Original Article

Chinese patients carrying two *CYP2C19* LOFs have a more active state of P2Y$_{12}$-G$_i$ signaling and larger MPV in post-clopidogrel platelets with acute coronary syndrome

Huajie Xu[1*], Hongyi Wu[1*], Xin Zhao[1], Yan Zhang[2], She Chen[2], Bing Fan[1], Si Zhang[2]

[1]Department of Cardiology, Shanghai Institute of Cardiovascular Diseases, Zhongshan Hospital, Fudan University, Shanghai, P. R. China; [2]Key Laboratory of Glycoconjugate Research Ministry of Public Health, Department of Biochemistry and Molecular Biology, School of Basic Medical Sciences, Fudan University, Shanghai, P. R. China. [*]Equal contributors.

Abstract: Clopidogrel is the most widely used P2Y$_{12}$-inhibitor in Chinese patients with acute coronary syndrome (ACS). It was previously found that carriers with two *CYP2C19* loss-of-function (LOF) alleles, but not one LOF allele, had poorer clinical outcomes. The current study further determined the link between *CYP2C19* genotype status and clopidogrel response, using P2Y$_{12}$-G$_i$ dependent signaling endpoints and mean platelet volume (MPV). A total of 196 patients with ACS were included. *CYP2C19* variant alleles were detected by gene sequencing. Platelet reactivity was assessed using thrombelastographs at 24 hours after 300 mg clopidogrel loading. P2Y$_{12}$-G$_i$ signaling was simultaneously measured using flow cytometric analysis of vasodilator-stimulated phosphoprotein phosphorylation-platelet reactivity (VASP-PRI) and Akt phosphorylation (P-Akt) indexes. Measurements of hematological parameters were carried out before and after one month of treatment with 75 mg clopidogrel. It was found that increases in VASP-PRI positively correlated with the number of LOF, significantly. However, only two LOF carriers showed a higher P-Akt index after clopidogrel treatment. VASP-PRI was positively associated with platelet aggregation (R = 0.661, *P* < 0.001), but not with P-Akt index. MPV was larger in patients with two LOFs after clopidogrel treatment. Results indicate that only two *CYP2C19* LOF carriers had a more active state of P2Y$_{12}$-G$_i$ signaling and larger MPV in post-clopidogrel platelets.

Keywords: *CYP2C19*, clopidogrel, P2Y$_{12}$, VASP, Akt, MPV

Introduction

Dual antiplatelet therapy has become the cornerstone of medical treatment in patients with acute coronary syndrome (ACS) undergoing percutaneous coronary intervention (PCI) [1]. Although more potent platelet P2Y$_{12}$-inhibitors (prasugrel or ticagrelor) have been developed to further reduce ischemic events when added to aspirin therapy, increased risks of severe bleeding have been shown and few East Asian patients have been included in trials [2, 3]. Furthermore, the availability of ticagrelor has been restricted to public medical insurance. Thus, clopidogrel is still the most widely used in patients with ACS in China [4]. Clopidogrel is

an inactive pro-drug that requires hepatic metabolism by cytochrome P450 to generate its active thiol metabolite, targeting and irreversibly inhibiting platelet ADP P2Y$_{12}$ receptors [5]. Insufficient P2Y$_{12}$ inhibition of clopidogrel is the most important concern in clinical practice.

Increasing evidence has demonstrated an association between *CYP2C19* polymorphisms and clopidogrel responsiveness [6]. However, there are considerable ethnic differences in distribution patterns and effects of *CYP2C19* LOF alleles. It should be noted that more than half of East Asians carry at least one *CYP2C19* LOF allele [7]. Although East Asians have a markedly higher prevalence over Westerners,

no evidence has been found that East Asians have worse response to clopidogrel than Westerners. Concerning whites with ACS, carriage of one or two *CYP2C19* LOF alleles may increase the risk of ischemic events during therapy with clopidogrel [8]. In contrast, in East Asians, the current study reported that only carriers with two *CYP2C19* LOF alleles, but not one LOF allele, have poor clinical outcomes after clopidogrel treatment [9, 10]. It should be defined which genotype with *CYP2C19* LOF allele is more likely to substantively influence clopidogrel responsiveness in Chinese patients.

Theoretically, platelet P2Y$_{12}$-G$_i$ signaling pathway assessment is a more special endpoint of clopidogrel effectiveness. The P2Y$_{12}$ receptor is coupled to G$_i$ proteins [11]. Binding of ADP to the P2Y$_{12}$ receptor liberates G$_i$ protein subunits α$_i$ and β$_γ$, resulting in the amplification and stabilization of platelet aggregation. The α$_i$ subunit leads to inhibition of adenylyl cyclase (AC), reducing cyclic adenosine monophosphate (cAMP) levels. This, in turn, diminishes cAMP-mediated phosphorylation of vasodilator-stimulated phosphoprotein (VASP) [12]. The β$_γ$ subunit activates phosphatidylinositol 3-kinase (PI3K) through activation of a serine-threonine protein kinase B (PKB) [12]. These complex series of intracellular signaling events, including the phosphorylation of VASP and Akt, compose P2Y$_{12}$-G$_i$ signaling pathways. To the best of our knowledge, in this study, two G$_i$-dependent events in post-clopidogrel platelets, including VASP and Akt, were simultaneously evaluated for the first time.

Mean platelet volume (MPV) is a routine measurement of platelet size. It has been proposed as a marker of platelet activity, as larger platelets are metabolically and enzymatically more active than normal-sized platelets, displaying greater prothrombotic potential than smaller ones [13]. The current study evaluated the effects of *CYP2C19* variant alleles on these two G$_i$-dependent events and MPV in post-clopidogrel platelets from Chinese patients with ACS undergoing PCI with drug-eluting stents (DES). The aim was to better explore which *CYP2C19* polymorphism is more susceptible to clopidogrel responsiveness.

Materials and methods

Study population

From September 2015 to February 2016, a total of 196 Chinese patients with ACS, under-going PCI with DES, were consecutively enrolled. Patients received a loading dose of 300 mg clopidogrel. This was followed by a daily regimen of 75 mg and a loading dose of 300 mg aspirin, followed by 100 mg daily. Exclusion criteria: Less than 18 years; Upstream or bail-out use of glycoprotein IIb/IIIa; Concomitant administration of cilostazol; Allergies or contraindications to either aspirin or clopidogrel; Malignancies; Pregnancy; Severe renal dysfunction or hepatic insufficiency; Total platelet count less than $100 × 10^9$/L; Increased risk of bleeding and hematologic disorder [14].

Hematologic routines were measured on admission using XE-5000 an automated hematology analyzer (Sysmex, Kobe, Japan). In these patients, measurements of hematologic routines were carried out on 2 occasions: 1) The first day before treatment with clopidogrel; and 2) One month after PCI, routinely treated with 75 mg clopidogrel.

The study protocol was approved by the Medical Ethics Committee of Zhongshan Hospital. Informed consent was obtained from all patients.

