

Rapid Evaluation of Platelet Function With T2 Magnetic Resonance

Adam Cuker, MD,^{1,2} Holleh Husseinzadeh, MD,¹ Tatiana Lebedeva, MD, PhD,² Joseph E. Marturano, PhD,⁴ Walter Masefski, PhD,⁴ Thomas J. Lowery, PhD,⁴ Michele P. Lambert, MD,⁵ Charles S. Abrams, MD,^{1,2} John W. Weisel, PhD,³ and Douglas B. Cines, MD^{1,2}

From the Departments of ¹Medicine, ²Pathology and Laboratory Medicine, and ³Cell and Developmental Biology, University of Pennsylvania, Philadelphia; ⁴T2 Biosystems, Lexington, MA; and ⁵Hematology Division, Department of Pediatrics, Children's Hospital of Philadelphia, Philadelphia, PA.

Key Words: Blood clotting; Blood platelets; Clot retraction; Platelet function tests; In vitro diagnostic devices; T2 magnetic resonance

Am J Clin Pathol December 2016;146:681-693

DOI: 10.1093/AJCP/AQW189

ABSTRACT

Objectives: *The clinical diagnosis of qualitative platelet disorders (QPDs) based on light transmission aggregometry (LTA) requires significant blood volume, time, and expertise, all of which can be barriers to utilization in some populations and settings. Our objective was to develop a more rapid assay of platelet function by measuring platelet-mediated clot contraction in small volumes (35 μ L) of whole blood using T2 magnetic resonance (T2MR).*

Methods: *We established normal ranges for platelet-mediated clot contraction using T2MR, used these ranges to study patients with known platelet dysfunction, and then evaluated agreement between T2MR and LTA with arachidonic acid, adenosine diphosphate, epinephrine, and thrombin receptor activator peptide.*

Results: *Blood from 21 healthy donors was studied. T2MR showed 100% agreement with LTA with each of the four agonists and their cognate inhibitors tested. T2MR successfully detected abnormalities in each of seven patients with known QPDs, with the exception of one patient with a novel mutation leading to Hermansky-Pudlak syndrome. T2MR appeared to detect platelet function at similar or lower platelet counts than LTA.*

Conclusions: *T2MR may provide a clinically useful approach to diagnose QPDs using small volumes of whole blood, while also providing new insight into platelet biology not evident using plasma-based platelet aggregation tests.*

Qualitative platelet disorders (QPDs) comprise a heterogeneous group of conditions characterized by diverse defects in platelet function and a variable tendency to bleed. Light transmission aggregometry (LTA) has remained the gold-standard method for diagnosing QPDs since its initial description by Born¹ in 1962 but is associated with practical and analytical shortcomings.

LTA is labor intensive, requires technical expertise, and is only available in specialized reference laboratories. Methods are not well standardized despite publication of international consensus recommendations.² A relatively large volume of whole blood (20-25 mL) is typically required, which may be problematic for small children. LTA is less reliable in samples with platelet counts less than $100 \times 10^9/L$ and cannot assess the contribution of other elements to hemostasis, such as the influence of erythrocytes and leukocytes on clot development, integrity, or contraction.³ Because LTA does not involve activation of coagulation, it assesses the relationship between platelets and fibrinogen rather than the more physiologically relevant relationship between platelets and fibrin.⁴ To overcome these limitations, novel modalities have emerged in recent years, such as whole-blood aggregometry, multiplate impedance aggregometry, turbidimetric aggregometry (VerifyNow, Accriva Diagnostics, San Diego, CA), and thromboelastography.⁵ However, these modalities have not shown close agreement ($r < 0.5$) with LTA in clinical settings.⁶

We previously described a novel platform to measure hemostasis in whole blood using T2 magnetic resonance (T2MR).⁷ This method requires a small blood volume

(35 μ L), is technically simple because it does not require separation of platelets, and yields results in minutes. The objective of the current study was to develop a method for assessing platelet function using the T2MR platform. Herein, we derive reference ranges for a panel of platelet agonists in healthy volunteers using T2MR, assess agreement between T2MR and LTA, describe the relative sensitivity of the two techniques in simulated thrombocytopenic samples, measure recovery of platelet function after aspirin ingestion, assess the effects of noncognate inhibitors, and evaluate platelet function in patients with known QPDs.

