Assessment of ADP-induced platelet aggregation with light transmission aggregometry and multiple electrode platelet aggregometry before and after clopidogrel treatment

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Summary

The level of platelet aggregation, measured with light transmission aggregometry (LTA) in platelet rich plasma (PRP), has been shown to predict outcomes after percutaneous coronary intervention (PCI). However, measuring parameters of platelet function with LTA is time consuming and weakly standardized. Thus, a fast and standardized method to assess platelet function after clopidogrel treatment would be of great value for clinical practice. A new method, multiple electrode platelet aggregometry (MEA), to rapidly measure platelet aggregation in whole blood has recently been developed. The aim of this study was to assess parameters of platelet function with MEA and LTA before and after administration of 600 mg clopidogrel. Blood samples from 149 patients scheduled for coronary angiography were taken after clopidogrel treatment; in addition, in 60 of the patients samples were available before clopidogrel treatment. ADP-induced platelet aggregation was measured with LTA and simultaneously in whole blood with MEA on the Multiplate analyzer. Platelet aggregation measured with MEA decreased significantly after clopidogrel treatment ($P<0.0001$). ADP-induced platelet aggregation assessed with MEA and LTA correlated significantly (Spearman rank correlation coefficient=0.71; $P<0.0001$). The results of MEA, a fast and standardized method to assess the platelet response to ADP prior to and after clopidogrel treatment, correlate well with LTA.

Keywords

Clopidogrel, platelet aggregation, whole blood aggregometry

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Introduction

Dual antiplatelet therapy consisting of aspirin and clopidogrel is the therapy of choice to prevent thrombotic complications in patients undergoing percutaneous coronary intervention (PCI) (1). Clopidogrel pretreatment with a single high loading dose has been shown to be advantageous (2). Several ex-vivo studies have demonstrated that the response to clopidogrel is largely variable (3). Prospective and retrospective studies have shown that enhanced platelet reactivity despite treatment with clopidogrel is associated with an increased risk for adverse cardiovascular events following PCI (4–8). In most of these studies platelet function was assessed using light transmission aggregometry (LTA) in platelet rich plasma (PRP) (6–9). LTA is considered to be the gold standard for assessing the platelet response to agonists such as adenosine diphosphate (ADP) and has been used to assess the drug response to clopidogrel in the initial dose-finding studies (4, 5, 10). However, LTA measurements are time consuming, weakly standardized and its logistical demands make it difficult to use it routinely in daily clinical practice (11). Thus, a simple, fast and standardized method to assess platelet function in clopidogrel treated patients would be of great value. Currently, besides LTA, different methods are used to analyze parameters of platelet function in whole blood (12–17). A new method, called multiple electrode platelet aggregometry (MEA), to rapidly measure platelet aggregation in whole blood, has been developed recently (18). MEA implements the principle of impedance aggregometry to measure platelet function in diluted whole blood (19). Its major advantage over LTA is that no centrifugation steps are needed and that assessment of platelet aggregation can be done in approximately 10 minutes. Moreover, as a whole blood method, MEA has the principle advantage that the cellular environment remains unchanged. Whether results obtained with MEA correlate with LTA is unknown. The aim of this study was to assess platelet aggregation with MEA and LTA before and after administration of a single high dose of 600 mg clopidogrel.

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Materials and methods

Consecutive patients (n=149) without clopidogrel treatment within the last four weeks and scheduled for coronary angiography were included in this study. All patients received a single high dose of 600 mg of clopidogrel which was recommended to be given at least two hours before catheterization. Whole blood was drawn by venipuncture before administration of clopidogrel from a subset of the study population (n=60) and from the arterial sheath of all patients (n=149) immediately after placement. For logistic reasons we were not able to obtain pretreatment blood samples from 89 of the 149 patients. The first tube drawn was labelled as a discard, and was not used for platelet function testing. The study was approved by the institutional ethics committee and patients gave written informed consent for participation.

