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Association of Immature Platelets With Adverse Cardiovascular Outcomes

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ABSTRACT

BACKGROUND Immature platelets are less responsive to the effects of antiplatelet drugs and contain messenger ribonucleic acid that is translationally active. They can be measured easily using an automated hematoanalyzer and reported as part of the complete blood count.

OBJECTIVES The purpose of this study was to determine the prognostic significance of elevated immature platelet count (IPC) in patients with coronary artery disease (CAD).

METHODS In this prospective cohort study in patients with CAD, patients underwent IPC measurement and were then followed up for the composite endpoint of major adverse cardiovascular events (MACE), defined as a composite of all-cause mortality, myocardial infarction, unplanned revascularization, or hospitalization for angina. For the purposes of analysis, patients were stratified into tertiles of IPC.

RESULTS Eighty-nine patients were followed up for a median of 31 months. Stratification to the high IPC tertile was associated with higher rates of MACE compared with the intermediate and low tertiles (60% vs. 24% vs. 16%, respectively; $p < 0.001$). Time-dependent receiver-operating characteristic analysis revealed that an IPC level ≥7,632 platelets/μl was 70.7% sensitive and 82.1% specific for MACE. After adjustment for age, admission diagnosis, index revascularization, heart failure, smoking, hematocrit, and baseline platelet count, patients with an IPC level ≥7,632 platelets/μl were more likely to experience a MACE (hazard ratio: 4.65; 95% confidence interval: 1.78 to 12.16; $p < 0.002$).

CONCLUSIONS IPC is a novel biomarker for MACE risk stratification in patients with CAD. Future studies should focus on the utilization of this marker for individualized antiplatelet therapy. (J Am Coll Cardiol 2014;64:2122–9)

A cornerstone event in the pathophysiology of intravascular thrombosis, platelet activation can lead to myocardial infarction (MI) and stroke (1,2). The population of circulating platelets is not homogeneous; different subpopulations of platelets can be categorized according to their activation patterns in response to different agonists (3). Immature platelets, also termed reticulated platelets (RPs) as they are analogous to erythroid reticulocytes, comprise the youngest component of the circulating platelet pool, and appear to participate most actively in thrombosis (4). They contain measurable amounts of cytosolic messenger ribonucleic acid (mRNA) that is translationally active (5–7). RPs also tend to be larger in size, contain more dense granules, and display a greater ex vivo reactivity profile in response to agonists compared with mature non-mRNA-containing platelets (8–10). There is concordance between the level of circulating RPs and the proportion of subjects who are hyporesponsive to aspirin or clopidogrel, indicating that RPs may play a significant role in attenuating the effects of these agents (8–10).

RPs were first described using light microscopy, which revealed a specific staining pattern of their nucleic acid content that is predominantly due to the presence of mRNA (11,12). The next significant

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advance was identifying RPs with flow cytometry after staining blood specimens with thiazole orange (13,14). However, flow cytometry is a time-consuming and costly procedure without a standardized protocol and not well suited for rapid screening in patients in clinical practice. More recently, newer assay techniques have been developed to allow for automated immature platelet determination as part of the standard complete blood count (15). The aim of the current study was to evaluate the ability of the automated method for immature platelet detection to further stratify patients with coronary artery disease (CAD) according to their risk for clinical outcomes.

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METHODS

STUDY DESIGN. In this prospective cohort study, the inclusion criteria were age ≥18 years, the ability to provide informed consent, diagnosis of CAD, and current treatment with dual antiplatelet therapy (unless coronary artery bypass graft [CABG] surgery was performed).

The study protocol was approved by the Houston Methodist Hospital institutional review board, and all patients provided written informed consent. Patients were excluded if they had received any blood products within the 30 days before immature platelet evaluation.

The primary study outcome was the occurrence of major adverse cardiovascular events (MACE), defined as the composite endpoint of all-cause mortality, MI (either ST-segment elevation myocardial infarction [STEMI] or non-ST-segment elevation myocardial infarction [NSTEMI]), unplanned revascularization, or recurrent angina requiring hospitalization. Myocardial infarction was defined as symptoms suggestive of myocardial ischemia (i.e., chest pain or discomfort) associated with biochemical evidence of myocardial necrosis, defined as the level of cardiac troponin >5 times the upper limit of normal (16). Unplanned revascularization was defined as the need for coronary revascularization that was not planned or staged at the index hospitalization. Angina was reported by patients and was defined as discomfort, pain, and/or tightness in the chest, jaw, shoulder, back, and/or arm that was aggravated by exertion and relieved by nitroglycerin or rest (17). To ensure the accuracy of the diagnosis of angina, categorization of an angina episode as an event required the patient to have had an overnight stay in the hospital for the evaluation of anginal symptoms.

