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Impaired Responsiveness to the Platelet $P2Y_{12}$ Receptor Antagonist Clopidogrel in Patients With Type 2 Diabetes and Coronary Artery Disease

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ABSTRACT

BACKGROUND Several studies have shown that patients with diabetes mellitus (DM) exhibit an impaired response to clopidogrel. This may contribute to their increased risk of recurrent atherothrombotic events, despite the use of dual-antiplatelet therapy. The mechanisms for impaired clopidogrel response in DM patients have not been fully elucidated.

OBJECTIVES The aim of this study was to explore the mechanisms for impaired clopidogrel-mediated platelet inhibition in patients with DM using a comprehensive methodological approach embracing both pharmacokinetic (PK) and pharmacodynamic (PD) assessments as well as ex vivo and in vitro investigations.

METHODS Patients (DM, n = 30; non-DM, n = 30) with stable coronary artery disease taking aspirin 81 mg/day and $P2Y_{12}$ antagonist naive were enrolled. Blood was collected before and at various times (0.5, 1, 2, 4, 6, and 24 h) after a 600-mg loading dose of clopidogrel. PD assessments included vasodilator-stimulated phosphoprotein, light transmission aggregometry, and VerifyNow $P2Y_{12}$ ex vivo, before and after dosing and following in vitro incubation with escalating concentrations (1, 3, and 10 μM) of clopidogrel's active metabolite (Clop-AM). Exposure to Clop-AM was also determined.

RESULTS PD assessments consistently showed that during the overall 24-h study time course, residual platelet reactivity was higher in DM patients compared with non-DM patients. In vitro incubation with Clop-AM revealed altered functional status of the $P2Y_{12}$ signaling pathway in DM platelets as measured by vasodilator-stimulated phosphoprotein, but not with other PD assays. Clop-AM exposure was ~40% lower in DM patients than in non-DM patients.

CONCLUSIONS The present study suggests that among DM patients, impaired $P2Y_{12}$ inhibition mediated by clopidogrel is largely attributable to attenuation of clopidogrel's PK profile. This is characterized by lower plasma levels of Clop-AM over the sampling time course in DM patients compared with non-DM patients and only modestly attributed to altered functional status of the $P2Y_{12}$ signaling pathway. (J Am Coll Cardiol 2014;64:1005–14) © 2014 by the American College of Cardiology Foundation.

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ABBREVIATIONS AND ACRONYMS

ACS = acute coronary syndrome(s)

ADP = adenosine diphosphate

ANCOVA = analysis of covariance

$AUC_{[0\text{-}tlast]}$ = area under the concentration-time curve through the sampling time of the last quantifiable clopidogrel active metabolite concentration

CAD = coronary artery disease

Clop-AM = clopidogrel's active metabolite

C_{max} = maximal observed plasma concentration

CYP = cytochrome P-450

DM = diabetes mellitus

LD = loading dose

LSM = least-square mean

LTA = light transmission aggregometry

MFI = mean fluorescence intensity

PD = pharmacodynamic

PGE_1 = prostaglandin E_1

PK = pharmacokinetic

PRI = platelet reactivity index

PRP = platelet-rich plasma

PRU = $P2Y_{12}$ reaction units

VASP = vasodilator-stimulated phosphoprotein

VASP-P = vasodilator-stimulated phosphoprotein phosphorylation

VN = VerifyNow

Dual-antiplatelet therapy with aspirin and an antagonist of the $P2Y_{12}$ receptor is the cornerstone of treatment in patients with acute coronary syndrome (ACS) and in those undergoing percutaneous coronary intervention (1–3). Despite the development of newer antiplatelet agents with more predictable pharmacodynamic (PD) response, clopidogrel is still the most broadly used $P2Y_{12}$ receptor antagonist (4). However, clopidogrel is characterized by a high interindividual variability in PD effects, and 5% to 44% of treated patients exhibit impaired responses (5,6). Importantly, inadequate clopidogrel-induced antiplatelet effect is associated with an increased risk of recurrent ischemic events, including stent thrombosis (5–9). Multiple factors can contribute to variations in individual responses to clopidogrel (5,6,10,11). Among these, PD investigations from our group and others have shown that patients with diabetes mellitus (DM) have impaired clopidogrel-mediated antiplatelet effects and higher rates of poor responsiveness than non-DM patients (12–16). This may contribute to the enhanced atherothrombotic risk that characterizes DM patients despite antiplatelet therapy (17).

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The mechanisms of impaired response to clopidogrel in DM patients remain to be fully elucidated. A post-hoc subgroup analysis suggested that the reduced ex vivo PD effects of clopidogrel among DM patients are secondary to a less favorable pharmacokinetic (PK) profile (18). In particular, DM patients have reduced plasma levels of clopidogrel's active metabolite (Clop-AM) compared with those of non-DM patients (18). An in vitro pilot study suggested that platelets from DM patients are characterized by upregulation of the $P2Y_{12}$ signaling pathway, which, in turn, can reduce the PD effects of clopidogrel (19). Prospective studies specifically designed to provide mechanistic insights of impaired clopidogrel response in DM are lacking. The aim of this prospective study was to explore the underlying mechanisms of impaired clopidogrel-mediated platelet inhibition in DM patients using a comprehensive methodological approach, embracing both PK and PD assessments as well as ex vivo and in vitro investigations.