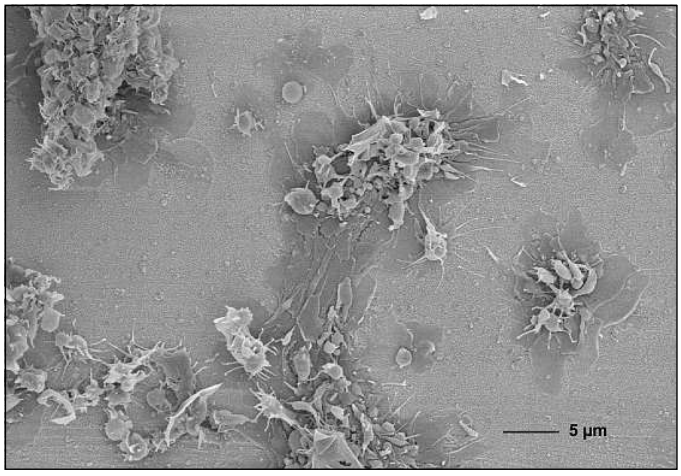

Figure 1: Electron microscopic imaging (2,000 times magnified) of a disposable Multiplate analyzer test cell showing platelets sticking on the surface of the electrode after a standard test.

Table 1: Baseline characteristics of the patients.

Mean age, years ± SD	**66.0 ± 10.0**
Woman (%)	41 (27.5)
Ejection fraction, % ± SD	59.7 ± 4.6
Diabetes mellitus (%)	50 (33.6)
Active smokers (%)	25 (16.8)
Arterial hypertension (%)	133 (89.3)
Hypercholesterolemia (%)	115 (77.2)
Family history of CAD[b] (%)	64 (43.0)
Previous PCI[c] (%)	72 (48.3)
Previous bypass surgery (%)	17 (11.4)
Previous myocardial infarction (%)	38 (25.5)
Platelet count x 10^9/L	204 ± 60.3
Hb (g/dl)	13.1 ± 1.5
Time from clopidogrel loading[d] (h)	3.3 [2.0–6.0]
Medication on admission	
Aspirin (%)	106 (71.1)
Statins (%)	96 (64.4)
Beta blockers (%)	95 (63.8)
ACE inhibitors (%)	85 (57.0)
Diuretics (%)	54 (36.2)

[a]Data presented are mean ± SD or number of patients (percentage). [b]CAD denotes coronary artery disease. [c]PCI denotes percutaneous coronary intervention. [d]Time from clopidogrel loading (h=hours) to blood sampling is expressed as median [interquartile range].

LTA was used to assess ADP-induced platelet aggregation in citrated PRP using the new 8-channel platelet aggregation profiler (PAP 8) aggregometer (Bio/Data) with a constant stirring rate of 1200 rpm at 37°C. The results obtained with this instrument are very similar to the results obtained with the 4-channel aggregometer from the same manufacturer (mean ± SD for 24 repeated measurements of one blood donor with different reagent lots of ADP in a concentration of 20 µM=74.3 ± 4.5% for PAP 8 vs. 79.5 ± 2.4% for PAP 4, data on file, Bio/Data) that has been used in a large study that showed an association between LTA measurements and clinical outcome (7). PRP was obtained as a supernatant after centrifugation of citrate- or hirudin-anticoagulated blood at room temperature with 750 rpm for 10 minutes. In the subset of the population with both prae and post clopidogrel measurements (n=60) ADP-induced platelet aggregation was also assessed in hirudin-anticoagulated PRP. The final platelet count was adjusted to 250 x 10^9/L with autologous platelet poor plasma (PPP). PRP (0% light transmission) and PPP (100% light transmission) served as references. After baseline adjustment, ADP in a final concentration of 5 or 20 µM was added and aggregation recorded for 6 min. The analyzed parameter was maximal aggregation (%) within the first six minutes after addition of ADP.

Parallel to LTA measurements, whole blood aggregation was assessed on a new generation impedance aggregometer with MEA (18, 20–23). The device is called Multiplate analyzer (Dynabyte, Munich, Germany), indicating the multiplicity of channels and sensors per channel of the device. One Multiplate test cell incorporates two independent sensor units. One unit consists of 2 silver-coated highly conductive copper wires with a length of 3.2 mm. After dilution (1:2 with 0.9% NaCl solution) of hirudin-anticoagulated whole blood (25 µg/ml, Refludan) and stirring for three minutes in the test cuvettes at 37°C, ADP in a final concentration of 6.4 µM was added and aggregation was continuously recorded for five minutes. The increase of impedance due to the attachment of platelets to the electrodes is detected for each sensor unit separately and transformed to arbitrary aggregation units (AU) that are plotted against time. Approximately 8 AU correspond to 1 Ohm. Aggregation measured with MEA is quantified as AU and area under the curve (AUC) of arbitrary units (AU*min). Aggregated platelets remaining on the surface of a Multiplate electrode after a standard test are shown in Figure 1. Electrical detection of impedance is calibrated by the manufacturer during production. Level 1 and 2 liquid controls are available for the user to enable on site quality control of impedance detection. All material used for MEA analysis, including ADP (6.4 µM), was obtained from Dynabyte (Munich, Germany). The ADP concentration for stimulation of platelets recommended by the manufacturer of the Multiplate device is