Patients were contacted between August and October of 2013 for the evaluation of the occurrence of the primary outcome. Follow-up was conducted by 2 investigators who were blinded to the immature platelet count (IPC) results and who made telephone calls to all of the patients or to their families if a patient was deceased. Follow-up ended at the time of the first contact with the patient or family.

IPC MEASUREMENT. Immature platelets were measured with an automated hematoanalyzer (Sysmex 2100 XE, Sysmex America Inc., Mundelein, Illinois) that uses fluorescent dyes containing polymethine and oxazine. Both dyes penetrate the cell membrane and stain the RNA in erythrocytes and leukocytes as well as platelets. Stained cells are then sorted as they pass through a beam of light created by a semiconductor laser diode. After measurements of cell volume (using forward scatter light) and RNA content (using fluorescence intensity), the device provides scattergrams using a proprietary nonadjustable software algorithm with a preset gate (Sysmex IPF Master). This system discriminates between mature and immature platelets and reports the immature platelet fraction (IPF). The IPC can then be determined by multiplying IPF by the platelet count from the complete blood count.

Immature platelet measurements were conducted on enrollment. In patients who underwent revascularization (either CABG or percutaneous coronary intervention), IPC was evaluated within 72 h of the index revascularization. In all other patients, IPC was evaluated within 72 h of admission to the hospital.

TURBIDIMETRIC PLATELET AGGREGATION. Light transmission aggregometry was used to measure agonist-induced platelet aggregation. We collected 20 ml of whole blood in 0.32% sodium citrate (final concentration). The tubes were centrifuged immediately at 1,700 *g* for 6 min to prepare platelet-rich plasma. The remaining whole blood fraction was further centrifuged at 3,200 *g* for 15 min to separate platelet-poor plasma. The platelet count in platelet-rich plasma was standardized between 200 and 250 × 10^3 cells/μl. Platelet aggregation was induced using 5 and 20 μM adenosine diphosphate (ADP) (final concentrations). The maximum aggregation achieved during a 6-min period was used for analysis.

Aggregation was assessed after an adequate period to ensure full antiplatelet effect (i.e., at least 6 h after 600 mg clopidogrel loading dose, at least 12 h after 300 mg clopidogrel loading dose, or long-term use of clopidogrel 75 mg daily for at least 5 days).

STATISTICAL ANALYSIS. Categorical variables were analyzed using the Fisher exact test. To evaluate the differences between normally distributed continuous

ABBREVIATIONS AND ACRONYMS

AUC = area under the curve
CAD = coronary artery disease
IPC = immature platelet count
IPF = immature platelet fraction
MACE = major adverse cardiovascular event(s)
NSTEMI = non-ST-segment elevation myocardial infarction
ROC = receiver-operating characteristic
RP = reticulated platelets
STEMI = ST-segment elevation myocardial infarction