Platelet function

At 24 hours after 300 mg clopidogrel loading, the magnitude of platelet reactivity was assessed using the Thrombelastograph (TEG) Hemostasis Analyzer (Haemoscope Corp, Niles, Illinois, USA). The Food and Drug Administration approved-TEG relies on measurements of activator-induced clot strength, enabling quantitative analysis of platelet function. Briefly, 1 mL of heparinized blood was transferred to a vial containing kaolin, mixed by inversion. Next, 500 μl activated blood was transferred to a vial containing heparinase, mixed to neutralize the heparin. Neutralized blood (360 μl) was immediately added to a heparinase-coated cup and assayed in the TEG analyzer, according to manufacturer instructions, generating the thrombin-induced platelet-fibrin clot, as previously described. Platelet aggregation in response to ADP was calculated with computerized software using this formula: % Aggregation = $[(MA_{ADP}-MA_{Fibrin})/(MA_{Thrombin}-MA_{Fibrin})] × 100\%$.

Genotyping

Genomic DNA was extracted from whole-blood samples using the Qiagen Blood Kit (Qiagen, Hilden, Germany), according to manufacturer recommendations. *CYP2C19*2* (rs4244285)

and *CYP2C19*3* (rs4986893) polymorphisms were genotyped using the Sanger sequencing method. For *CYP2C19*2*, a 435 bp DNA fragment containing the polymorphic site was amplified by PCR in the ABI 9600 (Applied Biosystems, Foster City, CA) using the forward primer 5'-CATCTTTGATTCTCTTGT-3' and the reverse primer 5'-TGAATCACAAATACGCAA-3'. PCR was carried out in a total volume of 30 µl, containing 3 µl 10 × PCR buffer, 0.5 µl 10 pMol each primer, 3 µl dNTP, 1.8 µl MgSO$_4$, 0.6 µl KOD-Plus-Neo polymerase (TOYOBO, Life Science Department OSAKA, JAPAN), 3 µl of genomic DNA, and 17.6 µl H$_2$O. PCR cycling parameters were 35 cycles of 30 seconds at 94°C, 55°C for 30 seconds, and 72°C for 45 seconds. Sanger sequencing of purified PCR products of selected samples was performed to confirm the different allelic variants of *CYP2C19*2*. Other gene polymorphisms were detected in the same way (forward primer 5'-CCAATCATTTAGCTTCAC-3' and the reverse primer 5'-ATATTCATTTCCTGTGCA-3' for *CYP2C-19*3*).

Participants were divided into 3 groups, based on the number of *CYP2C19* LOF alleles: 1) No LOF carriers (*CYP2C19*1/*1*); 2) 1 LOF carrier (*CYP2C19*1/*2* and *CYP2C19*1/*3*); and 3) 2 LOF carriers (*CYP2C19*2/*2*, *CYP2C19*2/*3*). No carriers of *CYP2C19*3/*3* were found in the present study population.

Flow cytometric analysis of VASP phosphorylation

Flow cytometric analysis of VASP phosphorylation was performed, according to the manufacturer recommendations (Platelet VASP; Diagnostica Stago/Biocytex, Asnie`res, France). Briefly, 10 µl samples of citrate anticoagulated blood were incubated at room temperature for 10 minutes with PGE1 alone or with PGE1 plus ADP (10 µM). Reactions were stopped by the addition of 10 µl of fixation reagent and were fixed for 5 minutes. Subsequently, cell suspensions were permeabilized and immunolabelled for 5 minutes using a VASP phosphorylation specific mouse monoclonal antibody or a negative isotypic control antibody, as well as a CD61 phycoerythrin-labeled platelet specific antibody. Finally, platelets were counterstained with a FITC-conjugated polyclonal anti-mouse IgG antibody for 5 minutes. Diluted samples were analyzed using a Coulter Epics XL-M flow cytometer (Beckman/Coulter, Fullerton,

CA, USA). VASP-PRI was calculated using corrected mean fluorescence intensities (MFIs) in the presence of PGE1 alone or PGE1+ADP, using the following calculation: VASP-PRI (%) = [(MFI$_{(PGE1)}$ - MFI$_{(PGE1+ADP)}$)/MFI$_{(PGE1)}$] × 100%, which was shown in Supplementary Figure 2.

Flow cytometric analysis of Akt (Ser^{473}) phosphorylation

Flow cytometric analysis of Akt Ser^{473} phosphorylation was performed, as described previously, with modification [14]. Briefly, 10 µl samples of citrate anticoagulated blood were diluted in 300 µl phosphate buffer saline (PBS) buffer and stimulated with 10 µM ADP for 5 minutes at 37°C. As a control, 10 µl blood was processed in the same way, but without ADP (resting). Reactions were stopped by the addition of 100 µl of 4% paraformaldehyde solution and were fixed for 2.5 hours. Subsequently, cell suspensions were permeabilized and immunolabeled with FITC-conjugated rabbit anti-Akt Ser^{473} antibody (abwiz bio, San Diego, CA, USA) and PE-conjugated anti-CD61 (eBioscience, San Diego, CA, USA) for 30 minutes. Cell suspensions were washed with PBS and analyzed using the FACS Calibur flow cytometer (BD Biosciences, San Jose, CA, USA), using dual-color analysis to acquire platelet (CD61)-positive events. P-Akt index was calculated using MFIs of resting and ADP-treated platelets, using the following calculation: P-Akt index (%) = [MFI$_{(ADP)}$/(MFI$_{(ADP)}$ + MFI$_{(resting)}$)] × 100%, which was indicated in Supplementary Figure 3.

To validate platelet Akt phosphorylation with Western blotting, washed platelets were stimulated with 10 µM ADP for 5 minutes at 37°C, without stirring. Platelet proteins were analyzed with Western blotting using rabbit monoclonal antibodies against Ser^{473}-phosphorylated Akt (Cell Signaling, Beverly, MA, USA) and total Akt (Cell Signaling, Beverly, MA, USA). As a control, platelets were processed in the same way, but without ADP (resting).

Statistical analyses

Continuous variables are expressed as mean ± standard deviation (SD) and were checked for normal distribution using Kolmogorov-Smirnov tests. Wilcoxon rank sum tests or *t*-tests for unpaired samples were used to compare any continuous variables with non-normal or normal-distribution, respectively. To test for differ-

Table 1. Characteristics of the study population according to *CYP2C19* genotype

Characteristics	No LOF carrier	1 LOF carrier	2 LOF carrier	*P* value
Number	80	88	28	
Male (%)	52 (65)	68 (77.3)	20 (71.4)	0.462
Age (years)	63.2 ± 9.8	64.2 ± 9.3	65.5 ± 11.1	0.738
BMI (kg/m^2)	24.8 ± 2.8	24.9 ± 2.2	25.3 ± 2.4	0.826
Current smoker (%)	24 (30)	26 (29.5)	8 (28.6)	0.802
Hypertension (%)	52 (65)	52 (59.1)	20 (71.4)	0.676
Dyslipidemia (%)	16 (20)	12 (13.6)	4 (14.3)	0.181
Diabetes (%)	38 (47.5)	28 (31.8)	8 (28.6)	0.249
Previous MI (%)	14 (17.5)	16 (18.2)	6 (21.4)	0.947
ALT (U/L)	22.2 ± 11.3	26.9 ± 21.7	19.4 ± 8.1	0.237
AST (U/L)	20.3 ± 6.0	21.9 ± 8.9	18.8 ± 5.5	0.353
ALP (U/L)	65.9 ± 18.0	67.7 ± 15.8	72.4 ± 27.5	0.544
GGT (U/L)	33.7 ± 17.3	35.2 ± 27.1	31.3 ± 15.4	0.838
eGFR (ml/min/1.73 m^2)	84.1 ± 17.6	83.0 ± 15.4	81.6 ± 22.4	0.9
Current medication				
PPI (%)	10 (12.5)	18 (20.5)	4 (14.3)	0.394
Statin (%)	80 (100)	88 (100)	28 (100)	1
β-blocker (%)	74 (92.5)	76 (86.4)	26 (92.9)	0.598
ACEI/ARB (%)	58 (72.5)	54 (61.4)	22 (78.6)	0.37
Ca-blocker (%)	22 (27.5)	18 (20.5)	5 (17.9)	0.839
Nitrates (%)	52 (65)	60 (68.2)	19 (67.9)	0.896