METHODS

PATIENT POPULATION. A total of 60 patients (30 DM and 30 non-DM) with coronary artery disease (CAD) taking low-dose aspirin were prospectively recruited. Patients were screened at the outpatient clinic of the Division of Cardiology-University of Florida College of Medicine Jacksonville. Patients were considered eligible for the study if they met all of the following inclusion criteria: between 18 and 80 years of age, had angiographically documented CAD, on treatment with low-dose aspirin (81 mg/day) for at least 30 days as part of standard of care, and treatment naive for $P2Y_{12}$ antagonism (ticlopidine, clopidogrel, prasugrel, or ticagrelor) for at least 30 days. Patients were stratified according to DM status, defined according to World Health Organization criteria. All subjects with DM needed to be on treatment with oral hypoglycemic agents and/or insulin for at least 2 months without any changes in their regimen (20). Exclusion criteria were any of the following: use of any antiplatelet therapy other than aspirin in the past 30 days, use of parenteral or oral anticoagulation in the past 30 days, active bleeding, hemodynamic instability, any clinical indication to be on a $P2Y_{12}$ receptor antagonist, hemoglobin A_{1c} >12%, use of any drug interfering with cytochrome P-450 (CYP) metabolism (fluconazole, ketoconazole, voriconazole, etravirine, felbamate, fluoxetine, fluvoxamine, omeprazole, esomeprazole, lansoprazole, rabeprazole), cerebrovascular accident in the past 3 months, any active malignancy, platelet count $<100 \times 10^6/\mu l$, hemoglobin <10 g/dl, creatinine >2.5 mg/dl, liver disease (bilirubin levels >2 mg/dl), or pregnant and lactating females. The study complied with the Declaration of Helsinki, was approved by the Institutional Review Board of the University of Florida College of Medicine-Jacksonville, and all patients gave their written informed consent.

STUDY DESIGN. This was a prospective study in which ex vivo and in vitro experiments were conducted. Blood samples for analysis were collected by the antecubital vein in anticoagulated tubes using a 19-gauge needle. The first 2 ml of blood sampled was discarded to avoid spontaneous platelet activation. In the ex vivo experimental component of our study, eligible patients were administered a 600-mg loading dose (LD) of clopidogrel; blood samples for PK and PD assessments were collected at a total of 7 time points: baseline (before LD administration) and 30 min, 1 h, 2 h, 4 h, 6 h, and 24 h after LD. In the in vitro experimental design of our study, blood samples collected at baseline only (before LD administration)

were used; PD testing was performed before and after incubation (for 30 min at 37°C) with escalating concentrations of Clop-AM (1, 3, and 10 μM). Daiichi Sankyo Co., Ltd. (Tokyo, Japan) provided Clop-AM. A flow diagram of the study design is presented in Figure 1.

PD ASSESSMENTS. Three different PD assays were used for both the ex vivo and in vitro experimental designs, as described below.

Vasodilator-stimulated phosphoprotein. The platelet reactivity index (PRI) was determined using standard protocols for the vasodilator-stimulated phosphoprotein (VASP) assay (21–23). Briefly, VASP phosphorylation (VASP-P) was measured by quantitative flow cytometry using commercially available labeled monoclonal antibodies (Biocytex Inc., Marseille, France). The mean fluorescence intensities (MFIs) of VASP-P levels were measured after challenge with prostaglandin E_1 (PGE_1) and PGE_1 + adenosine diphosphate (ADP). PGE_1 increases VASP-P levels through stimulation of adenylate cyclase; ADP binding to purinergic receptors leads to inhibition of adenylate cyclase. Thus, the addition of ADP to PGE1-stimulated platelets reduces the levels of PGE_1-induced VASP-P. The PRI was calculated as follows: $[(\text{MFI PGE}_1) - (\text{MFI PGE}_1 + \text{ADP})/(\text{MFI PGE}_1)] \cdot 100\%$. A reduced PRI indicates greater inhibition of the $P2Y_{12}$ signaling pathway (24).

Light transmission aggregometry. Light transmission aggregometry (LTA) was performed according to standard protocols as previously described (21–23). Briefly, blood was collected in sodium citrate (3.8%) tubes. Platelet aggregation was then assessed by the turbidimetric method in a 2-channel aggregometer (Chrono-Log 490 Model, Chrono-Log Corp., Havertown, Pennsylvania) using platelet-rich plasma (PRP). Platelet agonists included 5 and 20 μM ADP. PRP was obtained as a supernatant after centrifugation of citrated blood at 1,000 rpm for 10 min and was kept at 37°C before use. Platelet-poor plasma was obtained by a second centrifugation at 2,800 rpm for 10 min. For each measurement, light transmission was adjusted to 0% with PRP and to 100% with platelet-poor plasma. Curves were recorded for 6 min after ADP stimuli, and the level of platelet aggregation at 5 min was recorded, being more reflective of $P2Y_{12}$-mediated signaling (25).

