

CLINICAL RESEARCH

Antiplatelet Therapy

The ELAPSE (Evaluation of Long-Term Clopidogrel Antiplatelet and Systemic Anti-Inflammatory Effects) Study

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Objectives

This study was designed to evaluate the effects of long-term clopidogrel and aspirin administration on platelet aggregation, activation, and inflammation.

Background

Clopidogrel resistance was described in 15% to 30% of patients with short-term therapy, but its antiplatelet effects with long-term therapy is unknown.

Methods

We performed a prospective study of patients undergoing coronary stenting who were on aspirin for ≥ 5 days but not previously on clopidogrel. Clopidogrel 600 mg was given before stenting. Clopidogrel 75 mg/day and aspirin 325 mg/day were continued for 1 year. Light-transmittance aggregometry with 5- μ mol/l adenosine diphosphate and 1-mmol/l arachidonic acid stimulation; VerifyNow clopidogrel and aspirin assays; platelet activation receptor expression of CD40L, CD62P, and PAC-1 (antibody against activated glycoprotein IIb/IIIa); and inflammatory markers of soluble CD40L and P-selectin, high-sensitivity C-reactive protein, interleukin-10, and interleukin-18 were measured at baseline; 1 day; and 1, 6, and 12 months. Our primary analysis compared light-transmittance aggregometry aggregation at 1 versus 12 months.

Results

We enrolled 26 patients who completed a 1-year follow-up. Maximal platelet adenosine diphosphate-stimulated aggregation was $61.8 \pm 25.9\%$ at baseline, $22.1 \pm 18.3\%$ at 1 day, $30.6 \pm 16.8\%$ at 1 month, $29.0 \pm 13.3\%$ at 6 months, and $26.7 \pm 13.6\%$ at 12 months ($p = 0.099$ for 12 months vs. 1 month). VerifyNow clopidogrel platelet inhibition was similar at 12 months versus 1 month ($38.9 \pm 19.7\%$ vs. $45.6 \pm 26.7\%$, $p = 0.578$). Likewise, there was no difference in aspirin's effects on platelet aggregation at 12 months versus 1 month. In contrast, platelet activation receptor expression of CD40L, CD62P, and PAC-1 were higher at 12 months versus 1 month.

Conclusions

Our pilot study showed no attenuation of clopidogrel's effects on platelet aggregation with long-term administration. However, platelet activation receptor expression increased with time and should be further evaluated. (J Am Coll Cardiol 2008;52:1826-33) © 2008 by the American College of Cardiology Foundation

Aspirin and thienopyridines are indispensable pharmacologic arsenals in modern cardiovascular practices with several indications for long-term therapy: percutaneous coronary intervention (PCI) with drug-eluting stents, presentation with acute coronary syndrome, and patients with established myocardial infarction (MI), ischemic stroke, or symptomatic peripheral arterial disease (1). Thus, it is essential that clinicians comprehend the platelet response to long-term dual antiplatelet therapy.

Resistance to either aspirin or clopidogrel at baseline or development of resistance with prolonged administration could jeopardize their clinical efficacy. Although it is widely recognized that 15% to 30% of patients may be resistant to clopidogrel 75 mg/day, this is derived from short-term (≤ 30 days) studies (2,3), with no data pertaining to resistance prevalence with longer-term administration. Controversial results were reported on aspirin resistance (4-6), and no studies have addressed the development of resistance to both these agents with long-term administration.

We, therefore, designed a prospective pilot study to evaluate the effects of long-term clopidogrel and aspirin on platelet aggregation, activation, and inflammation.

Methods

Study population. We included patients ≥ 18 years of age undergoing PCI at Vancouver General Hospital, who

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received aspirin 81 mg/day for ≥ 5 days, but had not had clopidogrel within 2 weeks. Exclusion criteria were known allergy or intolerance to clopidogrel; platelet count $<100,000/\mu\text{l}$ or $>500,000/\mu\text{l}$; history of chronic inflammatory disease; clopidogrel, ticlopidine, dipyridamole, abciximab, steroidal and nonsteroidal anti-inflammatory drugs use within 2 weeks; eptifibatide or tirofiban within 48 h; MI within 1 week; active bleeding (excluding menses) or significant gastrointestinal bleed within 2 months; major surgery within 2 weeks; and pregnancy. Our study was approved by the Vancouver General Hospital and University of British Columbia Institutional Review Board, and written informed consent was obtained from all patients.

Study drugs and protocol. Clopidogrel 600 mg was given before PCI. Clopidogrel 75 mg/day and aspirin 325 mg/day were continued for 1 year. Procedural anticoagulant choice was at the discretion of the interventionalists. Eptifibatide or tirofiban was permitted, but had to be discontinued ≥ 10 h before the next day's blood work. Abciximab was not permitted due to its long effective half-life. The choice of bare-metal or drug-eluting stents was at the discretion of the interventionalists. The study protocol is depicted in Figure 1.

