In vivo and protease-activated receptor-1-mediated platelet activation but not response to antiplatelet therapy predict two-year outcomes after peripheral angioplasty with stent implantation

Thomas Gremmel¹; Sabine Steiner¹; Daniela Seidinger³; Renate Koppensteiner¹; Simon Panzer²∗; Christoph W. Kopp¹∗

¹Division of Angiology, Department of Internal Medicine II, Medical University of Vienna, Vienna, Austria; ²Department of Blood Group Serology and Transfusion Medicine, Medical University of Vienna, Vienna, Austria

Summary
Data linking the response to antiplatelet therapy with clinical outcomes after angioplasty and stenting for lower extremity artery disease (LEAD) are scarce. Moreover, associations of in vivo and thrombin-inducible platelet activation with the occurrence of adverse events have not been investigated in these patients, so far. We therefore assessed clinical outcomes and on-treatment platelet reactivity by four test systems in 108 patients receiving dual antiplatelet therapy after infragingual angioplasty and stenting for LEAD. Further, in vivo and thrombin receptor-activating peptide (TRAP)-6-inducible glycoprotein (GP) IIb/IIIa activation and P-selectin expression were measured as sensitive parameters of platelet activation. The primary endpoint was defined as the composite of atherothrombotic events and target vessel restenosis or reocclusion. Residual platelet reactivity to adenosine diphosphate and arachidonic acid was similar between patients without and with adverse outcomes within two-year follow-up (all p>0.05). Further, the occurrence of clinical endpoints did not differ significantly between patients without and with high on-treatment residual platelet reactivity by all test systems (all p>0.05). In contrast, in vivo and TRAP-6-inducible platelet activation were significantly more pronounced in patients with subsequent adverse events (all p<0.05), and high levels of platelet activation were independent predictors of the primary endpoint (adjusted hazard ratios: 3.5 for high in vivo activated GP IIb/IIIa, 2.9 for high TRAP-6-inducible activated GP IIb/IIIa, 2.3 for high in vivo P-selectin, and 3 for high TRAP-6-inducible P-selectin; all p<0.05). In conclusion, in vivo and protease-activated receptor-1-mediated platelet activation predict two-year clinical outcomes in stable patients undergoing angioplasty and stenting for LEAD.

Keywords
Peripheral arterial disease, angioplasty, stenting, platelet reactivity

Introduction
Dual antiplatelet therapy with aspirin and clopidogrel is the treatment of choice after angioplasty and stenting for lower extremity artery disease (LEAD) to prevent stent thrombosis and future adverse ischaemic events. The efficacy of both drugs in the secondary prevention of cardiovascular disease is well-documented by large-scale clinical trials (1, 2). However, a considerable number of patients still suffer ischaemic outcomes despite antiplatelet therapy, suggesting that aspirin and clopidogrel may not be equally effective in all patients. Indeed, platelet function tests revealed considerable interindividual variations of residual platelet reactivity during antiplatelet therapy with aspirin and clopidogrel (3–5). Further, patients with high on-treatment residual platelet reactivity (HRPR) were significantly more prone to stent thrombosis and adverse ischaemic events after coronary stenting (6–10). Currently, data linking the response to antiplatelet therapy with clinical outcomes after angioplasty and stenting for LEAD are scarce and limited to clopidogrel-mediated platelet inhibition. The different vascular bed with larger vessel diameters and the predominant use of bare metal stents in peripheral angioplasty may affect the role of residual platelet reactivity for short- and long-term prognosis of these patients.

The serine protease thrombin is considered the most potent platelet agonist. It acts mainly via protease-activated receptors (PAR)-1 and -4, and achieves rapid platelet activation even in the presence of dual antiplatelet therapy (11). We recently reported that patients with peripheral arterial disease (PAD) exhibit increased platelet activation in vivo and after in vitro stimulation with thrombin receptor activating peptide (TRAP)-6 compared to patients with coronary artery disease (CAD) (12). These findings suggest that many PAD patients have a high level of in vivo platelet
activation and are particularly susceptible to further activation by thrombin. However, associations of in vivo and thrombin-inducible platelet activation with the occurrence of adverse outcomes have not been investigated in patients with LEAD, so far. Since both are not targeted by current antiplatelet therapy, they may serve as independent risk predictors for future adverse events. We therefore hypothesized that in vivo and PAR-1-mediated platelet activation are superior to on-treatment residual adenosine diphosphate (ADP)- and arachidonic acid (AA)-inducible platelet activity in predicting two-year clinical outcomes after infragenual angioplasty and stenting for LEAD.