VerifyNow $P2Y_{12}$ assay. We used the VerifyNow (VN) system (Accumetrics, San Diego, California), a turbidimetric-based optical detection system that measures platelet-induced aggregation as increased light transmittance according to the manufacturer's instructions, as described (22,23). The VN system is a microbead agglutination assay with reagents specific for the pathways of interest. By combining ADP and PGE_1, the VN $P2Y_{12}$ assay measures platelet reactivity changes that are relatively specific for $P2Y_{12}$ antagonists. Optical signal changes are reported in $P2Y_{12}$ reaction units (PRUs).

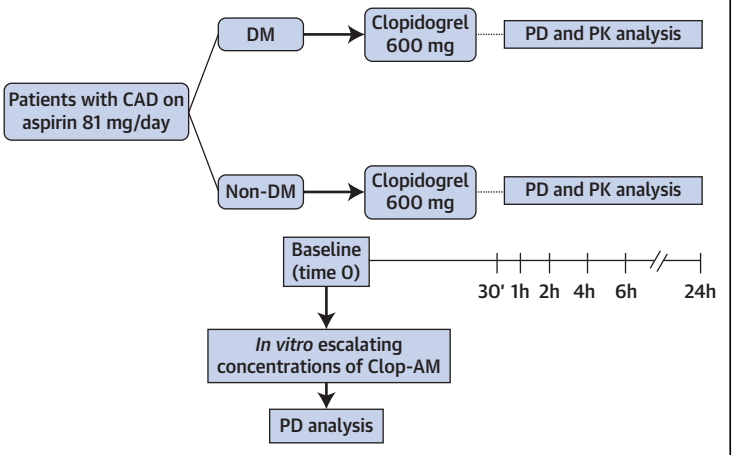


FIGURE 1 Study Design

CAD = coronary artery disease; Clop-AM = clopidogrel active metabolite; DM = diabetes mellitus; PD = pharmacodynamic; PK = pharmacokinetic.

PK ASSESSMENTS. A commercial laboratory (Advion Biosciences, Inc., Ithaca, New York) blinded to the nature of the samples determined the plasma concentration of Clop-AM using liquid chromatography with tandem mass spectrometry, according to standard protocols (26). Blood was drawn into standard ethylenediamine tetraacetic acid tubes. Within 30 s, a derivatizing agent (3′-methoxyphenacylbromide) was added to capture and stabilize the active metabolite. The geometric mean area under the concentration-time curve through the sampling time of the last quantifiable Clop-AM concentration ($AUC_{[0\text{-tlast}]}$) was calculated and the maximum observed plasma concentration (C_{max}) of Clop-AM was recorded.

Sample size estimation and study endpoints. The sample size was determined on the basis of assumptions derived for the ex vivo component of our experimental design, in particular, the comparison of PRI values from patients with and without DM at 6 h after administration of a 600-mg clopidogrel LD. Assuming a 13% SD and an ~10%, dropout rate, we would be able to detect a 10% difference in PRI with 60 patients (30 DM and 30 non-DM), with 95% power and a 2-tailed alpha value of 0.05. PRI was chosen

because it is the most specific marker for $P2Y_{12}$ receptor-mediated signaling (24,27). As part of our experimental plan, we justified our study's sample size to detect differences in our ex vivo PD experiments with established methods and then evaluated the other PK and PD study components. There is a paucity of published data, limiting the ability to define a sample size. The endpoints of our study included PD assessments (24 h overall time course) measured by VASP-PRI, LTA, and VN-P2Y12 as part of the ex vivo component of our experimental design; PK assessments (Clop-AM plasma concentrations, C_{max} and $AUC_{[0\text{-}tlast]}$) as part of the ex vivo component of our experimental design; and PD assessments as listed in the preceding text as part of the in vitro component of our experimental design.

STATISTICAL ANALYSIS. Conformity to the normal distribution was evaluated for continuous variables with the Kolmogorov-Smirnov test. For baseline characteristics, continuous variables are expressed as mean ± SD and categorical variables are expressed as frequency and percentage. The chi-square or Fisher exact test (if the expected value in any cell was <5) was used to compare categorical variables between 2 groups, whereas the Student *t* or Mann-Whitney *U* test was used to compare continuous variables, where appropriate. An analysis of covariance (ANCOVA) method with a general linear model was used to evaluate all between-group comparisons, using the baseline value of the corresponding platelet function test and obesity (defined as a body mass index ≥30 kg/m^2) as covariates. A repeated-measures ANCOVA model, also adjusted by baseline platelet function value and obesity status, was used to evaluate the overall difference between groups. ANCOVA models were performed for the previously mentioned analyses in line with other PK/PD studies (28,29); however, due to the small sample size (because of the PK/PD nature of the study), the dependent variable was not always normally distributed in every combination of values of the covariate and levels of the factor. PD results are reported as least-square mean (LSM) ± SE for the detailed analysis in the preceding text. A 2-tailed p value <0.05 was considered to indicate a statistically significant difference for all analyses performed. Statistical analysis was performed using SPSS version 21.0 software (SPSS Inc., Chicago, Illinois).

