AUTHOR QUERY FORM

ELSEVIER	**Journal: YJSCD** **Article Number: 3102**	**Please e-mail your responses and any corrections to:** **E-mail: corrections.essd@elsevier.toppanbestset.com**

Dear Author,

Please check your proof carefully and mark all corrections at the appropriate place in the proof (e.g., by using on-screen annotation in the PDF file) or compile them in a separate list. To ensure fast publication of your paper please return your corrections within 48 hours.

For correction or revision of any artwork, please consult http://www.elsevier.com/artworkinstructions.

We were unable to process your file(s) fully electronically and have proceeded by

☑ ~~Scanning (parts of) your article~~ ☐ Rekeying (parts of) your article ☐ Scanning the artwork

Any queries or remarks that have arisen during the processing of your manuscript are listed below and highlighted by flags in the proof. Click on the 'Q' link to go to the location in the proof.

Location in article	Query / Remark: click on the Q link to go Please insert your reply or correction at the corresponding line in the proof
Q1	If there are any drug dosages in your article, please verify them and indicate that you have done so by initialing this query.
Q2	Please confirm that given names and surnames have been identified correctly and are presented in the desired order and please carefully verify the spelling of all authors' names.
Q3	A short title running head has not been supplied. Please check if this is OK. Otherwise, please supply a short title that will be used as the running head.
Q4	Please confirm if modifications to the Conflict of interest statement are OK.
Q5	Throughout the Abstract and in the text, following distinction from gene to protein/enzymes, please italicize all instances of CYP2C19 if you refer to it as gene and leave it as roman (normal font) if you refer to it as protein or enzyme.
Q6	Please confirm this is 1 keyword rather than 2 or did you mean "Ischemic stroke"?
Q7	Is TIA spelled out as transient ischemic attack correct?
Q8	Elsewhere in the text you referred to Aspirin as ASA. Following journal style on abbreviations, subsequent instances of the full form "Aspirin" have been abbreviated to ASA. Please check throughout the text if correct.
Q9	Please spell out ADP.
Q10	Please supply complete manufacturer details for Aspirin, Clopidogrel and Ticlopidine, including company name, city, and two-letter abbreviated state if USA or city/province and country if non-USA.
Q11	Please supply complate manufacturing details, including company name, city, and two-letter abbreviated state if USA or city/province and country if non-USA for Impact-R cone and platelet analyzer; Platelet Function Assay-100; and VerifyNow Ultegra RPFA.
Q12	Should RPFA be spelled out? If so, please supply the full form.
Q13	Sentence beginning "We further compared. . ." OK as edited?

Thank you for your assistance.

Q14	Please spell out HRPR.
Q15	Please confirm that the group name Antithrombotic Trialists C is correct for Ref. 2.
Q16	Please confirm that the group name European Stroke Organisation Executive C is correct for Ref. 4.
Q17	Please check the row/column alignment of Table 1, and correct if necessary.
Q18	The captions for table parts A through C have been styled as the overall table caption, but the part labels are still reflected in the table as supplied. Please confirm if the presentation is OK.
Q19	Tables 1 and 2: You provided some of the *P* values in italic, apparently for emphasis. Please provide a table footnote to each table explaining what italicizing them means.
Q20	Table 1: For consistency with presentation in the text, references to "A" for "Aspirin" have been changed to "ASA." Is this OK?
	Please check this box or indicate your approval if you have no corrections to make to the PDF file ☐

Thank you for your assistance.

Platelet Function Testing in Patients with Acute Ischemic Stroke: An Observational Study

Francesca Rosafio, MD,* Nicoletta Lelli, MD,† Stefano Mimmi, PhD,‡ Laura Vandelli, MD,* Guido Bigliardi, MD,* Maria Luisa Dell'Acqua, MD,* Livio Picchetto, MD, PhD,* Roberta Pentore, MD,* Diana Ferraro, MD, PhD,* Tommaso Trenti, MD,† Paolo Nichelli, MD, PhD,* and Andrea Zini, MD*

Background: The measurement of platelet reactivity in patients with stroke undergoing antiplatelet therapies is not commonly performed in clinical practice. We assessed the prevalence of therapy responsiveness in patients with stroke and further investigated differences between patients on prevention therapy at stroke onset and patients naive to antiplatelet medications. We also sought differences in responsiveness between etiological subtypes and correlations between Clopidogrel responsiveness and genetic polymorphisms. *Methods:* A total of 624 stroke patients on antiplatelet therapy were included. Two different groups were identified: "non-naive patients", and ~~"naive patients."~~ Platelet function was measured with multiple electrode aggregometry, and genotyping assays were used to determine ~~CYP2C19~~ polymorphisms. *Results:* Aspirin (ASA) responsiveness was significantly more frequent in naive patients compared with non-naive patients (94.9% versus 82.6%, $P < .0010$). A better responsiveness to ASA compared with Clopidogrel or combination therapy was found in the entire population ($P < .0010$), in non-naive patients ($P < .0253$), and in naive patients ($P < .0010$). Multivariate analysis revealed a strong effect of Clopidogrel as a possible "risk factor" for unresponsiveness (odds ratio 3.652, $P < .0001$). No difference between etiological subgroups and no correlations between responsiveness and ~~CYP2C19~~ polymorphisms were found. *Conclusion:* In our opinion, platelet function testing could be potentially useful in monitoring the biological effect of antiplatelet agents. A substantial proportion of patients with stroke on ASA were ~~"resistant,"~~ and the treatment with

From the *Stroke Unit, Neurology Clinic, Department of Neuroscience, Nuovo Ospedale Civile S. Agostino-Estense, University Hospital of Modena, Modena, Italy; †Clinical Pathology-Toxicology, Nuovo Ospedale Civile S. Agostino-Estense, University Hospital of Modena, Modena, Italy; and ‡Local Health Unit, AUSL Modena, Modena, Italy.

Received January 25, 2017; accepted April 21, 2017.

Statistical analysis was performed by S.M., F.R., and A.Z.

Author contributions: Manuscript drafting/revising, study design, data analysis and interpretation, data acquisition, statistical analysis, study supervision: F.R.; manuscript drafting/revising, study design: N.L.; manuscript drafting/revising, data analysis and interpretation, statistical analysis: S.M.; manuscript revising, data acquisition: L.V.; manuscript revising, data acquisition: G.B.; manuscript revising, data acquisition: M.L.D.A.; manuscript revising, data acquisition: L.P.; manuscript revising, data acquisition: R.P.; manuscript revising, statistical analysis, native language speaker supervision: D.F.; manuscript revising, study design: T.T.; manuscript drafting/revising, study supervision: P.N.; manuscript drafting/revising, study design, data analysis and interpretation, data acquisition, statistical analysis, study supervision: A.Z. All authors approved the final version.

