Association of Cyclooxygenase-1–Dependent and –Independent Platelet Function Assays With Adverse Clinical Outcomes in Aspirin-Treated Patients Presenting for Cardiac Catheterization

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Background—Poor clinical outcome in aspirin-treated patients has been termed *aspirin resistance* and may result from inadequate inhibition of platelet cyclooxygenase-1 (COX-1) by aspirin. The objectives of this study were to determine prospectively whether COX-1–dependent and other platelet function assays correlate with clinical outcomes in aspirin-treated patients.

Methods and Results—Blood was collected before percutaneous coronary intervention from 700 consecutive aspirin-treated (81 or 325 mg for ≥3 days) patients. Platelet function was tested by (1) serum thromboxane B_2; (2) arachidonic acid–stimulated platelet surface P-selectin and activated glycoprotein IIb/IIIa and leukocyte–platelet aggregates; and (3) platelet function analyzer (PFA)-100 collagen-epinephrine and collagen-ADP closure time (CT). Adverse clinical outcomes of all-cause death, cardiovascular death, and major adverse cardiovascular events (cardiovascular death, myocardial infarction, hospitalization for revascularization, or acute coronary syndrome) were assessed by telephone interview and/or medical record review. Clinical outcomes information was obtained at 24.8±0.3 months after platelet function testing. By univariate analysis, COX-1–dependent assays, including serum thromboxane B_2 level, were not associated with adverse clinical outcomes, whereas the COX-1–independent assay, PFA-100 collagen-ADP CT <65 seconds, was associated with major adverse cardiovascular events ($P=0.0149$). After adjustment for covariables (including sex, aspirin dose, Thrombolysis in Myocardial Infarction risk score, clopidogrel use), both serum thromboxane B_2 >3.1 ng/mL and PFA-100 collagen-ADP CT <65 seconds were associated with major adverse cardiovascular events. In contrast, indirect measures of platelet COX-1 (arachidonic acid–stimulated platelet markers, shortened PFA-100 collagen-epinephrine CT) were not significantly associated with adverse clinical outcomes even after adjustment for covariables.

Conclusions—In this prospective study of 700 aspirin-treated patients presenting for angiographic evaluation of coronary artery disease, residual platelet COX-1 function measured by serum thromboxane B_2 and COX-1–independent platelet function measured by PFA-100 collagen-ADP CT, but not indirect COX-1–dependent assays (arachidonic acid–stimulated platelet markers, shortened PFA-100 collagen-epinephrine CT), correlate with subsequent major adverse cardiovascular events. This study suggests that multiple mechanisms, including but not confined to inadequate inhibition of COX-1, are responsible for poor clinical outcomes in aspirin-treated patients, and therefore the term *aspirin resistance* is inappropriate. (***Circulation*. 2009;120:2586-2596.**)

Key Words: aspirin ■ myocardial infarction ■ platelets ■ revascularization ■ thromboxane

Aspirin reduces thrombotic events in high-risk patients by 20% to 25%.[1,2] Nevertheless, 10% to 20% of aspirin-treated patients with an arterial thrombotic event have a recurrent thrombotic event during long-term follow-up.[2] In some studies, the occurrence of a thrombotic event despite aspirin treatment has been termed *aspirin resistance*, but, given the multifactorial pathogenesis of thrombosis, *treatment failure* may be a better term.[3,4] Aspirin irreversibly acetylates platelet cyclooxygenase-1 (COX-1), blocking the formation of the potent platelet agonist thromboxane (TX)

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A_2.[2] Thus, poor outcomes in aspirin-treated patients may result from inadequate inhibition of platelet COX-1. Consequently, the less-than-expected inhibition of platelet function by aspirin in an aspirin-sensitive laboratory test has also been termed *aspirin resistance*.[3,4] Estimates of the incidence of aspirin resistance as determined by these tests range from 2% to 65%.[3,5,6]

Clinical Perspective on p 2596

Studies have linked the lack of inhibition of platelet function in aspirin-sensitive in vitro tests with the occurrence of adverse clinical outcomes.[7–10] The magnitude of this risk is uncertain, with odds ratios (ORs) ranging from 1.64[10] to 3.85,[8] due in part to the use of different assays and different definitions of resistance in each assay. Furthermore, many of the assays used are influenced by factors other than inhibition of platelet COX-1 by aspirin. Therefore, it is unclear whether the results of these tests define true aspirin resistance, a term that Cattaneo[11] suggests should be reserved for situations in which a drug is unable to hit its pharmacological target (in this case COX-1). Whether or not poor clinical outcomes in aspirin-treated patients is specifically due to inadequate inhibition of COX-1 by aspirin or another mechanism is critical to the design of strategies to reduce the incidence of thrombosis in these patients.

We therefore undertook the present study to evaluate whether laboratory tests specific for platelet COX-1 activity predict adverse clinical outcomes. The COX-1–dependent assays used were (1) serum TXB_2; (2) arachidonic acid–stimulated platelet surface glycoprotein (GP) IIb/IIIa activation, platelet surface P-selectin, and leukocyte–platelet aggregation; and (3) platelet function analyzer (PFA)-100 collagen/epinephrine (CEPI) closure time (CT). Serum TXB_2 is the most specific test of platelet COX-1 activity because it is the stable metabolite of TXA_2.[11] In the arachidonic acid–stimulated platelet function assays, COX-1 is required to generate TXA_2, which then triggers the end point of the assay. In the PFA-100 CEPI cartridge, collagen, epinephrine, and shear activate multiple pathways including arachidonic acid release and generation of TXA_2. All of these activation pathways, along with other factors such as plasma von Willebrand factor (VWF) levels, contribute to the PFA-100 CEPI CT[12]; however, this assay is inhibited by aspirin, and, in small studies of selected patient populations,[13,14] a short PFA-100 CEPI CT was associated with poor clinical outcomes.

In the present single-site study of 700 consecutive aspirin-treated patients presenting for diagnostic cardiac catheterization, we prospectively determined whether the aforementioned COX-1–dependent assays and/or the COX-1–independent PFA-100 collagen/ADP (CADP) CT were independent predictors of major adverse cardiovascular events (MACE).