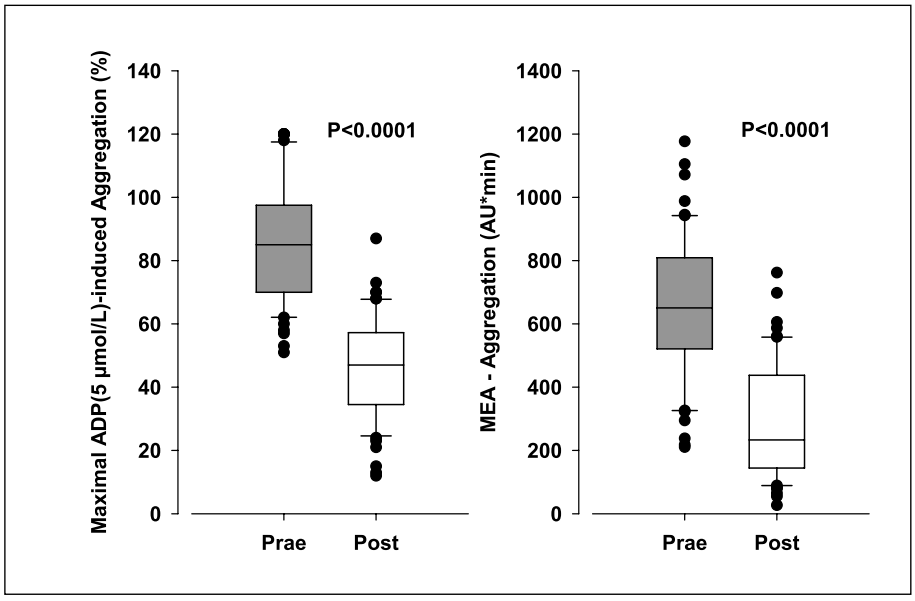

Figure 2: Box plot analyses (n=60 patients) of multiple electrode platelet aggregometry (MEA) and light transmission aggregometry (LTA) measurements. Boxes indicate 25th and 75th percentiles; whiskers denote 10th and 90th percentiles and outline values are shown as dots. Grey boxes signify measurements prior to clopidogrel treatment and white boxes signify post treatment values.

6.4 µM. Using the recommended ADP-concentration makes MEA results comparable and thus enables the user to take advantage of the standardization of this method. For LTA we chose the ADP-concentrations most commonly used to assess the antiplatelet effect of clopidogrel (7, 10, 24–26).

Coefficients of variations (CV) for MEA measurements were 11.4%, 14.9%, 9.7% and 8.4%. Each CV was calculated from 10 measurements with different samples of one donor. The donors were two healthy volunteers and two patients with chronic (>10 days) clopidogrel therapy. Coefficients of variations (CV) for LTA measurements were 9.2%, 12.8%, 14.8%, 5.9%. CVs for LTA were calculated in the same way as CVs for MEA.

Statistical analysis

We tested for normal distribution of continuous data with Kolmogorov-Smirnov Goodness-of-Fit test. Accordingly, continuous data are expressed as mean ± SD or median with interquartile range. Since some of the platelet function data were not normally distributed these data are uniformly reported as median with interquartile range. For the same reason Spearman rank correlation was used to test for the correlation of LTA with MEA values. P-values <0.05 were considered statistically significant.

