

A novel modification of the Thrombelastograph assay, isolating platelet function, correlates with optical platelet aggregation

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Flow cytometry, singlet platelet counting, and optical aggregation have been used to monitor clopidogrel and glycoprotein IIb/IIIa (GPIIb/IIIa) platelet antagonists. Optical aggregation is considered the gold standard, but neither it nor flow cytometry is convenient in larger-scale clinical studies or point-of-care systems. Singlet platelet counting, a point-of-care assay correlated with optical platelet aggregation, only provides a measurement of platelet function at a single point in time. The Thrombelastograph is used to assay whole blood for thrombin-generated maximal clot-shear elasticity, referred to as the maximal amplitude (MA). Although platelet dysfunction, thrombocytopenia, and the *in vitro* effect of strong inhibitors such as IIb/IIIa antagonists can be observed, with thrombin generation milder platelet inhibitors cannot be assessed. We modified the Thromboelastograph assay, using reptilase and factor XIIIa, to form a clot, without thrombin generation, in heparinized whole blood. The resulting clot MA is dependent on added platelet agonists such as ADP or arachidonic acid, is sensitive to platelet antagonists, and provides a continuous measure of platelet function more analogous and better correlated with optical aggregation. This novel modification of the Thromboelastograph assay should prove to be a useful point-of-care whole-blood assay with which to monitor the effects of GPIIb/IIIa, ADP, and thromboxane A₂-receptor-inhibiting drugs in patients. (*J Lab Clin Med* 2004;143:301-9)

Abbreviations: AA = arachidonic acid; GPIIb/IIIa = platelet glycoprotein IIb/IIIa; MA = maximal amplitude; MA₀ = MA without platelet activation; MA_{AA} = MA with AA activation; MA_{ADP} = MA with ADP activation; MA_{KH} = MA with kaolin/heparinase activation; NSAID = nonsteroidal antiinflammatory drug; OPA = optical platelet aggregation; PPACK = phenylalanine-proline-arginine chloromethyl ketone; PRP = platelet-rich plasma; PPP = platelet-poor plasma

Clopidogrel therapy, with or without NSAIDs, has been widely adopted for the prevention of thrombosis in patients undergoing interventional cardiology and those with unstable angina¹⁻⁴ and for the prevention stroke and other secondary ischemic events.^{5,6} However, these long-term therapies are

associated with increased risks of bleeding,⁶ especially in the surgical setting.⁷⁻⁹ One major problem is the variation in percent inhibition of platelet function and the need for point-of-care monitoring to correlate the effectiveness of therapy with patient outcome.^{10,11}

OPA was the main *ex vivo* assay used in clinical

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trials of IIb/IIIa antagonists¹² and clopidogrel.^{13,14} The authors of more recent studies have employed different whole-blood assays such as singlet platelet counting and flow cytometry with *p*-selectin as a marker of platelet activation.^{15,16}

Thromboelastograph analyzer standard assays have been used extensively in the management of hemostasis during major surgical interventions such as liver transplantation¹⁷ and cardiovascular procedures,¹⁸ as well as in obstetrics¹⁹ and the management of deep-vein thrombosis.²⁰ The use of these assays has resulted in reductions in the use of blood products¹⁸ and the rate of repeat exploration.²¹ The standard Thromboelastograph assay is designed to measure the development of clot-shear elasticity in response to the formation of thrombin and platelet activation.²² Several properties can be assessed with the standard assay, including the development of maximal clot-shear elasticity, referred to as the maximal amplitude (MA). Platelet dysfunction, thrombocytopenia, and the *in vitro* effects of strong inhibitors such as IIb/IIIa antagonists can readily be observed with this standard assay in terms of all the parameters it measures, including MA.²² Because the standard assay requires thrombin generation, it has not been possible to assess the effects of other platelet activators and sensitivity to milder inhibitors.

