Original Article

Platelet Function Profiles in Patients With Type 2 Diabetes and Coronary Artery Disease on Combined Aspirin and Clopidogrel Treatment

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To assess platelet function profiles in diabetic and nondiabetic patients on aspirin and clopidogrel therapy, two patient populations were included to investigate the *1*) acute effects of a 300-mg clopidogrel loading dose (group 1, $n = 52$) and *2*) long-term effects of clopidogrel (group 2, $n = 120$) on platelet function in diabetic compared with nondiabetic patients already on aspirin treatment. Patients were stratified according to the presence of type 2 diabetes. Platelet aggregation was assessed using light transmittance aggregometry (groups 1 and 2). Platelet activation (P-selectin expression and PAC-1 binding) was determined using whole-blood flow cytometry (group 2). Clopidogrel response was also assessed. In group 1, platelet aggregation was significantly increased in diabetic ($n = 16$) compared with nondiabetic ($n = 36$) patients at baseline and up to 24 h following a 300-mg loading dose ($P = 0.005$). In group 2, platelet aggregation and activation were increased in diabetic ($n = 60$) compared with nondiabetic ($n = 60$) subjects ($P < 0.05$ for all platelet function assays). Diabetic subjects had a higher number of clopidogrel nonresponders ($P = 0.04$). Diabetic patients have increased platelet reactivity compared with nondiabetic subjects on combined aspirin and clopidogrel treatment. Reduced sensitivity to antiplatelet drugs may contribute to the increased atherothombotic risk in diabetic patients. *Diabetes* 54:2430–2435, 2005

Diabetes is commonly associated with accelerated atherosclerosis, clinically resulting in premature coronary artery disease (CAD), increased risk of cerebrovascular disease, and severe peripheral vascular disease (1). Patients with type 2 diabetes have a two- to fourfold increase in the risk of CAD, and diabetic patients without prior myocardial infarction have the same risk for a subsequent acute coronary event as nondiabetic patients with a previous myocardial infarction (2,3). Recurrent ischemic events are also more frequent in patients with type 2 diabetes than in nondiabetic patients (4–6). Platelet dysfunction, among other mechanisms, contribute to the increased risk of atherothrombotic complications in the diabetic population (7–9). Such altered platelet function is revealed by hypersensitivity to aggregants observed in in vitro studies.

Platelets from diabetic subjects are also less sensitive to aspirin (10–11). Importantly, reduced sensitivity, or "poor response," to aspirin has been associated with an increased risk of ischemic events (12–15). Combining clopidogrel to aspirin enhances platelet inhibition and has been associated with a reduction in ischemic events compared with the use of aspirin alone (16–19). However, the magnitude of antiplatelet effects may be depressed in diabetic patients. The aim of this study was to compare platelet function profiles in diabetic and nondiabetic patients on combined aspirin and clopidogrel therapy.

RESEARCH DESIGN AND METHODS

Two patient populations were included to investigate the *1*) acute effects of a 300-mg loading dose of clopidogrel (group 1) and *2*) long-term effects of clopidogrel (group 2) on platelet function in diabetic compared with nondiabetic patients. Group 1 was composed of 52 patients, already on aspirin (250 mg/day), scheduled to undergo elective percutaneous coronary intervention and who received a 300-mg clopidogrel loading dose at the time of intervention. Group 2 was composed of 120 patients on long-term (>1 month) combined aspirin (100 mg/day) and clopidogrel (75 mg/day) treatment. All group 2 patients had a known history of CAD and were clinically stable. Patient compliance to antiplatelet treatment was assessed by interview and by pill counting. Patients were stratified according to the presence of type 2 diabetes, which was defined according to World Health Organization criteria (20). All patients were on hypoglyemic treatment (oral antidiabetic agents or insulin) for at least 1 month; diet-controlled diabetic subjects were not included. Exclusion criteria were the use of thrombolytics, platelet glycopro-

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CAD, coronary artery disease; GP, glycoprotein; PRP, platelet-rich plasma.

tein (GP) IIb/IIIa blockers, or thienopyridine pretreatment in group 1 and aspirin intolerance/allergy or platelet counts outside the range of 125–450 10^9/l in groups 1 and 2. This study complied with the Declaration of Helsinki and was approved by the ethical committee of the San Carlos University Hospital, and all patients gave their informed consent.

