

ORIGINAL ARTICLE

Diurnal variation in platelet inhibition by clopidogrel

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Abstract

Morning increase in the occurrence of myocardial infarction, stroke and sudden cardiac death is a well-recognized phenomenon, which is in line with a morning enhancement of platelet aggregation. We investigated whether platelet inhibition during clopidogrel and aspirin therapy varies during the day. Fifty-nine consecutive patients (45 men and 14 women) with first ST-segment elevation myocardial infarction (STEMI) treated with primary percutaneous coronary interventions (pPCI) on dual antiplatelet therapy were prospectively enrolled into the study. Blood samples were collected 4 days after start of clopidogrel treatment at 6.00 a.m., 10.00 a.m., 2.00 p.m. and 7.00 p.m. Arachidonic acid and adenosine diphosphate (ADP)-induced platelet aggregation were assessed by impedance aggregometry. Platelet inhibition by clopidogrel was lowest in the midmorning: median ADP-induced platelet aggregation was 55%, 17% and 27% higher at 10.00 a.m. compared to 6.00 a.m., 2.00 p.m. and 7.00 p.m., respectively ($p < 0.002$). Nonresponsiveness to clopidogrel defined according to the device manufacturer was 2.4-fold more frequent in the midmorning than in the early morning. We observed a more pronounced midmorning increase in ADP-induced platelet aggregation in diabetic patients when compared to non-diabetics. In contrast, no diurnal variation in the antiplatelet effect of aspirin was observed. In conclusion, in patients presenting with STEMI undergoing pPCI, platelet inhibition by clopidogrel is less strong in the midmorning hours. This periodicity in platelet aggregation in patients on dual antiplatelet therapy should be taken into consideration when assessing platelet function in clinical studies.

Keywords: Platelet aggregation, diurnal variation, dual antiplatelet therapy, acute myocardial infarction

Introduction

Antiplatelet agents are the mainstay of treatment to prevent and manage atherothrombotic events [1, 2]. However, despite extensive use of antiplatelet therapy, adverse cardiac events continue to occur in a substantial proportion of patients. Numerous reports have demonstrated considerable inter-individual variability in response to antiplatelet drugs [3, 4]. Insufficient platelet inhibition was suggested to account for many of the ischaemic cardiac events. Furthermore, clinical data linking both aspirin and

clopidogrel resistance with unfavourable outcomes are constantly growing [5–8]. Additionally, interventional cardiology successfully broadens its horizons to complex patients and challenging lesions with a high thrombotic risk. On the other hand, hyperresponsiveness to aspirin and to P2Y₁₂ receptor blockers such as thienopyridines may pose a serious threat of bleeding [9, 10].

Due to the limited number of events in these studies, routine monitoring of platelet function is not recommended [11]. In addition, an optimal method

of platelet testing as well as cut-off values associated with high cardiovascular risk remains to be determined. The recent widespread use of drug-eluting stents associated with the necessity for prolonged dual antiplatelet therapy further underscores the importance of these issues.

Most of the studies indicating a prognostic value of platelet hyperactivity do not specify the exact timing of blood sampling [5, 7, 8]. However, a previously performed pilot study suggested differences in diurnal platelet activity despite treatment with clopidogrel and aspirin [12]. This fact might have a profound impact on the interpretation of the obtained results, as previous observations have related increased morning platelet aggregation present in healthy subjects, as well as in patients with coronary artery disease without adequate antiplatelet treatment, with the phenomenon of morning excess of myocardial infarction, stroke and sudden cardiac death [13–17]. Furthermore, a recent study demonstrated that inhibition of platelets by clopidogrel and aspirin may be attenuated immediately after coronary stenting [18]. Although the authors suggested that the time of day of platelet function testing is important for the determination of cut-off points and the definition of nonresponsiveness to antiplatelet drugs, data supporting this hypothesis are scarce.

The aim of the study was to assess diurnal variation in platelet aggregation in patients with first ST-segment elevation myocardial infarction (STEMI) treated with primary percutaneous coronary intervention (pPCI) on dual antiplatelet therapy.

Methods

Study design and patients

Fifty-nine consecutive patients (45 men and 14 women) admitted to the Department of Cardiology and Internal Medicine of the Collegium Medicum in Bydgoszcz with a diagnosis of first STEMI and designated to undergo pPCI were prospectively recruited into the study. The enrolment criteria were: typical angina pain at rest for at least 20 min, symptom onset less than 12 h before admission to hospital, and electrocardiographic features of currently evolving STEMI (elevation of ST segment ≥ 0.1 mV in at least two limb leads or ≥ 0.2 mV in at least two precordial leads). Trial exclusion criteria consisted of:

- age less than 18 years and over 80 years;
- a history of previous myocardial infarction;
- prior coronary revascularization;
- cardiogenic shock at admission or initiation of the treatment with vasopressors before pPCI;

- bundle branch block;
- a history of chronic heart failure in functional class III or IV of the New York Heart Association, or haemodynamically significant valvular heart disease or idiopathic cardiomyopathy;
- a history of cardiac pacing or indications for temporal cardiac pacing;
- persistent atrial fibrillation or other indication for oral anticoagulants;
- thrombocytopenia ($<100\,000/\text{mm}^3$) or history of congenital or acquired bleeding disorder;
- a history of malignant neoplasm in the past 5 years;
- recent trauma or major surgery (within 2 months prior to enrolment);
- chronic obstructive pulmonary disease;
- any symptomatic concomitant infection;
- any concomitant immunosuppressive therapy including the use of steroids; and
- chronic kidney disease defined as serum creatinine >2 mg/dl or the need for renal replacement therapy.