Laboratory measurements. Blood samples were drawn at baseline (before clopidogrel); at 16 to 24 h (1 day) after PCI; and at 1, 6, and 12 months. Patients were fasting for ≥ 4 h prior to blood withdrawal. The first 3 ml of blood drawn were discarded. Baseline samples were collected from the arterial sheath into ethylenediaminetetraacetic acid, sodium citrate (3.2%), heparin, and serum-separator vacutainer tubes. Post-PCI samples were drawn from peripheral venipunctures. The following were assessed with each collection: 1) light-transmittance aggregometry (LTA) with 5- $\mu\text{mol/l}$ adenosine diphosphate (ADP) and 1-mmol/l arachidonic acid (AA) stimulation; 2) VerifyNow aspirin and clopidogrel assays; 3) flow cytometry platelet-bound CD40L, CD62P, and PAC-1 (antibody against activated glycoprotein IIb/IIIa); 4) soluble CD40L and P-selectin; 5) high-sensitivity C-reactive protein, interleukin (IL)-10, and IL-18; and 6) platelet count, hemoglobin, and hematocrit.

LTA preparation. Platelet-rich plasma and platelet-poor plasma were prepared at 24°C, and LTA was performed as previously described (7). The platelet-rich plasma platelet count was $316 \pm 66 \times 10^9/\text{l}$. Using a lumi-aggregometer (Chronolog Corporation, Havertown, Pennsylvania), platelet-rich plasma was stimulated with final concentrations of AA 1.0 mmol/l or ADP 5.0 $\mu\text{mol/l}$. The LTA was performed <2 h after blood withdrawal in duplicates (means reported as percent aggregation), and relative light transmission values were recorded at maximum and 6 min after agonist addition.

Flow cytometry preparation. Binding of phycoerythrin-labeled anti-CD40L (Beckman Coulter, Mississauga, Ontario), fluorescein isothiocyanate-labeled PAC-1 (BD Biosciences, Mississauga, Ontario), and anti-CD62P-phycoerythrin to platelets stimulated by 5.0- $\mu\text{mol/l}$

thrombin receptor activating peptide (Peninsula Laboratories Europe, Merseyside, England) was quantified by flow cytometry as previously described (7). Results were expressed as percent positive cells of the platelet population. Instrument stability was monitored by daily measurement of calibration beads (Flow-Check fluorospheres, Beckman Coulter). The mean antibody binding to platelets from 6 unmedicated normal controls were 30.9 ± 8.7 (CD40L), 83.9 ± 7.5 (CD62), and 93.7 ± 5.4 (PAC-1).

Enzyme-linked immunosorbent assay tests. Soluble CD40L, soluble P-selectin, IL-10, and IL-18

were measured using commercial enzyme-linked immunosorbent assay kits according to the manufacturer's instructions. Plasma CD40L and P-selectin were measured with kits from Bender MedSystems (Burlingame, California). Plasma IL-10 and -18 were measured with kits by BioSource (Invitrogen Canada, Burlington, Ontario).

VerifyNow assays. The VerifyNow (Accumetrics, San Diego, California) aspirin and clopidogrel (P2Y₁₂) assay cartridges contain fibrinogen-coated beads and platelet agonists (aspirin assay contains AA, and clopidogrel assay contains ADP/prostaglandin E1 and iso-thrombin receptor activating peptide). Activated platelets in the whole blood samples bind and aggregate the fibrinogen-coated beads in proportion to the number of expressed activated glycoprotein IIb/IIIa receptors, with resulting increase in light transmittance. This change in optical signal is reported as the aspirin reaction unit (ARU) and P2Y₁₂ reaction unit (PRU). The P2Y₁₂ assay also reports "percent inhibition" (percent change from baseline aggregation calculated from the PRU and the baseline thrombin receptor activating peptide channel value). Patients with ARU ≥ 550 were labeled as aspirin-resistant.

Clopidogrel and aspirin resistance definitions. Clopidogrel resistance was defined as an absolute difference between baseline and post-clopidogrel platelet aggregation $<10\%$ with LTA using 5.0- $\mu\text{mol/l}$ ADP (2). Aspirin resistance was defined as mean aggregation $>20\%$ with LTA using 1.0-mmol/l AA (8).