Materials and Methods

Participants

We describe the results of three studies in this article. In studies of healthy volunteers, adults (aged ≥ 18 years) were recruited. Participants were excluded if they were pregnant, had a history of abnormal clotting or bleeding, or had taken medication known to affect platelet function within 7 days. In the aspirin study, one adult meeting the same eligibility criteria ingested a 325-mg dose of aspirin. In the study of patients, participants with known QPDs were enrolled. Demographic and clinical information was collected, and the severity of bleeding was analyzed using the International Society on Thrombosis and Haemostasis Bleeding Assessment Tool.⁸ The protocols were approved by the Institutional Review Board of the University of Pennsylvania. All participants provided written informed consent.

Sample Collection

Upon enrollment, 30 mL of whole blood was collected into 3.2% sodium citrate evacuated tubes with an applied tourniquet and a 21-gauge needle (final citrate concentration, 0.32%). The first 3 mL was discarded. The remainder of the blood sample was separated for analysis of platelet function by T2MR and LTA, and measurements were completed within 4 hours after draw.

Measurement of Platelet Function by T2MR

Platelet-mediated clot contraction was measured with a small portable T2MR device (T2Plex prototype; T2 Biosystems, Lexington, MA) consisting of a 0.5 T permanent magnet and detection coil heated to 37°C.⁷ The coagulation reaction cuvette in this device was a custom disposable unit with a 0.2-mL volume. To run the assay, first, a 3- μ L total volume was added to the cuvette consisting of batroxobin and CaCl₂ to initiate fibrin formation, FXIIIa to crosslink fibrin molecules, and heparin (20 U/mL final) to block endogenous thrombin generation (T2

Table 1
Agonists, Antagonists, and Concentrations Used for Light Transmission Aggregometry and T2 Magnetic Resonance

Agonist or Cognate Antagonist	Final Concentration, μ mol/L
Agonist	
Arachidonic acid	500
Adenosine diphosphate	10
Epinephrine	10
Thrombin receptor activating peptide	10
Cognate antagonist	
Acetylsalicylic acid (aspirin)	600
MeSAMP	100
MRS2279	10
Yohimbine	10
Vorapaxar	5

Biosystems, Lexington, MA). Next, 2 μ L of various agonist solutions (Table 1) was added to the cuvette and then combined with 35 μ L of prewarmed whole blood. T2MR measurements were then initiated in a 40- μ L total volume.⁷ For inhibitor experiments, the cognate antagonist (on-target) was added along with its agonist, while in cross-inhibition experiments, noncognate antagonists (off-target) were added with agonists (Table 1). In these inhibitor and cross-inhibitor experiments, the whole-blood samples were preincubated with a small volume (1% total volume) of antagonist solution for approximately 15 minutes prior to T2MR measurements. In some experiments, we prepared “reconstructed blood” samples as described previously by isolating RBCs and platelets by centrifugation and recombining them in normal donor plasma at a hematocrit of 40% to vary the platelet count within a constant plasma environment and at a constant hematocrit.⁷

T2MR Data Acquisition

T2MR relaxation measurements were acquired using a prototype T2Plex reader and analyzed as described previously.⁷ A total of three readers were used in parallel.

T2MR measurements began with an initial 90° pulse and were followed by a Carr-Purcell-Meiboom-Gill (CPMG) echo train consisting of a series of 180° pulses, resulting in an exponential relaxation curve. At every measured time point, there may be up to three discrete populations of water molecules representing the serum (T₂₁), loose clot (T₂₂), and tight clot (T₂₃) during clot contraction.³ The exponential decay curve was fit using an adaptive algorithm to produce results that can be represented as a monoexponential, biexponential, or triexponential equation (equation 1):

$$V = I_1 e^{-t/T_{21}} + I_2 e^{-t/T_{22}} + I_3 e^{-t/T_{23}} \quad (1)$$

where V is voltage induced in the coil, T_{2i} is the T2 relaxation value of each water population (i = 1 to 3), I_i is the intensity or relative moles of water in each population (i = 1 to 3), and t

is time. A CPMG relaxation curve was obtained every 20 seconds and analyzed to produce T2 and intensity values.

