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P2Y12 Reaction Units and Clinical Outcomes in Acute Large Artery Atherosclerotic Stroke: A Multicenter Prospective Study

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Aims: We aimed to determine the association between acute platelet reactivity and clinical outcome in acute ischemic stroke (AIS) or transient ischemic attack (TIA) with large-artery atherosclerosis (LAA).

Methods: In this prospective, 16-multicenter study, we enrolled AIS/TIA patients with LAA receiving clopidogrel. We assessed the association of P2Y12 reaction units (PRU) 24 hours after initiation of antiplatelets with the CYP2C19 genotype and recurrent ischemic stroke within 90 days, and the difference between acute (≤ 7 days) and subacute (8–90 days) phases.

Results: Among the 230 AIS/TIA patients enrolled, 225 with complete outcome data and 194 with genetic results were analyzed. A higher PRU was significantly associated with recurrent ischemic stroke within 90 days (frequency, 16%), and within 7 days (10%). Twenty-nine patients (15%) belonged to a CYP2C19 poor metabolizer group (CYP2C19*2/*2, *2/*3, or *3/*3). Multivariable receiver-operating characteristic analysis showed a greater area-under-the-curve (AUC) in predicting recurrence within 7 days, compared to 8–90 days (AUC, 0.79 versus 0.64; $p=0.07$), with a cut-off PRU of 254. Multivariable analysis showed high PRU (≥ 254), which had a comparable predictive performance for recurrent ischemic stroke within 7 days (odds ratio, 6.82; 95% CI, 2.23–20.9; $p<0.001$) to the CYP2C19 poor metabolizer genotype. The net reclassification improvement, calculated by adding high PRU (≥ 254) to a model including the CYP2C19 poor metabolizer genotype in the prediction of recurrence within 7 days, was 0.83 ($p<0.001$).

Conclusions: Acute PRU evaluation possesses predictive value for recurrent ischemic stroke, especially within 7 days in AIS/TIA with LAA.

Key words: Atherosclerosis, Clopidogrel, CYP2C19, Platelet aggregation

Introduction

Clopidogrel, a platelet P2Y12 receptor antagonist, is commonly used with aspirin in patients undergoing acute ischemic stroke (AIS) or transient ischemic attack (TIA) with large artery atherosclerosis (LAA)[1]. Clopidogrel is also an inactive prodrug that must be converted to a biologically-active metabolite by hepatic cytochrome P450 (CYP) enzymes (particularly CYP2C19)[2]. Within CYP2C19 loss-of function variants, the prevalence of intermediate metabolizers (IM) or poor metabolizers (PM) is much higher in Asian than Caucasian populations[3]. Furthermore, the LAA subtype, especially intracranial LAA, is common in Asian populations[4, 5]. Thus, the impact of CYP2C19 polymorphisms and platelet reactivity on clinical outcomes in AIS/TIA with LAA is an important concern, especially in Asian populations.

The impact of the CYP2C19 polymorphism on stroke prevention in AIS/TIA has been controversial[6-9]. Recently, our paper[10] reviewed a difference in the effect of the CYP2C19 PM genotype for cerebrovascular events between the acute and subacute-chronic stroke phase; 10 of 11 (91%) studies recruiting patients during the acute phase (within 1 week after the index stroke) demonstrated a positive effect[11-20] but all three studies recruiting during the subacute-chronic phase (within >2 weeks) showed no significant effect[7, 8, 10]. Thus, the effect of the CYP2C19 polymorphism on cerebrocardiovascular events appears more pertinent during the acute than chronic stroke phase, an important finding given the difficulty in obtaining genetic testing results in the acute period.

P2Y12 reaction units (PRU), currently used as clinical indicators of responsiveness to P2Y12 receptor antagonists, are quickly measured, even in the acute stroke phase. PRU is an estimate of P2Y12 receptor-mediated platelet aggregation and influenced by genetic factors, including CYP2C19 polymorphisms. The impact of acute PRU evaluation on clinical outcomes in AIS/TIA with LAA has not been established. We therefore aimed to investigate the predictive value of acute PRU evaluation for recurrent ischemic stroke in patients with AIS/TIA with LAA, and the difference between acute (≤ 7days) and subacute (8–90 days) phases.

Methods

Platelet Reactivity Monitoring in Acute Ischemic StrokE (PRAISE) is a prospective, open-label, multicenter (16 sites), observational study. The study protocol and data collection were approved by the ethics committee of the National Cerebral and Cardiovascular Center (ethical approval number M25-073-4) and all other study centers. All procedures were conducted within ethical standards of relevant national guidelines on human experimentation and the Helsinki Declaration of 1975 (revised, 2008). All participants, or their representatives, provided written consent before study enrollment.

Patients who developed AIS/TIA with symptomatic atherosclerotic stenosis (≥ 50%) or occlusion of ipsilateral intracranial or extracranial arteries within 7 days after onset and treated with oral clopidogrel, were enrolled between October 2013 and December 2015. Other inclusion criteria consisted of (1) age greater than 20 years, (2) National Institutes of Health Stroke Scale (NIHSS) score of 0 to 20 before treatment, and (3) intra/extracranial arterial stenosis/occlusion of internal carotid, anterior cerebral (A1 and A2 segment), middle cerebral (M1 and M2 segment), posterior cerebral (P1 and P2 segment), vertebral, or basilar, artery. We selected patients with a pretreatment NIHSS score ranging from 0 to 20, as patients with a very high NIHSS score over 20 may have large ischemic lesions liable to hemorrhagic transformation, subsequently leading to early discontinuation of clopidogrel. The degree of arterial stenosis on MRA was graded as moderate (50%-69%), severe (70%-99% or focal flow void), or occlusion.

The exclusion criteria consisted of (1) prior modified Rankin Scale score >3, (2) cardio-embolic source, (3) contraindication to MRI scanning, (4) treatment with ozagrel (thromboxane synthetase inhibitor), (5) intracranial or severe systemic hemorrhage. Ischemic stroke was defined as a new neurological deficit of vascular origin lasting at least 24 hours, based on radiological evidence of stroke. TIA was defined as a transient episode of neurological dysfunction caused by focal brain ischemia, which improved within 24 hours.

This study included six visits: enrollment (baseline), 24 hours, 72 hours, 7 days, 30 days, and 90 days after the enrollment.

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Acute Treatment

The Japanese Guidelines for the Management of Patients with AIS recommends the use of dual antiplatelet therapy and argatroban, an injectable direct thrombin inhibitor, in patients with large artery atherosclerosis. Clopidogrel and ticlopidine are P2Y12 receptor antagonists approved for use in stroke in Japan. In this study, the acute treatment protocol included the use of clopidogrel ((i) continued at 75 mg/day standard dose used before admission, (ii) newly administered at 75 mg/day standard dose, (iii) newly administered at 300 mg loading and followed by 75 mg/day standard dose), with or without other antiplatelet agents (including aspirin at 200 mg/day and cilostazol at 200 mg/day), anticoagulant agents (including argatroban injection), optionally used by specialist stroke neurologists and neurosurgeons at each study center, who were blinded to the patients' genetic information and platelet reactivity (P2Y12 and aspirin reaction units), in line with the guidelines. Indications of thrombolysis with alteplase at 0.6 mg/kg, endovascular treatment (thrombectomy, angioplasty, intracranial and carotid artery stenting), and neurosurgical treatment (carotid endarterectomy and superficial temporal artery-middle cerebral artery bypass) were also in line with the guidelines.

