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Which platelet function test is suitable to monitor clopidogrel responsiveness?

A pharmacokinetic analysis on the active metabolite of clopidogrel

Short title: Bouman *et al.* – Monitoring clopidogrel responsiveness

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Summary

Background

Multiple platelet function tests claim to be P2Y12-pathway specific and capable of capturing the biological activity of clopidogrel.

Objectives

The aim of the present study was to determine which platelet function test provides the best reflection of the *in vivo* plasma levels of the active metabolite of clopidogrel (AMC).

Patients/Methods

Clopidogrel-naive patients scheduled for elective percutaneous coronary intervention received a 600 mg loading dose of clopidogrel and 100 mg of aspirin. For pharmacokinetic analysis, blood was drawn at 0, 20, 40, 60, 90, 120, 180, 240 and 360 minutes after clopidogrel loading and peak plasma concentrations (c_{max}) of the AMC were quantified with liquid chromatography-tandem mass spectrometry. Platelet function testing was performed at baseline and 360 minutes after clopidogrel loading.

Results

The VASP-assay, the VerifyNow P2Y12-assay and 20 µmol/L adenosine diphosphate (ADP)-induced light transmittance aggregometry (LTA) showed strong correlations with c_{max} of the AMC (VASP: R^2=0.56, $p<0.001$; VerifyNow platelet reactivity units (PRU): R^2=0.48, $p<0.001$; VerifyNow %inhibition: R^2=0.59, $p<0.001$; 20 µmol/L ADP-induced LTA: R^2=0.47, $p<0.001$). Agreement with c_{max} of the AMC was less evident for 5 µmol/L ADP-induced LTA or whole blood aggregometry (WBA), while the IMPACT-*R* ADP test did not show any correlation with plasmalevels of the AMC.

Conclusion

The flowcytometric VASP-assay, the VerifyNow P2Y12 assay and, though to a lesser extent, 20 µmol/L ADP induced LTA correlate best with the maximal plasma level of the AMC, suggesting these may be the preferred platelet function tests for monitoring the responsiveness to clopidogrel.

Key-words: active metabolite, clopidogrel, monitoring, pharmacokinetics, platelet function test, responsiveness.

Introduction

Clopidogrel is administered as an inactive prodrug requiring metabolization by the hepatic cytochrome P450 system for its antiplatelet activity[1]. The formed active thiol metabolite of clopidogrel (AMC) irreversibly inhibits adenosine diphosphate (ADP) mediated platelet activation and aggregation by antagonizing the P2Y12-receptor[2]. Although clopidogrel has proven its efficacy in reducing atherothrombotic events after PCI, a considerable interindividual variability in response has been reported[3]. Clinical, genetic and pharmacokinetic factors contribute to this wide variability in response and it has been demonstrated that the absolute magnitude of ADP-induced platelet reactivity at the time of PCI is associated with an increased risk of atherothrombotic events, including stent thrombosis[4-11]. It is therefore of utmost importance to have a confident parameter of the *in vivo* activity of clopidogrel.

Multiple platelet function tests claim to be P2Y12-pathway specific and to be capable of capturing the specific biological activity of clopidogrel[12]. Since the AMC is prone to rapid inactivation of its reactive thiol group[13], quantitative methods for the detection of the AMC require a sophisticated stabilization protocol. Data on the performance of different platelet function tests in relation to plasma levels of the AMC are therefore scarce.

In the present study we sought to investigate which of the currently available platelet function tests - if any - provides the best representation of the *in vivo* plasma levels of the active metabolite of clopidogrel.

Methods

Patient population

Consecutive patients with stable angina pectoris scheduled for PCI were eligible. All patients were on 80-100 mg aspirin therapy daily. Exclusion criteria were a history of bleeding

diathesis, presence of an acute coronary syndrome (ACS), platelet count <$150x10^9$/L, the use of a glycoprotein IIb/IIIa inhibitor or a coumarin within the last 14 days or any contraindication to clopidogrel or aspirin. The study protocol complied with the declaration of Helsinki and was approved by the ethical committee of our institution, and all patients gave written informed consent for participation.

Study protocol and blood sampling

All eligible patients visited the outpatient clinic for platelet function evaluation, physical examination and a standardized interview. All patients received a witnessed 600 mg loading dose of clopidogrel and 100 mg of aspirin.

Blood samples for platelet function evaluation were drawn from the antecubital vein with a loose tourniquet and collected in citrated (3.2%) non-vacuum tubes (Sarstedt, Nümbrecht, Germany) before and 6 hours after the clopidogrel loading dose. All blood samples were processed within 2 h after collection.