T2MR Data Analysis

A platelet activity metric (PAM) was developed to quantify platelet-induced clot contraction, which results in formation of clot and serum populations of water. The PAM was designed to evaluate the rate of serum formation weighted by the extent of serum formation. Here, the former is represented as the time summation of the difference in T2 values of the serum and clot and the latter as the serum intensity, from time 0 to 20 minutes (Figure 1A). If a tight clot (T2₃) was detected, the T2 of the loose clot (T2₂) and tight clot (T2₃) was combined into a single clot T2 (T2_{clot}) weighted by intensity for the purposes of calculating the PAM (equation 2).

$$T2_{clot} = \frac{I_2}{I_2 + I_3} T2_2 + \frac{I_3}{I_2 + I_3} T2_3 \quad (2)$$

This T2_{clot} value was developed as an empirical approximation and was not designed to derive a magnetic resonance value of the exact mean clot T2. Using T2_{clot}, the PAM for quantifying platelet function was defined as the summation of the difference in T2 value between the serum and clot, multiplied by the intensity of the serum (equation 3).

$$PAM = \sum_{i=0}^m (T2_{serum(n)} - T2_{clot(n)}) \times I_{serum(n)} \quad (3)$$

where i varies from 0 to 20 minutes in multiples of 20 seconds, m is the experiment length (ie, 20 minutes), and T2_{serum} and I_{serum} were given as T2₁ and I₁ in equation 1, respectively.

T2MR Activation Threshold

The T2MR activation thresholds were defined based on CLSI guidance of qualitative tests,⁹ with the overall goal of balancing the sensitivity and specificity of the test. First, PAM data were normalized into relative PAM units, such that 100% relative PAM represented the maximum healthy donor PAM value and 0% represented zero PAM. Next, PAM distributions were tested for normality using an Anderson-Darling normality test. If there was insufficient evidence for non-normality ($P > .05$), the activation thresholds were defined using the lower and upper 5% normal distribution of the activated and inhibited samples, respectively (equation 4).

$$\text{Threshold}_{normal} = ((\bar{x}_{active} - 1.645 \times SD_{active}) + (\bar{x}_{inhibited} + 1.645 \times SD_{inhibited}))/2 \quad (4)$$

where \bar{x}_{active} and $\bar{x}_{inhibited}$ are the means of the relative PAMs from the active and inhibited samples, respectively, and SD_{active} and SD_{inhibited} are the standard deviations of the

relative PAMs from the active and inhibited samples, respectively. This equation was designed to balance the sensitivity and specificity of the test. If the PAM distributions showed evidence of nonnormality ($P < .05$), then a nonnormal threshold equation was employed to define the lower and upper 5% distribution by rank of the activated and inhibited samples, respectively (equations 5 to 7).

$$\text{ActivatedRank} = 0.05 \times N + 0.5 \quad (5)$$

$$\text{InhibitedRank} = 0.95 \times N + 0.5 \quad (6)$$

$$\text{Threshold}_{nonnormal} = (\text{ActivatedRank} + \text{InhibitedRank})/2 \quad (7)$$

where ActivatedRank and InhibitedRank are the ranked PAM values from the activated and inhibited samples, respectively (lowest value assigned rank of “1”), and N is the number of samples. Again, this approach was designed to balance the sensitivity and specificity of the test.

Measurement of Platelet Function by LTA

Platelet-rich plasma (PRP) was prepared for LTA by centrifuging whole blood at 200g for 7 minutes. Platelet-poor plasma (PPP) was used as a blank control and prepared by centrifuging the whole-blood fraction left after removal of the PRP at 2,000g for 10 minutes. LTA was measured with an optical aggregometer (Model 700; Chrono-Log, Havertown, PA) using the same agonists and same concentrations that were used with T2MR (Table 1). When antagonists were used, whole blood was incubated with the inhibitor for approximately 15 minutes prior to PRP preparation.

For studies in healthy volunteers and in the aspirin fall-off study, the platelet count in PRP was not adjusted. For studies of samples from patients with known QPDs, a sample from a healthy control was run in parallel. If the platelet count in the patient’s PRP was below $250 \times 10^9/L$, the platelet count in the control was adjusted to match that in the patient sample; otherwise, it was not adjusted.

The LTA activation threshold was set to 60% or more transparency relative to the blank control after 6 minutes of runtime, per the established instrument threshold based on prior determination of normal ranges at the Hospital of University of Pennsylvania Special Coagulation Laboratory. A patient sample was classified as “abnormal” only if the corresponding control sample was classified as “normal.”