Data are expressed mean ± SD or number of patients (percentage). ACEI, angiotensin-converting-enzyme inhibitors; ARB, angiotensin II receptor blockers; ALT, alanine aminotransferases, AST, aspartate transaminase, ALP, alkaline phosphatase; BMI, body mass index; GGT, glutamyltransferase; LOF, loss-of-function; PPI, proton pump inhibitors; Previous MI, previous myocardial infarction; eGFR, estimated glomerular filtration rate.

ences across all groups, χ^2 and one-way analysis of variance tests were used. Association between VASP-PRI and platelet aggregation or P-Akt index was determined by Pearson's correlation analysis. Statistical analysis was performed using SPSS version 20.0 software (SPSS, Chicago, IL, USA) or Prism 5 software packages (GraphPad Inc, San Diego). All tests were two-sided with a significance level of *P* < 0.05.

Results

Baseline characteristics of study participants

Of the 196 ACS patients included in this study, 80 patients (40.8%) were classified as *CYP2C19*1/*1*, 88 patients (44.9%) were *CYP2C19*1/*2* and *CYP2C19*1/*3*, and 28 patients (14.3%) were *CYP2C19*2/*2* and *CYP2C19*2/*3*. Based on *CYP2C19* genotype, patients were categorized into three groups, no LOF carriers (*CYP2C19*1/*1*), one LOF carriers (*CYP2C19*1/*2* and *CYP2C19*1/*3*), and two LOF carriers (*CYP2C19*2/*2* and *CYP2C19*2/*3*), which can be found in Supplemen-

tary Figure 1. No carriers of *CYP2C19*3/*3* were found. As shown in **Table 1**, no significant differences in sex, age, body mass index, and baseline medications existed between the three groups. Likewise, the proportion of patients with hypertension, diabetes mellitus, hypercholesterolemia, current smoking, or previous myocardial infarction differed insignificantly. No significant differences were found in liver function, including alanine aminotransferases (ALT), aspartate transaminase (AST), alkaline phosphatase (ALP), and glutamyltransferase (GGT), between these three groups. Renal function of estimated glomerular filtration rates (eGFR) were not significantly different either. Demographic and baseline clinical characteristics are shown in **Table 1**, as well as procedural variables, suggesting that the three groups had similar baseline characteristics.

Post-clopidogrel platelet reactivity increased according to the numbers of CYP2C19 variant alleles

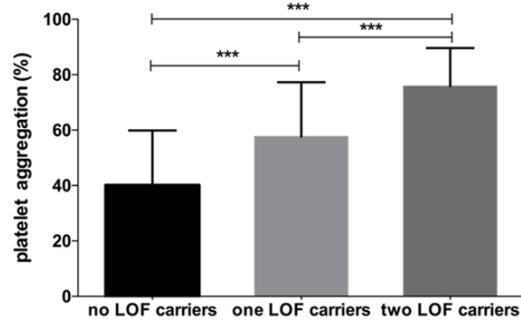As shown in **Figure 1**, mean platelet aggregation after clopidogrel treatment was significant-

Figure 1. Post-clopidogrel platelet reactivity increased according to the number of *CYP2C19* loss-of-function (LOF) alleles. *** as *P* < 0.001.
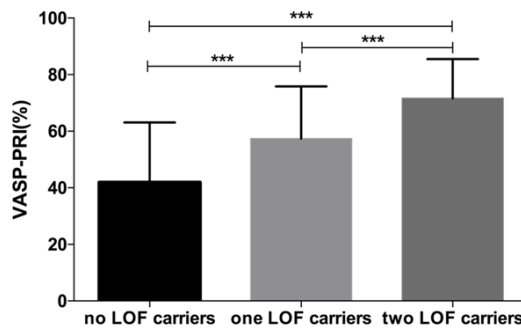

Figure 2. Post-clopidogrel vasodilator-stimulated phosphoprotein-platelet reactivity index (VASP-PRI) increased according to the number of *CYP2C19* loss-of-function (LOF) alleles. *** as *P* < 0.001.

ly higher in patients with 2 *CYP2C19* LOF alleles than in those carrying no LOF allele (75.9 ± 14.0% vs. 40.59 ± 19.9%, *P* < 0.001) or those with 1 LOF allele (75.9 ± 14.0% vs. 57.7 ± 17.7%, *P* < 0.001), as measured by TEG. Compared with subjects with no *CYP2C19* LOF allele, patients with one *CYP2C19* LOF allele had a significantly higher prevalence of post-clopidogrel platelet aggregation (57.7 ± 17.7% vs. 40.59 ± 19.9%, *P* < 0.001). Present data indicates that post-clopidogrel platelet aggregation increased depending on the numbers of the *CYP2C19* LOF allele, indicating that carriers with more CYP2C19 LOFs might have increased incidence of MACE.

Post-clopidogrel VASP-PRI was elevated according to the numbers of CYP2C19 variant alleles

As shown in **Figure 2**, patients with two *CYP2C19* LOF alleles had a significantly higher VASP-PRI index than those with one LOF allele (76.7 ± 9.2% vs. 57.4 ± 18.6%, *P* < 0.001) and

those with no LOF allele (76.7 ± 9.2% vs. 41.9 ± 21.3%, *P* < 0.001). Moreover, the VASP-PRI index of patients was higher in patients with one LOF allele than in patients with no LOF allele (57.4 ± 18.6% vs. 41.9 ± 21.3%, *P* < 0.001). Present data demonstrates that VASP-PRI was elevated depending on the number of *CYP2C19* LOF alleles, showing the same significance with platelet aggregation.

Post-clopidogrel P-Akt index was elevated in two CYP2C19 LOF carriers but not in one LOF carriers

Similar to VASP-PRI, patients with two *CYP2C19* LOF alleles had a significantly higher P-Akt index than those with 1 LOF allele (59.8 ± 12.4% vs. 49.0 ± 10.6%, *P* < 0.001) and those with no LOF allele (59.8 ± 12.4% vs. 47.2 ± 12.1%, *P* < 0.001) (**Figure 3A**). However, no significant differences were found in P-Akt indexes between patients with one LOF and patients with no LOF (49.0 ± 10.6% vs. 47.2 ± 12.1%, *P* = 0.701). Results suggest that only carriers with two *CYP2C19* LOFs had relatively higher P-Akt indexes.

