Am J Transl Res 2018;10(8):2712-2721
www.ajtr.org /ISSN:1943-8141/AJTR0076158

Original Article

Role of platelet α2-adrenoreceptor in biological low response to Clopidogrel for patients with non cardioembolic ischemic stroke or transient ischemic attack

Jérôme Varvat[2,3], Magali Epinat[2,3], Aurélie Montmartin[3], Sandrine Accassat[1,5], Claire Boutet[3,4], Arnauld Garcin[6], Guorong Li[7], Fabrice Malergue[8], Céline Chapelle[6], Silvy Laporte[3,5,6], Pierre Garnier[2,3], Claude Lambert[9], Nora Mallouk[1,10], Patrick Mismetti[1,2,3,6]

[1]Vascular and Therapeutic Medicine Department, Saint-Etienne University Hospital Center, North Hospital, Saint-Etienne F-42055, France; [2]Neurovascular Unit, Saint-Etienne University Hospital Center, North Hospital, Saint-Etienne F-42055, France; [3]University of Lyon, UJM-Saint-Etienne, Inserm, Sainbiose U1089, Saint-Etienne F-42023, France; [4]Radiology Department, Saint-Etienne University Hospital Center, North Hospital, Saint-Etienne F-42055, France; [5]Inserm, CIC1408, Saint-Etienne F-42055, France; [6]Clinical Research, Innovation and Pharmacology Unit, Saint-Etienne University Hospital Center, North Hospital, Saint-Etienne F-42055, France; [7]Urology Department, Saint-Etienne University Hospital Center, North Hospital, Saint-Etienne F-42055, France; [8]Beckman Coulter R&D-Research, 13009 Marseille, France; [9]Immunology Department, Saint-Etienne University Hospital Center, North Hospital, Saint-Etienne F-42055, France; [10]University of Lyon, UJM-Saint-Etienne, PRISMe, CMES, Saint-Etienne F-42023, France

Received March 16, 2018; Accepted July 4, 2018; Epub August 15, 2018; Published August 30, 2018

Abstract: Background and purpose: Low biological response to Clopidogrel prescribed after non cardioembolic ischemic stroke or transient ischemic attack (TIA) is a major clinical problem and could explain the recurrence of vascular events. Platelet α2-adrenoreceptors are involved in the high residual platelet reactivity in stable coronary artery disease patients on dual antiplatelet therapy. In the present study we investigated the impact of platelet α2-adrenoreceptors on ADP-induced platelet aggregation and on ADP-induced platelet membrane CD62P (P-selectin) expression, a marker of platelet activation on blood samples from patients hospitalized at the acute phase of a non cardioembolic ischemic stroke or TIA. Methods: 72 consecutive patients were prospectively recruited over the course of two years in a monocentric study. Patients received a daily 75 mg-dose of Clopidogrel. ADP-induced platelet aggregation was measured alone, with low dose epinephrine or with atipamezole, a selective α blocker of α2-adrenoreceptors, by Light Transmittance Aggregometry (LTA). Platelet membrane expression of P-selectin was measured by flow cytometry with either ADP alone or combined with epinephrine. Results: Epinephrine at low dose stimulated ADP-induced platelet membrane expression of CD62P whereas Atipamezole significantly inhibited 10 μM ADP-induced platelet aggregation. Conclusions: Our study showed the role of platelet α2-adrenoreceptors in biological low response to Clopidogrel for patients hospitalized for a non-cardioembolic ischemic stroke or TIA. Atipamezole could improve the status of biological response to Clopidogrel.

Keywords: Clopidogrel, light transmission aggregometry, flow cytometry, P-selectin, α2 adrenoreceptors, stroke

Introduction

Clopidogrel is one of the first-line treatments after non-cardioembolic ischemic stroke or transient ischemic attack (TIA) [1-3]. However, the recurrence rate of vascular events was 7.15% per year in CAPRIE trial (Clopidogrel versus Aspirin in Patients at Risk of Ischemic Events), 16.7% at 18 months in MATCH trial (Management of ATherothrombosis with Clopidogrel in High-risk patients having experienced either transient ischemic attack or stroke), and 13.1% in PRoFESS trial (Prevention Regimen for Effectively avoiding Second Strokes) after a mean follow-up of 2.5 years [4-6]. In addition, Clopidogrel did not provide

significant benefit in stroke patients when compared to aspirin treatment in CAPRIE trial [4]. In contrast, Clopidogrel was beneficial in patients with lower-limb occlusive arterial disease or diabetes, or with a history of stroke or myocardial infarction [4, 7, 8]. The well-known variability in biological response to Clopidogrel in coronary artery disease partly explains the recurrence of vascular events [9-11]. Indeed, in studies conducted specifically in ischemic stroke or TIA patients, the proportion of poor biological responders to Clopidogrel ranged from 8 to 55% according to the laboratory test and the cut-off value used [12-24].

The mechanisms underlying the variability in biological response to Clopidogrel have been studied. The involvement of platelet α2-adrenoreceptors in the high residual platelet reactivity has been investigated in stable coronary artery disease patients on dual antiplatelet therapy [25]. ADP-induced platelet aggregation was significantly potentiated by low-dose epinephrine (1.10^{-9} g/ml) whereas it was inhibited by atipamezole (2 μM), a selective α-2 adrenoreceptor blocker.