Disclosures: All Authors report no disclosures.

Conflict of interest: A.Z. has received speaker fees and consulting fees from Boehringer-Ingelheim and Medtronic, and serves as advisory board from Daiichi Sankyo.

Address correspondence to Andrea Zini, MD, Stroke Unit, Neurology Clinic, Department of Neuroscience, Nuovo Ospedale Civile S. Agostino-Estense, University Hospital of Modena, via Giardini 1355 Baggiovara, ZIP 41100 Modena, Italy. E-mail: andrea.zini@me.com.

1052-3057/$ - see front matter

http://dx.doi.org/10.1016/j.jstrokecerebrovasdis.2017.04.023

Clopidogrel was accompanied by even higher rates of unresponsiveness. Longitudinal studies are needed to assess whether aggregometry might supply individualized prognostic information and whether it can be considered a valid tool for future prevention strategies. **Key Words:** Stroke, ischemic—stroke prevention—platelet inhibitor—secondary prevention—aggregometry.

Introduction

Control of cerebrovascular risk factors and therapeutic secondary prevention are fundamental to prevent ischemic cerebrovascular events (transient ischemic attack [TIA]/stroke). Treatment of noncardiogenic strokes (atherosclerotic, lacunar, or cryptogenic infarcts) is based on antiplatelet agents, which reduce the relative risk of stroke or death on average by about 22%.[1,2] Regarding their mechanism of action, antiplatelet agents are classified in 3 groups: thromboxane inhibitors (~~Aspirin [ASA]~~, ASA-dipyridamole), ~~ADP~~ receptor antagonists (thienopyridines: ~~Clopidogrel~~, ~~Ticlopidine~~), and glycoprotein IIb/IIIa inhibitors. Oral antiplatelet therapy, with ASA, ASA-dipyridamole, and thienopyridines, showing similar effectiveness,[3] is strongly recommended in secondary stroke prevention.[4,5] The treatment of recurrences remains less clear; it is mandatory to exclude alternative causes of stroke and to improve control of risk factors, but no clear guidelines for alternative therapeutic strategies are available. In everyday clinical practice, after recurrence of ischemic stroke, a therapeutic shift to another antiplatelet agent is the most common option, although there is no evidence based on clinical trials indicating that this is associated with a reduction of the risk of recurrence.

In recent years, the clinical response variability to ASA or Clopidogrel treatment and the phenomenon of "low" or ~~"nonresponsiveness,"~~ ~~"antiplatelet resistance,"~~ or "high on-treatment platelet reactivity" (HTPR), defined as biochemical failure of the antiplatelet agent to inhibit tests of platelet function ex vivo, have been widely explored.[6,7] The mechanisms for resistance might include an insufficient dose, poor compliance, related gene polymorphisms, baseline platelet hyperactivity, and accelerated platelet turnover.[8,9]

Different methods of platelet function testing are available to assess inhibition of function induced by antiplatelet agents: light transmission aggregometry, the gold standard for monitoring antiplatelet effects ex vivo, but with difficult routine application; vasodilator-stimulated phosphoprotein assay, specific for evaluation of Clopidogrel responsiveness; ~~Impact R cone and platelet analyzer~~; Platelet Function Assay-100; and ~~VerifyNow Ultegra RPFA~~. A fairly new generation of impedance aggregometer, named multiple electrode platelet aggregometry (MEA; Multiplate, Roche Diagnostics International Ltd, Rotkreutz, Switzerland),[10,11] showed correlations with the estimates of Clopidogrel and ASA antiplatelet effect obtained by other methods.[12]

A greater risk of recurrence of cardiovascular events has been demonstrated in patients with resistance to ASA or Clopidogrel.[13-17] Furthermore, pharmacological interactions with other drugs (e.g., proton-pump inhibitor) have been associated with a diminished pharmacodynamic response to Clopidogrel.[18] Recently, a systematic review and meta-analysis of randomized clinical trials was performed to evaluate the clinical efficacy and safety of intensified antiplatelet therapy versus Clopidogrel at a standard dosage, on the basis of platelet reactivity testing, in patients undergoing percutaneous coronary intervention who presented HTPR.[19] The intensified therapy protocol was associated with a significant reduction in cardiovascular mortality, stent thrombosis, and myocardial infarction, with no difference in the rate of major bleeding between the 2 groups, although the net clinical benefit significantly depended on the risk of stent thrombosis with standard Clopidogrel dose. Similarly, another systematic review and meta-analysis on randomized trials, concerning tailored antiplatelet therapy in antiplatelet-resistant patients, showed a minor occurrence of death or clinical adverse events in personalized antiplatelet therapy compared with conventional treatment.[20]

Although several data are available on cardiovascular diseases, there are a small number of studies regarding monitoring of antiplatelet therapy in patients with ischemic stroke. In most of them, the evaluation of antiplatelet effect has been performed mainly with Platelet Function Assay-100, with evidence of a low responsiveness to low-dose or enteric-coated ASA in a significant proportion of patients (37%) in Alberts et al's study.[21] Other data pointed to limitations of platelet aggregation monitoring, particularly in terms of reliability of results.[22-24] The Trinity Antiplatelet Responsiveness study investigated the prevalence of ex vivo nonresponsiveness in patients with ischemic stroke/patients with TIA evaluated with a "longitudinal definition of HTPR" by comparing responsiveness to antiplatelet therapy at follow-up with patients' baseline values.[25] Payne et al reported a significant clinical impact of monitoring the intake of a single dose of Clopidogrel with flow cytometry and aggregometry before carotid endarterectomy to reduce postoperative embolization.[26] Recently, some studies evaluated the prevalence of antiplatelet responsiveness by using MEA in a cerebrovascular setting; Meves et al reported HTPR

to Clopidogrel therapy in 44% of 159 patients with ischemic stroke, and besides time-dependency of the antiplatelet effect, major risk factors reported for resistance were diabetes mellitus and higher HbA1c values.[27] Nevertheless, other evidence advises against a platelet function-guided modification of antiplatelet therapy after an ischemic stroke or TIA: Depta et al reported an association between increase of therapeutic dosages and rate of adverse clinical outcome, without differences in terms of ischemic events.[28] A more recent 2-phase pilot study assessed the reproducibility of results on ASA responsiveness using MEA between healthy volunteers, naive to ASA, and patients with acute ischemic stroke or TIA. Results demonstrated that the Multiplate device could feasibly supply reproducible and precise results in both groups, encouraging its implementation in routine clinical practice.[29] In conclusion, although several studies support its implementation, the routine measurement of platelet reactivity in patients with ischemic stroke has not been incorporated in clinical practice, mainly due to a lack of consensus on the optimal methods to use and of consistent data on the effective improvement of clinical outcome with tailored therapy. The aim of the current observational study was to assess the prevalence of antiplatelet therapy responsiveness in a large Italian acute stroke population, and to determine potential differences in responsiveness between patients already on antiplatelet secondary prevention therapy at stroke onset and patients who were naive to antiplatelet medications before the ischemic event.