Blood samples for determining the AMC plasma concentration were collected from the antecubital vein in tubes containing K_3-EDTA at 0, 20, 40, 60, 90 120, 180, 240 and 360 minutes after the clopidogrel loading dose. Samples were immediately centrifugated at 1500 g for 10 minutes and plasma was pipetted into tubes containing a stabilizing agent (Pat. No. DE 10 2004 046 159.7)[14] in order to prevent degradation of the AMC. After vortexing for 60 seconds, samples were stored at $–80^0$C until analysis of the AMC plasma concentration with liquid chromatography tandem mass spectrometry (LC-MS/MS).

Platelet Function testing

Platelet function was evaluated using five different ADP-induced platelet function tests. All tests were performed according to the manufacturer's recommendations or according to generally accepted standard procedures.

1. Light transmittance aggregometry (LTA)

LTA was quantified in non-adjusted platelet-rich plasma on a four-channel APACT 4004 aggregometer (LABiTec, Arensburg, Germany). Platelet-poor-plasma was set as 100% aggregation and after stimulation of platelet aggregation with ADP in final concentrations of 5 and 20 µmol/L, both peak (maximal) and late (at 360 seconds) aggregation (%) were measured[15,16].

2. Whole-blood Aggregometry (WBA)

Whole-Blood aggregometry (WBA) was performed in citrated whole blood as described previously[17]. In brief, citrated whole blood was diluted 1:1 with pre-warmed (37°C) saline. Platelet aggregation was induced by the addition of 10 µmol/L ADP and the increase in impedance caused by aggregated platelets between the two electrodes was recorded on a ChronoLog 700 model Aggregometer (ChronoLog, Havertown, PA, USA). The results are reported as the maximal amplitude of impedance (Ω) after 10 minutes.

3. IMPACT-*R* test

The Impact-*R* device (DiaMed, Cresier, Switzerland) is based on the cone and plate(let) analyzer technology[18]. Citrated whole-blood samples were incubated for 1 minute with 1.38 µmol/L ADP, causing the formation of micro platelet aggregates in nonresponsive samples. A volume of 130 µL of this ADP-pre-incubated whole blood sample was pipetted on a polystyrene well and subjected to shear (1800 s^{-1}) for 2 minutes, using a rotating cone. The wells were washed and stained followed by measurement of the percentage of surface coverage (SC) from shear-induced platelet adhesion and aggregation with an image analysis

system. The percentage SC is inversely correlated with the magnitude of ADP-induced platelet activation.

4. The VerifyNow® P2Y12 assay

The VerifyNow® P2Y12 assay (Accumetrics, San Diego, CA, USA) is based on optical detection of platelet aggregation in whole blood[19]. The assay contains 20 µmol/L ADP to induce platelet aggregation and 22 nmol/L prostaglandin E_1 to suppress the undesirable contribution from ADP-agonism of P2Y1-receptors to platelet aggregation. The magnitude of ADP-induced platelet activation is expressed as P2Y12 reaction units (PRU). In addition, the device calculates the percentage of P2Y12-inhibition, based on TRAP-induced platelet aggregation ('Base value') and PRU.

5. Flowcytometric Vasodilator-Stimulated Phosphoprotein (VASP)-analysis

Flowcytometric analysis of VASP phosphorylation was performed using a commercially available kit from Biocytex (Marseille, France)[10]. In brief, citrated blood was incubated with either PGE_1 or PGE_1+ADP and fixed with paraformaldehyde, after which platelets were permeabilized followed by immunolabeling with a CD61 phycoerythrin-labeled platelet specific antibody and a FITC-labeled VASP-P specific mouse monoclonal antibody or a negative isotopic control antibody. Samples were analyzed on a 500 MPL flowcytometer (Beckman Coulter). The magnitude of platelet activation was expressed as the platelet reactivity index (PRI), which can be calculated from the mean fluorescence intensity (MFI) of samples incubated with PGE1 or PGE1+ADP using the following formula:

$$PRI\ (\%) = [\ MFIc_{(PGE1)} - MFIc_{(PGE1\ +\ ADP)}\ ] / [\ MFIc_{(PGE1)}\ ] \times 100.$$

Liquid chromatography-tandem mass spectrometry (LC-MS/MS)

Analysis and detection of the active thiol metabolite were performed as previously described on a triple-quadruple tandem mass spectrometer (TSQ Quantum, Thermo Electron, Dreieich,

Germany)[20]. Measurements were performed in triplicate using a Surveyor HPLC system. Plasma concentration versus time data of each patient were fitted by a one-compartment first order model (Bateman function r > 0.94) using WinNolin^{TM} Software (Pharsight, Palo Alto, CA, USA). The maximal plasma concentration (c_{max}, ng/mL) was calculated from the individual regression fits.