Results

Assessment of clopidogrel response

Baseline characteristics of the patients included in this study are shown in Table 1. Assessed in the subset of the population with both prae and post clopidogrel measurements (n=60), administration of 600 mg of clopidogrel resulted in a significant suppression of maximal ADP-induced platelet aggregation assessed with LTA (from 85.0% [70.0–97.0] to 47.0% [35.0–56.5]; P<0.0001) when platelet aggregation was induced with 5 µM ADP and from 88.0% [81.5–101.5] to 50.5% [40.5–66.0] when platelet aggregation was induced with 20 µM ADP (P<0.0001). Correspondingly, clopidogrel treatment resulted in a significant reduction of multiple electrode platelet aggregometry AUC of arbitrary units (from 650.5 AU*min [523.0–807.0] at baseline to 233.5 AU*min [145.0–429.5] after clopidogrel; P<0.0001). Figure 2 demonstrates box plot analyses (n=60) of LTA and MEA aggregation values before and after clopidogrel treatment.

Correlation of LTA and MEA measurements

The correlations of all available values from all patients (n=149) obtained with LTA (5 and 20 µM ADP) with those obtained with MEA (AUC of arbitrary units) are demonstrated in Figure 3. The correlations were highly significant (Spearman rank correlation coefficient=0.71 and P<0.0001 for LTA with 5 and 20 µM ADP versus MEA). Using hirudin-anticoagulated blood for both LTA and MEA in the subset of the population with prae and post clopidogrel values (n=60), Spearman rank correlation coefficient for the correlation of MEA with maximal ADP(5 µM)-induced aggregation assessed with LTA was 0.66 (P<0.0001). Spearman rank correlation coefficient for inhibition of platelet aggregation [IPA=(platelet aggregation before clopidogrel-platelet aggregation after clopidogrel)/platelet aggregation before clopidogrel*100] in 60 patients with MEA (AUC of arbitrary units) and maximal ADP(5 µM)-induced aggregation assessed with LTA was 0.46 (P<0.0001).

Assessment of clopidogrel treatment failure with LTA and MEA

In the group of patients with prae and post values (n=60) we calculated the agreement between the lowest quartiles (patients with lowest response to clopidogrel) of inhibition of platelet aggregation (IPA) assessed with LTA and MEA. The quartile borders (lowest to highest) were 29.5%, 40.9% and 56.6% IPA for LTA and 41.9%, 58.9% and 72.5% IPA for MEA. Fifteen patients were in the lowest quartile according to LTA. Out of these 15 patients, seven patients (47%) were also in the lowest quartile according to MEA. According to LTA measurements the remaining 45 patients were assigned to the 2nd-4th quartile. Of these 45 patients, 37 (82 %) were also found in quartiles 2–4 according to MEA. Moreover, since platelet aggregation after treatment has