End points evaluated. Our primary analysis compared LTA platelet aggregation, for the 75-mg/day clopidogrel dosage, at 12 months versus 1 month. We chose 1 month for the comparison in order to achieve a steady state with the maintenance dose. Secondary assessments include the prevalence of clopidogrel and aspirin resistance, and the change in platelet activation and inflammatory markers. Death, stroke, MI, and target vessel revascularization at 12

Abbreviations and Acronyms

AA	= arachidonic acid
ADP	= adenosine diphosphate
ARU	= aspirin reaction unit
CK	= creatine kinase
IL	= interleukin
LTA	= light-transmittance aggregometry
MI	= myocardial infarction
PAC-1	= antibody against activated glycoprotein IIb/IIIa
PCI	= percutaneous coronary intervention
PRU	= P2Y ₁₂ reaction unit

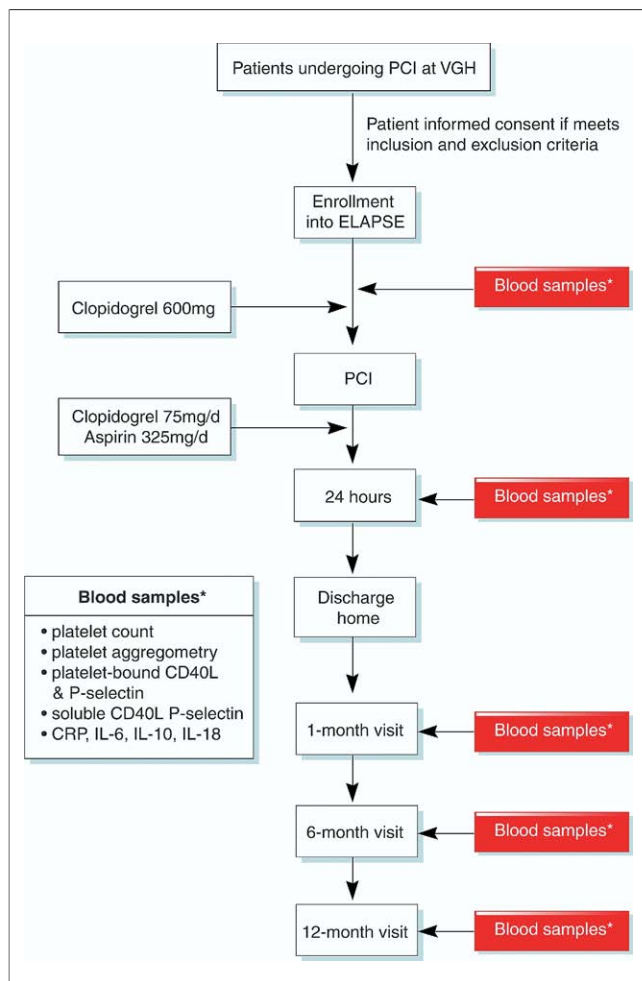


Figure 1 The ELAPSE Study Protocol

The chart shows the ELAPSE (Evaluation of Long-Term Clopidogrel Anti-Platelet and Systemic Anti-Inflammatory Effects) study protocol. The * indicates the blood samples tests done. CRP = high-sensitivity C-reactive protein; IL = interleukin; PCI = percutaneous coronary intervention; VGH = Vancouver General Hospital.

months were also assessed. MI was defined as chest pain with creatine kinase (CK) or CK-myocardial band elevation $>2\times$ the upper limit of normal or any troponin elevation. Peri-procedural MI was defined as CK or CK-myocardial band elevation $>3\times$ the upper limit of normal. Stroke was defined as new focal neurologic deficits lasting >24 h.

Statistical analysis. Hypothesis tests were done using 2-sided tests at the 5% significance level. Comparisons of binary variables were performed with the McNemar test, and continuous variables with the Wilcoxon signed rank test for paired samples. We hypothesized that LTA platelet aggregation would increase by an absolute 15% after 12 months of clopidogrel compared with 1 month of clopidogrel. Assuming a mean platelet aggregation of 30% at 1 month, we needed to enroll 21 patients to detect platelet aggregation increase to 45% at 12 months, with 90% power and 2-sided $\alpha = 0.05$ (estimated SD for mean change 20%). We anticipated 35% attrition, thus we planned to

enroll 33 patients. Statistical analyses were performed with SPSS (version 13, SPSS Inc., Chicago, Illinois).

Results

We enrolled 33 patients from September 2005 through August 2006, and 26 patients completed the 1-year follow-up after successful PCI. Table 1 shows the baseline demographics. Seven patients withdrew prematurely: 2 had contraindications to antiplatelet therapy (nosebleed and colon cancer surgery), and 5 withdrew on their own accord without medical contraindications.

LTA. The baseline maximal platelet aggregation measured by LTA 5.0- $\mu\text{mol/l}$ ADP was $61.8 \pm 25.9\%$. This was significantly reduced after the administration of clopidogrel at 1 day and at 1, 6, and 12 months (Table 2, Fig. 2). Our primary comparison showed no difference in platelet aggregation at 12 months versus 1 month (26.7% vs. 30.6%, $p = 0.099$). Likewise, no difference was shown in our comparison of platelet aggregation at 12 months versus 1 day after PCI (26.7% vs. 22.1%, $p = 0.093$). Similar results were obtained with 6-min aggregation.

