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ORIGINAL ARTICLE

Assessment of platelet-derived thrombogenicity with the total thrombus-formation analysis system in coronary artery disease patients receiving antiplatelet therapy

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Essentials

- Total thrombus-formation analysis system (T-TAS) quantitatively measures platelet thrombus formation.
- We examined the utility of T-TAS in patients with coronary artery disease.
- T-TAS can discriminate different types of the antiplatelet therapy in the same measuring method.
- Genetic background, cytochrome P-450 2C19 genotypes, also influenced T-TAS parameters.

Summary. *Background:* Accurate evaluation of thrombogenicity helps to prevent thrombosis and excessive bleeding. The total thrombus-formation analysis system (T-TAS) was developed for quantitative analysis of platelet thrombus formation by the use of microchips with thrombogenic surfaces (collagen, platelet chip [PL-chip]; collagen plus tissue factor, atherome chip [AR-chip]). We examined the utility of the T-TAS in the assessment of the efficacy of antiplatelet therapy in patients with coronary artery disease (CAD). *Methods and Results:* In this cross-sectional study, 372 consecutive patients admitted to the cardiovascular department were divided into three groups: patients not receiving any antiplatelet therapy (control, $n = 56$), patients receiving aspirin only ($n = 69$), and patients receiving aspirin and clopidogrel ($n = 149$). Blood samples were used for the T-TAS to measure the platelet thrombus-formation area under the curve (AUC) at various shear rates (1500 s^{-1} [PL_{18}-AUC_{10}] and 2000 s^{-1} [PL_{24}-AUC_{10}] for the PL-chip; 300 s^{-1} [AR_{10}-AUC_{30}] for the AR-chip). The on-clopidogrel platelet aggregation was measured by the use of P2Y12 reaction units (PRUs) with the VerifyNow system. The mean PL_{24}-AUC_{10} levels were 358 ± 111 (± standard deviation) (95% confidence interval [CI] 328.9–387.1) in the control group, 256 ± 108 (95% CI 230.5–281.5) in the aspirin group, and 113 ± 91 (95% CI 98.4–127.6) in the aspirin/clopidogrel group. In the aspirin/clopidogrel group, the PL_{24}-AUC_{10} was higher in poor metabolizers (PMs) with cytochrome P450 2C19(CYP2C19) polymorphisms (152 ± 112, 95% CI 103.4–200.6) than in the non-PM group (87 ± 74, 95% CI 73.8–100.2). *Conclusions:* Our findings suggest that the PL_{24}-AUC_{10} level measured by the T-TAS is a potentially suitable index for the assessment of antiplatelet therapy in CAD patients.

Keywords: antiplatelet therapy; clopidogrel; CYP2C19; new device; percutaneous coronary intervention.

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Introduction

Antiplatelet therapy is used for the prevention of ischemic heart disease. Aspirin is used as the mainstay drug for secondary prevention of ischemic cardiac events, and its concomitant use with thienopyridine can reduce the risk of thrombotic events after stent implantation [1–5]. Clopidogrel (Plavix) is a commonly used drug among the thienopyridines, and requires biotransformation to an active metabolite by cytochrome P450 2C19(CYP2C19) [6–9]. The efficacy of antiplatelet therapy can be assessed with several techniques [10]. The VerifyNow assay is an easy-to-use point-of-care system in which increased light transmission reflects platelet-induced aggregation

[11]. In this system, the platelet responses to aspirin and thienopyridines (inhibitors of P2Y12 receptor) are expressed as aspirin reaction units and P2Y12 reaction units (PRU), respectively; the PRU level is influenced by CYP2C19 genotype [12], and correlates with cardiovascular events [13].

The duration of dual antiplatelet therapy (DAPT) with the combined use of aspirin and clopidogrel after percutaneous coronary intervention (PCI) is controversial. Several trials have reported the safety of short-duration DAPT [14,15]; however, a recent randomized study showed that DAPT beyond 1 year reduced the risks of stent thrombosis and major adverse cardiovascular and cerebrovascular events [16]. Accurate assessment of thrombogenicity may allow evaluation of the most successful antiplatelet therapy in each patient. However, the use of a single conventional assay, such as the VerifyNow system, might not be suitable for comparison of the efficacies of different types of antiplatelet drugs.

A novel microchip flow chamber system, the total thrombus-formation analysis system (T-TAS), was recently developed for quantitative analysis of the thrombus-formation process [17,18]. This system can measure the process of platelet thrombus formation mediated by platelet–collagen interaction under selected shear rates in the microchip [17], and has broad utility as compared with conventional methods [19].

We hypothesized that the T-TAS is suitable for assessment of the effects of different antiplatelet therapies. In the present study, we examined the utility of the T-TAS in the assessment of the efficacy of antiplatelet therapy in patients with coronary artery disease (CAD).

Materials and methods

Study population

This study was a cross-sectional study that enrolled 372 consecutive patients who were admitted for the diagnosis or treatment of ischemic heart disease to Kumamoto University Hospital between September 2013 and April 2015. We excluded patients who were treated with other antithrombotic agents, such as heparin, warfarin, ticlopidine, sarpogrelate, cilostazol, and prasugrel. We also excluded patients with malignancies and acute coronary syndrome, defined as either acute myocardial infarction (with or without electrocardiographic evidence of ST-segment elevation) or those with unstable angina (class 2 or 3 of Braunwald's classification). The study patients were divided into three groups according to type of treatment: patients not receiving any antiplatelet agents (control group, $n = 56$), patients receiving 100 mg day^{-1} aspirin (aspirin group, $n = 69$), and patients receiving maintenance doses of aspirin and clopidogrel (aspirin/clopidogrel group, $n = 149$) (Fig. 1).