Sample size calculation and statistics

The t-test for point biserial correlation based on the noncentrality parameter >delta< was used for a priori sample size calculation assuming a two-sided significance level of a=0.05 and a power 1-b=0.95. From previous assessments (and from the clinical perspective that a diagnostic test should explain at least 50% of the variance of a variable) a determination coefficient of R^2=0.5 was deemed relevant[20]. The total required sample size was calculated to be 16. Platelet function testing results were related to the c_{max} of the AMC, since this appeared to be the pharmacokinetic parameter correlating best with inhibition of platelet aggregation (IPA) in a previous study[20]. Continuous variables are expressed as mean ± SD and categorical variables as frequencies (%). The individual clopidogrel responsiveness, defined as the absolute decrease in platelet reactivity from baseline to 6 hours after clopidogrel loading, was tested using a paired t-test. The coefficient of determination (R^2) was calculated to test the linear association between the c_{max} of the AMC and the absolute inhibition of platelet aggregation (IPA) at 6 h after clopidogrel loading. Additionally, a normalized ANOVA was performed on the ratio "% platelet inhibition ([baseline-T6]/baseline) / c_{max} of the AMC", to determine the statistical significance of the observed differences between platelet function tests with regard to their correlation with plasma levels of the AMC. All Statistical analyses were performed with SPSS (version 15.0, SPSS Inc., Chicago, IL, USA) and p-values < 0.05 were considered statistically significant.

Results

A total of 20 consecutive patients were enrolled. Clinical characteristics of the studied population are summarized in *table 1*. All patients were on aspirin therapy (80-100 mg/day) at the time of inclusion.

Responsiveness to a 600 mg loading dose of clopidogrel

All platelet function tests were able to detect a significant reduction in platelet function 6 h after clopidogrel loading dose administration ($p<0.001$ for all), except the IMPACT-*R* ADP-test ($p=0.13$). Despite the observed inhibitory shift in test results after clopidogrel administration, some overlap between baseline and post-clopidogrel values was notable for all platelet function tests.

Correlation between platelet function tests

Results of the VASP, VerifyNow P2Y12 assay and 20 µmol/L ADP induced LTA showed moderate to good correlation with one and another, but lacked significant correlation with WBA and the IMPACT-R ADP-test (*table 2*). Agreement between LTA induced by 5 µmol/L ADP and the other tests was less explicit, showing a moderate correlation with 20 µmol/L ADP induced LTA and the VerifyNow P2Y12 assay, but no correlation at all with the VASP-assay, nor with the WBA and the IMPACT-R ADP-test.

Correlation between IPA and plasmalevels of the AMC

Linear regression analysis revealed major differences between the various platelet function tests regarding correlations between IPA and the c_{max} of the AMC. The highest correlation coefficients were observed for the flowcytometric VASP-assay ($R^2=0.56$, $p<0.001$; *figure 1*) and the VerifyNow P2Y12 assay ($R^2=0.48$, $p<0.001$ for PRU and $R^2=0.59$, $p<0.001$ for %

inhibition). The absolute change in platelet reactivity when measured with 20 µmol/L ADP induced LTA also showed strong correlations with the c_{max} value of the AMC (R^2=0.46, *p=0.001* for peak and R^2=0.47, *p<0.001* for late IPA; *figure 2*). In contrast, no significant association was observed between the c_{max} value of the AMC and IPA when measured with LTA using a lower concentration of 5µmol/L ADP (peak: R^2=0.19, *p=0.06*; late: R^2=0.02, *p=0.56*) or WBA (r=0.27, *p=0.28*; *figure 2,3*). The IMPACT-R ADP-test did not show any correlation with c_{max} of the AMC (*oure 3*). The normalized ANOVA revealed that the IMPACT-R ADP-test was significantly inferior compared to all other platelet function tests (p values for comparisons with all other tests were <0.05), while no statistical differences were observed between all other platelet function tests (p>0.05).