With administration of aspirin at 325 mg/day, platelet aggregation measured with LTA 1.0-mmol/l AA was significantly reduced at each follow-up time point compared with platelet aggregation at baseline (aspirin 81 mg/day at baseline). However, we did not observe any difference in maximal platelet aggregation at 12 months versus 1 month ($p = 0.940$), or at 12 months versus 1 day after PCI ($p = 0.183$) (Table 3, Fig. 2). Similar results were obtained with 6-min aggregation.

VerifyNow assays. Baseline PRU was 292.4 ± 58.8 and platelet inhibition $4.9 \pm 8.1\%$. After clopidogrel adminis-

Table 1 Baseline Characteristics

Characteristics	Patients (n = 26)
Age, yrs	63.0 $\pm$ 8.6
Male sex	22 (84.6%)
Body mass index, kg/m ²	27.0 $\pm$ 4.4
Diabetes mellitus	4 (15.4%)
Hypertension	21 (80.8%)
Hyperlipidemia	25 (96.2%)
Current smoking	2 (7.7%)
Family history of coronary artery disease	13 (50.0%)
Acute coronary syndrome	2 (7.7%)
Baseline medications	
ACE inhibitor	12 (46.2%)
Beta-blockers	18 (69.2%)
Calcium-channel blockers	5 (19.2%)
Statin	22 (84.6%)
Procedural characteristics	
Number of treated vessels	1.3 $\pm$ 0.5
Drug-eluting stent use	18 (69.2%)
GP IIb/IIIa inhibitor use	18 (69.2%)

Values are n (%) or mean $\pm$ SD.

ACE = angiotensin-converting enzyme; GP = glycoprotein.

Table 2 Effects of Clopidogrel on Platelet Aggregation, Activation, and Inflammation

	Baseline	1 Day	p Value	1 Month	p Value	6 Months	p Value	12 Months	p Value
LTA with 5-μmol/l									
ADP platelet aggregation, %									
Maximum	61.8 $\pm$ 25.9	22.1 $\pm$ 18.3	<0.001	30.6 $\pm$ 16.8	<0.001	29.0 $\pm$ 13.3	<0.001	26.7 $\pm$ 13.6	<0.001
6 min	54.8 $\pm$ 29.8	7.9 $\pm$ 14.2	<0.001	11.7 $\pm$ 15.7	<0.001	10.5 $\pm$ 12.4	<0.001	10.7 $\pm$ 15.6	<0.001
VerifyNow P2Y₁₂ assay									
% inhibition	4.9 $\pm$ 8.1	40.7 $\pm$ 26.9	<0.001	45.6 $\pm$ 26.7	<0.001	39.1 $\pm$ 25.1	<0.001	38.9 $\pm$ 19.7	<0.001
PRU	292.4 $\pm$ 58.8	155.1 $\pm$ 64.4	0.001	147.8 $\pm$ 73.2	<0.001	145.6 $\pm$ 64.6	0.001	150.2 $\pm$ 61.6	0.001
Platelet positive, %									
CD40L	13.6 $\pm$ 10.1	11.7 $\pm$ 7.1	0.446	12.9 $\pm$ 7.0	0.275	21.1 $\pm$ 6.0	0.004	25.9 $\pm$ 10.7	<0.001
CD62P	40.0 $\pm$ 28.0	34.2 $\pm$ 21.8	0.066	31.1 $\pm$ 18.6	0.115	52.9 $\pm$ 16.1	0.041	60.4 $\pm$ 16.1	0.001
PAC-1	52.7 $\pm$ 28.5	33.8 $\pm$ 23.4	0.002	38.3 $\pm$ 23.7	0.010	49.5 $\pm$ 25.1	0.600	51.8 $\pm$ 22.5	0.819
Inflammatory markers									
Soluble CD40L, ng/ml	1.1 $\pm$ 1.2	1.2 $\pm$ 1.2	0.209	1.3 $\pm$ 1.3	0.761	0.9 $\pm$ 1.0	0.086	0.7 $\pm$ 0.7	0.004
Soluble P-selectin, ng/ml	46.2 $\pm$ 39.5	51.7 $\pm$ 44.6	0.072	47.6 $\pm$ 37.3	0.523	60.4 $\pm$ 27.5	0.043	72.8 $\pm$ 34.0	0.012
IL-10, pg/ml	0.5 $\pm$ 1.3	0.3 $\pm$ 1.2	0.161	0.3 $\pm$ 1.0	0.310	0.3 $\pm$ 0.9	0.674	0.5 $\pm$ 2.0	0.499
IL-18, pg/ml	299.3 $\pm$ 209.6	313.3 $\pm$ 198.8	0.230	319.5 $\pm$ 143.8	0.171	333.4 $\pm$ 151.9	0.049	342.2 $\pm$ 163.7	0.018
CRP, mg/dl	2.1 $\pm$ 2.4	8.7 $\pm$ 20.7	<0.001	1.5 $\pm$ 1.6	0.322	1.7 $\pm$ 1.6	0.451	1.8 $\pm$ 2.1	0.510

Values are presented as mean $\pm$ SD. The p values are for comparison to baseline.

