High on-treatment platelet reactivity in peripheral arterial disease – a pilot study to find the optimal test and cut-off values

Running head: Platelet reactivity in peripheral arterial disease

Tesse C. Leunissen[*1,2], Stefanie M.O. Peeters Weem[*1], Rolf T. Urbanus[2], Hester M. den Ruijter[3], Frans L. Moll[1], Folkert W. Asselbergs[4], Gert Jan de Borst[1]

*Authors contributed equally to the manuscript

[1] Department of Vascular Surgery, University Medical Center Utrecht, Utrecht, The Netherlands
[2] Department of Clinical Chemistry and Hematology, University Medical Center Utrecht, Utrecht, The Netherlands
[3] Laboratory of Experimental Cardiology, University Medical Center Utrecht, Utrecht, The Netherlands
[4] Department of Cardiology, Division Heart & Lungs, University Medical Center Utrecht, the Netherlands; Durrer Center for Cardiovascular Research, ICIN-Netherlands Heart Institute, Utrecht, the Netherlands; Institute of Cardiovascular Science, Faculty of Population Health Sciences, University College London, London, United Kingdom

Email addresses:
T.C.Leunissen@umcutrecht.nl
S.M.O.PeetersWeem-2@umcutrecht.nl
R.T.Urbanus@umcutrecht.nl
H.M.denRuijter-2@umcutrecht.nl
F.L.Moll@umcutrecht.nl
F.W.Asselbergs@umcutrecht.nl
G.J.deBorst-2@umcutrecht.nl

Corresponding author:
G.J. de Borst
UMC Utrecht, Department of Vascular Surgery
Heidelberglaan 100
3584 CX Utrecht, The Netherlands
G.J.deBorst-2@umcutrecht.nl

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Abstract

Objective:
Restenosis and stent thrombosis after endovascular intervention in patients with peripheral arterial disease (PAD) are potentially tackled by more intensive antiplatelet therapy, such as dual antiplatelet therapy (DAPT) consisting of aspirin and $P2Y_{12}$ inhibitor. Despite clopidogrel treatment, some patients still display high platelet reactivity (HCPR). Tailored antiplatelet therapy, based on platelet reactivity testing, might overcome HCPR. However, more data regarding the proportion of patients with HCPR in the PAD population, different platelet reactivity tests, their correlation and optimal timing for these tests is warranted as stepping stone for a future trial investigating the potential benefit of tailored antiplatelet therapy in PAD patients.

Methods:
Thirty patients on DAPT after percutaneous transluminal angioplasty underwent platelet reactivity testing by VerifyNow, vasodilator-stimulated phosphoprotein (VASP)- and platelet activation assay, and CYP2C19-polymorphism testing.

Results:
Proportion of patients with HCPR measured by VerifyNow varied between 43.3 and 83.3%, depending on the used cut-off values. Testing ≤24 hours after initiation of DAPT lead to a higher proportion of HCPR than testing >24 hours. According to DNA testing, 14.8% was CYP2C19*2 homozygote, 22.2% heterozygote and 63% was CYP2C19*2 negative. VASP-assay revealed 24% HCPR. The highest HCPR-rate was found with VerifyNow cut-off ≤40% inhibition, while the lowest HCPR-rate was found with the VASP-assay. There was a low correlation between the tests.

Conclusion:
HCPR is present in PAD patients and research on HCPR is needed in this population; timing of the tests is relevant and standardization of tests is needed. The optimal conditions for platelet function testing should be determined.

Key words
Platelet inhibitor; antiplatelet therapy; antiplatelet drug resistance; peripheral artery disease; clopidogrel

Introduction

Restenosis and stent thrombosis remain the main challenges after endovascular treatment of peripheral arterial disease (PAD). The one and five year patency of PTA alone is 71% and 49% and with additional stent placement the patency rates increase to 74% and 65% after one and three years.[1–6]

Because of the leading part of platelets in restenosis and stent thrombosis, antiplatelet therapy (APT) is given to prevent these complications. Numerous publications from the Antithrombotic Trialists' Collaboration have concluded the use of aspirin in patients with cardiovascular disease will result in a 25% odds reduction in subsequent cardiovascular events (CVE).[7] The prescription of dual antiplatelet therapy (DAPT), consisting of aspirin and $P2Y_{12}$ inhibitor, for patients undergoing endovascular treatment has increased, although the evidence for DAPT after peripheral endovascular procedures is lacking.[1], [unpublished data]