All procedures were conducted in accordance with the Declaration of Helsinki and its amendments. The study protocol was approved by the human ethics committee of Kumamoto University, and written informed consent was obtained from each patient or the family of the subject.

Measurement of thrombogenicity with the T-TAS

Blood samples were obtained on admission from patients. The T-TAS is an automated microchip-based flow chamber system developed for easy and rapid assessment of platelet thrombus formation under flow conditions [17–19]. This system analyzes different thrombus-formation processes with a simple procedure by using two disposable microchips with different thrombogenic surfaces. One chip, the platelet chip (PL-chip), contains 25 capillary channels (width, 40 μm; depth, 40 μm) coated with type I collagen. Briefly, a whole blood sample (330 μL) anticoagulated with hirudin was applied to the PL-chip at flow rates of 18 μL min^{-1} and 24 μL min^{-1}, corresponding to initial wall shear rates of 1500 s^{-1} and 2000 s^{-1}, respectively. Inside the microchip, platelets adhere and aggregate on the surface of collagen, and microchip capillaries are occluded. These thrombus-formation processes were analyzed by continuous monitoring of the flow pressure change resulting from capillary occlusion. The other chip, the atherome chip (AR-chip), contains a single capillary channel (width, 300 μm; depth, 80 μm) coated with type I collagen plus tissue thromboplastin. Whole blood taken into 3.2% sodium citrate was mixed with $CaCl_2$ and corn trypsin inhibitor immediately before testing. The 450-μL mixture was applied to the AR-chip at a flow rate of 10 μL min^{-1}, corresponding to an initial wall shear rate of 600 s^{-1}. Inside the microchip, activation of both the platelets and coagulation system is triggered simultaneously by collagen and tissue thromboplastin, respectively. Similarly, the fibrin-rich platelet thrombus-formation process inside the AR-chip was analyzed by monitoring the flow pressure change. The area under the flow pressure curve (AUC) was computed to assess platelet thrombogenicity inside the microchips. AUC_{10}s, i.e. the AUCs for the first 10 min for the PL-chip tested at flow rates of 18 min^{-1} and 24 μL min^{-1}, are termed PL_{18}-AUC_{10} and PL_{24}-AUC_{10}, respectively, and AUC_{30}, i.e. the AUC for the first 30 min for the AR-chip tested at 10 μL min^{-1}, is termed AR_{10}-AUC_{30}.

Measurement of residual platelet aggregation

PRUs were measured with the VerifyNow system (Ultegra rapid platelet function assay; Accumetrics, San Diego, CA, USA). Blood samples for the P2Y12 cartridge were drawn into 1.8-mL blood collection tubes containing 3.2% sodium citrate. In this assay, fibrinogen-coated microparticles were used in the VerifyNow P2Y12 cartridge for binding to available platelet receptors. The