P2Y12 and Aspirin Reaction Units

Platelet reactivity was measured by PRU and aspirin reaction units using the Verify Now® system (Instrumentation Laboratory, Bedford, Massachusetts, USA) at 24 and 72 hours after initiation of antiplatelet therapy. The VerifyNow system is a clinically available, rapid, and easy-to-use point-of-care system, suitable for evaluating acute stroke patients, thereby holding advantages over other assays, such as light transmittance aggregometry. The VerifyNow system was used by qualified personnel in each study center. Platelet reaction units were measured in each study center by specially trained and qualified personnel, according to a standardized procedure manual.

Genotyping

Among the several genetic variants of CYP2C19, the two most frequent variants[21], CYP2C19*2, and CYP2C19*3, are known as loss-of-function (LOF) variants; whereas, the gain-of-function variant, CYP2C19*17, is associated with increased CYP2C19 enzyme function[22].

Genomic DNA was extracted from peripheral blood leukocytes. The genotype of the *CYP2C19*2* (c.681G>A, rs4244285), **3* (c.636G>A, rs4986893), and **17* (c.-806C>T, rs12248560) variants was determined by the TaqMan genotype discrimination method (Applied Biosystems, Foster City, CA, USA) using commercially available primers and probes purchased from the Assay-on-Demand system. Patients were classified into three *CYP2C19* metabolizer groups, according to genotype, as follows: (1) extensive metabolizer (EM) not bearing the genetic variants *CYP2C19*2* or *CYP2C19*3* (**1/*1*); (2) intermediate metabolizer carrying one LOF allele (**1/*2*, **1/*3*); and (3) poor metabolizer carrying 2 LOF alleles (**2/*2*, **2/*3*, **3/*3*). There has been controversy regarding the phenotype associated with the CYP2C19*2/*17 and *3/*17 genotypes, seldom found in Asian populations[23, 24]. Therefore, patients with the CYP2C19*17 genotype (n=10) were excluded from the analysis. A single-nucleotide polymorphism of the *ABCB1* gene (3435C>T, rs1045642) was genotyped. Participants were classified as homozygous for the C allele (CC), heterozygous (CT), or homozygous for the T allele (TT) for ABCB1 3435C>T SNP.

Imaging Protocol

Magnetic resonance angiography and magnetic resonance imaging studies, including diffusion-weighted imaging, fluid-attenuated inversion recovery, and T2*-weighted images sequences, were performed at baseline (before treatment), between 7–14 days after enrollment, and at the time of neurological worsening (follow-up MRI scan was performed after the neurological worsening if necessary). All magnetic resonance images were visually assessed independently by at least two local specialist stroke neurologists or neurosurgeons blinded to patient genetic information and laboratory assay results.

Clinical Outcomes

The prespecified primary outcome was recurrent ischemic stroke within 90 days. Additionally, we divided primary outcome into acute phase (within 7 days) and subacute phase (during 8–90 days) as we assumed the effect of the CYP2C19 polymorphism and clopidogrel reactivity on cerebrocardiovascular events appears more pertinent during the acute than subacute-chronic stroke phase, as noted above.

Recurrent ischemic stroke was defined as the following (i) or (ii) below: (i) neurological deterioration with new acute ischemic lesions on brain MRI at the time of worsening within 90 days after enrollment; (ii) apparent neurological deterioration (≥ 4-point increase in NIHSS score compared to the admission score) without new lesions on brain MRI at the time of worsening and subsequent delayed responsible ischemic lesions on follow-up brain MRI within 30 days after enrollment. Delayed ischemic

lesions were assessed as recurrent ischemic stroke sometimes occurs without new ischemic lesions at the time of worsening, and confirmed by detecting responsible lesions with a delayed appearance on follow-up imaging[25]. Neurological deterioration due to symptomatic hemorrhagic transformation after cerebral infarction, hemorrhagic stroke, and other neurological deficits unrelated to ischemic stroke (e.g., infection) was excluded.

Secondary outcomes included all-cause death, major bleeding, and ST-elevated acute myocardial infarction. Major bleeding included intracranial hemorrhage, symptomatic bleeding (such as intraspinal, intraocular, retroperitoneal, intra-articular, pericardial or intramuscular bleeding with compartment syndrome), and episodes that caused ≥ 2 g/dL decline in hemoglobin level or required at least 280 ml of red cell transfusion.

All outcomes were assessed by at least two local expert stroke doctors, then verified by the events committee blinded to the patients' genetic information and laboratory assay results, including PRU.

Statistical Analysis

Patient characteristics were collected at baseline. Smoking habit was defined as consumption of at least one cigarette per day for at least 1 year. Estimated glomerular filtration rate was calculated using the abbreviated Modification of Diet in Renal Disease equation. Continuous variables are presented using the mean, standard deviation (SD), and median with interquartile range, whereas categorical variables are expressed as frequencies and percentages. Categorical variables were compared using χ^2 or Fisher's exact test. Continuous data were compared by Student-*t* or Wilcoxon rank-sum test. A probability value of less than 0.05 was considered significant.

The associations of recurrence with PRU, gene polymorphism, and other parameters were estimated using univariable analysis and multivariable logistic regression analysis adjusted for factors with a p value <0.05 in crude analysis or clinical significance in patients undergoing both PRU evaluation and genetic testing.

In the genetic testing population, the multivariable receiver operating characteristic curve (ROC) was used to evaluate the predictive performance of the P2Y12 assay for recurrence within 90 days and compared between the acute (within 7 days) and subacute phase (during 8–90 days). A scatter plot was created to show the association between PRU at 24 hours and the number of days to recurrence, comparing PM and IM/EM groups of CYP2C19. Risk prediction of high PRU and the CYP2C19 PM genotype for recurrence was quantified by comparison of area-under-the-curve (AUC) of multivariable ROC curves and by net reclassification improvement (NRI) analysis.

In selected patients who underwent genetic testing and clopidogrel loading dose administration, the associations of PRU at 24 hours with possible influencing factors of clopidogrel responsiveness, including gene polymorphism, combined drugs (cilostazol[26], statins[27], and proton pump inhibitors[28]), age, body-mass index, smoking habit, diabetes[27], renal failure[29], and platelet count[30], were analyzed by univariable and multiple logistic regression analysis. Statistical analysis was performed using JMP14 software (SAS Institute, Cary, NC, USA) and Stata version 15 (Stata Corp, Texas, USA).

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Results

The study flow is shown in **Fig. 1**. We screened 237 patients diagnosed with AIS or TIA with LAA and treated with clopidogrel from 1 October 2013 to 31 December 2015. Seven patients not satisfying the inclusion/exclusion criteria were excluded, meaning 230 patients were enrolled in the PRAISE study. A further 5 patients were excluded due to being lost at follow-up (n=4) and inadequate PRU evaluation (n=1). 225 patients with complete outcome and PRU data were analyzed as the study population, with 194 patients also assessed as the genetic testing population.