The P-Akt signal of resting platelets was low in all patients. Upon ADP stimulation, 12 different profiles of response were recorded. Compared to P-Akt indexes measured by flow cytometric, some patients (95/196) failed to display detectable P-Akt. Protein levels of P-Akt could scarcely be distinguished from resting and ADP-stimulated platelets in no and one LOF carriers. In accord with P-Akt indexes by flow cytometry, only two *CYP2C19* LOF carriers had higher P-Akt signals after ADP-simulation than resting, according to by Western blotting (**Figure 3B**). Results suggest that only two *CYP2C19* LOF carriers had relatively higher P-Akt indexes.

VASP-PRI did not correlate with P-Akt index in post-clopidogrel platelets

This study further determined correlation levels between signaling events mediated by two G$_i$ protein subunits α$_i$ and β$_\gamma$. Pearson's correlation analysis revealed no significant correlation between VASP-PRI and P-Akt index in post-clopidogrel platelets (*R* = 0.126, *P* = 0.075) (**Figure 4A**), indicating that these two G$_i$ protein subunits-dependent events did not strictly correlate to each other in post-clopidogrel patients.
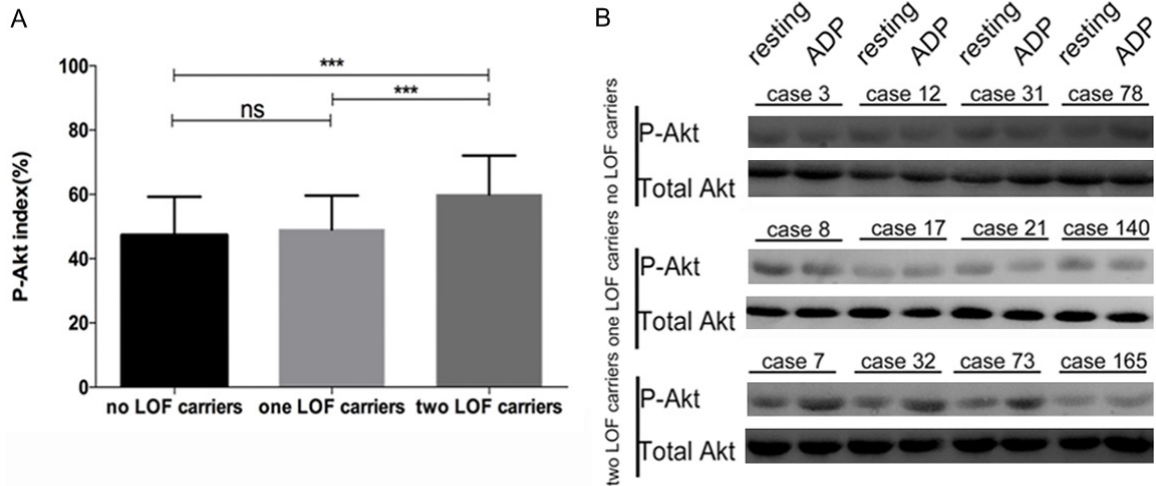
Figure 3. Post-clopidogrel Akt phosphorylation index (P-Akt index) was only elevated in two *CYP2C19* LOF carriers. A. Akt phosphorylation index (P-Akt index) was determined by flow cytometry. B. Western blotting analysis of P-Akt levels in resting and ADP-stimulated platelets of random 12 ACS patients including four patients with no LOF allele, four patients with one LOF allele, and four patients with two LOF alleles. *** as $P < 0.001$, ns as no significance.
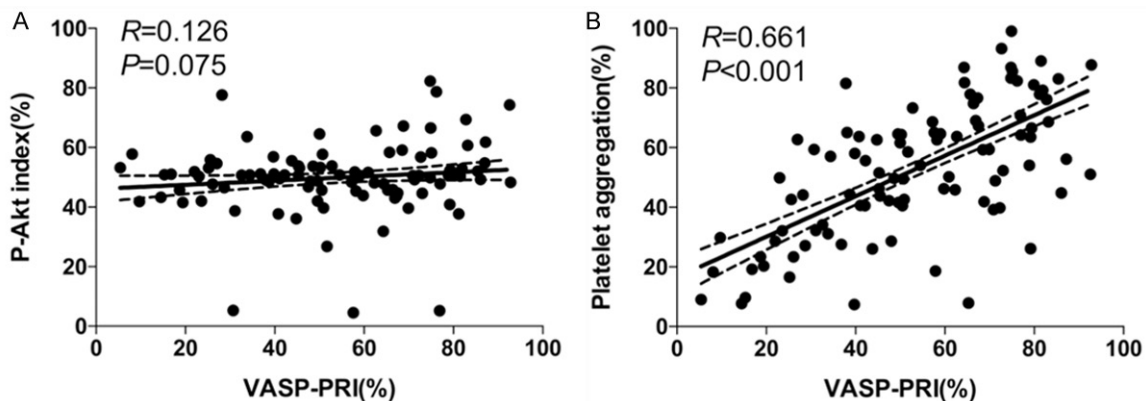

Figure 4. Relationship between post-clopidogrel phosphoprotein-platelet reactivity index (VASP-PRI), Akt phosphorylation index (P-Akt index), and post-clopidogrel platelet aggregation. A. Relationship between VASP-PRI and P-Akt index. B. Relationship between VASP-PRI and post-clopidogrel platelet aggregation.

In contrast, post-clopidogrel VASP-PRI was positively associated with post-clopidogrel platelet aggregation ($R = 0.661$, 95% CI: 0.545-0.777, $P < 0.001$) (**Figure 4B**). Therefore, no consistent phosphorylation with VASP and Akt occurred in post-clopidogrel platelets. Only two LOF carriers had similarly elevated VASP-PRI and P-Akt indexes, further explaining present results.

MPV was significantly larger in patients with two CYP2C19 LOFs than in patients with one LOF and no LOF after one month of clopidogrel treatment

As shown in **Table 2**, there were no significant differences in red blood cell (RBC), hemoglobin

(Hb), white blood cell (WBC), neutrophil percentages (N%), platelet counts (PLT), platelet large cell ratios (P-LCR), or platelet distribution width (PDW) between no LOF carriers, one LOF carriers, and two LOF carriers. Interestingly, there were significant differences in MPV between these three groups after clopidogrel treatment.

As demonstrated in **Figure 5**, before clopidogrel treatment, there were no significant differences in MPV between these three groups. Intriguingly, one month after clopidogrel treatment, two LOF carriers had a higher MPV than no LOF carriers (11.5 ± 1.2 vs. 10.3 ± 0.9 fl, $P < 0.001$) and one LOF carriers (11.5 ± 1.2 vs.