The aim of our study was to assess the influence of α2-adrenoreceptors on ADP-induced platelet aggregation and on ADP-induced platelet membrane P-selectin expression, in patients hospitalized at the acute phase of a non-cardioembolic ischemic stroke or a TIA and treated with a regular dose of Clopidogrel.

Materials and methods

Patients

Consecutive patients hospitalized in the Neurovascular Unit of Saint-Etienne University Hospital Centre following a non-cardioembolic ischemic stroke or TIA, and selected for treatment with Clopidogrel alone, were prospectively recruited between September 2013 and November 2015.

Patients were selected to receive Clopidogrel alone (Plavix 75 mg; Sanofi Pharma Bristol-Myers Squibb SNC, Paris, France) at the dose of 75 mg per day if this treatment was considered likely to confer greater benefit than aspirin.

Exclusion criteria comprised an abnormal laboratory test result (platelet count < 100 G/l, prothrombin time (PT) < 70% or > 130%, activated partial thromboplastin time (aPTT) < 27 s or > 39 s, aspartate aminotransferase or alanine aminotransferase > 2.5 N), need for continued treatment with aspirin, history of a stroke or TIA under Clopidogrel, or contraindication to Clopidogrel or to one of the excipients in the formulation. Ongoing treatment interacting with the α-adrenergic system was also an exclusion criterion.

Clinical and laboratory assessments

The following patients characteristics were recorded: age, sex, body mass index (BMI), medical history (hypertension, hypercholesterolemia, current smoking, diabetes, coronary artery disease, lower-limb occlusive arterial disease, ischemic stroke or TIA), ongoing treatment (β-blocker, calcium channel blocker, angiotensin converting enzyme inhibitor, angiotensin II receptor blocker, proton pump inhibitor, or statin). The definition of TIA established by the TIA Working group in 2002, the Trial of ORG 10172 in Acute Stroke Treatment (TOAST) etiological classification, the National Institutes of Health Stroke Score (NIHSS) at inclusion, and the modified Rankin handicap Score (mRS) at discharge were used to characterize the index ischemic event [26-29]. The laboratory parameters analyzed were creatinine clearance (determined by the Cockcroft-Gault formula), glycated hemoglobin (HbA1c), low-density (LDL)-cholesterol, high-density (HDL)-cholesterol and triglycerides. Radiological parameters, evaluated by an independent radiologist, comprised the extent of white matter lesions of vascular origin according to the Fazekas classification, and the presence of microbleeds [30].

Evaluation of the biological response to Clopidogrel with Light Transmission Aggregometry (LTA)

Whole blood samples were drawn with a 19-gauge needle in citrated tubes (Sodium Citrate 0.105 M/3.2%, Becton Dickinson, Plymouth, UK) from the patients after 5 to 8 days of Clopidogrel treatment in order to ensure that the drug had reached a steady state dose [31]. Pre-analytical recommendations were followed [32]. Platelet rich plasma (PRP) was used for LTA.

The interval between blood sampling and LTA did not exceed 2 h. Platelet aggregation was

Table 1. Patients characteristics

Patients characteristics			Ongoing treatments	
Age (y), mean (SD)		68.7 (9.6)	Beta-blocker	11 (15.3%)
Age ≥ 75 y		22 (30.6%)	Statin	50 (69.4%)
Male		49 (68.1%)	PPI	26 (36.1%)
BMI ≥ 30 kg/m^2		14 (20.0%)	ACE inhibitor/ARB	42 (58.3%)
			Calcium channel blocker	24 (33.3%)
Medical history			Laboratory test parameters	
Hypertension		48 (66.7%)	Clearance	12 (16.7%)
Hypercholesterolemia		39 (54.2%)	(Cockroft-Gault) < 60 ml/min	
Diabetes		25 (34.7%)	Glycated hemoglobin ≥ 6%	37 (52.9%)
Current smokers		18 (25%)	LDL Cholesterol ≥ 2.6 mmol/l	41 (56.9%)
Coronary artery disease		7 (9.7%)	HDL Cholesterol ≤ 1 mmol/l	24 (35.3%)
Lower-limb arterial disease		5 (6.9%)	Triglycerides ≥ 1.70 mmol/l	27 (37.5%)
Ischemic stroke or TIA		14 (19.4%)		
Characteristics of stroke/TIA			Radiological analysis	
Type of event	Ischemic stroke	63 (87.5%)	Simplified Fazekas score ≥ 2	29 (40.3%)
	TIA	9 (12.5%)	Presence of microbleed	16 (22.2%)
TOAST classification	I	28 (38.9%)		
	III	18 (25.0%)		
	V	26 (36.1%)		
NIHSS at admission	< 5	61 (84.7%)		
	≥ 5	11 (15.3%)		
mRS at discharge	< 3	63 (87.5%)		
	≥ 3	9 (12.5%)		

ACE inhibitor: angiotensin converting enzyme inhibitor; ARB: angiotensin II receptor blocker; BMI: body mass index; HDL: high-density lipoprotein; LDL: low-density lipoprotein; mRS: modified Rankin score; NIHSS: National Institutes of Health Stroke Score; NR patients: non-responder/low–responder/poor-responder patients; PPI: proton pump inhibitor; R patients: responder patients; SD: standard deviation; TIA: transient ischemic attack; TOAST: Trial of ORG 10172 in Acute Stroke Treatment.

measured using an aggregometer (TA4V; SD-Medical, Heillecourt, France) and according to LTA recommendations [33]. The percentage of maximal platelet aggregation (MPA) induced by ADP (Elitech) at final concentrations of 1.25, 5 and 10 µM was measured at 37°C [34].