TABLE 1 Baseline Demographics Stratified by MACE at Follow-Up

	Total (N = 89)	MACE at Follow-Up (+) (n = 30)	MACE at Follow-Up (–) (n = 59)	p Value
Demographics				
Age, yrs	68.1 ± 12.1	70 ± 11	67.2 ± 12.6	0.294
Male	57 (64%)	19 (63.3%)	38 (64.4%)	0.751
Ejection fraction, %	55 (39-60)	50 (31-60)	55 (40-60)	0.497
Medications at time of enrollment				
Aspirin	83 (93.3%)	30 (100%)	53 (89.8%)	0.093
Clopidogrel	70 (78.7%)	26 (86.7%)	44 (74.6%)	0.275
Beta-blocker	85 (95.5%)	29 (96.7%)	56 (94.9%)	1.000
Statin	80 (89.9%)	29 (96.7%)	51 (86.4%)	0.263
Medical history at time of enrollment				
Diabetes	37 (41.6%)	10 (33.3%)	27 (45.8%)	0.363
History of heart failure	29 (32.6%)	13 (43.3%)	16 (27.1%)	0.153
Hyperlipidemia	76 (85.4%)	28 (93.3%)	48 (81.4%)	0.205
Tobacco use	22 (24.7%)	5 (16.7%)	17 (28.8%)	0.299
Peripheral vascular disease	12 (13.5%)	5 (16.7%)	7 (11.9%)	0.534
Stroke	10 (11.2%)	4 (13.3%)	6 (10.2%)	0.789
Hypertension	84 (94.4%)	30 (100%)	54 (91.5%)	0.163
Laboratory analysis at time of enrollment				
WBC, x10^3 cell/μl	7.7 (6.1-9.2)	8.0 (6.1-9.1)	7.6 (6.1-9.2)	0.892
Hemoglobin, g/dl	11.3 ± 2.1	11.1 ± 2.5	11.3 ± 1.9	0.765
RDW, fl	46.6 (43.0-51.3)	48.0 (44.9-53.2)	45.2 (42.2-50.1)	0.020
Platelets, x 10^3 platelet/μl	180 (137-219)	197 (167-246)	179 (133-210)	0.117
BUN, mg/dl	20.0 (15.0-27.0)	20.0 (15.8-28.8)	19.0 (15.0-26.0)	0.581
Creatinine	1.0 (0.8-1.4)	1.0 (0.8-1.5)	1.0 (0.8-1.2)	0.976
Cardiac diagnosis at time of enrollment				0.797
Stable CAD	38 (42.7%)	13 (43.3%)	25 (42.4%)	
STEMI	4 (4.5%)	2 (6.7%)	2 (3.4%)	
Unstable angina/NSTEMI	47 (52.8%)	15 (50%)	32 (54.2%)	
Revascularization/treatment strategy at time of enrollment				0.590
CABG	26 (29.2%)	7 (23.3%)	19 (32.2%)	
Medical therapy only	33 (37.1%)	11 (36.7%)	22 (37.3%)	
Stent	30 (33.7%)	12 (40%)	18 (30.5%)	
Platelet assay results				
IPF, %	4.3 (3.0-5.8)	5.3 (4.3-6.4)	3.7 (3.0-5.1)	0.007
IPC, platelets/μl	7,353 (4,860-11,154)	10,507 (7,166-13,482)	6,322 (4,730-8,250)	0.002
MPV, fl	10.6 ± 1.0	10.8 ± 1.0	10.5 ± 1.0	0.224
5M-ADP aggregation, %	43.8 ± 19.3	45.9 ± 18.7	42.4 ± 19.8	0.542
20M-ADP aggregation, %	55.0 (42.0-65.5)	54.0 (37.2-67.5)	55.0 (47.0-63.0)	0.759

Values are mean ± SD, n (%), or median (interquartile range).

ADP = adenosine diphosphate; BUN = blood urea nitrogen; CAD = coronary artery disease; CABG = coronary artery bypass graft; IPC = immature platelet count; IPF = immature platelet fraction; MACE = major adverse cardiac event(s); MPV = mean platelet volume; NSTEMI = non-ST-segment elevation myocardial infarction; RDW = red blood cell distribution width; STEMI = ST-segment elevation myocardial infarction; WBC = white blood cell.

variables, the Student *t* test was used, and for variables that were not normally distributed, we employed the Wilcoxon rank sum test. Normality was evaluated using the Shapiro-Wilk test, with alpha ≤ 0.05 as the threshold to reject the assumption of normality. Time-dependent receiver-operating characteristic (ROC) curve analysis was performed to determine an IPC level that may have prognostic significance for MACE (18,19). The optimal cutoff was considered as the point that maximized sensitivity and specificity. Cox proportional hazards analysis was used to analyze the relationship between elevated IPC level and MACE. To evaluate the unadjusted relationship between clinical outcomes and IPC level, patients were stratified by IPC tertile. Following this, either the log-rank test of the Kaplan-Meier estimator or the Gray test of the cumulative incidence function (for outcomes with a competing risk) was used (20). Covariates for the model were selected on the basis of biological plausibility using directed acyclic graph analysis to model the hypothesized relationship between IPC level and MACE to identify those variables that were potential confounding factors (21,22). These identified factors were then used as covariates for the Cox proportional hazards model and included a dichotomous variable that indicated whether the IPC level was above the identified threshold by ROC analysis, the diagnosis at time of enrollment (stable CAD, STEMI, or NSTEMI), initial treatment (optimal medical therapy alone, stent placement, or CABG), age, history of heart failure, smoking, hematocrit, and baseline platelet count. A p value of <0.05 was considered statistically significant. All statistical analyses were done using R for Statistical Computing version 3.0.0 (R Foundation, Vienna, Austria). (23).