Table 2. Hematologic routines of the study population according to *CYP2C19* genotype

Hematologic routines	No LOF carriers n = 80	1 LOF carriers n = 88	2 LOF carriers n = 28	*P* value
Before therapy				
RBC (10^{12}/L)	4.5 ± 0.5	4.6 ± 0.4	4.5 ± 0.3	0.451
Hb (g/L)	136.0 ± 15.7	138.5 ± 15.0	135.9 ± 14.4	0.805
WBC (10^9/L)	6.7 ± 1.6	6.8 ± 2.3	7.5 ± 1.9	0.383
NEUT (%)	61.4 ± 9.5	61.2 ± 9.7	57.9 ± 10.7	0.429
PLT (10^9/L)	210.2 ± 65.9	193.1 ± 46.3	197.0 ± 34.9	0.801
P-LCR (%)	33.0 ± 7.2	33.0 ± 8.3	34.8 ± 4.9	0.665
PDW (fl)	13.4 ± 2.7	13.4 ± 2.5	13.5 ± 2.9	0.982
MPV (fl)	11.1 ± 0.9	11.3 ± 1.1	11.6 ± 0.3	0.281
After therapy				
RBC (10^{12}/L)	4.3 ± 0.5	4.6 ± 0.4	4.4 ± 0.5	0.167
Hb (g/L)	133.4 ± 16.0	137.4 ± 12.9	128.4 ± 10.6	0.062
WBC (10^9/L)	8.1 ± 3.5	6.4 ± 1.3	6.3 ± 1.3	0.654
NEUT (%)	60.6 ± 9.0	60.0 ± 9.3	63.9 ± 11.5	0.320
PLT (10^9/L)	222.0 ± 76.4	207.3 ± 58.1	197.0 ± 54.2	0.376
P-LCR (%)	36.4 ± 7.0	33.9 ± 8.0	34.1 ± 7.8	0.484
PDW (fl)	13.7 ± 1.7	13.6 ± 2.3	13.5 ± 1.6	0.928
MPV (fl)	10.3 ± 0.9	10.6 ± 0.8	11.5 ± 1.2	< 0.001

Data are expressed mean ± SD. RBC, red blood cell; Hb, hemoglobin; WBC, white blood cell, NEUT, neutrophil percentage; PLT, platelet counts; P-LCR, platelet large cell ratio; PDW, platelet distribution width.
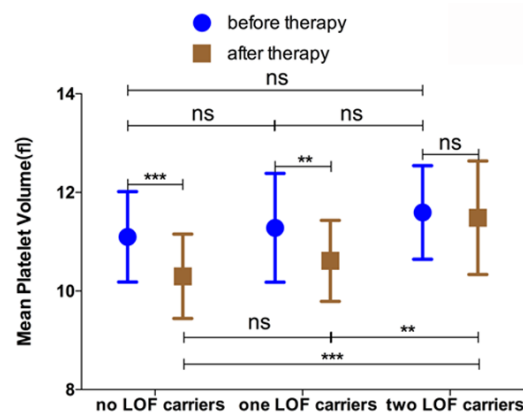

Figure 5. Clopidogrel therapy reduced MPV in patients carrying no *CYP2C19* LOF and patients carrying one *CYP2C19* LOF, but not in patients carrying two *CYP2C19* LOF. **P < 0.01, ***P < 0.001, ns: no significance.

10.6 ± 0.8 fl, *P* = 0.002). There were no differences in MPV between no LOF carriers and one LOF carriers (10.3 ± 0.9 vs. 10.6 ± 0.8 fl, *P* = 0.109). After clopidogrel treatment, MPV reduced in the group carrying no LOF alleles (11.1 ± 0.9 vs. 10.3 ± 0.9 fl, *P* < 0.001) and in the group carrying one LOF allele (11.3 ± 1.1 vs. 10.6 ± 0.8 fl, *P* = 0.002), but remained unchanged in the group carrying two LOF allel-

es (11.6 ± 0.3 vs. 11.5 ± 1.2 fl, *P* = 0.776). MPV remained unchanged in two LOF carriers after one month of clopidogrel treatment. Thus, only carrier with two CYP2C19 LOFs had HPR and poorer prognosis.

Discussion

To the best of our knowledge, the present study is the first to investigate the influence of *CYP2C19* LOF alleles on P2Y$_{12}$-G$_i$ signaling of post-clopidogrel platelets in Chinese patients with ACS. This study demonstrated that, although VASP-PRI increased depending on the numbers of the *CYP2C19* LOF allele, increased P-Akt indexes only existed in two *CYP2C19* LOF carriers (not in one *CYP2C19* LOF carriers) after clopidogrel treatment. A previous study reported that P-Akt index was inhibited by clopidogrel in parallel with VASP-PRI in whites [15]. However, the current study found that P-Akt index did not strictly correlate to VASP-PRI in the Chinese clinic setting.

Many studies have shown that carriage of *CYP2C19* LOF alleles is likely to increase the risk of adverse cardiovascular outcomes [16]. Certain alleles of *CYP2C19* have been associated with reduced enzymatic function and decreased conversion of clopidogrel to the active

metabolite [10]. There are considerable ethnic differences in distribution patterns and types of *CYP2C19* LOF alleles. The carriage prevalence of the *CYP2C19* LOF variant is 35%~45% and 25%~35% among blacks and whites, respectively. It is 55%~70% among East Asians [17]. Prevalence of *CYP2C19* poor metabolizers (subjects carrying 2 LOF alleles) is 5% among blacks and whites, while it is 10%~20% among East Asians [18]. In Asian patients, platelet reactivity increases, proportionally, according to the number of *CYP2C19* LOF alleles (*2 or *3). This is related to a high prevalence of the consensus-defined high on-treatment platelet reactivity (HPR) (more than 50%) [18]. A previous study showed that up to 60.6% of Chinese ACS patients carried one or two *CYP2C19* LOF alleles, while carriage of only two *CYP2C19* LOF alleles showed poorer clinical outcomes [9, 19]. Based on the evaluation of two G$_i$-dependent events, including VASP-PRI and P-Akt index, this study further demonstrated that, although carriers of one or two *CYP2C19* LOF alleles had higher VASP-PRI, only two LOF allele carriers showed higher P-Akt index and MPV after clopidogrel therapy. Thus, carriers with two LOFs might have a more active state of ADP P2Y$_{12}$ receptors, which could explain why only two LOF alleles carriage was associated with poorer clinical outcome in Chinese ACS patients.

Platelet activation resulting from plaque disruption plays a crucial role in pathological and clinical outcomes of ACS [20]. G$_i$ Subunits α$_i$-mediated VASP dephosphorylation favors GPIIb/IIIa in an activating conformation, leading to binding of fibrinogen to GPIIb/IIIa, promoting platelet aggregation [21] and adhesion [22]. VASP dephosphorylation has no effects on platelet secretion [23], spreading [23], and clot retraction [23]. In contrast, Subunits β$_γ$-mediated Akt phosphorylation plays a central role in mediating multiple platelet function, including platelet aggregation [24], secretion [24], adhesion [25], spreading [26], and clot retraction [27]. The integral roles of Akt phosphorylation in platelet activation, in combination with the present finding that P-Akt indexes only increase in two *CYP2C19* LOF carriers, further emphasizes the potential roles of Akt pathways in the pathobiology and prognosis of ACS.

Elevated MPV levels have been identified as an independent risk factor for myocardial infarction in patients with coronary artery disease, as well as for death or recurrent vascular events post ACS [28]. Asher et al. [29] found that non-responders to clopidogrel had significantly larger MPV. In the current study, MPV was significantly reduced in no LOF carriers and one LOF carriers after one month of clopidogrel treatment. However, MPV remained unchanged in two LOF carriers after one month of clopidogrel treatment. Larger platelets contain more granules and mitochondria per unit volume, having a higher capacity for production of thromboxane A2 and secretion of transforming growth factor β. They express more glycoprotein IIb/IIIa per unit area of plasma membrane than their smaller counterparts [30]. Unchanged MPV after clopidogrel treatment in two *CYP2C19* LOF carriers suggests that these patients have a poorer prognosis after clopidogrel treatment.