Potentiation of ADP-induced aggregation by a low-dose of epinephrine (1.10^{-9} g/l) was assessed with ADP 1.25 µM whereas the inhibition of ADP-induced aggregation was assessed with atipamezole (Antisedan®, Atipamezole hydrochloride, Orion Pharma) and with ADP 10 µM. Preincubation of PRP was performed with 2 µM of atipamezole for 2 minutes.

Clopidogrel response according to LTA is usually considered as low if a maximal platelet aggregation value is > 70% [9].

Flow cytometry

10 µl of PRP was activated with ADP at a final concentration of 1.25, 5 and 10 µM for 10 minutes and this activation was stopped with thrombofix platelet stabilizer kit (Beckman Coulter, Villepinte, France). After 60 minutes, 10 µl of this mixture was incubated with 5 µl of anti P-selectin-PE antibody (Phycoerythrine, clone CLB-Thromb/6, Beckman Coulter) and 10 µL of anti CD61-PE-Cy7 antibody (phycoerythrin-cyanine 7, clone SZ21, Beckman Coulter) for 15 minutes in a dark room, at room temperature. Then 500 µl of phosphate buffer saline (PBS) were added and the acquisition of data was performed on 10000 platelets with a Navios (Beckman Coulter, Brea, USA) [35]. The Mean Fluorescence Intensity or MFI was determined on CD61-positive platelets and on P-selectin positive platelets by using the Kaluza 1.5a flow cytometry analysis software and the percentage of P-selectin positive platelets was determined.

Potentiation of ADP-induced platelet membrane P-selectin expression by low-dose of epi-

Table 2. Maximal platelet aggregation values (Aggmax) by ADP group

		ADP (N=72)	ADP + epinephrine (N=72)	ADP + atipamezole (N=72)
Aggmax: ADP 1.25 µM (%)	N	71	70	66
	Missing data	1	2	6
	Mean (SD)	30.4 (22.0)	31.3 (23.4)	29.8 (22.0)
	Median	24.7	22.7	21.0
	Min-Max	4.7-94.2	3.1-88.8	5.8-99.0
	Q1-Q3	16.5-33.2	14.6-34.9	15.7-32.3
Aggmax: ADP 5 µM (%)	N	72	72	68
	Missing data	0	0	4
	Mean (SD)	67.9 (20.1)	62.0 (18.2)	62.9 (21.5)
	Median	73.7	66.3	67.2
	Min-Max	16.0-101.2	15.4-90.8	1.2-97.2
	Q1-Q3	51.7-83.6	50.4-76.6	44.6-78.7
Aggmax: ADP 10 µM (%)	N	72	71	66
	Missing data	0	1	6
	Mean (SD)	70.9 (16.4)	65.5 (16.6)	66.6 (15.8)
	Median	74.1	70.5	70.5
	Min-Max	22.0-101.8	11.2-93.8	33.2-97.1
	Q1-Q3	64.2-80.9	55.7-76.6	54.3-78.7

All values are expressed as Mean (SD), Median and inter-quantile range (Q1-Q3).

nephrine (1.10^{-9} g/l) was assessed with ADP 1.25 µM.

Clinical trials regulations

This study, registered at ClinicalTrials.gov (no. NCT01955642) was approved by the French health authorities and the local ethics committee. A signed consent form was provided by each patient included in the study.

Statistical analysis

Continuous data were expressed as mean and standard deviation or median and inter-quartile range (Q1-Q3) and categorical data as absolute and relative frequencies (expressed in percentages).

Maximal aggregation values induced by ADP 1.25 µM, 5 µM and 10 µM were compared using a Wilcoxon signed-rank test (paired data) as follows: i) ADP alone *versus* ADP in presence of low dose epinephrine; ii) ADP alone *versus* ADP combined with atipamezole.

All statistical tests were two-sided and *P*-values less than 0.05 were considered as statistical significant. Statistical analyses were performed with SAS (version 9.4).

Results

Seventy two patients were included in the study. The population characteristics are displayed in **Table 1**. The mean age was 68.7 +/- 9.6 years and 68.1% of the patients were males. The proportion of patients taking β-blocker, statin, PPI, ACE inhibitor/ARB and calcium channel blocker was respectively 15.3%, 69.4%, 36.1%, 58.3% and 33.3%. In our study, 38.9% of the patients were TOAST I. 25% of the patients were smokers.

Parameters of maximal aggregation induced by ADP 1.25, 5 and 10 µM are shown in **Table 2**. Median values of maximal aggregation values induced by ADP at 1.25 µM, 5 µM and 10 µM were 24.7%, 73.7% and 74.1% respectively.

Median values of maximal aggregation induced by 1.25 µM, 5 µM and 10 µM ADP in presence of low dose epinephrine were 22.7%, 66.3% and 70.5% (**Table 2**). A significant difference was found between values for ADP versus ADP in presence of low dose epinephrine with *P* values < 0.05 (**Figure 1**; P=0.01, n=70 for ADP 1.25 µM, P < 0.0001, n=72 for ADP 5 µM and P < 0.0001, n=71 for ADP 10 µM).

