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The prognostic value of multiple electrode aggregometry and light transmittance aggregometry in stable cardiovascular patients with type 2 diabetes mellitus

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ABSTRACT

Aim: Limited data are available regarding the clinical relevance of platelet function measurements in stable patients with coronary artery disease (CAD). Our aim is to evaluate the agreement between multiple electrode aggregometry (MEA) and light transmittance aggregometry (LTA) in detecting clopidogrel low responders and their prognostic value in CAD patients with type 2 diabetes mellitus (T2DM) on dual platelet inhibition.

Methods: LTA and MEA were performed in 122 stable cardiovascular patients with T2DM. The upper quartile of patients according to maximum LTA (LTAmax) and MEA measurements were defined as clopidogrel low responders. Agreement between the two methods was evaluated by kappa statistics. We assessed the potential correlation between antiplatelet response and clinical outcome and the optimal cutoff value according to ROC analysis to predict the occurrence of major adverse cardiovascular events (MACE), during 1-year follow-up period.

Results: Cohen's kappa coefficients (0.214) indicated fair agreement (70.2%) between LTA and MEA. A total of 25 MACE occurred in 108 patients (23.1%). Patients with MACE had higher LTAmax than those without (57.1 ± 16.5 vs 49.3 ± 18.3, respectively, p = 0.023). MEA measurements were similar between patients with and without MACE (30.1 ± 15.4 vs 30.6 ± 20.8, respectively; p = 0.84). Multiple logistic regression showed LTAmax response as an independent predictor of death from cardiovascular causes (Odds Ratio, adjusted:0.2;0.05–0.81). ROC analysis indicated that LTAmax cutoff of 62.5% best predicted death (AUC = 0.67, sensitivity = 78%, specificity = 61.5%).

Conclusions: The assessment of platelet responsiveness remains highly test-specific. Our results support the prognostic role of LTA, but not MEA testing, for death risk evaluation in stable cardiovascular T2DM patients.

Abbreviations: ACS, acute coronary syndromes; ASA, aspirin; AUC, area under the curve; CAD, coronary artery disease; CIs, confidence intervals; CR, clopidogrel resistance; DM, diabetes mellitus; HPR, high on-treatment platelet reactivity; IQR, interquartile ranges; LTA, light transmittance aggregometry; MACE, major adverse cardiovascular events; MEA, multiple electrode aggregometry; OR, odds ratios; PCI, percutaneous coronary intervention; POC, point of care; PPP, platelet-poor plasma; PRP, platelet-rich plasma; ROC, Receiver-operating characteristic; T2DM, type 2 diabetes mellitus

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1. Introduction

Antiplatelet therapy with amino-salicylic acid (ASA) - clopidogrel reduces the risk of cardiovascular episodes after percutaneous coronary intervention (PCI) in patients with acute coronary syndromes [1]. However, a significant number of patients experience recurrent events while on such therapy. The individual response to dual antiplatelet therapy is not uniform, and consistent findings across multiple investigations support the association between a lower degree of platelet inhibition, high on-treatment platelet reactivity (HPR), and the occurrence of atherothrombotic events [2,3]. The absolute level of platelet reactivity during treatment (i.e., on-treatment platelet reactivity) has been proposed as a potential measure of thrombotic risk. Thrombotic events may be prevented by decreasing platelet reactivity below certain thresholds. It should be noted that such cut points might depend on the subset of patients studied. In fact, to date, cutoff values have been mainly investigated in patients undergoing PCI and different targets may be obtained in other clinical settings [4,5]. For instance, the dual antiplatelet therapy is not considered to confer significant advantages in patients with stable coronary artery disease (CAD) [6] and increased body mass index, diabetes mellitus (DM), acute coronary syndromes (ACS), older age, reduced left ventricular ejection fraction, have also been associated with a diminished antiplatelet response to clopidogrel [7–10]. Particularly in diabetic patients, impaired clopidogrel-induced antiplatelet effects, leading to HPR, is more prevalent compared with non-diabetic [11–15].

HPR is a well-established marker of recurrent ischemic events, including stent thrombosis, and may thus contribute to the high event rates observed in DM patients undergoing PCI [16,17]. In most studies, platelet function was measured in patients administered clopidogrel during the immediate treatment of ACS and PCI. Limited data are available regarding platelet function measurements during the chronic steady state phase of dual antiplatelet therapy, although this approach is the most practical and feasible in real-world patients.

The aim of this study was to evaluate the capacity of a point-of-care (POC) device, the multiple electrode aggregometry (MEA) and light transmittance aggregometry (LTA), which is considered the gold standard of platelet function testing [18], to detect clopidogrel resistance and predict the clinical outcome in patients with type 2 diabetes mellitus (T2DM) and CAD, while in their chronic steady state phase of combined treatment with clopidogrel and ASA.

2. Methods

Patients with T2DM, between 18 and 75 years of age, treated with oral and/or parenteral hypoglycaemic therapy for at least 1 month, and angiographically established CAD on chronic clopidogrel maintenance therapy of 75 mg/day in combination with daily per os aspirin therapy, were enrolled from January 2015 to April 2017. Patients' enrollment took place in the Second University Department of Cardiology of the Attikon University Hospital in Athens, Greece. Baseline assessment included recording of demographic data, medical history, cardiovascular risk factors, hematological and biochemical parameters, number of diseased vessels, previous MI, ejection fraction and concomitant medications. Patients with renal (creatinine levels > 2.5 mg/dl) or hepatic (bilirubin level > 2 mg/dl) insufficiency, malignant disease, use of drugs known to affect platelet function, history of bleeding diathesis, platelet count $< 100 \times 10^9$/l and a hematocrit < 28% were excluded.