On the background of recent findings regarding the individual differences in Clopidogrel absorption and metabolization, we also studied genetic polymorphisms within the ~~CYP2C19~~ gene in our patients with stroke: ~~CYP2C19*2~~ (c.681G>A; Single Nucleotide Polymorphism ID number rs4244285) and ~~CYP2C19*17~~ (c.806C>T; rs12248560) allelic variants. Clopidogrel is a prodrug that requires conversion into an active metabolite by hepatic cytochrome P450 isoenzymes, in a 2-step sequential bioactivation. Many studies indicate that the isoenzyme CYP2C19 contributes to both of the oxidative steps required for conversion.[30,31] Hypotheses suggest that the variability of responsiveness to Clopidogrel, with absent or insufficient active metabolite generation, might be primarily due to limited intestinal absorption or to functional variability in P450 isoenzyme activity. Some studies have evaluated therefore the influence of polymorphisms of gene encoding CYP2C19 isoenzymes.[32-35] Two common and functionally relevant polymorphisms exist within the ~~CYP2C19~~ gene: ~~CPY2C19*2(*2)~~, resulting in complete loss of CYP2C19 enzyme activity, and ~~CYP2C19*17(*17)~~, resulting in increased enzyme function. Although the loss-of-function (LoF) allele confers a higher risk of adverse events by affecting the pharmacodynamics response to Clopidogrel, no single study has clearly demonstrated a link between the presence of LoF genetic polymorphism, decreased Clopidogrel responsiveness, and adverse clinical outcome. On the background of these findings, we additionally evaluated potential correlations between responsiveness and ~~CYP2C19~~ genotype in a group of Clopidogrel patients of the study population.

Materials and Methods

Study Design

In this observational study, we assessed the prevalence of antiplatelet therapy responsiveness, using impedance aggregometry, in a population of 624 patients with acute stroke treated with a standard dose of ASA (100 mg/day), Clopidogrel (75 mg/day), or with a combination of both drugs. In this sample, we further identified 2 different groups: patients who were already on antiplatelet treatment at the time of stroke onset (called "non-naive patients") and patients who started antithrombotic therapy because of the ischemic stroke that led to hospitalization (called "naive patients"). The platelet function was tested within a mean interval of 24-48 hours from admission in the former group, and a mean interval of 7-10 days after treatment initiation in the latter group. To avoid potential confounding effects, we excluded patients whose previous antiplatelet therapy was shifted to another antiplatelet medication because of the cerebrovascular event (e.g., shift from ASA to Clopidogrel during hospitalization). Associations between patients' clinical characteristics and responsiveness to antiplatelet therapy were sought. We further compared the responsiveness to antiplatelet therapy with the different antiplatelet medications (ASA, Clopidogrel, or a combination of both drugs) in the entire study population and in the 2 different groups.

In non-naive patients, we also assessed the prevalence of antiplatelet responsiveness within etiological subgroups of patients, identified on the basis of the underlying mechanism of the current ischemic event (large artery atherosclerosis, small artery disease, cardiogenic embolism, undetermined etiology, other determined causes). Each patient, therefore, underwent an extensive diagnostic workup to identify the correct etiopathogenetic mechanism of the index event.

We finally evaluated responsiveness among the Clopidogrel patients of the study population in whom we were able to assess the ~~CYP2C19~~ genotype (*1/*1, *1/*17, *17/*17, *2/*17, *2/*2, *1/*2).

Impedance Aggregometry

Platelet function in whole blood was measured by using Multiple Electrode Aggregometry on a Multiplate Analyzer® (Roche Diagnostics International Ltd CH-6343 Rotkreutz, Switzerland).[36,37] Description of the impedance aggregometry system and of cut-off values for responsiveness are reported in Method I in the online-only Supplementary data.

*CYP2C19*2 and CYP2C19*17 Genotyping*

Specific single nucleotide polymorphism (SNP) Genotyping TaqMan assays (C_25986767_70 and C_469857_10, Life Technologies, Grand Island, NY) were used for the detection of rs4244285 and rs12248560, respectively ("DMGA CYP2C19*2", rs4244285, assay ID C_25986767_70; "DMGA CYP2C19*17", rs12248560, assay ID C_469857_10). Description of SNP Genotyping TaqMan assays system is shown in Method II in the online-only Supplementary data.

Statistical Analysis

Descriptive statistical analyses, assessing the percentage of patients on ASA, Clopidogrel, or ASA plus Clopidogrel therapy, were performed in the entire population and in each of the study groups. Chi-square or Fisher exact tests were used to compare proportions between groups. Possible associations between antiplatelet therapy unresponsiveness and demographic and clinical factors were examined using stepwise logistic regression. Unadjusted and adjusted odds ratio (OR) and the corresponding *P* values were calculated for each variable included in the univariate and multivariate models. $P < .05$ was considered statistically significant. All calculations were conducted using SAS Enterprise Guide version 5.1 (SAS Institute Inc., Cary, NC).

Results

Clinical and Demographic Characteristics of Patients

This observational study analyzed 624 consecutive patients with acute ischemic stroke referred to our stroke unit. Two different groups were identified: non-naive patients, including 278 patients who were already treated with antiplatelet therapy at stroke onset (219 patients were treated with ASA, 33 patients with Clopidogrel, and 26 patients with a combination of both [ASA plus Clopidogrel]), and naive patients, including 346 patients who started antiplatelet therapy because of the ischemic event (196 patients started ASA, 89 patients Clopidogrel, and 61 patients ASA plus Clopidogrel). Demographic and clinical characteristics of patients are reported in Table 1, A. The group of patients who were already on antiplatelet secondary prevention therapy at stroke onset (non-naive patients) were significantly older ($P < .0001$); the frequencies of hypertension ($P = .0001$), previous cerebrovascular or myocardial ischemic events ($P < .0001$), relevant carotid stenosis ($P = .0165$), smoking ($P = .0148$), hyperlipidaemia and hyperhomocysteinemia ($P = .0040$ and $P = .0344$, respectively), and patency of the foramen ovale ($P = .0012$) were significantly higher in this group. In addition, the use of ASA was significantly more frequent in non-naive patients ($P < .0001$), whereas Clopidogrel or dual antiplatelet therapies were significantly more frequent among naive patients ($P < .0001$ and $P = .0030$, respectively). The etiological subgroups identified among the study population are reported in Table 1, B. Large artery atherosclerosis and cardiogenic embolism subgroups were significantly more frequent in the first study group (non-naive patients) ($P = .0309$ and $P < .0001$, respectively), whereas lacunar strokes and strokes of undetermined etiology were more frequent among naive patients ($P = .0016$ and $P = .0048$, respectively). In a smaller group of patients treated with Clopidogrel or with a combination of ASA and Clopidogrel, we were able to study the genetic polymorphisms within the ~~CYP2C19~~ gene. The expressed genotypes assigned to each patient in the entire population and in the 2 study groups are shown in Table 1, C. There were no statistical differences between study groups considering ~~CYP2C19*2~~ and ~~CYP2C19*17~~ genotypes, except for the ~~CPY2C19*1(*2)~~ allelic variant being slightly more frequent among non-naive patients.