Materials and methods

Study population

In this prospective cohort study, 108 patients undergoing successful infragenual angioplasty with endovascular stent implantation were enrolled consecutively at the Division of Angiology of the Medical University of Vienna between 2008 and 2010. All patients had intermittent claudication classified as Rutherford stages of PAD 2-3 due to sonographically confirmed infragenual artery stenosis and occlusion, respectively. All patients received long-term aspirin therapy (100 mg/day), and 75 mg of clopidogrel per day for three months following angioplasty and stenting. Clinical follow-up was assessed one and two years after the percutaneous intervention.

Exclusion criteria were a known aspirin or clopidogrel intolerance (allergic reactions, gastrointestinal bleeding), a therapy with vitamin K antagonists (warfarin, phenprocoumon, acenocoumarol), a treatment with ticlopidine, dipiridamol or nonsteroidal antiinflammatory drugs, a family or personal history of bleeding disorders, malignant paraproteinemias, myeloproliferative disorders, or hepatic-induced thrombocytopenia, severe hepatic failure, known qualitative defects in thrombocyte function, a major surgical procedure within one week before enrollment, a platelet count <100,000 or >450,000/µl and surgical procedure within one week before enrollment, platelet activity >450,000/µl and a haematocrit <30%. The study protocol was approved by the Ethics Committee of the Medical University of Vienna in accordance with the Declaration of Helsinki, and written informed consent was obtained from all study participants.

Blood sampling

Blood was drawn by clean venipuncture from an antecubital vein using a 21-gauge butterfly needle (0.8 x 19 mm; Greiner Bio-One, Kremsmünster, Austria) one day after the percutaneous intervention (13). To avoid procedural deviations all blood samples were taken by the same physician applying a light tourniquet, which was immediately released and the samples were mixed adequately by gently inverting the tubes. After the initial 3 ml of blood had been discarded to reduce procedurally induced platelet activation, blood was drawn into a 3.8% sodium citrate Vacuette tube (Greiner Bio-One; 9 parts of whole blood, 1 part of sodium citrate 0.129 M/L) for evaluations by light transmission aggregometry (LTA), the vasodilator-stimulated phosphoprotein (VASP) phosphorylation assay, and flow cytometric analyses of P-selectin and glycoprotein (GP) Iib/IIa expression, into a 3.2% sodium citrate Vacuette tube (Greiner Bio-One; 9 parts of whole blood, 1 part of sodium citrate 0.109 M/L) for the VerifyNow P2Y12 and aspirin assays, and into a Vacuette tube containing lithium heparin (18 IU/ml) for the determinations by multiple electrode aggregometry (MEA). The time interval between blood sampling and platelet function testing was at least 1 hour (h) and did not exceed 3 h. To avoid investigator-related variations of the results, each of the different tests was performed by just one corresponding operator, who was blinded to the results from the other operators.

Platelet function tests

All tests have been described in detail previously (3, 4).

Light transmission aggregometry (LTA)

Platelet counts were not adjusted as the median platelet count was 210 G/l (182–240 G/l) (14). Aggregation was performed using adenosine diphosphate (ADP; 10 µM; Rolf Greiner BioChemica, Flacht, Germany) or arachidonic acid (AA; final concentration of 0.5 mg/ml). Optical density changes were recorded photoelectrically for 10 minutes as platelets began to aggregate.

VerifyNow P2Y12 and aspirin assays

The VerifyNow P2Y12 and aspirin assays were performed as previously described (3). Higher P2Y12 Reaction Units (PRU) and higher Aspirin Reaction Units (ARU) reflect greater ADP- and AA-mediated platelet reactivity, respectively.

Vasodilator-stimulated phosphoprotein (VASP) phosphorylation assay

For determination of the platelet reactivity index (PRI), the extent of VASP phosphorylation was measured by geometric mean fluorescence intensity (MFI) values in the presence of PGE1 without (T1) or with ADP (T2) (3, 15). After subtraction of the background fluorescence from the corresponding fluorescence values, the PRI (%) was calculated according to the following formula:

PRI % = [T1 (PGE1) – T2 (PGE1+ADP)/ T1 (PGE1)] × 100

Multiple electrode platelet aggregometry (MEA)

In whole blood impedance aggregometry, ADP (6.4 µM) or AA (0.5 mM; both from Verum Diagnostica, Munich, Germany) activated platelets adhered to the electrodes leading to an increase of impedance, which was detected for each sensor unit separately and transformed to aggregation units (AU) that were plotted against time (3).
Activated glycoprotein (GP) IIb/IIIa and platelet surface expression of P-selectin