TABLE 1 Baseline Characteristics of the Study Population

Variable	DM (n = 30)	Non-DM (n = 30)	p Value
Age, yrs	58.9 ± 8.8	60.5 ± 9.3	0.496
Male	19 (63.3)	21 (70.0)	0.584
Obesity, BMI ≥30 kg/m^2	21 (70.0)	14 (46.7)	0.067
Race			0.786
Caucasian	17 (56.7)	18 (60.0)	
African American	10 (33.3)	9 (30.0)	
Hispanic	1 (3.3)	1 (3.3)	
Other	2 (6.7)	2 (6.7)	
Hypertension	28 (93.3)	26 (86.7)	0.671
Dyslipidemia	27 (93.1)	26 (86.7)	0.671
Active smoking	10 (33.3)	10 (33.3)	1.00
Previous MI	16 (53.3)	14 (46.7)	0.606
PAD	4 (13.3)	4 (13.3)	1.00
Previous PCI	17 (56.7)	15 (50.0)	0.597
Previous CABG	11 (36.7)	11 (36.7)	1.00
Previous stroke	6 (20.0)	1 (3.3)	0.103
LVEF, %	56.9 ± 12.6	56.3 ± 9.1	0.904
Creatinine, g/dl	1.04 ± 0.37	0.95 ± 0.34	0.409
Platelet count, ×10^3/μl	211.5 ± 69.7	244.0 ± 56.2	0.076
Medications			
Insulin therapy	12 (40.0)	–	–
OAD	21 (70.0)	–	–
Aspirin	30 (100)	30 (100)	1.00
Beta-blockers	25 (83.3)	24 (80.0)	0.706
ACEIs/ARBs	21 (70.0)	22 (73.3)	0.940
Statins	23 (76.7)	25 (83.3)	0.905
CCB	8 (26.6)	8 (26.6)	1.00
Nitrates	10 (30.0)	13 (43.3)	0.712

Values are mean ± SD or n (%).

ACEIs = angiotensin-converting enzyme inhibitors; ARBs = angiotensin receptor blockers; BMI = body mass index; CABG = coronary artery bypass surgery; CCB = calcium-channel blockers; DM = diabetes mellitus; LVEF = left ventricular ejection fraction; MI = myocardial infarction; OAD = oral antidiabetic drugs; PAD = peripheral artery disease, PCI = percutaneous coronary intervention.

RESULTS

PATIENT POPULATION. A total of 72 patients meeting study inclusion criteria were identified; 12 of these declined to participate. Thus, a total of 60 patients (DM, n = 30 and non-DM, n = 30) provided their written informed consent to participate and completed the study. Baseline characteristics are summarized in Table 1 and were similar between groups, except for obesity status, which was numerically higher in subjects with DM (p = 0.067). HbA_{1c} levels were 7.45 ± 1.13% in patients with DM.

EX VIVO PD ASSESSMENTS. PRI levels (measured by VASP) (Figure 2A) after a 600-mg clopidogrel LD were significantly higher in DM subjects compared with non-DM during the 24 h of crude analysis (p = 0.001) and after adjusting for baseline PRI values and obesity status (p = 0.016). Platelet reactivity measured with LTA with ADP 20 μM after the 600-mg clopidogrel LD was significantly higher in DM subjects than non-DM subjects (p = 0.005 in

the unadjusted analysis and p = 0.022 after adjusting for baseline platelet reactivity and obesity) (Figure 2B). LTA with 5 μM ADP showed higher platelet reactivity in patients with DM (p = 0.032 in the unadjusted analysis and p = 0.080 after adjusting for baseline platelet reactivity values and obesity) (data not shown). Parallel findings were observed with PRU values measured with VN-$P2Y_{12}$ during the overall 24 h after the clopidogrel LD in the crude analysis (p = 0.039 in the unadjusted analysis and p = 0.056 after adjusting for baseline PRU and obesity) (Figure 2C).

PK ASSESSMENTS. After the 600-mg clopidogrel LD, Clop-AM concentrations throughout the 24-h time course were lower in DM patients compared with non-DM patients (Figure 3). Overall exposure to Clop-AM and C_{max} was significantly reduced in DM patients (Table 2). In particular, the geometric LSM $AUC_{[0\text{-tlast}]}$ in DM patients was 62.7% of that observed for non-DM patients (32.81 ng · h/ml vs. 52.36 ng · h/ml; adjusted p = 0.02). Accordingly, the geometric LSM C_{max} in patients with DM was 53.0% of that observed for patients without DM (19.77 ng/ml vs. 37.32 ng/ml; adjusted p = 0.004).

