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Protease-activated receptor-mediated platelet aggregation in acute coronary syndrome patients on potent $P2Y_{12}$ inhibitors

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Abstract

Background: Despite the increasing use of potent $P2Y_{12}$ inhibitors, further atherothrombotic events still impair the prognosis of many acute coronary syndrome (ACS) patients. This may in part be attributable to intact platelet aggregation via the human thrombin receptors protease-activated receptor (PAR)-1 and PAR-4.

Objective: We studied PAR mediated platelet aggregation in ACS patients following percutaneous coronary intervention (PCI) with stent implantation in a cross-sectional study.

Methods: Platelet aggregation to ADP as well as to the PAR-1 agonist SFLLRN and the PAR-4 agonist AYPGKF was assessed by multiple electrode aggregometry in 194 ACS patients on dual antiplatelet therapy with aspirin and either prasugrel (n = 114) or ticagrelor (n = 80) 3 days after PCI.

Results: Based on the consensus cutoff value, high on-treatment residual platelet reactivity to ADP (HRPR ADP) was observed in only 2 prasugrel-treated patients. Both patients with HRPR ADP had also a normal response to SFLLRN and AYPGKF. Among the 112 prasugrel-treated patients with adequate $P2Y_{12}$ inhibition, 50 patients (45%) still had a normal response to SFLLRN, and 70 patients (63%) still had a normal response to AYPGKF. Among the 80 ticagrelor-treated patients with adequate $P2Y_{12}$ inhibition, 25 patients (31%) still had a normal response to SFLLRN, and 50 (63%) still had a normal response to AYPGKF.

Conclusion: Normal platelet aggregation via PAR-1 and PAR-4 is preserved in many patients with adequate $P2Y_{12}$ inhibition by prasugrel and ticagrelor. The present findings may at least in part explain adverse ischemic events despite potent $P2Y_{12}$ inhibition.

KEYWORDS

antiplatelet therapy, protease-activated receptor 1, protease-activated receptor 4, prasugrel, ticagrelor, platelet aggregation

Essentials

- Atherothrombotic events still impair the prognosis of many acute coronary syndrome (ACS) patients.
- This may in part be attributable to intact platelet aggregation via protease-activated receptor (PAR)-1 and PAR-4.
- Platelet aggregation was measured 3 days after percutaneous coronary intervention in 194 ACS patients on novel $P2Y_{12}$ blockers.
- PAR-mediated platelet aggregation is preserved despite adequate $P2Y_{12}$ inhibition.

1 | INTRODUCTION

Dual antiplatelet therapy with aspirin and an ADP $P2Y_{12}$ inhibitor is the current standard treatment for patients with an acute coronary syndrome (ACS) undergoing percutaneous coronary intervention (PCI) with stent implantation.[1–5] The newer $P2Y_{12}$ receptor antagonists prasugrel and ticagrelor offer more potent platelet inhibition than clopidogrel and were shown to significantly reduce adverse ischemic outcomes following an ACS compared with clopidogrel.[6,7] However, despite the increasing use of prasugrel and ticagrelor, further atherothrombotic events still impair the prognosis of many ACS patients.[4,8] Recently, Motovska et al[8] reported the 1-year outcomes of the PRAGUE-18 (Comparison of Prasugrel and Ticagrelor in the Treatment of Acute Myocardial Infarction) trial including 1230 patients on dual antiplatelet therapy with aspirin and prasugrel or ticagrelor. During the follow-up period of 12 months, adverse ischemic events occurred in 6.6% of prasugrel- and 5.7% of ticagrelor-treated patients.[8] The latter has been explained by the fact that a significant number of patients were switched to clopidogrel after hospital discharge, but might in part also be attributable to intact platelet aggregation via the human thrombin receptors protease-activated receptor (PAR)-1 and PAR-4.[9–11]