The present study had several limitations, however. First, this study was a single-center investigation. Results should be verified by multi-center studies. Second, this study did not analyze relationships between P2Y$_{12}$-G$_i$ signaling and clinical outcomes in ACS patients. Third, this study only concerned Chinese patients, a population characterized by a high prevalence of *CYP2C19* LOF alleles. Therefore, present results may not be generalized to the Western population.

In conclusion, in Chinese patients with ACS receiving PCI and clopidogrel treatment, only two *CYP2C19* LOF carriers showed a more active state of P2Y$_{12}$-G$_i$ signaling and larger MPV, containing increased VASP-PRI and P-Akt indexes.

Disclosure of conflict of interest

None.

Address correspondence to: Dr. Si Zhang, Key Laboratory of Glycoconjugate Research Ministry of Public Health, Department of Biochemistry and Molecular Biology, School of Basic Medical Sciences, Fudan University, 130 Dong'an Road, Xuhui District, Shanghai 200032, P. R. China. Fax: +86-21-64437203; E-mail: zhangsi@fudan.edu.cn; Dr. Bing

Fan, Department of Cardiology, Shanghai Institute of Cardiovascular Diseases, Zhongshan Hospital, Fudan University, 180 Fenglin Road, Xuhui District, Shanghai 200032, P. R. China. Fax: +86-21-642-23006; E-mail: fan.bing@zs-hospital.sh.cn

References

[1] Bagai A, Bhatt DL, Eikelboom JW, Mancini GJ, Cohen EA, Vijayaraghavan R, Cheema AN, Udell JA, Niznick J and Tanguay JF. Individualizing duration of dual antiplatelet therapy after acute coronary syndrome or percutaneous coronary intervention. Circulation 2016; 133: 2094-2098.

[2] Cayla G, Cuisset T, Silvain J, Leclercq F, Manzo-Silberman S, Saint-Etienne C, Delarche N, Bellemain-Appaix A, Range G and El Mahmoud R. Platelet function monitoring to adjust antiplatelet therapy in elderly patients stented for an acute coronary syndrome (ANTARCTIC): an open-label, blinded-endpoint, randomised controlled superiority trial. Lancet 2016; 388: 2015-2022.

[3] Aradi D, Kirtane A, Bonello L, Gurbel PA, Tantry US, Huber K, Freynhofer MK, Ten Berg J, Janssen P and Angiolillo DJ. Bleeding and stent thrombosis on P2Y12-inhibitors: collaborative analysis on the role of platelet reactivity for risk stratification after percutaneous coronary intervention. Eur Heart J 2015; 36: 1762-1771.

[4] Xin YG, Zhang HS, Li YZ, Guan QG, Guo L, Gao Y, Yu HJ, Zhang XG, Xu F and Zhang YL. Efficacy and safety of ticagrelor versus clopidogrel with different dosage in high-risk patients with acute coronary syndrome. Int J Cardiol 2017; 228: 275-279.

[5] Bhatt DL, Paré G, Eikelboom JW, Simonsen KL, Emison ES, Fox KA, Steg PG, Montalescot G, Bhakta N and Hacke W. The relationship between CYP2C19 polymorphisms and ischaemic and bleeding outcomes in stable outpatients: the CHARISMA genetics study. Eur Heart J 2012; 33: 2143-2150.

[6] Chan NC, Eikelboom JW, Ginsberg JS, Lauw MN, Vanassche T, Weitz JI and Hirsh J. Role of phenotypic and genetic testing in managing clopidogrel therapy. Blood 2014; 124: 689-699.

[7] Sorich MJ, Rowland A, McKinnon RA and Wiese MD. CYP2C19 genotype has a greater effect on adverse cardiovascular outcomes following percutaneous coronary intervention and in Asian populations treated with clopidogrel. Circ Cardiovasc Genet 2014; 7: 895-902.

[8] Scott SA, Sangkuhl K, Stein C, Hulot JS, Mega J, Roden D, Klein T, Sabatine M, Johnson J and Shuldiner A. Clinical pharmacogenetics implementation consortium guidelines for CYP2C19 genotype and clopidogrel therapy: 2013 update. Clin Pharmacol Ther 2013; 94: 317-323.

[9] Wu H, Qian J, Xu J, Sun A, Sun W, Wang Q and Ge J. Effects of CYP2C19 variant alleles on postclopidogrel platelet reactivity and clinical outcomes in an actual clinical setting in China. Pharmacogenet Genomics 2012; 22: 887-890.

[10] Hwang SJ, Jeong YH, Kim IS, Koh JS, Kang MK, Park Y, Kwak CH and Hwang JY. The cytochrome 2C19*2 and *3 alleles attenuate response to clopidogrel similarly in East Asian patients undergoing elective percutaneous coronary intervention. Thromb Res 2011; 127: 23-28.

[11] Lu WJ, Chang NC, Jayakumar T, Liao JC, Lin MJ, Wang SH, Chou DS, Thomas PA and Sheu JR. Ex vivo and in vivo studies of CME-1, a novel polysaccharide purified from the mycelia of Cordyceps sinensis that inhibits human platelet activation by activating adenylate cyclase/cyclic AMP. Thromb Res 2014; 134: 1301-1310.

[12] Angiolillo DJ, Fernandez-Ortiz A, Bernardo E, Alfonso F, Macaya C, Bass TA and Costa MA. Variability in individual responsiveness to clopidogrel. J Am Coll Cardiol 2007; 49: 1505-1516.

[13] Verdoia M, Pergolini P, Rolla R, Nardin M, Barbieri L, Schaffer A, Bellomo G, Marino P, Suryapranata H and De Luca G. Mean platelet volume and high-residual platelet reactivity in patients receiving dual antiplatelet therapy with clopidogrel or ticagrelor. Expert Opin Pharmacother 2015; 16: 1739-1747.

[14] Perez OD and Nolan GP. Simultaneous measurement of multiple active kinase states using polychromatic flow cytometry. Nat Biotechnol 2002; 20: 155-162.

[15] Carrié D, Garcia C, Gratacap M, Voisin S, Payrastre B and Sié P. Assessment of platelet response to clopidogrel through measurement of ADP-induced Akt phosphorylation. J Thromb Haemost 2009; 7: 1411-1413.

[16] Man M, Farmen M, Dumaual C, Teng CH, Moser B, Irie S, Noh GJ, Njau R, Close S and Wise S. Genetic variation in metabolizing enzyme and transporter genes: comprehensive assessment in 3 major East Asian subpopulations with comparison to Caucasians and Africans. J Clin Pharmacol 2010; 50: 929-940.

[17] Lee JM, Park S, Shin DJ, Choi D, Shim CY, Ko YG, Kim JS, Shin ES, Chang CW and Lee JE. Relation of genetic polymorphisms in the cytochrome P450 gene with clopidogrel resistance after drug-eluting stent implantation in Koreans. Am J Cardiol 2009; 104: 46-51.

[18] Jeong YH, Tantry US, Kim IS, Koh JS, Kwon TJ, Park Y, Hwang SJ, Bliden KP, Kwak CH and

Hwang JY. Effect of *CYP2C19*2* and *3 loss-of-function alleles on platelet reactivity and adverse clinical events in East Asian acute myocardial infarction survivors treated with clopidogrel and aspirin. Circ Cardiovasc Interv 2011; 4: 585-594.