Analysis of the Study Population

At baseline, the 225 patients had a median age of 72 years and comprised 31% females. The stroke subtype was composed of AIS (83%) and TIA (17%) cases. At registration, continued use of standard dose (75 mg), newly administered standard dose (75 mg), and newly administered loading dose (300 mg) of clopidogrel were identified in 11 (4.9%), 74 (33%), and 140 (62%) of patients, respectively. Recurrent ischemic stroke occurred in 36 patients (16%) within 90 days, and in 22 patients (10%) within 7 days. The secondary outcomes are shown in **Table 1**. All-cause death occurred in 1 patient and major bleeding in 4 patients; acute myocardial infarction did not occur in any patients. Details of primary and secondary outcomes and endovascular/neurosurgical treatment are shown in the Data Supplement.

The baseline characteristics in patients with non-recurrence, and recurrence within 90 days and within 7 days are shown in **Table 2**. In univariable analysis,

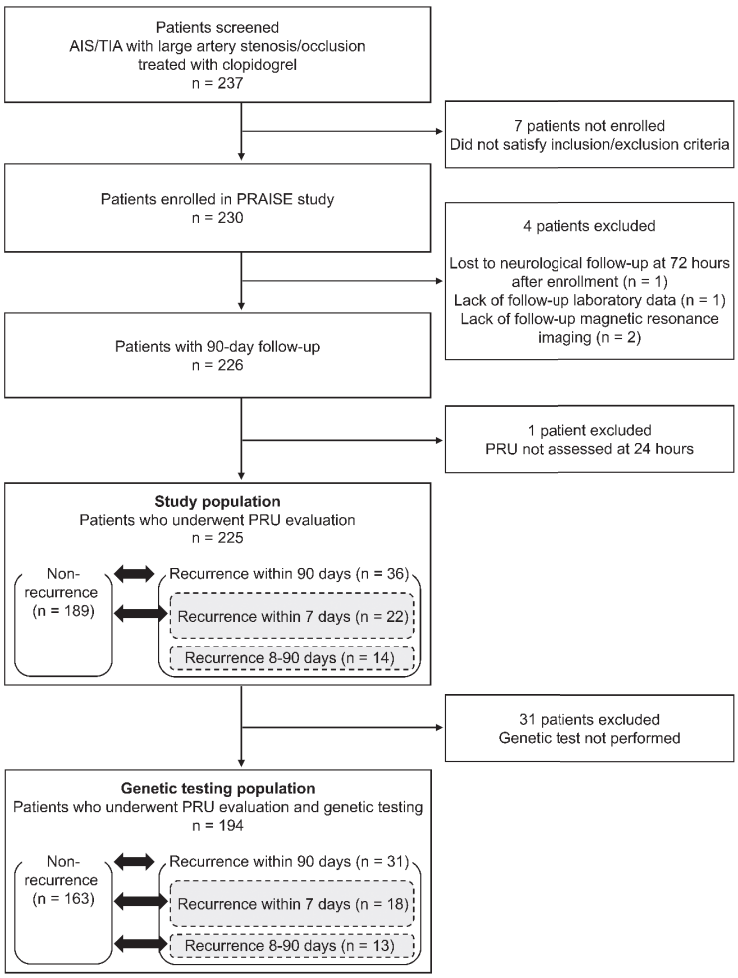

Fig. 1. Flowchart of patient participation in the study

AIS/TIA, Acute ischemic stroke or transient ischemic attack; PRU, P2Y12 reaction units.

Table 1. Secondary outcomes (n=225)

Secondary outcomes	N	Event details	PRU at 24h	PRU at 72h	ARU at 24h	ARU at 72h	CYP2C19 variants	Medication at events	Days to event
All-cause death	1	Obstructive hydrocephalus due to multiple infarctions including cerebellar hemisphere	317	247	527	538	EM	CLP 75 mg	9
Major bleeding	4	Lower gastrointestinal tract bleeding	145	128	493	491	PM	CLP 75 mg, ASA 100 mg	34
		Bleeding from gastric cancer	231	156	393	474	IM	CLP 75 mg, ASA 100 mg	22
		Intracerebral hemorrhage	244	230	579	541	NA	CLP 75 mg, ASA 200 mg	19
		Femoral hematoma after occurring after bruise	254	325	497	414	PM	CLP 75 mg, ASA 100 mg	4
Myocardial infarction	0								

Abbreviations: ARU, aspirin reaction units; ASA, aspirin; CLP, clopidogrel; EM, extensive metabolizer; h, hours; IM, intermediate metabolizer; NA, not assessed; N, number of patients; and PRU, P2Y12 reaction units.

Table 2. Patient characteristics

Study population (n=225)	Non-recurrence (n=189)	Recurrence within 90 days (n=36)	P	Recurrence within 7 days (n=22)	P
Female	58 (31)	11 (31)	0.99	8 (36)	0.59
Age, years	72 ± 11	73 ± 10	0.53	74 ± 9	0.63
Body mass index, kg/m^2	23 ± 4	22 ± 3	0.06	22 ± 3	0.18
Smoking habit	54 (29)	10 (28)	0.93	7 (32)	0.75
NIHSS on admission, median (IQR)	2 (0–4)	3.5 (1–6)	0.03	4 (1–7.25)	0.05
Medical history					
Hypertension	157 (83)	31 (86)	0.65	19 (86)	>0.99
Dyslipidemia	123 (65)	19 (53)	0.16	12 (55)	0.33
Diabetes mellitus	66 (35)	9 (25)	0.25	6 (27)	0.47
Past ischemic stroke	31 (16)	8 (22)	0.40	2 (9.1)	0.54
Past coronary heart disease	25 (13)	4 (11)	0.73	2 (9.1)	0.75
Prior medication					
Anti-platelet drug	61 (32)	15 (42)	0.27	8 (36)	0.70
- Clopidogrel	8 (4.2)	3 (8.3)	0.39	1 (4.5)	>0.99
Anti-coagulant drug	4 (2.1)	0 (0)		0 (0)	
Statin	49 (25)	8 (22)	0.64	4 (18)	0.43
Proton pump inhibitor	37 (20)	8 (22)	0.72	3 (14)	0.78
Acute medication					
Dual anti-platelet drug	150 (79)	32 (89)	0.19	20 (91)	0.19
Loading of clopidogrel	112 (59)	28 (78)	0.04	18 (83)	0.04
Anti-coagulant drug	143 (76)	29 (81)	0.53	19 (86)	0.26
- Argatroban	137 (73)	28 (78)	0.51	18 (82)	0.35
Thrombolysis	8 (4.2)	3 (8.3)	0.20	1 (4.5)	0.59
Laboratory data					
PRU value at 24h	219 ± 70	251 ± 78	0.04	273 ± 77	0.003
PRU value at 72h	204 ± 84	224 ± 72	0.09	242 ± 65	0.01
ARU value at 24h	502 ± 96	505 ± 82	0.62	505 ± 76	0.59
ARU value at 72h	498 ± 100	498 ± 79	0.93	511 ± 73	0.43
Hemoglobin level, g/dL	13.8 ± 1.8	13.6 ± 1.9	0.86	13.3 ± 1.9	0.49
Platelet count, 10^3/μL	220 ± 76	198 ± 60	0.17	201 ± 64	0.39
eGFR, ml/min	65 ± 20	67 ± 20	0.66	67 ± 23	0.87
LDL-C level, mg/dL	124 ± 38	121 ± 35	0.69	119 ± 36	0.54
HDL-C level, mg/dL	50 ± 13	50 ± 12	0.78	50 ± 14	0.84
HbA1c, %	6.3 ± 1.1	6.4 ± 1.6	0.46	6.5 ± 1.6	0.92
Imaging findings					
Large artery occlusion	38 (20)	12 (33)	0.22	12 (54)	0.002
Severe stenosis	84 44)	13 (36)		5 (23)	
Moderate stenosis	67 (35)	11 (31)		5 (23)	
Genetic testing population (n=194)					
Gene polymorphism	Non-recurrence (n=163)	Recurrence within 90 days (n=31)	P	Recurrence within 7 days (n=18)	P
CYP2C19 variants					
- PM	20 (12)	9 (29)	0.04	6 (33)	0.04
- IM	94 (58)	16 (52)		7 (39)	
- EM	49 (30)	6 (19)		5 (28)	
CYP2C19*17 (C806T) (rs12248560)					
- CT (versus CC)	2 (1.2)	0 (0)		0 (0)	
ABCB1 (C3435T) (rs1045642)					
- TT (versus CC/CT)	24 (15)	6 (19)	0.81	4 (22)	0.69