Thrombin is a strong endogenous platelet agonist, leading to platelet activation at subnanomolar concentrations.[12] It acts as a multifunctional serine protease, which activates human platelets predominantly via PAR-1 and, to a lesser extent, PAR-4.[13–15] PAR-1 is activated by low thrombin concentrations and amplified by PAR-4 signaling as the thrombin concentration rises.[11,16] Thus, both PAR-1 and PAR-4 need to be blocked for full inhibition of thrombin-induced platelet activation. The activation of platelets by thrombin leads in turn to ADP release, promoting further recruitment, adhesion, and aggregation of activated platelets via stimulation of $P2Y_1$ and $P2Y_{12}$ receptors. Accordingly, $P2Y_{12}$ inhibition also affects PAR-mediated platelet aggregation.[17]

In the past, we have demonstrated that platelets can still be activated via PAR-1 and PAR-4 despite $P2Y_{12}$ inhibition by clopidogrel.[18] In the current study, we sought to investigate if platelet aggregation via PAR-1 and PAR-4 persists in ACS patients receiving the newer and more potent $P2Y_{12}$ antagonists following PCI with stent implantation.

2 | METHODS

2.1 | Study population

The study population consisted of 194 ACS patients on daily aspirin (100 mg/d), and either prasugrel (10 mg/d, n = 114), or ticagrelor (180 mg/d, n = 80) therapy. All study patients were of Caucasian ethnicity. Blood sampling was performed 72 hours after acute PCI with stent implantation. Due to the short half-life of unfractionated heparin, all patients were free of heparin from PCI.[19]

Exclusion criteria were a known $P2Y_{12}$ inhibitor or aspirin intolerance (manifested as allergic reactions or gastrointestinal bleeding); a therapy with vitamin K antagonists (phenprocoumon, acenocoumarol, warfarin), rivaroxaban, apixaban, dabigatran, or edoxaban; treatment with nonsteroidal anti-inflammatory drugs, ticlopidine, or dipyridamole; known bleeding disorders; severe hepatic failure; known qualitative defects in platelet function; heparin-induced thrombocytopenia; malignant myeloproliferative disorders; a platelet count <100 000 or >450 000/μL; a hematocrit <30%; and a major surgical procedure within 1 week before enrollment,

The study protocol was approved by the local Ethics Committee of the Medical University of Vienna and was in accordance with the Declaration of Helsinki. Written informed consent was obtained from all study participants.

2.2 | Blood sampling

Blood was drawn by aseptic venipuncture from an antecubital vein using a butterfly needle (21-gauge, 0.8 × 19 mm; Greiner Bio-One, Kremsmünster, Austria) 72 hours after PCI. To avoid procedural deviations, blood sampling was performed by the same physician applying a light tourniquet, which was immediately released, and the samples were mixed by gently inverting the tubes. The initial 3 mL of blood were discarded to reduce periprocedural platelet activation. Afterwards, blood was drawn into hirudin-coated tubes (Roche Diagnostics, Mannheim, Germany) for multiple electrode aggregometry (MEA).

2.3 | Multiple electrode aggregometry

Whole blood impedance aggregometry was performed using the Multiplate analyzer (Roche Diagnostics) as previously described.[17,18] In brief, hirudin-anticoagulated whole blood was diluted 1:2 with 0.9% NaCl solution and stirred in the test cuvettes for 3 minutes at 37°C. Thereafter, ADP ($P2Y_{12}$ agonist, 6.5 μmol/L), SFLLRN (PAR-1 agonist, 32 μmol/L) or AYPGKF (PAR-4 agonist, 645 μmol/L, all from Roche Diagnostics) was added and aggregation was recorded for 6 minutes. Titration experiments were carried out, increasing the dosages of SFLLRN and AYPGKF, respectively, until both agonists induced platelet aggregation >60 aggregation units (AU) by MEA, but less than maximal response in healthy Caucasian individuals (n = 30). The determined dosages corresponded to the concentrations

recommended by the manufacturer. The increase of impedance that was evoked by the adhesion of activated platelets to the electrodes was detected by each sensor unit separately, transformed to AU and plotted against time.