The study was performed in accordance with the Declaration of Helsinki, and the protocol was approved by the institutional review board of Attikon University Hospital (893/03-12-2013). All patients gave written informed consent before undergoing any study procedure.

3. Platelet function analysis

Platelet function tests were performed on the same day and within two hours of sampling. Blood samples were collected from an antecubital vein 2 to 4 h after antiplatelet therapy intake. The first 2 to 4 ml of blood were discarded to avoid spontaneous platelet activation.

3.1. Light transmittance aggregometry (LTA)

The whole blood specimen was collected in 3.8% trisodium citrate and centrifuged at 200*g* for 10 min to obtain platelet-rich plasma (PRP). The remaining specimen was re-centrifuged at 2000*g* for 15 min to obtain platelet-poor plasma (PPP). The platelet count was adjusted to between 200,000/μl and 300,000/μl with PPP. Aggregation was performed using a Biodata-PAP-4 aggregometer (Bio/Data Corporation, PA, USA). The 100% line was set using PPP and a 0% baseline established with PRP before addition of the agonist. ADP 2.0×10^{-5} M (20 μM/l) (Bio/Data Corporation, PA, USA) was the agonist used to induce aggregation. Test procedure was performed as previously described [19]. In brief, 0.45 ml PRP was transferred into a cuvette incubated at 37 °C for 3 min. Then 0.05 ml of the agonist was added into the PRP and the aggregation pattern was allowed to generate for 6 min. Curves were recorded for 6 min, and platelet aggregation was determined as the maximal percent change in light transmittance from baseline using PPP as a reference. In addition to maximum platelet aggregation (LTA max), late (final or residual) aggregation (LTA late) measured 6 min after the addition of agonist, a time when platelet disaggregation normally appears, has been proposed as a better indicator of clopidogrel responsiveness. Although Collet et al. have correlated late aggregation with the antiplatelet response to clopidogrel [20], Gurbel et al. suggested that clopidogrel nonresponders may be similarly identified by both maximal and late aggregation [21]. Aggregation was measured at peak (Aggmax) and at 6 min (Agglate).

3.2. Multiple electrode aggregometry (MEA)

Platelet aggregation in whole blood were assessed by MEA using an impedance aggregometer (Multiplate, Dynabyte, Munich, Germany). Samples were collected into 3.2% citrate tubes and analyzed within the period of half to two hours after blood collection according to manufacturer's instructions. Platelet aggregation was induced by ADP in final concentration 6.5 μM. Each disposable test cell contains two pairs of electrodes, thus enabling two simultaneous measurements. Aggregation was reported as area under the curve (AUC, units), an integrated measure of aggregation velocity and maximal aggregation.

Platelet function analysis was performed in the Laboratory of Haematology and Blood Bank Unit of Attikon University Hospital.

Patients with the upper quartile of LTAmax and MEA measurements were defined as clopidogrel low responders [3,15,17].

Agreement between the two functional platelet methods was evaluated. We assessed the potential correlation between response to antiplatelet treatment and clinical outcome. We also estimated the optimal cutoff value according to ROC analysis to predict the occurrence of atherothrombotic complications during 1-year follow-up period.

The primary end point was defined as a major adverse-cardiovascular event (MACE), a composite of death from cardiovascular causes, nonfatal myocardial infarction, stent thrombosis, ischemic stroke, urgent rehospitalisation for acute coronary syndrome and/or revascularisation during 1-year follow-up period. Episodes of major bleeding were recorded during follow-up [22].

4. Statistical methods

Descriptive statistics are presented as means ± SD, medians and interquartile ranges (IQR), or percentages when appropriate. Depending on the variables' distribution, parametric (two-sample Student's *t*-test) and non-parametric methods (chi-squared test, and the two-sample Wilcoxon rank-sum [Mann-Whitney] test) were employed for the statistical evaluations. Agreement between the two methods

(MEA and ADP-induced LTA max) in detecting resistance to clopidogrel was determined by kappa statistic and the respective p-value. Kappa values of < 0.20 are considered to indicate poor agreement, 0.21 to 0.40 indicate fair agreement, 0.41 to 0.60 indicate moderate agreement, 0.61 to 0.80 indicate good agreement, and > 0.81 indicate very good agreement. Multiple logistic regression analysis was applied to assess whether MEA and LTA max measurements were associated with the clinical outcome (dependent variable). Multivariable analysis was performed (i) by including platelet aggregation measurements as a continuous independent variable, and (ii) in a second model as a dichotomous independent variable (presence of enhanced clopidogrel responsiveness). To control for possible confounding variables, the model included certain covariates that are well-known risk factors, as well as variables with a difference between normal and low responders at a p-value < 0.10. The crude and the adjusted odds ratios (ORs), and the corresponding 95% confidence intervals (CIs) are presented.

Receiver-operating characteristic (ROC) analysis was performed to define sensitivity and specificity of both platelet function methods. It was also used for an exploratory evaluation of the best cutoff point of LTA max and MEA to predict clinical outcome in our study population. The diagnostic odds ratio (DOR), positive and negative predictive values were also estimated using this cutoff point. For hypothesis testing, a probability level of < 0.05 was considered as statistically significant. All statistical tests were two-sided. The R software was used for statistical analyses.