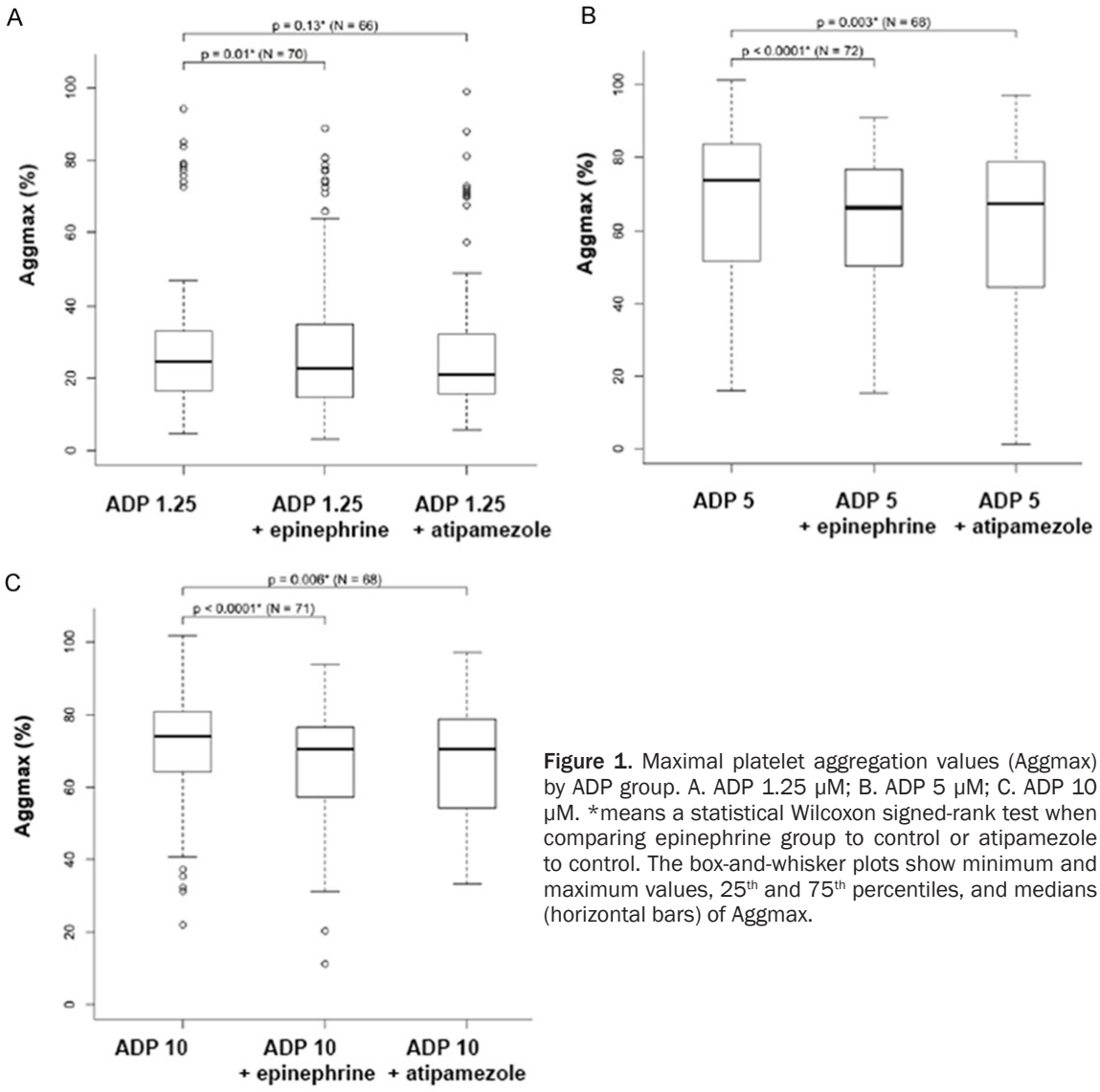

Figure 1. Maximal platelet aggregation values (Aggmax) by ADP group. A. ADP 1.25 µM; B. ADP 5 µM; C. ADP 10 µM. *means a statistical Wilcoxon signed-rank test when comparing epinephrine group to control or atipamezole to control. The box-and-whisker plots show minimum and maximum values, 25th and 75th percentiles, and medians (horizontal bars) of Aggmax.

In representative dotplots (**Figure 2A-F**), low dose epinephrine potentiated ADP 1.25 µM induced platelet membrane P-selectin expression. On platelets from 67 patients, the median values of the percentage of P-selectin positive platelets induced by ADP and by ADP with low dose epinephrine (+ epi) were as follows: 16% with ADP 1.25 µM, 18.1% with ADP 1.25 µM + epi, 28.2% with ADP 5 µM, 31% with ADP 5 µM + epi, 30% with ADP 10 µM, 36.5% with ADP 10 µM + epi (**Figure 2G**).

Median maximal aggregation values induced by 1.25 µM, 5 µM and 10 µM of ADP on PRP pretreated with atipamezole were: 21%, 67.2% and 70.5% respectively (**Table 2**). A significant difference was found between values for ADP versus ADP + atipamezole for ADP 5 µM and ADP 10 µM but not for ADP 1.25 µM (P=0.13, n=66 for ADP 1.25 µM, P=0.003, n=68 for ADP 5 µM and P=0.006, n=68 for ADP 10 µM). Furthermore, 33.3% of Clopidogrel low responder became responder in the "atipamezole" condition with an Aggmax value < 70% (n=14/42).