2.4 | Statistical analysis

Statistical analysis was performed using the Statistical Package for Social Sciences (SPSS version 24.0; SPSS, Chicago, IL). Median and interquartile range of continuous variables are shown. Categorical variables are given as number (%). We performed the nonparametric Mann-Whitney *U*-tests to detect differences in continuous variables. The chi-square test was used to assess differences in categorical variables. Spearman correlation was used to test for correlations between platelet aggregation in response to the different agonists. Two-sided *P* values <0.05 were considered statistically significant.

3 | RESULTS

Clinical, laboratory, and procedural characteristics of the study population are given in Table 1. As expected, ticagrelor-treated patients (n = 80) were older than prasugrel-treated patients (n = 114; *P* = 0.02) and had significantly higher serum creatinine levels (*P* = 0.001).

Residual ADP-inducible platelet aggregation was similar in prasugrel- and ticagrelor-treated patients (19 AU [15-23 AU] vs. 20 AU [14-25 AU], *P* = 0.4; Figure 1A). Likewise, SFLLRN- and AYPGKF-inducible platelet aggregation was similar between prasugrel- and ticagrelor-treated patients (SFLLRN: 68 AU [48-85 AU] vs. 62 AU [47-80], *P* = 0.2; AYPGKF: 60 AU [44-83 AU] vs. 64 AU [45-78 AU], *P* = 0.96; Figure 1B,C; Table 2).

Adenosine diphosphate inducible platelet aggregation correlated significantly with both SFLLRN and AYPGKF inducible platelet aggregation in the overall study population (SFLLRN: *r* = 0.55, *P* < 0.001; AYPGKF: *r* = 0.48, *P* < 0.001; Figure 2A and B) as well as in prasugrel- and ticagrelor-treated patients alone (prasugrel: SFLLRN: *r* = 0.52, *P* < 0.001; AYPGKF: *r* = 0.48, *P* < 0.001; ticagrelor: SFLLRN: *r* = 0.6, *P* < 0.001; AYPGKF: *r* = 0.5, *P* < 0.001). All patients with PAR-mediated platelet aggregation in the first quartile also had suppressed platelet aggregation via the $P2Y_{12}$ receptor. Patients with PAR-mediated platelet aggregation in the first quartile were defined as patients with low PAR-1 (n = 50) and low PAR-4 (n = 51) mediated platelet aggregation, respectively. Patients with low PAR-mediated platelet aggregation had significantly less platelet aggregation in

TABLE 1 Patient characteristics

Characteristics	Prasugrel (n = 114)	Ticagrelor (n = 80)	*P* value
Age, y	57 (49-64)	60 (51-70)	0.02
Male sex, n (%)	93 (82)	62 (78)	0.49
BMI, kg/m^2	28 (25-31)	28 (25-30)	0.72
Medical history			
Previous myocardial infarction, n (%)	18 (16)	13 (16)	0.87
Hypertension, n (%)	76 (67)	57 (71)	0.47
Hyperlipidemia, n (%)	87 (76)	58 (73)	0.79
Diabetes mellitus, n (%)	18 (16)	15 (19)	0.15
Active smoking, n (%)	66 (58)	38 (48)	0.16
Stent implantation, n (%)	114 (100)	80 (100)	1
Number of stents/patient	1 (1-2)	1 (1-2)	0.21
Laboratory data			
Serum creatinine, mg/dL	0.9 (0.76-1.02)	1 (0.82-1.2)	0.001
Platelet count, g/L	222 (194-252)	226 (187-269)	0.86
High sensitivity C-reactive protein, mg/dL	1.3 (0.7-4.3)	1.2 (0.5-3.4)	0.18
Hemoglobin, g/dL	13.9 (13.1-14.7)	13.6 (12.7-14.6)	0.42
WBC, g/L	8.9 (7.9-10.4)	8.7 (7-10.6)	0.53
Medication			
Statins, n (%)	113 (99)	79 (99)	0.8
Beta blockers, n (%)	110 (96)	78 (98)	0.69
ACE inhibitors, n (%)	96 (84)	60 (75)	0.11
Calcium channel blockers, n (%)	10 (9)	9 (11)	0.57
Angiotensin receptor blockers, n (%)	16 (14)	18 (23)	0.13

Continuous data are shown as median (interquartile range). Dichotomous data are shown as n (%).
ACE, angiotensin-converting-enzyme; BMI, body mass index; WBC, white blood cells.