For the sample size calculation, we hypothesized that clopidogrel resistance is more prevalent among T2DM patients compared with non-DM patients [7]; at the same time, the DM group of patients with clopidogrel resistance (CR) suffer an increased likelihood of adverse cardiovascular events [17]. Clopidogrel resistance [called, as previously, CR(+) group] among T2DM patients can be estimated to reach approximately 40% [23,24]. It has been shown that 13% of T2DM patients suffer a major adverse cardiovascular event; this number increases to 38% among T2DM patients with clopidogrel resistance [17]. We estimated that the sample size needed to detect this difference in adverse event rates between CR(+) and CR(−) DM patients ($\alpha = 0.05$, two-sided; power = 80%) was 101 patients [40 in CR(+) group and 61 in CR(−) group]. Assuming an attrition rate of 10%, the necessary sample size should be increased to 111 patients [44 in CR(+) group and 67 in CR(−) group]. The statistical software Power and Precision 4.0 (Biostat Inc., NJ, USA) was used for the sample size analysis.

5. Results

During the study period, a total of 122 consecutive patients meeting the inclusion criteria were enrolled. LTA and MEA were not performed in four patients each, due to technical reasons or transient lack of reagents. Clinical follow-up was not completed in 14 patients.

The cut off values for post-treatment LTA and MEA measurements defining the upper quartile of patients were 63.75% and 44.75 units, respectively. Clinical and laboratory characteristics of the whole study population, normal and low responders based on LTA are presented in Tables 1 and 2, respectively, while similar data based on MEA are shown in Tables 3 and 4, respectively. The agreement between MEA and ADP-induced LTA was approximately 70%. Table 5 gives Cohen's kappa for LTA and MEA. Twelve (10.5%) patients were defined as clopidogrel low responders by both assays, and 68 (59.6%) patients were detected as clopidogrel normal responders by both methods (Fig. 1).

A total of 25 MACE occurred in 108 patients (23.1%) during the one-year study follow-up period. Patients with MACE had higher LTA max (57.1 ± 16.5% vs 49.3 ± 18.3%, p = 0.023) and LTA late (45.6 ± 19.6% vs 35.8 ± 20.7%, p = 0.045) than those with an uneventful follow-up. On the contrary, MEA measurements were similar between patients with and without MACE (30.1 ± 15.4 vs 30.6 ± 20.8 units, p = 0.84). Among low responders based on LTA max, 29.6% suffered a MACE, while 19.2% of MEA low responders reported a MACE.

Multiple logistic regression analysis showed LTA max response to be an independent predictor of death from cardiovascular causes (adjusted Odds Ratio, 0.21; 0.05–0.81), unlike MEA measurements which showed no correlation with clinical outcome (Table 6).

The ROC analysis indicated that a LTA max cutoff value of 62.5% best predicted death due to cardiovascular events in our study population. With this cutoff value, the LTA assay had an area under the ROC curve (AUC) of 0.67 (95% CI: 0.5–0.84), 61.5% sensitivity, 78% specificity, and this corresponded to a DOR of 5.68 (95% CI: 1.67–19.29) (Fig. 2). The positive and negative predictive value of 62.5% LTA max were 28.6% and 93.4%, respectively.

Regarding MEA, 28.5 units was the best threshold to predict death. This cutoff value showed a sensitivity and specificity of 78.6% and 48.9%, respectively, an AUC of 0.57 (95% CI 0.44–0.71) and a corresponding diagnostic odds ratio of 3.51 (95% CI: 0.92–13.42). The positive and negative predictive value of this threshold (i.e. 28.5 units MEA) were 19.3% and 93.6%, respectively.

6. Discussion

In the current study, we investigated the association of on treatment platelet reactivity, as measured by LTA and MEA, with clinical outcome in T2DM patients with CAD. Our findings showed a fair only agreement between the two methods in detecting clopidogrel low responders. Moreover, HPR, as estimated by LTA, seems to have an impact on prognosis in stable cardiovascular T2DM patients. On the contrary, MEA was not found to be correlated with the occurrence of MACE.

In most cases, association of HPR with adverse clinical outcome was based on platelet functional methods performed early after initiation of clopidogrel treatment [4,25,26]. Although the prognostic utility of HPR is more clear in patients undergoing PCI, its clinical relevance in medically-managed, stable CAD patients is less apparent and available data are controversial [17,27,28]. Furthermore, it has been reported that a significant number of patients with decreased platelet inhibition early after initiation of dual antiplatelet therapy, improves during treatment [29,30]. These findings probably indicate that the predictive value of several platelet function tests which have been mainly assessed on stent implanted ACS populations, may differ in several other clinical scenarios.

Regarding T2DM patients with CAD, increased platelet reactivity compared to patients without diabetes seems to be a constant finding, despite combined treatment with clopidogrel and ASA [7,15,31,32]. Whether HPR could have an impact on clinical outcome in these patients remains to be investigated. Cohen's kappa coefficients, as a measure of agreement between the methods, indicated fair agreement between LTA and MEA. The lack of agreement among platelet function methods and absence of consensus on the optimal assays to measure HPR and the cutoff values associated with clinical risk is a common issue in the clinical practice regarding measurements of platelet reactivity in cardiovascular patients [19,33]. However, the most remarkable finding in the current study, is the difference in prognostic value between the two tested methods. Antiplatelet drug responsiveness measured by LTA was significantly associated with clinical prognosis. Likewise, Angiolillo et al. exhibited that HPR, as assessed by LTA, was associated with a higher risk of long-term adverse cardiovascular events in T2DM patients with CAD [17]. It is noteworthy that the authors reported a max cutoff value, best predicted MACE, which is almost the same with that predicted death due to cardiovascular events in our study population (62% vs 62.5%, respectively). Moreover, similar percentages of MACE were reported in these two studies (19.7% vs 23.1%). In the current study patients with MACE had higher LTA max and LTA late than those with an uneventful follow-up, while LTA measurements were found to be significant prognostic variable in the multivariate adjusted analysis. Correlation of laboratory resistance with

Table 1
Baseline demographic and clinical characteristics of the entire cohort and according to response to clopidogrel based on LTA (non-responders > 3rd quartile).