All participants provided informed written consent. The study protocol was approved by the Local Ethics Committee in accordance with the Declaration of Helsinki.

Pharmacotherapy

At the first contact with health care providers immediately after the diagnosis of STEMI, all patients were pre-treated with an intravenous bolus of unfractionated heparin (70 IU/kg, but not more than 5000 IU) and oral loading doses of clopidogrel (600 mg) and aspirin (300 mg). At the catheterization laboratory, a second dose of unfractionated heparin was intra-arterially administered in a weight-adjusted manner (up to 100 IU/kg) or under activated clotting time guidance (to the target range 200–250 s) when abciximab was intended. Abciximab was given at the discretion of the invasive cardiologist. The drug was injected as an intracoronary bolus 0.25 mg/kg with a subsequent intravenous infusion of 0.125 $\mu\text{g}/\text{min}$ for 12 h according to the manufacturer's recommendations. Throughout the hospitalization period, clopidogrel and acetylsalicylic acid were continued in single doses of 75 mg given at 8.00 a.m. Post-discharge antiplatelet therapy was planned in accordance with the current European recommendations. Concomitant medications in all patients included ramipril and bisoprolol, provided at 8.00 a.m. in doses adjusted for resting heart rate and blood pressure, and atorvastatin administered at 8.00 p.m. Additionally, six (10.2%) patients were treated with

pantoprazole while one (1.7%) participant received amlodipine.

Percutaneous coronary interventions

Coronary angiography and pPCI procedures were performed using the standard technique via the femoral artery with the aid of an Integris Allura device (Philips, the Netherlands). Non-ionic low-osmolar contrast media were applied. During angiography, at least five left coronary artery and three right coronary artery projections were taken after previous administration of 0.3 mg nitroglycerine into the coronary vessels, if arterial pressure was sufficient. Epicardial coronary flow was assessed according to the Thrombolysis in Myocardial Infarction (TIMI) scale. In all patients, bare metal stents were implanted. Optimal direct effect of the intervention was assigned when no residual stenosis or a stenosis of less than 20% of the reference segment diameter along with TIMI 3 flow in the infarct-related artery were observed.

Measurement of platelet aggregation

Blood samples were collected into hirudin-containing tubes (Dynabyte Medical, Munich, Germany) at 6.00 a.m., 10.00 a.m., 2.00 p.m. and 7.00 p.m. The fourth day of hospitalization was chosen for blood sampling because at this time the patient with acute myocardial infarction is usually mobile, usually leaves the coronary care unit, and both aspirin and clopidogrel reach the steady state [8, 19]. If a patient was admitted after 7.00 p.m., the next day was counted as the first day of hospital stay.

Whole blood aggregation was determined using multiple electrode aggregometry (MEA) on a new generation impedance aggregometer (Multiplate Analyzer, Dynabyte Medical, Munich, Germany) according to the manufacturer's instructions [20]. The principle of impedance aggregometry is based on the fact that platelets get sticky upon activation, and therefore have a tendency to adhere and aggregate on metal sensor wires in the test cell. One Multiplate[®] test cell incorporates two independent sensor units, each consisting of two silver-coated highly conductive wires. When activated platelets adhere onto the sensor wires, the electrical resistance between the wires rises, which is continuously registered. The instrument detects the impedance change of each sensor separately and transforms it into arbitrary aggregation units (AU) that are plotted against time. The area under the aggregation curve (AUC) is an estimator of platelet aggregation that was evaluated in our study. It is affected by the total height of the AUC as well as by its slope and is best suited to express overall platelet activity. Aggregation, quantified as the area under the curve,

is displayed in arbitrary units ($10 \text{ AU} \times \text{min} = 1 \text{ U}$). In previous studies, AUC highlighted as the parameter with the highest diagnostic power [20, 21]. To assess a platelet response to aspirin and clopidogrel, we applied arachidonic acid (AA) and adenosine diphosphate (ADP)-induced aggregation. AA serves as the substrate of cyclooxygenase, blocked by aspirin and necessary for the synthesis of a potent platelet agonist, thromboxane A₂. ADP stimulates platelet activation by the ADP receptors that are blocked by clopidogrel. Using this fast and standardized method, comprehensive information on platelet function and antiplatelet therapy can be obtained. Reported intra-assay coefficients of variations (CVs) were 11.5% for AA-test and 14.1% for ADP-test, while intra-individual CVs were 11.4% for AA-test and 13.7% for ADP-test, respectively [22]. The manufacturer recommends 30 and 50 U as the cut-off values associated with platelet hyperreactivity in patients on aspirin and clopidogrel therapy, respectively.

Statistical analysis

In order to achieve a 31% difference in ADP-induced platelet aggregation, we estimated that a sample size of 55 would have 92% power to detect an absolute difference in means of 6.6 U (e.g. a first condition mean of 21.4 U [23] and a second condition mean of 28.0 U), assuming a standard deviation of differences of 14.0, with a 0.05 two-sided significance level. To compensate for potential withdrawal of consent, lack of aggregation assessed due to clot formation, or other reasons, we enrolled four additional patients.

Use of the Shapiro–Wilk test demonstrated that the investigated variables were not normally distributed. Therefore, continuous results were reported throughout the manuscript as median values and interquartile ranges. However, in the results section, we also provided mean values and standard deviations of AA- and ADP-induced platelet aggregation assessed at different sampling points. Multiple comparisons were analysed with the ANOVA Friedman test, whereas the Wilcoxon matched-paired rank sum test was used for comparisons between two sampling points. Dependent qualitative data were assessed with the use of Cochran's *Q*-test. Correlations were tested with the Spearman rank correlation test. A value of two sided $p < 0.05$ was considered statistically significant; $0.05 \geq p < 0.1$ was regarded as a trend towards statistical significance, while $p \geq 0.1$ was marked as NS. All statistical computations except power analysis were carried out with Statistica, version 8.0 (StatSoft, Tulsa, USA) while sample size calculation was performed with nQuery advisor, version 7.0 (Statistical Solutions, Cork, Ireland).