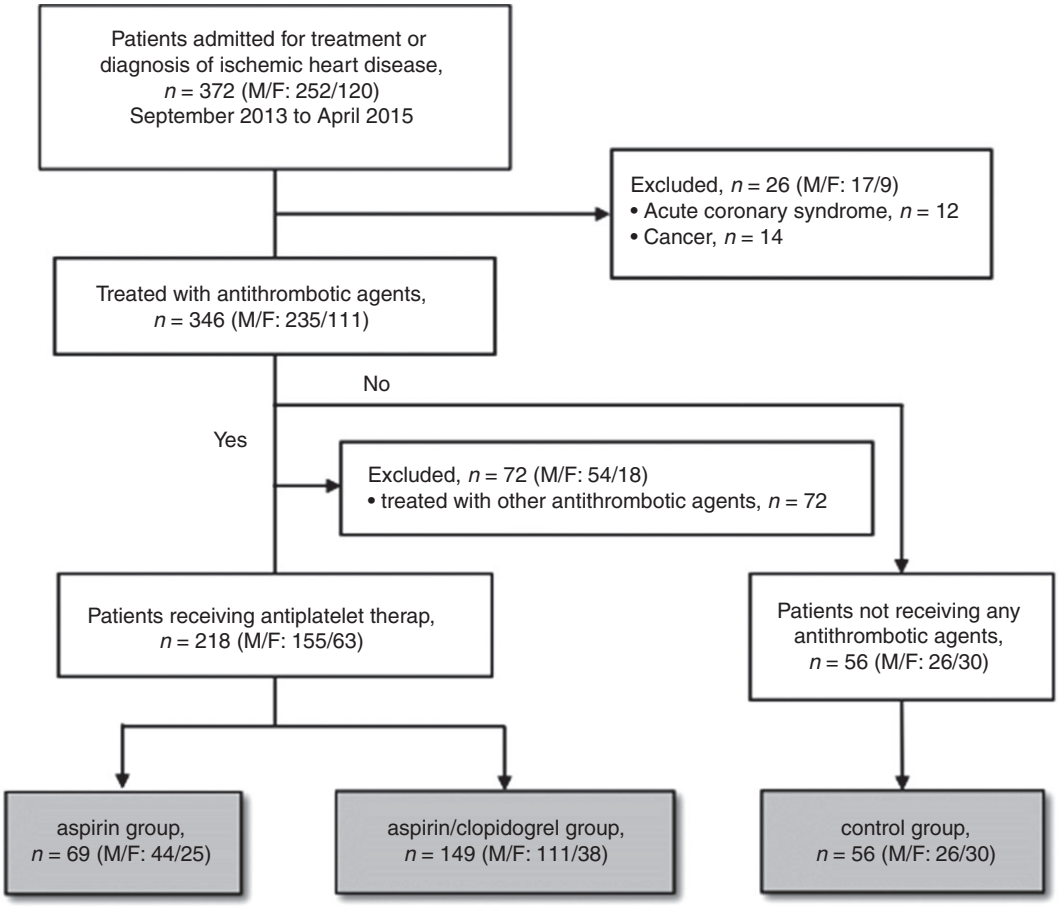

Fig. 1. Patient recruitment process. M, male; F, female.

results were reported in PRUs, which represented the amount of ADP-mediated aggregation specific to the platelet P2Y12 receptor. PRUs were determined on the basis of the rate and extent of platelet reactivity in the ADP channel.

Determination of CYP2C19 genotype and phenotype

Genomic DNA was extracted from whole blood with a DNA Extractor WB kit (Wako Pure Chemical Industries, Osaka, Japan) according to the protocol modified by Richards *et al*. [20]. PCR restriction fragment length polymorphisms for CYP2C19*2 (681G>A) and CYP2C19*3 (636G>A) were detected as described previously [21,22]. CYP2C19*2 and CYP2C19*3 are considered to account for > 99% of the alleles generating the null-activity enzyme protein in the Japanese population [22]. CYP2C19 genotypes were therefore classified into three phenotypes: (i) extensive metabolizers (EMs) carrying normal-function alleles (CYP2C19*1/CYP2C19*1); (ii) intermediate metabolizers (IMs) carrying one loss-of-function allele (CYP2C19*1/CYP2C19*2; CYP2C19*1/CYP2C19*3); and (iii) poor metabolizers (PMs) carrying two loss-of-function alleles (CYP2C19*2/CYP2C19*2; CYP2C19*2/CYP2C19*3; CYP2C19*3/CYP2C19*3).

Statistical analysis

The sample size was calculated on the basis of previous reports that assessed PRU levels in Japanese CAD patients [23,24]. On the basis of the proportions of patients in our hospital, we estimated that the study subjects should include eight patients who were not treated with clopidogrel for every 10 patients treated with aspirin/clopidogrel. In previous studies, the PRU levels in different subject groups showed normal distribution patterns, with a standard deviation (SD) value of 90. If the true difference in PRU levels between the experimental and control mean values is 80, there is a need to enroll 31 subjects treated with clopidogrel and 25 control subjects in order to reject the null hypothesis that the population means of the aspirin/clopidogrel and aspirin groups are equal with a probability (power) of 0.9. The type I error probability associated with this test of this null hypothesis is 0.05.

Data are expressed as mean ± SD. Categorical data are presented as frequencies and percentages. We compared subject baseline characteristics by patient group and by CYP2C19 phenotype by using the χ^2 test for categorical variables, and one-way ANOVA for continuous variables, as appropriate. Pearson's correlation coefficient

was used to evaluate the association between parameters of the T-TAS and VerifyNow system. The associations between treatment type and PL_{18}-AUC_{10}, PL_{24}-AUC_{10}, AR_{10}-AUC_{30}, PRUs and other significant parameters known to influence platelet function [25–29] were analyzed by multiple logistic regression analysis with the forced entry method. Statistical analyses were performed with SPSS version 22 (SPSS, Tokyo, Japan) and the free software R (version 3.0.2) (http://www.R-project.org/).

Results

Patient characteristics

The clinical characteristics of the aspirin and aspirin/clopidogrel groups are shown in Table 1. The proportions of patients with a history of PCI and receiving treatment with statins and proton pump inhibitors were lower in the aspirin group than in the aspirin/clopidogrel group. None of the other parameters was significantly different between the two groups.

Differences between the T-TAS and the VerifyNow P2Y12 assay in assessment of thrombogenicity

First, we examined the relationship between the T-TAS and the VerifyNow P2Y12 assay. As shown in Fig. S1, the relationships between PL_{18}-AUC_{10}, PL_{24}-AUC_{10}, AR_{10}-AUC_{30} and PRUs were weak although significant (PL_{18}-AUC_{10} versus PRUs, $r = 0.364$, $P < 0.01$; PL_{24}-AUC_{10} versus PRUs, $r = 0.310$, $P < 0.01$; AR_{10}-AUC_{30} versus PRUs, $r = -0.154$, $P < 0.01$). However, PL_{18}-AUC_{10} correlated strongly with PL_{24}-AUC_{10} ($r = 0.908$, $P < 0.01$). In the present study, the intrachip flow rate had no significant effect on platelet thrombogenicity. In the T-TAS system, a high shear rate corresponds to stenosed arteries. Accordingly, we used PL_{24}-AUC_{10} in further analysis of PL-AUCs.

The T-TAS assesses both types of antiplatelet therapy

Using the T-TAS system, we assessed differences in platelet-based thrombogenicity among the three treatment groups. First, we compared the videos recorded during the measurement of PL_{24}-AUC_{10}, and noticed different levels of platelet aggregation and thrombi among the three groups (Fig. S2A). Patients receiving antiplatelet therapy had fewer thrombi and longer patent capillary paths than patients in the control group. These findings reflected flattening of the slope of the pressure–time curves with resultant low PL_{24}-AUC_{10} values (Fig. S2B).