Methods

Patient Study Population

The study was approved by the Committee for the Protection of Human Subjects at the University of Massachusetts Medical School and has been described previously.[6] All patients presenting to University of Massachusetts Memorial Medical Center for diagnostic cardiac catheterization for the evaluation of coronary artery disease (CAD) between the hours of 7 AM and 3 PM on weekdays from July 2002 through July 2004 were evaluated, and those who met the enrollment criteria of self-reported intake of either 81 or 325 mg of aspirin per day for ≥3 days were invited to participate. Patients receiving GPIIb/IIIa antagonists were excluded. A total of 700 consecutive patients were enrolled. Less than 3% of eligible patients declined participation. After patients provided written informed consent, peripheral arterial or venous blood was drawn before angiography from the femoral artery or vein after sheath insertion. The blood was immediately placed in evacuated tubes containing either 3.2% sodium citrate or no anticoagulant (BD Biosciences, San Jose, Calif). As described previously,[6] 17 patients were excluded because of blood collection issues, and 1 patient withdrew after consenting. Evaluable results were therefore obtained from 682 subjects.[6] Thrombolysis in Myocardial Infarction (TIMI) risk score[15] was evaluated at study entry. The aspirin-free healthy control subjects were described previously.[6] Briefly, the healthy subjects (n=36; 18 male, age 37.4±1.7 years [mean±SEM]) had normal blood cell counts, no history of a bleeding disorder, and, during the previous 10 days, had abstained from aspirin and other antiplatelet drugs (including nonsteroidal antiinflammatory drugs).

Laboratory Tests of Aspirin Inhibition and Platelet Function

Serum TXB_2

Nonanticoagulated blood was incubated at 37°C for 1 hour, resulting in thrombin generation, platelet activation, and in vitro release of TXA_2, followed by clot formation. Serum was separated and stored at −80°C until analysis. Serum TXB_2 (the stable metabolite of TXA_2) was measured by enzyme-linked immunosorbent assay according to the manufacturer's (R&D Systems) recommendations. Because there are no large prospective studies demonstrating association of a particular level of serum TXB_2 with adverse clinical outcomes, we used the highest combination of specificity and sensitivity obtained by receiver operating characteristic analysis of the present data to identify the optimal serum TXB_2 cutoff for distinguishing patients with MACE from those without MACE. A benefit of this approach is that the cutoff is optimized for this patient population's demographics, medical histories, and comedications.

Flow Cytometric Detection of Arachidonic Acid–Stimulated Leukocyte–Platelet Aggregates, Platelet Surface Activated GPIIb/IIIa, and Platelet Surface P-Selectin

Monocyte– and neutrophil–platelet aggregates were analyzed as described previously.[16] Within 15 minutes of collection, 3.2% citrated whole blood was incubated at 37°C for 15 minutes with fluorescein isothiocyanate–conjugated antihuman CD14 monoclonal antibody (directed against the lipopolysaccharide receptor, a monocyte identifier; Becton Dickinson, Franklin Lakes, NJ) and phycoerythrin conjugates of either CD42a monoclonal antibody (directed against GP IX, a platelet identifier; Pharmingen, San Jose, Calif) or antihuman IgG_{1kappa} (isotype control; Pharmingen) and either buffer or 250 μg/mL arachidonic acid (Bio/Data Corporation, Horsham, Pa). After incubation, samples were fixed with FACSLyse solution (Becton Dickinson) and analyzed in a FACSCalibur flow cytometer (Becton Dickinson). Monocytes and neutrophils were identified by forward and side light scatter and differential CD14 expression. The presence of CD42a on monocytes and neutrophils indicated formation of heterotypic aggregates with activated platelets. The CD42a fluorescence intensity of the heterotypic aggregates was recorded as an approximate indicator of the number of platelets per aggregate. The percentages of monocytes and neutrophils with adherent platelets were recorded.

Platelet surface fibrinogen receptor (GPIIb/IIIa, integrin $\alpha_{IIb}\beta_3$) activation and platelet surface P-selectin were measured by flow cytometry as described previously.[6] Fluorescein isothiocyanate–conjugated PAC1 (a monoclonal antibody specific for the activated conformation of GPIIb/IIIa),[17] phycoerythrin-conjugated CD62P (a

P-selectin–specific monoclonal antibody), peridinin chlorophyll protein–conjugated CD61 (a GPIIIa-specific monoclonal antibody), and control IgG-phycoerythrin were from Becton Dickinson. Aliquots of 3.2% citrate anticoagulated blood were incubated for 5 minutes at 37°C and then stimulated with 250 μg/mL arachidonic acid for 5 minutes in the presence of PAC1–fluorescein isothiocyanate. Samples were then fixed by the addition of 1% formaldehyde in 10 mmol/L HEPES, 0.15 mol/L sodium chloride, pH 7.4. After 30 minutes' fixation, the samples were diluted with 0.5% bovine serum albumin in 10 mmol/L HEPES, 0.15 mol/L sodium chloride (pH 7.4) and stained with CD61-peridinin chlorophyll protein (as a platelet identifier) and CD62P-phycoerythrin. Sample analysis was performed in the FACSCalibur flow cytometer with CellQuest software (Becton Dickinson), as described previously.[18] Positive analysis regions for P-selectin and activated GPIIb/IIIa, respectively, were set with appropriate nonspecific controls. Platelet surface P-selectin and activated GPIIb/IIIa were also analyzed by mean fluorescence intensity.

Platelet Function Analyzer-100

Blood collected in 3.8% sodium citrate was tested in PFA-100 (Siemens Healthcare Diagnostics, Deerfield, Ill) CEPI cartridges (duplicate determinations), according to the manufacturer's recommendations. Blood collected in 3.2% sodium citrate was tested in the PFA-100 CADP cartridge (single determination), according to the manufacturer's recommendations. Results are reported as CT in seconds or as the percentage of subjects with CEPI CT ≤193 seconds or CADP CT <65 seconds; these cutoffs represent the upper limit (CEPI CT) and the lower limit (CADP CT) of the 90% central interval of duplicate results measured in an aspirin-free healthy population.[19,20] Plasma VWF antigen and Ristocetin cofactor activity levels, which affect the PFA-100 assay,[12] were measured with the use of a commercial kit from Dade Behring and the Behring Coagulation Timer (Dade Behring, Marburg, Germany).