	Total	Responders	Non-responders	p-value
Age	67.2 ± 10.1, 68.0 (42.0–92.0)	66.8 ± 9.9, 67.5 (42.0–92.0)	68.7 ± 11.2, 68.5 (42.0–88.0)	0.32
Women	27/122, 22.1%	17/88, 19.3%	9/30, 30.0%	0.31
Cardiovascular history				
STEMI	19/122, 15.6%	12/88, 13.6%	5/30, 16.7%	0.76
Non-STEMI	35/122, 28.7%	24/88, 27.3%	10/30, 33.3%	0.64
Unstable angina	40/122, 32.8%	32/88, 36.4%	7/30, 23.3%	0.26
Previous coronary artery disease	57/122, 46.7%	44/88, 50.0%	12/30, 40.0%	0.4
Number of diseased vessels	2.2 ± 0.9, 2.0 (0.0–3.0)	2.1 ± 0.9, 2.0 (0.0–3.0)	2.4 ± 0.7, 3.0 (1.0–3.0)	0.28
Ejection fraction (%)	43.9 ± 10.2, 45.0 (20.0–65.0)	43.6 ± 10.4, 45.0 (20.0–60.0)	44.3 ± 9.5, 45.0 (20.0–65.0)	0.88
Comorbidities				
Diabetes mellitus	122/122, 100.0%	88/88, 100.0%	30/30, 100.0%	N/A
HBA1c (%)	7.4 ± 2.4, 7.0 (4.9–29.0)	7.3 ± 2.7, 6.8 (4.9–29.0)	7.4 ± 1.1, 7.2 (5.3–10.2)	0.06
Arterial hypertension	111/122, 91.0%	78/88, 88.6%	29/30, 96.7%	0.29
Dyslipidaemia	81/122, 66.4%	56/88, 63.6%	23/30, 76.7%	0.26
Peripheral vascular disease	17/122, 13.9%	12/88, 13.6%	5/30, 16.7%	0.76
BMI	28.9 ± 3.8, 29.0 (0.0–40.0)	28.6 ± 4.1, 29.0 (0.0–40.0)	29.6 ± 3.0, 30.0 (23.0–35.0)	0.14
Smoking status				
Never-smokers	26/122, 21.3%	19/88, 21.6%	7/30, 23.3%	0.8
Ex-smokers	23/122, 18.9%	17/88, 19.3%	6/30, 20.0%	1
Current smokers	73/122, 59.8%	52/88, 59.1%	17/30, 56.7%	0.83
Non-current smokers	49/122, 40.2%	36/88, 40.9%	13/30, 43.3%	0.83
Treatment				
ASA	122/122, 100.0%	88/88, 100.0%	30/30, 100.0%	N/A
Clopidogrel	122/122, 100.0%	88/88, 100.0%	30/30, 100.0%	N/A
Beta blockers	108/122, 88.5%	76/88, 86.4%	28/30, 93.3%	0.51
Statins	116/122, 95.1%	84/88, 95.5%	28/30, 93.3%	0.64
ACE inhibitors	66/122, 54.1%	47/88, 53.4%	17/30, 56.7%	0.83
Acenocoumarol	12/122, 9.8%	10/88, 11.4%	2/30, 6.7%	0.73
Dabigatran	1/122, 0.8%	0/88, 0.0%	1/30, 3.3%	0.25
Rivaroxaban	1/122, 0.8%	1/88, 1.1%	0/30, 0.0%	1
Apixaban	1/122, 0.8%	1/88, 1.1%	0/30, 0.0%	1
Calcium channel blockers	35/122, 28.7%	25/88, 28.4%	10/30, 33.3%	0.65
Protein pumb inhibitors	79/122, 64.8%	58/88, 65.9%	18/30, 60.0%	0.66
Insulin antidiabetics	58/122, 47.5%	41/88, 46.6%	16/30, 53.3%	0.53
Non-insulin antidiabetics	78/122, 63.9%	55/88, 62.5%	19/30, 63.3%	1

Continuous variables are presented as mean ± SD, median and (range), and compared with the Mann-Whitney test. Categorical variables are presented as numerator/denominator and (percentages), and compared with the Fisher test.
Abbreviations: LTA = Light transmittance aggregometry; STEMI = ST-segment elevation myocardial infarction; HBA1c = glycated hemoglobin A1c; BMI = body mass index; ASA = amino-salicylic acid; ACE = angiotensin converting enzyme.

Table 2
Laboratory data of the entire cohort and according to response to clopidogrel based on LTA (non-responders > 3rd quartile).