Results

Study population and pPCI

The study participants reflected a typical population of patients with first STEMI referred for pPCI. Age of patients ranged from 35 to 83 years, with a median value of 55. Slightly more than three-quarters of subjects were male gender. Most of participants suffered from overweight or obesity (median body mass index 27.4 kg/m^2), arterial hypertension (52.5%) and moderate hypercholesterolemia (median total cholesterol and LDL cholesterol levels on hospital admission 228.5 and 153.0 mg/dl, respectively). Diabetes mellitus was diagnosed in 28.8% of study participants. The majority of patients were smokers including 67.8% of current smokers and 10.2% of former smokers. The comprehensive clinical characteristics of the study population are presented in Table I.

All patients underwent bare metal coronary stenting that was in most cases limited to the culprit lesion. Direct stenting was frequently applied (59.3% of subjects). Optimal direct effect of the intervention was achieved in all patients. Abciximab was administered in 16 (27.1%) participants. Relatively high inflation pressures with a median value of 18.0 atm were used to adequately implant typically wide and medium length stents.

Platelet aggregation

Platelet counts evaluated at baseline and 4 days after start of dual antiplatelet therapy were comparable in the overall study population (233 [197–278] vs. 227

[184–263] $\times 10^3 \times \mu\text{l}^{-1}$; $p = \text{NS}$) as well as in the subgroup treated with abciximab (217 [198–270] vs. 222 [185–261] $\times 10^3 \times \mu\text{l}^{-1}$; $p = \text{NS}$). Drops in the platelet count greater or equal to 25% on the fourth day of hospitalization with respect to the baseline level occurred in four patients (none of them received abciximab). However, in all participants the platelet count was at least $100 \times 10^3 \times \mu\text{l}^{-1}$ when aggregation was assessed.

A substantial diurnal variation in ADP-induced platelet aggregation in subjects with STEMI treated in line with contemporary clinical practice was observed. Platelet inhibition by clopidogrel was lowest in the midmorning: median ADP-induced platelet aggregation was 55%, 17% and 27% higher at 10.00 a.m. compared to 6.00 a.m. (median value [interquartile range] 28.0 [15.0–48.0] vs. 18.0 [10.0–29.0] U; mean value \pm standard deviation 33.9 ± 25.6 vs. 22.5 ± 17.2 U; $p < 0.001$), 2.00 p.m. (24.0 [10.0–34.0] U; 27.8 ± 25.1 U; $p < 0.007$) and 7.00 p.m. (22.0 [12.5–36.5] U; 28.4 ± 24.7 U; $p < 0.02$), respectively. The ANOVA Friedman test indicated a significant heterogeneity among diurnal levels of ADP-dependent platelet aggregation ($p < 0.002$; Figure 1). After exclusion of standing-off values, a considerable heterogeneity of ADP-induced platelet aggregation at different sampling points was maintained ($p < 0.02$). Nonresponsiveness to clopidogrel defined as $\text{AUC} > 50$ U was 2.4-fold more frequent in the midmorning than in the early morning (20.3% at 10.00 a.m. vs. 8.5% at 6.00 a.m.; Table II). The magnitude of the midmorning increase in platelet aggregation after ADP stimulation was not associated with the level of platelet reactivity in the early morning. Furthermore, we

Table I. Clinical characteristics of the study population ($n = 59$).

Age (years)	55.0 (50.0–65.0)
Gender (male/female)	45 (76.3%)/14 (23.7%)
Infarct location:	14 (23.7%)/43 (72.9%)/2 (3.4%)
anterior/inferior/lateral wall	
Time from symptom onset (h)	3.2 (2.0–7.0)
CK-max (U/L)	1852.0 (765.0–2890.0)
CK-MBmax (U/L)	230.0 (96.0–354.0)
TnImax (ng/ml)	48.9 (11.0–50.0)
LVEF (%)	47.0 (41.0–50.0)
Risk factors of coronary artery disease	
Body mass index (kg/m^2)	27.4 (25.3–30.0)
Arterial hypertension	31 (52.5%)
Diabetes mellitus	17 including 6 newly diagnosed patients
Current smokers	40 (67.8%)
History of smoking	6 (10.2%)
Positive family history	14 (23.7%)
Total cholesterol (mg/dl)	228.5 (189.0–257.0)
LDL (mg/dl)	153.0 (127.0–187.0)
HDL (mg/dl)	43.5 (34.0–51.0)
Triglycerides (mg/dl)	97.5 (75.0–130.0)

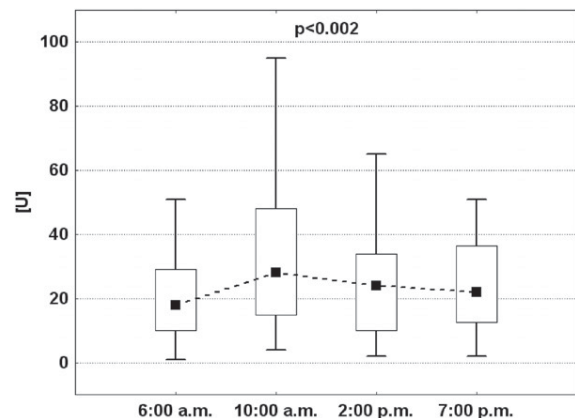


Figure 1. Diurnal variation of ADP-induced platelet aggregation assessed 4 days after the start of dual antiplatelet therapy in patients with STEMI. Data are presented as medians, interquartile ranges and non-outlier ranges. Statistical significance p was calculated for heterogeneity among diurnal levels of ADP-induced platelet aggregation.