In this article we report a modification of the Thromboelastograph assay in which reptilase (batroxobin) and factor XIIIa are used to generate a whole blood–crosslinked clot in the absence of thrombin generation or platelet activation. The Thromboelastograph MA parameter is then sensitive to platelet activation by ADP or AA. In addition, we report the sensitivity of this novel Thromboelastograph assay compared with OPA in measuring the effects of platelet inhibitors of GPIIb/IIIa, ADP, and thromboxane A₂ receptors.

METHODS

Materials. Reptilase (batroxobin maranhao; CenterChem, Norwalk, Ct) and factor XIIIa (Nu-Millennium Research, Manhasset, NY) were obtained separately or as a mixture through Hameoscope Corp (Niles, Ill). Monoject sodium heparin and sodium citrate tubes were obtained from Sherwood Medical (St Louis, Mo). We used the GP IIb/IIIa inhibitors eptifibatide (Integrilin injection; Millennium Pharmaceuticals, Cambridge, MA) and abciximab (ReoPro; Eli Lilly, Indianapolis, Ind). PPACK anticoagulant was purchased from BioMol Research Laboratories (Plymouth Meeting, Pa). ADP and AA (Chrono-Log, Havertown, Pa) were used as platelet agonists. All other reagents were obtained through Sigma-Aldrich (St Louis, Mo).

Blood sampling. Blood samples were collected, with the use of standard phlebotomy techniques, in Vacutainer tubes anticoagulated with 14.7 U/mL heparin (Becton Dickinson Vacutainer Systems, Franklin Lakes, NJ). All subjects in this study gave informed consent as approved by an institutional review board operating under the principles of the Declaration of Helsinki.

Modified Thromboelastograph assay. We placed cups and pins in the Thromboelastograph model 5000 instrument in accordance with the standard procedure recommended by the manufacturer (Haemoscope Corp). To each standard Thromboelastograph cup, placed in the 37°C instrument holder, we added 10 μL of the mixture of reptilase plus factor XIIIa, then added ADP or AA as indicated from stock solutions made and stored in accordance with the manufacturer's instructions. We started the reaction by adding 0.35 mL of heparinized whole blood and rapidly stirring the mixture 3 times with a pipette. The Thromboelastograph monitored the reaction until an MA parameter value was obtained. One sample in each set was assayed without platelet activation (MA₀), 1 sample was assayed with ADP activation (MA_{ADP}), and 1 sample was assayed with AA (MA_{AA}). An additional sample was assayed with a standard assay to generate thrombin. We then pipetted the blood sample into a heparinase vial to neutralize the heparin. The same sample was next transferred to a vial of kaolin and added to a heparinase-coated Thromboelastograph cup and monitored for the thrombin-clot MA_{KH}.

To compare the MA results with percent OPA, we used a normalized formula to derive a percent MA aggregation response to agonist, as follows:

$$\%MA_{ADP} = ([MA_{ADP} - MA_0] / [MA_{KH} - MA_0]) \cdot 100$$

$$\%MA_{AA} = ([MA_{AA} - MA_0] / [MA_{KH} - MA_0]) \cdot 100$$

OPA. Whole blood collected in heparinized Vacutainer tubes as described above was centrifuged for 20 minutes at 100g to separate PRP. We obtained PPP from the same sample after removing the PRP layer through further centrifugation at 1000g for 10 minutes. We monitored PRP aggregation in 0.35-mL aliquots after adding the same agonists as described above in accordance with a standard protocol²³ on a Chrono-Log dual-channel aggregometer (Chrono-Log, Havertown, Pa) at 37°C with constant stirring. Calibration and percent aggregation were monitored with Aggrolink software in accordance with manufacturer recommendations. Percent aggregation is calculated with a formula similar to the one derived for the Thromboelastograph in which light transmission from PRP equals 0% and light transmission from PPP equals 100% aggregation. Percent aggregation is calculated after the PPP baseline is subtracted from both the test sample and PRP and divided. The result is multiplied by 100 to yield a percentage.