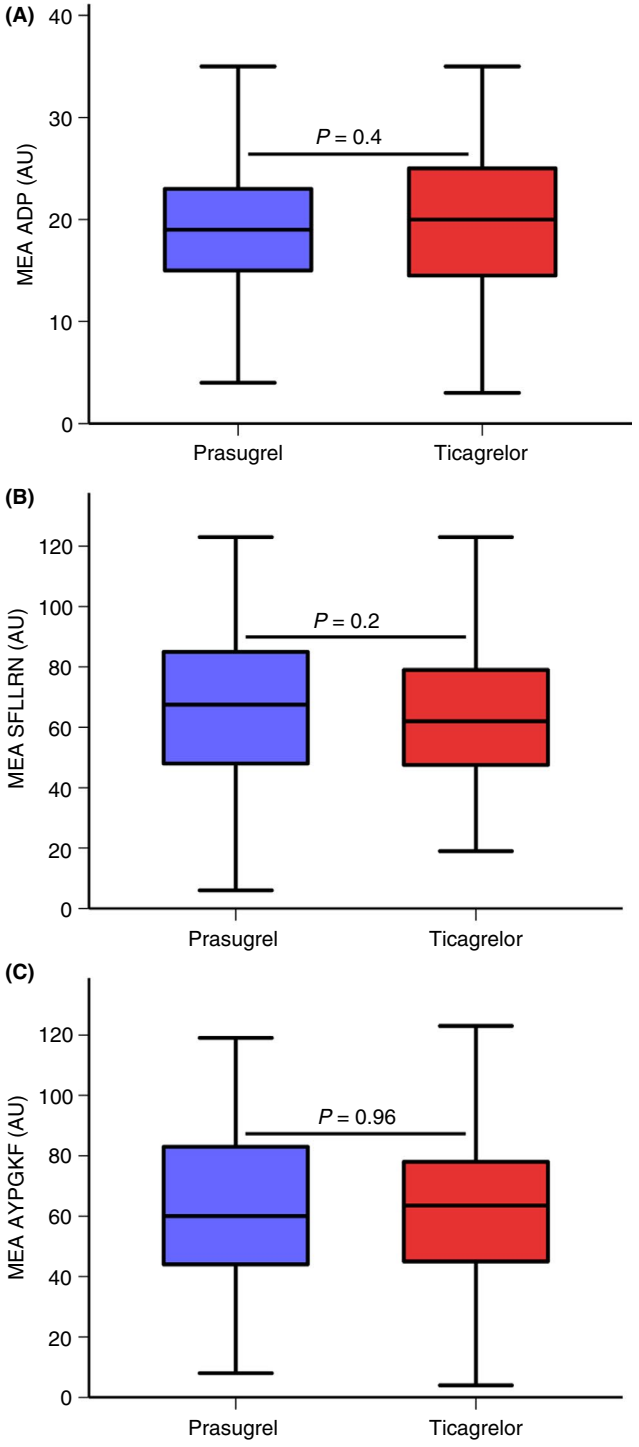

FIGURE 1 Platelet aggregation following stimulation with (A) ADP, (B) protease-activated receptor (PAR)-1 agonist SFLLRN, or (C) PAR-4 agonist AYPGKF in patients receiving prasugrel or ticagrelor. The boundaries of the box show the lower and upper quartile of data, and the line inside the box represents the median. Whiskers are drawn from the edge of the box to the highest and lowest values that are outside the box but within 1.5 times the box length. AU, aggregation units; MEA, multiple electrode aggregometry

response to ADP than the remaining patients (PAR-1: 15 AU (10-19 AU) vs. 20 AU (16-25 AU), $P < 0.001$; PAR-4: 16 AU (10-20 AU) vs 21 AU (16-25 AU), $P < 0.001$). Further, we observed a strong correlation between SFLLRN- and AYPGKF-inducible platelet aggregation in the overall study population ($r = 0.7$, $P < 0.001$; Figure 2C) as well as in prasugrel- and ticagrelor-treated patients alone (prasugrel: $r = 0.74$, $P < 0.001$; ticagrelor: $r = 0.63$, $P < 0.001$).