tried to explore which factors might have an impact on the magnitude of morning increase in ADP-induced platelet aggregation. We took into account all variables reported in Table I as well as administration of abciximab, pantoprazole and amlodipine. We observed significantly higher values of platelet aggregation after ADP stimulation only at 10.00 a.m. in diabetics in comparison to non-diabetics, without any differences at other sampling points (6.00 a.m. 18.0 [15.0–32.5] vs. 17.0 [9.0–29.0] U, $p = \text{NS}$; 10.00 a.m. 40.0 [23.0–61.0] vs. 24.5 [12.0–41.0] U, $p < 0.03$; 2.00 p.m. 30.0 [9.0–43.0] vs. 21.5 [10.0–33.0] U, $p = \text{NS}$; 7.00 p.m. 34.0 [13.0–39.0] vs. 20.0 [11.0–36.0] U, $p = \text{NS}$). Additionally, we compared characteristics of patients with and without a peak of ADP-induced platelet aggregation at 10.00 a.m. The prevalence of diabetes was considerably higher ($p < 0.05$) in patients with enhanced platelet aggregation in response to ADP in the midmorning period (Table III).

In contrast, platelet inhibition by aspirin was well distributed without any diurnal variation (Figure 2). Although median AA-induced platelet aggregation in the morning was higher (10.5 [6.0–21.0] U; 14.8 ± 14.2 U at 6.00 a.m. and 11.0 [4.0–19.0] U; 15.0 ± 15.8 U at 10.00 a.m.) than in the afternoon (7.0 [3.0–16.0] U; 11.4 ± 13.2 U at 2.00 p.m.) and in the evening (7.0 [3.0–15.0] U; 10.6 ± 11.9 U at 7.00 p.m.), absolute differences were modest and did not reach statistical significance. Additionally, when considering courses of diurnal AA-induced platelet aggregation in individual cases, no visual trends were present (data not shown). Furthermore, the prevalence of nonresponsiveness to aspirin (AUC > 30 U) did not differ significantly throughout the day (Table II).

An analysis of patients treated with and without abciximab did not reveal any significant differences in both ADP- and AA-induced platelet aggregation at any of the sampling points (ADP: 6.00 a.m. 16.5 [9.0–22.0] vs. 18.0 [10.0–35.0] U, $p = \text{NS}$; 10 a.m. 21.5 [12.0–54.0] vs. 29.0 [15.0–48.0] U, $p = \text{NS}$; 2.00 p.m. 18.5 [6.0–30.5] vs. 26.0 [14.0–40.0] U, $p = \text{NS}$; 7.00 p.m. 15.0 [10.5–29.5] vs. 25.5

[13.5–36.5] U, $p = \text{NS}$; AA: 6.00 a.m. 8.5 [6.0–12.0] vs. 11.0 [4.0–19.0] U, $p = \text{NS}$; 10.00 a.m. 9.0 [3.0–13.5] vs. 11.5 [5.0–20.5] U, $p = \text{NS}$; 2.00 p.m. 7.5 [3.0–12.0] vs. 7.0 [3.0–16.0] U, $p = \text{NS}$; 7.00 p.m. 9.5 [3.5–16.0] vs. 6.5 [3.0–14.0] U, $p = \text{NS}$).

Measurements of platelet aggregation inhibited by clopidogrel and aspirin at each time point were unrelated to platelet count or mean platelet volume.

Discussion

The main finding of our study is that ADP-dependent platelet aggregation is increased at 10.00 a.m. compared to values at 2.00 p.m. in patients with first STEMI when assessed at day four of hospitalisation. To our knowledge, we demonstrate for the first time that heightened platelet aggregation in the midmorning persists despite dual antiplatelet therapy. Combined treatment with aspirin and clopidogrel was proven to provide considerable benefits in patients undergoing coronary stenting and those with acute coronary syndromes regardless of the applied therapeutic strategy. On the other hand, we failed to show any statistically significant diurnal variation in platelet aggregation in response to AA in this group. Interestingly, the magnitude of the midmorning rise in platelet aggregation after ADP stimulation was not associated with its baseline level.

Morning increase in the occurrence of myocardial infarction, stroke, and sudden cardiac death is a well-recognized phenomenon, and the period from 6.00 a.m. to noon covers a time when a number of physiological processes that could contribute to the onset of coronary thrombosis or fatal arrhythmia are intensified. The phenomenon can be due to the morning increase in platelet aggregation accompanied by a decrease in fibrinolytic activity, an increased morning thrombin formation, with a morning peak in blood pressure triggered by sympathetic overactivity and cortisol hypersecretion [24]. In particular, the extensive morning release of catecholamines promotes thrombus formation and

Table II. Comparison of clopidogrel and aspirin nonresponsiveness at different times of day.

Prevalence of clopidogrel nonresponsiveness				
6.00 a.m. <i>n</i> (%)	10.00 a.m. <i>n</i> (%)	2.00 p.m. <i>n</i> (%)	7.00 p.m. <i>n</i> (%)	<i>p</i>
5 (8.5%)	12 (20.3%)	6 (10.2%)	6 (10.2%)	<0.02
Prevalence of aspirin nonresponsiveness				
6.00 a.m. <i>n</i> (%)	10.00 a.m. <i>n</i> (%)	2.00 p.m. <i>n</i> (%)	7.00 p.m. <i>n</i> (%)	<i>p</i>
4 (6.8%)	6 (10.2%)	4 (6.8%)	3 (5.1%)	NS

Notes: Nonresponsiveness was defined according to the Multiplate[®] manufacturer as ADP-induced platelet aggregation ≥ 50 U for clopidogrel and AA-induced platelet aggregation ≥ 30 U for aspirin, respectively.