Discussion

The magnitude of platelet inhibition varies widely according to the platelet function assay used in monitoring responsiveness to clopidogrel. The current recommendations issued by the American College of Cardiology (ACC), the American Heart Association (AHA), and the Society for Cardiovascular Angiography and Interventions (SCAI) state the following: "In patients in whom stent thrombosis may be catastrophic or lethal (unprotected left main, bifurcating left main, or last patent coronary vessel), platelet aggregation studies may be considered and the dose of clopidogrel increased to 150 mg per day if <50% inhibition of platelet aggregation is demonstrated"[21]. However, these guidelines do not further specify any details on which test or test conditions should be used. This puts the clinician in a difficult situation since multiple platelet function tests are now widely available but little is known about their ability to provide a reliable reflection of the *in vivo* biological activity of clopidogrel.

The present study demonstrates that the flowcytometric VASP-assay and the VerifyNow P2Y12-assay are the most appropriate platelet function tests to monitor peak plasma levels of the AMC, achieved by a 600mg loading dose of clopidogrel. Therefore, these platelet function tests are likely to be the most accurate in measuring the actual *in vivo* biological activity of clopidogrel.

LTA is commonly recognized as the "gold standard" platelet function test and showed fine correlations with peak plasma levels of the AMC as well, though only when platelet aggregation was stimulated with a high concentration of ADP (20 µmol/L). LTA induced by 5 µmol/L lacks significant correlation with peak plasmalevels of the AMC, and should therefore not be used to determine clopidogrel responsiveness. Both the flowcytometric VASP-assay and the VerifyNow P2Y12-assay incorporate PGE_1 to decrease the contribution of ADP-agonism at the P2Y1-receptor to platelet aggregation, thereby increasing the selectivity of these tests for the P2Y12-pathway[22, 23]. This might indeed be reflected by the high agreement between IPA and the c_{max} of the AMC when measured with these tests. Using a higher concentration of the agonist ADP (20 µmol/L) appears to improve the assessment of the responsiveness to clopidogrel as well, since 20 µmol/L ADP-induced LTA showed a higher degree of correlation with peak plasma levels of the AMC than 5 µmol/L ADP-induced LTA.

Although the VASP-assay has the advantage of providing stable test results until 24 hour after blood drawing, both the VASP-assay and LTA are relatively labour intensive[24]. In contrast, the point-of-care VerifyNow P2Y12 assay is suitable for use in daily clinical practice, since the semi-automated technique allows rapid assessment of platelet inhibition in whole blood without the need for expert laboratory personnel. The accuracy of the Impact-*R* ADP-test in monitoring clopidogrel-responsiveness might be seriously hampered by the requirement of multiple sample preparation proceedings that are sensitive to introduction of variation in test

results by the technician performing the test. Furthermore, the Impact-*R* ADP-test relies on a mechanism of platelet activation induced by shear stress, which is a different aspect of platelet reactivity, and apparently not a very suitable method for monitoring inhibition of the P2Y12-pathway.

The present study shows that the flowcytometric VASP, the VerifyNow P2Y12-assay and 20 µmol/L ADP induced LTA are the most appropriate tests for determining the *in vivo* plasma levels of the AMC. In addition, these tests have previously been shown to predict clinical outcome in patients treated with clopidogrel[5-10]. Poor responsiveness to clopidogrel is however one out of multiple factors contributing to the development of atherothrombotic events. Furthermore, the magnitude of post-treatment platelet reactivity is a composite of both clopidogrel responsiveness as well as pre-treatment (baseline) platelet reactivity, resulting in a high on-treatment platelet reactivity in some patients showing a sufficient response to clopidogrel[25, 26]. Hence, other platelet function tests evaluating different aspects of platelet function, may as well be capable of predicting clinical outcome in patients on clopidogrel, despite the lack of correlation with plasma levels of the AMC.

The present study describes the level of correlation of different ADP-induced platelet function tests with peak plasma levels of the AMC. This important message might resolve the ongoing debate on which platelet function test is suitable for monitoring clopidogrel responsiveness. Two aspects have to be addressed that need to be explored to further improve the clinical applicability of this message. First, the present study does not cover the complete subset of platelet function tests available for monitoring clopidogrel-responsiveness. Additional tests include the Multiplate and the thromboelastography, which might show correlation with the plasma levels of the AMC as well. Second, although the present study contains a sufficient number of patients for a pharmacokinetic analysis, it does not allow an answer to the question whether patients with low plasma levels of the AMC are the ones to develop atherothrombotic

complications after PCI. Nonetheless, combining the results of the present study with the already available evidence on the clinical efficacy of clopidogrel in reducing atherothrombotic events after PCI suggests a positive answer to that question.