IN VITRO PD ASSESSMENTS. In vitro incubation of DM blood samples collected at baseline with escalating concentrations of Clop-AM showed attenuated $P2Y_{12}$ inhibition, reflected in significantly higher PRI levels measured by VASP than those from patients without DM, both in the unadjusted analysis (p = 0.006) and after adjustment for baseline PRI and obesity (p = 0.034) (Figure 4A). Furthermore, PRI after incubation with 1 μM (p = 0.047) of Clop-AM was significantly higher in patients with DM, but did not reach statistical significance with 3 μM (p = 0.254) and 10 μM (p = 0.062) after adjustment for baseline PRI and obesity.

LTA with 20 μM ADP showed a higher, but nonsignificant, platelet aggregation in DM patients in the global analysis (p = 0.068 in the unadjusted analysis and p = 0.228 after adjustment for baseline platelet reactivity and obesity), and at each Clop-AM concentration, platelet reactivity did not significantly differ between groups (Figure 4B). Similar trends were observed for LTA with 5 μM ADP (data not shown). PRU values measured with VN-$P2Y_{12}$ after in vitro incubation with escalating concentrations of Clop-AM were also similar between patients with and without DM, both in the global analysis (p = 0.389 in the unadjusted analysis and p = 0.567 after adjustment for baseline PRU and obesity) and at each active metabolite concentration (Figure 4C).

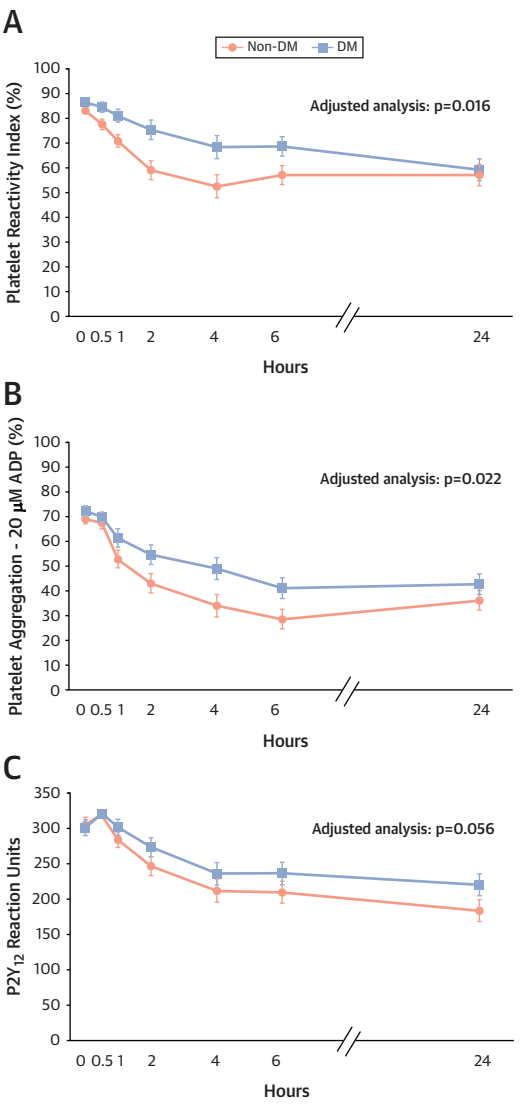


FIGURE 2 Ex Vivo Pharmacodynamic Assessments After 600-mg Loading Dose of Clopidogrel

(A) Platelet reactivity index measured by the vasodilator-stimulated phosphoprotein assay. **(B)** Platelet aggregation measured by light transmission aggregometry after stimulation with 20 μM adenosine diphosphate (ADP). **(C)** $P2Y_{12}$ reaction units measured by the VerifyNow-$P2Y_{12}$ assay. Values are expressed as least-square means. **Error bars** indicate SE. The p values indicate the overall differences between groups assessed by repeated-measures analysis of covariance and adjusted for baseline values and obesity. DM = diabetes mellitus.

DISCUSSION

The results of the present study, which used a comprehensive methodological approach embracing PK and PD assessments and ex vivo and in vitro

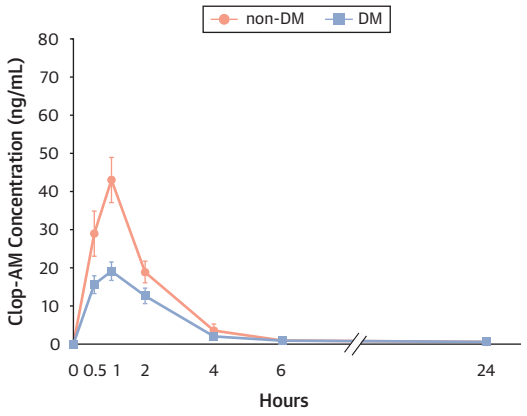


FIGURE 3 Pharmacokinetics of Clop-AM After 600-mg Loading Dose

Mean plasma levels of Clop-AM during the 24 h after a 600-mg loading dose of clopidogrel. **Error bars** indicate SE. Clop-AM = clopidogrel active metabolite; DM = diabetes mellitus.

