

# Platelet Mapping Assay Interference Due to Platelet Activation in Heparinized Samples

Nicole J. Nelles, MD, and Wayne L. Chandler, MD

From the Department of Pathology and Genomic Medicine, Houston Methodist Hospital, Houston, TX.

**Key Words:** Platelet activation; Heparin; Microvesicles; Thrombelastography

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## ABSTRACT

**Objectives:** *Thromboelastography Platelet Mapping (TEGPM) is an assay designed to detect platelet inhibition due to aspirin or clopidogrel-like drugs. The purpose of this study was to evaluate potential causes of error in the design or operation of the assay.*

**Methods:** *We evaluated percent inhibition of platelets due to aspirin or clopidogrel using TEGPM, which measures clot viscoelastic maximum amplitude (MA) after activation with adenosine diphosphate (ADP) or arachidonic acid (AA) and subtraction of MA due to fibrin ( $MA_{Fibrin}$ ).*

**Results:**  *$MA_{Fibrin}$  measured in heparinized blood showed an unstable increasing pattern in 28% of samples (16 of 58). The platelet aggregation inhibitor eptifibatid corrected increasing  $MA_{Fibrin}$  in 14 of 16 cases, while the thrombin inhibitor argatroban corrected increasing  $MA_{Fibrin}$  in six of 16 cases, suggesting that unanticipated platelet activation/aggregation was a more important cause of unstable rising  $MA_{Fibrin}$  than uninhibited thrombin. The unstable increased  $MA_{Fibrin}$  falsely increased percent ADP inhibition on average from 19% to 38% and percent AA inhibition from 29% to 58%. Heparinized samples showed platelet clumping and had procoagulant platelet microvesicle levels double those in citrate anticoagulant.*

**Conclusions:** *Unanticipated platelet activation/aggregation occurring in the heparinized TEGPM samples lead to erroneous percent inhibition results.*

Upon completion of this activity you will be able to:

- describe how the Platelet Mapping assay measures percent adenosine diphosphate (ADP) and percent arachidonic acid (AA) platelet inhibition.
- discuss how problems with the maximum amplitude (MA) fibrin measurement in the assay can lead to erroneous results.
- summarize how problems with  $MA_{ADP}$  and  $MA_{AA}$  compared to  $MA_{Thrombin}$  measurement in the assay can lead to erroneous results.

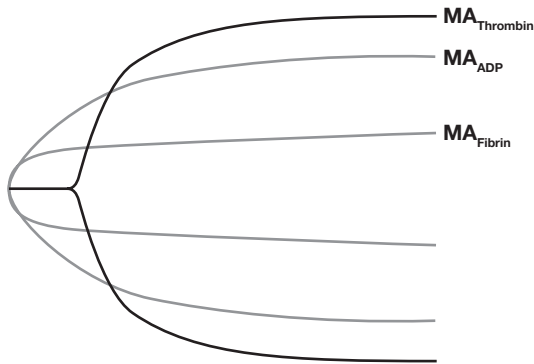
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The authors of this article and the planning committee members and staff have no relevant financial relationships with commercial interests to disclose. Questions appear on p 420. Exam is located at [www.ascp.org/ajcpeme](http://www.ascp.org/ajcpeme).

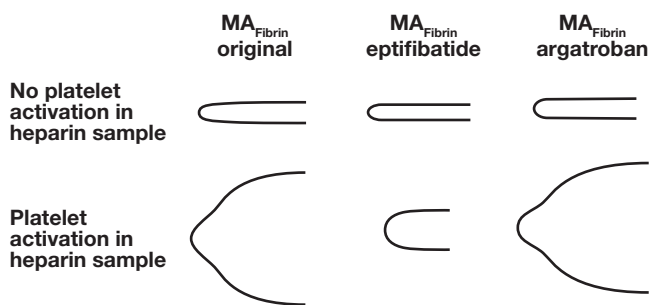
Platelet function tests are commonly performed to help predict risk of bleeding and to monitor therapies that alter platelet function.<sup>1-3</sup> Thromboelastography Platelet Mapping (TEGPM; Haemonetics, Braintree, MA) uses thromboelastography to estimate platelet function and to monitor platelet inhibition by aspirin or clopidogrel. Thromboelastography evaluates hemostatic function by measuring changes in blood elasticity due to coagulation and platelet activation. The maximum clot elasticity generated is termed the *maximum amplitude* (MA).

The overall design of the TEGPM assay is to compare maximum platelet activation produced by thrombin ( $MA_{Thrombin}$ ) in a citrated blood sample with platelet activation by adenosine diphosphate ( $MA_{ADP}$ ) or arachidonic acid ( $MA_{AA}$ ) in samples anticoagulated with heparin to prevent thrombin generation. Thrombin is the most potent platelet

agonist, directly activating platelets through the PAR1 receptor, which is unaffected by aspirin or clopidogrel.<sup>4</sup> In the TEGPM assay, therefore, it is assumed that  $MA_{Thrombin}$  represents maximum platelet function and is not affected by platelet-inhibiting drugs such as aspirin or clopidogrel.<sup>5,6</sup> Any decrease in  $MA_{ADP}$  or  $MA_{AA}$  compared with  $MA_{Thrombin}$  is assumed to be due to platelet inhibition by either a P2Y12 inhibitor such as clopidogrel for  $MA_{ADP}$  or a cyclooxygenase inhibitor such as aspirin for  $MA_{AA}$ .