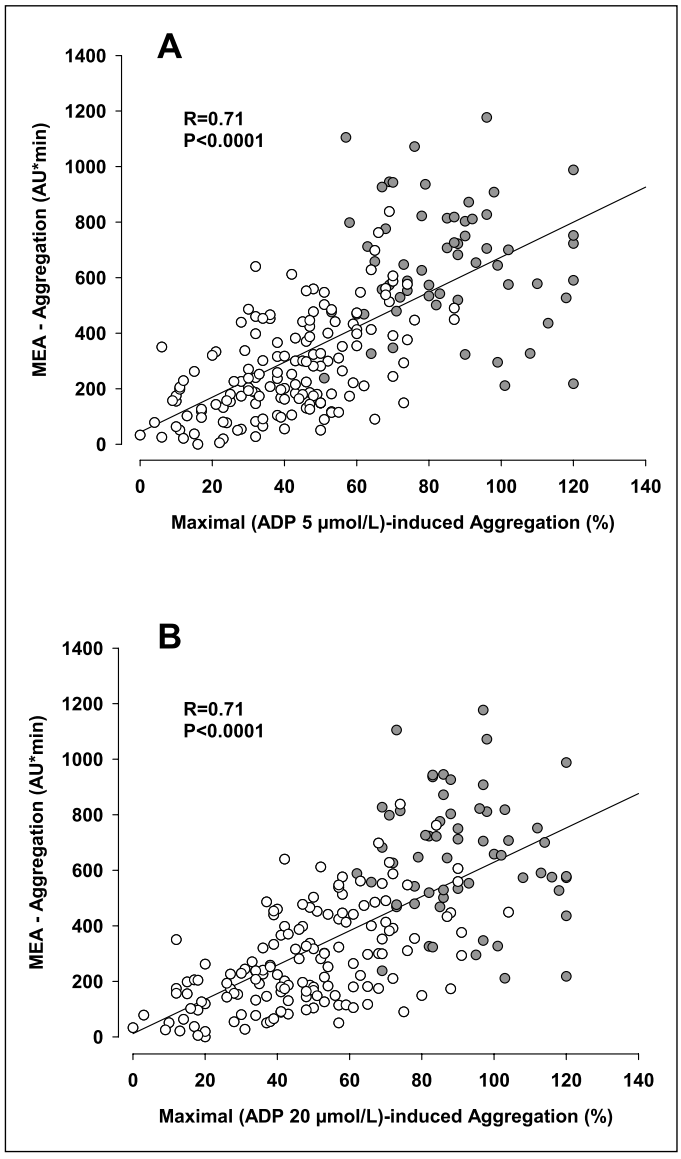

Figure 3: Scatter plots (n=149 patients) of maximal ADP(5 and 20 µM)-induced aggregation (LTA) and aggregation of multiple electrode platelet aggregometry (MEA) quantified as area under the curve (AUC) of arbitrary units (AU*min). Filled circles represent individual measurements. Measurements before clopidogrel treatment are depicted as grey circles (n=60). Measurements after clopidogrel treatment are depicted as white circles (n=149). ADP denotes adenosine diphosphate.

been shown to be the best predictor for clinical outcome (6, 7, 27), we calculated the agreement between the highest quartiles of ADP(5 µM)-induced platelet aggregation (LTA) and MEA after administration of clopidogrel from all patients (n=149). The quartile borders (lowest to highest) were 28.8%, 42.0% and 53.0% for LTA and 145.5 AU*min, 229.0 AU*min and 398.5 AU*min for MEA. Thirty-eight patients were in the upper quartile according to LTA. Out of these 38 patients, 21 patients (55%) were also in the upper quartile according to MEA. According to LTA measurements the remaining 111 patients were assigned to the 1st-3rd quartile. Of these 111 patients, 94 (85%) were also found in quartiles 1–3 according to MEA.

One major adverse cardiac event occurred within 30 days in all patients studied. This event was a periprocedural myocardial infarction due to occlusion of a side branch (diagonal branch). Creatinkinase rose to 611 U/l (MB isoenzyme fraction=13 %) within 24 hours after PCI and then declined. Recanalization of this side branch was neither attempted during the index PCI nor afterwards. In this patient, ADP(5 µM)-induced platelet aggregation (LTA) was 43 % and ADP-induced platelet aggregation (MEA) was 215 AUC.

Discussion

To the best of our knowledge, this is the first study that compares platelet aggregation assessed by MEA in whole blood with platelet aggregation assessed with conventional LTA in PRP. In addition, suppression of platelet function in clopidogrel-treated patients has never been tested with MEA and LTA to correlate the results of the two methods in this setting. This important and so far lacking information is provided by the present study. LTA is the only method that has been shown in large prospective trials to predict the risk of thrombotic events after percutaneous coronary intervention (6, 7). The main result of the study is that MEA is capable of detecting the effect of clopidogrel treatment and that values assessed with MEA in whole blood and expressed as AUC of arbitrary units correlate with maximal ADP-induced platelet aggregation assessed with LTA in PRP. The observed correlation coefficient of 0.71 is comparable to those observed in other studies investigating the correlation of the results of different clopidogrel-sensitive tests with LTA such as the flow-cytometry based test that measures vasodilator-stimulated phosphoprotein (VASP) phosphorylation (PLT VASP/P2Y12, Biocytex; correlation coefficient=0.66) (15) and the cartridge-based point-of-care assay that measures ADP-induced aggregation/agglutination in whole blood (VerifyNow® P2Y12 Assay, Accumetrics, correlation coefficient=0.73) (16). Correlation of IPA (n=60) with both methods was lower than the correlation found for the absolute values obtained with both methods in the entire study population of 149 patients. In MEA the adhesion and aggregation of platelets leads to an increased impedance signal; the more platelets adhere to the electrode the higher the impedance signal. Hence, MEA measurements have no inherent upper limit. When calculating the relative reduction for both methods, differences in prae values for both methods highly influence the relative reduction obtained. The coefficients of variation for LTA and MEA that we assessed in the present study suggest that the two methods are equally reliable to test for clopidogrel non-responsiveness, although, this has not been tested directly.