Statistical analysis. We carried out statistical analysis using Statview 4.0 software (SAS Institute, Cary, NC). Data were examined for normal distribution and, when appropriate, nonparametric testing was performed, with *P* values corrected

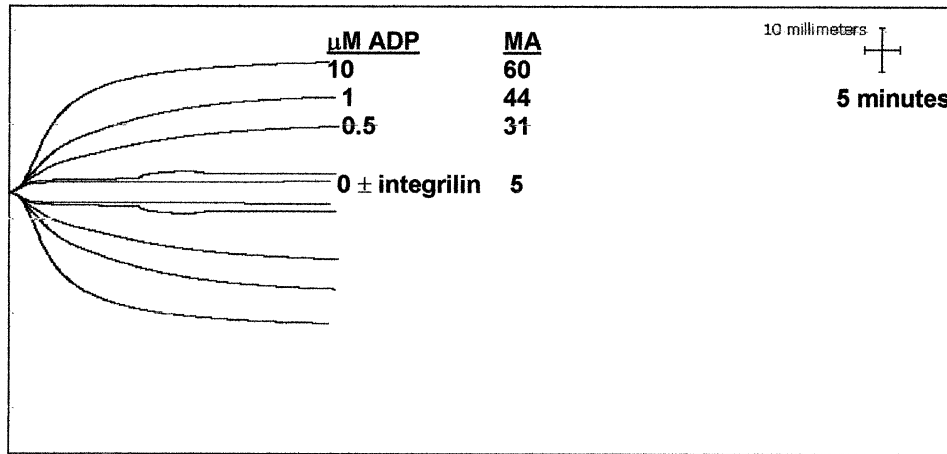


Fig 1. ADP dose responses on modified Thromboelastograph assay. Vertical bar = 10 mm; horizontal bar = 5 minutes. ADP dose and MA responses are indicated next to each trace.

for multiple testing with the use of the sequential Bonferroni method. We performed linear regression to obtain the least-squares fit and r^2 value, but significance testing was based on Spearman rank correlations.

RESULTS

Development of the modified Thromboelastograph assay. Several properties can be measured with the standard Thromboelastograph assay, including time to clot formation, rate of clot formation, and development of MA. The *in vitro* effect of strong inhibitors of platelet function such as GPIIb/IIIa antagonists can be observed with this standard assay in terms of all the parameters measured, including a reduction in MA.²² To measure platelet activation by other agonists and make the assay sensitive to weaker platelet inhibitors such as NSAIDs or clopidogrel, it is necessary to avoid thrombin generation yet generate a fibrin network in which the platelets can interact. We accomplished this with the use of reptilase, which generates fibrin through a thrombin-like activity in the absence of platelet activation.²⁴ It was also necessary to crosslink this fibrin network with factor XIIIa to give it sufficient rigidity for us to observe the platelet interaction.²⁵

This novel modification of the Thromboelastograph assay that isolates platelet function is sensitive to a dose of an activator such as ADP, as shown in Fig 1. We collected blood from a normal volunteer who was not taking any platelet inhibitors into standard heparin-treated Vacutainer blood tubes. In the absence of platelet activation, we noted a low MA. This baseline MA was a result of the fibrin network and was not substantially reduced by 20 $\mu\text{g/mL}$ integrilin, which we added in advance to the Thromboelastograph cup to block

platelet IIb/IIIa interaction. Maximal ADP dose-response increases in MA were comparable to a thrombin-clot MA generated from the same blood sample with reversal of heparin anticoagulation by heparinase and activation with kaolin (data not shown). We obtained identical results with 12.5 $\mu\text{g/mL}$ PPACK-anticoagulated blood samples but not with citrate anticoagulants, possibly because of a calcium concentration of less than 50 $\mu\text{mol/L}$, which would greatly reduce the rate of factor XIIIa crosslinking²⁶ (data not shown).