In conclusion, the flowcytometric VASP-assay, the VerifyNow P2Y12 assay and, though to a lesser extent, 20 µmol/L ADP induced LTA correlate best with the maximal plasma level of the AMC, suggesting these may be the preferred platelet function tests for monitoring the responsiveness to clopidogrel. Further distinction between these tests should be based on their labour intensiveness, costs, and most importantly their sensitivity and specificity in predicting the occurrence of atherothrombotic events after PCI.

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b) Acknowledgements

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c) Disclosure

Conflict of Interest: none declared.

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Figure Titles and Legends

Figure 1: Correlation between the c_{max} of the AMC (ng/mL) and IPA assessed using the flowcytometric VASP-assay (upper graph) and the VerifyNow P2Y12 assay, both PRU (middle graph) and % inhibition (lower graph). IPA was calculated as Δ [baseline-T6] of the results for each platelet function test. Solid lines represent the linear regression fit to the data, dashed lines indicate 95% confidence intervals. The coefficient of determination (R^2) and the corresponding p-values are represented in the separate graphs.

Figure 2: Correlation between the c_{max} of the AMC (ng/mL) and IPA assessed using the ADP-induced LTA using 5 µmol/L and 20 µmol/L (upper and lower panels, respectively) ADP-induced peak and late (at 360 sec.) aggregation (left and right panels, respectively). IPA was calculated as Δ [baseline-T6] of the results for each platelet function test. Solid lines represent the linear regression fit to the data, dashed lines indicate 95% confidence intervals. For each

test, the coefficient of determination (R^2) and the corresponding p-values are represented in the separate graphs.

Figure 3: Correlation between the c_{max} of the AMC (ng/mL) and IPA assessed using the ADP-induced whole blood impedance aggregometry (WBA) and the IMPACT-*R* ADP test. IPA was calculated as Δ [baseline-T6] of the results for each platelet function test. Solid lines represent the linear regression fit to the data, dashed lines indicate 95% confidence intervals. For each test, the coefficient of determination (R^2) and the corresponding p-values are represented in the separate graphs.

Tables

Table 1: Baseline characteristics

Baseline characteristics (n=20)

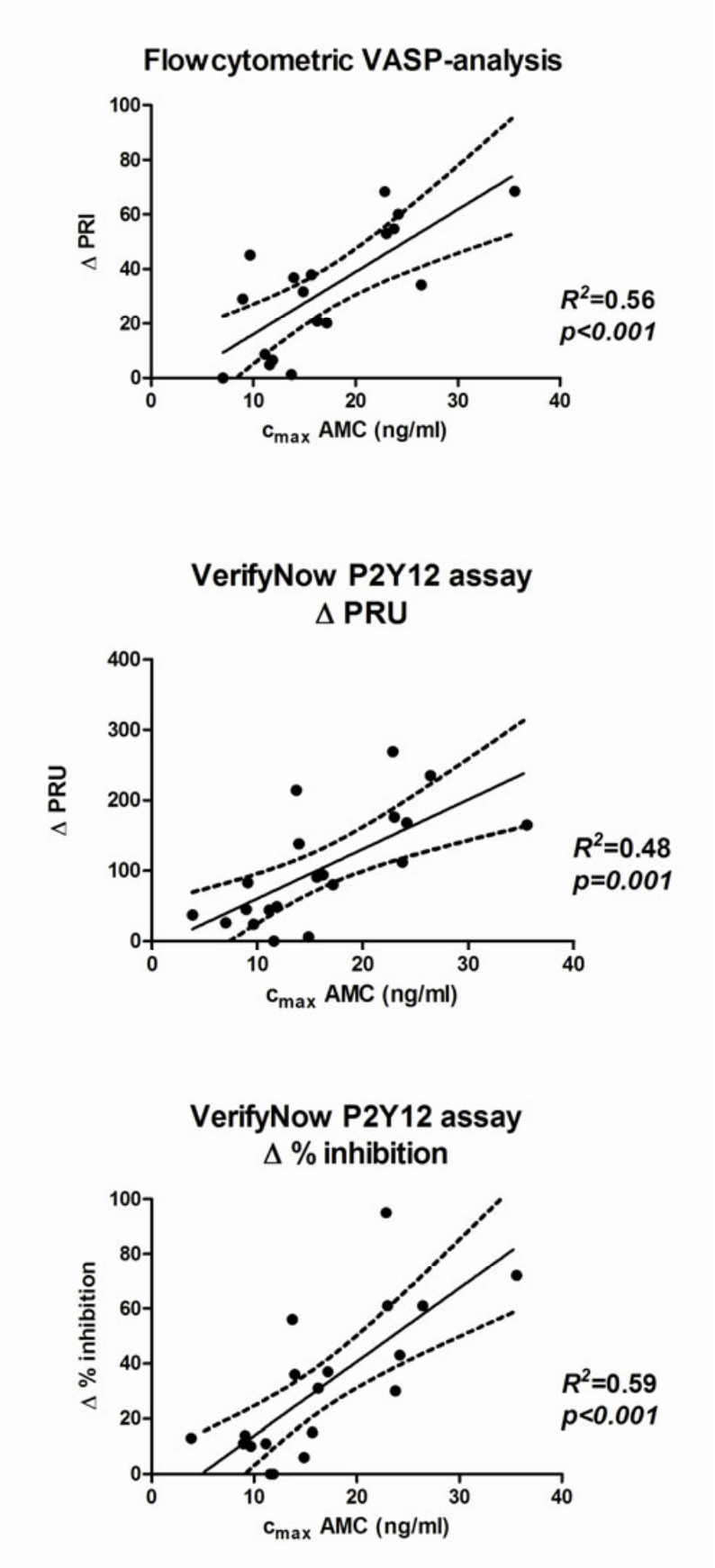
Age (yrs)	60.2 ± 10.3
Men	19 (95%)
Risk Factors	
Hypertension	13 (65%)
BMI (kg/m^2)	27.90 ± 3.31
Diabetes Mellitus	2 (10%)
Dyslipidemia	13 (65%)
Current smoker	12 (60%)
Familial history of CAD	12 (60%)
Previous MI	9 (45%)
Previous PCI	9 (45%)
Concomitant medication	
Aspirin	20 (100%)
ACE inhibitor	8 (40%)
Beta-blocker	14 (70%)
Statin	18 (90%)
Laboratory parameters	
Platelet count in whole blood (x 10^9/L)	242 ± 53
Platelet count in PRP (x 10^9/L)	356 ± 96
Hemoglobin (mmol/L)	8.9 ± 0.5