In a previous study, Hochholzer et al. compared different whole blood assays, including impedance aggregometry measured on a four-channel impedance aggregometer (Chronolog Series 590) (28). The study comprised 27 patients. Similar to our study, platelet function was assessed before and after administration of a single high dose of clopidogrel. The main result of that study was that relative inhibition (%) calculated from the results of both methods did not correlate significantly. Spearman rank correlation coefficient was 0.257 for impedance aggregometry versus LTA (P=0.196) and 0.135 for the ULTEGRA assay

versus LTA (P=0.504). However, multiple electrode platelet aggregometry performed on the new Multiplate analyzer is different from whole blood aggregometry performed on the chronolog device, which is likely to account for the different results obtained.

MEA was performed with hirudin-anticoagulated blood as recommended by the manufacturer. Hirudin as a non-calcium-chelating anticoagulant has potential advantages compared to citrate (18, 29, 30). First, agonist-induced platelet aggregation is attenuated in citrated platelet rich plasma and citrated whole blood most likely due to the lack of divalent ions that are important for several aspects of platelet function (29, 31). Second, platelet secretion and thus the secondary wave of agonist-induced aggregation is artificially enhanced in low calcium environment due to enhanced thromboxane formation (32, 33). We used hirudin as an anticoagulant for LTA in addition to the most commonly used citrate in a subset of the patients to be able to compare MEA with LTA measurements in an environment with similar ion concentrations. Furthermore, MEA implements disposable test cells with duplicate impedance sensors (18). The duplicate sensors serve as an internal control implying the potential to reduce the occurrence of systematic errors. During MEA Pearson´s correlation coefficient of single measurements of the curves assessed by the two electrode pairs and the difference of the two AUCs is calculated (23). The result is flagged if the values are outside of the acceptance range (correlation coefficient <0.98, difference to the mean curve >20%). In general, disposable test cells are beneficial compared to the reusable test cells commonly used for the Chronolog aggregometer, as it is obvious that washing the same cell several times may change surface properties of the electrodes. Moreover, it has been demonstrated by electron microscopic imaging, that platelets, once aggregated during a whole blood aggregometry test, remain on the surface of the electrode (Fig. 1). It is likely that even thorough washing of test cells might not remove them completely. Consequently, reusing the cells has the potential to influence test results. Single use electrodes have since also recently become available for the Chronolog aggregometer (34); however they are not widely used. Notably, MEA measures platelet aggregation as AUC, which is the result of the total height of the aggregation curve as well as its time course.

In general, the assessment of platelet function in whole blood has several advantages over conventional LTA in PRP. There is no need for time-consuming centrifugation steps to separate platelets from other blood cells and to obtain PRP and PPP. Moreover, compared to in-vivo conditions, PRP is an artificial milieu lacking erythrocytes, leukocytes and larger platelet subspecies, all of which are likely to influence the amount of platelet aggregation (35). Additionally, aggregation in MEA takes place on surfaces, which is a major difference compared to LTA, where aggregation occurs in a liquid phase. *In vivo*, coagulation and platelet aggregation take place on surfaces, such as on atheromatous plaques or at sites of vascular injury. Only under certain circumstances like disseminated intravascular coagulopathy (DIC) or heparin-induced thrombocytopenia (HIT) type II do these processes take place in liquid phase. Due to these elementary differences of MEA and LTA the results of both methods are comparable but not identical. It is unknown whether the information obtained with MEA is just as, or maybe even more, relevant for the outcome of patients after PCI.

In conclusion, MEA is a fast and standardized method to individually assess platelet function prior to and after clopidogrel treatment. MEA may be helpful in tailoring antiplatelet regimes in patients scheduled for PCI. However, prognostic implications of measuring platelet function in response to clopidogrel treatment have been demonstrated convincingly only for LTA in PRP (4–8, 24). Clinical validation of the method was not the objective of the present study. The sample size in the present study is far too small to sufficiently analyze a possible association of measurements obtained with MEA and LTA at the time of the intervention with rare thrombotic events after PCI. Whether the information on the effect of clopidogrel obtained with MEA is as or even more predictive for the occurrence of thrombotic events after PCI than the information obtained with LTA, has to be clarified in a specifically designed, large prospective study.

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