Clinical Outcomes

Adverse clinical outcomes of all-cause death, cardiovascular death, and MACE (cardiovascular death, acute myocardial infarction [MI], hospitalization for revascularization [coronary artery bypass grafting {CABG} or percutaneous coronary intervention {PCI}], or acute coronary syndrome) were assessed by telephone interview and/or medical record review. Revascularization during the index procedure at enrollment was not counted as a MACE. Follow-up contact was initiated at 18 to 24 months after enrollment; no study-related patient contact occurred between enrollment and follow-up. Patients were identified as lost to follow-up if contact was not made within 5 telephone attempts and medical records were unavailable. All-cause death was assessed for all subjects by review of medical records and the Social Security Death Index (online database) (Provo, Utah: The Generations Network, Inc, 2007. Original data: Social Security Administration, SSDI, Master File; Social Security Administration accessed via www.ancestry.com). For some individuals, cause of death was obtained from Massachusetts Registry of Vital Records and Statistics. Cause of death could not be identified for 2 patients identified as deceased through the Social Security Death Index but for whom medical records and death certificate were not available. Patients not listed as expired were presumed to be living. All clinical outcomes data were obtained by research personnel who were blinded to the results of the laboratory tests of aspirin inhibition and platelet function.

Statistical Analysis

Statistical analyses were performed with the statistical package SAS 9.1.3 (SAS Institute Inc, Cary, NC). This study was powered (α=0.05, β=0.8) to detect a difference of 20% in the frequency of MACE in subjects with high versus low serum TXB_2. A multivariable logistic regression model for the influence of demographic factors and laboratory results on clinical outcome was developed by the following steps. First, demographic factors were assessed for independent association with MACE by using stepwise logistic regression, with entry and retention in the model set at a significance level of 0.15 and 0.05, respectively. If multiple factors were highly correlated with each other, only one was included in the model. Factors commonly considered related to adverse cardiovascular outcomes (sex, body mass index [BMI], platelet count, use of oral hypoglycemic agents) were also included in the model, even if not statistically different between subjects with and without MACE. Laboratory results were then individually added to the model to evaluate their association with outcomes while controlling for demographic differences. Cox proportional-hazard analysis was performed to take into account time to event and was adjusted for the same demographic factors as the logistic regression model. In all instances, the assumption of proportionality was tested and found to be valid. Results of laboratory assays in aspirin-free subjects compared with aspirin-treated patients were compared with an unpaired *t* test with Welch's correction. Kruskal-Wallis test with Dunn posttest was used for analysis of ≥3 groups containing nonparametric data. Categorical data were analyzed with χ^2 or, if the number of events in any category was <5, by Fisher exact test. Results are reported as mean±SE unless indicated otherwise. All tests were 2 sided, and a *P* value of 0.05 was considered significant.

Results

Patient Demographics

Clinical characteristics at the time of entry into the study for all patients and those with and without follow-up for MACE are shown in Table 1. The average time of follow-up was 24.8±0.3 months.

COX-1–Dependent Tests

As expected, serum TXB_2 was significantly reduced in aspirin-treated patients compared with aspirin-free healthy controls (2.04±11.8 versus 247±106 ng/mL [mean±SD]; $P<0.001$; Table 2). However, the level of residual platelet COX-1 function, as evidenced by serum TXB_2 levels, varied widely (range, 0.01 to 235 ng/mL). Two patients had serum TXB_2 levels in the range observed for aspirin-free healthy controls, and their platelet function was therefore consistent with aspirin noncompliance. Because "resistance" cannot be distinguished from noncompliance, these subjects were not excluded from follow-up.

Receiver operator characteristic analysis of serum TXB_2 levels with regard to MACE identified 3.1 ng/mL as the optimal cutoff. Demographic characteristics of patients followed up for MACE who had low serum TXB_2 (≤3.1 ng/mL) versus those with high serum TXB_2 (>3.1 ng/mL) are shown in Table 3. Aspirin-treated patients with high serum TXB_2 were more often on low-dose aspirin (81 mg/d), COX-2 antagonists, antidepressants, and oral hypoglycemic agents than were patients with low serum TXB_2. In addition, patients with high serum TXB_2 had higher BMI and platelet counts than those of patients with low serum TXB_2 (Table 3).

As expected, arachidonic acid–stimulated (1) monocyte–platelet aggregates, (2) neutrophil–platelet aggregates, (3) platelet surface activated GPIIb/IIIa, and (4) platelet surface P-selectin were all significantly reduced in aspirin-treated patients compared with aspirin-free healthy controls (Table 2). Like serum TXB_2 levels, however, the response to arachidonic acid in aspirin-treated patients varied widely (Table 2).

Although serum TXB_2 and the arachidonic acid–induced platelet function tests are clearly COX dependent, the PFA-100 CEPI CT is more complex, with collagen, epinephrine,

Table 1. Patient Characteristics Grouped by Follow-Up

	All Patients (n=682)	Patients With Follow-Up (n=562)	Patients Lost to Follow-Up (n=120)	*P**
CAD risk factors				
Age, y	60.7±0.44	61.6±0.48	56.4±1.05	0.001
Sex, % male	68	65	80	0.002
BMI, kg/m^2	30.1±0.23	30.0±0.25	30.6±0.59	0.357
Family history of CAD, %	64	65	58	0.273
Hyperlipidemia, %	84	84	84	0.384
Hypertension, %	71	72	65	0.073
Diabetes mellitus, %	27	28	20	0.053
Current smoker, %	22	19	34	0.001
Prior smoker, %	72	72	72	0.989
TIMI risk score	2.90±0.05	2.98±0.05	2.57±0.11	0.001
History, %				
Prior MI	24	24	26	0.878
Prior PCI	30	30	32	0.769
Prior CABG	11	12	6	0.121
Prior stroke	4	4	3	0.670
Prior pulmonary embolism	0.6	1	0	1.000
Prior deep vein thrombosis	0.6	1	0	1.000
Reason for catheterization, %				
Positive exercise tolerance test	38.0	37.4	40.9	0.834
Chest pain	17.7	17.2	20.0	0.469
Stable angina	17.1	17.7	13.9	0.324
Unstable angina	14.5	15.2	11.3	0.280
Non–ST-segment–elevation MI	6.5	6.4	7.0	0.838
ST-segment–elevation MI	1.2	0.7	3.5	0.032
Other	5.0	5.4	3.5	0.490
Hematology values				
White blood cells, $\times10^9$/L	7.1±0.1	7.1±0.1	7.2±0.2	0.537
Hematocrit, %	40.0±0.17	39.8±0.18	41.1±0.39	0.003
Platelets, $\times10^9$/L	227.7±2.6	228.7±2.9	222.6±5.7	0.366
Medications, %				
COX-2 antagonists	6	6	5	0.657
Nonsteroidal antiinflammatory drugs	10	10	0	<0.0001
Clopidogrel	32	34	23	0.026
Heparin	11	10	18	0.010
Warfarin	4	5	3	0.264
Low-molecular-weight heparin	8	9	7	0.461
β-blocker	74	74	78	0.404
Statin	69	69	72	0.496
Angiotensin-converting enzyme inhibitor	41	42	33	0.068
Calcium channel blocker	23	24	18	0.206
Diuretic	29	31	22	0.039
Insulin	7	8	4	0.143
Oral hypoglycemics	19	20	16	0.321
Antidepressants	19	17	27	0.013
Angiography result				
CAD, %	80.9	81.7	77.5	0.291
Stent, % (n)	35.3 (241)	36.5 (205)	30.0 (36)	0.178
Bare metal stent, % (n)	53.9 (130)	52.7 (108)	61.1 (22)	0.349
Drug-eluting stent, % (n)	40.3 (97)	41.5 (85)	33.3 (12)	0.359
Bare metal stent and drug-eluting stent, % (n)	5.8 (14)	5.9 (12)	5.6 (2)	0.944