Agreement Between LTA and T2MR in Patients

To compare the diagnostic agreement between LTA and T2MR, patients with a genetic or acquired QPD were recruited. For LTA, “normal” was defined as a transparency of 60% or more compared with blank PPP after 6 minutes of incubation for all agonists (Table 1). For T2MR, “normal” was defined as a PAM value greater than the PAM

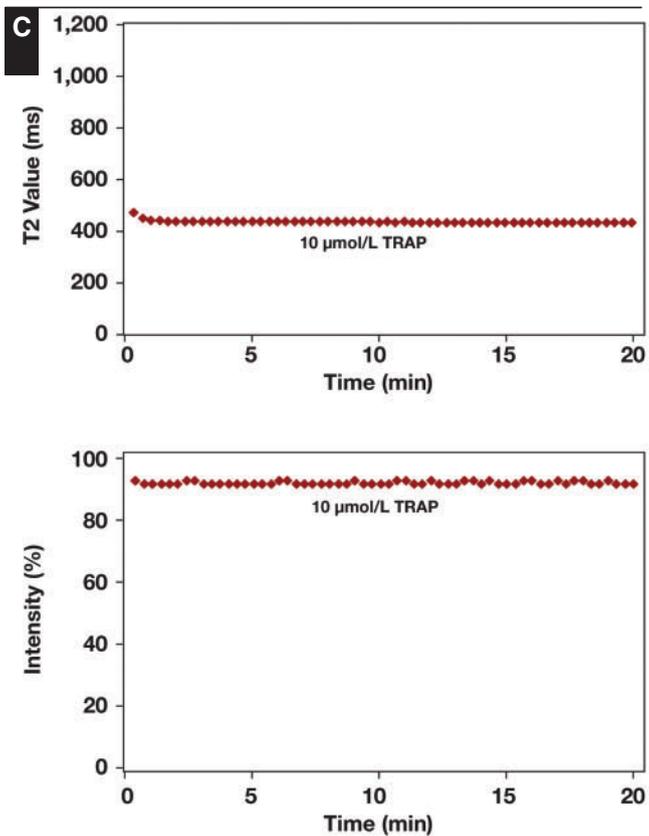
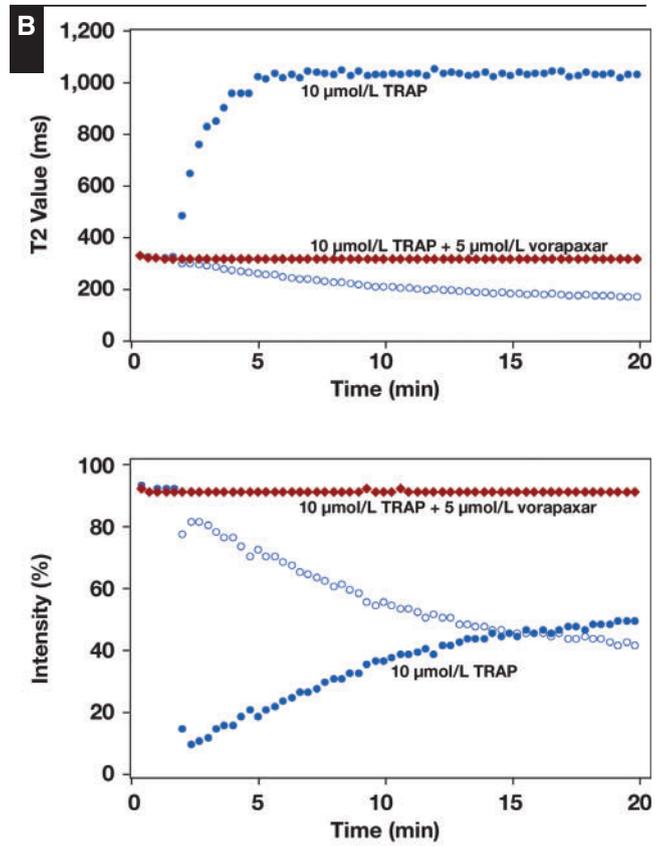
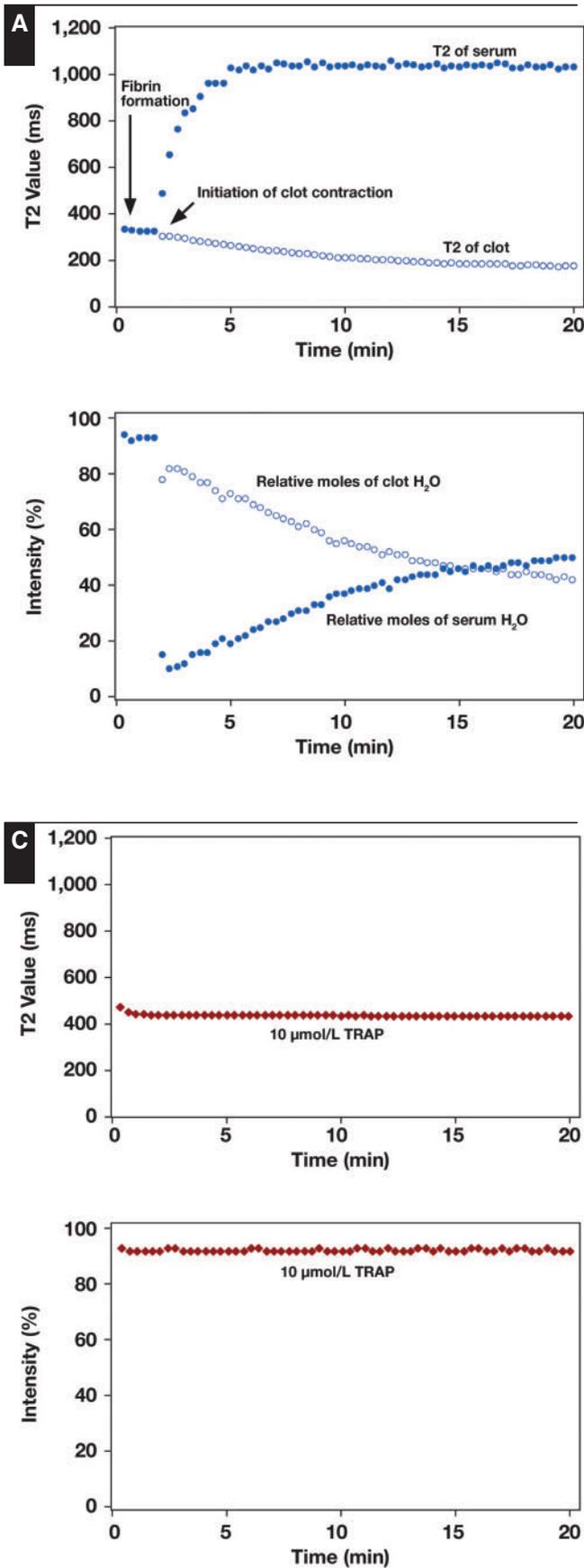