The binding of the monoclonal antibody PAC-1 to activated GPIIb/IIa and the expression of P-selectin were determined in citrate anticoagulated blood, as previously published (11, 16). In brief, whole blood was diluted in phosphate buffered saline to obtain 20x10º platelets and incubated without agonists, and after in vitro exposure to suboptimal concentrations of TRAP-6 (5.7 µM; Bachem, Bubendorf, Switzerland) for 10 minutes (min). The concentration of TRAP-6 was determined in previous titration experiments in 10 healthy controls and was further evaluated in 50 other healthy individuals. Thereby, in non-activated platelets activated GPIIb/IIa was 2.3 MFI (2.1-4.7 MFI), and P-selectin expression was 2.8 MFI (2.1-5.8 MFI). The data for TRAP-6-inducible activated GPIIb/IIa and P-selectin were 3.0 MFI (2.1-8.5 MFI) and 27 MFI (6.7-108 MFI), respectively. The platelet population was identified by staining with anti-CD42b (clone HIPI, allopheocya- nin labelled; Becton Dickinson (BD), San Jose, CA, USA), and activated GPIIb/IIa and P-selectin expression were determined by the binding of the monoclonal antibodies PAC-1-fluorescein (BD) and anti-CD62p-phycoerythrin (clone CLB-Thromb6; Immuno- tech, Beckman Coulter, Fullerton, CA, USA), respectively. After 15 min of incubation in the dark, the reaction was stopped by adding 500 µl phosphate-buffered saline (PBS) and samples were acquired immediately on a FACSCalibur flow cytometer (BD) with excitation by an argon laser at 488 nm and a red diode laser at 635 nm at a rate of 200-600 events per second. Platelets were gated in a side scatter versus FL3 dot plot. A total of 10,000 events were acquired within this gate. The gated events were further analysed in histograms for FL-1 and FL-2 for PAC-1 and P-selectin, respectively. Standard BD calibrite beads were used for daily calibration of the cytometer.

Clinical endpoints

The primary endpoint was defined as the composite of the first occurrence of any of the following events: non-fatal myocardial infarction (MI), non-fatal stroke or transient ischaemic attack (TIA), cardiovascular death, and recurrent symptoms of LEAD, i.e. claudication or critical limb ischaemia, due to >80% target vessel restenosis or reoclusion within two years after peripheral angioplasty.

The composite of the first occurrence of nonfatal MI, nonfatal stroke or TIA, and cardiovascular death within two-year follow-up was defined as secondary endpoint.

Statistical analysis

A sample size calculation was based on the observed mean ± SD (49 ± 22 AU) of residual ADP-inducible platelet reactivity by MEA in a former population of 50 patients (27 male, 23 female; median age 66 years, interquartile range 61–73 years) under dual antipla- telet treatment 24 h after peripheral angioplasty with stent implantation. We calculated that we needed to include 100 patients to be able to detect a 30% relative difference of ADP inducible platelet aggregation between patients without and with the primary end- point with a power of 90% (using a two-sided alpha level of 0.05). To compensate for potential loss to follow up, we included eight additional patients.

Statistical analysis was performed using the Statistical Package for Social Sciences (IBM SPSS version 19, Armonk, NY, USA). Median and interquartile range of continuous variables are shown. Categorical variables are given as number (%). We performed Mann Whitney U tests to detect differences in continuous variables. The Chi-square and the Fisher’s exact test were used to detect differences in categorical variables, respectively. Survival curves were generated using the Kaplan-Meier method, and the differences between the groups were assessed by the log-rank test. Receiver-operating characteristic (ROC) curve analyses were used to determine the ability of in vivo and TRAP-6 inducible platelet activation to distinguish between patients without and with the primary endpoint. The optimal cut-off values were calculated by determining in vivo and TRAP-6 inducible platelet activation values that provided the greatest sum of sensitivity and specificity among the cut-off values with a sensitivity >80%. Cox regression analysis was used to adjust for patient characteristics that were different between patients without and with the primary endpoint. Covariates for adjustment were selected on the basis of univariate analyses (p-value ≤0.1), including age, sex, body mass index (BMI), hypertension, hypercholesterolaemia, diabetes, active smoking, haemoglobin, white blood cell count, platelet count, serum creatinine, C-reactive protein (CRP), number of implanted stents, use of statins, angiotensin converting enzyme inhibitors or angiotensin receptor blockers, beta-blockers, proton pump inhibitors and calcium channel blockers. Two-sided p-values <0.05 were considered statistically significant.