**Figure 1** Platelet mapping adenosine diphosphate (ADP) inhibition assay maximum amplitude (MA) tracings.  $MA_{ADP}$ , heparinized sample activated with reptilase, factor XIIIa, and ADP;  $MA_{Fibrin}$ , heparinized sample activated with reptilase and factor XIIIa;  $MA_{Thrombin}$ , citrate sample activated with kaolin-producing thrombin. The width of each curve represents the MA.



**Figure 2** Platelet mapping maximum amplitude (MA) showing fibrinogen to fibrin activation by reptilase and factor XIIIa in heparinized samples (maximum amplitude fibrin,  $MA_{Fibrin}$ ). The tracings show the effect of eptifibatide (platelet aggregation blocker) and argatroban (direct thrombin inhibitor) on  $MA_{Fibrin}$  tracings in two patients compared with the original  $MA_{Fibrin}$ . When no platelet activation is present in the heparinized sample, addition of eptifibatide has no effect (upper three tracings). When the sample shows platelet activation in the heparinized sample, as indicated by a biphasic curve, addition of eptifibatide blocks the platelet aggregation, resulting in a monophasic fibrin activation—only curve while addition of argatroban had no effect.

The MA measurements performed in the TEGPM assay are actually the sum of clot strength due to fibrin formation plus the clot strength due to platelet activation. To determine the platelet-specific MA, MA due to fibrin must be subtracted (Figure 1).<sup>2</sup> The maximum amplitude due to fibrin formation alone ( $MA_{Fibrin}$ ) is measured in heparinized whole blood through the addition of reptilase and activated factor XIII (FXIIIa). Heparin prevents thrombin generation while reptilase activates only fibrin and FXIIIa crosslinks the fibrin. It is assumed that no platelet activation is occurring in this heparinized sample and that the MA is due only to fibrin formation. ADP or AA plus reptilase and FXIIIa are added to heparinized whole blood to give measures of ADP maximum amplitude ( $MA_{ADP}$ ) and AA maximum amplitude ( $MA_{AA}$ ). Platelet-specific amplitude is estimated by subtracting  $MA_{Fibrin}$  from  $MA_{Thrombin}$ ,  $MA_{ADP}$ , and  $MA_{AA}$ . To evaluate platelet inhibition by aspirin or clopidogrel,  $MA_{ADP}$  and  $MA_{AA}$  results are compared with  $MA_{Thrombin}$  after subtracting  $MA_{Fibrin}$ . It is assumed that in a patient not receiving platelet inhibitors, all three platelet agonists produce the same level of amplitude change in the assay—that is,  $MA_{Thrombin}$  equals  $MA_{ADP}$ , which equals  $MA_{AA}$ . For example, if  $MA_{ADP}$  minus  $MA_{Fibrin}$  is the same as  $MA_{Thrombin}$  minus  $MA_{Fibrin}$ , then ADP platelet function is 100% and percent ADP inhibition is 0%. If  $MA_{ADP}$  minus  $MA_{Fibrin}$  is 40% of  $MA_{Thrombin}$  minus  $MA_{Fibrin}$ , then ADP platelet function is 40% and percent ADP inhibition is 60%. Potential problems in the assay exist if the  $MA_{Fibrin}$  result is not due to fibrin activation alone or if baseline  $MA_{ADP}$  and  $MA_{AA}$ , in the absence of inhibitors, do not equal  $MA_{Thrombin}$ .

TEGPM is a complex test requiring two samples collected in different anticoagulants (citrate and heparin) with different sample dilution effects, multiple reagents, and a complex calculation with many assumptions to arrive at an estimate of percent inhibition of platelet function stimulated by ADP or AA. Errors in assumptions, assay design, or technique at any step may reduce the accuracy of the test. When working correctly,  $MA_{Fibrin}$  produced by addition of reptilase and FXIIIa reaches MA quickly and remains stable. On many samples, we observed that  $MA_{Fibrin}$  was not stable but continued to increase over time, typically showing a biphasic curve (Figure 2). In a review article, it has been suggested that increased  $MA_{Fibrin}$  may be due to high fibrinogen, circulating activated platelets, or low antithrombin activity, resulting in uninhibited thrombin in the sample, but no data were provided.<sup>2</sup> If antithrombin deficiency is present, it has been suggested that a thrombin inhibitor should be used in the TEGPM assay, but this has not been tested.<sup>2</sup> No solutions were given for other possible causes of falsely increased  $MA_{Fibrin}$ . If the  $MA_{Fibrin}$  is falsely elevated, estimates of platelet function using TEGPM may be inaccurate.