TABLE 2 PK Profile of Clopidogrel Active Metabolite in Diabetic and Nondiabetic Patients

Parameter	DM Geometric LS Means	Non-DM Geometric LS Means	Ratio of Geometric LS Means, %	p value
$AUC_{[0\text{-}tlast]}$, ng·h/ml	32.81	52.36	62.7	0.02
C_{max}, ng/ml	19.77	37.32	53.0	0.004

Geometric least-square (LS) means and p values were calculated on log-transformed pharmacokinetic (PK) parameters using an analysis of covariance method with a general linear model and obesity status as a covariate. Geometric LS means are presented after back-transformation to the original scale.

$AUC_{[0\text{-}tlast]}$ = area under the concentration-time curve through the sampling time of the last quantifiable clopidogrel active metabolite concentration; C_{max} = maximal observed plasma concentration; DM = diabetes mellitus.

evaluations, confirm that clopidogrel-mediated platelet $P2Y_{12}$ receptor blockade responses are impaired in patients with DM compared with non-DM patients. In particular, we found that residual platelet reactivity after treatment with clopidogrel is higher in patients with DM than in non-DM patients, as corroborated by multiple PD assays. In vitro incubation with escalating concentrations of Clop-AM showed that, as measured by VASP, the functional status of the $P2Y_{12}$ signaling pathway in platelets from DM patients is significantly inhibited. In contrast, with other PD assays, only trends toward up-regulation of the $P2Y_{12}$ signaling pathway were observed. Our study shows that Clop-AM exposure was ~40% lower in DM patients than in non-DM patients. These findings suggest that overall impaired platelet $P2Y_{12}$ receptor blockade mediated by clopidogrel may largely be attributable to abnormalities in the Clop-AM PK profile ("drug exposure") and only to a much lesser degree to platelet dysfunction (drug response) (**Central Illustration**).

Several studies have demonstrated the clinical benefit associated with platelet $P2Y_{12}$ receptor inhibition by clopidogrel, particularly in high-risk settings (1–3). However, a significant number of patients continue to experience poor response to clopidogrel and are at increased risk of adverse outcomes (4–9). Multiple factors can contribute to these findings (5–11). Among these, PD investigations (from our group and others) have shown that patients with DM have impaired clopidogrel-induced antiplatelet effects and higher rates of poor responsiveness compared with non-DM patients (12–16). This may explain the characteristic enhanced ischemic risk in DM patients, including high rates of stent thrombosis, despite clopidogrel treatment (17). The mechanisms contributing to inadequate clopidogrel-induced antiplatelet effects in DM patients are likely multifactorial, although some may be specific to this patient population, such as differences in plasma levels of procoagulant factors, oxidative stress, and cellular function (17,30,31). In particular, platelets from patients with type 2 DM have lost responsiveness to insulin, leading to increased $P2Y_{12}$-mediated suppression of cyclic adenosine monophosphate and decreased response to $P2Y_{12}$ inhibitors (32–34). A previous investigation by our group demonstrated that DM platelets exposed in vitro to escalating concentrations of Clop-AM had persistently higher PRI levels than non-DM platelets, suggesting that $P2Y_{12}$-mediated signaling is dysfunctional in DM patients (19). Although the present results are consistent with these previous in vitro findings with VASP, no significant differences were found with LTA and VN-$P2Y_{12}$, which showed only numerical increases in platelet reactivity in DM patients compared with non-DM patients. These findings may also be due to the greater specificity of VASP-PRI for the $P2Y_{12}$ signaling pathway (24,27).

Inadequate clopidogrel-induced platelet inhibition among DM patients has also been attributed to suppressed Clop-AM circulating levels. Erlinge et al. (18) conducted a post-hoc analysis of a small DM subgroup (n = 9) derived from a prospective PK/PD investigation and showed that DM patients were overrepresented among poor responders identified in a prospective evaluation comparing prasugrel and clopidogrel and had significantly lower Clop-AM levels. However, this study did not find any differences between DM and non-DM patients in either PD

effects after oral administration of clopidogrel or in vitro PD effects after incubation with a single high concentration (10 μM) of Clop-AM. Furthermore, only a trend toward a lower Clop-AM AUC among DM patients was observed (18). Overall, these findings were likely due to the small size of this analysis. Our investigation expands on this previous post-hoc analysis with our prospective, comprehensive PK and PD assessments and ex vivo and in vitro experiments using an array of assays in a larger study population. We showed that C_{max} and the AUC of Clop-AM were lower in DM patients than in non-DM patients. We also used a range of Clop-AM concentrations for the in vitro tests. Overall, our study findings suggest that impaired platelet $P2Y_{12}$ receptor blockade following clopidogrel is largely attributed to abnormalities in clopidogrel's PK profile and only in "small" part attributed to platelet dysfunction. The mechanisms leading to abnormalities in clopidogrel's PK profile among patients with DM are not fully understood and likely imply multiple contributing factors. Indeed, it is well-known that DM patients are affected by gastrointestinal and hepatic abnormalities that can affect drug absorption and metabolism (35–37). Therefore, factors leading to abnormalities in clopidogrel's PK profile among patients with DM may include: 1) decreased gastrointestinal absorption of clopidogrel prodrug; 2) increased clopidogrel prodrug hydrolysis to an inactive carboxylic acid metabolite; 3) reduced hepatic CYP activity; and 4) increased Clop-AM hydrolysis.