Clopidogrel, a $P2Y_{12}$ inhibitor, is often the first choice APT added to aspirin, although previous trials have shown that 40% of patients show high platelet reactivity, despite additional clopidogrel treatment.[8] The few trials reporting on high on-clopidogrel platelet reactivity (HCPR) in PAD patients indicate an even higher incidence.[11–11] This phenomenon can in some patients be caused by a mutation in the genes coding for cytochrome P450 2C19 (CYP2C19) activity, a liver enzyme that converts the clopidogrel pro-drug into its active metabolite.[12] Other causes include non-compliance, diabetes mellitus, renal failure and non-smoking.[13–16]

The existence of HCPR has led to the concept of tailored APT, the idea that simply testing platelet reactivity in response to APT and adjusting the regimen based on the results, will lead to improved clinical outcomes.[17] Most research on this concept is performed in cardiac patients undergoing percutaneous coronary intervention. Up to now, clinical trials on tailored APT showed diverging results. Two large clinical trials (GRAVITAS[18], n=2214 and ARCTIC[19], n=2240) showed no difference in primary outcome (composite of cardiovascular death, nonfatal acute myocardial infarction and ST-elevation) or bleeding complications after tailored APT compared to standard therapy. However, few smaller studies did show a beneficial effect of tailored APT compared to standard therapy.[20–22] No studies regarding tailored APT for PAD patients have been performed.

Currently, there is a large need for randomized trials investigating the benefit of (tailored) APT in PAD patients after endovascular treatment. To perform such trials, more knowledge is warranted concerning the proportion of patients with HCPR in this population, the optimal timing, test and cut-off values to identify HCPR .

Therefore, the aim of this pilot study was to display the proportion of patients with HCPR in the PAD population and to evaluate different platelet reactivity tests, their correlation and optimal timing for these tests.

Materials and methods

Study design

The present study was a prospective, observational pilot study with 30 patients. Since no previous trial results were available regarding PAD patients with HCPR, an adequate power calculation was not possible. The study was conducted with approval of the local ethics committee and in accordance with the declaration of Helsinki. All patients gave written informed consent prior to the procedure.

Patient selection

Patients were included if they were adults planned to undergo an endovascular revascularization (PTA, percutaneous transluminal angioplasty) of the superficial femoral artery or popliteal artery and were on aspirin treatment prior to the intervention.
Exclusion criteria were treatment with heparin, oral anticoagulants or $P2Y_{12}$ inhibitors at the time of the PTA, since these patients would not be suitable to receive DAPT post intervention.

Procedures

Participating patients underwent regular PTA with or without additional stenting. Procedures were performed by either the interventional radiologist or vascular surgeon and aspirin was continued during endovascular treatment. Blood was drawn from the arterial access sheath directly prior to the angioplasty for platelet reactivity testing. Prescription of DAPT after intervention was left to the treating physician's discretion. Patients with an indication for DAPT received a loading dose (LD) of 300mg clopidogrel at the same day of the procedure and platelet reactivity tests were performed between 1 and 5 days after the LD. In addition to the platelet reactivity tests, a CYP2C19 polymorphism DNA test was performed using the Spartan RX CYP2C19 DNA testing system (Spartan Bioscience Inc, Ottawa, Canada), to determine the presence of CYP2C19*2 loss of function alleles.

Platelet reactivity measurements

VerifyNow

Platelet reactivity was assessed using the VerifyNow $P2Y_{12}$ assay (Accumetrics®, San Diego, CA, USA), which is a cartridge-based optical detection system utilizing whole blood. Blood was collected in a Greiner Bio-One 3.2% citrate Vacuette tube. Although the VerifyNow is a widely used point-of-care test, different cut-off values are used in clinical and research setting. Some researchers advocate the use of $P2Y_{12}$ reaction units (PRU), while others use the percentage of platelet inhibition as measurement for sufficient response to $P2Y_{12}$ inhibitors. In current literature the most frequent used cut-off value is post PRU <235,[23] although the manual of the VerifyNow advises a cut-off value of <208 PRU.[24] A third commonly used cut-off value is >40% inhibition.[22],[25] We therefore compared these three cut-offs to evaluate the differences in (non)-responders to clopidogrel. At the other end of the spectrum, low platelet reactivity due to clopidogrel treatment, increases the risk of bleeding. Low platelet reactivity is defined as PRU <95 by the VerifyNow manual.