*P value for patients with follow-up vs patients lost to follow-up, determined by t test for continuous variables or by χ^2 analysis for categorical variables. Fisher exact test was used when there were ≤5 events in a category. Ninety-nine percent of individuals enrolled and 100% of individuals with follow-up were white.

Table 2. Comparison of COX-1–Dependent and COX-1–Independent Platelet Function Assays in Aspirin-Treated Patients vs Healthy Aspirin-Free Controls

Test	Aspirin-Free Controls, Mean±SD, n or %, n/N	Aspirin-Treated Patients (81 or 325 mg × ≥3 days)		*P**
		Mean±SD, n or %, n/N	Range	
Serum TXB_2, ng/mL	247±106, 33	2.04±11.8, 562	0.01–235	<0.0001
AA-stimulated monocyte-platelet aggregates, %	73.1±20.8, 36	13.6±10.4, 561	2.9–82.5	<0.0001
AA-stimulated neutrophil-platelet aggregates, %	53.6±22.0, 36	7.6±5.6, 561	2.3–52.9	<0.0001
AA-stimulated monocyte-platelet aggregates, fluorescence	226±151, 36	50.5±16, 561	11.5–254.2	<0.0001
AA-stimulated neutrophil-platelet aggregates, fluorescence	132±70, 36	33.2±8.6, 561	6.2–95.8	<0.0001
AA-stimulated P-selectin–positive platelets, %	80.8±12.5, 36	13.9±7.7, 561	2.7–67.7	<0.0001
AA-stimulated PAC1-positive platelets, %	81.0±12.0, 36	39.3±17.4, 561	0.3–86.8	<0.0001
AA-stimulated platelet surface P-selectin, fluorescence	122±113, 36	4.2±1.7, 561	2.6–26.8	<0.0001
AA-stimulated platelet surface PAC1 binding, fluorescence	40.7±32.8, 36	7.5±3.5, 561	2.5–28.3	<0.0001
PFA-100 CEPI CT ≤193 s, %	94, 34/36	21.7%, 122/562		<0.0001
PFA-100 CADP CT <65 s, %	2.8, 1/35	2.8%, 16/562		NS

AA indicates arachidonic acid.

*P value for aspirin-free controls *vs* aspirin-treated patients determined by t test for continuous variables or by χ^2 test for categorical variables.

and shear contributing to platelet activation. The PFA-100 CEPI CT was prolonged in aspirin-treated patients compared with aspirin-free healthy controls (ie, PFA-100 CEPI CT was more often >193 seconds [the manufacturer's lower limit of the normal range for aspirin-free healthy controls]) (Table 2). The frequency of CEPI CT >193 seconds in the study population with aspirin treatment was 78.3% (440 of 562). Thus, the CEPI CT is partially COX-1 dependent.

COX-1–Independent Tests

The PFA-100 CADP CT for patients treated with aspirin alone (no clopidogrel) was not significantly different from that observed in aspirin-free healthy controls (median [25th to 75th percentile], 92.0 [80 to 112] seconds, n=370, versus 88.5 [70 to 96] seconds, n=36, respectively; $P>0.05$), which confirms that the PFA-100 CADP CT is COX-1 independent. Similarly, the frequency of CADP CTs less than the lower limit of the normal range (65 seconds) was not different between aspirin-free controls and all evaluable aspirin-treated patients (Table 2). In contrast, compared with either aspirin-free healthy controls or aspirin-treated patients, patients treated with both aspirin and clopidogrel had significantly prolonged PFA-100 CADP CT (102.0 [83 to 133] seconds; n=191; $P<0.001$ for each comparison, Kruskal-Wallis test with Dunn multiple comparison), demonstrating the partial dependence of the PFA-100 CADP CT on $P2Y_{12}$ function.

Clinical Outcomes

Major Adverse Cardiovascular Events

MACE (cardiovascular death, MI, hospitalization for revascularization [CABG or PCI], or acute coronary syndrome) were observed in 19.8% (111 of 562) of the patients. This outcome was driven mainly by revascularization (10.1%; n=57) and secondarily by acute coronary syndrome (5.3%; n=30) and MI or stroke (3.0%; n=17). Cardiovascular death was infrequent (1.2%; n=7). One MACE occurred on day 2 after enrollment and catheterization, whereas all other MACE occurred at >7 days after enrollment and catheterization. Therefore, no censoring of early MACE events was required.

Demographic Factors and MACE

The association of individual demographic factors with MACE was evaluated, and those with a $P<0.15$ were included in a multivariable regression model to correct for the influence of covariables. The covariables adjusted for were sex, TIMI risk score, aspirin dose, clopidogrel use, platelet counts, white blood counts, and the use of low-molecular-weight heparin. When multiple covariables were associated very closely with one another, adjustment was made for only one, and the others were excluded (eg, the TIMI risk score and current smoking were very closely associated, and therefore adjustment was made for the TIMI risk score but not for current smoking status). Figure 1 shows the ORs for demographic factors at enrollment found to be associated with subsequent MACE after adjustment for covariables. As expected, aspirin dose of 325 mg/d, clopidogrel use, statin use, and high TIMI risk score were each independently associated with an increased risk for subsequent MACE, consistent with their use or presence in a subset of patients with more severe cardiovascular disease at enrollment.