Blood sampling. Blood samples for platelet function assays were collected before intervention (baseline sample), while patients were only on aspirin, and 10 min and 4 and 24 h following clopidogrel administration in group 1. Blood was drawn from a 6 French arterial sheet for baseline, 10-min, and 4-h samples and from an antecubital vein using a 21-gauge needle for the 24-h samples. Blood samples were collected from an antecubital vein 2–4 h after aspirin and clopidogrel intake in group 2. The initial milliliters of all blood samples were discarded to avoid spontaneous platelet activation. Hematocrit and platelet count was assessed at all time points to ensure that the degree of platelet reactivity was not influenced by cell counts in each group.

Assessment of platelet aggregation. Blood was collected in tubes containing 3.8% trisodium citrate. Platelet aggregation was assessed using platelet-rich plasma (PRP) by the turbidimetric method in a two-channel aggregometer (Chrono-Log 490 Model; Chrono-Log, Havertown, PA), as previously described (21–24). Platelet agonists included 6 μmol/l ADP (Chrono-Log) in group 1 and 6 μmol/l ADP, 20 μmol/l ADP, and 6 μg/ml collagen (Chrono-Log) in group 2 (21–24). PRP was obtained as a supernatant after centrifugation of citrated blood at 800 rpm for 10 min. Platelet-poor plasma was obtained by a second centrifugation of the blood fraction at 2,500 rpm for 10 min. The platelet count in PRP was adjusted to the range of 250.000/μl by dilution with autologous plasma when the platelet count was out of range. Light transmission was adjusted to 0% with PRP and to 100% for platelet-poor plasma for each measurement. Platelet aggregation was assessed within 2 h from blood sampling. PRP was kept at 22°C before use and at 37°C 1 min before running the aggregatory test. Aggregation was assessed in siliconized tubes at 37°C in constant stirring conditions, and curves were recorded for 5 min. Platelet aggregation was determined as the maximal percent change in light transmittance from baseline using platelet-poor plasma as a reference.

Platelet activation. Platelet activation was determined by assessing platelet surface expression of activated GP IIb/IIIa and P-selectin following 2 μmol/l ADP (Chrono-Log) stimuli through flow cytometry in group 2 (23–25). GP IIb/IIIa activation was assessed using a PAC-1 antibody (PAC1-fluorescein isothiocyanate conjugated; Becton Dickinson, Rutherford, NJ), which binds directly to the activated GP IIb/IIIa receptor. P-selectin expression was assessed using a phycoerithrin-conjugated anti-CD62P (0.3 mg/ml; Becton Dickinson, San José, CA). An EPICS-XL PROFILE II Coulter flow cytometer (Coulter, Miami, FL) was used for the assessment. After discard of the initial millilitres of blood to avoid spontaneous platelet activation, whole blood was drawn into sterile tubes containing 3.8% trisodium citrate and then diluted with HEPES-tyrodes buffer (5 mmol/l HEPES, 137 mmol/l NaCl, 2.7 mmol/l $NaHCO_3$, 0.36 mmol/l NaH_2PO_4, 2 mmol/l $CaCl_2$, 5 mmol/l glucose, and 0.2% BSA) to a final volume of 1:8:1 (blood:HEPES-tyrodes:citrate), resulting in a 1/10 dilution of whole blood during sampling. Previously, HEPES-tyrodes buffer was filtered through 0.22-μm sterile filters to avoid interference from particles. Then, 50 μl diluted whole blood was stimulated in vitro with 2 μmol/l ADP before immunolabeling. The corresponding antibody was then added and incubated for 20 min in the dark at room temperature. After incubation, 300 μl of 0.5% PBS-buffered paraformaldehyde was added for fixation. Samples were analyzed within 2 h by flow cytometry, and platelets were identified based on particle size (forward scatter) and complexity (side scatter). Light scatter and fluorescence data from 10,000 platelet events were collected, with all detectors in logarithmic mode. Acquisition and processing data were analyzed with XL2 software (Coulter). Platelet activation was expressed as the percentage of platelets positive for antibody binding.

Definition of clopidogrel response. Response to clopidogrel treatment was assessed according to the degree of inhibition of platelet aggregation defined as the absolute reduction in platelet aggregation 24 h after 300 mg clopidogrel administration compared with baseline values. Baseline values refer to platelet aggregation before clopidogrel administration while patients were only on aspirin. Inhibition of platelet aggregation was expressed as percent inhibition of baseline aggregation according to the following equation: % inhibition = $(PA_{baseline} - PA_{24\ h\ after\ treatment}/PA_{baseline}) \times 100$. Patients were classified as nonresponders, low responders, and responders when platelet inhibition was <10, 10–29, and ≥30%, respectively, as previously reported (23,26,27).