VASP assay

Vasodilator-stimulated phosphoprotein (VASP) is an intracellular platelet protein that is not phosphorylated when the $P2Y_{12}$ receptors are active. Persistent VASP phosphorylation, as measured with flow cytometry, correlates with $P2Y_{12}$ receptor inhibition, reflecting the effect of antiplatelet therapy. Blood was collected in a 0.105 M tri-sodium citrate tube. Flow-

cytometry analysis of VASP phosphorylation was performed using a commercial kit (PLT VASP/P2Y$_{12}$ Test Kit, Biocytex, Marseille, France) and FACS Canto flow cytometer (BD Biosciences, San Jose, USA). Platelet reactivity index (PRI) was calculated and expressed as continuous percentage value (%). PRI >50% is regarded to predict major adverse cardiac events of clinical interest with sensitivity of 100% and is therefore used as cut-off of (non)-responder in this study.[26,27]

PACT

The platelet activation (PACT) assay is a flow cytometry based test, stimulating platelets in whole blood with increasing concentrations of the agonists adenosine diphosphate (ADP) (Roche, Almere, the Netherlands) and SFLLRN (TRAP-6) (Bachem, Weil am Rhein, Germany) in a hydroxyethyl-piperazineethane-sulfonic (HEPES) buffered saline mixture which contains a fixed concentration (1:25) R-Phycoerythrin (RPE)-conjugated anti-P-selectin (BD Pharmingen™, Franklin Lakes, NJ, USA) and (1:50) fluorescein isothiocyanate (FITC)-conjugated antifibrinogen (Dako, Glostrup, Denmark).

Per agonist, wells were filled with a 50 µl assay mixture wherein 5 µl whole blood was pipetted. The mix was homogenised and incubated at room temperature. The reaction was stopped after 20 minutes by pipetting 500 µl fixative solution (0.148% formaldehyde, 137 mM NaCl, 2.7 mM KCl, 1.12 mM NaH2PO4, 1.15 mM KH2PO4, 10.2 mM NaHPO4, 4 mM EDTA, pH 6.8) and analysed on a BD FACS Canto II flow cytometer (BD-biosciences, San Jose, United States) on the same day of processing. Single platelets were gated based on forward and side scatter properties. Fibrinogen binding was used as a measure of αIIbβ3 activation and P-selectin expression as marker of granule release.

The median fluorescent intensity (MFI) of the highest concentration agonist was used to calculate the percentage inhibition between platelet reactivity assessed prior and post intervention (and consequently the LD clopidogrel).

End points

Primary end point was the proportion of patients with high platelet reactivity, based on different platelet reactivity tests and different cut-off values as explained in the previous paragraphs. Secondary aim was to determine the correlation between the different tests.

Statistical analysis

Data were analyzed using Statistical Package for Social Science (IBM SPSS, version 22, IBM Corp., Armonk, NY, USA). Statistical significance was considered at a double-sided $p < .05$. Non-normally distributed data were displayed as median (interquartile range, IQR) and normally distributed data were displayed as mean (standard deviation, SD). Correlation between different platelet reactivity tests was tested using Spearman's rank-order correlation coefficient (rho). Inhibition rates after ADP and TRAP-6 stimulation in the PACT test were compared using the Wilcoxon signed rank test. Results of the CYP2C19 test were considered as ordinal data, with 0 being CYP2C19*2 loss of function allele negative and 2 being two CYP2C19*2 loss-of-function alleles. Bonferroni's correction was used to adjust for multiple testing, when necessary.

Results

Patients and procedures

Thirty-five out of 50 consecutive patients undergoing endovascular treatment for superficial femoral or popliteal atherosclerosis received postoperative DAPT. Thirty of them were tested for platelet reactivity and could be included in the analyses. Median time between LD and platelet reactivity testing was 1 day (IQR 1 - 3). A summary of baseline characteristics is displayed in table 1.

VerifyNow

Proportion of patients with high on clopidogrel platelet reactivity

The cut-off value PRU ≥235 identified 13 out of 30 included patients (43.3%) as non-responders. The cut-off value PRU ≥208 identified 18 patients (60.0%) as non-responders while the cut-off value ≤40% identified 25 patients (83.3%) as non-responders (table 2).