Prevalence of Antiplatelet Therapy Responsiveness in the Study Population

Based on platelet aggregation testing, 122 patients (19.6%) were determined to be nonresponders in the entire study population, considering all antiplatelet medications. Fifty-six patients (20.1%) were nonresponders in the first group (non-naive patients) and 66 patients (19.1%) in the second group (naive patients). There were no significant differences between the 2 groups by chi-square testing ($P = .7379$). When considering ASA therapy, in the entire population, 48 patients (11.6%) were nonresponders: 38 patients (17.4%) among non-naive patients and 10 patients (5.1%) among naive patients. There was a significantly larger prevalence of therapy responsiveness in the second group (patients who started ASA treatment during hospitalization) by chi-square testing ($P < .0010$). When considering Clopidogrel therapy, 44 patients (36.1%) of the entire population were determined to be nonresponders: 9 patients (27.3%) were nonresponders among non-naive patients, 35 patients (39.3%) among naive patients, and there were no significant differences between the 2 groups by chi-square testing ($P = .2181$). In the entire population on combination therapy with ASA and Clopidogrel, 30 patients (34.5%) were determined to be nonresponders to both of them. Nine patients (34.6%) were nonresponders among non-naive patients, and 21 patients (34.4%) among naive patients. No significant differences between the 2 groups by chi-square testing were found ($P = .9864$).

Measures of Association between Clinical Characteristics and Antiplatelet Therapy Unresponsiveness

The entire study population was examined to determine if there was any association between antiplatelet therapy unresponsiveness and demographic and clinical characteristics reported in Table 1, A; the univariate analysis revealed that the severity of stroke (onset National

Table 1. *(A) Demographic and clinical characteristics of study population, (B) etiological subgroups in study population, and (C) CYP2C19 genotype in Clopidogrel patients of study population*

(A)

	Non-naive patients (n = 278)	Naive patients (n = 346)	Total (N = 624)	*P* value
Variable				
Age (SD)	72.1 (11.5)	65.9 (15.1)	68.6 (13.9)	*<.0001*
Male (%)	185 (66.5)	215 (62.1)	400 (64.1)	.2539
Onset NIHSS (±SD)	6.7 (±7.1)	6.4 (±6.7)	6.5 (±6.8)	.6449
Discharge NIHSS (±SD)	3.6 (±5.8)	2.9 (±5.4)	3.2 (±5.6)	.1217
Hypertension (%)	236 (84.9)	249 (72)	485 (77.7)	*.0001*
Previous stroke (%)	63 (22.7)	30 (8.7)	93 (14.9)	*<.0001*
Previous TIA (%)	31 (11.2)	21 (6.1)	52 (8.3)	*.0224*
ICA stenosis >50% (%)	115 (41.4)	111 (32.1)	226 (36.2)	*.0165*
Atrial fibrillation (%)	61 (21.9)	24 (6.9)	85 (13.6)	*<.0001*
Diabetes mellitus (%)	76 (27.3)	72 (20.8)	148 (23.7)	.0567
Prior myocardial infarction (%)	90 (32.4)	34 (9.8)	124 (19.9)	*<.0001*
Cigarette smoking (%)	72 (25.9)	121 (35.0)	193 (30.9)	*.0148*
Hyperlipidemia (%)	214 (77)	230 (66.5)	444 (71.2)	*.0040*
Hyperhomocysteinemia (%)	51 (18.3)	88 (22.3)	139 (22.3)	*.0344*
History of neoplasia (%)	20 (7.2)	30 (8.7)	50 (8.0)	.4996
Obesity (%)	36 (12.9)	40 (11.6)	76 (12.2)	.5980
Foramen ovale patency	8 (2.9)	32 (9.2)	40 (6.4)	*.0012*
ASA	219 (78.8)	196 (56.6)	415 (66.5)	*<.0001*
Clopidogrel	33 (11.9)	89 (25.7)	122 (19.6)	*<.0001*
ASA plus Clopidogrel (ASA + C)	26 (9.3)	61 (17.6)	87 (13.9)	*.0030*

(B)

Large artery atherosclerosis (%)	90 (32.4)	85 (24.6)	175 (28)	*.0309*
Small artery disease (%)	40 (14.4)	85 (24.6)	125 (20)	*.0016*
Cardiogenic embolism (%)	70 (25.2)	36 (10.4)	106 (17)	*<.0001*
Undetermined etiology (%)	61 (21.9)	111 (32.1)	172 (27.6)	*.0048*
Other causes (%)	14 (5.0)	26 (7.5)	40 (6.4)	.2090
Mimics (%)	3 (1.1)	3 (.9)	6 (1.0)	.7531

(C)

	Non-naive patients (n = 38) C = 21, ASA + C = 17	Naive patients (n = 92) C = 54, ASA + C = 38	Total (N = 130)	*P* value
CPY2C19*1(*1)	11 (28.9)	40 (43.5)	51 (39.2)	.1228
CPY2C19*1(*17)	10 (26.3)	28 (30.5)	38 (29.2)	.6386
CPY2C19*1(*2)	11 (28.9)	13 (14.2)	24 (18.5)	*.0477*
CPY2C19*17(*17)	2 (5.3)	4 (4.3)	6 (4.6)	.7623
CPY2C19*2(*17)	3 (7.8)	4 (4.3)	7 (5.4)	.4162
CPY2C19*2(*2)	1 (2.8)	3 (3.2)	4 (3.1)	.7540

Abbreviations: ICA, internal carotid artery; NIHSS, National Institute of Health Stroke Scale; SD, standard deviation; TIA, transient ischemic attack.