RESULTS

A total of 95 patients were enrolled in the study between December 2009 and September 2013. Two patients (2.1%) withdrew consent after enrollment, and 4 patients (4.2%) were lost to follow-up despite at least 3 separate attempts to contact the patient and/or family with a review of inpatient and outpatient medical records. The remaining 89 patients (93.7%) were followed up for a median of 31 months (interquartile range: 10 to 40 months). Baseline characteristics are shown in Table 1. With the exception of the platelet assay parameters, there were no significant differences in baseline characteristics between patients who had MACE at follow-up and those who did not.

Both the IPC (10,507 vs. 6,322 platelets/μl; p = 0.002) and the IPF (5.2% vs. 4.1%; p = 0.007) were greater in patients who had MACE at follow-up (Figure 1). The total platelet count was higher in patients in the high IPC tertile compared with those in the intermediate and low tertiles (226.2 ± 65.6 vs. 190.1 ± 63.0 vs. 152.2 ± 38.9, respectively; p = 0.001). Follow-up events in patients in each IPC tertile are shown

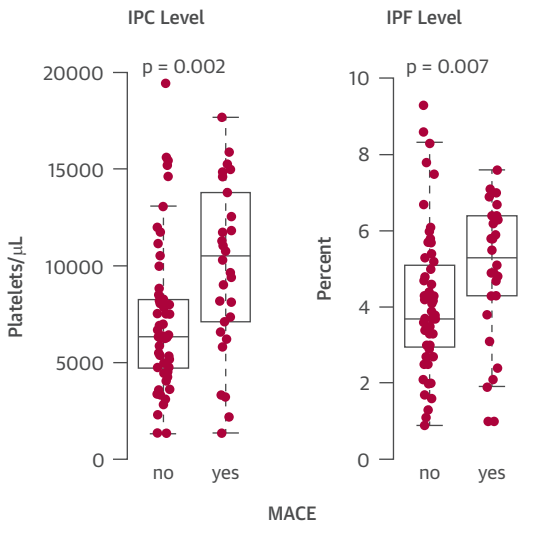


FIGURE 1 Box-and-Whiskers Plot of IPC and IPF Levels, Stratified by MACE at Follow-Up

A comparison using Wilcoxon rank sum test shows higher immature platelet count (IPC) (**left**; p = 0.002) and immature platelet fraction (IPF) (**right**; p = 0.007) in patients with major adverse cardiovascular events (MACE) at 31 months. **Center band** represents median, **box hinges** represent the first and the third quartiles, and **whiskers** extend to the most extreme value, which is no more than 1.5 times the interquartile range from the box edges.

in Table 2. At follow-up, 30 patients (33.7%) had experienced an event at a mean of 10.4 ± 7.9 months after enrollment. Eleven patients (12.4%) had experienced an NSTEMI, 10 (11.2%) had died, 7 (7.9%) were hospitalized for chest pain, 6 (6.7%) had undergone an unplanned revascularization, and 0 had experienced a STEMI. Furthermore, death from cardiovascular causes (advanced heart failure, cardiogenic shock, or ischemic or arrhythmic death) was reported in 8 patients. The one death among patients in the low IPC tertile was attributed to advanced pulmonary fibrosis. One death among the patients in the high IPC tertile was attributed to septic shock.

Time-dependent ROC curve analysis demonstrated an area under the curve (AUC) of 0.774 (Figure 2A). Further evaluation revealed that a cut point of 7,632 platelets/μl maximized discrimination and was 70.7% sensitive and 82.1% specific for MACE. Throughout follow-up, AUC ranged from 0.630 to 0.799, with a median level of 0.746 (Figure 2B). Cox proportional hazards analysis demonstrated that even after adjustment for confounding variables, an IPC level of ≥7,632 platelets/μl was associated with an increased risk for MACE (Central Illustration) (hazard ratio [HR]: 4.65; 95% confidence interval [CI]: 1.78 to 12.16; p < 0.002).

There was a modest correlation between IPC and platelet count (r^2 = 0.338) (Figure 3). The performances of IPF and IPC in the discrimination of high-risk patients are compared in Figure 2. Throughout follow-up, the time-dependent ROC curves for IPC and IPF were not significantly different (Figure 2B), with the exception of early at 4 months, when IPC had a higher AUC (73.2% vs. 64.5%; p = 0.044).