Table comparing non-recurrence with recurrence in the study population within 90 days and 7 days groups, respectively (as shown in Fig. 1).
Data are presented as n (%) or means ± standard deviation, unless otherwise indicated.
Abbreviations: ARU, aspirin reaction units; BMI, body mass index; eGFR, estimated glomerular filtration rate; EM, extensive metabolizer; h, hours; HDL-C, high-density lipoprotein-cholesterol; IM, intermediate metabolizer; IQR, interquartile range; LDL-C, low-density lipoprotein-cholesterol; NIHSS, National Institutes of Health stroke scale; PM, poor metabolizer; and PRU, P2Y12 reaction units.

Table 3. Multivariable logistic regression analysis of P2Y12 reaction units and clinical variables associated with recurrence (n=225)

	N	Unadjusted OR (95% CI)	P	Model 1[§] OR (95% CI)	P	Model 2[†] OR (95% CI)	P
PRU at 24 hours, 10 units							
- Recurrence within 90 days	36	1.07 (1.01–1.13)	0.01	1.08 (1.02–1.15)	0.004	1.08 (1.02–1.15)	0.004
- Recurrence within 7 days	22	1.12 (1.05–1.21)	<0.001	1.14 (1.06–1.24)	<0.001	1.15 (1.07–1.25)	<0.001
- Recurrence during 8–90 days	14	1.00 (0.92–1.08)	0.90	1.00 (0.92–1.09)	0.97	NA[‡]	NA[‡]

[§]Model 1: adjusted for NIHSS and clopidogrel loading.
[†]Model 2: adjusted for NIHSS, clopidogrel loading, and large artery occlusion.
[‡]The multivariable analysis using model 2 of recurrence during 8–90 days could not be performed as large artery occlusion perfectly predicted recurrence within 7 days.
Abbreviations: CI, confidence interval; N, number of patients; NA, not applicable; OR, odds ratio; and PRU, P2Y12 reaction units.

clopidogrel loading and higher PRU at 24 hours were significantly related to recurrence within 7 and 90 days. Large artery occlusion was significantly related to recurrence within 7, but not 90, days. NIHSS on admission was significantly related to recurrence within 90 days.

The results of multivariable logistic regression analysis for recurrence during the three time periods, are shown in **Table 3**. This was performed using two models: model 1 was adjusted for NIHSS and clopidogrel loading; model 2 was adjusted for NIHSS, clopidogrel loading, and large artery occlusion. A higher PRU at 24 hours was significantly associated with recurrence within 90 days (adjusted odds ratio (OR) per 10 units, 1.08; 95% CI, 1.02–1.15; p=0.01) and 7 days (adjusted OR per 10 units, 1.14; 95% CI, 1.06–1.24; p<0.001) for model 1, with similar associations observed for model 2. There was no significant association of PRU at 24 hours with recurrence during 8–90 days. Large artery occlusion was significantly associated with recurrence within 7 days in model 2 only (adjusted OR, 5.63; 95% CI, 2.03–15.62; p=0.001). The subgroup analysis of 140 patients receiving clopidogrel loading dose and 182 patients receiving dual antiplatelet therapy, including clopidogrel, revealed similar results **(Supplemental Tables 1 and 2)**.

Analysis of the Genetic Testing Population

In the population of 194 patients undergoing genetic testing, the characteristics were similar to the entire study population (not shown). The genetic results are shown in **Table 2**. The distribution of the clopidogrel-metabolizing groups, according to CYP2C19 genotype, was as follows: 28%, 57%, and 15% for EM (n=55; *1/*1), IM (n=110; *1/*2, *1/*3), and PM (n=29; *2/*2, *2/*3, *3/*3) groups, respectively. The univariable and multivariable ROC analysis, adjusted for NIHSS and clopidogrel loading, found a PRU of 254 as the best cut-off value for predicting recurrence within 90 and 7 days. The multivariable ROC analysis showed the AUC for predicting recurrence within 7 days was greater, though non-significantly, than found during the 8–90-day period (AUC, 0.79; 95% CI, 0.69–0.88 versus AUC, 0.64; 95% CI, 0.50–0.77; p=0.08) **(Fig. 2A, B, and C)**. **Fig. 2D** shows a scatter plot of the 31 patients who experienced recurrence in the genetic testing population. High PRU (≥ 254) was identified in 13 of 18 patients (72%) who experienced recurrence within 7 days but in only 3 of 13 patients (23%) during 8–90 days. PM of CYP2C19 was shown in 6 of the 13 patients (46%) with high PRU (≥ 254) who experienced recurrence within 7 days.

Multivariable logistic regression analysis, adjusted for NIHSS and clopidogrel loading, showed recurrence within 7 days, and was significantly related to high PRU (≥ 254) (adjusted OR, 6.82; 95% CI, 2.23–20.9; p<0.001) and PM of CYP2C19 (adjusted OR, 5.84; 95% CI, 1.75–19.5; p=0.006). In the multivariable ROC curve adjusted for the same variables, the AUC of high PRU (≥ 254) for predicting recurrence within 7 days was greater (AUC, 0.79; 95% CI, 0.69–0.88 versus AUC, 0.71; 95% CI, 0.59–0.84; p=0.24), though not significantly, than PM of CYP2C19. NRI was also used to evaluate potential predictive improvement. The modified model, adding high PRU (≥ 254) to the basic model, including PM of CYP2C19, NIHSS, and clopidogrel loading, significantly improved the prediction of recurrence within 7 days: NRI, 0.83 (95% CI, 0.34–1.32; p<0.001) **(Table 4)**.

In the subgroup of 121 patients receiving clopidogrel loading dose, multivariable logistic regression analysis showed high PRU (≥ 254) was significantly affected by the CYP2C19 PM genotype, older age, and proton pump inhibitors **(Supplemental Fig. 1)**.