	Total	Responders	Non-responders	p-value
HCT (%)	38.8 ± 5.3, 39.0 (25.5–54.7)	39.2 ± 5.6, 39.5 (27.9–54.7)	37.6 ± 4.5, 37.1 (25.5–44.6)	0.24
HGB (g/dl)	13.1 ± 1.9, 13.3 (8.8–18.9)	13.3 ± 2.0, 13.4 (9.0–18.9)	12.7 ± 1.6, 12.4 (8.8–15.3)	0.17
WBC (/μl)	8196.3 ± 2784.8, 7490.0 (4050.0–20,720.0)	7919.9 ± 2777.8, 7305.0 (4050.0–20,720.0)	8685.0 ± 2655.9, 7725.0 (5300.0–18,000.0)	0.07
PLTs (/μl)	239,352.5 ± 74,992.8, 228,000.0 (94,000.0–520,000.0)	243,545.5 ± 83,214.0, 233,500.0 (94,000.0–520,000.0)	229,833.3 ± 48,715.3, 217,500.0 (152,000.0–339,000.0)	0.62
CREATININE (mg/dl)	1.1 ± 0.5, 0.9 (0.4–5.5)	1.1 ± 0.6, 0.9 (0.6–5.5)	1.0 ± 0.4, 0.9 (0.4–2.5)	0.6
AST (U/l)	26.9 ± 23.8, 19.0 (6.0–164.0)	27.7 ± 24.8, 20.0 (6.0–164.0)	25.0 ± 22.3, 18.5 (10.0–123.0)	0.59
ALT (U/l)	25.3 ± 17.6, 21.0 (4.0–112.0)	26.2 ± 18.9, 21.0 (5.0–112.0)	21.9 ± 14.3, 17.5 (4.0–58.0)	0.14
LDH (U/l)	252.1 ± 161.8, 201.0 (99.0–1288.0)	249.4 ± 133.4, 212.0 (99.0–693.0)	220.2 ± 115.3, 181.5 (121.0–626.0)	0.32
TSH (μIU/ml)	2.4 ± 3.5, 1.7 (0.1–37.0)	2.1 ± 1.3, 1.8 (0.1–6.8)	3.2 ± 6.5, 1.7 (0.3–37.0)	0.79
PT (sec)	12.5 ± 2.8, 11.7 (9.8–26.1)	12.8 ± 3.2, 11.8 (9.8–26.1)	11.8 ± 0.9, 11.6 (10.1–13.8)	0.23
INR	1.1 ± 0.2, 1.0 (0.8–2.3)	1.1 ± 0.3, 1.0 (0.8–2.3)	1.0 ± 0.1, 1.0 (0.9–1.2)	0.38
aPTT (sec)	32.1 ± 4.6, 31.5 (22.6–50.9)	32.2 ± 4.9, 31.5 (22.6–50.9)	31.4 ± 3.2, 31.4 (24.7–37.6)	0.8
FIBRINOGEN (mg/dl)	426.2 ± 116.1, 421.0 (22.3–924.0)	425.6 ± 116.3, 420.5 (22.3–696.0)	425.7 ± 117.2, 430.5 (273.5–924.0)	0.66
D-DIMERS (ng/ml)	934.5 ± 1150.2, 539.6 (4.0–7894.0)	965.0 ± 1305.1, 525.5 (4.0–7894.0)	878.1 ± 610.2, 738.1 (176.7–2308.9)	0.31
LTA MAX (%)	50.3 ± 18.7, 53.5 (6.0–93.0)	43.0 ± 15.6, 49.0 (6.0–63.0)	71.7 ± 6.3, 70.0 (64.0–93.0)	< 0.001
LTA LATE (%)	37.6 ± 20.6, 39.5 (0.0–90.0)	29.1 ± 15.9, 30.5 (0.0–61.0)	62.4 ± 10.2, 63.5 (42.0–90.0)	< 0.001
MEA (AUC, units)	31.6 ± 19.6, 30.0 (0.0–81.0)	29.1 ± 20.2, 26.0 (0.0–81.0)	41.9 ± 13.9, 41.5 (11.0–70.0)	< 0.001

Data are presented as mean ± SD, median and (range). Continuous variables are presented as mean ± SD, median and (range), and compared with the Mann-Whitney test. Categorical variables are presented as numerator/denominator and (percentages), and compared with the Fisher test.
Abbreviations: LTA = light transmittance aggregometry; HCT = hematocrit; HGB = hemoglobin; WBC = white blood cells; PLT = platelets; AST = aspartate transaminase; ALT = alanine transaminase; LDH = l*actate dehydrogenase*; *TSH = thyroid-stimulating hormone*; *PT = prothrombin time*; *INR* = international normalized ratio; aPTT = *activated partial thromboplastin time*; LTA MAX = maximum platelet aggregation; LTA LATE = late platelet aggregation; MEA = multiple electrode aggregometry.

Table 3
Baseline demographic and clinical characteristics of the entire cohort and according to response to clopidogrel based on MEA (non-responders > 3rd quartile).