Table III. Comparison of characteristics of patients with and without a peak of ADP-induced platelet aggregation at 10.00 a.m.

	Patients with a peak of ADP-induced platelet aggregation at 10.00 a.m. (n=26)	Patients without a peak of ADP-induced platelet aggregation at 10.00 a.m. (n=33)	p
Age (years)	54.0 (50.0–65.0)	56.0 (50.0–62.0)	NS
Gender (male/female)	21 (80.8%)/5 (19.2%)	24 (72.7%)/9 (27.3%)	NS
Infarct location:	5 (19.2%)/20 (76.9%)/1 (3.9%)	9 (23.7%)/23 (72.9%)/1 (3.4%)	NS
anterior/inferior/lateral wall			
Time from symptom onset (h)	3.5 (2.5–8.0)	3.0 (2.0–5.5)	NS
CK-max (U/l)	1869.0 (787.0–2676.0)	1852.0 (655.0–2908.0)	NS
CK-MBmax (U/l)	192.5 (96.0–358.0)	255.0 (97.0–337.0)	NS
TnImax (ng/ml)	47.9 (13.2–50.0)	49.9 (8.7–50.0)	NS
LVEF (%)	47.2 (41.0–50.0)	46.0 (41.0–50.0)	NS
Risk factors of coronary artery disease			
Body mass index (kg/m ²)	26.8 (24.7–30.0)	27.5 (24.7–29.9)	NS
Arterial hypertension	12 (46.1%)	19 (57.6%)	NS
Diabetes mellitus	11 (42.3%)	6 (18.2%)	<0.05
Current smokers	18 (69.2%)	22 (66.7%)	NS
History of smoking	3 (11.5%)	3 (9.1%)	NS
Positive family history	6 (23.1%)	8 (24.2%)	NS
Total cholesterol (mg/dl)	216.0 (188.0–252.0)	232.5 (197.0–260.0)	NS
LDL (mg/dl)	143.0 (124.0–187.0)	164.0 (130.5–189.0)	NS
HDL (mg/dl)	40.0 (32.0–50.0)	45.5 (35.5–51.5)	NS
Triglycerides (mg/dl)	99.0 (76.0–172.0)	96.5 (73.0–122.5)	NS
Abciximab	9 (34.6%)	7 (21.2%)	NS
Pantoprazole	3 (11.5%)	3 (9.1%)	NS
Amlodipine	0 (0.0%)	1 (3.0%)	NS

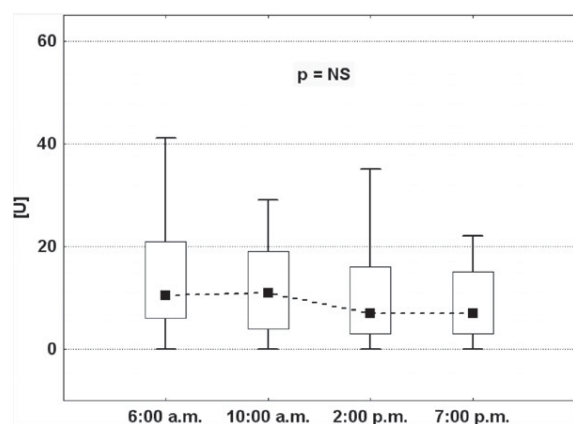


Figure 2. Diurnal variation of AA-induced platelet aggregation assessed 4 days after the start of dual antiplatelet therapy in patients with STEMI. Data are presented as medians, interquartile ranges and non-outlier ranges. Statistical significance *p* was calculated for heterogeneity among diurnal levels of AA-induced platelet aggregation.

enhances myocardial vulnerability. It has been suggested that a higher rate of acute cardiac events in the morning is frequently provoked by waking and commencing physical activity.

Sympathovagal circadian variation may explain a midmorning increase in ADP-induced platelet aggregation observed in our study despite ongoing clopidogrel treatment. The physiologic basis of the relationship between autonomic system and platelet

reactivity is supported by the evidence that human platelet membrane contains both alpha-2 A adrenoceptors and dopamine receptors [25]. The alpha-2 A receptors are most prominent and probably responsible for the physiological and pathological changes in platelet function associated with the cardiovascular system. Increases in circulating catecholamine levels (example e.g. caused by physical or mental exercise) initiates responses which include platelet aggregation, secretion of serotonin from the storage granules and activation of the arachidonate pathway. In support of this hypothesis, we observed a more pronounced midmorning increase in ADP-induced platelet aggregation in diabetic patients vs. non-diabetics. In the former group, autonomic neuropathy is frequently diagnosed.

Previous studies on platelet aggregation indicated that the upright posture and initiation of daily activities could play a crucial role in morning platelet hyperaggregability in healthy subjects [17, 26]. Particularly pronounced platelet hyperresponsiveness to physical exercise appears in habitually inactive subjects [27]. Andrews et al. [28], in a sophisticated flow cytometric study, proved that the morning increase in platelet aggregation does not occur with expression of activation-dependent platelet surface receptors. The researchers hypothesized that the increase in whole blood aggregation may be primarily due to the increases in catecholamine levels, platelet count and haemoconcentration.