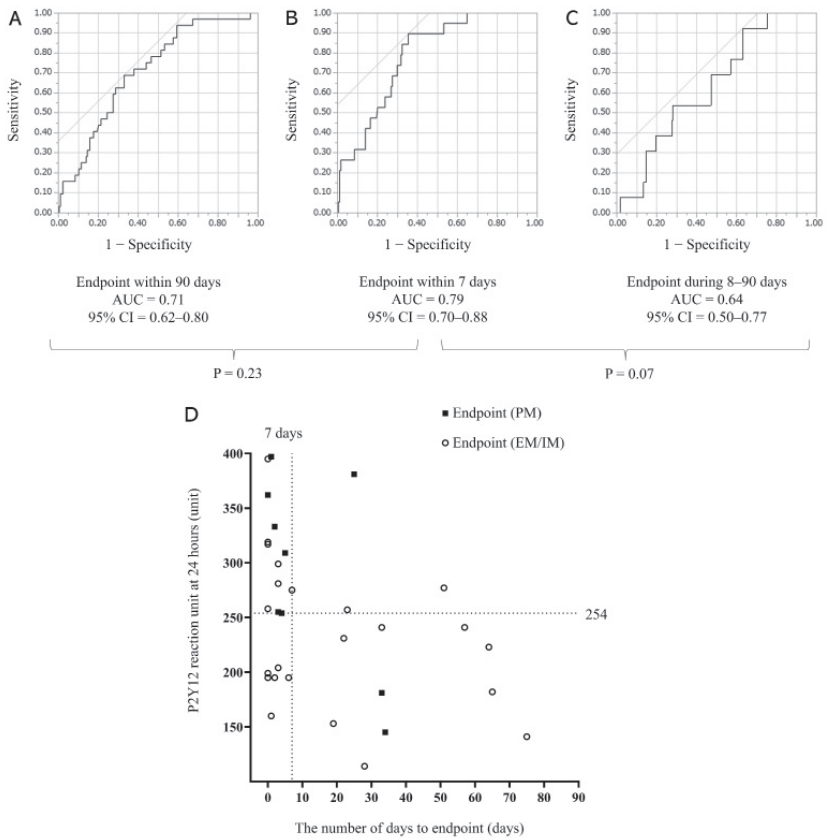

Fig. 2. Multivariable receiver operating characteristic curves and scatter plot showing the relationship between P2Y12 reaction units at 24 hours and recurrence (n=194)

Multivariable receiver operating characteristic curves depicting predictive performance of the P2Y12 reaction units (PRU) assays for recurrence within 90 days (A), within 7 days (B), and during 8–90 days (C).
Scatter plot showing correlation between PRU and the number of days to recurrence (D). Black squares show a value of PRU at 24 hours in the CYP2C19 poor metabolizer (PM) group. Empty circles show a value of PRU at 24 hours in the CYP2C19 extensive or intermediate metabolizer (EM/IM) group.

Table 4. Prediction performance of the P2Y12 assay and CYP2C19 genotyping for recurrence (n=194)

	Recurrence within 7 days (n=18)		Recurrence during 8-90 days (n=13)	
Multivariable logistic regression[§]	Adjusted OR (95% CI)	*P*	Adjusted OR (95% CI)	*P*
PM of CYP2C19	5.84 (1.75–19.5)	0.006	2.94 (0.69–12.5)	0.23
PRU value ≥ 254	6.82 (2.23–20.9)	<0.001	1.34 (0.35–5.15)	0.67
Comparison of receiver operating characteristic[†]	Adjusted AUC (95% CI)	*P*	Adjusted AUC (95% CI)	*P*
PM of CYP2C19 (Control)	0.71 (0.59–0.84)	-	0.64 (0.49–0.79)	-
PRU value ≥ 254	0.79 (0.70–0.88)	0.24	0.65 (0.49–0.80)	0.96
Assessment of risk reclassification[‡]	NRI (95% CI)	*P*	NRI	*P*
PM of CYP2C19 adjusted for NIHSS and CLP loading (Basic model)	-	-	-	-
Basic model + PRU value ≥ 254	0.83 (0.34–1.32)	<0.001	0.15 (-0.44–0.72)	0.60

[§]Adjusted for NIHSS and clopidogrel loading.
[†]Adjusted for NIHSS and clopidogrel loading.
[‡]Net reclassification improvement was calculated by adding high PRU (≥ 254), with a basic model including PM of CYP2C19, NIHSS, and clopidogrel loading, improving prediction of recurrence.
Abbreviations: AUC, area-under-the-curve; CI, confidence interval; CLP, clopidogrel; h, hours; NIHSS, National Institutes of Health stroke scale; NRI, net reclassification improvement; OR, odds ratio; PM, poor metabolizer; PRU, P2Y12 reaction units; and ROC, receiver operating characteristic.

Discussion

This study yielded two main results in AIS or TIA with LAA. Firstly, high PRU (≥ 254) at 24 hours was significantly associated with recurrent ischemic stroke, especially in acute phase (within 7 days). Secondly, high PRU (≥ 254) had a comparable, or conditionally better, predictive performance for recurrence within 7 days, compared to PM of CYP2C19. To our knowledge, this is the first study to demonstrate the predictive performance of acute PRU evaluation for recurrence in AIS/TIA with LAA.

PRU is a predictive marker related to thromboembolic or hemorrhagic events in patients treated with P2Y12 receptor antagonists. Previous studies have shown acute thrombotic events are associated with high PRU measured early after percutaneous coronary intervention for coronary artery disease[31] or endovascular therapy for cerebrovascular disorders[32]. In contrast, little is known about the association between clinical events and PRU in patients with AIS not undergoing endovascular therapy. A recent randomized clinical trial showed DAPT using ticagrelor/aspirin reduced the frequency of high PRU (>208) at 90 days (i.e. subacute PRU evaluation) and stroke recurrence within 90 days (i.e. during the acute and subacute stroke phase), compared with clopidogrel/aspirin, in patients with minor AIS or TIA, including patients with LAA[33]. This trial possesses similarities to the present study as the majority of study patients had mild neurological dysfunction at enrollment. The present study is relevant and complementary to the previous report, being the first to demonstrate the predictive value of acute PRU evaluation for recurrence in patients with high-risk AIS/TIA with LAA.

In this study, high PRU at 24 hours was independently significantly associated with recurrence, especially within 7 days. The ROC analysis showed a PRU of 254 as the best cut-off value for predicting recurrence, and greater AUC value for the PRU assay in predicting recurrence within 7 days compared to the 8–90-day period (AUC, 0.79 versus 0.64; p=0.08). Specifically, the association of recurrence with high PRU at 24 hours was affected by recurrence within 7, rather than 8–90, days. These results are consistent with our recent report[10], which showed a difference in the effect of the CYP2C19 PM genotype for cerebrovascular events between the acute and subacute-chronic stroke phase, with a negative effect in the acute, but no significant effect in the chronic, stroke phase. In addition, a randomized clinical trial for AIS/TIA in Asian populations carrying the CYP2C19 PM genotype showed acute DAPT using a combination of aspirin and clopidogrel with loading dose was superior to aspirin alone for reducing the risk of recurrent stroke, which mainly occurred during the acute stroke phase[19, 34], suggesting the clinical importance of adequate antiplatelet therapy in AIS/TIA. Thus, we predict the current and previous studies emphasize the importance of the antiplatelet effects of clopidogrel and the CYP2C19 gene polymorphism for antiplatelet therapy in AIS. The PRU assay findings might suggest effective antiplatelet therapy should be initiated and continued for at least 1 week after AIS/TIA with LAA onset.