ADP = adenosine diphosphate; CRP = high-sensitivity C-reactive protein; IL = interleukin; LTA = light-transmittance aggregometry; PAC-1 = antibody against activated glycoprotein IIb/IIIa; PRU = P2Y₁₂ reaction unit.

tration, repeat PRU measurements were significantly reduced and platelet inhibitions were significantly increased at each follow-up. However, there were no significant differences in PRU ($p = 0.520$) or platelet inhibitions ($p = 0.578$) at 12 months versus 1 month (Table 2, Fig. 2). Likewise, there were no differences in PRU ($p = 0.551$) or platelet inhibitions ($p = 0.446$) at 12 months versus 1 day after PCI. Similarly, with the aspirin assays, there was no difference in ARU measurements at 12 months versus 1 month ($p = 0.212$) or at 12 months versus 1 day after PCI ($p = 0.687$) (Fig. 3).

Clopidogrel and aspirin resistance. Using our LTA definition, the proportion of patients who were clopidogrel-resistant was 16.0% at 1-day, 12.0% at 1-month, 8.0% at 6-month, and 12.0% at 12-month follow-ups ($p = 1.0$ for 12 months vs. 1 month). Among the 3 who were clopidogrel-resistant at 12 months, all were previously labeled clopidogrel-resistant at 1 day or 1 month. Using LTA with 1.0-mmol/l AA, no patient was identified as aspirin-resistant at any time point. However, with the VerifyNow aspirin assay, 4% of patients were aspirin-resistant at baseline, 0% at 1-day, 0% at 1-month, 0% at 6-month, and 15% at 12-month follow-ups ($p = 0.625$ for 12 months vs. baseline; $p = 0.250$ for 12 months vs. 1 month).

Platelet activation markers. Platelet expression of PAC-1 was significantly lower after starting clopidogrel at 1 day and 1 month after PCI. In contrast, CD40L and CD62P expressions appeared lower at 1 day and 1 month, but were not statistically significant compared with baseline expressions. Nevertheless, platelet expressions of all 3 markers were significantly higher at 12 months compared with expressions 1 month or 1 day after clopidogrel administration (Table 2, Fig. 2).

Inflammatory markers. Soluble P-selectin remained unchanged at 1 day and 1 month following clopidogrel administration, but became significantly higher at 6 and 12 months. Conversely, soluble CD40L levels were stable at 1 day and 1 month, but became progressively and significantly lower at 6 and 12 months. Serum IL-18 levels were stable at 1 day and 1 month, but became significantly higher at 6 and 12 months compared with baseline levels. However, the 12-month IL-18 level was not significantly higher compared with the 1-month level. Serum IL-10 levels were unchanged from baseline to all subsequent visits. High-sensitivity C-reactive protein was significantly elevated 1 day after PCI, but returned to baseline values at all subsequent visits (Table 2, Fig. 3).

Clinical events. Three non-ST-segment elevation MIs occurred during the 1-year follow-up (1 had procedural-related MI requiring target vessel revascularization). None of these patients were resistant to aspirin or clopidogrel. There were no death or stroke events.

Discussion

Our prospective study showed no increase in platelet aggregation or prevalence of resistance with long-term clopidogrel and aspirin administration. However, the inhibitory effects of clopidogrel on platelet expression of CD40L, CD62P, and PAC-1 diminished over the course of long-term treatment.

Our study is the first to evaluate the long-term effects of clopidogrel on platelet aggregation. With rising concerns of clopidogrel resistance and the associated risks of atherothrombotic complications, it is prudent to assess the longer-term platelet response, even among patients who were initially clopidogrel-responsive. In our primary analysis, we

found no increase in platelet aggregation with 1-year clopidogrel administration. This should alleviate some lingering concerns among clinicians who manage patients with drug-eluting stents, because the risk of stent thrombosis is in part related to heightened platelet reactivity (4).

Likewise, our study showed no increase in platelet aggregation or resistance prevalence with 1-year aspirin administration. These results differed from prior studies (4–6), which may be explained by the use of different agonists and doses, lack of clopidogrel use, and different patient populations.