TABLE 2 Platelet aggregation following stimulation with SFLLRN and AYPGKF in prasugrel- and ticagrelor-treated patients

	Prasugrel (n = 114)	Ticagrelor (n = 80)	*P* value
Multiplate SFLLRN, AU	68 (48-85)	62 (47-80)	0.19
Multiplate AYPGKF, AU	60 (44-83)	64 (45-78)	0.96

Continuous data are shown as median (interquartile range). AU, aggregation units.

Based on the consensus cutoff value of AU ≥47,[20] only 2 prasugrel-treated patients had HRPR ADP. In contrast, 112 patients on prasugrel therapy (98%) and all patients on ticagrelor therapy (100%) had an adequately suppressed response to ADP. The cutoff values for PAR-mediated platelet aggregation were derived from a group of 55 healthy Caucasian volunteers (male/female, 21/34) aged 42 ± 13 years, who served as the control population in a previously published study.[18] For PAR-1 and PAR-4–mediated platelet aggregation, the upper 95% of data obtained in the healthy control population were considered as normal uninhibited platelet aggregation to eliminate possible low outliers. The corresponding cutoff values were AU ≥ 71 for normal PAR-1–mediated platelet aggregation (SFLLRN as agonist) and AU ≥ 54 for normal PAR-4–mediated platelet aggregation (AYPGKF as agonist).[18]

The 2 prasugrel-treated patients with HRPR ADP by MEA also had a normal response to SFLLRN and AYPGKF. Among the 112 prasugrel-treated patients with adequate $P2Y_{12}$ inhibition, 50 patients (45%) still had a normal platelet response to SFLLRN, 70 patients (63%) still had a normal platelet response to AYPGKF, and 45 patients (40%) had a normal response to both SFLLRN and AYPGKF. Among the 80 ticagrelor-treated patients with adequate $P2Y_{12}$ inhibition, 25 patients (31%) still had a normal platelet response to SFLLRN, 50 patients (63%) had a normal platelet response to AYPGKF, and 22 patients (28%) had a normal response to both SFLLRN and AYPGKF.

4 | DISCUSSION

Our study demonstrates that ACS patients with adequate ADP $P2Y_{12}$ inhibition during antiplatelet therapy with prasugrel or ticagrelor frequently have a normal aggregation response to PAR-1 or PAR-4 stimulation.

We decided to assess platelet aggregation by MEA because MEA is a fast and standardized platelet function assay that can be easily applied in daily clinical routine. Moreover, results obtained by MEA have been associated with adverse outcomes following PCI.[20–23]