The study also found high PRU at 24 hours (≥ 254) had a predictive performance for recurrence within 7 days (adjusted OR, 6.82; 95% CI, 2.23–20.9), which was comparable to PM of CYP2C19 (adjusted OR, 5.84; 95% CI, 1.75–19.5) (AUC, 0.79; 95% CI, 0.69–0.88 versus 0.71; 95% CI, 0.59–0.84; p=0.24). Furthermore, NRI analysis showed significantly improved risk prediction with the addition of PRU evaluation to the CYP2C19 genotyping model (0.83; 95% CI, 0.34–1.32; p< 0.001). Influencing factors for platelet reactivity other than the CYP2C19 polymorphism could explain this effect. Our results showed not only PM of CYP2C19, but also PPIs and older age, were significantly associated with high PRU (≥ 254) at 24 hours. In addition, the administration of statins and cilostazol tended to be inversely associated with PRU at 24 hours in univariable analysis. PRU evaluation therefore appears favorable to genetic testing, as it allows a more rapid and direct measure of the consequent antiplatelet effect and how this is affected by influencing factors. In AIS/TIA with LAA, acute PRU evaluation directly measuring platelet reactivity is acceptable for clinical use as AIS/TIA patients are usually exposed to such influencing factors and may experience difficulty receiving genetic testing during the acute phase.

This study has several limitations. Firstly, it is an observational design, with the chosen antiplatelet and anticoagulant agents dependent on the discretion of the patient's stroke neurologist or neurosurgeon. However, most patients received relatively uniform treatment with DAPT and argatroban through adherence to the Japanese Guidelines for AIS. In addition, the reason for the association between recurrence and clopidogrel loading may lie in the choice of high-risk AIS/TIA by physicians treating the patients. Secondly, the study had no data on novel P2Y12 receptor antagonists. Nevertheless, our results indicate that acute PRU evaluation can identify patients at high risk of recurrence who should be considered for change in P2Y12 receptor antagonists

(e.g. clopidogrel to novel P2Y12 receptor antagonists) and concomitant drugs (e.g. PPIs to novel acid-inhibitory drugs, such as vonoprazan, whose metabolism is independent of CYP2C19 genotype). Further research is needed to confirm whether a change in P2Y12 receptor antagonists (i.e. clopidogrel to novel P2Y12 receptor antagonists) can reduce recurrence during the acute phase in ischemic stroke/TIA with LAA with high PRU. Thirdly, this study included participants from Asian populations only. Whilst limiting the study's scope, this did provide advantages in obtaining evidence of acute PRU evaluation because of the high frequency of intracranial LAA and PM of CYP2C19 in Asian populations[3]. In addition, our recent study demonstrated that the Asian-specific RNF213 p.R4810K variant partly explains the high frequency of the intracranial LAA subtype in Asian populations[35]. A future treatment strategy for IS/TIA with LAA in Asian populations may be tailored to platelet reactivity monitoring and variants of the CYP2C19 and RNF213 genotype.

Conclusions

This study demonstrates acute PRU evaluation has predictive value for recurrent ischemic stroke, especially within 7 days in high-risk AIS/TIA with large artery stenosis/occlusion. High PRU (≥ 254) has greater reliability in predicting recurrence within 7 days than CYP2C19 genetic testing, as rapidly assessed PRU can monitor the antiplatelet effects of P2Y12 receptor antagonists affected not only by gene polymorphism but also environmental factors. This finding suggests potential for tailored treatments with novel P2Y12 receptor antagonists based on platelet reactivity monitoring in AIS/TIA with LAA.

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Conflicts of Interest/Competing Interests

Dr. Hiroshi Yamagami and Dr. Kazunori Toyoda have received speaker's honoraria from Bayer and Daiichi Sankyo. Dr. Ryo Itabashi has received speaker's honoraria from Daiichi Sankyo. Dr. Shigeki Miyata has received research support and speaker honoraria from Daiichi Sankyo Co., Ltd. and Mitsubishi Tanabe Pharma Corporation, both of whom manufacture argatroban, used in the treatment of patients with acute ischemic stroke in this study. Dr. Shigeki Miyata is currently working at the Central Blood Institute, Japanese Red Cross Society, which manufactures blood products related to the secondary outcome of this study. The other authors report no conflicts of interest in connection with this study.

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Authors' Contributions

HY and KaN. conceived and designed the study, with additional input from KF, MI, TT, TM, SM, KK, HY, MH, KK, YE, RI, EF, YM, ME, KT, KH, SU, and KT. All authors contributed to data acquisition, analysis, and interpretation. TM, SM, and KK contributed to the experimental studies. KuN and YN performed the statistical analysis. KF, HY, TT and MI contributed to drafting of text and preparation of figures. All authors reviewed and commented on the manuscript.

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Supplemental Results

Details of Primary Outcomes

Among the 32 patients with recurrent ischemic stroke, 30 patients presented neurological deterioration, with new acute ischemic lesions on brain MRI at the time of worsening within 90 days after enrollment. The remaining two patients presented apparent neurological deterioration (≥ 4-point increase in NIHSS score compared to the admission score), without new lesions on brain MRI at the time of worsening and subsequent delayed responsible ischemic lesions, referable to the large artery stenosis/occlusion, on follow-up brain MRI within 30 days after enrollment. The median (range, interquartile range) number of days to recurrence was 4.5 days (0–75 days, 1-31.75 days).

Details of Secondary Outcomes

All-cause death occurred in a patient who had obstructive hydrocephalus due to multiple infarctions, including in the cerebellar hemisphere. Major bleeding occurred in 4 patients treated with clopidogrel and aspirin; 2 of the 4 patients had a low PRU value at 72h (156 and 128, respectively), and no drugs and comorbidities affecting PRU reduction. Possible causes of serious bleeding may be low platelet reaction, dual antiplatelet therapy or organic cause/ bruise injury. No patient had an acute myocardial infarction.

Endovascular/Neurosurgical Treatment

Fifty patients underwent endovascular/ neurosurgical treatment: 3 for thrombectomy, 5 for angioplasty, 1 for intracranial artery stenting, 8 for carotid artery stenting, 27 for carotid endarterectomy, and 8 for superficial temporal artery-middle cerebral artery bypass. Two patients underwent two procedures: one in thrombectomy and angioplasty, the other in thrombectomy and intracranial stenting. In the 50 patients who underwent endovascular/ neurosurgical treatment, all recurrence of ischemic stroke occurred before endovascular/neurosurgical treatment. The median number from recurrence to endovascular/neurosurgical treatment was 1 day (range, 0–56 days).

Subgroup Analysis of the Patients who Received Clopidogrel Loading Dose (n=140)

The results of multivariable logistic regression analysis for the primary outcomes divided by time period in the patients who received clopidogrel loading dose, are shown in **Supplemental Table 1**. This was performed using two models: model 1 was adjusted for NIHSS; model 2 was adjusted for NIHSS and large artery occlusion. A higher PRU at 24 hours was significantly associated with recurrence within 90 days (adjusted odds ratio (OR) per 10 units, 1.07; 95% CI, 1.01–1.14; p=0.03) and 7 days (adjusted OR per 10 units, 1.12; 95% CI, 1.03–1.21; p=0.003) for model 1, with similar associations observed for model 2. There was no significant association of PRU at 24 hours with recurrence during 8–90 days.