To our knowledge, no studies to date have evaluated the cause of or effects due to increasing  $MA_{Fibrin}$  on

percent ADP inhibition or percent AA inhibition in the TEGPM assay. The purpose of this study was to evaluate the mechanism of increasing  $MA_{\text{Fibrin}}$  and determine the effect of increasing  $MA_{\text{Fibrin}}$  on TEGPM assay accuracy. We hypothesized that if the  $MA_{\text{Fibrin}}$  in the heparinized whole-blood sample is elevated due to uninhibited thrombin activity, then thrombin inhibitors such as argatroban should lower  $MA_{\text{Fibrin}}$ . Alternatively, if platelet activation is causing increasing  $MA_{\text{Fibrin}}$ , then agents that inhibit platelet aggregation, such as the GPIIb/IIIa receptor antagonist eptifibatide, should lower  $MA_{\text{Fibrin}}$ . To evaluate whether platelet activation might be occurring in the samples prior to TEGPM analysis, we reviewed microscopic slides for evidence of platelet clumping and measured platelet microvesicle levels in the citrated and heparinized samples. When platelets are activated, they release platelet microvesicles.<sup>7</sup> We hypothesized that if platelet activation were occurring more in one type of sample vs the other, then platelet microvesicle levels would be substantially higher in that sample type.

## Materials and Methods

### Human Participants

The study was approved by the Houston Methodist Research Institute Human Subjects Review Committee. Blood samples were anticoagulated with 0.109 mol/L citrate or 15 U/mL unfractionated heparin. Studies were performed on leftover samples. Additional studies included repeating the  $MA_{\text{Fibrin}}$  test with addition of 21  $\mu\text{g}/\text{mL}$  eptifibatide to block platelet aggregation or 28  $\mu\text{g}/\text{mL}$  argatroban to inhibit thrombin, microscopic blood smear evaluation for the presence of platelet clumps, and microvesicle analysis as described below. Platelet clumping was defined as moderate if small platelet aggregates could be seen on smear evaluation and as severe if many large platelet aggregates could be seen on smear evaluation. Additional TEGPM studies with eptifibatide and argatroban were performed in parallel with clinical studies. Smear preparation and flow cytometry studies were performed within 3 hours of sample collection.

TEGPM was performed according to the manufacturer's instructions. ActivatorF reagent (reptilase and FXIIIa), ADP, and AA were prepared by reconstitution with distilled water. Four cups/pins, one heparinase and three plain, were loaded into four channels on two instruments. Into one plain cup, 10  $\mu\text{L}$  of ActivatorF and 360  $\mu\text{L}$  of heparinized whole blood were added to determine  $MA_{\text{Fibrin}}$ . Into a second plain cup, 10  $\mu\text{L}$  of ActivatorF, 10  $\mu\text{L}$  of ADP, and 360  $\mu\text{L}$  of heparinized whole blood were added to determine  $MA_{\text{ADP}}$ .

Into the third plain cup, 10  $\mu\text{L}$  of ActivatorF, 10  $\mu\text{L}$  of arachidonic acid, and 360  $\mu\text{L}$  of heparinized whole blood were added to determine  $MA_{\text{AA}}$ . Finally, in the heparinase cup, 20  $\mu\text{L}$  of 0.2 mol/L calcium chloride was added. Next, 1 mL of citrated whole blood was added to a kaolin activation vial and mixed well, followed by addition of 340  $\mu\text{L}$  of kaolin-activated, citrated whole blood to the heparinase cup containing calcium to determine  $MA_{\text{Thrombin}}$ . Heparinase was used in this cup to eliminate any heparin activity that may have been present in the patient, which would reduce  $MA_{\text{Thrombin}}$ .

For our study, two additional assays were performed on every patient. Into one cup, 10  $\mu\text{L}$  eptifibatide (750  $\mu\text{g}/\text{mL}$ ), 10  $\mu\text{L}$  ActivatorF, and 350  $\mu\text{L}$  heparinized blood were added and the MA determined ( $MA_{\text{Eptifibatide}}$ ). Into a second cup, 10  $\mu\text{L}$  argatroban (1,000  $\mu\text{g}/\text{mL}$ ), 10  $\mu\text{L}$  ActivatorF, and 350  $\mu\text{L}$  heparinized blood were added and MA determined ( $MA_{\text{Argatroban}}$ ). Using the  $MA_{\text{Eptifibatide}}$  and  $MA_{\text{Argatroban}}$  to replace the  $MA_{\text{Fibrin}}$ , a new percent inhibition was calculated for ADP and AA.