ADP dose response and sensitivity to clopidogrel for Thromboelastograph percent MA relative to OPA. OPA measures the percent change in light transmittal after the addition of platelet agonist to PRP; Thromboelastograph MA parameter measures the percent change in clot strength after the addition of platelet agonist to the Thromboelastograph cup with reptilase and FXIIIa. We used normalization formulas to derive percent MA and percent OPA so that we might compare the Thromboelastograph MA parameter results with those of OPA, as described in the Methods. Fig 2, A and B, shows that the dose-dependent ADP-induced increase in Thromboelastograph percent MA is comparable to percent OPA with PRP obtained from the same heparinized blood sample. Also shown in the figure is the effect of a 6 days' treatment with clopidogrel, which has been shown to yield a steady-state percent inhibition of 5 $\mu\text{mol/L}$ ADP-dependent OPA of heparin-anticoagulated PRP.¹⁴

The dose responses of both assays were found to have returned to pretreatment values by 10 days after the last clopidogrel dose (data not shown). Repeat treatments of the same subject at the same dosage and

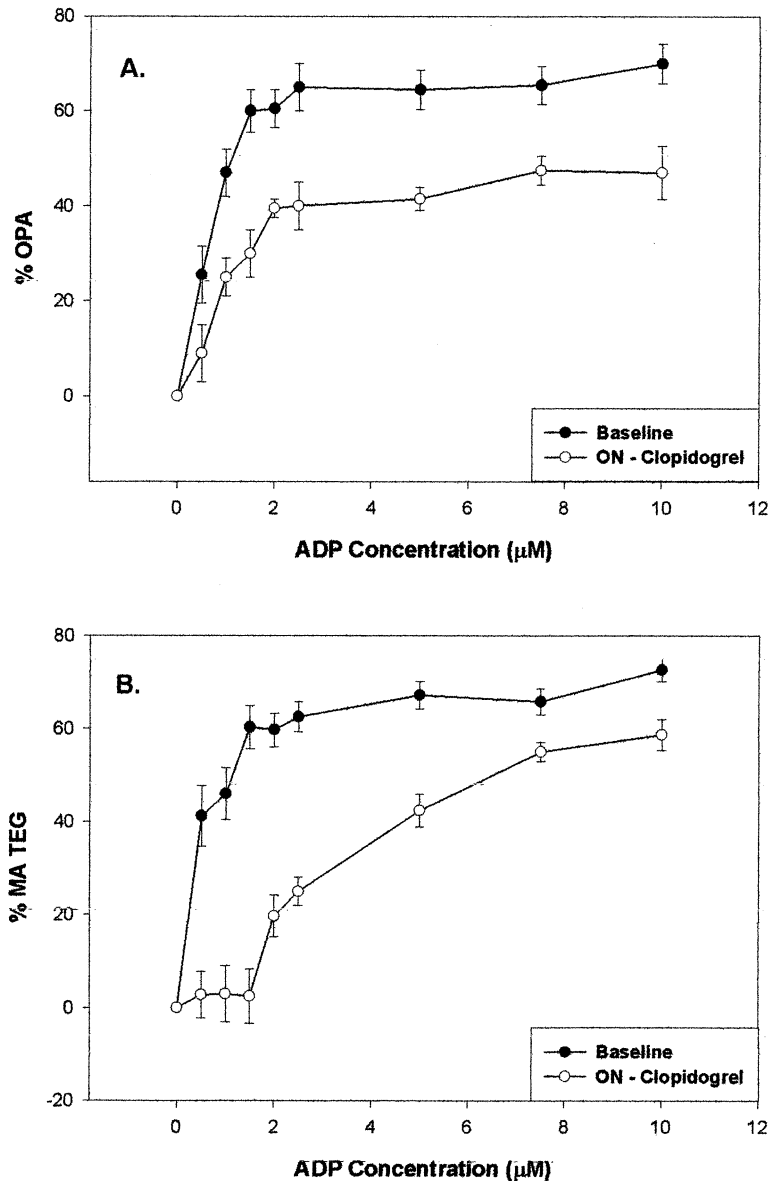


Fig 2. ADP dose-response curves for Thromboelastograph and OPA without and with clopidogrel treatment. A normal volunteer donated blood for baseline Thromboelastograph and OPA (black circles), then began a regimen of clopidogrel 75 mg/day for 6 days. The postclopidogrel samples were assayed with both Thromboelastograph and OPA (white circles). Assays were performed in quadruplicate, with SDs indicated by the error bars.