~~Continuous variables are indicated as mean ± SD and categorical variables as frequencies and percentages.~~

Institute of Health Stroke Scale) and the residual disability (discharge National Institute of Health Stroke Scale) slightly increased the odds of being nonresponsive to antiplatelet therapy, but multivariate logistic regression analysis did not confirm these associations. With regard to the other tested variables, univariate analysis revealed that nonresponders were more frequently reported under Clopidogrel (OR = 2.89, $P < .0001$) and dual

Table 2. *Association of unresponsiveness with demographic and clinical data antiplatelet therapy responsiveness*

	Univariate analysis		Multivariate analysis	
Factor	OR	*P* value	OR	*P* value
Gender (M versus F)	.67	.0465	.73	.1555
Age	1.01	.1905	.99	.7132
Onset NIHSS	1.03	*.0383*	1.01	.5750
Discharge NIHSS	1.03	*.0366*	1.02	.3379
Hypertension	1.24	.3755	1.18	.5626
Previous stroke	1.06	.8299	.70	.2450
Previous TIA	1.31	.4220	.99	.9808
ICA stenosis >50%	1.30	.1972	1.03	.8874
Atrial fibrillation	1.04	.9011	.89	.7284
Diabetes mellitus	1.53	.0518	1.48	.1080
Prior myocardial infarction	1.22	.4049	.91	.7467
Cigarette smoking	.65	.0518	.71	.1723
Hyperlipidemia	1.17	.4837	1.17	.5331
Hyperhomocysteinemia	1.17	.4837	.99	.9759
History of neoplasia	.96	.9035	1.13	.7604
Obesity	1.22	.4896	1.11	.7520
Foramen ovale patency	.80	.6092	1.24	.6642
Clopidogrel versus ASA	2.89	*<.0001*	3.65	*<.0001*
ASA plus Clopidogrel versus ASA	2.96	*<.0001*	3.60	*<.0001*
Study group (non-naive patients versus naive patients)	.80	.2720	.55	.0169

~~Abbreviations: ASA, aspirin; F, female; ICA, internal carotid artery; M, male; NIHSS, National Institute of Health Stroke Scale; OR, odds ratio; TIA, transient ischemic attack.~~

antiplatelet therapy (OR = 2.96, *P* = .0002). Further multivariate logistic regression analysis, including the variable "study group" as an independent factor, confirmed this association: the odds of being nonresponders was increased by 3.65-fold with Clopidogrel therapy (*P* < .0001) and by 3.60-fold with dual antiplatelet therapy (*P* < .0001). Overall results are summarized in Table 2.

Comparison between Responsiveness to Different Antiplatelet Medications (ASA, Clopidogrel, or ASA Plus Clopidogrel) in the Study Population and in the 2 Groups

In the entire study population, there was a significant difference in responsiveness to the different antiplatelet therapies; in particular, patients had a better responsiveness to ASA therapy compared with Clopidogrel or combination therapy by chi-square testing (*P* < .0010) (Fig 1, A). In the group of patients who was already on antiplatelet treatment at the time of the acute stroke (non-naive patients), there was a significant difference in responsiveness to the different antiplatelet therapies; in particular, patients had a better responsiveness to ASA therapy compared with Clopidogrel or combination therapy by chi-square testing (*P* = .0253) (Fig 1, B). In the group of patients who started antithrombotic therapy following the ischemic stroke (naive patients), the prevalence of responsiveness was, again, significantly different among different types of antiplatelet agents; in particular, patients had a better responsiveness to ASA compared with Clopidogrel or combination therapy by chi-square testing (*P* < .0010) (Fig 1, C).

Prevalence of Responsiveness within Etiological Subgroups in Non-Naive Patients

Prevalence of antiplatelet responsiveness to all medications and to ASA, Clopidogrel, ASA plus Clopidogrel separately, in each etiological subgroup of patients, is shown in Table I in the online-only Supplementary data. There were no significant differences in the proportion of nonresponders across etiological subgroups considering all antiplatelet medications, Clopidogrel, or dual antiplatelet therapy by chi-square testing (*P* = .2509, *P* = .7281, *P* = .3360, respectively). A significant difference was found considering ASA therapy (*P* = .0479): in this group of patients (N = 219) the prevalence of ASA responsiveness was 90%, 74%, and 86% in large artery atherosclerosis, lacunar, and cardiogenic embolism subgroups, respectively. No statistical difference was found between lacunar and nonlacunar subgroups (*P* = .1266), between large artery atherosclerosis subgroup and others (*P* = .0596), and between cardiogenic embolism and noncardiogenic embolism subgroups (*P* = .4420). Prevalence of responsiveness to all antiplatelet medications within noncardiogenic subgroups overall and within the cardiogenic embolism subgroup was 78.4% and 84.3%, respectively, without statistical differences (*P* = .2854).

Prevalence of Responsiveness in Clopidogrel Patients according to CYP2C19 Genotype

Prevalence of responsiveness among the 130 Clopidogrel patients of the study population, and separately in each study group, according to the genetic polymorphism within the ~~CYP2C19~~ gene is shown in Table II in the online-only Supplementary data. No significant difference was found by Fisher exact testing (*P* = .5721). Particularly, no difference was found in prevalence of Clopidogrel responsiveness between patients carrying the *2 allelic variant (homozygous *2/*2 or heterozygous variants *2/*17, *1/*2) and patients carrying other allelic variants in our 130 patients (*P* = .2715).

Discussion

In the present observational study, we assessed the prevalence of antiplatelet therapy responsiveness by using MEA in a population of 624 patients with acute ischemic stroke.