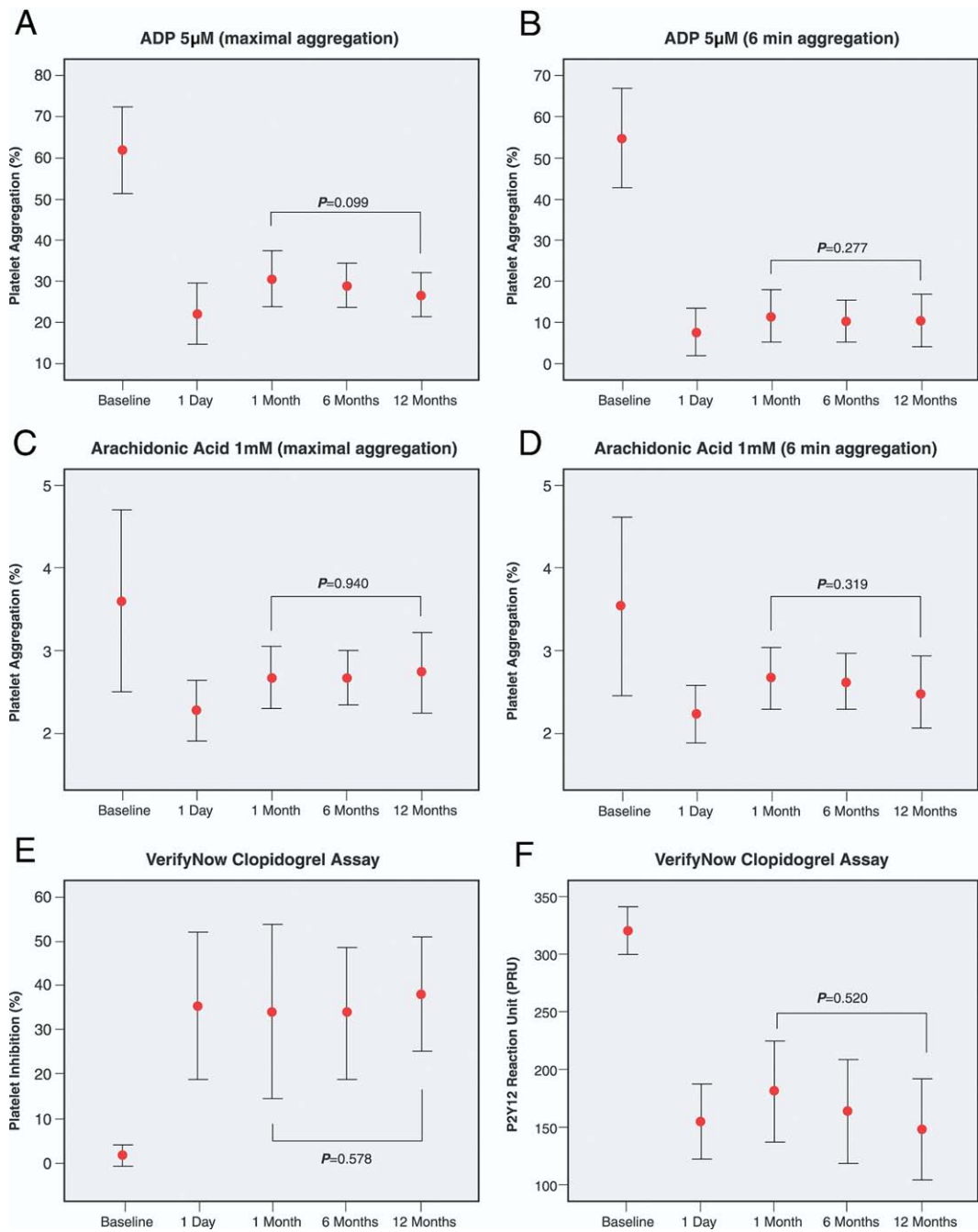


Figure 2 Platelet Aggregation and Activation Markers

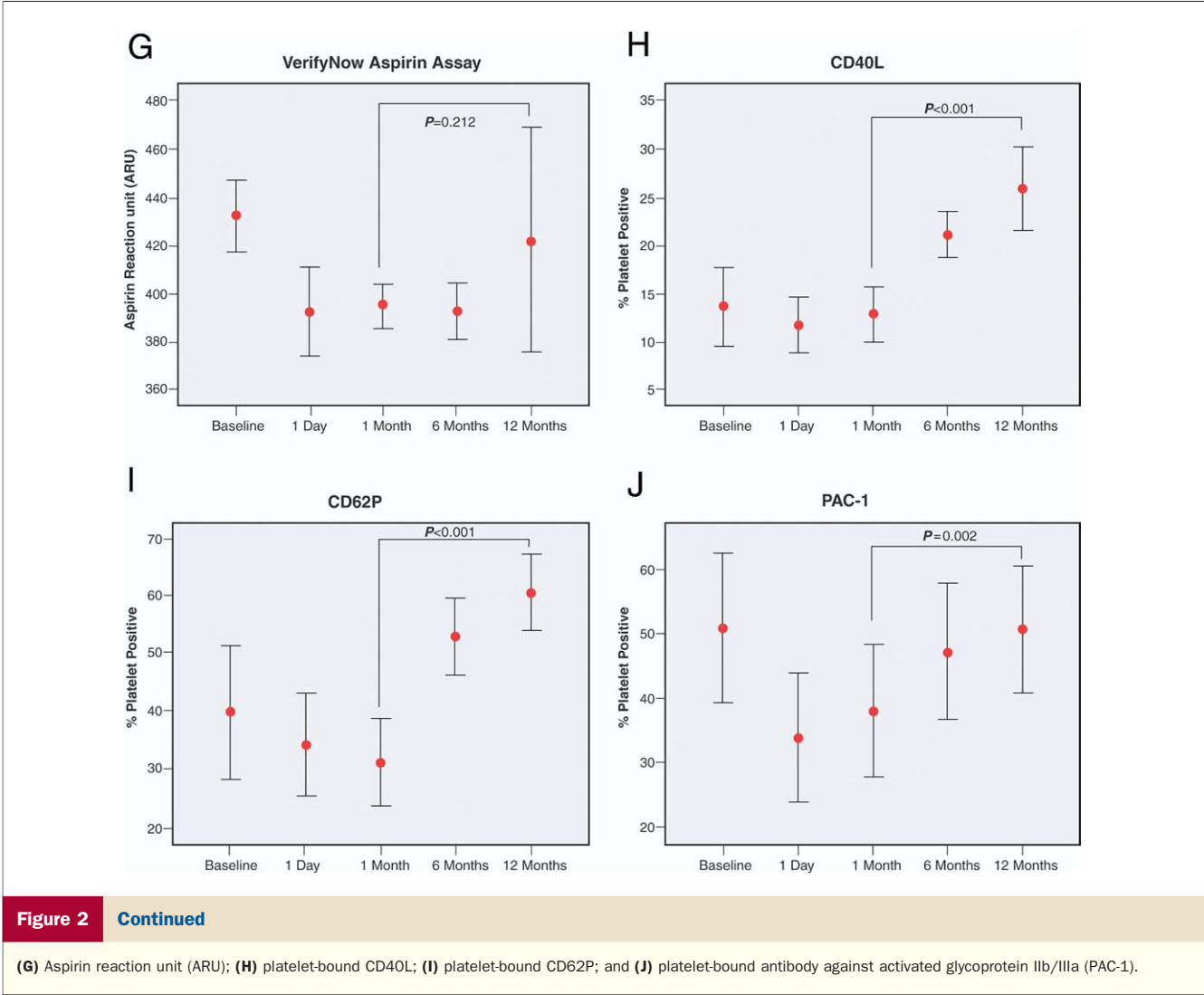
Laboratory measurements (mean with 95% confidence interval) for platelet aggregation and activation: (A) maximal platelet aggregation with light-transmittance aggregometry (LTA) 5-μmol/l adenosine diphosphate (ADP); (B) 6-min platelet aggregation with LTA 5-μmol/l ADP; (C) maximal platelet aggregation with LTA 1-mmol/l arachidonic acid; (D) 6-min platelet aggregation with LTA 1-mmol/l arachidonic acid; (E) VerifyNow clopidogrel assay platelet inhibition; and (F) P2Y₁₂ reaction unit (PRU). Continued on next page.

Table 3	Effects of Aspirin on Platelet Aggregation								
	Baseline	1 Day	p Value	1 Month	p Value	6 Months	p Value	12 Months	p Value
LTA with 1-mmol/l									
AA platelet aggregation, %									
Maximum	3.6 ± 2.5	2.4 ± 0.9	0.010	2.7 ± 0.9	0.030	2.6 ± 0.8	0.042	2.6 ± 1.2	0.047
6 min	3.6 ± 2.4	2.3 ± 0.9	0.009	2.7 ± 0.99	0.047	2.6 ± 0.8	0.060	2.4 ± 1.1	0.014
VerifyNow aspirin assay									
ARU	444.6 ± 46.7	402.5 ± 37.7	0.001	406.5 ± 39.3	0.011	413.5 ± 48.1	0.009	424.3 ± 71.1	0.215

Values are presented as mean ± SD. The p values are for comparison to baseline.
AA = arachidonic acid; ARU = aspirin reaction unit; other abbreviations as in Table 2.

The expression of platelet activation markers CD40L, CD62P, and PAC-1 were significantly higher at 12 months compared with expression at 1 month after PCI. Expressions of these receptors are known to promote binding and mediate interactions among platelets, the endothelium, and leukocytes, which may play important roles in inducing inflammation, atherosclerosis, and thrombosis (9). However, in our study, this increase in platelet receptor expression did not correspond to an increase in platelet aggrega-

tion. This apparent discrepancy is baffling, and our study is too small to assess for potential clinical events that may correspond to these contradictory effects.
The inflammatory marker findings in our study were atypical and discordant with the different markers assessed. Although the high-sensitivity C-reactive protein, IL-10, and IL-18 levels remained unchanged at 12 months versus 1 month, suggesting that the overall systemic inflammatory status remained stable in our patients, we could not explain the