number of days, with at least 1 month's recovery between treatments, yielded a consistent percent inhibition for both assays (1 $\mu\text{mol/L}$ ADP response, 91% \pm 11% inhibition of Thromboelastograph, 52% \pm 11% inhibition of OPA; mean \pm SD, $n = 4$ separate treatments).

Measurement of IIb/IIIa inhibitors by Thromboelastograph. We assessed the usefulness of the Thromboelastograph assay in monitoring IIb/IIIa therapies with the use of the dose-response assays depicted in Fig

3. Dose-response curves comparing percent MA and percent OPA are shown for ReoPro. The data again show a correspondence between the assays, even though in the Thromboelastograph assay the IIb/IIIa interaction is with a fibrin network as opposed to soluble fibrinogen molecules. An advantage of the Thromboelastograph assay is that it maintains a normal calcium concentration, influencing the effectiveness of different IIb/IIIa inhibitors.²⁷

We also conducted assays in patients undergoing

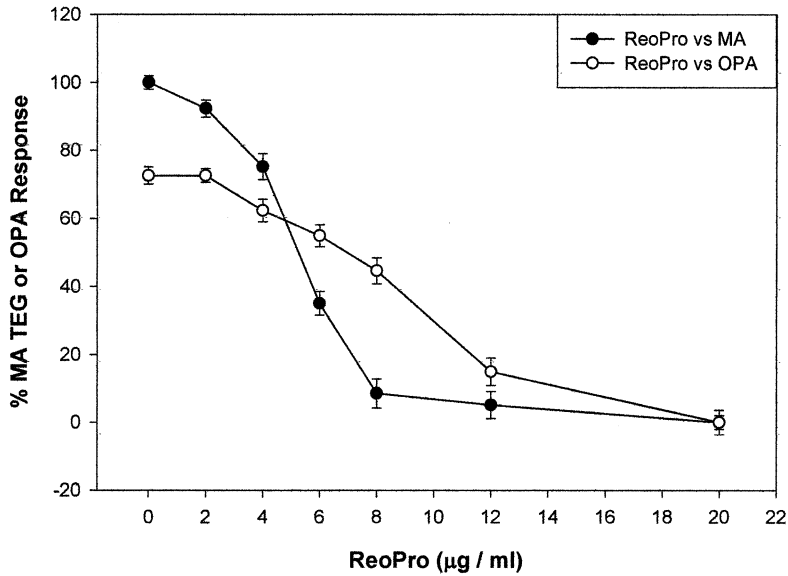


Fig 3. ReoPro inhibition dose-response curves for 100 $\mu\text{mol/L}$ ADP-activated whole blood (Thromboelastograph) or PRP (OPA). Blood or PRP obtained from the same sample was titrated in vitro with the indicated concentration of ReoPro (abciximab) added in advance to the Thromboelastograph cup before activation from a 100-fold concentrated stock solution. Samples were then assayed on OPA (white circles) or Thromboelastograph (black circles) in triplicate with activation by 100 $\mu\text{mol/L}$ ADP as described earlier. Data expressed as means \pm SD (error bars).