Statistical analysis. Continuous variables are expressed as means ± SD. Categorical variables are expressed as frequencies and percentages. Comparisons between categorical variables were performed using two-tailed Fisher's exact test or the Pearson's χ^2 test, as appropriate. Student's t test was used to compare continuous variables, since these were normally distributed. A MANOVA was used to assess differences between groups during the overall study time course in group 1. $P < 0.05$ was considered statistically significant. Statistical analysis was performed using a SPSS v. 11.0 software (SPSS, Chicago, IL).

TABLE 1
Demographics of group 1

	Diabetic subjects	Nondiabetic subjects	P
n	16	36	
Age (years)	62 ± 10	62 ± 11	0.89
Male	13 (81)	32 (89)	0.66
Risk factors			
Cigarette smoking	8 (50)	26 (72)	0.20
Hyperlipemia	9 (56)	22 (61)	0.77
Hypertension	10 (62)	17 (47)	0.38
Treatment			
β-Blockers	12 (75)	23 (64)	0.53
Nitrates	9 (56)	25 (69)	0.37
ACE inhibitors	5 (31)	12 (33)	1.00
Statins	6 (37)	13 (36)	1.00
Calcium blockers	4 (25)	4 (11)	0.23

Data are means ± SD or n (%).

RESULTS

Acute response to clopidogrel loading dose (group 1). There were 16 diabetic and 36 nondiabetic patients in group 1. Baseline demographic data are reported in Table 1. HbA_{1c} (A1C) levels in individuals with diabetes were 7.4 ± 0.8%. There were no procedural complications, and patients were discharged uneventful.

ADP-induced platelet aggregation was significantly increased in diabetic compared with nondiabetic patients at baseline (patients only on aspirin) and 4 and 24 h after clopidogrel front loading (Fig. 1). No differences in hematocrit and platelet count and mean platelet volume were observed between the two groups (Table 2). Platelet aggregation during the 24 h following clopidogrel loading dose was significantly higher (MANOVA, $P = 0.005$) in diabetic compared with nondiabetic patients (Fig. 1). A significantly higher number of clopidogrel nonresponders were observed 24 h after drug administration among diabetic patients ($P = 0.04$, Fig. 2).

Long-term response to combined clopidogrel and aspirin therapy (group 2). There were 60 diabetic and 60 nondiabetic patients in group 2. Patients were on dual antiplatelet treatment for 8.3 ± 4.6 months. There were no differences in baseline demographics between diabetic and nondiabetic patients (Table 3). A1C levels in individuals with diabetes were 7.1 ± 1.3%. Hematocrit and platelet count and mean platelet volume were also similar in the two groups (Table 3). Diabetic patients had significantly increased platelet aggregation compared with nondiabetic patients, following stimuli with 6 μmol/l ADP (36.5 ± 13.5 vs. 31.2 ± 14.6), 20 μmol/l ADP (51.0 ± 12.1 vs. 45.5 ± 15.8), and 6 μg/ml collagen (41.9 ± 17.3 vs. 31.9 ± 18.5) (Fig. 3*A*). In addition, GP IIb/IIIa activation (39.5 ± 20.4 vs. 30.4 ± 18.6) and P-selectin expression (24.2 ± 16.0 vs. 16.7 ± 11.8) were significantly increased in diabetic compared with nondiabetic patients (Fig. 3*B*). No differences in platelet function were observed between diabetic patients on oral antidiabetic agents ($n = 43$) and insulin treatment ($n = 17$) (Table 4).