Proportion of patients with low platelet reactivity (PRU <95)

Three patients displayed low platelet reactivity (PRU 30, 30 and 35 and inhibition 89%, 90% and 85%, respectively).

Timing of platelet reactivity measurements

VerifyNow was mostly performed within 24 hours after LD clopidogrel (60%). Of the 18 patients tested at day one, 8 patients (44.4%) were non-responder with cut-off value PRU ≥235, 13 patients (72.2%) with cut-off value PRU ≥208 and all 18 patients (100%) were non-responder when applying the cut-off level of 40% inhibition. Of the 12 patients measured beyond day one, five patients (41.7%) were non-responders with both cut-off values PRU ≥235 and PRU ≥208 and seven patients (58.3%) were non-responder with cut-off value ≤40% inhibition. Exact numbers of non-responders at different time points are displayed in table 3. The three patients with low platelet reactivity were measured at day 3, 4 and 5 post intervention.

Four patients were tested at two different time intervals. The first measurement was on the first day postoperative and the second VerifyNow between day 5 and 21. They were tested twice because there was a suspicion that the interval between the LD and VerifyNow was insufficient and the treating physician didn't want to switch regime based on these test results. In three patients, the second test showed substantially more inhibition than the first test, the fourth patient persistently displayed 0% inhibition after 21 days. Results of patients tested at different intervals are summarized in table 4. All four patients were CYP2C19*2 negative.

Vasodilator-stimulated phosphoprotein (VASP) assay

proportion of patients with high on clopidogrel platelet reactivity

In the 25 tested patients, the Platelet Reactivity Index varied widely (median 71.3%, IQR 53.2 – 84.5). Only six patients (24%) were identified as good responders to clopidogrel. From these responders, three patients were acknowledged by VerifyNow cut-off value inhibition ≤40%, five by cut-off PRU ≥235 and four PRU ≥208 as good responders. One patient showed very low platelet reactivity (PRI=0%), which was confirmed by VerifyNow PRU 35 and inhibition of 85%.

PACT results

The median inhibition of fibrinogen binding was 53.8% (IQR 14.5 - 75.1%) after stimulation with ADP and 40.3% (IQR 21.7- 61.6%) after stimulation with TRAP-6, which is not

significantly different (p=0.459). Granule release, measured as P-selectin expression, after stimulation with ADP was inhibited significantly stronger (median inhibition 40.7%, IQR 12.5 - 69.6%) than granule release after stimulation with TRAP-6 (median 1.5%, IQR 0.0- 34.0%, P<0.001). Despite the $P2Y_{12}$ treatment, no inhibition of granule release after TRAP-6 stimulation was observed in 11 patients (42.3%). The inhibitory effect of clopidogrel was more evident in the $P2Y_{12}$ signaling pathway than in the thrombin signaling pathway.

CYP2C19*2 polymorphism

CYP2C19*2 polymorphism DNA testing was performed in 29 patients. Two patients displayed inconclusive results and were counted as missing values. Of 27 remaining patients, 17 had no CYP2C19*2 variant (63.0 %), six patients were heterozygous for the CYP2C19 *2 allele (22.2 %), and 4 patients were homozygous for CYP2C19*2 (14.8 %). Results of the platelet reactivity and DNA tests for all included patients are displayed in supplementary table 1 (online only).

Strategies in patients with HCPR

Ten out of 30 included patients switched to prasugrel during follow-up, based on the VerifyNow results with cut-off level <40% inhibition. Two of them had two CYP2C19*2 alleles, four had one CYP2C19*2 allele, three were negative and one was missing.

Correlation between different platelet reactivity test results

There was a significant correlation between the percentage inhibition of the VerifyNow and inhibition of fibrinogen binding after TRAP stimulation, measured with PACT (rho = 0.541, p = 0.004, table 5), but no significance was found after correction for multiple testing. No other significant correlations between VerifyNow inhibition and PACT test results could be demonstrated. A significant correlation was seen between the results of the VASP assay and fibrinogen binding after ADP stimulation (rho = -0.606, p = 0.002, table 5 and figure 1).