	Total	Responders	Non-responders	p-value
Age	67.2 ± 10.1, 68.0 (42.0–92.0)	66.8 ± 10.1, 66.5 (42.0–92.0)	68.3 ± 9.8, 70.0 (42.0–85.0)	0.31
Women	27/122, 22.1%	13/88, 14.8%	12/30, 40.0%	0.008
Cardiovascular history				
STEMI	19/122, 15.6%	14/88, 15.9%	5/30, 16.7%	1
Non-STEMI	35/122, 28.7%	26/88, 29.5%	7/30, 23.3%	0.64
Unstable angina	40/122, 32.8%	28/88, 31.8%	11/30, 36.7%	0.66
Previous coronary artery disease	57/122, 46.7%	42/88, 47.7%	12/30, 40.0%	0.53
Number of diseased vessels	2.2 ± 0.9, 2.0 (0.0–3.0)	2.2 ± 0.9, 2.0 (0.0–3.0)	2.2 ± 0.8, 2.0 (1.0–3.0)	0.95
Ejection fraction (%)	43.9 ± 10.2, 45.0 (20.0–65.0)	43.4 ± 10.0, 45.0 (20.0–65.0)	44.7 ± 10.9, 47.5 (20.0–60.0)	0.4
Comorbidities				
Diabetes mellitus	122/122, 100.0%	88/88, 100.0%	30/30, 100.0%	N/A
HBA1c (%)	7.4 ± 2.4, 7.0 (4.9–29.0)	7.2 ± 1.3, 7.0 (5.0–12.1)	7.8 ± 4.2, 7.0 (4.9–29.0)	0.89
Arterial hypertension	111/122, 91.0%	78/88, 88.6%	29/30, 96.7%	0.29
Dyslipidaemia	81/122, 66.4%	59/88, 67.0%	19/30, 63.3%	0.82
Peripheral vascular disease	17/122, 13.9%	13/88, 14.8%	4/30, 13.3%	1
BMI	28.9 ± 3.8, 29.0 (0.0–40.0)	28.9 ± 4.1, 29.0 (0.0–40.0)	28.7 ± 2.8, 29.0 (23.0–33.0)	0.6
Smoking status				
Never-smokers	26/122, 21.3%	15/88, 17.0%	10/30, 33.3%	0.07
Ex-smokers	23/122, 18.9%	17/88, 19.3%	4/30, 13.3%	0.59
Current smokers	73/122, 59.8%	56/88, 63.6%	16/30, 53.3%	0.39
Non-current smokers	49/122, 40.2%	32/88, 36.4%	14/30, 46.7%	0.39
Treatment				
ASA	122/122, 100.0%	88/88, 100.0%	30/30, 100.0%	N/A
Clopidogrel	122/122, 100.0%	88/88, 100.0%	30/30, 100.0%	N/A
Beta blockers	108/122, 88.5%	76/88, 86.4%	28/30, 93.3%	0.51
Statins	116/122, 95.1%	83/88, 94.3%	29/30, 96.7%	1
ACE inhibitors	66/122, 54.1%	47/88, 53.4%	17/30, 56.7%	0.83
Acenocoumarol	12/122, 9.8%	7/88, 8.0%	5/30, 16.7%	0.18
Dabigatran	1/122, 0.8%	1/88, 1.1%	0/30, 0.0%	1
Rivaroxaban	1/122, 0.8%	1/88, 1.1%	0/30, 0.0%	1
Apixaban	1/122, 0.8%	0/88, 0.0%	1/30, 3.3%	0.25
Calcium channel blockers	35/122, 28.7%	24/88, 27.3%	11/30, 36.7%	0.36
Protein pumb inhibitors	79/122, 64.8%	56/88, 63.6%	21/30, 70.0%	0.66
Insulin antidiabetics	58/122, 47.5%	40/88, 45.5%	17/30, 56.7%	0.3
Non-insulin antidiabetics	78/122, 63.9%	59/88, 67.0%	15/30, 50.0%	0.13

Continuous variables are presented as mean ± SD, median and (range), and compared with the Mann-Whitney test. Categorical variables are presented as numerator/denominator and (percentages), and compared with the Fisher test.
Abbreviations: MEA = multiple electrode aggregometry; STEMI = ST-segment elevation myocardial infarction; HBA1c = glycated hemoglobin A1c; BMI = body mass index; ASA = amino-salicylic acid; ACE = angiotensin converting enzyme.

Table 4
Laboratory data of the entire cohort and according to response to clopidogrel based on MEA (non-responders > 3rd quartile).

	Total	Responders	Non-responders	p-value
HCT (%)	38.8 ± 5.3, 39.0 (25.5–54.7)	39.6 ± 5.3, 39.8 (25.5–54.7)	36.5 ± 4.5, 35.5 (28.5–44.6)	0.003
HGB (g/dl)	13.1 ± 1.9, 13.3 (8.8–18.9)	13.4 ± 2.0, 13.4 (8.8–18.9)	12.3 ± 1.6, 12.2 (9.6–15.2)	0.004
WBC (/μl)	8196.3 ± 2784.8, 7490.0 (4050.0–20,720.0)	8080.2 ± 2535.6, 7490.0 (4050.0–17,240.0)	8478.3 ± 3415.5, 7400.0 (4390.0–20,720.0)	0.82
PLTs (/μl)	239,352.5 ± 74,992.8, 228,000.0 (94,000.0–520,000.0)	219,909.1 ± 60,950.1, 216,500.0 (94,000.0–520,000.0)	295,566.7 ± 86,015.9, 280,500.0 (152,000.0–503,000.0)	< 0.001
CREAT (mg/dl)	1.1 ± 0.5, 0.9 (0.4–5.5)	1.1 ± 0.6, 0.9 (0.4–5.5)	1.0 ± 0.4, 0.9 (0.6–2.5)	0.66
AST (U/l)	26.9 ± 23.8, 19.0 (6.0–164.0)	26.9 ± 25.0, 19.5 (8.0–164.0)	27.7 ± 21.8, 19.0 (6.0–104.0)	0.98
ALT (U/l)	25.3 ± 17.6, 21.0 (4.0–112.0)	23.8 ± 16.0, 20.5 (4.0–101.0)	29.3 ± 22.2, 20.5 (6.0–112.0)	0.4
LDH (U/l)	252.1 ± 161.8, 201.0 (99.0–1288.0)	263.2 ± 170.5, 218.0 (109.0–1288.0)	224.9 ± 138.2, 179.0 (99.0–626.0)	0.11
TSH (μIU/ml)	2.4 ± 3.5, 1.7 (0.1–37.0)	2.1 ± 1.5, 1.6 (0.3–9.6)	3.3 ± 6.5, 2.2 (0.1–37.0)	0.36
PT (sec)	12.5 ± 2.8, 11.7 (9.8–26.1)	12.5 ± 2.7, 11.7 (9.8–26.1)	12.6 ± 3.2, 11.6 (10.2–24.4)	0.49
INR	1.1 ± 0.2, 1.0 (0.8–2.3)	1.0 ± 0.2, 1.0 (0.8–2.3)	1.1 ± 0.3, 1.0 (0.8–2.0)	0.44
aPTT (sec)	32.1 ± 4.6, 31.5 (22.6–50.9)	32.2 ± 4.9, 31.4 (22.6–50.9)	31.4 ± 3.7, 31.1 (24.7–41.1)	0.54
FIBR (mg/dl)	426.2 ± 116.1, 421.0 (22.3–924.0)	422.3 ± 113.3, 413.6 (22.3–696.0)	437.9 ± 128.7, 432.0 (230.0–924.0)	0.52
D-DIMERS (ng/ml)	934.5 ± 1150.2, 539.6 (4.0–7894.0)	1008.4 ± 1307.4, 537.0 (4.0–7894.0)	781.9 ± 536.3, 542.3 (220.0–2200.0)	0.88
LTA MAX (%)	50.3 ± 18.7, 53.5 (6.0–93.0)	46.9 ± 18.6, 50.0 (7.0–80.0)	58.1 ± 14.9, 57.5 (6.0–86.0)	0.004
LTA LATE (%)	37.6 ± 20.6, 39.5 (0.0–90.0)	34.5 ± 20.2, 35.5 (0.0–71.0)	43.8 ± 18.5, 43.0 (4.0–75.0)	0.038
MEA (AUC, units)	31.6 ± 19.6, 30.0 (0.0–81.0)	22.6 ± 13.0, 25.0 (0.0–44.0)	58.1 ± 8.8, 56.5 (45.0–81.0)	< 0.001