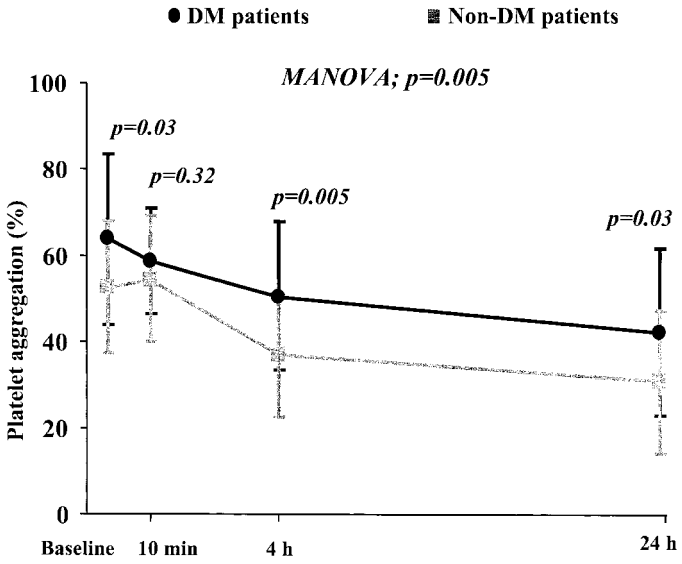

FIG. 1. ADP (6 μmol/l)-induced platelet aggregation in diabetic (DM) (n = 16) compared with nondiabetic (n = 36) patients at baseline (while only on aspirin) and 10 min, 4 h, and 24 h following a 300-mg clopidogrel loading dose.

DISCUSSION

This is the first study to assess platelet function profiles in diabetic patients on combined aspirin and clopidogrel treatment. Our findings demonstrate increased platelet aggregation and activation in diabetic patients compared with nondiabetic patients. In particular, platelet dysfunction in diabetic patients was observed acutely after a 300-mg clopidogrel loading dose and persisted after long-term combined clopidogrel and aspirin therapy. A high proportion of nonresponders to the standard clopidogrel loading dose was also observed in the diabetic population.

Type 2 diabetes has been considered an equivalent to CAD (28). The recurrence rate of ischemic events is higher in diabetic than in nondiabetic patients (1–6). Several mechanisms account for such increased atherothrombotic risk in diabetic patients (28). Individuals with diabetes frequently present with other cardiovascular risk factors (hypertension, dyslipedemia, and obesity). However, this accounts for no more than 25% of the excess cardiovascular risk in this population. Other factors, such as hyperglycemia, insulin resistance, and proinflammatory and prothrombotic status, specific for the diabetic subjects, also contribute to the increased atherthombotic risk. The prothrombotic status is related to endothelial dysfunction, impaired fibrinolysis, increased coagulation factors, and altered platelet function, which decrease response to antiplatelet agents (7–11).

Several mechanisms may account for the reduced sensitivity to aspirin in diabetic patients (7–9). A poor response to aspirin may occur despite adequate suppression of the thromboxane A2 pathway (29). In particular, increased cell-cell interactions (i.e., platelet-erythrocytes) and platelet exposure or reactivity to ADP observed in diabetic patients may contribute to this phenomenon (30–33). This may explain why the use of the ADP receptor antagonist clopidogrel is superior to aspirin in reducing ischemic events in diabetic patients (34). This also suggests that the addition of clopidogrel to aspirin may improve the overall response to antiplatelet therapy in these patients. Indeed, the present study corroborates the synergistic effects on platelet inhibition of dual (aspirin and clopidogrel) antiplatelet therapy. However, diabetic patients still experienced higher platelet aggregation and activation compared with nondiabetic subjects.

Clopidogrel and aspirin synergistically inhibit platelet function through two different pathways: the $P2Y_{12}$ subtype ADP receptor for clopidogrel and the cyclooxygenase-1 enzyme for aspirin (16,17). The addition of clopidogrel to aspirin was accompanied by a significant reduction in platelet aggregation compared with baseline values; however, this still resulted in suboptimal platelet inhibition in diabetic patients. Although the relative reduction in platelet reactivity following clopidogrel administration was lower in diabetic patients, one may argue that at 24 h the absolute reduction in platelet reactivity from baseline was similar (~21%) in both groups, suggesting equivalent sensitivity to clopidogrel. However, when sensitivity is defined according to the degree of inhibition of platelet function following clopidogrel administration, as suggested by several authors (23,26,27), patients with diabetes showed reduced responsiveness (Fig. 2). In addition, when applying another proposed definition for clopidogrel responsiveness (35), which defines clopidogrel nonresponders as those with absolute changes in platelet reactivity <10%, the result is that diabetic patients are still less responsive to clopidogrel (44 vs. 17%, P = 0.04). Also, one may argue that the degree of response to clopidogrel