Next, we compared the results of quantitative analysis. PRU levels were significantly lower in the aspirin/clopidogrel group than the control and aspirin groups, but there were no significant differences in PRU levels between the control and aspirin groups (control, 264 ± 50; aspirin,

Table 1 Comparison of baseline demographics, clinical parameters and medication use between patients in the aspirin and aspirin/clopidogrel groups

Baseline characteristic	Aspirin group ($n = 69$)	Aspirin-clopidogrel group ($n = 149$)	*P*-value
Age (years), mean ± SD	70.6 ± 9.3	69.3 ± 10.3	0.356
Male, *n* (%)	44 (63.8)	111 (74.5)	0.111
Mean body mass index (kg m^{-2}), mean ± SD	24.1 ± 3.3	24.0 ± 3.6	0.851
Hypertension, *n* (%)	60 (87.0)	131 (87.9)	0.841
Dyslipidemia, *n* (%)	57 (82.6)	129 (86.8)	0.441
Diabetes, *n* (%)	36 (52.2)	86 (57.7)	0.443
Stroke, *n* (%)	8 (11.6)	19 (12.8)	0.809
Current smoking, *n* (%)	12 (17.4)	14 (9.4)	0.090
Family history of IHD, *n* (%)	17 (24.6)	44 (29.5)	0.518
History of PCI, *n* (%)	16 (23.2)	119 (79.9)	< 0.001
CCB, *n* (%)	50 (72.3)	90 (60.4)	0.020
β-Blocker, *n* (%)	37 (53.6)	102 (68.5)	0.034
ARB/ACE-I, *n* (%)	36 (52.2)	98 (65.8)	0.055
Statins, *n* (%)	53 (76.8)	142 (95.3)	< 0.001
PPI, *n* (%)	39 (56.5)	117 (78.5)	< 0.001
EF (%), mean ± SD	61.5 ± 8.0	61.3 ± 7.3	0.840
Hb (g dL^{-1}), mean ± SD*	13.4 ± 1.7	13.1 ± 1.7	0.172
Hct (%),mean ± SD*	39.5 ± 5.2	38.8 ± 4.6	0.305
Platelet count (103 μL^{-1}), mean ± SD*	197.4 ± 54.2	210.5 ± 59.3	0.121
eGFR (mL min^{-1} 1.73 m^{-2}), mean ± SD*	64.9 ± 16.8	63.6 ± 17.4	0.591

ACE-I, angiotensin-converting enzyme inhibitor; ARB, angiotensin II receptor blocker; CCB, calcium channel blocker; EF, left ventricular ejection fraction; eGFR, estimated glomerular filtration rate; Hb, hemoglobin; Hct, hematocrit; IHD, ischemic heart disease; PCI, percutaneous coronary intervention; PPI, proton pomp inhibitor; SD, standard deviation. *Data for this parameter were measured at the point of admission.

278 ± 57; aspirin/clopidogrel, 213 ± 67; $P < 0.001$ for control and aspirin versus aspirin/clopidogrel) (Fig. 2A). Furthermore, PL_{24}-AUC_{10} values were significantly lower in the two antiplatelet therapy groups than in the control group, and the value was significantly lower in the aspirin/clopidogrel group than in the aspirin group (control, 358 ± 111; aspirin, 256 ± 108; aspirin/clopidogrel, 113 ± 90; $P < 0.001$ for control versus aspirin and aspirin/clopidogrel; $P < 0.001$ for aspirin versus aspirin/clopidogrel) (Fig. 2B). AR_{10}-AUC_{30} values were identical among the three groups (control, 1687 ± 355; aspirin, 1710 ± 221; aspirin/clopidogrel, 1662 ± 266; $P = 0.671$ for control versus aspirin; $P = 0.194$ for aspirin versus aspirin/clopidogrel; $P = 0.580$ for control versus aspirin/clopidogrel, aspirin) (Fig. 2C).

Correlation between treatment type and T-TAS parameters

To confirm the association between T-TAS parameters and antiplatelet therapy, we performed single and multiple logistic regression analyses on the data of patients receiving antiplatelet therapy (including those treated with aspirin and those treated with aspirin/clopidogrel) (Table 2). Simple regression analysis showed that aspirin/clopidogrel therapy correlated significantly with PRU level, PL_{24}-AUC_{10}, and AR_{10}-AUC_{30} (respectively: [odds ratio [OR] 10.09, 95% confidence interval [CI] 4.704–21.65, $P < 0.001$; OR 14.529, 95% CI 6.965–30.31, $P < 0.001$; OR 2.648, 95% CI 1.438–4.876, $P = 0.002$). Multivariate logistic regression analysis demonstrated that the type of antiplatelet therapy (aspirin therapy and aspirin/clopidogrel) significantly influenced PRU and PL_{24}-AUC_{10} values (respectively: OR 14.609, 95% CI 5.109–41.77, $P < 0.0001$; OR 10.552, 95% CI 4.214–26.42, $P < 0.0001$). In receiver operating characteristic (ROC) analysis (*c*-statistics), the PRU, PL_{24}-AUC_{10} and the combined PRU and PL_{24}-AUC_{10} AUC values were 0.802 (95% CI 0.741–0.863), 0.871 (95% CI 0.820–0.922), and 0.915 (95% CI 0.875–0.955), respectively (Fig. 3). The PRU AUC was significantly different from the combination AUC ($P < 0.0001$). Furthermore, the net reclassification improvement (NRI) was significantly different between PRU and PL_{24}-AUC_{10} values (NRI − 0.3786 [95% CI − 0.6735 to − 0.0836], $P = 0.012$). These data suggest that PL24-AUC_{10} is a useful parameter for discrimination between the aspirin and aspirin/clopidogrel groups, and its use with the PRU level could improve the discriminative capacity.

