-5100, CS-2500, CS-2400,

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CS-2100*i* and CS-2000*i*), which are used for coagulation tests such as PT, APTT, AT, and D-dimer.<sup>3)</sup>

Other than diagnosing platelet dysfunction, the test is now frequently used to confirm patient pharmacological response to antiplatelet drugs (unresponsiveness caused by metabolic enzymes or insufficient dose or overdose at administration). In clinical practice, dual antiplatelet therapy (DAPT) is often prescribed<sup>4)</sup> as a method of administration of antiplatelet drugs, such as the P2Y12 receptor antagonists clopidogrel, prasugrel, or cangrelor, with the COX-1 inhibitor acetylsalicylic acid (aspirin). Resistance to clopidogrel has been reported because the genetic variability in CYP2C19

Notes: This article is based on current regulatory requirements in Japan. (as of Nov. 2018)

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reduces enzyme activity during metabolism. Clopidogrel resistance has been detected in approximately 20% of Japanese individuals, which is more prevalent compared to the 3% prevalence in individuals of European descent.<sup>5)</sup> Thus, determination of drug resistance is critical for guided decision-making with antithrombotic therapy and for studies on the cerebrovascular region, and is being actively investigated worldwide.

Maximal aggregation rate and aggregation waveform have been reported as the results of LTA. However, a certain skillset and level of experience are required to interpret these results. To circumvent this problem, a scoring method using "2 or 4 reagent concentrations" was developed,<sup>6,7)</sup> which was introduced to semi-automated analyzers commonly used in Japan to determine the effect of antiplatelet drugs. The frequency of use of the "2 or 4 concentration method" is second to that of the maximal aggregation rate and aggregation waveform method according to the results of a Japanese survey on platelet aggregation (**Table 1**). Although the CS is a routine instrument for performing blood coagulation tests that can also measure platelet aggregation, the above-mentioned scoring method has not yet been installed in this system.

Hence, we have developed a new scoring method called platelet aggregation level (PAL) to establish an index for confirming the effect of antiplatelet drugs that can be installed on the CS-5100, CS-2500 and CS-2400 analyzers (hereinafter "CS-series" on this paper). PAL is newly designed for two reagents, namely, adenosine diphosphate (ADP), which specifically measures the P2Y12 receptor antagonist (clopidogrel and prasugrel), and collagen, which specifically measures COX-1 inhibitors (aspirin) for dual antiplatelet therapy. These two reagents are used at two different concentrations. We termed the values calculated from the measurements obtained using two concentrations of ADP as ADP-induced PAL (APAL), and those calculated from the measurements obtained using two concentrations of collagen as collagen-induced PAL (CPAL). The score ranged from 0.0 to 10.0 with 100 levels, which was designed to decrease with suppression of platelet aggregation by the antiplatelet drugs (Fig. 1). In this study, we report results related to PAL evaluation in in vitro experiments using samples from healthy volunteers. PAL is for research use only and is not cleared for clinical diagnostic use.

**Table 1** Results of a Japanese survey on platelet aggregation test parameters reported from laboratories to clinical doctors. (n = 210)



Fig. 1 Examples of PAL score calculated from wave form results.

APAL and CPAL were calculated from the wave form results obtained using two concentrations of agonist (ADP for APAL, collagen for CPAL). These scores changed with platelet aggregation activity.

### EVALUATION OF PAL FUNCTION USING SAMPLES OF HEALTHY VOLUNTEERS

### 1. Subjects

Blood samples were collected using blood collection tubes containing 3.2% sodium citrate from 79 healthy Sysmex employees (volunteers) after approval by the ethics committee of Sysmex Corporation. The samples were centrifuged at 200 × g for 10 minutes and the supernatant was collected and labeled as platelet-rich plasma (PRP). The remaining blood was centrifuged at 1,500 × g for 15 minutes and the supernatant was collected and labeled as plateletpoor plasma (PPP).

PRP without any additives post-collection was labeled as normal sample. For the P2Y12 receptor antagonist, an active metabolite with a similar effect as cangrelor (Adooq Bioscience, CA, USA) was added to the normal sample to prepare the cangrelor-added sample. For the COX-1 inhibitor (aspirin), acetylsalicylic acid (Wako Pure Chemical Industries, Ltd., Osaka, Japan) was added to prepare the aspirin-added sample, and cangrelor and aspirin were added to prepare the cangrelor/aspirin-added sample. The antiplatelet drugs were added to normal PRP at a ratio of 1:49. The final concentrations of antiplatelet drugs are listed in the Methods section and the figures.