DISCUSSION

This is the first prospective study to show a relationship between IPC—a readily available parameter that can be determined as part of the complete blood count and is unlikely to be affected by previous antiplatelet treatment—and clinical events. The relationship was present even after adjustment for multiple baseline variables. Additionally, we were able to detect a cutoff level of IPC with good discriminative power to predict clinical outcomes (Central Illustration). IPC, which is easily obtained by multiplying the IPF by the platelet count, may prove to be a better tool than IPF, as it incorporates absolute platelet count as well as the proportion of platelets that are immature and rich in mRNA.

It is likely that the association we observed between immature platelets and thromboembolic events reflects the increased ability of immature platelets to participate in thrombosis compared with senescent platelets. Hyperactivity of immature platelets in response to multiple agonists might be, in part, due to the presence of mRNA (24). Although this mRNA was at one time considered inconsequential, a growing body of evidence indicates that spliceosome complexes are present in platelets, even though they are anucleate, and that activation-dependent splicing and translation of pre-mRNA can occur in these platelets, resulting in the de novo synthesis of

TABLE 2 Outcome Events Stratified by IPC Tertile

		IPC Tertile (platelets/μl)			
		Lowest	Middle	Highest	
	Total (N = 89)	(1,364–5,836) (n = 30)	(5,836–9,272) (n = 29)	(9,272–27,520) (n = 30)	p Value*
Death	10 (11.2)	1 (3.3)	3 (10.3)	6 (20)	0.047
NSTEMI	11 (12.4)	1 (3.3)	3 (10.3)	7 (23.3)	0.023
Hospitalization for angina	7 (7.9)	2 (6.7)	1 (3.4)	4 (13.3)	0.175
Revascularization	6 (6.7)	1 (3.3)	1 (3.4)	4 (13.3)	0.116
MACE (composite)	30 (33.7)	5 (16.7)	7 (24.1)	18 (60)	<0.001

Values are n (%). *For each tertile, the time to event curves were created and compared using the log-rank test or Gray's test (for outcomes where death was a competing risk).

Abbreviations as in Table 1.

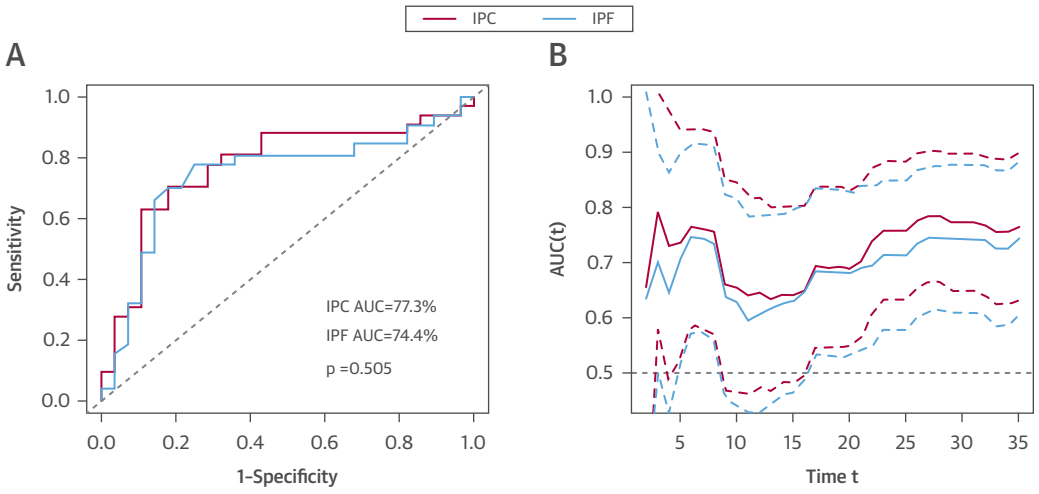


FIGURE 2 Time-Dependent ROC Curve

(A) Time-dependent receiver-operating characteristic (ROC) curve for IPC **(red)** and IPF **(blue)** at 30 months. **(B)** Time-dependent area under the curve (AUC) changes over time in months and the 95% confidence interval **(solid lines)**. Abbreviations as in Figure 1.

prothrombotic and proinflammatory proteins (25,26). Mechanistic support for the increased thrombotic potential of immature platelets is provided by observations from our laboratory and others of a relationship between IPC and high residual platelet reactivity (RPR) despite dual antiplatelet therapy with aspirin and clopidogrel or with aspirin and prasugrel. This relationship is present in healthy subjects as well as in patients with CAD (9,10,27,28). Alternatively, as the platelet count is also an acute phase reactant, an increased IPC may also be a marker of systemic inflammatory activity.