TABLE 2
Biological data in group 1

	Baseline	10 min	4 h	24 h
Platelet count (10^9/l)				
Diabetic patients	203.4 ± 67.3	180.9 ± 43.8	201.8 ± 69.5	199.2 ± 70.1
Nondiabetic patients	177.9 ± 42.1	173.3 ± 41.2	179.4 ± 36.6	178.5 ± 51.0
P value	0.18	0.73	0.24	0.63
Hematocrit (%)				
Diabetic patients	40.1 ± 3.8	36.2 ± 4.3	37.7 ± 3.4	40.0 ± 4.3
Nondiabetic patients	41.3 ± 4.5	38.3 ± 3.8	40.1 ± 4.8	41.6 ± 3.4
P value	0.37	0.12	0.09	0.16
Mean platelet volume (fl)				
Diabetic patients	9.7 ± 1.0	9.5 ± 0.9	9.7 ± 1.2	9.4 ± 0.9
Nondiabetic patients	9.4 ± 0.7	9.4 ± 0.7	9.3 ± 0.6	9.5 ± 1.2
P value	0.35	0.60	0.13	0.86

Data are means ± SD.

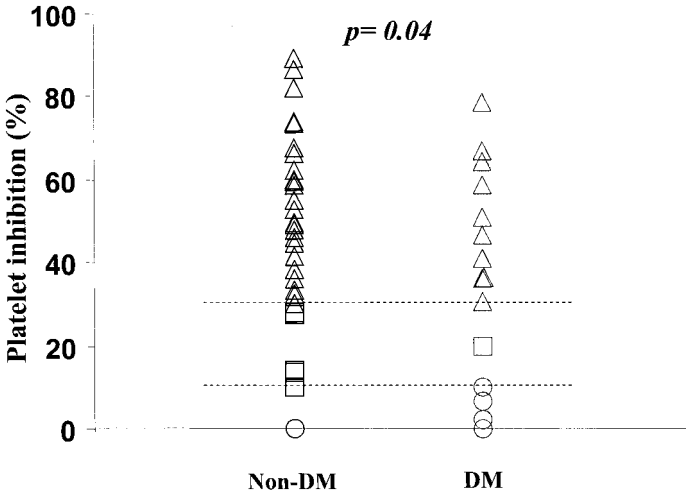

FIG. 2. Inhibition of ADP (6 μmol/l)-induced platelet aggregation to define clopidogrel responsiveness 24 h after loading dose. Clopidogrel nonresponders (○), low responders (□), and responders (△) where defined when platelet inhibition was <10, 10–29, and ≥30%, respectively. Dashed lines indicate 10 and 30% inhibition of platelet aggregation. A significantly higher number of clopidogrel nonresponders were observed among diabetic patients (DM) (nondiabetic vs. diabetic subjects; responders: 78 vs. 56%, low responders: 14 vs. 6%, nonresponders: 8 vs. 38%, $P = 0.04$).

improves beyond 24 h after clopidogrel front loading (35). However, platelet function remained increased in diabetic compared with nondiabetic patients, even after prolonged treatment (Fig. 3*A* and *B*).

Mechanisms, other than increased ADP exposure, may be involved in the reduced sensitivity of diabetic platelets to dual antiplatelet therapy observed in our study. Increased platelet turnover (36), cytosolic levels of calcium (37), oxidative stress resulting in aspirin-insensitive thromboxane biosynthesis (38), altered structure of platelet membrane due to impaired lipid metabolism, and enhanced protein glycation reducing interaction with drug target (39) all affect response to antiplatelet agents. The latter may explain why poor metabolic control may play a role in reduced platelet sensitivity to antiplatelet drugs

TABLE 3
Demographics and biological data of group 2

	Diabetic subjects	Nondiabetic subjects	*P*
n	60	60	
Age (years)	65 ± 10	62 ± 11	0.10
Male	38 (63)	46 (77)	0.16
Risk factors			
Cigarette smoking	4 (7)	8 (13)	0.36
Hyperlipemia	30 (50)	38 (63)	0.19
Hypertension	33 (55)	30 (50)	0.72
Treatment			
β-Blockers	34 (57)	38 (63)	0.58
Nitrates	33 (55)	35 (59)	0.71
ACE inhibitors	25 (42)	18 (30)	0.25
Statins	36 (60)	37 (62)	1.00
Calcium blockers	23 (39)	24 (40)	1.00
Biological markers			
Hematocrit (%)	49 ± 4.5	41.2 ± 3.7	0.24
Platelet count (10^9/ml)	213.1 ± 63.7	208.4 ± 52.7	0.67
Mean platelet volume (fl)	9.2 ± 1.1	9.3 ± 0.9	0.56

Data are means ± SD or *n* (%).