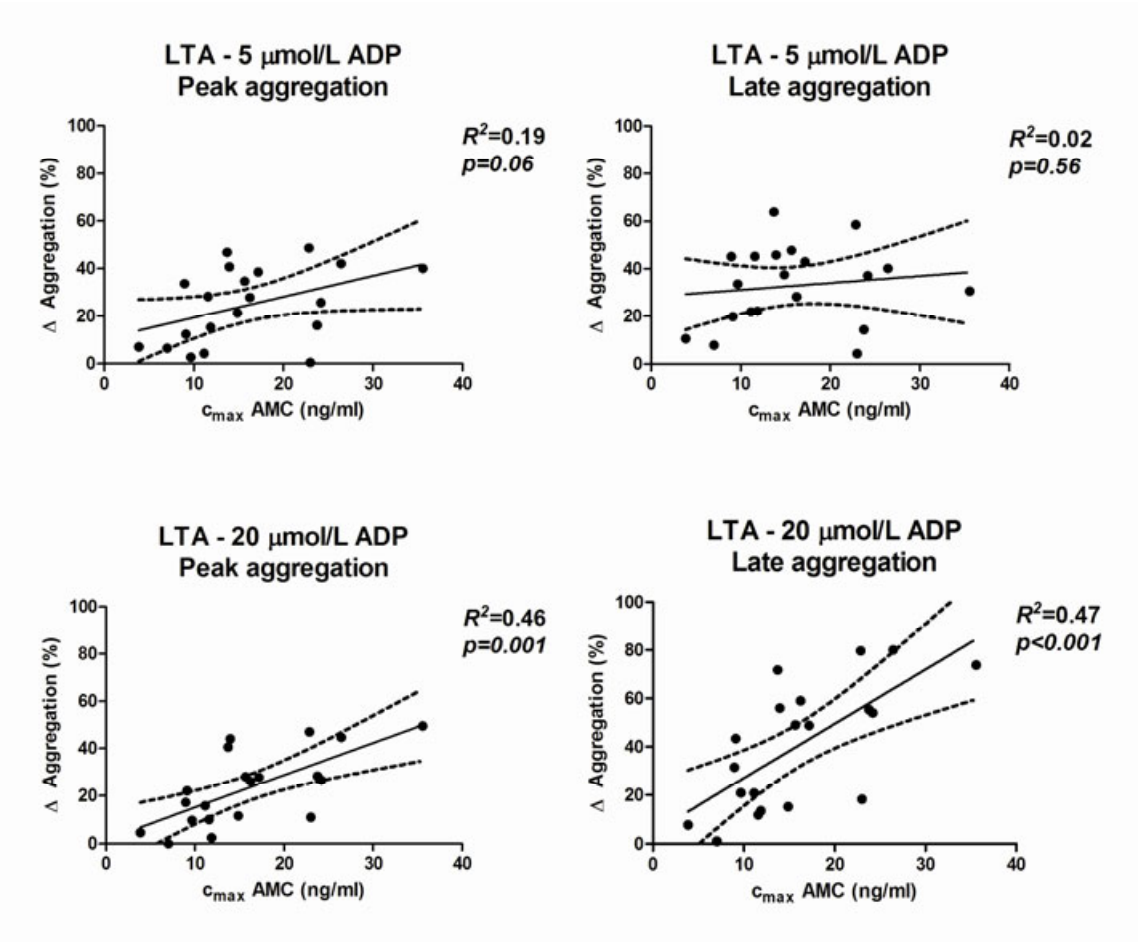
Continuous variables are presented as mean ± SD, and categorical variables as counts (%). BMI=body mass index, CAD=coronary artery disease, MI= myocardial infarction, PCI= percutaneous coronary intervention, PRP= platelet-rich plasma