Table I. Responses to ADP in patients treated ($n = 12$) and not treated ($n = 5$) with integrilin

ADP dose	%MA			%OPA		
	Not treated	Treated	P	Not treated	Treated	P
1 $\mu\text{mol/L}$	6 \pm 1	4 \pm 2	.114	43 \pm 7	0	<.0001
2 $\mu\text{mol/L}$	31 \pm 12	6 \pm 3	.005	57 \pm 6	0	<.0001
100 $\mu\text{mol/L}$	91 \pm 4	51 \pm 16	.004	59 \pm 3	0	<.0001

Data expressed as median \pm median absolute deviation, Mann-Whitney U test. P values corrected for multiple testing with the sequential Bonferroni method.

interventional cardiology procedures with or without integrilin, another GPIIb/IIIa antagonist. All of these patients had been prophylactically treated with a 300-mg loading dose followed by 75 mg/d clopidogrel and 80 mg/d aspirin 1 to 25 days before their procedures. Heparinized blood samples were taken at baseline (on the patients' admission to the catheterization laboratory but before the administration of additional anticoagulants or antiplatelet agents). Some of the patients were then administered integrilin in an 180 $\mu\text{g/kg}$ bolus and a 2 $\mu\text{g/kg/min}$ infusion. Other patients in the study received bivalirudin or additional heparin. Whereas bivalirudin and heparin had no effect on the Thromboelastograph percent MA response to ADP, these extra anticoagulants

inhibited the MA_{KH} used for normalization of the assay. We therefore calculated the percent MA_{ADP} using the baseline MA_{KH} values obtained with the admission samples. Table I shows the percent MA and OPA for groups of patients, treated or not treated with integrilin, and the Mann-Whitney U P value yielded by comparison of the 2 groups. The extra inhibition by integrilin is not significant at the Thromboelastograph 1 $\mu\text{mol/L}$ ADP dose response. This may be a result of the clopidogrel and aspirin inhibition at this low ADP dose for all the patients (both groups averaged 6 days' clopidogrel treatment with no significant difference, $P = .881$).

NSAID inhibition of ADP response. We assayed normal subjects using the Thromboelastograph before and 1

Table II. Effect of aspirin on Thromboelastograph %MA and %OPA responses before and after various doses of ADP ($n = 15$)

ADP dose	%MA			%OPA		
	Before	After	<i>P</i>	Before	After	<i>P</i>
1 $\mu\text{mol/L}$	77 \pm 14	51 \pm 18	.016	37 \pm 5	29 \pm 10	.015
10 $\mu\text{mol/L}$	100 \pm 7	96 \pm 5	.036	66 \pm 9	57 \pm 8	.071
100 $\mu\text{mol/L}$	107 \pm 4	105 \pm 3	.061	67 \pm 6	63 \pm 7	.051

Data expressed as median \pm median absolute deviation, Wilcoxon signed-rank test. *P* values corrected for multiple testing with the sequential Bonferroni method.

'hour after ingestion of a standard 325-mg aspirin tablet. As shown in Table II, Wilcoxon's signed-rank test, corrected for multiple testing with the sequential Bonferroni method, indicated significant inhibition of the ADP response at 1 $\mu\text{mol/L}$ ADP, whereas higher doses yielded less significant inhibition. We detected no significant effect of aspirin on MA_0 or MA_{KH} ($P > .3$).

A more sensitive assay for the monitoring of aspirin inhibition by OPA typically involves the use of AA as an activator; an aggregation response of less than 5% is observed after ingestion of aspirin, compared with aggregation of greater than 50% with 1 mmol/L AA.²⁸ A comparable Thromboelastograph assay for whole-blood studies is shown in Fig 4, A and B. We also compared 30 cardiology and surgery patients receiving low-dose (81 mg/d) aspirin therapy with 14 patients who were not taking NSAIDs (Table III). The Mann-Whitney *U* *P* value yielded by comparison of the 2 groups indicates significant inhibition of the Thromboelastograph response to 1 mmol/L AA compared with the inhibition seen on OPA.