The findings of the current study expand those reported by other investigators. Grove et al. (29) examined the relationship between IPC and high RPR among patients treated with aspirin and clopidogrel. IPC was reported to reflect RPR more precisely than IPF. When arachidonic acid, collagen, or ADP was used to induce platelet aggregation, significantly higher IPC levels were found in patients with high RPR compared with those without high RPR for each of the agonists. Similar trends were not present for IPF. Interestingly, the median IPC value that was associated with RPR in the study by Grove et al. (29) was 8,400 platelets/μl, which is similar to the cutoff value of 7,632 platelets/μl observed using ROC curve analysis in the current study.

Previous cross-sectional studies have reported an increase in the proportion of immature platelets in the setting of acute thrombotic events (30,31). However, in those studies, the measurement of immature platelets took place after the event had occurred, and no longitudinal follow-up was included. In the one other longitudinal study that examined the relationship between immature platelets and clinical events, Cesari et al. (32) reported that in patients with acute coronary syndromes, IPF was a predictor of cardiovascular death after adjustment for the Global Registry of Acute Coronary Events risk score (odds ratio: 4.15; 95% CI: 1.24 to 13.9; $p = 0.02$). In comparison, the current study had a longer follow-up period (31 months), a diverse composite endpoint (including all-cause mortality), and adjustment for multiple baseline variables using a Cox proportional hazards analysis to assess the relationship between the initial observation and clinical outcomes.

Given that IPC is the product of the IPF and total platelet count, it might be expected that the higher the platelet count, the higher the IPC. However, we observed only modest correlation between those 2 parameters ($r^2 = 0.338$), perhaps reflecting the persistence of a systemic stimulus for continued platelet production.

Knowing that patients' responses to antiplatelet therapy are not uniform, multiple investigators have tried to link high RPR to the occurrence of atherothrombotic events (33–37). In a study in patients undergoing drug-eluting stent placement who are treated with clopidogrel, high RPR was strongly related to the risk for stent thrombosis (HR: 2.4; 95% CI: 1.4 to 4.3; $p = 0.001$), whereas bleeding risk was

inversely related to high RPR (HR: 0.73; 95% CI: 0.61 to 0.89; p = 0.02) (38). In our study, when RPR was evaluated using light transmission aggregometry with ADP (5 or 20 μM) as the agonist, no significant difference was found between patients who had experienced an event versus those who did not.

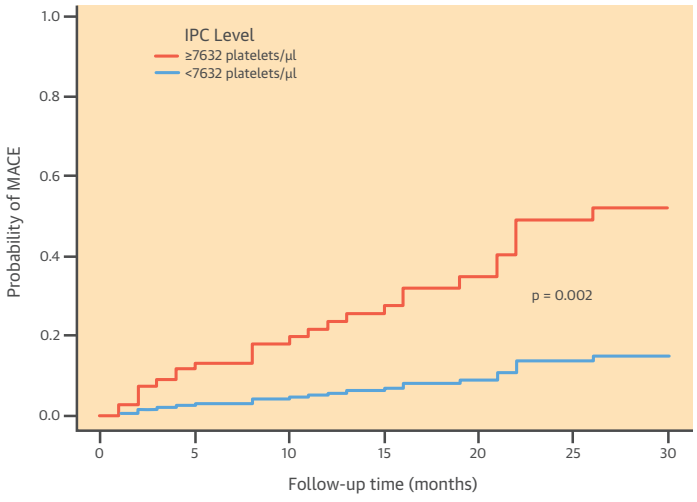
We believe that antiplatelet therapy is shifting into a new paradigm of personalized therapy. Thus far, targeting RPR on the basis of aggregometry assays has not proven to alter clinical outcomes (39-41). The current data offer a potential new and easily ascertained target for such a strategy. IPC may become an important tool for investigating personalized antiplatelet therapy, perhaps by selecting patients who might be more likely to benefit from drugs with longer half-lives or from dosing antiplatelet drugs more frequently than once daily.