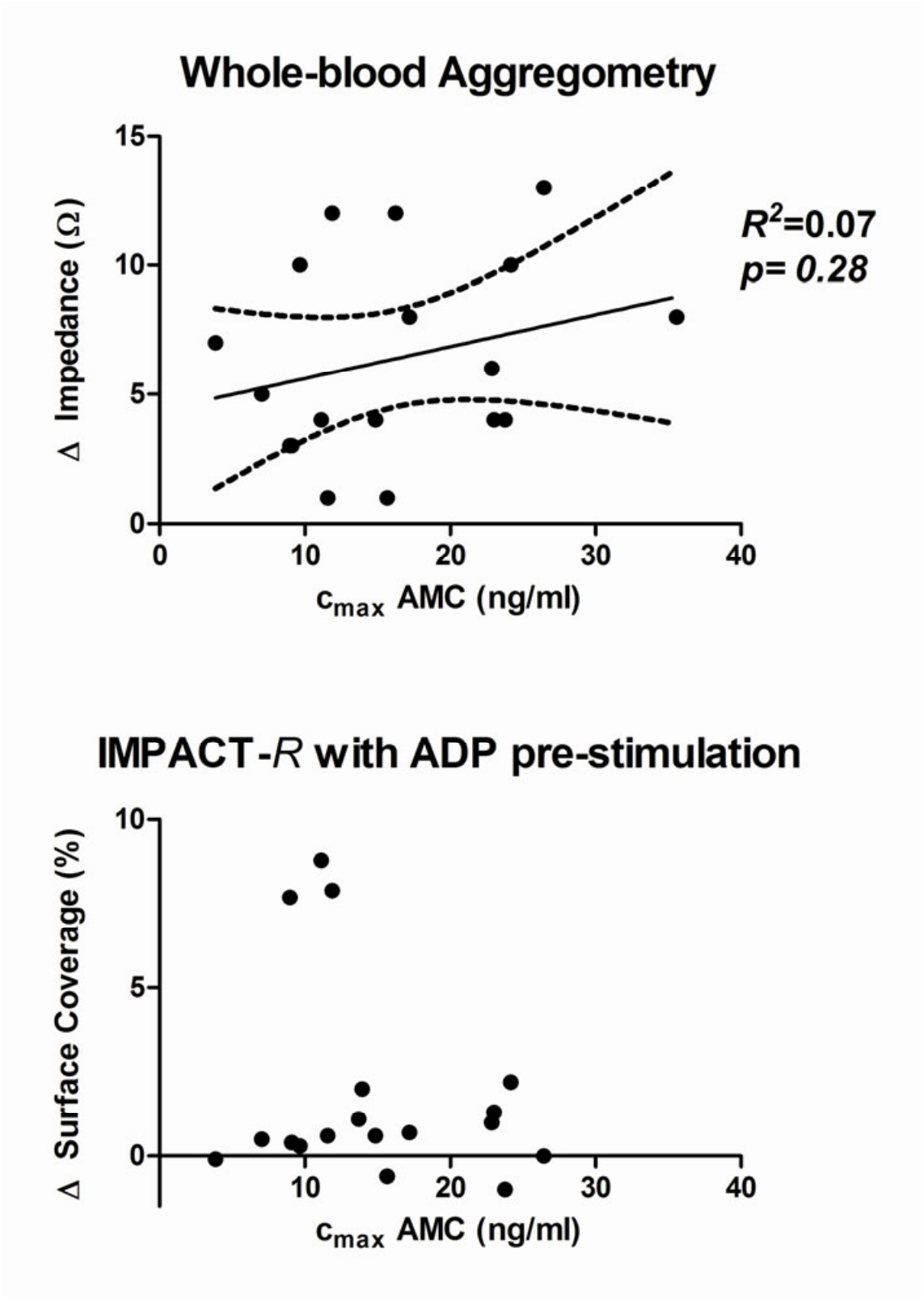
Table 2. Correlation between different platelet function tests.