### Flow Cytometry

Activation of platelets releases platelet microvesicles that can be detected using antibodies against the platelet fibrinogen receptor, integrin  $\alpha_{\text{IIb}}\beta_3$  (GPIIb/IIIa). In this study, we used antibodies directed toward the integrin  $\alpha_{\text{IIb}}$  chain (anti-CD41a) to identify microvesicles derived from platelets.<sup>7</sup> When platelets are activated, they expose negatively charged phospholipids on their surface and on the surface of microvesicles the platelets produce. The anionic phospholipids bind calcium and coagulation factors, enhancing the procoagulant activity of the platelet and microvesicle surface. Exposure of anionic phospholipids on the microvesicle surface can be detected using annexin V, which binds phosphatidyl serine. Microvesicles that bind CD41a and annexin V are thought to be of platelet origin and procoagulant in nature.<sup>8</sup>

Annexin V labeled with phycoerythrin (PE) and anti-human CD41a labeled with PE-Cy5 were obtained from BD Biosciences (San Jose, CA). Phe-pro-arg chloromethyl ketone (PPACK) was obtained from Haematologic Technologies (Essex Junction, VT). Silica microsphere size standards were obtained from Polysciences (Warrington, PA). Megamix fluorescent dye-labeled polystyrene microspheres (0.5  $\mu\text{m}$ , 0.9  $\mu\text{m}$ , and 3.0  $\mu\text{m}$ ) were obtained from BioCytex (Marseille, France).

Platelet-poor plasma was prepared by double centrifugation for 20 minutes at 1,500g. Plasma was used fresh without freezing. Platelet-poor plasma was stained by addition of 3.6  $\mu\text{L}$  of anti-CD41a-PE-Cy5, 1.8  $\mu\text{L}$  of annexin-PE, and 9.6  $\mu\text{L}$  of HEPES dilution buffer (10 mmol/L HEPES, 140 mmol/L NaCl, 4.5 mmol/L KCl, 1%

bovine serum albumin, and 0.1% sodium azide, pH 7.4) to 15  $\mu$ L of the sample, followed by incubation at room temperature for 30 minutes protected from light. Samples were further incubated for 10 minutes at room temperature after a 1:40 dilution in HEPES buffer containing 2.5 mmol/L calcium to promote annexin binding and 15  $\mu$ mol/L PPACK to prevent clotting in the recalcified diluted plasma. All antibodies and buffers were 0.1  $\mu$ m filtered prior to use.

Samples were run on an Apogee A50 (Apogee Flow Systems, Hertfordshire, UK) flow cytometer. Flow cytometer sheath solutions were 0.1  $\mu$ m filtered prior to use. Microvesicle size calibration on the Apogee A50 was performed in a manner similar to that described for the Apogee A40 instrument.<sup>9</sup> On the Apogee A50, 1- $\mu$ m cellular microvesicles forward scattered approximately the same amount of 488-nm light as 1- $\mu$ m silica microspheres. Total microvesicle counts were defined as particles that forward scattered 488 nm of light at intensities above the noise background of the instrument (equivalent to a 0.3- $\mu$ m lipid microvesicle) but less light than a 0.4- $\mu$ m silica microsphere (equivalent to a 1.0- $\mu$ m lipid microvesicle). Microvesicles that stained with CD41 and annexin V were termed *platelet procoagulant microvesicles*. Microvesicles that stained with annexin V but not CD41 were termed *nonplatelet procoagulant microvesicles*. Forward and side 488-nm light scatter intensities from Megamix fluorescent dye-labeled polystyrene microspheres 0.5  $\mu$ m, 0.9  $\mu$ m, and 3.0  $\mu$ m in diameter were also measured for comparison.

## Statistics

Significant differences between paired samples were determined using the paired *t* test. Differences were considered statistically significant if *P* was less than .05. SigmaStat 3.1 software (Systat Software, San Jose, CA) was used for statistical comparisons.

## Results

TEGPM, with and without eptifibatide or argatroban added, was performed on 58 samples. Forty-eight samples were from male patients and 10 samples were from female patients. The mean  $\pm$  SD age of the patients was 62  $\pm$  13 years. Most patients (57/58) were receiving Coumadin, clopidogrel, aspirin, or a combination of these. One patient had no anticoagulant therapy. Forty-six (79%) patients had a left ventricular assist device, five patients underwent aortic valve replacement, four patients had coronary artery disease, one patient was on extracorporeal membrane oxygenation, one patient had an intracranial aneurysm, and one patient underwent mitral valve repair. MA<sub>Fibrin</sub> showed an unstable increasing biphasic pattern in 16 (28%) of 58 samples tested. Of these patients, 15 had a left ventricular assist device while one had coronary artery disease. An example from one patient is shown in Figure 2.

When the platelet aggregation blocker eptifibatide was added, 14 samples showed a 10-mm or greater decrease in the MA<sub>Fibrin</sub> compared with the baseline MA<sub>Fibrin</sub> with no eptifibatide (*P* < .0001) (Table 1 and Figure 3). The addition of eptifibatide to these samples decreased the percent ADP inhibition on average from 38% to 19% (*P* < .004). Individual patients, though, showed larger changes. In one patient, addition of eptifibatide caused percent ADP inhibition to decrease from 100% to 29%. Addition of eptifibatide also decreased the percent AA inhibition on average from 58% to 29% (*P* < .0004). In one patient, eptifibatide addition decreased percent AA inhibition from 97% to 26%. In samples with less evidence of platelet activation (less than 10-mm decrease in the MA<sub>Fibrin</sub> after addition of eptifibatide), the mean  $\pm$  SD MA<sub>Fibrin</sub> still decreased significantly from 14.7  $\pm$  6.6 mm to 11.9  $\pm$  5.7 mm (*P* < .0001). In these samples, the addition of eptifibatide also minimally, yet significantly, decreased the percent ADP inhibition (*P* < .0001) and decreased the percent AA inhibition (*P* < .0001).