Results

Four patients were lost to follow-up, the remaining 104 patients entered statistical analysis. Clinical, laboratory, and procedural characteristics of the overall study population, and of patients without and with the primary endpoint are given in ▶ Table 1. The use of proton pump inhibitors was significantly more common in patients without the primary endpoint (p=0.04). The remaining characteristics were similar between patients without and with the primary endpoint within two-year follow-up. Results from the VerifyNow assays were available for all patients, results from LTA and the VASP assay were available for 103 patients (99%), results from flow cytometry were available for 100 patients (96.2%), and results from MEA were available for 99 patients (95.2%).

The primary endpoint occurred in 41 patients (39.4%), and comprised two patients (4.9%) with non-fatal MI, three patients (7.3%) with ischaemic stroke or TIA, and 36 patients (87.8%) with recurrent symptoms of PAD due to >80% target vessel restenosis or reoclusion. The latter underwent target vessel revascularisation in 83.3% (30 out of 36 patients). The secondary endpoint occurred in seven patients (6.7%) within the two-year follow-up,
and comprised 1 cardiovascular death (14.3%), three patients with non-fatal MI (42.85%), and three patients with non-fatal stroke or TIA (42.85%).

Residual platelet reactivity to ADP and AA by all test systems was similar between patients without and with the primary endpoint (Table 2A; all p>0.05). In contrast, in vivo and TRAP-6 inducible activated GPIIb/IIIa and P-selectin expression were significantly higher in patients with the primary endpoint (Table 2B; all p<0.05).

In a second step, we stratified the study population in patients without and with HRPR by the different platelet function tests. HRPR in response to ADP (HRPR ADP) was defined according to the recently published consensus document on the definition of high on-treatment platelet reactivity to ADP (10). The respective cut-off values for HRPR ADP were a maximal aggregation >67% for LTA, PRU >235 for the VerifyNow P2Y12 assay, a PRI >50% for the VASP assay, and AU ≥47 for MEA. Thereby, HRPR ADP was seen in 16 (15.5%), 30 (28.8%), 54 (52.4%), and 35 (35.4%) patients by LTA, the VerifyNow P2Y12 assay, the VASP assay, and MEA, respectively. The occurrence of the primary endpoint did not differ significantly between patients without and with HRPR ADP by all test systems (Figure 1). ROC curve analyses revealed that even other thresholds of residual ADP-inducible platelet reactivity would not be able to distinguish between patients without and with the primary endpoint. Likewise, the occurrence of the secondary endpoint was similar between patients without and with HRPR ADP by all platelet function tests (all p>0.05).

Moreover, we selected thresholds, which were previously associated with the occurrence of adverse ischaemic events, to define HRPR in response to AA (HRPR AA) by LTA and the VerifyNow