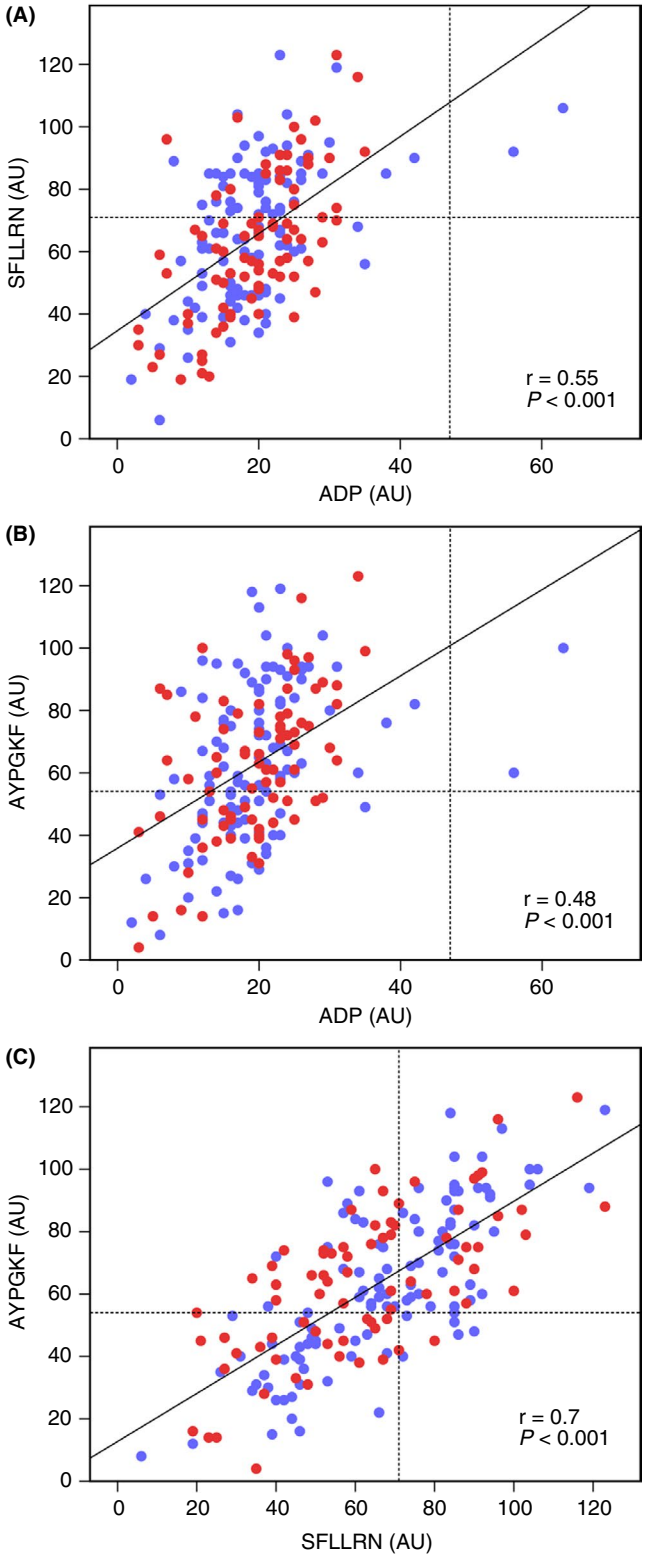

FIGURE 2 Platelet aggregation following stimulation with (A) ADP and the protease-activated receptor (PAR)-1 agonist SFLLRN, (B) ADP and the PAR-4 agonist AYPGKF, and (C) the PAR-1 and PAR-4 agonists SFLLRN and AYPGKF, respectively, in patients receiving prasugrel (blue circles) and in patients receiving ticagrelor (red circles). Cutoff values for high on-treatment residual platelet reactivity to ADP[20] and for normal platelet aggregation in response to SFLLRN and AYPGKF (data from healthy controls as published previously)[18] are represented by the dotted lines. AU, aggregation units

In accordance with data from others, the rate of HRPR ADP was low in prasugrel-treated patients, and HRPR ADP was not seen in patients on ticagrelor,[24–26] findings that differ from patients receiving clopidogrel.[18] Clopidogrel-treated subjects exhibit inadequate $P2Y_{12}$ inhibition in up to 15% if ADP-inducible platelet aggregation is assessed by MEA.[21,22] This is most likely due to the fact that clopidogrel is a prodrug requiring 2 steps of hepatic biotransformation to become pharmacologically active, while prasugrel needs only 1 step of hepatic metabolization and ticagrelor acts directly without prior modification.[5,27] Its complex metabolism predisposes clopidogrel-mediated platelet inhibition to alteration by various factors like genetic polymorphisms, co-medication, age, sex, hemoglobin levels, renal function, smoking, and obesity.[27–29] In contrast to clopidogrel, prasugrel and ticagrelor provide a stronger and more consistent antiplatelet effect. However, Bonello et al[30,31] reported HRPR ADP in 25.2% of 301 prasugrel-treated ACS patients. The discrepancy between their findings and ours may be explained by the timing of testing: While Bonello et al[30,31] measured platelet response to ADP immediately after administration of the prasugrel loading dose, we determined on-treatment platelet aggregation 72 hours after PCI. We chose this approach to capture the steady state of platelet inhibition by prasugrel and ticagrelor.[5,32] Moreover, we assessed the response to prasugrel and ticagrelor by MEA, while Bonello et al used the vasodilator-stimulated phosphoprotein phosphorylation assay. As previously shown, different test systems for the determination of on-treatment platelet reactivity correlate at best moderately with each other.[33,34]