Correlation of percent MA on Thromboelastograph and OPA. Linear-regression analysis yielded a r^2 value of 0.65 for percent MA performed with whole blood compared with that yielded by OPA performed with PRP (Fig 5). This analysis involved 5 different ADP doses (1, 2, 5, 10, and 100 $\mu\text{mol/L}$ ADP) and was carried out in 30 normal volunteers, 55 cardiology patients, and 35 surgery patients ($N = 120$). Subjects were variously receiving aspirin, clopidogrel, both therapies, or neither therapy at the time of assay. The Spearman rank test comparing the 2 assays yielded a *P* value of less than .0001.

DISCUSSION

Reptilase and thromboelastography have been used previously to study the interaction of platelets with the fibrin network.²⁹ The development of MA has

been shown to depend on platelet activation and to be correlated with clot retraction. However, the MA obtained with reptilase and ADP in the absence of factor XIIIa crosslinking was relatively small and slow to develop compared with a thrombin clot.

In this study, the addition of purified factor XIIIa to a reptilase-generated fibrin network permitted an assay in which the platelet contribution to the shear elasticity of the clot, after activation by ADP or AA, could be easily and rapidly measured in whole blood. It is important to note that this platelet interaction takes place in an unmodified-calcium environment, which may have affected the strength of fibrin(ogen) platelet IIb/IIIa interaction and sensitivity to IIb/IIIa antagonists.^{27,30} Complete inhibition of the percent OPA responses of patients undergoing interventional cardiology procedures are seen at therapeutic doses of integrilin for all doses of ADP, whereas the inhibition of the Thromboelastograph percent MA is partially overcome at 100 $\mu\text{mol/L}$ ADP. This may be a result of the relative difficulty of blocking the interaction with a fibrin network compared with individual fibrinogen molecules. The physiologic significance of this partial inhibition can only be determined through further clinical studies.

Thromboelastograph also provides a point-of-care assay with which to monitor NSAID and clopidogrel therapy. Clopidogrel inhibition of platelet function has been reported to be quite variable,^{15,16} possibly as a result of metabolic differences among patients. We recently compared Thromboelastograph with OPA and singlet platelet counting assays in the monitoring of platelet inhibition correlated with patient variables such as length of clopidogrel therapy and coincident atorvastatin therapy.³¹ Because this was a small pilot study, we could not make any significant observations with regard to how patient outcomes correlated with a lack of clopidogrel inhibition.