Subgroup Analysis of the Patients who Received Dual Antiplatelet Therapy including Clopidogrel (n=182)

The results of multivariable logistic regression analysis for the primary outcomes divided by time period in the patients who received dual antiplatelet therapy including clopidogrel, are shown in **Supplemental Table 2**. This was performed using two models: model 1 was adjusted for NIHSS; model 2 was adjusted for NIHSS and large artery occlusion. A higher PRU at 24 hours was significantly associated with recurrence within 90 days (adjusted OR per 10 units, 1.09; 95% CI, 1.02–1.16; p=0.004) and 7 days (adjusted OR per 10 units, 1.16; 95% CI, 1.07–1.26; p<0.001) for model 1, with similar associations observed for model 2. There was no significant association of PRU at 24 hours with recurrence during 8–90 days.

Association between PRU and Recurrent Ischemic Stroke according to the Severity of Stenosis of the Responsible Artery

The results of multivariable logistic regression analysis for the primary outcomes according to the severity of stenosis of the responsible artery, are shown in **Supplemental Table 3**. This model was adjusted for NIHSS and clopidogrel loading. Within the 147 patients with arterial occlusion or severe stenosis, a higher PRU at 24 hours was significantly associated with recurrence within 90 days (adjusted odds ratio (OR) per 10 units, 1.10; 95% CI, 1.03–1.18; p=0.005) and 7 days (adjusted OR per 10 units, 1.17; 95% CI, 1.07–1.28; p<0.001); however, there was no significant association of PRU at 24 hours with recurrence during 8–90 days. In contrast, of the 78 patients with moderate arterial stenosis, there was no significant association of PRU at 24 hours with recurrence during any periods in multivariable analysis. Similar associations were obtained in univariable analysis.

Subgroup Analysis of Patients with Transient Ischemic Attack (n=39)

Thirty-nine patients with transient ischemic attack (TIA) were included in the present study. While

not collecting data on the ABCD2 score itself, three of five factors for the score (age, blood pressure, and diabetes) were compiled. Within the 39 TIA patients, there was no difference in: (i) age over 60 years (100% versus 79%, p=0.56), (ii) blood pressure over 140/90 mmHg (100% versus 82%, p=0.57), (iii) diabetes (40% versus 24%, p=0.59) and (iv) subtotal score using the three factors (median, 2 (IQR, 2–3) versus median, 2 (IQR, 2–2)) between the patients with and without recurrence (shown in **Supplemental Table 4**). A subgroup analysis of the 39 TIA patients showed that administration types of clopidogrel were not different between those with and without severe stenosis/occlusion in terms of continued use of standard dose (12% versus 0%), newly administered standard dose (23% versus 0%), and newly administered loading dose (65% versus 100%) (χ^2 test, p=0.054).

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Supplemental Table 1. Multivariable logistic regression analysis of P2Y12 reaction units and clinical variables associated with recurrent ischemic stroke in patients receiving clopidogrel loading dose (n=140)

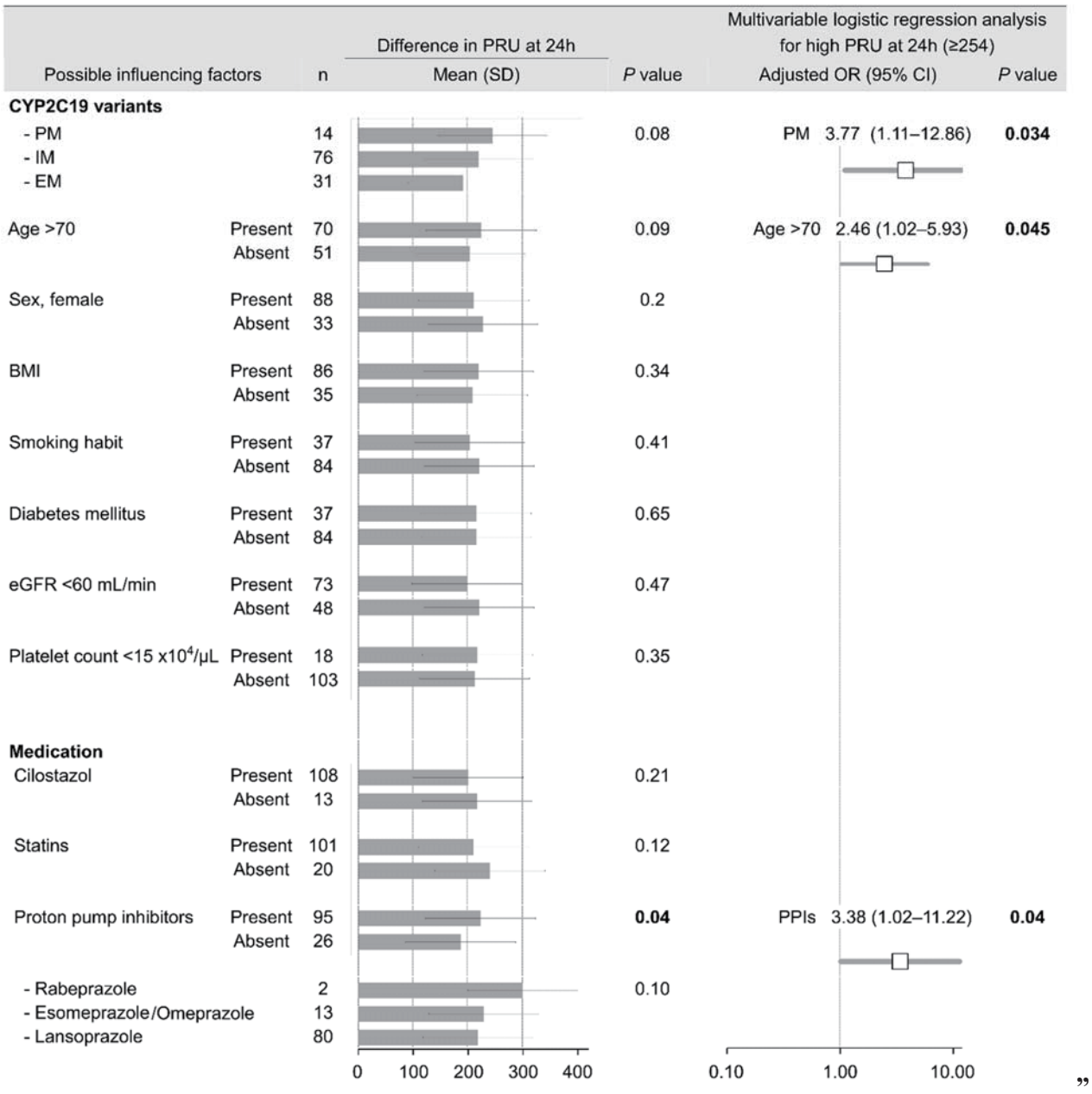
	N	Unadjusted OR (95% CI)	*P*	Model 1[§] OR (95% CI)	*P*	Model 2[†] OR (95% CI)	*P*
PRU at 24 hours, 10 units							
- Recurrence within 90 days	24	1.07 (1.01–1.14)	0.03	1.07 (1.00–1.14)	0.03	1.07(1.00–1.14)	0.03
- Recurrence within 7 days	14	1.12 (1.03–1.21)	0.003	1.12 (1.03–1.21)	0.03	1.12 (1.04–1.22)	0.002
- Recurrence during 8–90 days	10	0.99 (0.90–1.09)	0.90	0.99 (0.90–1.09)	0.97	NA[‡]	NA[‡]

[§]Model 1: Adjusted for NIHSS.
[†]Model 2: Adjusted for NIHSS and large artery occlusion.
[‡]The multivariable analysis using model 2 of recurrence during 8–90 days could not be performed as large artery occlusion perfectly predicted recurrence within 7 days.
Abbreviations: CI, confidence interval; N, number of patients; NA, not applicable; OR, odds ratio; and PRU, P2Y12 reaction units.