Discussion

Values of ADP-induced platelet aggregation for 5 and 10 µM ADP were close to the normal reference laboratory aggregation values previously published [36] from 100 healthy volunteers despite Clopidogrel treatment. Several hypotheses could explain this: a) the patients of our

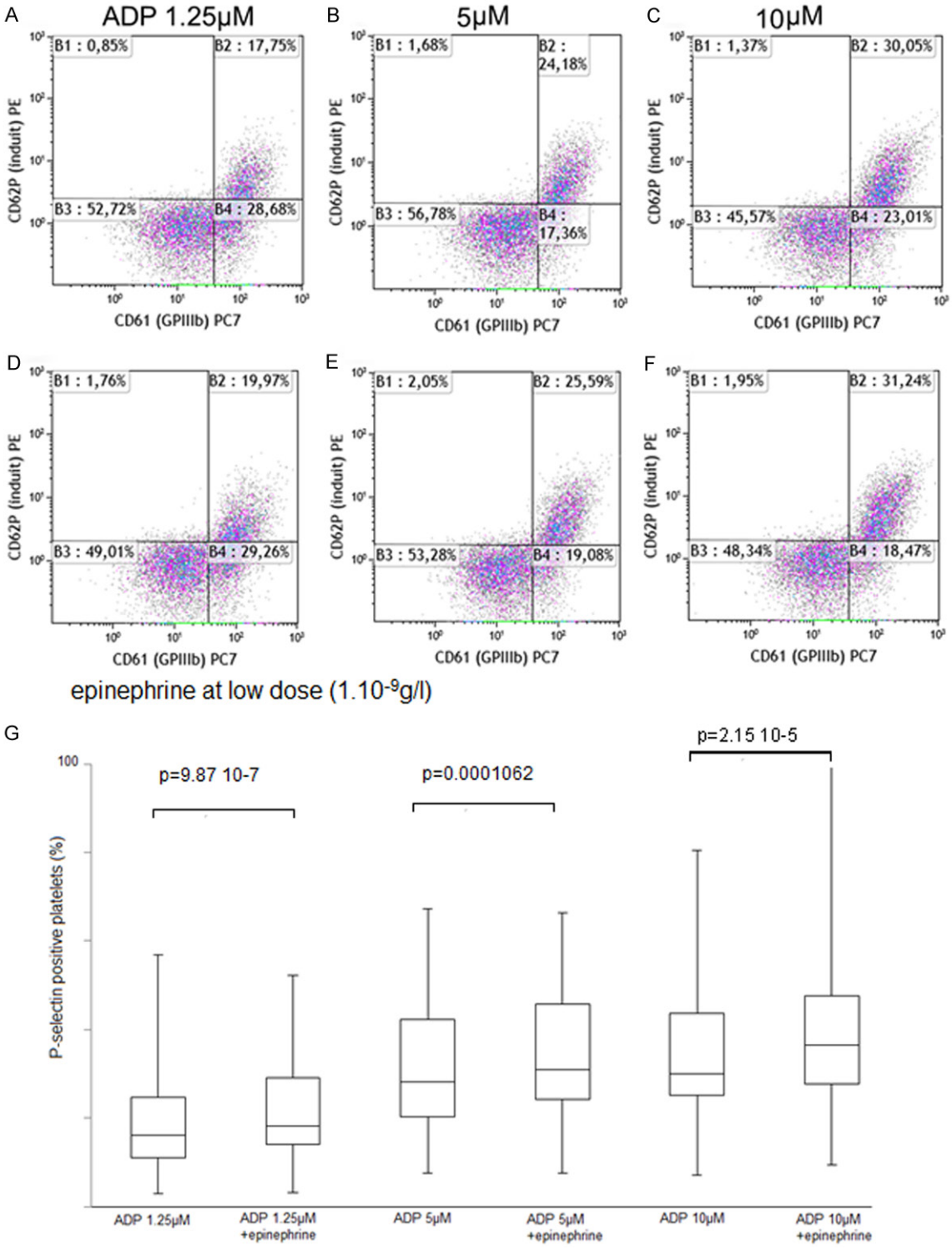

Figure 2. Effect of a low-dose of epinephrine on ADP-induced P-selectin expression assessed with flow cytometry. Representative density plots for ADP (A-C) versus ADP+low dose of epinephrine (1.10^{-9} g/l) (D-F). Median values of the percentage of P-selectin positive platelets induced by ADP and by ADP with low dose epinephrine (G).

study were included at the acute phase of their ischemic stroke which favors the activation of platelets, especially for the patients with large-vessel cerebral infarction [37]; b) they received only one antiaggregant treatment without loading dose because of the risk of bleeding; c) a small number of patients received β-blocker, which is known to modulate the platelet reac-

tivity. In addition, the conditions used in our study are significantly different from those previously published on patients hospitalized for a stable angor: all patients were treated by 100 mg aspirin, and 75 mg Clopidogrel and 92.6% were taking β-blocker [25].

Median values were taken into consideration because the number of patients included was low and because all the data were not normally distributed.

It is worth noting that epinephrine was applied at a dose falling within the range of catecholamines physiological concentrations. Indeed, at the nanomolar range, epinephrine cannot initiate platelet aggregation but is able to potentiate the effect of other agonist like ADP. A low concentration of ADP (1.25 μM) has been chosen to optimize the potentiating effect of low dose epinephrine on ADP-induced aggregation and ADP-induced platelet membrane P-selectin expression consecutively to translocation from alpha-granules following activation.