[19] Wu H, Qian J, Sun A, Wang Q and Ge J. Association of CYP2C19 genotype with periprocedural myocardial infarction after uneventful stent implantation in Chinese patients receiving clopidogrel pretreatment. Circ J 2012; 76: 2773-2778.

[20] Topol EJ. Toward a new frontier in myocardial reperfusion therapy. Circulation 1998; 97: 211-218.

[21] Aszódi A, Pfeifer A, Ahmad M, Glauner M, Zhou XH, Ny L, Andersson KE, Kehrel B, Offermanns S and Fässler R. The vasodilator-stimulated phosphoprotein (VASP) is involved in cGMP- and cAMP-mediated inhibition of agonist-induced platelet aggregation, but is dispensable for smooth muscle function. EMBO J 1999; 18: 37-48.

[22] Massberg S, Grüner S, Konrad I, Arguinzonis MIG, Eigenthaler M, Hemler K, Kersting J, Schulz C, Müller I and Besta F. Enhanced in vivo platelet adhesion in vasodilator-stimulated phosphoprotein (VASP)-deficient mice. Blood 2004; 103: 136-142.

[23] Eliautou S, Mangin PH, Walter U, Gachet C and Lanza F. Normal filopodia extension in VASP-deficient platelets upon activation by adhesive matrices or soluble agonists. Thromb Haemost 2009; 102: 792-794.

[24] Li Z, Zhang G, Le Breton GC, Gao X, Malik AB and Du X. Two waves of platelet secretion induced by thromboxane A2 receptor and a critical role for phosphoinositide 3-kinases. J Bioll Chem 2003; 278: 30725-30731.

[25] Martin V, Guillermet-Guibert J, Chicanne G, Cabou C, Jandrot-Perrus M, Plantavid M, Vanhaesebroeck B, Payrastre B and Gratacap MP. Deletion of the p110β isoform of phosphoinositide 3-kinase in platelets reveals its central role in Akt activation and thrombus formation in vitro and in vivo. Blood 2010; 115: 2008-2013.

[26] O'Brien KA, Gartner TK, Hay N and Du X. ADP-stimulated activation of Akt during integrin outside-in signaling promotes platelet spreading by inhibiting glycogen synthase kinase-3β. Arterioscler Thromb Vasc Biol 2012; 32: 2232-2240.

[27] Schoenwaelder SM, Ono A, Nesbitt WS, Lim J, Jarman K and Jackson SP. Phosphoinositide 3-kinase p110β regulates integrin αIIbβ3 avidity and the cellular transmission of contractile forces. J Biol Chem 2010; 285: 2886-2896.

[28] Endler G, Klimesch A, Sunder-Plassmann H, Schillinger M, Exner M, Mannhalter C, Jordanova N, Christ G, Thalhammer R and Huber K. Mean platelet volume is an independent risk factor for myocardial infarction but not for coronary artery disease. Br J Haematol 2002; 117: 399-404.

[29] Asher E, Fefer P, Shechter M, Beigel R, Varon D, Shenkman B, Savion N, Hod H and Matetzky S. Increased mean platelet volume is associated with non-responsiveness to clopidogrel. Thromb Haemost 2014; 112: 137-141.

[30] Martin JF, Kristensen SD, Mathur A, Grove EL and Choudry FA. The causal role of megakaryocyte-platelet hyperactivity in acute coronary syndromes. Nat Rev Cardiol 2012; 9: 658-670.
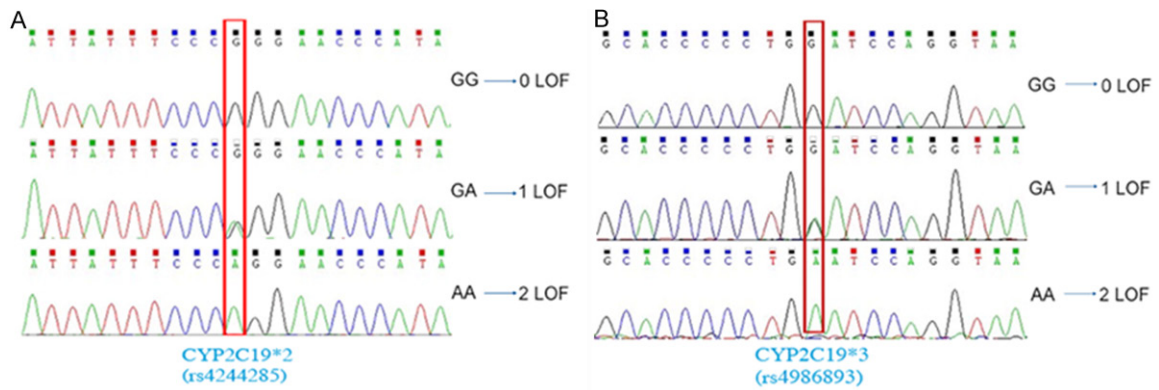
Supplementary Figure 1. Diagram of *CYP2C19* gene sequencing. A. At the point of rs4244285, there are two variants of *CYP2C19*2*, which can be differentiated to 0, 1, and 2 *CYP2C19* LOF; B. At the point of rs4986893, there are two variants of *CYP2C19*3*, which can be differentiated to 0, 1, and 2 CYP2C19 LOF.
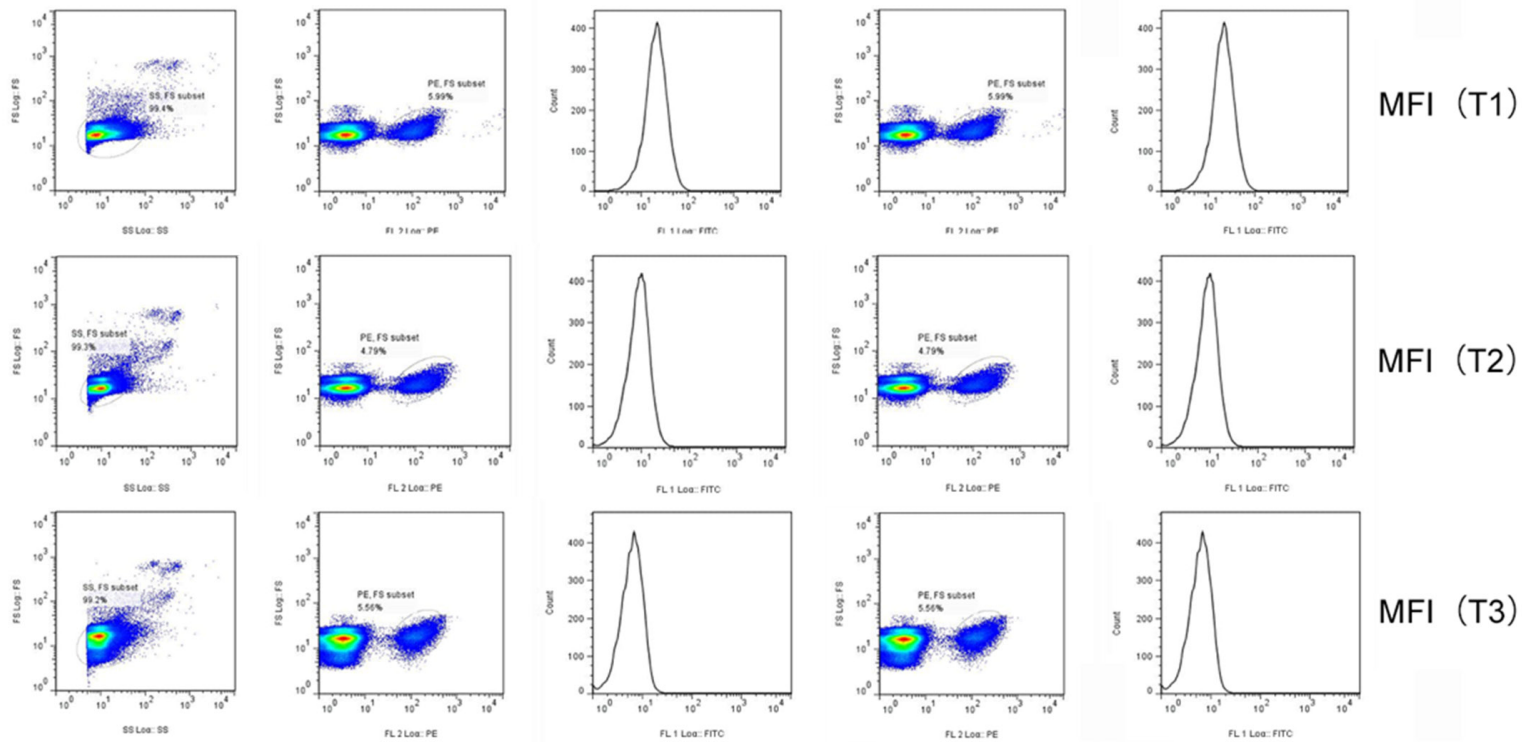
$$MFI_{(PGE1)}=MFI(T1)-MFI(T3) \quad MFI_{(PGE1+ADP)}=MFI(T2)-MFI(T3) \quad MFI_{(PGE1+ADP)}=MFI(T2)-MFI(T3)$$