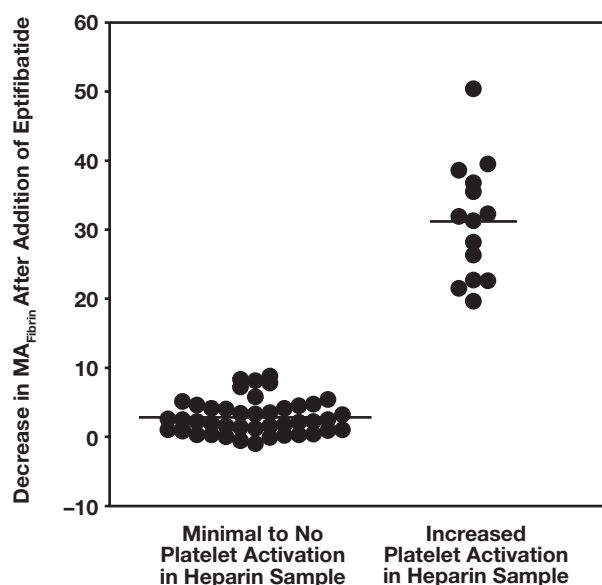
**Table 1**  
Effect of Eptifibatide on MA<sub>Fibrin</sub>, Percent ADP Inhibition, and Percent AA Inhibition

Characteristic	Baseline, Mean $\pm$ SD	Eptifibatide, Mean $\pm$ SD	<i>P</i> Value <sup>a</sup>
$\Delta$ MAFPLT <10 mm (minimal or no platelet activation in heparin sample, n = 44)			
MA <sub>Fibrin</sub> , mm	14.7 $\pm$ 6.6	11.9 $\pm$ 5.7	<.0001
%ADP inhibition	37.6 $\pm$ 25.9	35.6 $\pm$ 24.7	<.0001
%AA inhibition	49.3 $\pm$ 41.4	46.8 $\pm$ 39.7	<.0001
$\Delta$ MAFPLT $\geq$ 10 mm (substantial platelet activation in heparin sample, n = 14)			
MA <sub>Fibrin</sub> , mm	47.6 $\pm$ 6.6	16.3 $\pm$ 6.0	<.0001
%ADP inhibition	38.3 $\pm$ 38.0	19.1 $\pm$ 20.6	<.004
%AA inhibition	58.1 $\pm$ 42.1	28.8 $\pm$ 23.4	<.0004

%AA inhibition, reduction in maximum amplitude response attributed to platelets activated with arachidonic acid =  $1 - (MA_{AA} - MA_{Fibrin}) / (MA_{Thrombin} - MA_{Fibrin})$ ; %ADP inhibition, reduction in maximum amplitude response attributed to platelets activated with adenosine diphosphate =  $1 - (MA_{ADP} - MA_{Fibrin}) / (MA_{Thrombin} - MA_{Fibrin})$ ; MA<sub>Fibrin</sub>, maximum amplitude fibrin;  $\Delta$ MAFPLT, change in maximum amplitude attributed to fibrin, baseline without vs with addition of eptifibatide, which blocks platelet aggregation (measured in heparinized whole blood activated with reptilase and factor XIIIa).

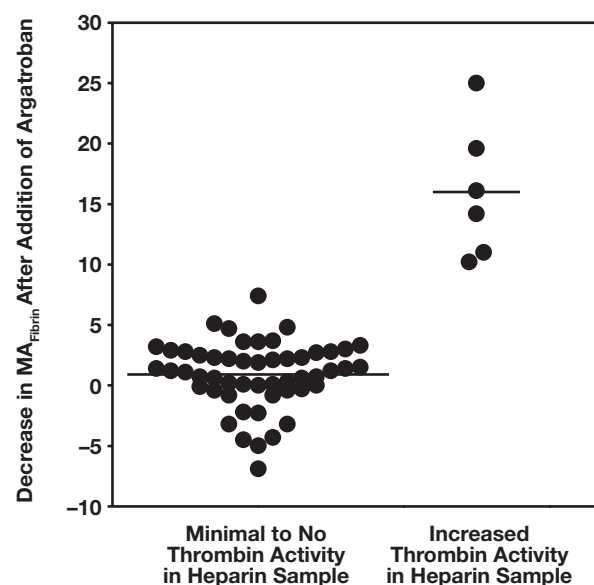
<sup>a</sup> Paired *t* test.





**Figure 3** Decrease in maximum amplitude fibrin ( $MA_{\text{Fibrin}}$ ) after addition of eptifibatide. Figure shows change in maximum amplitude attributed to fibrin, baseline without vs with addition of eptifibatide, which blocks platelet aggregation. Measured in heparinized whole blood activated with reptilase and factor XIIIa. First column shows samples with change less than 10 mm (minimal or no platelet activation) vs samples with change 10 mm or more (increased platelet activation in sample).