No potentiation of ADP induced aggregation by low-dose of epinephrine was observed. This is different from previous results likely because of the clinical settings [25]. A slight platelet inhibition has been described for patients with non-ST elevation acute coronary syndrome carrying the α2A-adrenoreceptor 6.3 kb variant and treated with 250 mg aspirin and 600 mg Clopidogrel [38]. This paradoxical result could be explained by a possible overstimulation of α2-adrenoreceptors in the context of an activated sympathetic nervous system and a desensitization of the α2-adrenoreceptors [38]. The sympathetic nervous system of our patients could be highly active because they were stressed at the acute phase of the ischemic stroke or of the TIA. During this phase like in non-ST elevation acute coronary syndrome a high level of blood catecholamines could induce an overstimulation and a desensitization of α2-adrenoreceptors [39].

However a potentiation by a low-dose of epinephrine of the ADP-induced P-selectin expression was observed. Thus the pathway linking α2-adrenoreceptor activation to the platelet membrane P-selectin expression is still active. This result confirms the hypothesis of Motulsky: the desensitization of α2-adrenoreceptors pathway occurs without change in the alpha 2-adrenoreceptors or in their coupling to an inhibition of adenylate cyclase [39].

A link has been suggested among smoking, serotonin and catecholamine signaling resulting in an increased tonic level of platelet activation in smokers [40]. We did not confirm these results (data not shown).

We found a significant inhibitory effect of atipamezole on 10 μM ADP-induced platelet maximal aggregation. Interestingly, after atipamezole incubation ADP-induced platelet maximal aggregation values were lowered. This could have a potential therapeutic interest for Clopidogrel low responders. This work could be further investigated by studying the impact of the autonomous nervous system on platelet function.

Conclusions

Our study showed the role of platelet α2-adrenoreceptors in biological low response to Clopidogrel for patients hospitalized for a non-cardioembolic ischemic stroke or TIA, and treated with Clopidogrel alone. Atipamezole could have a potential therapeutic effect for Clopidogrel low responder.

This transverse study involved clinical and research teams. This paper was written by NM and JV. English was improved by GL and FM. NM was in charge of the LTA and flow cytometry tests with AM. AM and CL provided the aggregometer and the flow cytometer. AG was in charge of the clinical research management. CC and SL were in charge of the statistical tests. CB was in charge of the radiological analysis. JV, SA and ME were in charge of patients recruitment; PG and PM supervised the clinical and pharmacological aspects of the study. The clinical team was also involved in the interpretation of the results.

Acknowledgements

This research has received funding support from the Centre Hospitalier Universitaire from Saint Etienne and was promoted by the University Hospital of Saint Etienne.

Disclosure of conflict of interest

None.

Address correspondence to: Nora Mallouk, Vascular and Therapeutic Medicine Department, Saint-Etienne University Hospital Center, North Hospital, Saint-Etienne F-42055, France; University of Lyon, UJM-Saint-Etienne, PRISMe, CMES, Saint-Etienne F-42023, France. E-mail: nora.mallouk@chu-st-etienne.fr

References

[1] European Stroke Organisation (ESO) Executive Committee; ESO Writing Committee. Guidelines for management of ischaemic stroke and transient ischaemic attack 2008. Cerebrovasc Dis 2008; 25: 457-507.

[2] Lansberg MG, O'Donnell MJ, Khatri P, Lang ES, Nguyen-Huynh MN, Schwartz NE, Sonnenberg FA, Schulman S, Vandvik PO, Spencer FA, Alonso-Coello P, Guyatt GH, Akl EA; American College of Chest Physicians. Antithrombotic and thrombolytic therapy for ischemic stroke: antithrombotic therapy and prevention of thrombos is, 9th edition: American college of chest physicians evidence-based clinical practice guidelines. Chest 2012; 141: e601S-e636S.

[3] Kernan WN, Ovbiagele B, Black HR, Bravata DM, Chimowitz MI, Ezekowitz MD, Fang MC, Fisher M, Furie KL, Heck DV, Johnston SC, Kasner SE, Kittner SJ, Mitchell PH, Rich MW, Richardson D, Schwamm LH, Wilson JA; American Heart Association Stroke Council, Council on Cardiovascular and Stroke Nursing, Council on Clinical Cardiology, and Council on Peripheral Vascular Disease. Guidelines for the prevention of stroke in patients with stroke and transient ischemic attack: a guideline for healthcare professionals from the American heart association/American stroke association. Stroke 2014; 45: 2160-2236.

[4] CAPRIE Steering Committee. A randomised, blinded, trial of Clopidogrel versus aspirin in patients at risk of ischaemic events (CAPRIE). Lancet 1996; 348: 1329-1339.

[5] Diener HC, Bogousslavsky J, Brass LM, Cimminiello C, Csiba L, Kaste M, Leys D, Matias-Guiu J, Rupprecht HJ; MATCH investigators. Aspirin and Clopidogrel compared with Clopidogrel alone after recent ischaemic stroke or transient ischaemic attack in high-risk patients (MATCH): randomised, double-blind, placebo-controlled trial. Lancet 2004; 364: 331-337.