Serum TXB_2 Levels and MACE

MACE occurred more frequently in patients with high (>3.1 ng/mL) than in patients with low (≤3.1 ng/mL) serum TXB_2 (28.9% [13 of 45] versus 19.0% [95 of 510], respectively; Table 4). Although this univariate analysis was not significant ($P=0.108$, χ^2), multivariable logistic regression analysis

Table 3. Demographics of Patients With Low (≤3.1 ng/mL) vs High (>3.1 ng/mL) Serum TXB_2

	Serum TXB_2 ≤3.1 ng/mL (n=517)	Serum TXB_2 >3.1 ng/mL (n=45)	P*
CAD risk factors			
Age, y	61.9±11.2	58.7±12.4	0.070
Sex, % male	65.2	66.7	0.841
BMI, kg/m^2	29.8±5.83	32.9±6.58	<0.001
Family history of CAD, %	64.5	71.1	0.535
Hyperlipidemia, %	83.5	84.4	0.909
Hypertension, %	72.7	68.9	0.541
Diabetes mellitus, %	27.9	35.6	0.425
Current smoker, %	19.5	17.8	0.775
Prior smoker, %	71.9	73.3	0.832
TIMI risk score	2.97±1.26	3.11±1.47	0.488
History, %			
Prior MI	23.8	22.2	0.453
Prior PCI	31.0	20.0	0.271
Prior CABG	12.8	4.4	0.149
Prior stroke	4.1	4.4	0.707
Prior pulmonary embolism	1.0	0.0	1.000
Prior deep vein thrombosis	1.0	0.0	1.000
Reason for catheterization, %			
Positive exercise tolerance test	37.0	42.2	0.485
Chest pain	17.7	11.1	0.261
Stable angina	17.3	22.2	0.408
Unstable angina	16.0	6.7	0.128
Non–ST-segment–elevation MI	6.2	8.9	0.520
ST-segment–elevation MI	0.6	2.2	0.286
Other	5.3	6.7	0.687
Hematology values			
White blood cells, ×10^9/L	7.1±2.30	7.4±2.29	0.372
Hematocrit, %	39.8±4.31	40.0±4.55	0.678
Platelets, ×10^9/L	226.9±67.99	249.5±65.78	0.033
Medications, %			
COX-2 antagonists	27.9	68.9	<0.001
Nonsteroidal antiinflammatory drugs	6.2	4.4	1.000
Clopidogrel	8.7	15.1	0.128
Heparin	34.4	26.7	0.291
Warfarin	9.5	11.1	0.721
Low-molecular-weight heparin	4.5	8.9	0.261
β-blocker	8.9	6.7	0.787
Statin	74.5	66.7	0.253
Angiotensin-converting enzyme inhibitor	67.9	75.6	0.288
Calcium channel blocker	41.8	48.9	0.355
Diuretic	22.8	33.3	0.112
Insulin	31.0	33.3	0.740
Oral hypoglycemics	7.7	11.4	0.424
Antidepressants	18.6	33.3	0.017
Angiography result			
CAD, %	81.6	82.2	0.921
Stent, % (n)	36.2 (187)	40.0 (18)	0.609
Bare metal stent, % (n)	50.0 (99)	52.9 (9)	0.811
Drug-eluting stent, % (n)	44.4 (77)	41.2 (8)	0.788
Bare metal stent and drug-eluting stent, % (n)	5.6 (11)	5.9 (1)	1.000

*P value for patients with serum TXB_2 ≤3.1 vs patients with serum TXB_2 >3.1, determined by t test for continuous variables or by χ^2 analysis for categorical variables. Fisher exact test was used when there were ≤5 events in a category.

showed a significant association between serum TXB_2 >3.1 ng/mL and MACE when factors known to be different between patients with versus without MACE (aspirin dose, TIMI score, use of clopidogrel or statins) and factors thought to affect MACE (platelet count, BMI, sex, use of oral hypoglycemic agents) were included in the model (OR, 2.399; 95% confidence interval [CI], 1.053 to 5.463; P=0.0372; Figure 2). Figure 3A shows the timing of MACE with respect to serum TXB_2 levels. Cox proportional-hazard analysis, which takes into account time to event, also showed a significant association of serum TXB_2 ≤3.1 ng/mL with MACE (hazard ratio, 2.22; 95% CI, 1.208 to 4.091; P=0.0103; adjusted for sex, BMI, TIMI score, aspirin dose, platelet count, and use of clopidogrel, statins, or oral hypoglycemic agents). The χ^2 analysis showed no significant association of the occurrence of all-cause death or cardiovascular death with high versus low serum TXB_2, although the occurrence of these events was infrequent (Table 4).

COX-1–Dependent and –Independent Assays and MACE

To determine whether residual COX-1–dependent activity, as reflected by end points downstream from arachidonic acid–stimulated thromboxane production (platelet surface acti-

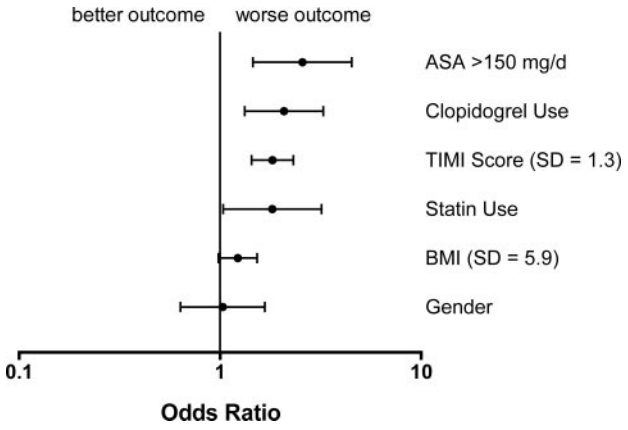

Figure 1. Demographic factors at enrollment associated with subsequent MACE are consistent with a subset of patients known to be at risk for a worse outcome. ASA indicates acetylsalicylic acid.