STUDY LIMITATIONS. First, the sample size was small, mainly due to the exclusion of patients who received blood product transfusions before their platelet studies. The sample size limited the number of variables that could be examined to determine the independence of IPC as a predictor of events, as well as the robustness of the primary observation. However, despite the sample size, the observed relationship between IPC and clinical outcomes was statistically significant. With the number of covariates considered, there was a risk for overfitting the data, which may have limited the model's predictive performance.

Second, the design of collecting follow-up information via phone calls introduced a potential recall bias. However, in this study, we conducted a full review of medical records to ensure event accuracy, and we used a strict definition of *recurrent angina*. Moreover, the fact that hard endpoints (i.e., death, NSTEMI with biomarker evidence) were driving the composite endpoint also reduced the effect of recall bias.

Third, data are lacking as to the stability of the IPC over the course of time. Such studies are currently underway.

Finally, the current study does not provide a mechanistic explanation of the association that was observed between IPC and clinical outcomes in patients with CAD. Thus, whether IPC is a modifiable risk marker is still unknown. Platelet count is, in part, an acute phase reactant, and the proportion of immature platelets may also increase in response to a systemic inflammatory process. Markers of inflammation were not measured in the current study. However, the current study demonstrates that a single measurement of IPC at the point of care may be a useful predictor of subsequent cardiac events.



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CENTRAL ILLUSTRATION Cox Proportional Hazards Model for MACE at Follow-Up

After adjustment for diagnosis at time of enrollment, initial treatment, age, history of heart failure, smoking, hematocrit, and platelet count, the level of immature platelet count (IPC) was found to be significantly associated with major adverse cardiovascular events (MACE).

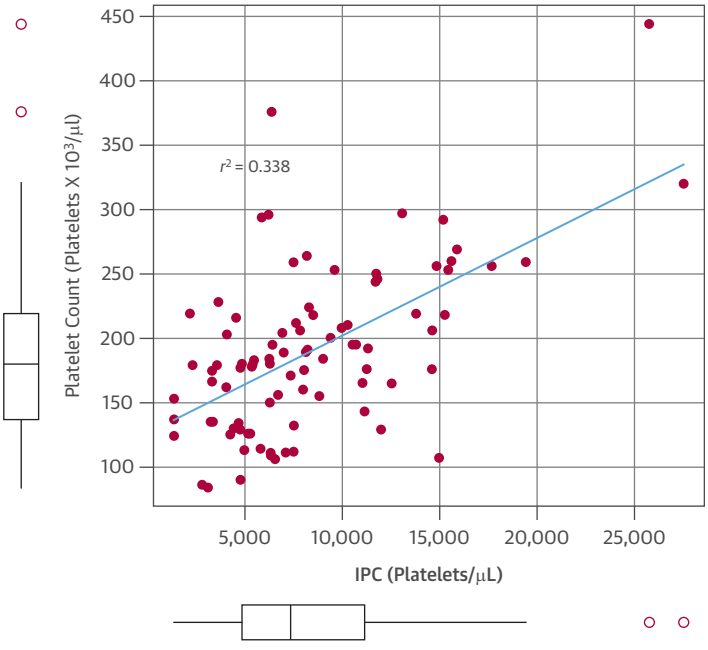

FIGURE 3 Scatterplot With Marginal Box-and-Whiskers Plots to Explore the Relationship Between IPC and Total Platelet Count

The **blue line** represents linear regression. The Pearson coefficient of determination of 0.338 reflects poor correlation between immature platelet count (IPC) and total platelet count.

CONCLUSIONS

IPC is an easily obtained laboratory test that can be incorporated into a routine complete blood count with little additional cost and allows for further stratification of patients with CAD in terms of risk for future MACE. Future studies should focus on personalizing antiplatelet therapy guided by IPC level.

REPRINT REQUESTS AND CORRESPONDENCE: Dr. Neal S. Kleiman, Houston Methodist DeBakey Heart and Vascular Center, 6565 Fannin, MS F10-035, Houston, Texas 77030. E-mail: nkleiman@houstonmethodist.org.

PERSPECTIVES

COMPETENCY IN MEDICAL KNOWLEDGE: Immature platelets are associated with adverse clinical outcomes in patients with coronary artery disease.

TRANSLATIONAL OUTLOOK: Better understanding of mechanisms by which immature platelets escalate risk, such as drug resistance, may aid in developing more individualized antiplatelet therapy for prevention of ischemic events.

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