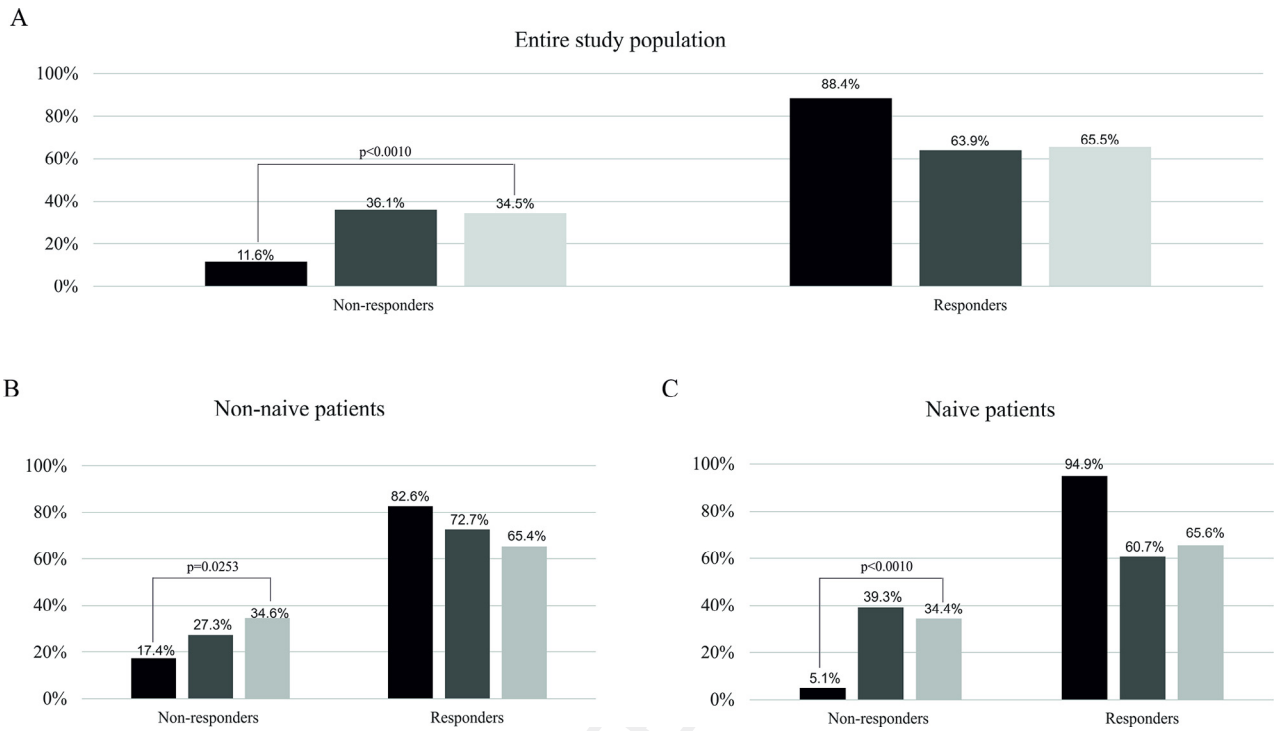

Figure 1. *Graphic presentations of responsiveness to each tested antiplatelet therapy (ASA—black columns, Clopidogrel—dark gray columns, ASA plus Clopidogrel—light gray columns) and related P values, in entire study population (A), in "non-naive patients" (B), and in "naive patients" (C).*

Our findings showed that the prevalence of ASA responsiveness was significantly higher ($P < .0010$) in patients who started antithrombotic therapy during hospitalization (naive patients) compared with patients who were already on antiplatelet prevention therapy with ASA at the time of stroke onset (non-naive patients). As previously reported, the vascular event risk reduction in patients taking ASA amounts to 22%, but the benefit in cerebrovascular patients, specifically, seems to be more modest. Furthermore, it has been estimated that between 5% and 60% of patients on secondary prevention therapy with ASA present a heterogeneous phenomenon known as "aspirin resistance,"[38] with possible associations between aspirin resistance and repeated cerebrovascular ischemic events.[39,40] The evidence that a consistent proportion of patients on ASA, including patients with acute stroke (as shown by the present study and by others[7,38-40]), are ~~"ASA resistant,"~~ raises the question as to whether antiplatelet activity testing should be recommended in patients with risk factors for ischemic stroke on ASA because up to one third of subjects may be taking a biologically ineffective medication. Multiple potential mechanisms have been reported with regard to ASA resistance, but poor therapeutic adherence and factors affecting a faster platelet turnover (such as underlying chronic inflammatory states depending on atherosclerosis or type 2 diabetes mellitus) are likely to contribute to a good part of cases.[38] In our study, the higher prevalence of ASA responsiveness (94.9%) observed in naive patients might certainly be due to a global improvement of therapeutic compliance, but it might be conceivable that the tight control of concomitant cerebrovascular risk factors, especially hyperglycemia or hyperlipidemia, in these patients may account for the better response. Furthermore, time-dependent changes in individual platelet reactivity have been reported in patients with a previous ischemic stroke, with inconstant effects of a fixed dose of ASA over time.[41] Recently, other evidence showed an independent association between the increase of platelet reactivity over time and early neurological deterioration in patients with ischemic stroke.[42,43]

Logistic regression performed on the entire study population does not show any significant association between patients' demographic characteristics and comorbidities and unresponsiveness to antiplatelet therapy; nevertheless, it revealed a strong effect of Clopidogrel therapy (alone or in combination with ASA) as a "risk factor" for unresponsiveness to antiplatelet therapy, regardless of the duration of treatment, as this association is even more significant if the variable "study group" is included in the analysis. Our results, moreover, showed differences in the responsiveness between antiplatelet medications (ASA, Clopidogrel, or a combination of both drugs) in the entire population and in the 2 subgroups: we found a significantly better responsiveness to ASA therapy compared with Clopidogrel or combination therapy in the entire study population ($P < .0010$), both in non-naive patients ($P < .0253$) and in naive patients ($P < .0010$). Although

our results did not show any significant differences regarding Clopidogrel responsiveness between the study groups (P = .2181), the proportion of Clopidogrel nonresponders was higher than the proportion of ASA nonresponders both in non-naive patients (27.3% versus 17.4%) and in naive patients (39.3% versus 5.1%). The finding of a lower responsiveness to Clopidogrel therapy in our stroke population is consistent with a wide variety of previous studies.[19,27,44] The phenomenon named "Clopidogrel resistance," highly variable in different populations (ranging from 15.9% to 49.5%),[45] has been widely reported in association with worse clinical outcomes in patients with cardiovascular events. The cause of this phenomenon is known to be multifactorial because it seems to be dependent on several issues: choice of assay and cutoff value,[6] timing of testing, pharmacokinetic, drug–drug interactions, comorbidities, age, genetic polymorphisms, and poor compliance. We found similarities between the prevalence of Clopidogrel resistance in our population and other studies on patients with ischemic stroke tested with MEA.[27] We further hypothetically may assume that the question of therapeutic adherence is less prominent in patients treated with Clopidogrel than those in therapy with ASA, as there were no significant differences between study groups (non-naive patients and naive patients), and the odds of being nonresponders is even higher if the analysis is adjusted also for this factor. With regard to the hypothesis that Clopidogrel nonresponsiveness in the naive patients group might have been time-dependent, this is not likely to have been an issue as mean testing interval from stroke onset was 7-10 days, and evidence has previously shown that the steady state of the Clopidogrel antiplatelet effect is usually achieved in 3-5 days.[46]