### 2. Instrument

The automated blood coagulation analyzer CS-5100 (Sysmex Corporation, Kobe, Japan) was used as the evaluation instrument. APAL and CPAL, calculated from the results obtained using two concentrations each of ADP and collagen, respectively, were confirmed while screening for platelet aggregation (*Fig. 2*).

A conventional platelet aggregation analyzer developed by Manufacturer A (Conventional Analyzer) was used as the reference instrument. Natural standard range-II (NSR-II) equipped on the Conventional Analyzer was used for the calculation of the results of the "2-concentration method", which was used as reference. As NSR-II shows the results in 9-levels from 1 to 9 on the screen, the results were extracted up to the lowest one digit from the CSV data.



Fig. 2 Example of PAL result displayed on the instrument

### 3. Agonists for measurement

Revoluem ADP and Revoluem collagen (Sysmex Corporation, Kobe, Japan) were used as evaluation reagents. The final concentrations of ADP were 1  $\mu$ M and 10  $\mu$ M, whereas those of collagen were 1  $\mu$ g/mL and 5  $\mu$ g/mL (*Table 2*).

ADP and collagen used in the Conventional Analyzer were used as reference reagents, and their final concentrations were 1  $\mu$ M and 10  $\mu$ M, and 2  $\mu$ g/mL and 5  $\mu$ g/mL, respectively.

### 4. Methods

- 1) Confirming reactivity by adding antiplatelet drugs
- 1. Concentration-dependent variation of PAL for single drug

A normal sample and two concentrations of cangreloradded samples (0.05 and 0.5  $\mu$ M) were prepared, and aggregation waveform and APAL were obtained from the measurement results. A normal PRP and two concentrations of aspirin-added samples (1.0 and 1.5 mM) were prepared, and aggregation waveform and CPAL were obtained from the measurement results. 2. Variation of PAL for single and dual drugs

A normal sample,  $0.05 \mu$ M cangrelor-added sample, and  $0.05 \mu$ M cangrelor/1.0 mM aspirin-added sample were prepared, and aggregation waveform and APAL were obtained. A normal sample, 1.0 mM aspirin-added sample, and 0.05  $\mu$ M cangrelor/1.0 mM aspirin-added sample were prepared, and aggregation waveform and CPAL were obtained.

# 2) Comparison of PAL before and after adding antiplatelet drugs to a normal sample

APAL was obtained by preparing normal PRP and 0.05  $\mu$ M cangrelor-added samples from 51 blood samples. CPAL was obtained by preparing normal PRP and 1.0  $\mu$ M aspirinadded samples from 51 blood samples. Student's t-test was used for statistical calculations.

### 3) Correlation with the existing index (NSR-II)

We prepared a normal sample, multiple concentration cangrelor-added samples, aspirin-added samples, and cangrelor/aspirin-added samples, and measurement was conducted using the CS-5100 and the Conventional Analyzer. Each correlation between APAL and NSR-II of ADP and between CPAL and NSR-II of collagen was calculated.

Table 2	Reagent	information	for APAL and	CPAL
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Assay	Reagent	Adjusted concentraion	Final concentration
APAL	Boucham ADD	8µM	1μM
	Revollent ADP	80μΜ	10µM
CPAL	Davaham Callagan	8µg/mL	1µg/mL
	Revonem Collagen	40µg/mL	5µg/mL

### 5. Results

- 1) Reactivity by adding antiplatelet drugs
- 1. Concentration-dependent variation of PAL for single drug

Regarding the P2Y12 receptor antagonist, APAL decreased with cangrelor concentration from 10.0 (0.0  $\mu$ M) to 4.4 (0.5  $\mu$ M) (*Fig. 3A*). Regarding the COX-1 inhibitor, CPAL decreased with aspirin concentration from 10.0 (0.0 mM) to 6.6 (1.5 mM) (*Fig. 3B*).



Fig. 3 Examples of change in PAL score with single drug concentration

A. APAL score of normal sample, and 0.05 μM and 0.5 μM cangrelor-treated samples. B. CPAL score of normal sample, and 1.0 mM and 1.5 mM aspirin-treated samples.

### 2. Variation of PAL for single and dual drugs

APAL of the normal sample, 0.05  $\mu$ M cangrelor-added sample, and 0.05  $\mu$ M cangrelor/1.0 mM aspirin-added sample were 10.0, 8.3, and 5.6, respectively, showing decrease in dual-drug added sample by 2.7 compared to the single-drug added sample (*Fig. 4A*).