When the thrombin inhibitor argatroban was added, six samples had a decrease in  $MA_{\text{Fibrin}}$  10 mm or more ( $P < .0009$ ) (Table 2 and Figure 4). The addition of argatroban to these samples decreased the percent ADP inhibition on average from 48% to 39% ( $P < .02$ ), while the percent AA inhibition was not significantly different in these samples after argatroban addition. In samples with less evidence of uninhibited thrombin activity (decrease in  $MA_{\text{Fibrin}}$  of less than 10 mm with addition of argatroban), the mean  $MA_{\text{Fibrin}}$



**Figure 4** Decrease in maximum amplitude fibrin ( $MA_{\text{Fibrin}}$ ) after addition of argatroban. Figure shows change in maximum amplitude attributed to fibrin, baseline without vs with addition of argatroban, which inhibits thrombin. Measured in heparinized whole blood activated with reptilase and factor XIIIa. First column shows samples with change less than 10 mm (minimal or no thrombin activity in sample) vs samples with change 10 mm or more (increased thrombin activity in sample).

still decreased significantly, but the change was minimal and the change in percent ADP inhibition and percent AA inhibition was not significant.

### Flow Cytometry

Reduced  $MA_{\text{Fibrin}}$  in heparinized samples after the addition of eptifibatide suggested that unanticipated platelet activation was occurring in this part of the assay, even though it was intended to measure MA due to fibrin formation only.

**Table 2**  
Effect of Argatroban on  $MA_{\text{Fibrin}}$ , Percent ADP Inhibition, and Percent AA Inhibition

Characteristic	Baseline, Mean $\pm$ SD	Argatroban, Mean $\pm$ SD	<i>P</i> Value <sup>a</sup>
$\Delta$ MAFTHR <10 mm (minimal or no thrombin activity in heparin sample, n = 52)			
$MA_{\text{Fibrin}}$ , mm	21.0 $\pm$ 15.8	20.0 $\pm$ 16.5	<.02
%ADP inhibition	36.5 $\pm$ 29.1	36.1 $\pm$ 28.9	NS
%AA inhibition	50.9 $\pm$ 41.6	51.0 $\pm$ 41.6	NS
$\Delta$ MAFTHR $\geq$ 10 mm (substantial thrombin activity in heparin sample, n = 6)			
$MA_{\text{Fibrin}}$ , mm	37.0 $\pm$ 10.2	21.0 $\pm$ 9.2	<.0009
%ADP inhibition	48.3 $\pm$ 27.2	38.9 $\pm$ 31.1	<.02
%AA inhibition	55.7 $\pm$ 43.3	45.9 $\pm$ 38.5	NS

%AA inhibition, reduction in maximum amplitude response attributed to platelets activated with arachidonic acid =  $1 - (MA_{\text{AA}} - MA_{\text{Fibrin}}) / (MA_{\text{Thrombin}} - MA_{\text{Fibrin}})$ ; %ADP inhibition, reduction in maximum amplitude response attributed to platelets activated with adenosine diphosphate =  $1 - (MA_{\text{ADP}} - MA_{\text{Fibrin}}) / (MA_{\text{Thrombin}} - MA_{\text{Fibrin}})$ ;  $MA_{\text{Fibrin}}$ , maximum amplitude fibrin;  $\Delta$ MAFTHR, change in maximum amplitude attributed to fibrin, baseline without vs with addition of argatroban, which blocks thrombin activity (measured in heparinized whole blood activated with reptilase and factor XIIIa); NS, not statistically significant,  $P > .05$ .

<sup>a</sup> Paired *t* test.

To further evaluate the possibility of platelet activation in the heparinized vs citrated samples, we prepared blood smears for evaluation of platelet clumping and measured platelet and nonplatelet procoagulant microvesicle levels in subsequent pairs of heparinized and citrated samples. Heparinized whole-blood samples showed moderate to severe platelet clumping in 75% of blood smears reviewed, while paired citrate anticoagulated samples on the same patients showed little or no clumping (11 of 12 samples), indicating platelet activation in the heparinized samples. On average, platelet procoagulant microvesicle levels were more than twice as high in heparinized samples compared with citrate anticoagulated samples (Table 3), while nonplatelet procoagulant microvesicle levels were similar in the two sample types.

### MA<sub>Thrombin</sub> vs MA<sub>ADP</sub> and MA<sub>AA</sub>

To calculate the estimated percent inhibition of ADP and AA activity, the TEGPM assay compares MA<sub>ADP</sub> and MA<sub>AA</sub> with MA<sub>Thrombin</sub> after subtracting MA<sub>Fibrin</sub>. This assumes that in a patient not receiving any platelet inhibitor, MA<sub>ADP</sub> and MA<sub>AA</sub> are equal to MA<sub>Thrombin</sub>. To determine whether this assumption was valid in our patients, we compared MA<sub>ADP</sub> and MA<sub>AA</sub> with MA<sub>Thrombin</sub> in 19 patients who were not taking any antiplatelet agents (Table 4). All 19 of these patients were on left ventricular assist devices. MA<sub>ADP</sub> was less than MA<sub>Thrombin</sub> in 100% of patients not taking antiplatelet agents. On average, MA<sub>ADP</sub> was 18 mm or 28% lower than MA<sub>Thrombin</sub>. Percent ADP inhibition in patients not receiving any antiplatelet agents was on average 45% (range, 8%-100%), even though all should have been 0%. MA<sub>AA</sub> was less than MA<sub>Thrombin</sub> in 84% of patients not taking antiplatelet agents. On average, MA<sub>AA</sub> was 10 mm or 15% lower than MA<sub>Thrombin</sub>. Percent AA inhibition in patients not receiving antiplatelet agents was on average 28% (range, 0%-100%), even though all should have been 0%.