Effects of CYP2C19 phenotype on PL_{24}-AUC_{10} levels

To assess the effects of CYP2C19 phenotype on PL_{24}-AUC_{10} levels, we compared the PL_{24}-AUC_{10} and PRU values in patients in the aspirin/clopidogrel group classified as EMs carrying normal-function alleles (CYP2C19*1/CYP2C19*1), IMs carrying one loss-of-function allele (CYP2C19*1/CYP2C19*2; CYP2C19*1/CYP2C19*3), and PMs carrying two loss-of-function alleles (CYP2C19*2/CYP2C19*2; CYP2C19*2/CYP2C19*3; CYP2C19*3/CYP2C19*3). Table S2 summarizes the

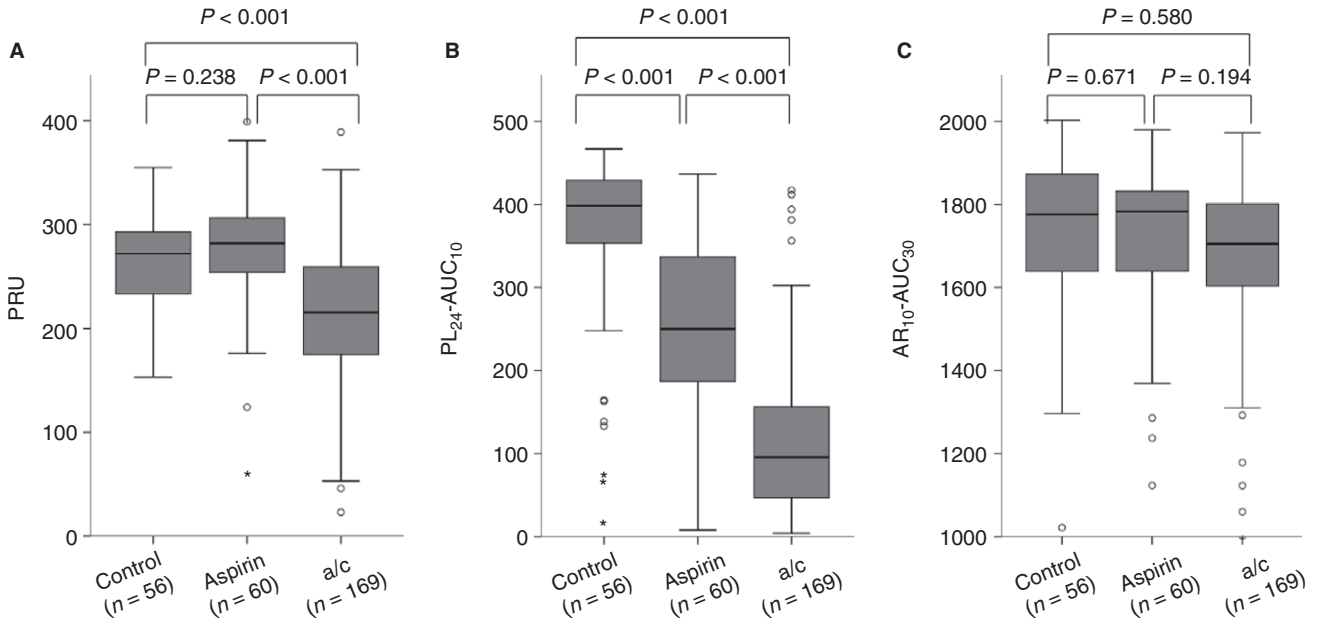

Fig. 2. Changes in total thrombus-formation analysis system parameters and P2Y12 reaction unit (PRU) levels under different types of antiplatelet therapy. (A) PRUs. (B)PL_{24}-AUC_{10}. (C) AR_{10}-AUC_{30}. The graphs show data of each parameter as box-and-whisker plots. In these plots, lines within the boxes represent median values. The upper and lower lines of the boxes represent the 25th and 75th percentiles, respectively. The upper and lower bars outside the boxes represent the 90th and 10th percentiles, respectively. a/c, aspirin/clopidogrel.