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Overall (n=104)</th>
<th>No primary endpoint (n=63)</th>
<th>Primary endpoint (n=41)</th>
<th>P-value</th>
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<tr>
<td><strong>Demographics</strong></td>
<td>----------------</td>
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</tr>
<tr>
<td>Age, years</td>
<td>65 (58 – 73)</td>
<td>63 (58 – 70)</td>
<td>66 (59 – 74)</td>
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<td>Male sex</td>
<td>64 (61.5)</td>
<td>42 (66.7)</td>
<td>22 (53.7)</td>
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<tr>
<td>BMI, kg/m²</td>
<td>26.8 (24.6 – 29.2)</td>
<td>27.6 (24.8 – 29.7)</td>
<td>25.7 (24.5 – 28.4)</td>
<td>0.2</td>
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<td><strong>Medical history</strong></td>
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<td></td>
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<tr>
<td>Hypertension</td>
<td>96 (92.3)</td>
<td>58 (92.1)</td>
<td>38 (92.7)</td>
<td>1</td>
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<tr>
<td>Hypercholesterolemia</td>
<td>96 (92.3)</td>
<td>61 (96.8)</td>
<td>35 (85.4)</td>
<td>0.06</td>
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<td>Diabetes mellitus</td>
<td>38 (36.5)</td>
<td>19 (30.2)</td>
<td>19 (46.3)</td>
<td>0.09</td>
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<tr>
<td>Active smoking</td>
<td>46 (44.2)</td>
<td>31 (49.2)</td>
<td>15 (36.6)</td>
<td>0.2</td>
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<td><strong>Laboratory data</strong></td>
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<tr>
<td>Haemoglobin, g/dl</td>
<td>13.7 (12.5 – 14.6)</td>
<td>13.8 (12.6 – 14.8)</td>
<td>13.5 (11.9 – 14.2)</td>
<td>0.4</td>
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<tr>
<td>White blood cell count, G/l</td>
<td>8.9 (6.9 – 10.3)</td>
<td>9.3 (6.9 – 10.5)</td>
<td>8.5 (6.9 – 10.1)</td>
<td>0.5</td>
</tr>
<tr>
<td>Platelet count, G/l</td>
<td>210 (182 – 240)</td>
<td>210 (175 – 257)</td>
<td>210 (193 – 229)</td>
<td>0.9</td>
</tr>
<tr>
<td>Serum creatinine, mg/dl</td>
<td>1 (0.9 – 1.2)</td>
<td>1 (0.9 – 1.1)</td>
<td>1 (0.9 – 1.2)</td>
<td>0.7</td>
</tr>
<tr>
<td>C-reactive protein, mg/dl</td>
<td>1.1 (0.4 – 0.8)</td>
<td>1.2 (0.6 – 1.9)</td>
<td>0.9 (0.4 – 1.8)</td>
<td>0.3</td>
</tr>
<tr>
<td><strong>Procedure</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Stent implantation</td>
<td>104 (100)</td>
<td>63 (100)</td>
<td>41 (100)</td>
<td>1</td>
</tr>
<tr>
<td>Number of stents/patient</td>
<td>2 (1 – 2)</td>
<td>2 (1 – 2)</td>
<td>1 (1 – 2)</td>
<td>0.3</td>
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<td><strong>Medication pre-intervention</strong></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Clopidogrel</td>
<td>104 (100)</td>
<td>63 (100)</td>
<td>41 (100)</td>
<td>1</td>
</tr>
<tr>
<td>Aspirin</td>
<td>104 (100)</td>
<td>63 (100)</td>
<td>41 (100)</td>
<td>1</td>
</tr>
<tr>
<td>Statins</td>
<td>94 (90.4)</td>
<td>59 (93.7)</td>
<td>35 (85.4)</td>
<td>0.2</td>
</tr>
<tr>
<td>ACE inhibitors/ARB</td>
<td>89 (85.6)</td>
<td>52 (82.5)</td>
<td>37 (90.2)</td>
<td>0.3</td>
</tr>
<tr>
<td>Beta blockers</td>
<td>61 (58.7)</td>
<td>37 (58.7)</td>
<td>24 (58.5)</td>
<td>1</td>
</tr>
<tr>
<td>Calcium channel blockers</td>
<td>40 (38.5)</td>
<td>21 (33.3)</td>
<td>19 (46.3)</td>
<td>0.2</td>
</tr>
<tr>
<td>Proton pump inhibitors</td>
<td>46 (44.2)</td>
<td>33 (52.4)</td>
<td>13 (31.7)</td>
<td>0.04</td>
</tr>
</tbody>
</table>

Continuous data are shown as median (interquartile range). Dichotomous data are shown as n (%). BMI, body mass index; ACE, angiotensin converting enzyme; ARB, angiotensin receptor blockers.
aspirin assay, respectively (6, 17). For MEA, on-treatment residual AA-inducible platelet reactivity was considered as HRPR AA. The corresponding cut-off values were a maximal aggregation ≥20% for LTA, ARU ≥550 for the VerifyNow aspirin assay, and AU ≥21 for MEA. Thereby, HRPR AA was seen in 11 (10.7%), 11 (10.6%), and 29 (29.3%) patients by LTA, the VerifyNow aspirin assay, and MEA, respectively. The occurrence of the primary endpoint did not differ significantly between patients without and with HRPR AA by all test systems (see Suppl. Figure 1, available online at www.thrombosis-online.com). ROC curve analyses revealed that even other thresholds of residual AA-inducible platelet reactivity would not be able to distinguish between patients without and with the primary endpoint. Likewise, the occurrence of the secondary endpoint was similar between patients without and with HRPR AA by all platelet function tests (all p>0.05).