CPAL of the normal sample, 1.0 mM aspirin-added sample, and 0.05  $\mu$ M cangrelor/1.0 mM aspirin-added sample were 10.0, 7.4, and 4.2, respectively, showing decrease in dual-drug added sample by 3.2 compared to the single-drug added sample (*Fig. 4B*).



Fig. 4 Examples of change in PAL score with single or double drug addition

A. APAL score of normal sample and drug samples that contained cangrelor (0.05 μM) or cangretor and aspirin (0.05 μM, 1.0 mM) B. CPAL score of normal sample and drug samples that contained aspirin (1.0 mM) or cangretor and aspirin (0.05 μM, 1.0 mM)

A. APAL

# 2) Comparison of PAL before and after adding antiplatelet drugs to normal sample

The Mean  $\pm$  standard deviation (SD) of APAL was 9.4  $\pm$  0.9 for the normal sample without any addition and 6.7  $\pm$  1.4 for 0.05 µM cangrelor-added sample, and the difference was significant (p < 0.001). The Mean  $\pm$  SD of CPAL was 9.8  $\pm$ 0.9 for the normal sample without any addition and 6.0  $\pm$ 1.3 for the 1.0 mM aspirin-added sample, and the difference was significant (p < 0.001) (**Fig. 5**).

#### 3) Correlation with the existing index (NSR-II)

The correlation between APAL and NSR-II was expressed by the equation y = 0.79x + 1.60; r = 0.94, whereas that between CPAL and NSR-II was expressed by y = 0.83x + 0.95, r = 0.90 (*Fig. 6*).



Fig. 5 Comparison of PAL scores between normal samples and anti-platelet drug-treated samples

Fifty-one normal samples were used for measurement and anti-platelet drug samples were prepared by adding cangrelor or aspirin to the normal sample. These PAL scores were compared using the box-and-whisker plots.



Fig. 6 Comparison between APAL or CPAL and the reference method (NSR-II)

## DISCUSSION

In this study, we developed a new method of analysis called PAL, which aims at confirming the effect of antiplatelet drugs. We have set two indexes called APAL for clopidogrel and CPAL for aspirin, for determining the effect of these drugs that are commonly used in clinical practice in dual antiplatelet therapy (clopidogrel + aspirin). We confirmed reactivity to antiplatelet drugs in the *in vitro* system and evaluated correlation with the existing index.

In addition, in an experiment simulating single antiplatelet therapy, the index varied in a dose-(concentration) dependent manner, reflecting the efficacy of each drug (**Fig. 4A, B**). Stronger platelet aggregation was observed in the system simulating dual antiplatelet therapy, than that simulating single drug therapy (**Fig. 5A, B**). Results showed that APAL and CPAL decreased with the effect of antiplatelet drugs in the *in vitro* system. Next, we determined the reactivity using actual samples of patients receiving antiplatelet drugs to determine the utility and practical feasibility of this index.

Comparison of this index with the existing index NSR-II revealed a promising correlation between ADP-induced APAL and NSR-II, and between collagen-induced CPAL and NSR-II. Thus, this index was highly reliable (*Fig. 6A*, *B*).

Clinical doctors are interested in monitoring the effect of antiplatelet drugs mentioned in the practice guidelines  $^{8-10)}$ 

although the recommendation level is low. A recent publication describes protocols of antiplatelet therapy in the field of cerebral intravascular therapy<sup>11</sup> (*Fig. 7*). Therefore, platelet aggregation tests are expected to be used by more institutes in the future.

Several instruments available on the market that use whole blood samples to measure platelet aggregation are easy to operate but expensive. Thus, they are not suitable for high volume aggregation testing, considering the elevated cost of medical treatment in several countries. In addition, each manufacturer has its own format of reporting results, and antiplatelet drug monitoring is considered basic research. The setting of cut-off values and test protocols for enabling the clinical application of antiplatelet drug monitoring in daily practice was suggested several years ago; however, nothing has been published or recommended to date.<sup>12</sup>

In conclusion, we have developed a new RUO analysis index for the CS-series, confirmed reactivity to antiplatelet drugs in the *in vitro* system, and evaluated the correlation with NSR-II which is already in use. As the CS-series is a routine automated blood coagulation analyzer, platelet aggregation can be measured without purchasing another instrument. In addition, since the CS-series is distributed worldwide, further evidence on the clinical utility and practical use of PAL is expected to be collected. Thus, these evidence may help standardize future methods for confirming the effect of antiplatelet drugs.



Fig. 7 Example of testing the anti-platelet drug effect using the platelet aggregateon test<sup>11</sup>

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