In another experimental work, the same researchers successfully attenuated the morning orthostatic increase in platelet aggregation by an α_2 -adrenergic blockade with yohimbine [29]. The effect was present, despite a concomitant rise in plasma nor-epinephrine levels associated with its enhanced release from presynaptic nerves. Furthermore, it has been reported that guanabenz, a centrally acting α_2 -agonist exerting antihypertensive properties, suppressed morning elevations in aggregation of human platelets [30]. However, Willich et al. [31] showed that α_2 -adrenergic receptor density and agonist binding affinity assessed simultaneously did not change in the morning. They also suggested that the increase in platelet aggregability is due to factors extrinsic to the platelets or to an intra-platelet mechanism distal to the receptor level. In another study, metoprolol, a β_1 -selective blocker commonly administered in patients with myocardial infarction, did not alter the basal level nor blunt the morning increase of platelet aggregability in patients with stable coronary artery disease, despite successful suppression of silent ischaemic episodes [32].

Our observations are somewhat surprising: we noted the highest ADP-induced platelet aggregation at 10.00 a.m. (2 h after morning drug intake) instead of 6.00 a.m. (2 h before morning drug intake and 22 h after last dose), probably due to sympathetic stimulation overcoming the antiplatelet effect of clopidogrel maintenance dose. Additionally, proplatelets are released from bone marrow in a circadian rhythm, with a peak within a few hours after sunrise [33] when a number of platelets in the peripheral circulation is increasing from early morning till afternoon [34].

Our results are in apparent contrast with the data provided by Husted et al. [35] who reported a decrease in ADP-induced platelet aggregation for up to 12 h after administration of clopidogrel maintenance dose. However, the lack of information on the time of day of drug administration in that article, together with different methods of platelet assessment, and the lack of pre-treatment assessment in this study preclude direct comparison between the studies.

McCall et al. [36] observed that enteric-coated aspirin markedly reduced baseline platelet thromboxane A2 production in healthy males and eliminated its increase after the subjects got up. It also abolished biphasic platelet aggregation in response to epinephrine and ADP. The lack of a morning increase in thromboxane A2 synthesis revealed in the study corresponds with our results, indicating suppression of the circadian variation in thromboxane A2-dependent platelet aggregation. Similarly, Tison et al. [37] noted that aspirin, when co-administered with nitrendipine in patients with

essential hypertension, triggered a significant decrease and flattening of the beta-thromboglobulin curve suggesting attenuation of morning surge in platelet activity. To our knowledge there is no previous study assessing the diurnal variation in AA-induced platelet aggregation in patients on dual antiplatelet therapy.

The clinical significance of our finding remains to be demonstrated. The immediate consequence of these observations is the emphasis that should be placed on the pharmacologic protection of patients during the morning hours. Pepine [24] suggests that attention to the morning vulnerable period is merited in the timing and choice of medication, both to prevent or reduce ischaemia and to modify potential disease-triggering mechanisms. Due to the relatively short half-life and duration of the therapeutic effect (<24 h) of many agents applied in contemporary cardiology, it is likely that single day agents taken in the morning will have reached subtherapeutic levels by the time of waking and commencing activity the following morning. We also speculate that suppression of the morning increase in platelet aggregation by novel potent antiplatelet agents may further improve the long-term prognosis in survivors of myocardial infarction. Furthermore, in our opinion the periodicity in platelet aggregation present in antiplatelet therapy should be adjusted for the discriminative ability of various methods applied in the analysis of platelet function.

In our study, we applied impedance aggregometry. This method is capable of detecting the effect of clopidogrel and aspirin treatment and its results, prior to, and after antiplatelet treatment, and it correlates well with light transmission aggregometry [38]. Whole blood, which was utilized in our study, is the physiological environment where platelet function takes place *in vivo*. Moreover, the use of whole blood for *in vitro* testing eliminates the need for the time-consuming centrifugation steps required to obtain the platelet-rich plasma necessary for light transmission aggregometry. Therefore, it must be stressed that impedance aggregometry and light transmission aggregometry measure different aspects of platelet function. Impedance aggregometry results reflect interactions between platelets, red and white cells, while light transmission aggregometry does not [22]. Dyszkiewicz-Korpanty et al. [39] even suggest that whole blood aggregation appears to be more sensitive in detecting clopidogrel effects than platelet-rich plasma methods.

Despite the fact that the Multiplate[®] is a newly invented device, the data on its diagnostic power are constantly accumulating. In a study by Mengistu et al. [40], impedance aggregometry with the Multiplate[®] device, but not thromboelastography, successfully predicted postoperative requirements

for blood transfusion in patients undergoing cardiac surgery. Furthermore, platelet assessment with MEA in a cohort of 1608 subjects treated with drug-eluting coronary stenting sufficiently predicted early stent thrombosis [41]. Finally, a recent trial demonstrated that platelet hyperreactivity in MEA might be even a better risk predictor for stent thrombosis than the assessment of the specific clopidogrel effect with the VASP assay [42].

However, we have to acknowledge that AA-induced platelet aggregation is rather a weak tool to adequately detect responsiveness to aspirin. Measurement of thromboxane B₂ levels represents a more sensitive method. Other study limitations include administration of abciximab during pPCI in 27% of our patients as a potential confounder and lack of adjustment in our analyses for the time between the administration of antiplatelet drugs and serial measurements of platelet activity.

To conclude, ADP-induced platelet aggregation increases in the midmorning compared to other times of day despite treatment with aspirin and clopidogrel in patients with first STEMI undergoing pPCI. Substantial periodicity in platelet aggregation present on dual antiplatelet therapy should be taken into consideration when various methods of platelet function monitoring are applied to clinical studies.