Table 2 Results of univariate and multivariate logistic regression analyses on factors that influence the outcome of dual antiplatelet therapy

	Univariate			Multivariate		
	OR	95% CI	*P*-value	OR	95% CI	*P*-value
Age (> 75 years)	1.120	0.609–2.059	0.716	1.046	0.411–2.663	0.926
Male (yes)	1.660	0.898–3.066	0.106	0.874	0.343–2.230	0.779
Body mass index (> 25 kg m^{-2})	1.234	0.681–2.237	0.489	1.208	0.479–3.047	0.688
Hypertension (yes)	0.841	0.464–2.571	0.841	1.089	0.305–3.897	0.895
Diabetes (yes)	1.251	0.705–2.220	0.443	1.527	0.645–3.612	0.335
Dyslipidemia (yes)	1.358	0.622–2.964	0.442	1.274	0.366–4.428	0.704
Stroke (yes)	0.809	0.462–2.688	0.809			
EF (> 60%)	1.187	0.625–2.257	0.600			
Hct (%)*	1.467	0.826–2.606	0.191			
Platelet count (< 150 000 L^{-1})†	1.532	0.693–3.389	0.292			
eGFR (< 60 mL min^{-1} 1.73 m^{-2})†	1.033	0.573–1.864	0.913			
BNP (> 100 pg mL^{-1})†	0.728	0.292–1.814	0.496			
CRP (> 0.1 mg dL^{-1})†	0.990	0.541–1.813	0.974			
Current smoker (yes)	0.493	0.215–1.131	0.095			
AR_{10}-AUC_{30}*	2.648	1.438–4.876	0.002	2.991	1.208–7.406	0.018
PL_{24}-AUC_{10}*	14.529	6.965–30.31	< 0.001	10.552	4.214–26.42	< 0.0001
PRU*	10.091	4.704–21.65	< 0.001	14.609	5.109–41.77	< 0.0001

BNP, B-type natriuretic peptide; CI, confidence interval; CRP, C-reactive protein; EF, left ventricular ejection fraction; eGFR, estimated glomerular filtration rate; Hct, hematocrit; OR, odds ratio; PRU, P2Y12 reaction unit. *Data for this parameter were divided into two groups on the basis of the median value. †Data for this parameter were measured at the point of admission.

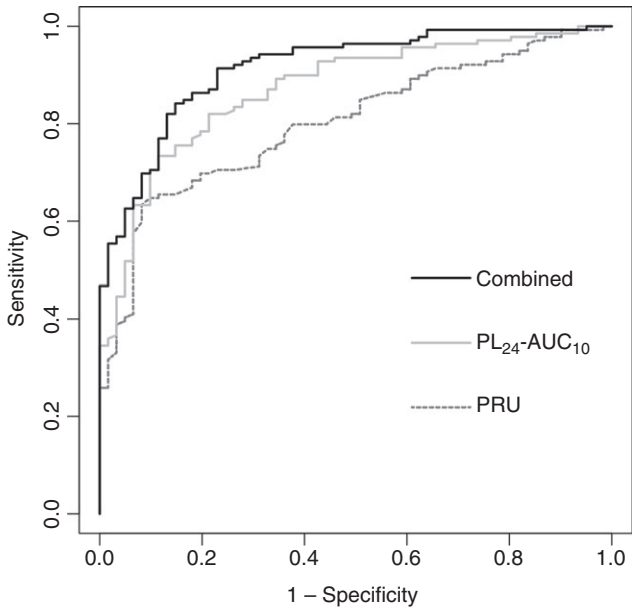

Fig. 3. Receiver operating characteristic curves of P2Y12 reaction units (PRUs), PL_{24}-AUC_{10} and PRUs + PL_{24}-AUC_{10} to discriminate the aspirin group from the aspirin/clopidogrel group.

clinical characteristics of the three groups. PL_{24}-AUC_{10} values were significantly higher in the PM group than in the IM and EM groups, whereas the values were similar in the IM and EM groups (PMs, 152 ± 112; IMs, 83.8 ± 72.2; EMs, 92.2 ± 77.3; $P = 0.002$ for PMs versus IMs; $P = 0.014$ for EMs versus PMs; $P = 0.546$ for IMs versus EMs; Fig. 4A). On the other hand, PRU levels were significantly higher in IMs and PMs than in EMs, whereas they were similar in IMs and PMs (PMs, 232 ± 64; IMs, 232 ± 65; EMs, 196 ± 54; $P = 0.994$ for PMs versus IMs; $P = 0.022$ for EMs versus PMs; $P = 0.02$ for IMs versus EMs) (Fig. 4B). The CYP2C19 phenotype had no effect on AR_{10}-AUC_{30} (data not shown).

We also divided the enrolled patients into PM and non-PM groups. Table S3 summarizes the clinical background of these patients. PL_{24}-AUC_{10} was significantly higher in the PM than in the non-PM groups, whereas PRU levels were not significantly different between the two groups (PL_{24}-AUC_{10} PM, 152 ± 111; PL_{24}-AUC_{10} non-PM, 87 ± 74; [$P = 0.001$]; PRUs PM, 232 ± 64; PRUs non-PM, 218 ± 63 [$P = 0.370$]) (Fig. 4C,D).

PL_{24}-AUC_{10} correlates with PM status

The above analyses showed the usefulness of PL_{24}-AUC_{10} and that this parameter is different from the PRU level. Finally, we performed simple and multiple regression analyses to determine the factors that could influence the CYP2C19 PMs. In simple regression analysis, high PL_{24}-AUC_{10} values correlated with PM status (OR 3.923, 95% CI 1.339–11.49, $P = 0.013$). Multiple regression analysis identified high PL_{24}-AUC_{10} as an independent and significant determinant of PM status (OR 3.868, 95% CI 1.314–11.39, $P = 0.014$) (Table 3). The PRU, PL_{24}-AUC_{10} and PRU + PL_{24}-AUC_{10} values determined by ROC analysis (*c*-statistics) were 0.578 (95% CI 0.437–0.720), 0.708 (95% CI 0.595–0.822), and 0.703 (95% CI 0.574–0.832), respectively (Fig. 5). The NRI was not significantly different among the three groups (PRUs versus PL_{24}-AUC_{10}, NRI – 0.3176 [95% CI – 0.7994 to

0.1642, $P = 0.196$]; PRUs versus combined, NRI – 0.4202 [95% CI – 0.8998 to 0.0595, $P = 0.0860$]; PL_{24}-AUC_{10} versus combined, NRI – 0.1125 [95% CI – 0.5966 to 0.3716, $P = 0.64887$]). The *c*-statistics of PL_{24}-AUC_{10} had higher discriminative ability than those for PRUs; however, all ROC AUC values were modest, and they did not show significant differences to allow discrimination of PMs from non-PMs.