Table 4. Frequency of Adverse Outcomes by Serum TXB_2 Level and PFA-100 CADP CT

	≤3.1 ng/mL	>3.1 ng/mL	*P*
TXB_2			
MACE	98/510 (19.0)	13/45 (28.9)	0.108 (χ^2)
All-cause death	26/630 (4.1)	3/52 (5.8)	0.4785 (Fisherexact)
Cardiovascular death	5/609 (0.82)	2/51 (3.9)	0.0957 (Fisherexact)
	≥65 s	<65 s	*P*
CADP CT			
MACE	104/544 (19.1)	7/16 (43.7)	0.0149 (χ^2)
All-cause death	24/652 (3.7)	2/28 (7.1)	0.2909 (Fisherexact)
Cardiovascular death	6/652 (0.9)	1/28 (3.5)	0.2560 (Fisherexact)

Numbers in parentheses are percentages.

vated GPIIb/IIIa, P-selectin expression, monocyte–platelet aggregates and neutrophil–platelet aggregates, and PFA-100 CEPI CT), was associated with MACE, these responses to arachidonic acid were evaluated in a multivariable logistic regression model, controlling for TIMI risk score, aspirin dose, platelet count, BMI, and use of clopidogrel, statins, and oral hypoglycemic agents. The resultant ORs are shown in Figure 2. In this population of 682 aspirin-treated patients, none of the COX-1–dependent downstream end points evaluated (serum TXB_2, arachidonic acid–stimulated GPIIb/IIIa activation, arachidonic acid–stimulated platelet surface P-selectin, arachidonic acid–stimulated leukocyte–platelet aggregates, and PFA-100 CEPI CT) was associated with MACE.

In contrast, by univariate analysis of the PFA-100 CADP CT, a COX-1–independent assay, MACE occurred more frequently in patients with a CT <65 seconds (7 of 16; 43.7%) than in patients with a CT ≥65 seconds (104 of 544; 19.1%; $P=0.0149$, χ^2; Table 4). The association of PFA-100 CADP CT <65 seconds with MACE remained significant after adjustment for sex, BMI, TIMI score, aspirin dose, platelet count, VWF antigen (because VWF is known to affect the PFA-100 assay[12]), and use of clopidogrel, statins, or oral hypoglycemic agents (OR, 3.5; 95% CI, 1.2 to 10.4; $P=0.0265$) (Figure 2). Likewise, when time to event was taken into account, Cox proportional-hazard analysis showed a significant association of PFA-100 CADP CT <65 seconds with MACE (hazard ratio, 2.343; 95% CI, 1.039 to 5.284; $P=0.0402$; covariables age, sex, BMI, TIMI score, aspirin dose, platelet count, VWF antigen, and use of clopidogrel, statins, or oral hypoglycemic agents). Figure 3B shows the timing of MACE events with respect to the PFA-100 CADP CT.

Discussion

The present study is the largest prospective study to date to use serum TXB_2 to evaluate the relationship between aspirin inhibition of platelet COX-1 and subsequent adverse clinical outcomes. The main findings of this study of aspirin-treated patients presenting for angiographic evaluation of CAD are as follows: (1) Serum TXB_2, a direct measure of platelet COX-1 platelet function, is associated with an increased risk for MACE (OR, 2.399; 95% CI, 1.053 to 5.463; $P=0.0372$); (2) 5 separate indirect measures of platelet COX-1 function (arachidonic acid–stimulated, platelet surface activated GPIIb/IIIa; arachidonic acid–stimulated platelet surface P-selectin; arachidonic acid–stimulated monocyte–platelet aggregates; arachidonic acid–stimulated neutrophil–platelet

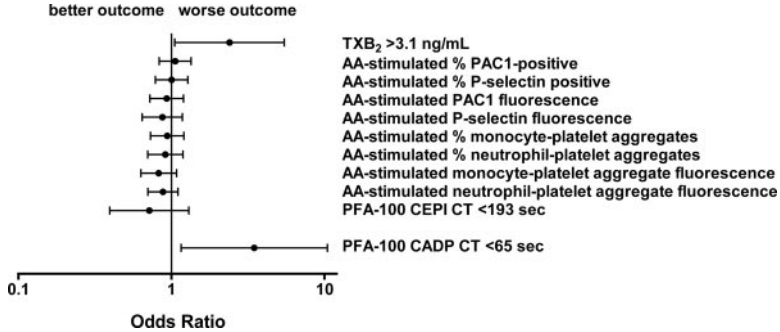

Figure 2. ORs for subsequent MACE as independently predicted by COX-1–independent (PFA-100 CADP CT) and COX-1–dependent (all other) assays at study enrollment. Results shown are multivariable logistic ORs by category or per SD unit (defined below) adjusted for differences in sex, BMI, TIMI score, aspirin dose, platelet count, and use of clopidogrel, statins, or oral hypoglycemic agents. One SD unit for each assay is as follows: arachidonic acid (AA)–stimulated % PAC1 positive, 17.4%; AA-stimulated % P-selectin positive, 7.7%; AA-stimulated PAC1 fluorescence, 3.5 arbitrary fluorescence units; AA-stimulated P-selectin fluorescence, 1.7 arbitrary fluorescence units; AA-stimulated % monocyte–platelet aggregates, 10.4%; AA-stimulated % neutrophil–platelet aggregates, 5.6%; AA-stimulated monocyte–platelet aggregate fluorescence, 16.0 arbitrary fluorescence units; and AA-stimulated neutrophil–platelet aggregate fluorescence, 8.6 arbitrary fluorescence units.

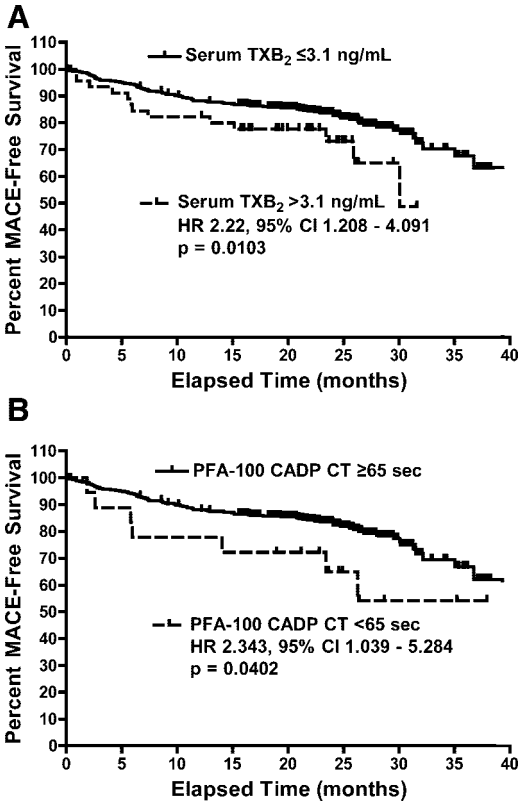

Figure 3. Kaplan–Meier MACE-free survival curves for subjects with serum TXB_2 ≤3.1 versus >3.1 ng/mL (A) and subjects with PFA-100 CADP CT <65 versus ≥ 65 seconds (B). Hazard ratios (HRs) were calculated by Cox proportional hazard analysis with adjustment for age, sex, BMI, TIMI score, aspirin dose, platelet count, and use of clopidogrel, statins, or oral hypoglycemic agents. For PFA-100 CADP, VWF antigen level was also included as a covariable because VWF is known to affect the PFA-100 assay.