Data are presented as mean ± SD, median and (range).Continuous variables are presented as mean ± SD, median and (range), and compared with the Mann-Whitney test. Categorical variables are presented as numerator/denominator and (percentages), and compared with the Fisher test.
Abbreviations: MEA = multiple electrode aggregometry; HCT = hematocrit; HGB = hemoglobin; WBC = white blood cells; PLT = platelets; AST = aspartate transaminase; ALT = alanine transaminase; LDH = l*actate dehydrogenase*; *TSH = thyroid-stimulating hormone*; *PT = prothrombin time*; *INR* = international normalized ratio; aPTT = *activated partial thromboplastin time*; LTA MAX = maximum platelet aggregation; LTA LATE = late platelet aggregation.

Table 5
Agreement between LTA and MEA determined by kappa statistics and the respective p-values. We defined the responses based on quantile cutoffs (non-responders > 3rd quartile).

	MEA Non-responders	MEA Responders
LTA non-responders	12	16
LTA Responders	18	68

Agreement (%) = 70.2%, Kappa = 0.214, p-value = 0.0221.
Abbreviations: LTA = light transmittance aggregometry; MEA = multiple electrode aggregometry.

clinical outcome was positive only when clopidogrel responsiveness was tested as a dichotomous independent variable (presence or not of enhanced clopidogrel responsiveness) and not as a continuous one. This is probably in line, with the notion that ischemic event occurrences, are not linearly related to on treatment platelet reactivity, but instead occurred above a moderate level of on-treatment platelet reactivity [25]. Another notable finding is the significant association of LTA max with death from cardiovascular causes but not with all MACE, despite higher maximum and late LTA in all patients with MACE, possibly indicating the intense impact of HPR on clinical outcome. Thus, in spite of the weak positive predictive value and the inconvenience of LTA, an aggregation max value of about 62% seems to be a critical predictor variable of clinical prognosis in T2DM patients with CAD.

On the contrary, MEA measurements were not different between patients with and without MACE and no correlation was detected between HPR, as assessed by MEA, and adverse cardiovascular events. MEA high on-treatment platelet responsiveness has been reported as an important independent risk factor for the occurrence of thrombotic/ischemic events after PCI [3]. This does not seem to be the case in stable cardiovascular patients treated with aspirin and clopidogrel, which is in keeping with the perception that prognostic value of HPR in steady-state phase of dual antiplatelet therapy is less overt [27]. Taking into account that in our hands, MEA was found to be considerably affected by platelet count and hematocrit, it seems that as a whole blood assay, it offers convenience but its ability to effectively isolate platelet function from other hematologic parameters is rather doubtful.

We acknowledge that our study has some significant limitations.

Table 6
Crude and adjusted[a] logistic regression estimates regarding the association between clinical outcome and platelet responsiveness to clopidogrel as assessed by LTA and MEA.

	Odds ratio (crude).	Odds ratio (adjusted).
Death, LTA response	0.24 (0.07–0.80)	0.21 (0.05–0.81)
Death, MEA response	1.26 (0.32–4.91)	1.39 (0.31–6.25)
Death, either LTA or MEA response	0.38 (0.06–2.21)	0.34 (0.05–2.30)
Death, both LTA and MEA response	0.33 (0.07–1.55)	0.33 (0.06–1.75)
Death, LTA (continuous)	1.03 (0.99–1.07)	1.03 (0.99–1.08)
Death, MEA (continuous)	1.01 (0.98–1.04)	1.00 (0.96–1.04)
MACE, LTA response	0.44 (0.17–1.19)	0.45 (0.15–1.39)
MACE, MEA response	1.26 (0.42–3.82)	1.04 (0.26–4.10)
MACE, either LTA or MEA response	0.45 (0.10–2.13)	0.28 (0.04–1.78)
MACE, both LTA and MEA response	0.49 (0.13–1.90)	0.35 (0.07–1.82)
MACE, LTA (continuous)	1.03 (1.00–1.06)	1.03 (0.99–1.06)
MACE, MEA (continuous)	1.00 (0.98–1.02)	0.99 (0.96–1.03)

Abbreviations: LTA = light transmittance aggregometry; MEA = multiple electrode aggregometry; MACE = major adverse cardiovascular events; BMI = body mass index; WBC = white blood cells; PLT = platelets.
[a] Adjusted for age, sex, BMI, HbA1c, WBC count, PLT count, hemoglobin, ejection fraction, smoking status and number of diseased coronary vessels.