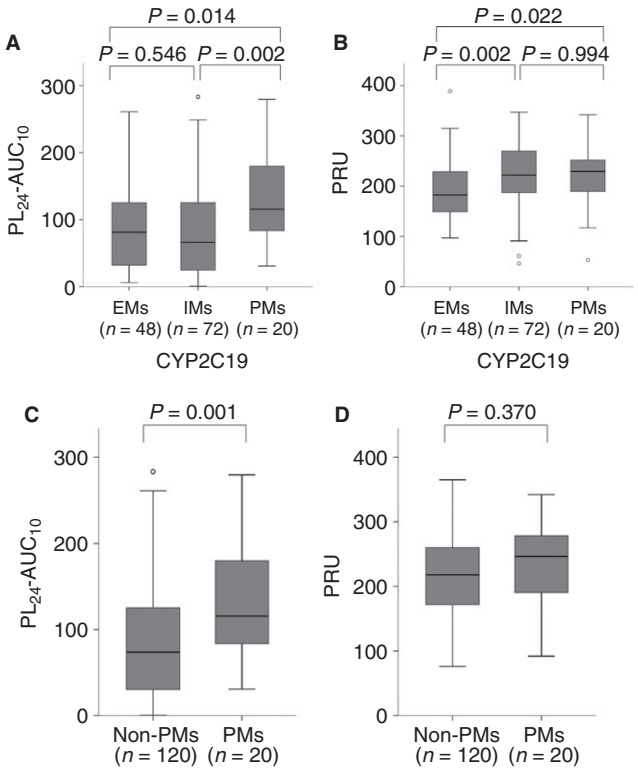

Fig. 4. PL_{24}-AUC_{10} and P2Y12 reaction units (PRUs). (A) PL_{24}-AUC_{10} and (B) PRUs according to the cytochrome 2C19 (CYP2C19) genotype. (C) PL_{24}-AUC_{10} and (D) PRUs for the poor metabolizer (PM) and non-PM groups. EMs, extensive metabolizers; IMs, intermediate metabolizers.

Discussion

In the present study, we tested the utility of the T-TAS in CAD patients receiving or not receiving antiplatelet therapies. Although the correlation between the T-TAS and the VerifyNow P2Y12 assay was weak, PL_{24}-AUC_{10} accurately assessed the therapeutic effects of antiplatelet therapy. In addition, high PL_{24}-AUC_{10} values correlated with CYP2C19 PM status. These data indicated that the T-TAS is potentially useful for the assessment of antiplatelet therapy. To the best of our knowledge, this is the first report to describe the usefulness of the T-TAS as a tool for the global assessment of antiplatelet therapy in CAD patients.

The VerifyNow P2Y12 assay was designed to measure the rate and extent of platelet aggregation induced by ADP [30]. This system had high diagnostic accuracy for the inhibition rate of P2Y12 inhibitors; however, the need for a specific method has limited its general applicability, and it is often difficult to use the VerifyNow P2Y12 assay to compare different types of antiplatelet therapy. On the other hand, the T-TAS was developed to assess thrombogenicity in a situation closer to the *in vivo* condition. The system is based on the use of a preselected blood flow rate and shear stress designed to trigger platelet activation followed by serial reactions. These features allowed us to broaden the use of this system to assess blood samples without any reference to the drugs used. The utility of the T-TAS for evaluating

Table 3 Results of simple and multiple regression analyses for determinants of poor metabolizer status

	Simple regression analysis			Multiple regression analysis		
	OR	95% CI	*P*-value	OR	95% CI	*P*-value
Age (> 75 years)	1.238	0.469–3.266	0.666			
Male (yes)	1.393	0.433–4.486	0.578			
Body mass index (> 25 kg m^{-2})	0.619	0.210–1.822	0.384			
Hypertension (yes)	1.189	0.249–5.677	0.828			
Diabetes (yes)	0.935	0.361–2.422	0.889			
Dyslipidemia (yes)	0.571	0.168–1.939	0.369			
Stroke (yes)	1.893	0.554–6.471	0.309			
Current smoker (yes)	1.101	0.225–5.383	0.905			
EF (> 60%)	1.000	0.356–2.809	1.000			
Hct (%)*	1.796	0.670–4.815	0.244			
Platelet count (< 150 000 L^{-1})†	0.841	0.176–4.018	0.828			
eGFR (< 60 mL min^{-1} 1.73 m^{-2})†	1.072	0.408–2.822	0.887			
CRP (> 0.1 mg dL^{-1})†	0.796	0.285–2.223	0.663			
High PL_{24}-AUC_{10}*	3.923	1.339–11.49	0.013	3.868	1.314–11.39	0.014
High PRU*	1.307	0.505–3.381	0.582	1.139	0.428–3.032	0.794

CI, confidence interval; CRP, C-reactive protein; EF, left ventricular ejection fraction; eGFR, estimated glomerular filtration rate; Hct, hematocrit; OR, odds ratio. *Data for this parameter were divided into two groups on the basis of the median value. †Data for this parameter were determined at the point of admission.