## Discussion

The TEGPM assay compares maximum platelet activation produced by thrombin in citrated blood with ADP and AA

platelet activation in heparinized samples to prevent thrombin generation. The MA<sub>Fibrin</sub> is subtracted from MA<sub>Thrombin</sub>, MA<sub>ADP</sub>, and MA<sub>AA</sub> to give the platelet-specific change in amplitude. Potential problems in the assay exist if the MA<sub>Fibrin</sub> result is not due to fibrin activation alone or if baseline MA<sub>ADP</sub> and MA<sub>AA</sub> in the absence of inhibitors does not equal MA<sub>Thrombin</sub>. In 28% of patient samples, we observed an unstable increasing biphasic MA<sub>Fibrin</sub> curve, indicating problems with the TEGPM assay (see Figure 2). When the MA<sub>Fibrin</sub> was falsely elevated, the percent ADP inhibition and percent AA inhibition were also falsely increased, potentially leading to inappropriate patient treatment. No studies to date have evaluated the mechanism of increasing biphasic MA<sub>Fibrin</sub>. If MA<sub>Fibrin</sub> is elevated due to unanticipated thrombin generation, thrombin inhibitors, such as argatroban, should lower MA<sub>Fibrin</sub> values. Alternatively, if unanticipated platelet activation is causing the problem, then agents that inhibit platelet aggregation, such as the GPIIb/IIIa receptor antagonist eptifibatide, should correct the MA<sub>Fibrin</sub> values.

Blocking platelet aggregation with the GPIIb/IIIa inhibitor eptifibatide resulted in a greater than 10-mm decrease in MA<sub>Fibrin</sub> in 14 of 16 samples showing an increasing biphasic MA<sub>Fibrin</sub> curve, suggesting that platelet activation and aggregation were occurring in the sample even though no platelet activators had been added. The erroneously high MA<sub>Fibrin</sub> values led to overestimates of percent ADP inhibition and percent AA inhibition on average by 19% for ADP and 29% for AA. In some patients, though, the error was much greater. In one patient sample, percent ADP inhibition was estimated to be 100% originally, but after addition of eptifibatide this decreased to only 29% ADP inhibition, essentially normal. A statistically significant decrease in MA<sub>Fibrin</sub> was seen in all 58 samples with the addition of eptifibatide, suggesting that some degree of platelet activation was occurring in most if not all heparinized samples. This was supported by further studies showing moderate to severe platelet clumping in 75% of heparinized samples vs only 8% of citrated samples and, on average, a twofold increase in procoagulant platelet microvesicles in heparinized samples vs citrate samples.

Addition of argatroban decreased MA<sub>Fibrin</sub> in six of 16 samples showing an increasing biphasic MA<sub>Fibrin</sub> curve. In only two of these samples was the decrease in MA<sub>Fibrin</sub> with argatroban greater than the decrease with eptifibatide,

**Table 3**  
Procoagulant Microvesicle Levels in Plasma From Heparinized and Citrated Samples (n = 19)

Microvesicle <sup>a</sup>	Citrate Sample, Mean ± SD	Heparin Sample, Mean ± SD	P Value <sup>b</sup>
Platelet procoagulant, /μL	91,000 ± 55,000	189,000 ± 98,000	<.0001
Nonplatelet procoagulant, /μL	24,000 ± 11,000	25,000 ± 16,000	NS

NS, not statistically significant,  $P > .05$ .

<sup>a</sup> Platelet procoagulant microvesicles are defined as vesicles less than 1 μm in diameter that stain for CD41 and annexin V binding. Nonplatelet procoagulant microvesicles are vesicles less than 1 μm in diameter that show annexin V binding but do not stain for CD41.

<sup>b</sup> Paired *t* test.

**Table 4**  
**Comparison of MA<sub>Thrombin</sub>, MA<sub>ADP</sub>, and MA<sub>AA</sub> in Patients Not Taking Antiplatelet Medications (n = 19)**

Characteristic	Value, Mean ± SD, mm	P Value <sup>a</sup>
MA <sub>Thrombin</sub>	64.4 ± 64.3	
MA <sub>ADP</sub>	46.4 ± 46.3	<.0001
MA <sub>AA</sub>	54.4 ± 54.2	<.004

MA<sub>AA</sub>, heparinized sample activated with reptilase, factor XIIIa, and arachidonic acid; MA<sub>ADP</sub>, heparinized sample activated with reptilase, factor XIIIa, and adenosine diphosphate; MA<sub>Fibrin</sub>, maximum amplitude fibrin.