Within the PACT, there was a significant correlation between inhibition in fibrinogen binding after ADP stimulation and all three other PACT tests separately (ADP stimulated granule relase: rho= 0.931, P <0.001; fibrinogen binding after TRAP stimulation: rho= 0.458, p=0.019 and TRAP stimulated granule release: rho= 0.464, p=0.017). After correction for multiple testing only the correlation between inhibition on fibrinogen and granule release after ADP stimulation remained statistically significant. Results of the correlation tests are summarized in table 5.

Discussion

Our prospective explorative study on thirty patients with PAD undergoing endovascular treatment with platelet reactivity testing revealed that the proportion of HCPR was highly dependent on the utilized platelet reactivity tests and cut-off values. Prevalence of HCPR varied between 43.3-83.3% using different cut-offs for the VerifyNow. The instructions state that the optimal cut-off value is PRU ≥208,[25] but in current literature ≥234 or ≥235 are more common.[9, 10, 23, 28] The VASP-assay displayed HCPR in 76% of the patients. Based on this pilot study, HCPR is common in PAD patients undergoing endovascular treatment.

An extensive meta-analysis evaluating platelet reactivity in various patients on antiplatelet therapy, assessed with numerous platelet reactivity tests showed HCPR in 40.4% of the pooled patients, based on 59 studies with 34.776 patients. [8] The few studies investigating platelet reactivity in PAD, all showed a higher incidence of HCPR compared to patients with coronary artery disease, confirming the relevance of HCPR in PAD patients.[9–11]

In this study, the test results seemed to be highly dependent on timing after LD clopidogrel. Most patients were tested <24 hours after the LD of 300 mg clopidogrel, according to the advised minimum of 8 hours after LD.[24] This advice is based on a single study examining platelet inhibition after different loading dosages of clopidogrel, tested hourly up to 7 hours post LD and daily for 5 days after discontinuation of daily treatment. However, when re-evaluating these study results, we agree that maximum platelet inhibition was reached after 6 hours post loading with 600 or 900 mg clopidogrel, but we disagree that maximum platelet inhibition is reached in patients after loading with 300 mg.[29] However, it is unclear whether maximum inhibition is necessary for clinically effective inhibition.

This study shows that most non-responders were diagnosed in patients tested <24 hours after LD and even reached 100% non-responders with cut-off value <40% inhibition. However, four patients displaying 0% inhibition at day 1, were tested a few days later and three showed notably higher inhibition levels (PRU<208). We suspect that testing <24 hours after LD clopidogrel does not reflect maximal achieved platelet inhibition. Different time periods of platelet reactivity testing need to be investigated. Since restenosis mostly occurs within the first six months after PTA, adequate platelet inhibition during the first days after intervention is of particular importance.[30],[31] We therefore suggest trials investigating platelet reactivity testing within 3 to 5 days after DAPT initiation.

The different tests performed in this study showed negligible correlation. Partially this might be explained by suboptimal timing and lack of power, but even more by the different mechanisms of action. Previous studies have evaluated the agreement between VerifyNow and VASP-assay with divergent conclusions.[32,33] Although both tests reflect $P2Y_{12}$-receptor blockage, the tests results might not be interchangeable, since different aspects of platelet activation are measured. A trial investigating the superiority of either of these tests is desirable.

Furthermore, a combination of tests might be necessary for accurate monitoring of the platelet function. Platelets do not only play part in thrombus formation but also in wound healing, angiogenesis and inflammation. In the acute phase after intervention, the main goal of antiplatelet therapy is preventing thrombus formation. However, APT is also prescribed to prevent secondary CVEs.[34],[35] With regard to the latter, other expressions of platelet activation than aggregate formation (VerifyNow) might be more relevant and should be tested in platelet reactivity testing. More studies on differential platelet activation and the clinical consequences should be assessed.

The study holds some limitations: since the study design was observational, there was no consistent policy for the patients with HCPR. Some patients switched to prasugrel

and some continued the clopidogrel treatment without adjustment, according to physician's discretion. This study is underpowered for conclusions about the optimal treatment strategy in HCPR. Previous studies showed that increasing the dose of clopidogrel is less effective than switching to prasugrel in patients with HCPR, so switching to another drug is recommended.[20,36]

Conclusion

This pilot study in patients with PAD undergoing PTA shows a high percentage of HCPR. Large variance in proportion HCPR exists between the different platelet reactivity tests (VerifyNow, VASP-assay and PACT). Future studies are needed to determine the timing of testing and the optimal combination of platelet reactivity testing before studies regarding tailored antiplatelet therapy in PAD patients can be performed.