	LTA 5 ADP peak	LTA 5 ADP late	LTA 20 ADP peak	LTA 20 ADP late	WBA	VerifyNow PRU	VerifyNow % inhibition	Impact- *R* ADP	VASP PRI
LTA 5 ADP peak	1	0.84 ‡	0.81 ‡	0.78 ‡	0.13	0.56 *	0.56 †	-0.19	0.16
LTA 5 ADP late	0.71‡	1	0.59 †	0.56 *	-0.01	0.36	0.34	-0.03	0.02
LTA 20 ADP peak	0.66‡	0.35†	1	0.96 ‡	0.21	0.82 ‡	0.80 ‡	-0.36	0.50 *
LTA 20 ADP late	0.61‡	0.31*	0.92‡	1	0.32	0.83 ‡	0.79 ‡	-0.25	0.49*
WBA	0.02	0.00	0.04	0.10	1	0.20	0.23	0.16	0.08
VerifyNow PRU	0.32*	0.13	0.67‡	0.69‡	0.04	1	0.94 ‡	-0.30	0.55*
VerifyNow % inhibition	0.32†	0.11	0.64‡	0.62‡	0.05	0.89‡	1	-0.38	0.64†
Impact-*R* ADP	0.04	0.00	0.13	0.06	0.03	0.09	0.14	1	-0.48 *
VASP PRI	0.03	0.00	0.25*	0.24*	0.00	0.31*	0.41†	0.23*	1

The correlation coefficient (r, above the diagonal axis) and the coefficient of determination (R^2, below the diagonal axis) are presented for each mutual comparison, *p<0.05, †p<0.01, ‡p<0.001. ADP=adenosine diphosphate, LTA=light transmittance aggregometry, PRI=platelet reactivity index, PRU=P2Y12 reaction units, VASP= vasodilator-stimulated phosphoprotein, WBA=whole blood impedance aggregometry.


Flowcytometric VASP-analysis
Δ PRI
100
80
60
40
20
0
0
10
20
30
40
c_{max} AMC (ng/ml)
R^2=0.56
p<0.001
VerifyNow P2Y12 assay
Δ PRU
Δ PRU
400
300
200
100
0
0
10
20
30
40
c_{max} AMC (ng/ml)
R^2=0.48
p=0.001
VerifyNow P2Y12 assay
Δ % inhibition
Δ % inhibition
100
80
60
40
20
0
0
10
20
30
40
c_{max} AMC (ng/ml)
R^2=0.59
p<0.001


LTA - 5 µmol/L ADP
Peak aggregation
R^2=0.19
p=0.06
Δ Aggregation (%)
c_{max} AMC (ng/ml)
LTA - 5 µmol/L ADP
Late aggregation
R^2=0.02
p=0.56
Δ Aggregation (%)
c_{max} AMC (ng/ml)
LTA - 20 µmol/L ADP
Peak aggregation
R^2=0.46
p=0.001
Δ Aggregation (%)
c_{max} AMC (ng/ml)
LTA - 20 µmol/L ADP
Late aggregation
R^2=0.47
p<0.001
Δ Aggregation (%)
c_{max} AMC (ng/ml)


Whole-blood Aggregometry
Δ Impedance (Ω)
15
10
5
0
0
10
20
30
40
c_{max} AMC (ng/ml)
R^2=0.07
p= 0.28
IMPACT-R with ADP pre-stimulation
Δ Surface Coverage (%)
10
5
0
10
20
30
40
c_{max} AMC (ng/ml)