ROC curve analyses demonstrated that in vivo and TRAP-6 inducible platelet activation were able to distinguish between patients without and with subsequent adverse events. Regarding in vivo platelet activation, an activated GPIIb/IIIa >2.56 MFI was identified as the best cut-off value to predict the primary endpoint, providing a sensitivity of 82.1% and a specificity of 54.1% (►Figure 2A), and was therefore defined as high TRAP-6-inducible GPIIb/IIIa. With use of this cut-off value high in vivo GPIIb/IIIa was seen in 60 patients (60%). Moreover, in vivo P-selectin expression >2.93 MFI was identified as cut-off value to predict the primary endpoint, providing a sensitivity of 82.1% and a specificity of 41%. High in vivo P-selectin was seen in 68 patients (68%). Regarding TRAP-6 inducible platelet activation, an activated GPIIb/IIIa >3.23 MFI was identified as the best cut-off value to predict the primary endpoint, providing a sensitivity of 87.2% and a specificity of 42.6% (►Figure 2B), and was therefore defined as high TRAP-6-inducible GPIIb/IIIa. With use of this cut-off value high TRAP-6 inducible GPIIb/IIIa was seen in 69 patients (69%). Moreover, TRAP-6-inducible P-selectin expression >40.2 MFI was identified as cut-off value to predict the primary endpoint, providing a sensitivity of 82.1% and a specificity of 47.5%. High TRAP-6-inducible P-selectin was seen in 64 patients (64%). Positive and negative predictive values of all thresholds are given in ►Table 3.

The primary endpoint occurred significantly more frequent in patients with high in vivo and high TRAP-6-inducible platelet activation, respectively, than in patients with lower levels of platelet activation (►Figure 3). Both, high in vivo and high TRAP-6 inducible platelet activation remained significantly associated with the primary endpoint after adjustment for age, hypercholesterolaemia, diabetes, and the use of proton pump inhibitors by multivariable Cox regression analysis (Suppl. Table 1A-D, available online at www.thrombosis-online.com). In detail, high in vivo GPIIb/IIIa

Table 2A: Residual adenosine diphosphate (ADP) and arachidonic acid (AA) inducible platelet reactivity by light transmission aggregometry (LTA), the VerifyNow P2Y12 and aspirin assays, and multiple electrode platelet aggregometry (MEA), and residual ADP inducible platelet reactivity by the vasodilator-stimulated phosphoprotein (VASP) phosphorylation assay in the overall study population, and in patients without and with the primary endpoint.

<table>
<thead>
<tr>
<th>Test system</th>
<th>Overall (n=104)</th>
<th>No primary endpoint (n=63)</th>
<th>Primary endpoint (n=41)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>LTA ADP (maximal aggregation %)</td>
<td>45 (31.5 – 60.3)</td>
<td>44.5 (31 – 60.3)</td>
<td>45 (33.6 – 57.8)</td>
<td>0.7</td>
</tr>
<tr>
<td>LTA AA (maximal aggregation %)</td>
<td>3.9 (1.4 – 7.6)</td>
<td>3.2 (1.1 – 7.5)</td>
<td>4.3 (2 – 7.6)</td>
<td>0.3</td>
</tr>
<tr>
<td>VerifyNow P2Y12 assay (PRU)</td>
<td>186 (120 – 242)</td>
<td>191 (107 – 245)</td>
<td>180 (129 – 214)</td>
<td>0.6</td>
</tr>
<tr>
<td>VerifyNow aspirin assay (ARU)</td>
<td>398 (387 – 485)</td>
<td>395 (385 – 485)</td>
<td>400 (388 – 485)</td>
<td>0.6</td>
</tr>
<tr>
<td>MEA ADP (AU)</td>
<td>41 (30 – 56)</td>
<td>41 (29 – 57)</td>
<td>42 (31 – 56)</td>
<td>0.8</td>
</tr>
<tr>
<td>MEA AA (AU)</td>
<td>17 (10 – 21)</td>
<td>17 (9 – 22)</td>
<td>17 (14 – 21)</td>
<td>0.6</td>
</tr>
<tr>
<td>VASP assay (PRI %)</td>
<td>51.5 (28.8 – 66)</td>
<td>50.8 (28 – 63.7)</td>
<td>53.3 (32.5 – 67.3)</td>
<td>0.7</td>
</tr>
</tbody>
</table>

Continuous data are shown as median (interquartile range). Dichotomous data are shown as n (%). PRU, P2Y12 reaction units; ARU, aspirin reaction units; AU, aggregation units, PRI, platelet reactivity index.

Table 2B: In vivo and thrombin receptor-activating peptide (TRAP)-6 inducible glycoprotein (GP) IIb/IIIa and P-selectin in the overall study population, and in patients without and with the primary endpoint.