Supplemental Table 2. Multivariable logistic regression analysis of P2Y12 reaction units and clinical variables associated with recurrent ischemic stroke in patients receiving dual antiplatelet therapy including clopidogrel (n=182)

	N	Unadjusted OR (95% CI)	*P*	Model 1[§] OR (95% CI)	*P*	Model 2[†] OR (95% CI)	*P*
PRU at 24 hours, 10 units							
- Recurrence within 90 days	32	1.09 (1.03–1.16)	0.003	1.09 (1.02–1.16)	0004	1.09 (1.02–1.15)	0.005
- Recurrence within 7 days	20	1.16 (1.07–1.26)	0.004	1.16 (1.07–1.26)	<0.001	1.16 (1.06–1.26)	0.002
- Recurrence during 8–90 days	12	1.00 (0.92–1.10)	0.93	1.00 (0.92–1.09)	0.96	NA[‡]	NA[‡]

[§]Model 1: Adjusted for NIHSS.
[†]Model 2: Adjusted for NIHSS and large artery occlusion.
[‡]The multivariable analysis using model 2 of recurrence during 8–90 days could not be performed as large artery occlusion perfectly predicted recurrence within 7 days.
Abbreviations: CI, confidence interval; N, number of patients; NA, not applicable; OR, odds ratio; and PRU, P2Y12 reaction units.



Supplemental Fig. 1. The association between P2Y12 reaction units and possible influencing factors of clopidogrel responsiveness in patients undergoing genetic testing and receiving clopidogrel loading dose ($n=121$)

In the group of 121 patients undergoing genetic testing and receiving clopidogrel loading dose, the association between PRU and possible factors influencing platelet reactivity was analyzed. In univariable analysis, PRU at 24 hours was significantly higher in the patients treated with proton pump inhibitors (PPIs) on admission than those not (221.1 ± 59.9 versus 185.1 ± 80.4; $p=0.04$). There was no significant difference in PRU at 24 hours between the three types of PPIs: (i) rabeprazole, (ii) esomeprazole/omeprazole, and lansoprazole, (iii) (299.5 ± 0.71, 0.10, 228.6±62.7, versus 218.7±59.0). Furthermore, PRU at 24 hours tended to be higher in patients over 70 years (222.1 ± 65.3 versus 201.3±66.0; $p=0.09$) and in the order of PM, IM, and EM for CYP2C19 (242.9±77.2, 217.6±60.9, and 189.5 ± 68.0, respectively). In multivariable logistic regression analysis adjusted for the possible influencing factors with a p value <0.05 in the crude analysis or clinical significance (PM of CYP2C19, age over 70 years, and PPIs at the treatment starting point), PM of CYP2C19 (adjusted OR, 3.77; 95% CI, 1.11–12.86; $p=0.034$), age over 70 years (adjusted OR, 2.46; 95% CI, 1.02–5.93; $p=0.045$), and PPIs (adjusted OR, 3.38; 95% CI, 1.0–11.22; $p=0.04$) were significantly associated with high PRU (≥ 254) at 24 hours.

BMI, body mass index; CI, confidence interval; eGFR, estimated glomerular filtration rate; EM, extensive metabolizer; h, hour; IM, intermediate metabolizer; OR, odds ratio; PM, poor metabolizer; PPIs, proton pump inhibitors; PRU, P2Y12 reaction units; and SD, standard deviation.

Supplemental Table 3. Multivariable logistic regression analysis for recurrent ischemic stroke, according to the severity of the responsible artery

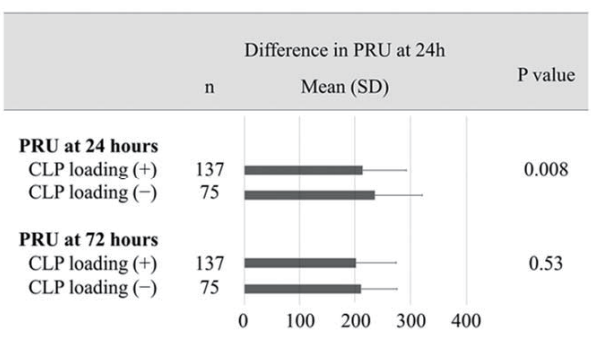
	Severe stenosis/occlusion (n=147)			Moderate stenosis (n=78)		
	N	Adjusted OR (95% CI)	*P*	N	Adjusted OR (95% CI)	*P*
PRU at 24 hours, 10 units						
- Recurrence within 90 days	25	1.10 (1.03–1.18)	0.005	11	1.04 (0.92–1.17)	0.54
- Recurrence within 7 days	17	1.17 (1.07–1.28)	<0.001	5	1.05 (0.88–1.26)	0.61
- Recurrence during 8–90 days	8	0.99 (0.89–1.09)	0.79	6	1.03 (0.89–1.18)	0.71

The model was adjusted for NIHSS and clopidogrel loading.
Abbreviations: CI, confidence interval; N, number of patients; OR, odds ratio; and PRU, P2Y12 reaction units.

Supplemental Table 4. Subgroup analysis of the patients with transient ischemic attack (n=39)

	Non-recurrence (n=34)	Recurrence within 90 days (n=)	*P*	Recurrence within 7 days (n=2)	*P*
Factors in the ABCD2 score					
Age over 60 years	27 (79)	5 (100)	0.56	2 (100)	>0.99
Blood pressure over 140/90 mmHg	28 (82)	5 (100)	0.57	2 (100)	>0.99
Diabetes mellitus	8 (24)	2 (40)	0.59	1 (50)	0.44
Imaging findings for the responsible artery					
Severe stenosis/occlusion	23 (68)	3 (60)	>0.99	0 (0)	>0.99
Moderate stenosis	11 (32)	2 (40)		2 (100)	

Table comparing non-recurrence with recurrence in the study population within 90 days and 7 days groups, respectively (as shown in Fig. 1).
Data are presented as n (%).



Supplemental Fig. 2. The association between P2Y12 reaction units and clopidogrel loading

In the group of 212 patients undergoing PRU evaluation at 24 and 72 hours after initiation of antiplatelet therapy and receiving newly administered loading dose (300 mg) or newly administered standard dose (75 mg) of clopidogrel, the association between PRU and clopidogrel loading was analyzed using by Wilcoxon rank-sum test. Patients with clopidogrel loading showed a significantly higher mean value of PRU at 24 hours compared with those without clopidogrel loading (213.0±72.6 versus 235.3±65.1; P=0.008). In contrast, there was no significant difference in PRU at 72 hours between the two groups (201.1±79.0 versus 210.2±85.4; p=0.53).