Our study demonstrates that this modification of the

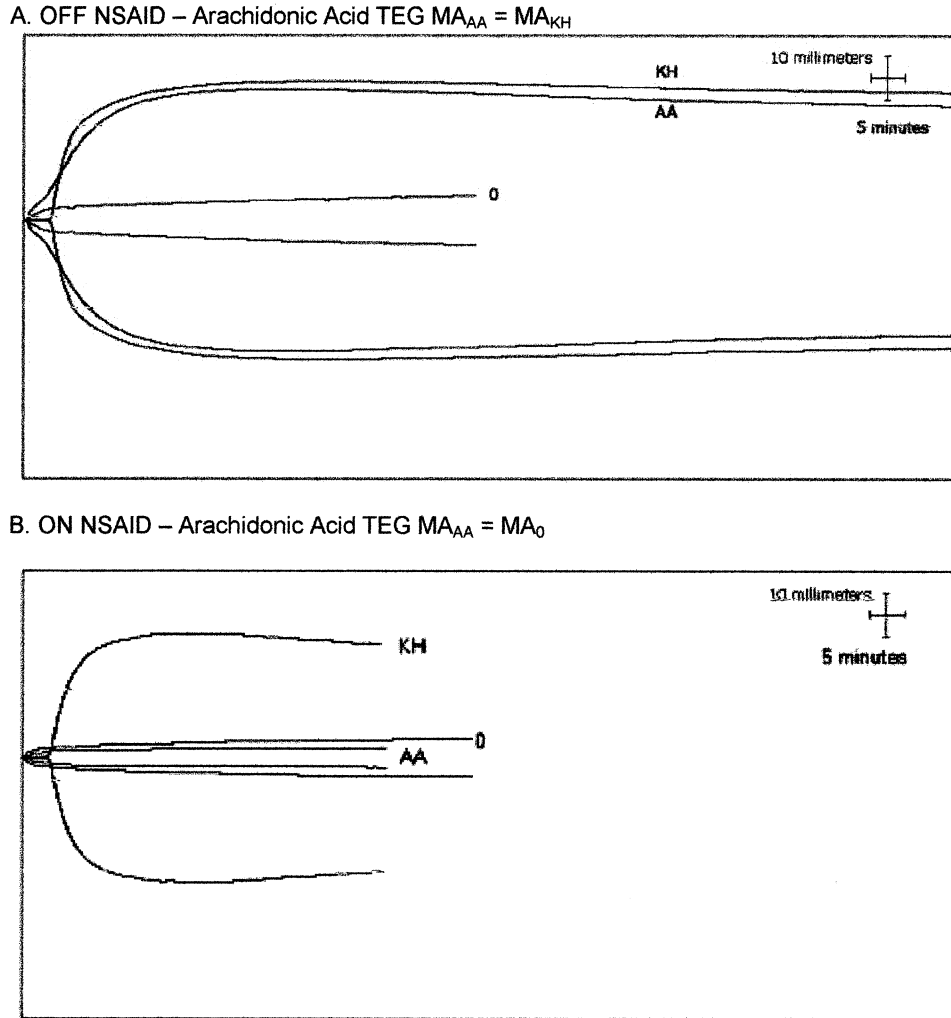


Fig 4. Thromboelastograph traces from patients (A) not receiving or (B) receiving aspirin therapy. Each set of Thromboelastograph traces shows the waveforms for MA_0 , MA_{AA} , and MA_{KH} .

Table III. %MA and %OPA responses to 1 mmol/L AA in patients treated (n = 30) or not treated (n = 14) with NSAIDS

AA treatment	%MA _{AA}			%OPA _{AA}		
	No NSAIDs	NSAIDs	P	No NSAIDs	NSAIDs	P
1 mmol/L	94 ± 4	11 ± 11	<.0001	69 ± 6	2 ± 1	<.0001

Data expressed as median ± median absolute deviation; P values calculated with Mann-Whitney U test.

Thromboelastograph provides a convenient whole-blood assay that correlates well with OPA, providing an analogous profile of platelet function over time, and that can accurately monitor inhibition by GPIIb/IIIa, ADP, and thromboxane A₂-receptor antago-

nists. Such a point-of-care assay permits convenient monitoring of patients receiving such platelet antagonists and makes it possible to conduct large scale correlative studies on the degree of platelet inhibition and patient outcomes.

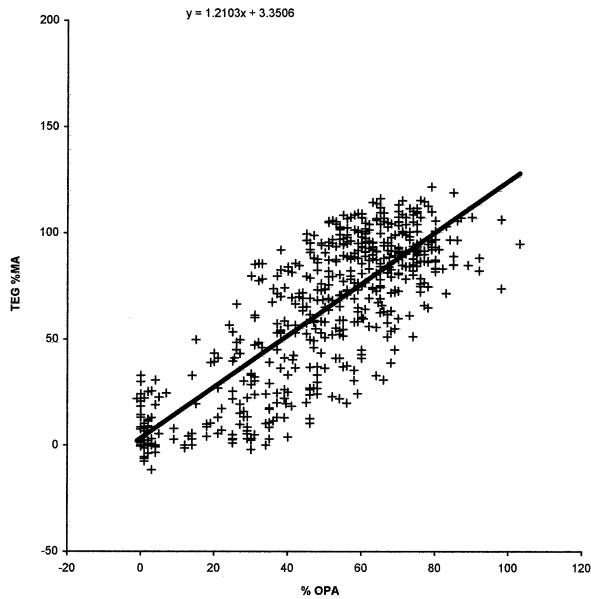


Fig 5. Linear-regression plot of Thromboelastograph percent MA and percent OPA. $N = 600$; confidence limits not displayed because of lack of normality.

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