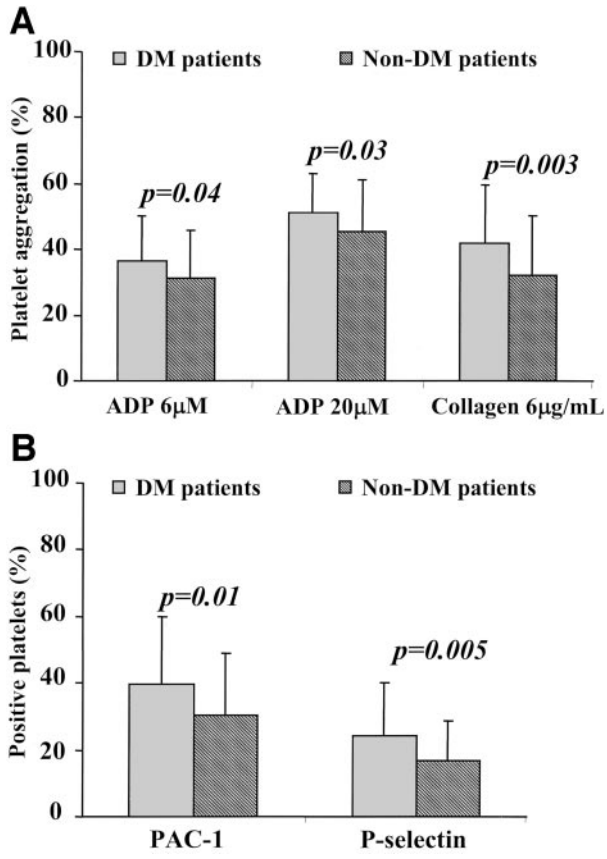

FIG. 3. Platelet aggregation following ADP (6 and 20 μmol/l) and collagen (6 μg/ml) stimuli (*A*) and platelet activation (PAC-1 binding and P-selectin expression) following ADP (2 μmol/l) stimuli (*B*) in diabetic (DM) ($n = 60$) compared with nondiabetic ($n = 60$) patients on sustained aspirin and clopidogrel treatment.

(10). However, in our study, we did not observe any correlation between A1C levels and platelet reactivity (data not shown). This is likely related to the limited variability in A1C levels in our patients. In fact, our study patients all had CAD; therefore, metabolic control was reinforced. Overall, it appears that a more generalized dysfunctional status of the diabetic platelet with multiple pathways is involved in the suboptimal response of diabetic patients to dual antiplatelet therapy.

Increased platelet reactivity is associated with cardiovascular risk (40–43) and may be associated with the increased atherothrombotic risk in the diabetic patient

TABLE 4
Platelet function profiles in diabetic patients according to hypoglycemic treatment

	Insulin	Oral antidiabetic agents	*P*
n	17	43	
Platelet aggregation (%)			
6 μmol/l ADP	38.9 ± 16.5	35.6 ± 12.6	0.39
20 μmol/l ADP	53.3 ± 14.0	50.2 ± 11.3	0.35
6 μg/ml collagen	42.6 ± 17.7	41.7 ± 17.4	0.86
Platelet activation (%)			
PAC-1	43.2 ± 24.3	38.3 ± 19.0	0.48
P-selectin	23.1 ± 11.9	24.6 ± 17.5	0.74

Data are means ± SD.

population. Several reports (12–15) have described that aspirin-resistant patients have an increased long-term risk of ischemic events. Recently, nonresponders to clopidogrel treatment have also been shown to be associated with an increased risk of adverse cardiac events (27,44). Further studies are warranted to define the clinical implications of the poor response to both clopidogrel and aspirin in diabetic patients, as observed in our study. The ongoing CHARISMA (Clopidogrel for High Atherothrombotic Risk and Ischemic Stabilization, Management, and Avoidance) trial has randomized >15,000 patients to aspirin plus placebo or aspirin plus clopidogrel; of these patients, >6,000 have diabetes (45). This trial also included a substudy assessing responsiveness to antiplatelet therapy. Therefore, CHARISMA will not only provide a prospective assessment of whether clopidogrel plus aspirin versus aspirin alone reduces ischemic events in diabetic patients and how this risk reduction compares with that seen in nondiabetic subjects but will also better define the clinical implications of suboptimal response to antiplatelet agents in these patients. Whether optimization of antiplatelet therapy for diabetic patients, either by using higher clopidogrel doses or addition of other antithrombotic drugs, will further decrease the atherothrombotic risk of diabetic patients also remains to be demonstrated.

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