<table>
<thead>
<tr>
<th>Platelet activation marker</th>
<th>Overall (n=104)</th>
<th>No primary endpoint (n=63)</th>
<th>Primary endpoint (n=41)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>GPIIb/IIIa (MFI)</td>
<td>2.7 (2.3 – 3.2)</td>
<td>2.5 (2.2 – 2.9)</td>
<td>3 (2.6 – 3.8)</td>
<td>0.001</td>
</tr>
<tr>
<td>GPIIb/IIIa TRAP-6 (MFI)</td>
<td>4 (3.1 – 6)</td>
<td>3.5 (3 – 5.7)</td>
<td>4.8 (3.5 – 6.4)</td>
<td>0.01</td>
</tr>
<tr>
<td>P-selectin (MFI)</td>
<td>3.3 (2.9 – 3.8)</td>
<td>3.2 (2.6 – 3.5)</td>
<td>3.4 (3 – 4.2)</td>
<td>0.03</td>
</tr>
<tr>
<td>P-selectin TRAP-6 (MFI)</td>
<td>54 (29.8 – 81.7)</td>
<td>46.5 (28.3 – 74.7)</td>
<td>59.1 (43.2 – 91.3)</td>
<td>0.04</td>
</tr>
</tbody>
</table>

Continuous data are shown as median (interquartile range). Dichotomous data are shown as n (%). MFI, mean fluorescence intensity.
was associated with a 3.5-fold (95% confidence interval [CI] 1.5 – 8.1; p=0.003) increased risk, and high TRAP-6 inducible GPIIb/IIIa was associated with a 2.9-fold (95% CI 1.1 – 7.5; p=0.04) increased risk of the primary endpoint. High in vivo P-selectin was associated with a 2.3-fold (95% CI 1.02 – 5.3; p=0.045) increased risk, and high TRAP-6 inducible P-selectin was associated with a three-fold (95% CI 1.3 – 7; p=0.009) increased risk of the primary endpoint.

The secondary endpoint occurred significantly more frequent in patients with high in vivo GPIIb/IIIa and in patients with high TRAP-6-inducible P-selectin than in patients with low in vivo GPIIb/IIIa and low TRAP-6-inducible P-selectin, respectively (7 vs...
0 patients; both p<0.05 with the log-rank test). Further, the secondary endpoint occurred numerically more often in patients with high TRAP-6-inducible GPIIb/IIIa and in patients with high in vivo P-selectin than in patients with low TRAP-6-inducible GPIIb/IIIa and low in vivo P-selectin, respectively (6 vs 1 patients; both p>0.05 with the log-rank test).

### Discussion

To the best of our knowledge, our study is the first to investigate the associations of on-treatment platelet reactivity by multiple platelet function tests, in vivo platelet activation, and PAR-1-mediated platelet activation with adverse events after infrainguinal angioplasty with stent implantation. In our population, response to dual antiplatelet therapy by all test systems was not associated with two-year outcomes after angioplasty and stenting. In contrast, in vivo and TRAP-6-inducible GPIIb/IIIa activation and P-selectin expression were significantly more pronounced in patients with subsequent adverse events. Moreover, high levels of platelet activation were independent predictors of the composite endpoints.

Previous studies revealed a considerable heterogeneity of on-treatment platelet reactivity in response to ADP and AA between the various platelet function tests (3-5). This is most likely due to the fact that each method is based on a different principle, and therefore captures different aspects of agonists’-inducible platelet reactivity (18). Previous studies have shown that some influencing factors for platelet reactivity are identified by one test system, but not by another one (19). Likewise, platelet reactivity by one method may be associated with clinical outcome after peripheral angioplasty and stenting, while other test systems may not be able to discriminate between patients without and with subsequent adverse events. Therefore, in order to thoroughly investigate the association between platelet reactivity and adverse outcomes, we decided to assess residual platelet reactivity with the four most widely used methods, namely LTA, the VerifyNow assays, MEA, and the VASP assay. Thereby, we found no significant associations of on-
treatment platelet reactivity by all platelet function tests with the composite endpoints, suggesting that a clinically relevant role of HRPR for long-term outcomes after infrainguinal angioplasty with stent implantation is unlikely in patients with intermittent claudication. In contrast to our observations, Spiliopoulos et al. recently reported a significant association between HRPR ADP by the VerifyNow P2Y12 assay and adverse events after peripheral angioplasty and stenting (20). The discrepancy between their results and our study may be explained by differences in the antiplatelet regimens, the clinical presentation scenarios of the study patients, and the study design: While our patients received 75 mg of clopidogrel for three months in addition to long-term aspirin therapy (100 mg/day), their patients were prescribed aspirin (100 mg/day) for six months in addition to long-term clopidogrel therapy (75 mg/day) after the percutaneous intervention. Moreover, we exclusively enrolled stable patients with intermittent claudication classified as Rutherford stages of PAD 2-3, whereas Spiliopoulos et al. enrolled mainly patients with critical limb ischaemia (70% of patients with Rutherford stages of PAD 4-6). Finally, Spiliopoulos et al. reported one-year clinical outcomes. In contrast, our primary endpoint was the occurrence of adverse events within two years of follow-up.