$$VASP\text{-}PRI(\%)=[(MFI_{(PGE1)}-MFI_{(PGE1+ADP)})/MFI_{(PGE1)}]\times 100\% FI_{(PGE1)}-MFI_{(PGE1+ADP)})/MFI_{(PGE1)}]\times 100\%$$

Supplementary Figure 2. Flow cytometric analysis of VASP phosphorylation. The platelet population was gated by the specific surface marker CD61-PE [4]. The geometric mean fluorescence intensities (MFIs) for VASP-phosphorylation of platelets incubated with iloprost alone (upper right panel) was corrected by subtracting the isotype control MFI. The MFI of platelets incubated with iloprost plus ADP was calculated in the same way. Finally, both MFI values are reported as VASP-PRI (VASP-PRI = [(MFI$_{(PGE1)}$ - MFI$_{(PGE1+ADP)}$)/MFI$_{(PGE1)}$] × 100%).
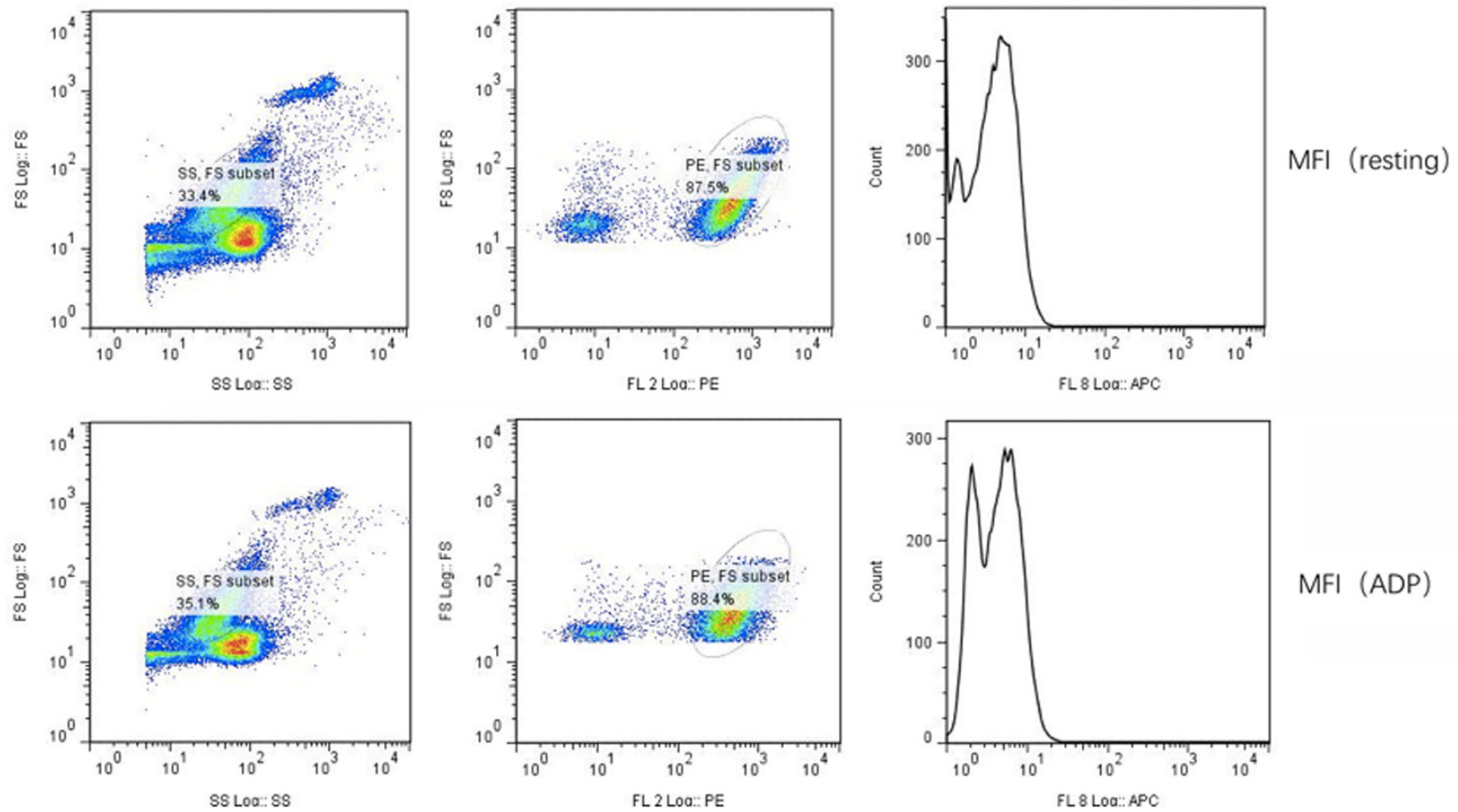
$$\text{P-Akt index(\%)} = [\text{MFI}_{(ADP)} / (\text{MFI}_{(ADP)} + \text{MFI}_{(resting)})] \times 100\%$$

Supplementary Figure 3. The platelet population was gated by the specific surface marker CD61-PE. Twenty thousand platelets were gated. Geometric mean fluorescence intensities (MFIs) for Akt-phosphorylation of resting platelets (upper right panel) was control MFI. The MFI of platelets incubated with ADP was calculated in the same way. Finally, both MFI values are reported as P-Akt index (P-Akt index (%) = [MFI$_{(ADP)}$/MFI$_{(ADP)}$ + MFI$_{(resting)}$] × 100%).