aggregates; and PFA-100 CEPI CT) are not associated with adverse clinical outcomes; and (3) COX-1–independent platelet function, as determined by the PFA-100 CADP CT, is associated with an increased risk for MACE (OR, 3.5; 95% CI, 1.2 to 10.4; $P=0.0265$). On the basis of these results, we conclude that, in this patient population, poor clinical outcomes of aspirin-treated patients are due in part to incomplete COX-1 inhibition but are also due in part to COX-1–independent platelet hyperreactivity.

Mechanism of Platelet Hyperreactivity in "Aspirin-Resistant" Patients

The term *aspirin resistance* has been used frequently in the recent literature to describe the less-than-expected inhibition in aspirin-sensitive laboratory tests of platelet function.[3,7,8,21] This definition, however, does not provide insight into the specific mechanism(s) of recurrent thrombosis despite aspirin treatment and may be misleading.[11] In the present study, we measured serum TXB_2, a product directly dependent on COX-1 activity, as well as other COX-1–dependent and –independent platelet function assays, enabling us to address whether poor inhibition of COX-1, as opposed to another assay variable(s), is related to subsequent poor clinical outcomes. In the present aspirin-treated patient population, mean serum TXB_2 was inhibited by >99% compared with serum TXB_2 levels in aspirin-free normal donors (Table 2). Thus, consistent with other studies,[21–23] a poor response to aspirin, as measured by serum TXB_2, was infrequent. Less than 2% of subjects had serum TXB_2 >10 ng/mL, and only 8% had a serum TXB_2 level >3.1 ng/mL. Because there is no prospectively defined level of TXB_2 associated with MACE, this latter cutoff was generated by receiver operator characteristic analysis to optimally distinguish patients with MACE from those without MACE. Despite this approach introducing a bias toward identifying a significant association between serum TXB_2 and MACE, high residual COX-1 function, as reflected by high residual serum TXB_2 (>3.1 ng/mL), was not associated with subsequent MACE ($P=0.108$, χ^2; Table 4) unless the effects of confounding covariables were taken into account (OR, 2.4; 95% CI, 1.05 to 5.46; $P=0.0372$). Less direct measures of residual COX-1 function, 4 separate arachidonic acid–dependent assays evaluating platelet activation markers downstream from COX-1, were not associated with subsequent MACE, even after adjustment for covariables (Figure 2). Finally, using the more complex but still partially COX-1–dependent (Table 2) end point of PFA-100 CEPI CT, we also found no association with subsequent MACE (Figure 2). That a direct measure (serum TXB_2 >3.1 ng/mL) but not indirect measures of residual platelet COX-1 function are associated with subsequent MACE may be due to the influence on the indirect assays of variables downstream of COX-1 or to differences in assay sensitivity. Given a potential for bias based on the method used to define the cutoff for high residual serum TXB_2, the need to adjust for covariables to show a significant association between serum TXB_2 and subsequent MACE, and the failure of indirect assays of residual platelet COX-1 function to confirm an association with MACE, the link between residual platelet COX-1 function as reported by serum TXB_2 >3.1 ng/mL and MACE must be considered tentative until validated in a larger, multicenter study.

In contrast, very short CTs (<65 seconds) in the PFA-100 CADP test, a COX-1-independent assay (Table 2 and other data presented in Results), were associated with subsequent MACE both before ($P=0.0149$, χ^2) and after (OR, 3.5; 95% CI, 1.2 to 10.4; $P=0.0265$) adjustment for covariables. These results suggest that poor clinical outcomes in aspirin-treated patients are better correlated with platelet reactivity to collagen, ADP, and shear (all of which are measured by the PFA-100 CADP CT[13]) rather than with residual COX-1 function. In the same patients studied here, we previously reported[6] a residual arachidonic acid–induced platelet activation via a COX-1– and COX-2–independent (but partly ADP-dependent) pathway. However, this pathway of arachidonic acid–induced platelet activation is not associated with MACE, as shown by the results obtained with the 4 separate arachidonic acid–dependent assays (Figure 2).

Comparison of the Present Results With Previous Studies Associating Laboratory Measures of Aspirin Resistance With Poor Clinical Outcomes

Our results are consistent with recent meta-analyses associating poor inhibition of aspirin-sensitive tests of platelet

function with poor clinical outcomes.[7–10] However, the majority of the studies cited in these meta-analyses did not evaluate the relationship between serum TXB_2, the most direct measure of residual platelet COX-1 function,[11] and the occurrence of MACE but instead used assays that, although sensitive to inhibition by aspirin, are influenced by many other factors. Thus, although these meta-analyses[7–10] suggest that patients with an underlying platelet hyperreactivity have worse clinical outcomes than those who do not, our results represent the most direct evidence to date that this hyperreactivity may be explained in part by poor inhibition of COX-1 by aspirin. However, of the 13 patients with high residual serum TXB_2 and subsequent MACE, 2 had PFA-100 CADP CT <65 seconds, raising the possibility that in these subjects COX-1–independent factors may account for the poor clinical outcome. Other significant differences between the present study and previous studies include the patient population (the present study included all aspirin-treated patients presenting for diagnostic catheterization, whereas many other studies were restricted to patients with acute coronary syndromes); timing of collection of test samples (pre-PCI compared with post-PCI[13]); and differing definitions of adverse outcome (as documented in the meta-analyses[7–10]).