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References

- Meadows TA, Bhatt DL. Clinical aspects of platelet inhibitors and thrombus formation. *Circ Res* 2007;100:1261–1275.
- Bryniarski L, Pelc-Nowicka A, Zabojszcz M, Mirek-Bryniarska E. Dual antiplatelet therapy and antithrombotic treatment: Recommendations and controversies. *Cardiol J* 2009;16:179–189.
- Crescente M, Di Castelnuovo A, Iacoviello L, Vermuyen J, Cerletti C, de Gaetano G. Response variability to aspirin as assessed by the platelet function analyzer (PFA)-100. A systematic review. *Thromb Haemost* 2008;99:14–26.
- Serebruany VL, Steinhubl SR, Berger PB, Malinin AI, Bhatt DL, Topol EJ. Variability in platelet responsiveness to clopidogrel among 544 individuals. *J Am Coll Cardiol* 2005;45:246–251.
- Buonamici P, Marcucci R, Migliorini A, Gensini GF, Santini A, Panizza R, Moschi G, Gori AM, Abbate R, Antoniucci D. Impact of platelet reactivity after clopidogrel administration on drug-eluting stent thrombosis. *J Am Coll Cardiol* 2007;49:2312–2317.
- Eikelboom JW, Hankey GJ, Thom J, Bhatt DL, Steg PG, Montalescot G, Johnston SC, Steinhubl SR, Mak KH, Easton JD, et al. Clopidogrel for high atherothrombotic risk and ischemic stabilization, management and avoidance (CHARISMA) investigators. Incomplete inhibition of thromboxane biosynthesis by acetylsalicylic acid: Determinants and effect on cardiovascular risk. *Circulation* 2008;118:1705–1712.
- Gum PA, Kottke-Marchant K, Welsh PA, White J, Topol EJ. A prospective, blinded determination of the natural history of aspirin resistance among stable patients with cardiovascular disease. *J Am Coll Cardiol* 2003;41:961–965.
- Matetzky S, Shenkman B, Guetta V, Shechter M, Bienart R, Goldenberg I, Novikov I, Pres H, Savion N, Varon D, et al. Clopidogrel resistance is associated with increased risk of recurrent atherothrombotic events in patients with acute myocardial infarction. *Circulation* 2004;109:3171–3175.
- Chen L, Bracey AW, Radovancevic R, Cooper Jr JR, Collard CD, Vaughn WK, Nussmeier NA. Clopidogrel and bleeding in patients undergoing elective coronary artery bypass grafting. *J Thorac Cardiovasc Surg* 2004;128:425–431.
- Sibbing D, Schulz S, Braun S, Morath T, Stegherr J, Mehilli J, Schömig A, von Beckerath N, Kastrati A. Antiplatelet effects of clopidogrel and bleeding in patients undergoing coronary stent placement. *J Thromb Haemost* 2010;8:250–256.
- Kuliczkowski W, Witkowski A, Polonski L, Kuliczkowski W, Witkowski A, Polonski L, Watala C, Filipiak K, Budaj A, Golanski J, et al. Interindividual variability in the response to oral antiplatelet drugs: A position paper of the working group on antiplatelet drugs resistance appointed by the section of cardiovascular interventions of the polish cardiac society, endorsed by the working group on thrombosis of the European society of cardiology. *Eur Heart J* 2009;30:426–435.
- Koziński M, Bielis L, Wiśniewska-Szmyt J, Sukiennik A, Grabczewska Z, Swiatkiewicz I, Ziołkowski M, Rośc D, Kubica J. Increased morning ADP-dependent platelet aggregation persists despite dual antiplatelet therapy in patients with first ST-segment elevation myocardial infarction: Preliminary report. *Cardiol J* 2008;15:530–536.
- Beisiegel B, Treese N, Hafner G, Meyer J, Darius H. Increase in endogenous fibrinolysis and platelet activity during exercise in young volunteers. *Agents Actions Suppl* 1992;37:183–189.
- Mores N, Martire M, Pistrutto G, Volpe AR, Menini E, Folli G, Cardillo C. Platelet alpha 2-adrenoceptors and diurnal changes of platelet aggregability in hypertensive patients. *J Hypertens* 1994;12:939–945.
- Muller JE, Stone PH, Turi ZG, Rutherford JD, Czeisler CA, Parker C, Poole WK, Passamani E, Roberts R, Robertson T. Circadian variation in the frequency of onset of acute myocardial infarction. *N Engl J Med* 1985;313:1315–1322.
- Muller JE, Tofler GH, Stone PH. Circadian variation and triggers of onset of acute cardiovascular disease. *Circulation* 1989;79:733–743.
- Tofler GH, Brezinski D, Schafer AI, Czeisler CA, Rutherford JD, Willich SN, Gleason RE, Williams GH, Muller JE. Concurrent morning increase in platelet aggregability and the risk of myocardial infarction and sudden cardiac death. *N Engl J Med* 1987;316:1514–1518.
- Siller-Matula JM, Haberl K, Prillinger K, Panzer S, Lang I, Jilma B. The effect of antiplatelet drugs clopidogrel and aspirin is less immediately after stent implantation. *Thromb Res* 2009;123:874–880.
- Karon BS, Wockenfus A, Scott R, Hartman SJ, McConnell JP, Santrach PJ, Jaffe AS. Aspirin responsiveness in healthy volunteers measured with multiple assay platforms. *Clin Chem* 2008;54:1060–1065.
- Toth O, Calatzis A, Penz S, Losonczy H, Siess W. Multiple electrode aggregometry: A new device to measure platelet