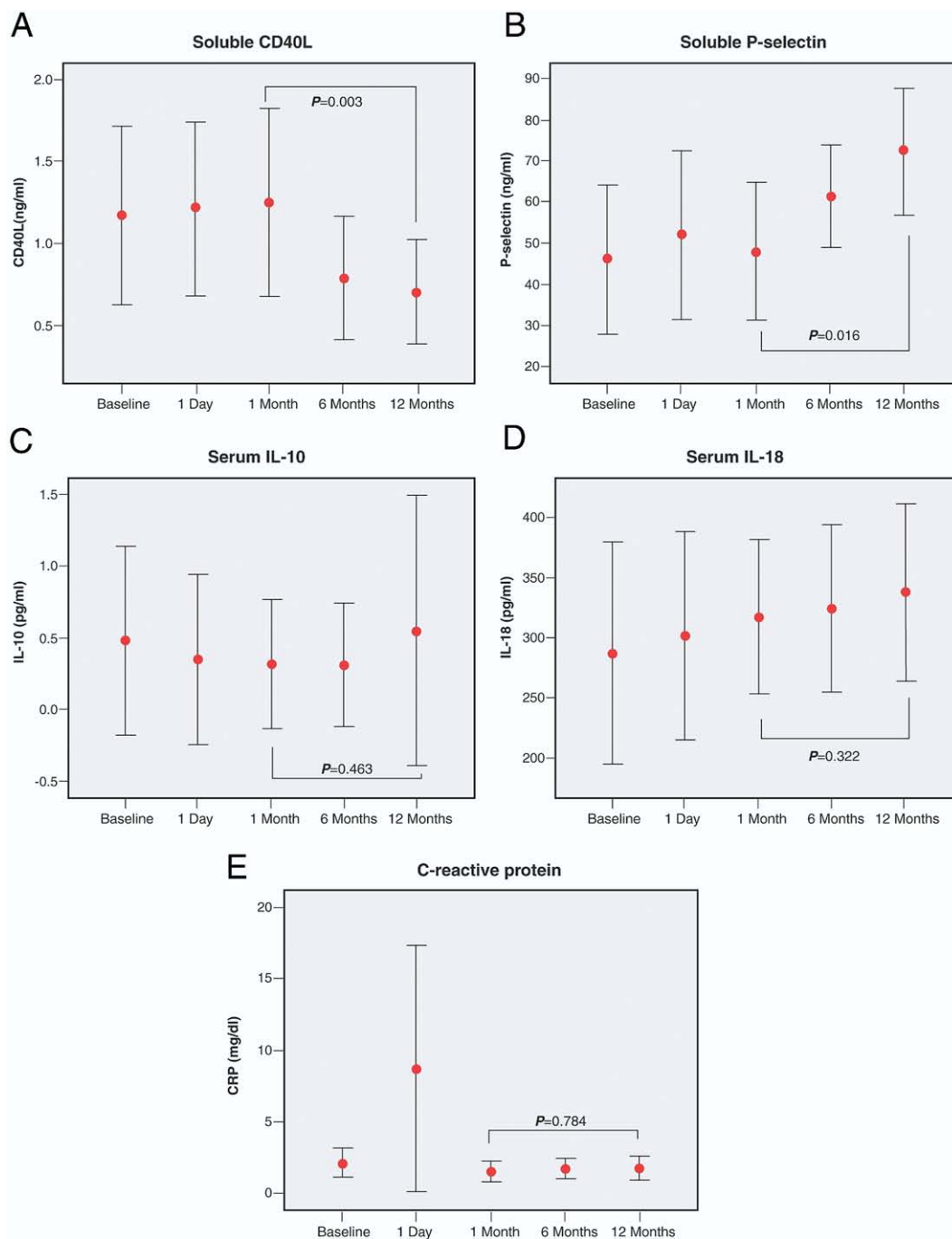


Figure 3 Inflammatory Markers

Laboratory measurements (mean with 95% confidence interval) for inflammatory markers: (A) soluble CD40L; (B) soluble P-selectin; (C) serum IL-10; (D) serum IL-18; and (E) C-reactive protein. Abbreviations as in Figure 1.

contradicting increase in soluble P-selectin and decrease in soluble CD40L levels at the 1-year follow-up. These findings are perplexing and should be further explored in larger studies. **Study limitations.** Our study has several limitations. Although prospective, our study population is small, and we

could not adequately assess clinical events corresponding to platelet function tests. However, we rigorously followed our patients with laboratory and clinical assessments longitudinally and exceeded our power calculation with 26 patients completing the 1-year follow-up. The definition that we

used for clopidogrel resistance, although not universally accepted, had been widely used (2,3).

Conclusions

Our prospective pilot study showed that long-term therapy with clopidogrel and aspirin did not attenuate the initial inhibitory effects on platelet aggregation. In contrast, platelet activation receptor expression increased with long-term therapy and should be further evaluated in large-scale prospective studies corresponding laboratory findings with clinical end points.

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Key Words: aspirin ■ clopidogrel ■ platelet aggregation ■ platelet activation ■ inflammation.