Current literature rarely addresses PAR-mediated platelet aggregation in patients with adequate inhibition of the $P2Y_{12}$ receptor.[17,35] In 2012, Kreutz et al[17] showed that clopidogrel nonresponders also exhibit an increased platelet response to PAR-1 stimulation in 55 patients undergoing elective PCI. Likewise, we previously observed higher levels of PAR-1–mediated platelet surface P-selectin expression, activated glycoprotein IIb/IIIa and monocyte-platelet aggregate formation in thienopyridine nonresponders compared to patients with adequate clopidogrel and prasugrel mediated platelet inhibition.[36] In addition, we found preserved PAR-1 and PAR-4 mediated platelet aggregation in the majority of clopidogrel-treated patients and in about 20% of prasugrel-treated patients with adequate ADP $P2Y_{12}$ inhibition.[18] However, our previous studies comprised only 14 and 19 prasugrel-treated patients,[18,36] respectively, and no patients on ticagrelor therapy. Accordingly, our previous results had to be considered as hypothesis generating only regarding platelet aggregation via PAR-1 and PAR-4 in patients on potent $P2Y_{12}$ inhibitors. Moreover, in contrast to the present investigation in ACS patients, the patients examined in the previous studies by others and us were all stable and underwent elective angioplasty and stenting.[17,18,36]

Interestingly, in the present investigation, more prasugrel- and ticagrelor-treated patients showed a normal aggregation response following PAR-4 stimulation than following PAR-1 stimulation. This could partially be explained by the different concentrations of the PAR-1 agonist SFLLRN and the PAR-4 agonist AYPGKF used in our study. However, since PAR-4 requires higher concentrations of thrombin than PAR-1 in order to be activated, a higher AYPGKF concentration is necessary to achieve detectable PAR-4 mediated platelet aggregation.[11,37] Another aspect is that PAR-4–mediated platelet aggregation differs with respect to ethnicity: In black populations, a high frequency of the gene variant PAR4-Thr120 has been reported, which is associated with greater signaling and platelet aggregation via PAR-4.[38] However, all patients included in our study were of Caucasian ethnicity.

Vorapaxar is a highly selective antagonist of PAR-1, reversibly blocking thrombin-inducible platelet activation.[39–41] In the phase III TRACER (Thrombin Receptor Antagonist for Clinical Event Reduction in Acute Coronary Syndrome) trial, vorapaxar on top of dual antiplatelet therapy did not reduce the primary composite end point of myocardial infarction (MI), urgent coronary revascularization, stroke, recurrent ischemia with rehospitalization, and death from cardiovascular causes in 12 944 patients with non–ST-elevation ACS.[42] However, the key secondary end point consisting of death from cardiovascular causes, MI, or stroke was significantly reduced in patients on vorapaxar compared to placebo (14.1% vs. 12.7%; $P = 0.02$).[42]

In the TRA°2P (Thrombin Receptor Antagonist in Secondary Prevention of Atherothrombotic Ischemic Events)-TIMI 50 trial, vorapaxar in addition to standard antiplatelet therapy significantly reduced the composite end point of cardiovascular death, MI and stroke at 3 years in 26 449 patients with a history of ACS, ischemic stroke, or peripheral artery disease (PAD).[43] On the other hand, major bleeding events including intracranial hemorrhage were significantly increased in patients receiving vorapaxar in both trials.[42,43] Consequently, vorapaxar should not be prescribed in all ACS patients but may become a treatment option for patients with a high risk of ischemic cardiovascular events and a low bleeding risk.