Among patients who were already on prevention antiplatelet therapy at baseline, we tried to assess the prevalence of responsiveness within etiological subgroups of stroke to evaluate whether the information provided by platelet function testing might help in the prediction of the underlying stroke etiology. We found no significant difference in responsiveness to all antiplatelet medications, Clopidogrel, and combination therapy between etiological subgroups, whereas significant differences were found considering ASA therapy (prevalence of ASA responsiveness was 90%, 74%, and 86% in large artery atherosclerosis, lacunar, and cardiogenic embolism subgroups, respectively, P = .0479); this result, however, is difficult to interpret. These findings were also reported in a recent study in which ASA/Clopidogrel resistance was not associated with a specific subgroup of stroke mechanism.[47] We noticed a higher prevalence of nonresponsiveness to all medications, ASA, and combination of ASA and Clopidogrel (respectively 27%, 26%, and 50%) in the small artery disease etiological subgroup, but without statistical relevance. Greater ASA resistance in lacunar strokes was reported in a previous study,[48] in which authors suggested that, rather than anterior or posterior circulation strokes, which are often caused by embolic mechanism in large vessels, in this type of stroke, small arteries, already narrowed by intrinsic disease, might be more sensitive to the formation of microthrombi due to lack of inhibition of platelet activation induced by ASA. More recently, Cha et al reported a significant correlation between large artery atherosclerosis subtype of ischemic stroke and poor long-term outcome in presence of ~~HRPR~~ after ADP stimuli.[49] Q14

The main limit of the present study is its cross-sectional design, which did not permit us to evaluate the long-term outcome of patients with an inadequate inhibition of platelet function, although data collection is ongoing.

We finally evaluated the responsiveness among 130 patients of the study population on Clopidogrel therapy, in which we assessed the ~~CYP2C19~~ genotype. Notwithstanding previous and recent reports of a higher prevalence of unresponsiveness or adverse clinical events in patients carrying the LoF alleles,[32,33] in our particular population we did not find any statistical differences in homozygous or heterozygous *2 allelic variants carriers. One possible limitation of this result might be the small size of our sample, because hypothetical "slow metabolizers" (*2/*2 and *1/*2) were only 11. Conversely, it might be hypothesized that in the Italian population, other polymorphisms play a more significant role in Clopidogrel pharmacokinetic than CYP2C19 alone. Other clinical or biological factors rather than ~~CYP2C19~~ polymorphisms could be related to the high prevalence of unresponsiveness observed in the entire group of our Clopidogrel patients. Further analyses on Clopidogrel therapy and relevant functional genetic variants are needed.

Conclusion

The present observational study confirms that a substantial proportion of patients with acute stroke on ASA showed biological resistance and that treatment with Clopidogrel is accompanied by even higher rates of nonresponsiveness. Monitoring the effect of antiplatelet medications with platelet function testing in patients with ischemic stroke could be potentially useful for promptly detecting any problems of poor compliance, which is known to play a significant role in the phenomenon of Aspirin resistance,[50] in those patients who were already on antiplatelet prevention therapy at the time of the stroke. Furthermore, the possibility of testing the biological effectiveness of Clopidogrel in particular, which is widely considered as equal to or even more effective than ASA, although associated with higher rates of biological nonresponsiveness, might supply helpful information to clinicians because the therapeutic shift from 1 antiplatelet agent to another is currently not supported by positive evidence concerning the reduction of the risk of ischemic stroke recurrence.

Longitudinal studies are needed to assess whether aggregometry might supply individualized prognostic information and whether it can be considered a valid tool in the development of tailored therapies in the future.

Appendix: Supplementary Material

Supplementary data to this article can be found online at doi:10.1016/j.jstrokecerebrovasdis.2017.04.023.

References

1. Goldstein LB, Bushnell CD, Adams RJ, et al. Guidelines for the primary prevention of stroke: a guideline for healthcare professionals from the American Heart Association/American Stroke Association. Stroke 2011;42:517-584.
2. ~~Antithrombotic Trialists C,~~ Collaborative meta-analysis of randomised trials of antiplatelet therapy for prevention of death, myocardial infarction, and stroke in high risk patients. BMJ 2002;324:71-86.
3. Dutch TIA Trial Study Group, van Gijn J, Algra A, et al. A comparison of two doses of aspirin (30 mg vs. 283 mg a day) in patients after a transient ischemic attack or minor ischemic stroke. N Engl J Med 1991;325:1261-1266.
4. ~~European Stroke Organisation Executive C, Committee ESOW.~~ Guidelines for management of ischaemic stroke and transient ischaemic attack 2008. Cerebrovasc Dis 2008;25:457-507.
5. Furie KL, Kasner SE, Adams RJ, et al. Guidelines for the prevention of stroke in patients with stroke or transient ischemic attack: a guideline for healthcare professionals from the American Heart Association/American Stroke Association. Stroke 2011;42:227-276.
6. Sofi F, Marcucci R, Gori AM, et al. Clopidogrel non-responsiveness and risk of cardiovascular morbidity. An updated meta-analysis. Thromb Haemost 2010;103:841-848.
7. Hankey GJ, Eikelboom JW. Aspirin resistance. Lancet 2006;367:606-617.
8. Wang TH, Bhatt DL, Topol EJ. Aspirin and clopidogrel resistance: an emerging clinical entity. Eur Heart J 2006;27:647-654.
9. Siller-Matula J, Schror K, Wojta J, et al. Thienopyridines in cardiovascular disease: focus on clopidogrel resistance. Thromb Haemost 2007;97:385-393.
10. Toth O, Calatzis A, Penz S, et al. Multiple electrode aggregometry: a new device to measure platelet aggregation in whole blood. Thromb Haemost 2006;96:781-788.
11. Mueller T, Dieplinger B, Poelz W, et al. Utility of whole blood impedance aggregometry for the assessment of clopidogrel action using the novel multiplate analyzer—comparison with two flow cytometric methods. Thromb Res 2007;121:249-258.
12. Siller-Matula JM, Gouya G, Wolzt M, et al. Cross validation of the multiple electrode aggregometry. A prospective trial in healthy volunteers. Thromb Haemost 2009;102:397-403.
13. Aradi D, Komocsi A, Vorobcsuk A, et al. Prognostic significance of high on-clopidogrel platelet reactivity after percutaneous coronary intervention: systematic review and meta-analysis. Am Heart J 2010;160:543-551.
14. Bonello L, Camoin-Jau L, Arques S, et al. Adjusted clopidogrel loading doses according to vasodilator-stimulated phosphoprotein phosphorylation index decrease rate of major adverse cardiovascular events in patients with clopidogrel resistance: a multicenter randomized prospective study. J Am Coll Cardiol 2008;51:1404-1411.
15. Snoep JD, Hovens MM, Eikenboom JC, et al. Clopidogrel nonresponsiveness in patients undergoing percutaneous coronary intervention with stenting: a systematic review and meta-analysis. Am Heart J 2007;154:221-231.
16. Sofi F, Marcucci R, Gori AM, et al. Residual platelet reactivity on aspirin therapy and recurrent cardiovascular events—a meta-analysis. Int J Cardiol 2008;128:166-171.
17. Frossard M, Fuchs I, Leitner JM, et al. Platelet function predicts myocardial damage in patients with acute myocardial infarction. Circulation 2004;110:1392-1397.
18. Ho PM, Maddox TM, Wang L, et al. Risk of adverse outcomes associated with concomitant use of clopidogrel and proton pump inhibitors following acute coronary syndrome. JAMA 2009;301:937-944.
19. Aradi D, Komocsi A, Price MJ, et al. Efficacy and safety of intensified antiplatelet therapy on the basis of platelet reactivity testing in patients after percutaneous coronary intervention: systematic review and meta-analysis. Int J Cardiol 2013;167:2140-2148.
20. Li J, Jian Z, Song M, et al. Tailored antiplatelet therapy and clinical adverse outcomes. Heart 2014;100:41-46.
21. Alberts MJ, Bergman DL, Molner E, et al. Antiplatelet effect of aspirin in patients with cerebrovascular disease. Stroke 2004;35:175-178.
22. von Lewinski F, Riggert J, Paulus W. Towards a rationale of platelet aggregation monitoring in stroke prophylaxis? J Stroke Cerebrovasc Dis 2009;18:111-115.
23. Harrison P, Segal H, Blasbery K, et al. Screening for aspirin responsiveness after transient ischemic attack and stroke: comparison of 2 point-of-care platelet function tests with optical aggregometry. Stroke 2005;36:1001-1005.
24. Kinsella JA, Tobin WO, Cox D, et al. Prevalence of ex vivo high on-treatment platelet reactivity on antiplatelet therapy after transient ischemic attack or ischemic stroke on the PFA-100 (R) and VerifyNow (R). J Stroke Cerebrovasc Dis 2013;22:e84-e92.
25. Tobin WO, Kinsella JA, Coughlan T, et al. High on-treatment platelet reactivity on commonly prescribed antiplatelet agents following transient ischaemic attack or ischaemic stroke: results from the Trinity Antiplatelet Responsiveness (TRAP) study. Eur J Neurol 2013;20:344-352.
26. Payne DA, Jones CI, Hayes PD, et al. Therapeutic benefit of low-dose clopidogrel in patients undergoing carotid surgery is linked to variability in the platelet adenosine diphosphate response and patients' weight. Stroke 2007;38:2464-2469.
27. Meves SH, Schroder KD, Endres HG, et al. Clopidogrel high-on-treatment platelet reactivity in acute ischemic stroke patients. Thromb Res 2014;133:396-401.
28. Depta JP, Fowler J, Novak E, et al. Clinical outcomes using a platelet function-guided approach for secondary prevention in patients with ischemic stroke or transient ischemic attack. Stroke 2012;43:2376-2381.
29. Mannu GS, Macartney A, Lambert JR, et al. The clinical utility of multiplate analyser measurement in platelet function testing following stroke and transient ischaemic attack. Eur J Haematol 2015;94:138-144.
30. Sibbing D, Gebhard D, Koch W, et al. Isolated and interactive impact of common CYP2C19 genetic variants on the antiplatelet effect of chronic clopidogrel therapy. J Thromb Haemost 2010;8:1685-1693.