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Table 1. Baseline characteristics of included patients

Baseline characteristics of included patients (n=30)	
Male sex, n (%)	20 (67)
Age, years (SD)	70.5 (8.61)
Cardiovascular risk factors, n (%)	
Diabetes mellitus	13 (43)
Dyslipidemia	23 (77)
Hypertension	28 (93)
Smoking, current or previous	26 (87)
History of coronary artery disease	15 (50)
History of CVA / TIA	7 (24)
Fontaine classification, n (%)	
Fontaine IIa	2 (7)
Fontaine IIb	19 (63)
Fontaine III	4 (13)
Fontaine IV	5 (17)
Medication at baseline, n (%)	
Aspirin	30 (100)
Dipyridamole	3 (10)
Statins	24 (80)
ACE-inhibitors	19 (63)
β-blockers	14 (47)
Diuretics	16 (53)
Proton pump inhibitors	15 (50)

Table 2. Numbers of non-responders with different cut-off values of VerifyNow

Cut-off method	Responders (%)	Non-responders (%)
Cut-off PRU < 235	17 (56.7)	13 (43.3)
Cut-off PRU < 208	12 (40.0)	18 (60.0)
Cut-off inhibition > 40%	5 (16.7)	25 (83.3)

PRU indicates $P2Y_{12}$ reactive units

Table 3. Days between start dual antiplatelet therapy (DAPT) and VerifyNow with corresponding numbers of non-responders and responders

Cut-off method		Days between start DAPT and VerifyNow							
		1	**2**	**3**	**4**	**5**	**12**	**16**	**24**
PRU <235	non-responder	8	2	2	0	0	1	0	0
	responder	10	1	1	1	2	0	1	1
PRU<208	non-responder	13	2	2	0	0	1	0	0
	responder	5	1	1	1	2	0	1	1
>40% inhibition	non-responder	18	3	2	0	1	1	0	0
	responder	0	0	1	1	1	0	1	1

PRU indicates $P2Y_{12}$ reactive units

Table 4. VerifyNow results of patients tested at different intervals

Day	VN baseline	VN post	VN inhibition	Day	VN baseline	VN post	VN inhibition
1	226	222	2%	**13**	289	54	81%
1	272	249	9%	**5**	255	187	27%
1	223	277	0%	**21**	252	253	0%
1	255	273	0%	**6**	228	187	18%

VN indicates VerifyNow

Table 5. Correlation between different platelet reactivity tests according to Spearman's correlation coefficient (rho)

		VN (%)	ADP fibrinogen	ADP granule release	TRAP fibrinogen	TRAP granule release	CYP2C19 DNA test	VASP
VN (%)	Rho		0.369	0.385	0.541	-0.019	-0.476	-0.224
	p		0.063	0.052	0.004	0.926	0.014	0.282
	N		26	26	26	26	26	25
ADP fibrinogen	Rho	0.369		0.931	0.458	0.464	-0.394	-0.606
	p	0.063		**<0.001**	0.019	0.017	0.057	**0.002**
	N	26		26	26	26	24	23
ADP granule release	Rho	0.385	0.931		0.335	0.537	-0.456	-0.493
	p	0.052	**<0.001**		0.094	0.005	0.025	0.017
	N	26	26		26	26	24	23
TRAP fibrinogen	Rho	0.541	0.458	0.335		0.010	-0.231	-0.351
	p	0.004	0.019	0.094		0.960	0.277	0.101
	N	26	26	26		26	24	23
TRAP granule release	Rho	-0.019	0.464	0.537	0.010		-0.124	-0.491
	p	0.926	0.017	0.005	0.960		0.564	0.017
	N	26	26	26	26		24	23
CYP2C19 DNA test	Rho	-0.476	-0.394	-0.456	-0.231	-0.124		0.272
	p	0.014	0.057	0.025	0.277	0.564		0.209
	N	26	24	24	24	24		23
VASP	Rho	-0.224	-0.606	-0.493	-0.351	-0.491	0.272	
	p	0.282	**0.002**	0.017	0.101	0.017	0.209	
	N	25	23	23	23	23	23	

VN indicates VerifyNow; VASP, vasodilator-stimulated phosphoprotein phosphorylation

Figure 1. Correlation between the results of the VASP assay and fibrinogen binding after ADP stimulation