Our results are also consistent with the results of 2 of the largest published studies of the effects of aspirin inhibition of COX-1–dependent thromboxane synthesis, in which Eikelboom et al[24,25] measured 11-dehydro-TXB_2, a urinary metabolite of TXB_2, and identified an association of increasing risk for MI, stroke, or cardiovascular death with increasing quartiles of urinary 11-dehydro-TXB_2 (OR, 1.8 and 1.7 in each study, respectively,[24,25] for the highest quartile relative to the lowest quartile). Although both our study and those of Eikelboom et al[24,25] measured products of the COX-1 pathway, important mechanistic differences exist. First, our measurement of serum TXB_2 evaluates the capacity of maximally activated platelets to produce thromboxane via COX-1, whereas generation of urinary 11-dehydro-TXB_2 depends on in vivo platelet activation, which may be a reflection of the total atherothrombotic burden. In addition, urinary 11-dehydro-TXB_2, unlike serum TXB_2, may be derived in part from nonplatelet sources.[11,26] Nevertheless, although the effect observed both in the present study and in Eikelboom's 2 studies[24,25] is relatively weak (OR, 2.4, 1.8, and 1.7, respectively), taken together, these studies indicate that residual platelet COX-1 function is in part responsible for subsequent MACE. However, the magnitude of this COX-1–dependent effect appears to be small relative to the presently demonstrated ability of a COX-1–independent assay (the PFA-100 CADP CT) to predict clinical outcomes (OR, 3.5). Furthermore, although much smaller than our present study, Campo et al[27] reported that, in 70 consecutive aspirin-treated patients with ST-segment–elevation MI undergoing PCI, the PFA-100 CADP CT predicts clinical outcomes (hazard ratio, 11; 95% CI, 1.5 to 78; $P=0.02$).

Implications for Treatment of Aspirin Resistance

Because the proper treatment, if any, of aspirin resistance is unknown, several expert groups have stated that, other than in research studies, it is not currently recommended to test for aspirin resistance.[2,3,28] Two major factors contribute to these recommendations: (1) No published studies address the clinical effectiveness of altering therapy on the basis of a finding of aspirin resistance; and (2) the mechanism of aspirin "resistance" is unknown. With regard to the latter, our present results suggest that increasing aspirin dosage would not be very effective in altering clinical outcomes because the underlying cause does not appear to be related solely to aspirin's inhibition of COX-1. Furthermore, higher doses of aspirin may be associated with increased risk of bleeding without any additional antithrombotic effect, as seen in the observational analyses of the Blockade of the IIb/IIIa Receptor to Avoid Vascular Occlusion (BRAVO) trial and Clopidogrel in Unstable Angina to Prevent Recurrent Events (CURE)[29,30] and in meta-analyses.[31]

Our present study, together with previous studies of clinical outcomes and the PFA-100 CADP cartridge,[32,33] raise the possibility that the PFA-100 CADP CT may be useful as a clinical predictor of outcomes in patients with CAD. Moreover, the finding of an association of the PFA-100 CADP CT with adverse clinical events suggests the possibility that greater inhibition of ADP-induced platelet activation would result in improved outcomes in this patient group. This possibility is supported by the results of the recent Trial to Assess Improvement in Therapeutic Outcomes by Optimizing Platelet Inhibition with Prasugrel–Thrombolysis in Myocardial Infarction 38 (TRITON-TIMI 38),[34,35] which demonstrated that greater and more consistent inhibition of ADP-induced platelet activation results in a significant reduction in thrombotic events. However, the PFA-100 CADP CT reflects the combined effects of platelet activation by collagen, ADP, and high shear.[13] Therefore, it remains unknown what additional treatment would be of most benefit for aspirin-treated patients with hyperreactive platelets as judged by the PFA-100 CADP CT.

Study Limitations

This was a single-center study, and, although it is the largest prospective study to date evaluating serum TXB_2 (n=700), it may still be underpowered to detect a relationship between COX-1 inhibition and clinical outcomes. A large, multicentered study would overcome this limitation, but properly controlling such a study would be logistically and technologically difficult, whereas strict standardization is more readily achievable in a single-center study such as ours. Inclusion of patients on either 81 or 325 mg aspirin daily as well as patients treated with clopidogrel contributes to population heterogeneity. Nevertheless, given this real-world population, our findings are more broadly applicable than if these variables had been restricted.

Limitations with respect to our observation of a significant OR (3.5) for MACE in patients with a PFA-100 CADP CT of <65 seconds include the low frequency of patients (n=16) and the large CI of the OR (95% CI, 1.2 to 10.4).

Conclusions

In this prospective study of 700 aspirin-treated patients presenting for angiographic evaluation of CAD, residual platelet COX-1 function as measured by serum TXB_2 and COX-1–independent platelet function measured by PFA-100

CADP CT, but not by indirect COX-1–dependent assays (arachidonic acid–stimulated platelet markers, shortened PFA-100 CEPI CT), correlate with subsequent MACE. This study suggests that multiple mechanisms, including but not confined to inadequate inhibition of COX-1, are responsible for poor clinical outcomes in aspirin-treated patients, and therefore the term *aspirin resistance* is inappropriate. Measurement of platelet function by ADP, collagen, and/or shear deserves further study as a marker(s) of adverse clinical outcomes.

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Disclosures

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CLINICAL PERSPECTIVE

The term *aspirin resistance* has been used to describe either the clinical observation of a thrombotic event despite aspirin therapy or the laboratory observation of less-than-expected inhibition in aspirin-sensitive laboratory tests of platelet function. Whether major adverse cardiovascular events that occur in aspirin-treated patients are the result of inadequate inhibition of platelet cyclooxygenase-1 (COX-1), and therefore may be amenable to alterations of aspirin therapy, or are independent (or partly independent) of platelet COX-1 activity is unclear because clinical outcomes have not been determined in the same patients in whom platelet COX-1 function has been specifically measured. In the present prospective study, we measured serum thromboxane B_2, a product directly dependent on platelet COX-1 activity, as well as other COX-1–dependent and –independent platelet function assays. In 700 aspirin-treated patients presenting for angiographic evaluation of coronary artery disease, residual platelet COX-1 function measured by serum thromboxane B_2 and COX-1–independent platelet function measured by the platelet function analyzer-100 collagen/ADP closure time, but not indirect COX-1–dependent assays (arachidonic acid–stimulated platelet markers [platelet surface activated glycoprotein IIb/IIIa and P-selectin, leukocyte–platelet aggregates], platelet function analyzer-100 collagen/epinephrine closure time), correlated with subsequent major adverse cardiovascular events. This study suggests that multiple mechanisms, including but not confined to inadequate inhibition of COX-1, are responsible for poor clinical outcomes in aspirin-treated patients. Therefore, the term *aspirin resistance* is inappropriate, and increasing aspirin dosage would not be expected to be very effective in altering clinical outcomes in these patients.