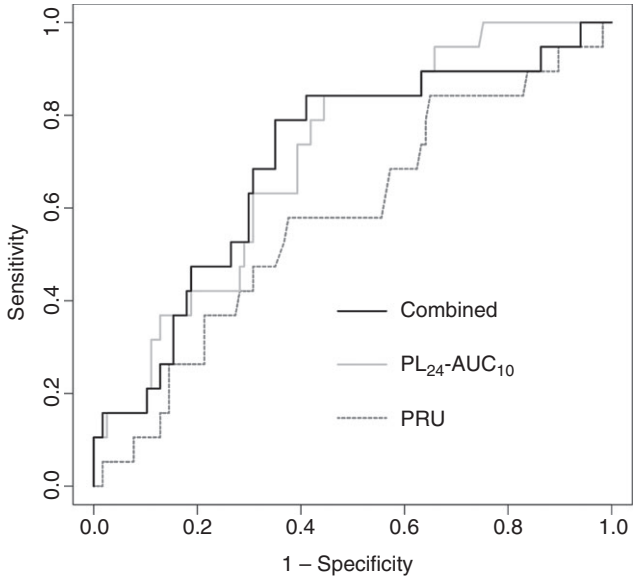

Fig. 5. Receiver operating characteristic curves of P2Y12 reaction units (PRUs), PL_{24}-AUC_{10} and PRUs + PL_{24}-AUC_{10} for discrimination of poor metabolizers (PMs) from non-PMs in the aspirin/clopidogrel group.

clotting status has been reported in experimental animals [31], healthy subjects [32–34], and a small population of patients [19,35]. The present study confirmed its usefulness in CAD patients.

Previous studies described the prognostic value of the PRU level in predicting cardiovascular events after stent implantation [36,37]. However, another group showed the poor predictive values of parameters derived from the currently available devices, such as the VerifyNow P2Y12 assay for bleeding events [38]. The results of the present study showed that the T-TAS compared well with the VerifyNow P2Y12 assay in distinguishing the aspirin group from the aspirin/clopidogrel group. Furthermore, the combination of PL24-AUC_{10} and PRU level enhanced the reliability of discrimination. These data highlight the potential of T-TAS in predicting cardiovascular events. Further prospective large-scale studies are needed to confirm this hypothesis.

Several studies have reported the effects of carriers of CYP2C19 reduced-function polymorphism on on-clopidogrel platelet aggregation and clinical outcome in patients with acute and chronic CAD [9,39–41]. The prevalence of CYP2C19 loss-of-function alleles is race-related, with a higher frequency of PM status in East Asians, including Japanese, than in Caucasians [42,43]. Recent reports showed the worse clinical outcome in Asian CYP2C19 PMs with acute myocardial infarction [44]. In the present study, platelet thrombus formation was highest in PMs, as measured with the T-TAS, whereas the PRU level did not show any statistical difference between the PM and non-PM groups. Our data indicated that the PL_{24}-AUC_{10} values computed by the T-TAS could be useful for detecting high-risk patients such as CYP2C19 PMs among CAD patients treated with clopidogrel.

The present study has several limitations. First, it was performed in a single center on a relatively small number of patients. Therefore, it might be underpowered to detect differences in clinical event rate. Further studies of larger populations are needed to examine the relationship between PL_{24}-AUC_{10} measured with the T-TAS and an increased risk of clinical cardiovascular events. Second, we did not measure aspirin reaction units with the VerifyNow Aspirin assay, and therefore could not compare the PL_{24}-AUC_{10} measured with the T-TAS and aspirin reaction units measured with the VerifyNow Aspirin assay. Third, because we did not measure serial changes in PL_{24}-AUC_{10} values in the aspirin/clopidogrel group after clopidogrel loading, it is possible that the on-clopidogrel platelet thrombus formation might not be sufficiently downregulated in the aspirin/clopidogrel group treated with a maintenance dose of clopidogrel.

In conclusion, we have demonstrated in the present study that the T-TAS is a potentially reliable system for the assessment of platelet thrombus-formation capacity in patients receiving various types of antiplatelet therapy. Further examination of the utility of PL_{24}-AUC_{10} measured with the T-TAS is warranted in patients receiving other antiplatelet therapies.

Addendum

Y. Arima, K. Kaikita, and H. Ogawa contributed to the conception and design of the study, and analysis and interpretation of data. M. Ishii, M. Ito, and D. Sueta contributed to interpretation of data. Y. Oimatsu, K. Sakamoto, K. Tsujita, S. Kojima, K. Nakagawa, and S. Hokimoto contributed data collection.

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Disclosure of Conflict of Interests

The authors state that they have no conflict of interest.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1. STROBE Statement – checklist of items that should be included in reports of observational studies.
Table S2. Baseline demographic, clinical parameters, and medication use according to the CYP2C19 type.
Table S3. Baseline demographic, clinical parameters, and medication use of the CYP2C19 EM, IM and PM groups.
Figure S1. Correlation between T-TAS parameters and PRU.
Figure S2. Effects of antiplatelet therapies on total thrombus formation in the AR-chip.

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