- aggregation in whole blood. *Thromb Haemost* 2006;96:781–788.
21. Calatzis A, Wittwer M, Krueger B. A new approach to platelet function analysis in whole blood – The multiplate analyzer. *Platelets* 2004;15:485–486.
 22. Seyfert UT, Haubelt H, Vogt A, Hellstern P. Variables influencing multiplate (TM) whole blood impedance platelet aggregometry and turbidimetric platelet aggregation in healthy individuals. *Platelets* 2007;18:199–206.
 23. Althoff TF, Fischer M, Langer E, Ziemer S, Baumann G. Sustained enhancement of residual platelet reactivity after coronary stenting in patients with myocardial infarction compared to elective patients. *Thromb Res* 2010;125:e190–e196.
 24. Pepine CJ. Circadian variations in myocardial ischemia. Implications for management. *JAMA* 1991;265:386–390.
 25. Anfossi G, Trovati M. Role of catecholamines in platelet function: Pathophysiological changes of clinical significance. *Eur J Clin Invest* 1996;26:353–370.
 26. Brezinski DA, Tofler GH, Muller JE, Pohjola-Sintonen S, Willich SN, Schafer AI, Czeisler CA, Williams GH. Morning increase in platelet aggregability. Association with assumption of the upright posture. *Circulation* 1988;78:35–40.
 27. Kestin AS, Ellis PA, Barnard MR, Errichetti A, Rosner BA, Michelson AD. Effect of strenuous exercise on platelet activation state and reactivity. *Circulation* 1993;88:1502–1511.
 28. Andrews NP, Gralnick HR, Merryman P, Vail M, Quyyumi AA. Mechanisms underlying the morning increase in platelet aggregation: A flow cytometry study. *J Am Coll Cardiol* 1996;28:1789–1795.
 29. Andrews NP, Goldstein DS, Quyyumi AA. Effect of systemic alpha-2 adrenergic blockade on the morning increase in platelet aggregation in normal subjects. *Am J Cardiol* 1999;84:316–320.
 30. Hayashi J, Sato H, Tanaka Y, Tokue J, Ishida N, Watanabe K, Kitamoto K. Guanabenz, an antihypertensive centrally acting alpha 2-agonist, suppresses morning elevations in aggregation of human platelets. *J Cardiovasc Pharmacol* 2001;37:89–93.
 31. Willich SN, Tofler GH, Brezinski DA, Schafer AI, Muller JE, Michel T, Colucci WS. Platelet alpha 2 adrenoceptor characteristics during the morning increase in platelet aggregability. *Eur Heart J* 1992;13:550–555.
 32. Willich SN, Pohjola-Sintonen S, Bhatia SJ, Shook TL, Tofler GH, Muller JE, Curtis DG, Williams GH, Stone PH. Suppression of silent ischemia by metoprolol without alteration of morning increase of platelet aggregability in patients with stable coronary artery disease. *Circulation* 1989;79:557–565.
 33. Behnke O, Forer A. From megakaryocytes to platelets: Platelet morphogenesis takes place in the bloodstream. *Eur J Haematol Suppl* 1998;61:3–23.
 34. Haus E, Cusulos M, Sackett-Lundeen L, Swoyer J. Circadian variations in blood coagulation parameters, alpha-antitrypsin antigen and platelet aggregation and retention in clinically healthy subjects. *Chronobiol Int* 1990;7:203–216.
 35. Husted S, Emanuelsson H, Heptinstall S, Sandset PM, Wickens M, Peters G. Pharmacodynamics, pharmacokinetics, and safety of the oral reversible P2Y12 antagonist AZD6140 with aspirin in patients with atherosclerosis: A double-blind comparison to clopidogrel with aspirin. *Eur Heart J* 2006;27:1038–1047.
 36. McCall NT, Tofler GH, Schafer AI, Williams GH, Muller JE. The effect of enteric-coated aspirin on the morning increase in platelet activity. *Am Heart J* 1991;121:1382–1388.
 37. Tison P, Ulicna L, Jakubovska Z, Oravcova J, Fetkowska N. Effects of dihydropyridines and their combination with aspirin on blood pressure and circadian platelet activity in patients with essential hypertension. *Am J Hypertens* 1994;7:46 S–49 S.
 38. Sibbing D, Braun S, Jawansky S, Vogt W, Mehilli J, Schömig A, Kastrati A, von Beckerath N. Assessment of ADP-induced platelet aggregation with optical aggregometry and multiple electrode platelet aggregometry before and after clopidogrel loading. *Thromb Haemost* 2008;99:121–126.
 39. Dyszkiewicz-Korpanty A, Olteanu H, Frenkel EP, Sarode R. Clopidogrel anti-platelet effect: An evaluation by optical aggregometry, impedance aggregometry, and the platelet function analyzer (PFA-100). *Platelets* 2007;18:491–496.
 40. Mengistu AM, Wolf MW, Boldt J, Röhm KD, Lang J, Piper SN. Evaluation of a new platelet function analyzer in cardiac surgery: A comparison of modified thromboelastography and whole-blood aggregometry. *J Cardiothorac Vasc Anesth* 2008;22:40–46.
 41. Sibbing D, Braun S, Morath T, Mehilli J, Vogt W, Schömig A, Kastrati A, von Beckerath N. Platelet reactivity after clopidogrel treatment assessed with point-of-care analysis and early drug-eluting stent thrombosis. *J Am Coll Cardiol* 2009;53:849–856.
 42. Siller-Matula JM, Christ G, Lang IM, Delle-Karth G, Huber K, Jilma B. Multiple electrode aggregometry better predicts stent thrombosis than the VASP assay. *J Thromb Haemost* 2010;8:351–359.