The need for more effective platelet-inhibiting strategies is underscored by the findings of the present investigation and the established knowledge that patients with DM remain at risk of ischemic recurrences (17). To achieve this goal, several pilot PD studies have been conducted specifically in DM patients (21–23,38). Indeed, among currently available strategies, use of the novel and more potent $P2Y_{12}$ receptor inhibitors prasugrel and ticagrelor are the most promising. Recently, Alexopoulos et al. (39) showed that DM patients with ACS undergoing percutaneous coronary intervention and pretreated with clopidogrel achieved higher inhibition when switched to ticagrelor compared to when switched to prasugrel. Although prasugrel achieves potent PD effects, largely attributed to its more favorable PK profile (i.e., ability to generate its active metabolite) than that of clopidogrel, DM status also affects plasma levels of prasugrel active metabolite (18,28). Whether this is a characteristic of all oral $P2Y_{12}$ receptor inhibitors or just of thienopyridines remains to be established, as it is unknown whether DM status modulates plasma levels of ticagrelor and its CYP3A4-derived metabolite (AR-C124910XX). Furthermore, it is possible that because ticagrelor is administered twice daily, which may be more optimal for patients with high platelet turnover

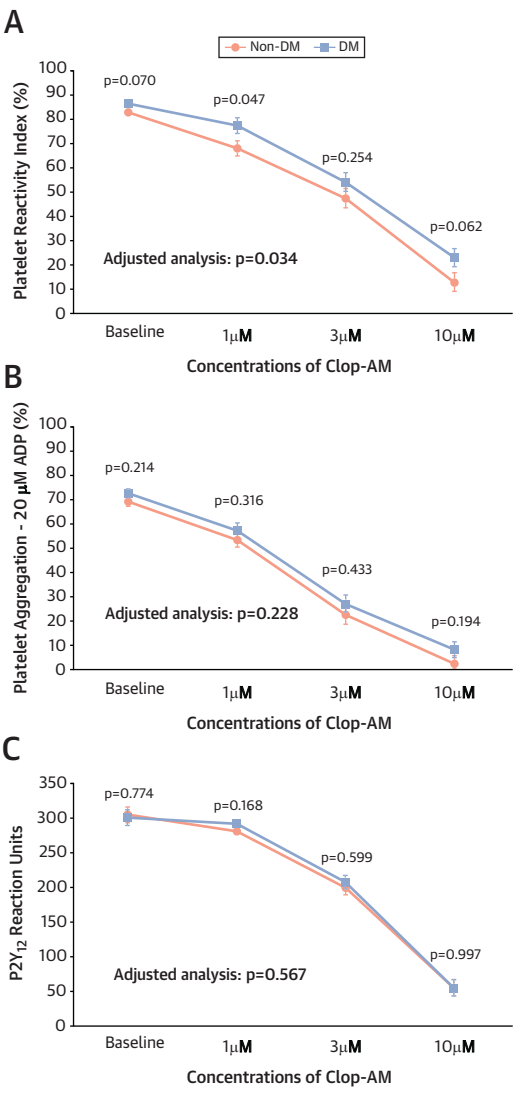
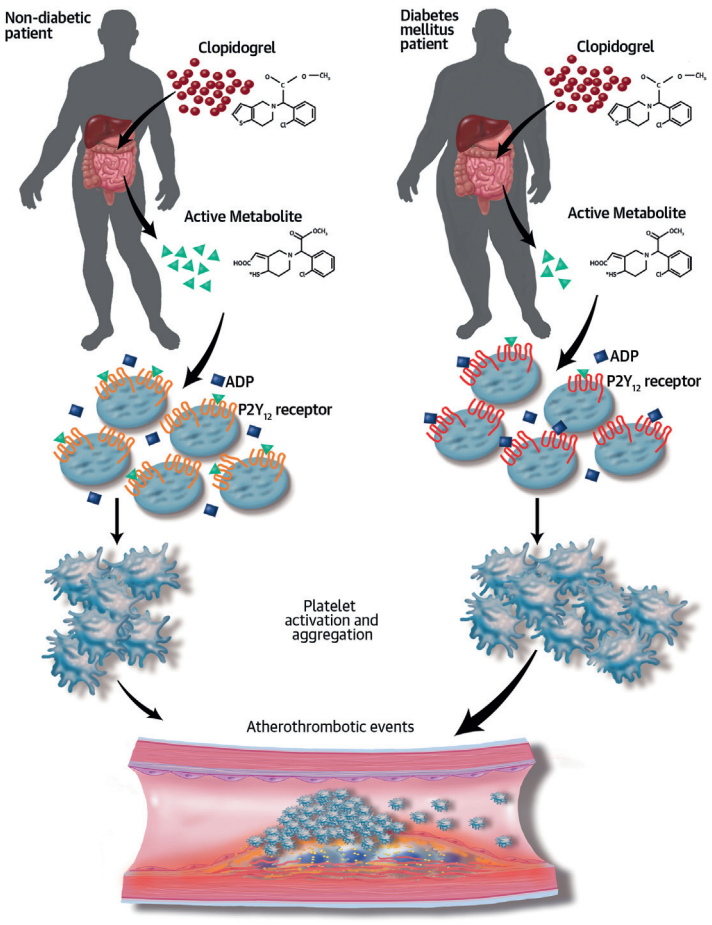