31. Sibbing D, Stegherr J, Latz W, et al. Cytochrome p450 2c19 loss-of-function polymorphism and stent thrombosis following percutaneous coronary intervention. Eur Heart J 2009;30:916-922.
32. Mega JL, Close SL, Wiviott SD, et al. Cytochrome p-450 polymorphisms and response to clopidogrel. N Engl J Med 2009;360:354-362.
33. Wang Y, Zhao X, Lin J, et al. for the CHANCE investigators. Association between CYP2C19 loss-of-function allele status and efficacy of clopidogrel for risk reduction among patients with minor stroke or transient ischemic attack. JAMA 2016;316:70-78.
34. Collet JP, Hulot JS, Pena A, et al. Cytochrome p450 2c19 polymorphism in young patients treated with clopidogrel after myocardial infarction: a cohort study. Lancet 2009;373:309-317.
35. Simon T, Verstuyft C, Mary-Krause M, et al. Genetic determinants of response to clopidogrel and cardiovascular events. N Engl J Med 2009;360:363-375.
36. ADPtest Package Insert (06673686001V2), Roche Diagnostics GmbH.
37. ASPItest Package Insert (06673821001V2), Roche Diagnostics GmbH.
38. Floyd CN, Ferro A. Mechanisms of aspirin resistance. Pharmacol Ther 2014;141:69-78.
39. Bennett D, Yan B, Macgregor L, et al. A pilot study of resistance to aspirin in stroke patients. J Clin Neurosci 2008;15:1204-1209.
40. Ozben S, Ozben B, Tanrikulu AM, et al. Aspirin resistance in patients with acute ischemic stroke. J Neurol 2011;258: 1979.
41. Helgason CM, Bolin KM, Hoff JA, et al. Development of aspirin resistance in persons with previous ischemic stroke. Stroke 1994;25:2331-2336.
42. Kim JT, Heo SH, Choi KH, et al. Clinical implications of changes in individual platelet reactivity to aspirin over time in acute ischemic stroke. Stroke 2015;46:2534-2540.
43. Yi X, Wang C, Liu P, et al. Antiplatelet drug resistance is associated with early neurological deterioration in acute minor ischemic stroke in the Chinese population. J Neurol 2016;263:1612.
44. Mallouk N, Labruyere C, Reny JL, et al. Prevalence of poor biological response to clopidogrel: a systematic review. Thromb Haemost 2012;107:494-506.
45. Zhou BR, Shi HT, Wang R, et al. Dynamic changes and associated factors of clopidogrel resistance in patients after cerebral infarction. J Neurol 2013;260: 2928.
46. Maree AO, Fitzgerald DJ. Variable platelet response to aspirin and clopidogrel in atherothrombotic disease. Circulation 2007;115:2196-2207.
47. Coignion C, Poli M, Sagnier S, et al. Interest of antiplatelet drug testing after an acute ischemic stroke. Eur Neurol 2015;74:135-139.
48. Englyst NA, Horsfield G, Kwan J, et al. Aspirin resistance is more common in lacunar strokes than embolic strokes and is related to stroke severity. J Cereb Blood Flow Metab 2008;28:1196-1203.
49. Cha JK, Park HS, Nah HW, et al. High residual platelet reactivity (HRPR) for adenosine diphosphate (ADP) stimuli is a determinant factor for long-term outcomes in acute ischemic stroke with anti-platelet agents: the meaning of HRPR after ADP might be more prominent in large atherosclerotic infarction than other subtypes of AIS. J Thromb Thrombolysis 2015;42:107-117.
50. Alberts MJ. Platelet function testing for aspirin resistance is reasonable to do: yes! Stroke 2010;41:2400-2401.