In a previous study, we showed that high on-treatment PAR-1–mediated platelet activation is a strong predictor of ischemic outcomes in PAD patients following peripheral angioplasty and stenting.[44] Furthermore, in a post hoc analysis of the TRA°2P-TIMI-50 trial, vorapaxar demonstrated its positive effects on adverse ischemic events vs. placebo in PAD patients.[45]

Based on our observation of preserved platelet aggregation via PAR-1 in many prasugrel- and ticagrelor-treated patients, one may speculate that the measurement of PAR-1–mediated platelet aggregation may allow the identification of ACS patients who might benefit from additional vorapaxar therapy despite being already treated with potent $P2Y_{12}$ inhibitors. Moreover, PAR-4 might be a potential new target for antiplatelet therapy in patients with high PAR-mediated platelet aggregation.[4,11] Indeed, it has recently been shown that PAR-4 antagonism reduces thrombin response by >50%.[4,46–48] It must be taken into account that a combination of vorapaxar with stronger $P2Y_{12}$ inhibitors might impact on bleeding as also a combination of vorapaxar with glycoprotein IIb/IIIa inhibitors was associated with increased bleeding events.[49] Accordingly, prospective clinical trials are necessary before such combination therapies can be considered.

Finally, inhibition of thrombin generation by addition of a low dose of the factor Xa inhibitor rivaroxaban to dual antiplatelet therapy with aspirin and clopidogrel was shown to reduce ischemic outcomes and death in 15 526 ACS patients in the ATLAS ACS 2-TIMI 51 (An Efficacy and Safety Study for Rivaroxaban in Patients With Acute Coronary Syndrome) trial.[50] As in the phase III trials investigating vorapaxar, the rates of major and intracranial bleeding were higher in the rivaroxaban groups than in patients receiving placebo.[50]

Given the above-described literature on adding antithrombotic medications to standard treatment, proper patient selection (high ischemic and low bleeding risk) will be key for the success of an intensified antithrombotic regimen following ACS and PCI. Testing for PAR responsiveness may therefore become a future option to identify patients at high risk of ischemic events during state-of-the-art antiplatelet therapy, who might benefit from additional antithrombotic treatment. For this approach, however, clinical studies associating PAR-mediated platelet aggregation with adverse outcomes after PCI are needed.

Limitations of our study are the lack of clinical outcome data, its observational design, and the fact that we measured platelet aggregation at only 1 time point using a single test system. Moreover, we did not perform in vivo analyses. However, MEA is a highly standardized platelet function test ensuring a good comparability of the obtained results with other laboratories, and platelet aggregation by MEA has repeatedly been linked to cardiovascular outcomes following PCI.[20,21] Another limitation of our study is that the cutoff values for PAR-1– and PAR-4–mediated platelet aggregation were derived from measurements in healthy volunteers, who were not on any medication and not age-matched to the patient population. However, by defining the upper 95% of PAR-mediated platelet aggregation in the control group as normal uninhibited platelet aggregation, we eliminated possible low outliers.

Finally, it must be mentioned that studies trying to individualize antiplatelet therapy based on platelet function testing have failed so far.

In conclusion, normal platelet aggregation via PAR-1 and PAR-4 is preserved in many ACS patients despite adequate $P2Y_{12}$ inhibition by prasugrel and ticagrelor. The present findings may in part explain the occurrence of adverse ischemic events despite potent $P2Y_{12}$ inhibition. Future trials are warranted to investigate the association of PAR-mediated platelet aggregation with clinical outcomes in ACS and to study potential therapeutic approaches.

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RELATIONSHIP DISCLOSURE

The authors report nothing to disclose.

AUTHOR CONTRIBUTIONS

PPW: data collection, performance of measurements, and writing of the manuscript. JP and CW: data collection, critical revision, and final approval of the manuscript. BE: performance of measurements, critical revision, and final approval of the manuscript. BP: data collection, writing of the manuscript, critical revision, and final approval of the manuscript. KH, IML and RK: critical revision and final approval of the manuscript. SP: study design, critical revision, and final approval of the manuscript. TG: study design, statistical analysis, writing of the manuscript, critical revision, and final approval of the manuscript.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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