FIGURE 4 In Vitro PD Assessments After Incubation With Escalating Concentrations of Clop-AM

(A) Platelet reactivity index measured by the vasodilator stimulated phosphoprotein assay. **(B)** Platelet aggregation measured by light transmission aggregometry after stimulation with 20 μM adenosine diphosphate. **(C)** $P2Y_{12}$ reaction units measured by the VerifyNow-$P2Y_{12}$ assay. Values are expressed as least-square means. **Error bars** indicate SE. The p values indicate the overall differences between groups assessed by repeated-measures analysis of covariance and adjusted for baseline values and obesity. Abbreviations as in Figure 1.



CENTRAL ILLUSTRATION Mechanism of Impaired Clopidogrel-Induced $P2Y_{12}$ Receptor Blockade in Patients With DM

Adenosine diphosphate (ADP) **(blue squares)**–driven platelet activation and aggregation contribute to atherothrombotic events. Clopidogrel **(red dots)**, after conversion to its active metabolite **(green triangles)**, antagonizes $P2Y_{12}$, the platelet ADP receptor. In diabetes mellitus (DM) patients treated with clopidogrel, impaired platelet $P2Y_{12}$ blockade is largely attributed to marked reductions (~40% lower than nondiabetic subjects) in the pharmacokinetic profile of clopidogrel's active metabolite (drug exposure) and attributed to a much lesser degree to altered functional status of the $P2Y_{12}$ signaling pathway (drug response).

rates such as those with DM, it may lead to more consistent levels of platelet inhibition (40). However, the rate of high platelet reactivity was extremely low without a significant difference between prasugrel and ticagrelor (39). These findings may explain why both prasugrel and ticagrelor were beneficial in the DM cohorts of the TRITON-TIMI 38 (Trial to Assess Improvement in Therapeutic Outcomes by Optimizing Platelet Inhibition with Prasugrel-Thrombolysis In Myocardial Infarction 38) and PLATO (Platelet Inhibition and Patient Outcomes) trials, respectively, although prasugrel appeared to have a greater net clinical benefit in DM patients (41,42).

STUDY LIMITATIONS. Our study demonstrates that impaired clopidogrel-mediated $P2Y_{12}$ inhibition among DM patients is mainly caused by lower Clop-AM exposure. However, we were not able to determine whether this reflects impaired absorption or metabolism or both (35–37). Plasma levels of clopidogrel prodrug would have helped to differentiate between these possibilities. Nevertheless, this does not affect the conclusions of our study. Because our investigation was performed in patients with stable CAD, these findings require confirmation in the setting of DM patients with ACS who are characterized by a hyperreactive platelet phenotype and are more susceptible to absorption and metabolism abnormalities (43,44). However, in the ACS setting, the novel $P2Y_{12}$ receptor antagonists prasugrel and ticagrelor are now more frequently used, and clopidogrel remains the standard of care for stable CAD patients, the target population of the present investigation (4). Although this is among the most comprehensive explorations of the mechanisms associated with differences in clopidogrel response profiles between DM and non-DM platelets, the complex nature of the experiments limited our study to a relatively small number of patients. Indeed, the inherent differences between DM and non-DM patients indicate that additional confounders, others than those already accounted for in our statistical adjustments, may emerge in a larger study. Finally, we did not genotype for CYP2C19 polymorphisms associated with differences in PK/PD profiles in our study; thus, we cannot exclude an allelic frequency imbalance between the DM and non-DM cohorts (10,11).

CONCLUSIONS

Patients with DM exhibit an impaired platelet inhibitory response to the $P2Y_{12}$ receptor inhibitor clopidogrel. The present mechanistic study suggests that this can largely be attributed to abnormalities in clopidogrel's PK profile, characterized by reduced plasma levels of Clop-AM that lead to reduced PD effects, and can only be attributed to a much lesser degree to dysfunctional $P2Y_{12}$ signaling pathway status.

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PERSPECTIVES

COMPETENCY IN MEDICAL KNOWLEDGE: Impaired responsiveness to the $P2Y_{12}$ receptor inhibitor clopidogrel contributes to the increased risk of recurrent atherothrombotic events in patients with diabetes mellitus.

TRANSLATIONAL OUTLOOK: Development of methods to enhance the pharmacokinetic profile of clopidogrel and increase drug exposure in patients with diabetes mellitus will likely prove more effective than interventions that alter the $P2Y_{12}$ signaling pathway in an effort to improve platelet inhibition in response to the drug.

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