Since platelet function assays are not interchangeable, more of them could have been evaluated in order to define their prognostic utility in this clinical setting. Many studies have demonstrated a link between the presence of genetic polymorphisms associated with decreased clopidogrel responsiveness and adverse clinical outcomes. For instance, it was observed that 12% of the variation in the response to clopidogrel can be attributed to the *CYP2C19*2* loss-of-function allele [34]. Thus, platelet function testing combined with CYP2C19 genetic testing might be more effective in identifying high-risk individuals. Finally, the recruitment of a control group i.e. patients with CAD but without T2DM, could have provided a more deep insight on how diabetes mellitus affect the association between platelet reactivity and clinical prognosis in CAD patients.

Conclusively, prevention of thrombotic events may be attainable by

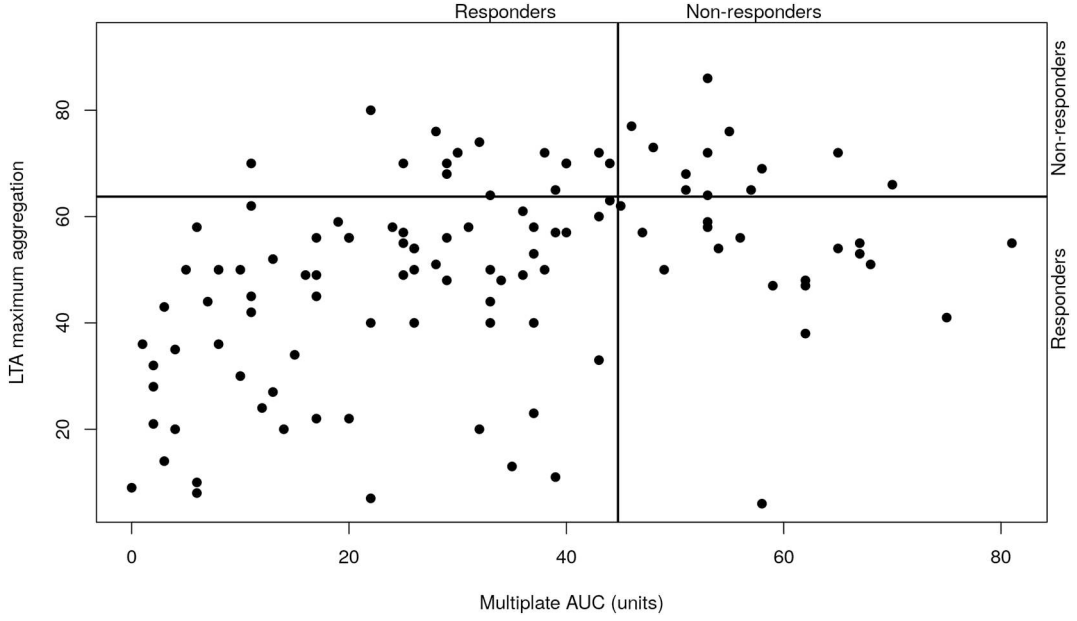

Fig. 1. Comparison of platelet response as determined by LTA and Multiplate. Patients with LTA > 63.75 (%) and Multiplate > 44.75 (units) were defined as clopidogrel non responders.
Abbreviations: LTA = light transmittance aggregometry.

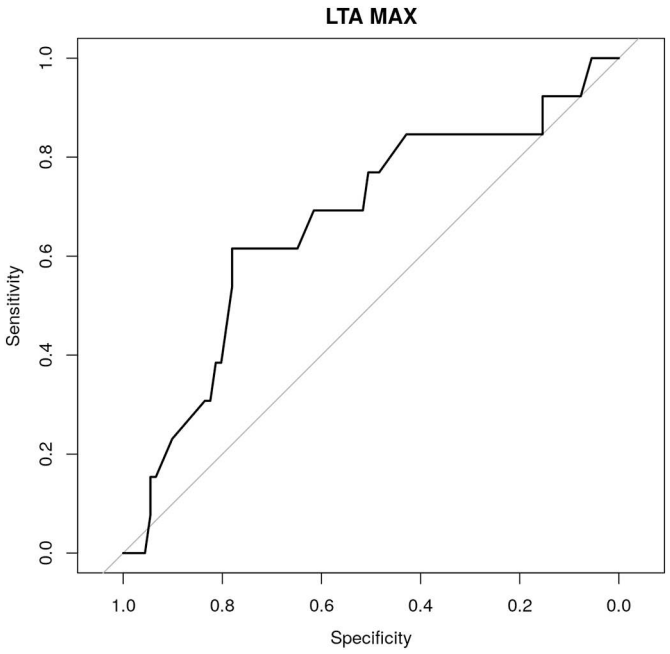

Fig. 2. The ability of LTA to predict death due to cardiovascular events in patients with T2DM and CAD. Area under ROC curve for LTA is 0.67, 95% CI (0.5–0.84).
Abbreviations: LTA = light transmittance aggregometry; T2DM = type 2 diabetes mellitus; CAD = coronary artery disease.

achieving platelet reactivity below certain thresholds. Such cutoff points probably vary according the subset of patients studied, the timing and the platelet function methods performed. The assessment of platelet responsiveness remains highly test-specific, with poor agreement between tests in discriminating patients with and without ischemic events. Our results do not support MEA testing for MACE risk evaluation in stable cardiovascular T2DM patients. On the contrary, the prognostic role of LTA in T2DM patients with CAD probably deserves further evaluation in large-scale trials in order to elucidate whether certain threshold for on-treatment platelet reactivity can be used to stratify patient risk for death due to ischemic/thrombotic events.

Acknowledgments section

A.T. is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

Data and resource availability

The datasets generated during and/or analyzed during the current study are available from the corresponding author upon reasonable request.

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Declaration of Competing Interest

None.

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