<sup>a</sup> Paired *t* test.

suggesting that platelet activation was more important than uninhibited thrombin generation in most heparinized samples. Repeat testing of patients showed that the decrease in MA<sub>Fibrin</sub> with eptifibatide was a reproducible pattern, but that this was not always the case for MA<sub>Fibrin</sub> decreases with argatroban. The two patients who showed a decrease in MA<sub>Fibrin</sub> for argatroban and not eptifibatide did not show this pattern on repeat testing, suggesting that this may have been due to sample or assay variability rather than uninhibited thrombin activity without platelet activation.

This suggests that platelet aggregation has a greater effect on rising MA<sub>Fibrin</sub> in heparinized samples than uninhibited thrombin activity. Unanticipated platelet activation in heparinized samples is an important cause of interference in the TEGPM assay. This was not anticipated in the design of the assay, since the test uses heparinized whole blood that is activated with reptilase and factor XIIIa only.

Heparin causes platelet aggregation, activation, and clinical thrombocytopenia by at least two different mechanisms termed *heparin-induced thrombocytopenia* (HIT) types 1 and 2.<sup>10,11</sup> HIT type 1 is an asymptomatic self-limiting decrease in platelet count due to the direct action of heparin on platelets; it does not involve an immune mechanism. HIT type 2 is the more clinically significant immune-mediated form associated with thrombosis. HIT type 1 is relatively common, occurring in 10% to 25% of patients receiving heparin. It is generally asymptomatic, causing more problems in vitro than in vivo. Unfractionated heparin is well known to cause platelet aggregation in vitro so it is curious why a heparinized sample would have been selected for use in a platelet function assay.<sup>12-14</sup> Unfractionated heparin causes a dose-dependent aggregation of platelets in vitro through a GPIIb/IIIa-related mechanism. Heparin-induced platelet aggregation can be blocked with antibodies to GPIIb/IIIa and is absent in platelets from patients with Glanzmann thrombasthenia (missing GPIIb/IIIa) but occurs in patients with Bernard-Soulier syndrome (missing GPIb). Heparin-induced platelet aggregation has been reported to be more common in burn, peripheral vascular disease, and intensive care patients compared with healthy controls.<sup>13</sup> The strength of the aggregation response to heparin is variable among patients and controls. Our finding of an

unanticipated increased MA<sub>Fibrin</sub> in heparinized samples that could be blocked with the GPIIb/IIIa inhibitor eptifibatide suggests that direct heparin-induced platelet aggregation is occurring in the heparinized samples, leading to erroneous percent ADP inhibition and percent AA inhibition.

Another potential problem with the assay is the assumption that, in the absence of platelet inhibitors such as aspirin and clopidogrel, ADP and AA produce the same intensity of platelet activation and aggregation as thrombin does. This is a basic assumption in the assay. To get 0% inhibition for ADP and AA requires the MA<sub>ADP</sub> and MA<sub>AA</sub> to equal MA<sub>Thrombin</sub>, yet when we compared them in patients not receiving any platelet-inhibiting drugs, we found that MA<sub>ADP</sub> was lower than MA<sub>Thrombin</sub> in all patients tested, and MA<sub>AA</sub> was lower than MA<sub>Thrombin</sub> in 84% of patients. Again, this led to falsely high estimates of percent ADP inhibition and percent AA inhibition in patients not taking inhibitor drugs. Collyer et al<sup>15</sup> reported similar findings in their study. They compared TEGPM in control patients not taking any platelet inhibitors, patients taking aspirin, and patients receiving clopidogrel. Average percent ADP inhibition in controls not receiving any antiplatelet medication was 48%, with some showing more than 80% inhibition. Average percent AA inhibition in controls not taking any antiplatelet medication was 18%, with some showing greater than 70% inhibition. Collyer et al concluded that “the overlap in platelet receptor inhibition between the three groups is likely to limit the clinical usefulness of this test.”<sup>15</sup>

In summary, the design of the TEGPM assay appears to be flawed in several respects. First, it uses a heparinized sample as a baseline for the absence of platelet activation in the MA<sub>Fibrin</sub> measurement, even though heparin is a known platelet-aggregating agent and there is evidence of unanticipated platelet activation occurring in the heparinized sample. This potentially leads to clinically significant overestimation of ADP and AA inhibition. A second flaw in the design of the TEGPM assay is the assumption that ADP and AA produce the same degree of platelet activation and aggregation as thrombin, even though ADP and AA have been shown to be weaker agonists than thrombin in general. This is another cause of overestimation of ADP and AA inhibition in patients who are not taking either drug and potentially overestimation in patients receiving the drug. These design flaws in the assay may in part be responsible for the lower predictive ability of the assay clinically compared with other platelet function methods.<sup>16,17</sup>

*Address reprint requests to Dr Chandler: Methodist Pathology Associates, 6565 Fannin, Suite B-490, Houston, TX 77030; wlchandler@houstonmethodist.org.*

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