[6] Sacco RL, Diener HC, Yusuf S, Cotton D, Ounpuu S, Lawton WA, Palesch Y, Martin RH, Albers GW, Bath P, Bornstein N, Chan BP, Chen ST, Cunha L, Dahlöf B, De Keyser J, Donnan GA, Estol C, Gorelick P, Gu V, Hermansson K, Hilbrich L, Kaste M, Lu C, Machnig T, Pais P, Roberts R, Skvortsova V, Teal P, Toni D, Vandermaelen C, Voigt T, Weber M, Yoon BW; PRoFESS Study Group. Aspirin and extended-release dipyridamole versus Clopidogrel for recurrent stroke. N Engl J Med 2008; 359: 1238-51.

[7] Bhatt DL, Marso SP, Hirsch AT, Ringleb PA, Hacke W, Topol EJ. Amplified benefit of Clopidogrel versus aspirin in patients with diabetes mellitus. Am J Cardiol 2002; 90: 625-628.

[8] Ringleb PA, Bhatt DL, Hirsch AT, Topol EJ, Hacke W; Clopidogrel Versus Aspirin in Patients at Risk of Ischemic Events Investigators. Benefit of clopidogrel over aspirin is amplified in patients with a history of ischemic events. Stroke 2004; 35: 528-532.

[9] Mallouk N, Labruyère C, Reny JL, Chapelle C, Piot M, Fontana P, Gris JC, Delavenne X, Mismetti P, Laporte S. Prevalence of poor biological response to Clopidogrel: a systematic review. Thromb Haemost 2012; 107: 494-506.

[10] Combescure C, Fontana P, Mallouk N, Berdague P, Labruyere C, Barazer I, Gris JC, Laporte S, Fabbro-Peray P, Reny JL; CLOpidogrel and Vascular ISchemic Events Meta-analysis Study Group. Clinical implications of clopidogrel nonresponse in cardiovascular patients: a systematic review and meta-analysis. J Thromb Haemost 2010; 8: 923-933.

[11] Brar SS, ten Berg J, Marcucci R, Price MJ, Valgimigli M, Kim HS, Patti G, Breet NJ, DiSciascio G, Cuisset T, Dangas G. Impact of platelet reactivity on clinical outcomes after percutaneous coronary intervention. A collaborative meta-analysis of individual participant data. J Am Coll Cardiol 2011; 58: 1945-1954.

[12] 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.

[13] Fong J, Cheng-Ching E, Hussain MS, Katzan I, Gupta R. Predictors of biochemical aspirin and Clopidogrel resistance in patients with ischemic stroke. J Stroke Cerebrovasc Dis 2011; 20: 227-230.

[14] Fukuoka T, Furuya D, Takeda H, Dembo T, Nagoya H, Kato Y, Deguchi I, Maruyama H, Horiuchi Y, Tanahashi N. Evaluation of Clopidogrel resistance in ischemic stroke patients. Intern Med 2011; 50: 31-35.

[15] Maruyama H, Takeda H, Dembo T, Nagoya H, Kato Y, Fukuoka T, Deguchi I, Horiuchi Y, Tanahashi N. Clopidogrel resistance and the effect of combination cilostazol in patients with ischemic stroke or carotid artery stenting using the verifynow P2Y12 assay. Intern Med 2011; 50: 695-698.

[16] Depta JP, Fowler J, Novak E, Katzan I, Bakdash S, Kottke-Marchant K, Bhatt DL. Clinical outcomes using a platelet function-guided ap-