We assessed platelet surface expressions of activated GPIIb/IIa and P-selectin without addition of agonists and after in vitro exposure to TRAP-6 to determine in vivo and PAR-1-mediated platelet activation. Upon activation platelets undergo a shape change, which leads to the exposure of the fibrinogen binding site on GPIIb/IIa, thereby enabling the interaction with coagulation factors and other platelets. Moreover, P-selectin, which is stored in the alpha granules of unactivated platelets, is rapidly released and expressed on the platelet surface. The binding of P-selectin to its counterreceptor P-selectin glycoprotein ligand-1 on leukocytes facilitates the formation of monocyte-platelet aggregates, which were shown to be elevated in several pathological circumstances, including MI (21). Through their interaction with other platelets, coagulation factors and leukocytes, activated platelets exert proinflammatory and prothrombotic effects at the site of endothelial injury leading to intimal hyperplasia and atherothrombosis, respectively (22). Consequently, the activation of GPIIb/IIa and P-selectin expression initiate processes that may result in target vessel restenosis, reocclusion, or thrombotic events following angioplasty and stenting. Indeed, in vivo and TRAP-6-inducible platelet activation were independently associated with ischaemic outcomes within two-year follow-up in our study population. The best predictors for future adverse events were high in vivo GPIIb/IIa and high TRAP-6-inducible P-selectin, which were significantly linked to both, the primary and secondary endpoint. Our observations are in line with the peripheral endpoints of PAD patients treated with the PAR-1 antagonist vorapaxar in the TRA2*P-TIMI 50 trial (23). While vorapaxar did not reduce the risk of cardiovascular death, MI, or stroke in patients with PAD, it significantly reduced acute limb ischaemia and the need for peripheral revascularisation. Thus, together with our findings, these data suggest that cyclooxygenase-1 and/or P2Y12-targeted inhibition might not be the adequate pharmacological approach in patients with PAD, and that these patients may particularly benefit from PAR-1 inhibition.

We previously reported that patients with PAD exhibit increased platelet activation compared to patients with coronary artery disease (CAD) (12). In consideration of our current findings, detrimental platelet activation may at least in part be responsible for the worse long-term prognosis of patients with PAD compared to CAD patients (24). It remains to be established if platelet activation can also serve as risk marker in medically treated PAD, and in other manifestations of atherosclerosis. Moreover, future studies are warranted to clarify whether alternative antiplatelet strategies like thrombin inhibition or thrombin receptor blockade are beneficial in patients with atherosclerotic cardiovascular disease exhibiting high platelet activation.

We did not perform serial measurements of residual platelet reactivity and platelet activation markers. Therefore, we cannot disclose variations of these parameters over time.

In conclusion, in vivo and PAR-1-mediated platelet activation predict two-year clinical outcomes in stable patients undergoing angioplasty and stenting for LEAD, whereas response to dual antiplatelet therapy is not associated with adverse events in these patients. The assessment of platelet activation may serve as risk stratification tool in patients with PAD.

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What is known about this topic?

- Residual platelet reactivity during antithrombotic treatment with aspirin and clopidogrel shows a great interindividual variability.
- High on-treatment residual platelet reactivity is associated with adverse outcomes after percutaneous coronary intervention.
- Data linking residual platelet reactivity with clinical outcomes after peripheral angioplasty are scarce.
- Associations between platelet activation and clinical outcomes after peripheral angioplasty have not been investigated.

What does this paper add?

- First study investigating the associations of on-treatment platelet reactivity by multiple platelet function tests, in vivo platelet activation, and protease activated receptor-1-mediated platelet activation with adverse events after infrainguinal angioplasty.
- Response to dual antiplatelet therapy by all test systems was not associated with two-year outcomes after peripheral angioplasty and stenting.
- In vivo and thrombin receptor activating peptide-6-inducible glycoprotein IIb/IIIa activation and P-selectin expression were significantly more pronounced in patients with subsequent adverse events.
- High levels of platelet activation were independent predictors of the composite endpoints.
Conflicts of interest
None declared.

References