proach for secondary prevention in patients with ischemic stroke or transient ischemic attack. Stroke 2012; 43: 2376-2381.
[17] Kinsella JA, Tobin WO, Cox D, Coughlan T, Collins R, O'Neill D, Murphy RP, McCabe DJ. Prevalence of ex vivo high on-treatment platelet reactivity on antiplatelet therapy after transient ischemic attack or ischemic stroke on the PFA-100(®) and VerifyNow(®). J Stroke Cerebrovasc Dis 2013; 22: e84-e92.
[18] Zhou BR, Shi HT, Wang R, Zhang M, Guan HT, Liu ZF, Deng YH. Dynamic changes and associated factors of Clopidogrel resistance in patients after cerebral infarction. J Neurol 2013; 260: 2928-2937.
[19] Jover E, Rodríguez JM, Bernal A, Arroyo AB, Iniesta JA, Guiú IS, Martínez C, Vicente V, Lozano ML, Rivera J. High on-treatment platelet reactivity in patients with ischemic cerebrovascular disease: assessment of prevalence and stability over time using four platelet function tests. Blood Coagul Fibrinolysis 2014; 25: 604-611.
[20] Meves SH, Schröder KD, Endres HG, Krogias C, Krüger JC, Neubauer H. Clopidogrel high-on-treatment platelet reactivity in acute ischemic stroke patients. Thromb Res 2014; 133: 396-401.
[21] Zhang S, Lai X, Li W, Xiong Z, Xu A, Xu A, Huang L. VASP phosphorylation and genetic polymorphism for Clopidogrel resistance in Chinese patients with non-cardioembolic ischemic stroke. Thromb Res 2014; 134: 1272-1277.
[22] Lundström A, Laska AC, Von Arbin M, Jörneskog G, Wallén H. Glucose intolerance and insulin resistance as predictors of low platelet response to Clopidogrel in patients with minor ischemic stroke or TIA. Platelets 2014; 25: 102-110.
[23] Lundström A, Wallén H, von Arbin M, Jörneskog G, Gigante B, Höeg Dembrower K, Laurencikas E, Laska AC. Clopidogrel resistance after minor ischemic stroke or transient ischemic attack is associated with radiological cerebral small-vessel disease. J Stroke Cerebrovasc Dis 2015; 24: 2348-2357.
[24] Qiu LN, Wang L, Li X, Han RF, Xia XS, Liu J. Predictive value of high residual platelet reactivity by flow cytometry for outcomes of ischemic stroke patients on Clopidogrel therapy. J Stroke Cerebrovasc Dis 2015; 24: 1145-1152.
[25] Béres BJ, Tóth-Zsámboki E, Vargová K, László A, Masszi T, Kerecsen G, Préda I, Kiss RG. Analysis of platelet α2-adrenergic receptor activity in stable coronary artery disease patients on dual antiplatelet therapy. Thromb Haemost 2008; 100: 829-838.
[26] Albers GW, Caplan LR, Easton JD, Fayad PB, Mohr JP, Saver JL, Sherman DG; TIA Working Group. Transient ischemic attack-proposal for a new definition. N Engl J Med 2002; 347: 1713-1716.
[27] Adams HP Jr, Bendixen BH, Kappelle LJ, Biller J, Love BB, Gordon DL, Marsh EE 3rd. Classification of subtype of acute ischemic stroke. Definitions for use in a multicenter clinical trial. TOAST. Trial of org 10172 in acute stroke treatment. Stroke 1993; 24: 35-41.
[28] Meyer BC, Hemmen TM, Jackson CM, Lyden PD. Modified national institutes of health stroke scale for use in stroke clinical trials: prospective reliability and validity. Stroke 2002; 33: 1261-1266.
[29] Van Swieten JC, Koudstaal PJ, Visser MC, Schouten HJ, van Gijn J. Interobserver agreement for the assessment of handicap in stroke patients. Stroke 1988; 19: 604-607.
[30] Fazekas F, Kleinert R, Offenbacher H, Schmidt R, Kleinert G, Payer F, Radner H, Lechner H. Pathologic correlates of incidental MRI white matter signal hyperintensities. Neurology 1993; 43: 1683-1689.
[31] Savcic M, Hauert J, Bachmann F, Wyld PJ, Geudelin B, Cariou R. Clopidogrel loading dose regimens: kinetic profile of pharmacodynamic response in healthy subjects. Semin Thromb Hemost 1999; 25 Suppl 2: 15-19.
[32] Polack B, Schved JF, Boneu B; Groupe d'Etude sur l'Hémostase et la Thrombose' (GEHT). Pre-analytical recommendations of the 'Groupe d'Etude sur l'Hémostase et la Thrombose' (GEHT) for venous blood testing in hemostasis laboratories. Haemostasis 2001; 31: 61-68.
[33] Cattaneo M, Cerletti C, Harrison P, Hayward CP, Kenny D, Nugent D, Nurden P, Rao AK, Schmaier AH, Watson SP, Lussana F, Pugliano MT, Michelson AD. Recommendations for the standardization of light transmission aggregometry: a consensus of the working party from the platelet physiology subcommittee of SSC/ISTH. J Thromb Haemost 2013.
[34] Hayward CP, Moffat KA, Raby A, Israels S, Plumhoff E, Flynn G, Zehnder JL. Development of North American consensus guidelines for medical laboratories that perform and interpret platelet function testing using light transmission aggregometry. Am J Clin Pathol 2010; 134: 955-963.
[35] Mallouk N, Varvat J, Berger A, Epinat M, Accassat S, Garcin A, Montmartin A, Li G, Garnier P, Mismetti P, Lambert C. Assessment of a flow cytometry technique for studying signaling pathways in platelets: monitoring of VASP phosphorylation in clinical samples. Pract Lab Med 2018; 11: 10-18.
[36] Buonamici P, Marcucci R, Migliorini A, Gensini GF, Santini A, Paniccia R, Moschi G, Gori AM, Abbate R, Antoniucci D. Impact of platelet reac-

tivity after clopidogrel administration on drug-eluting stent thrombosis. J Am Coll Cardiol 2007; 49: 2312-2317.

[37] Tsai NW, Chang WN, Shaw CF, Jan CR, Chang HW, Huang CR, Chen SD, Chuang YC, Lee LH, Wang HC, Lee TH, Lu CH. Levels and value of platelet activation markers in different subtypes of acute non-cardio-embolic ischemic stroke. Thromb Res 2009; 124: 213-218.

[38] Cuisset T, Hamilos M, Delrue M, Frère C, Verhamme K, Bartunek J, Saut N, Bonnet JL, Eijgelsheim M, Wijns W, Alessi MC, Barbato E. Adrenergic receptor polymorphisms and platelet reactivity after treatment withdual antiplatelet therapy with aspirin and clopidogrel in acute coronary syndrome. Thromb Haemost 2010; 103: 774-9.

[39] Motulsky HJ, Shattil SJ, Ferry N, Rozansky D, Insel PA. Desensitization of epinephrine-initiated platelet aggregation does not alter binding to the alpha 2-adrenergic receptor or receptor coupling to adenylate cyclase. Mol Pharmacol 1986; 29: 1-6.

[40] Lowery CL 3rd, Elliott C, Cooper A, Hadden C, Sonon RN, Azadi P, Williams DK, Marsh JD, Woulfe DS, Kilic F. Cigarette smoking-associated alterations in serotonin/adrenalin signaling pathways of platelets. J Am Heart Assoc 2017; 6.