Figure 1 Raw data curves from T2 magnetic resonance (T2MR) platelet function measurements: effect of protease-activated receptor 1 (PAR-1) inhibitor and defect in glycoprotein IIb/IIIa. **A**, Sample from healthy donor showing initiation of clot contraction and changes in T2 value and intensity during clot contraction after addition of 10 μmol/L thrombin receptor activator peptide (TRAP). **B**, Effect of TRAP and inhibitor of PAR-1 activation (vorapaxar) on clot formation and contraction compared with normal donor response. **C**, Absence of contraction in sample from patient with Glanzmann thrombasthenia.

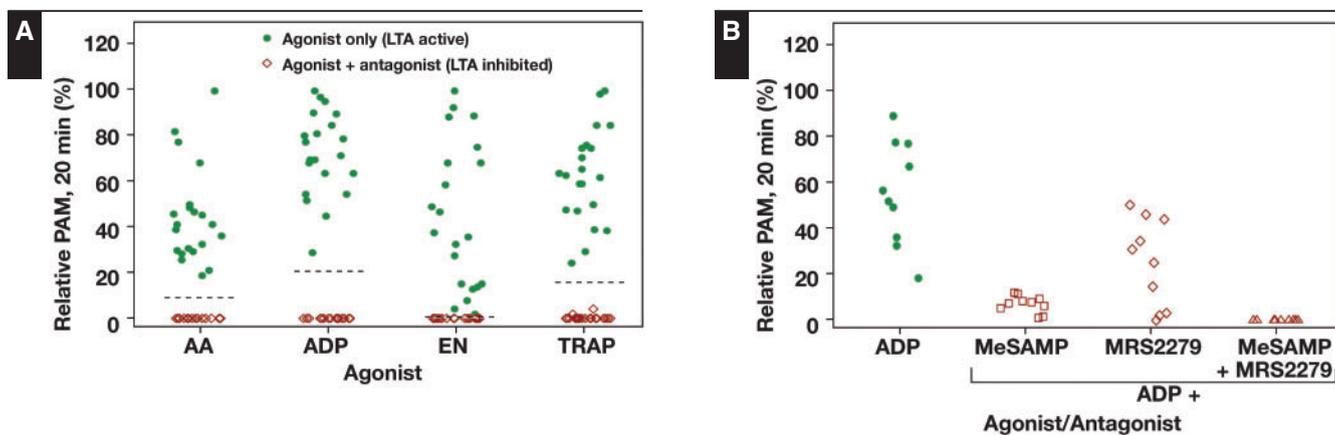


Figure 2 Agreement of T2 magnetic resonance (T2MR) platelet assays with light transmission aggregometry (LTA) and T2MR sensitivity to adenosine diphosphate (ADP) activation pathways. **A**, T2MR-relative platelet activity metric (PAM) for four agonists and corresponding thresholds with healthy normal donors ($n = 21$). T2MR-relative PAM values are color coded to indicate LTA responsiveness, either activated (green) or inhibited (red). The active samples included the agonist alone, and the inhibited samples included the agonist and cognate antagonist. **B**, Dual inhibition of response to ADP using 100 $\mu\text{mol/L}$ MeSAMP, 10 $\mu\text{mol/L}$ MRS2279, or 100 $\mu\text{mol/L}$ MeSAMP and 10 $\mu\text{mol/L}$ MRS2279 ($n = 9$).

activation threshold developed using 21 adult healthy donors (equations 4 to 7). Classifications of “normal” or “abnormal” were then compared for each agonist and donor.

Statistical Analysis and Testing

All statistical tests were performed using Minitab version 17 (Minitab, State College, PA). Statistical significance was defined as $P < .05$.

Results

Measurement of Platelet-Mediated Clot Contraction With T2MR

T2MR provides dynamic measurements of platelet-mediated clot contraction in whole blood. Prior to fibrin formation, T2MR detects a single population of water molecules represented by a single T2 curve.⁷ However, when fibrin forms by the addition of batroxobin in the presence of heparin to block endogenous thrombin, platelets activated by specific agonists contract the fibrin network and two T2MR curves are generated, representing the clot and serum phases. This effect is illustrated by addition of thrombin receptor activator peptide (TRAP) to whole blood as an agonist in the presence of batroxobin, FXIIIa, and heparin (Figure 1A). The distinction in T2 values between these phases is attributable to differences in erythrocyte and protein concentrations.⁷ In addition to T2 values, T2MR also measures the intensity or relative moles of water in each phase. During platelet contraction, the intensity or moles of water in the serum phase

increases with a corresponding decrease in intensity of the clot phase, as expected (Figure 1A).

As a demonstration of the differences in raw data between platelet activation and inhibition, platelets were activated using TRAP, a protease-activated receptor 1 (PAR-1) receptor agonist. T2MR demonstrated clot contraction after addition of TRAP; however, when TRAP was added along with vorapaxar (a PAR-1 inhibitor), no contraction was detected over 20 minutes, as indicated by horizontal curves for T2 value and intensity (Figure 1B). Similarly, no T2MR response was detected in blood from a patient with Glanzmann thrombasthenia after addition of TRAP, as expected, due to a defect in the glycoprotein $\alpha_{\text{IIb}}\beta_3$ complex that is necessary for clot contraction¹⁰ (Figure 1C).

Defining Thresholds for Platelet Activation and Inhibition With T2MR

Samples from 21 healthy volunteers were analyzed using T2MR after activation with each of four platelet agonists, in the absence or presence of their cognate inhibitors (Table 1). The difference in mean PAM between activated and inhibited samples was significant for all agonists ($P < .001$; paired t test) (Figure 2A). In addition, there was no overlap in measured PAM values between samples with agonist alone and samples with both agonist and antagonist added. Activation with epinephrine produced the broadest distribution in the PAM (Figure 2A), likely due to the low number and high variation in platelet α -adrenergic receptor density.^{11,12} On a

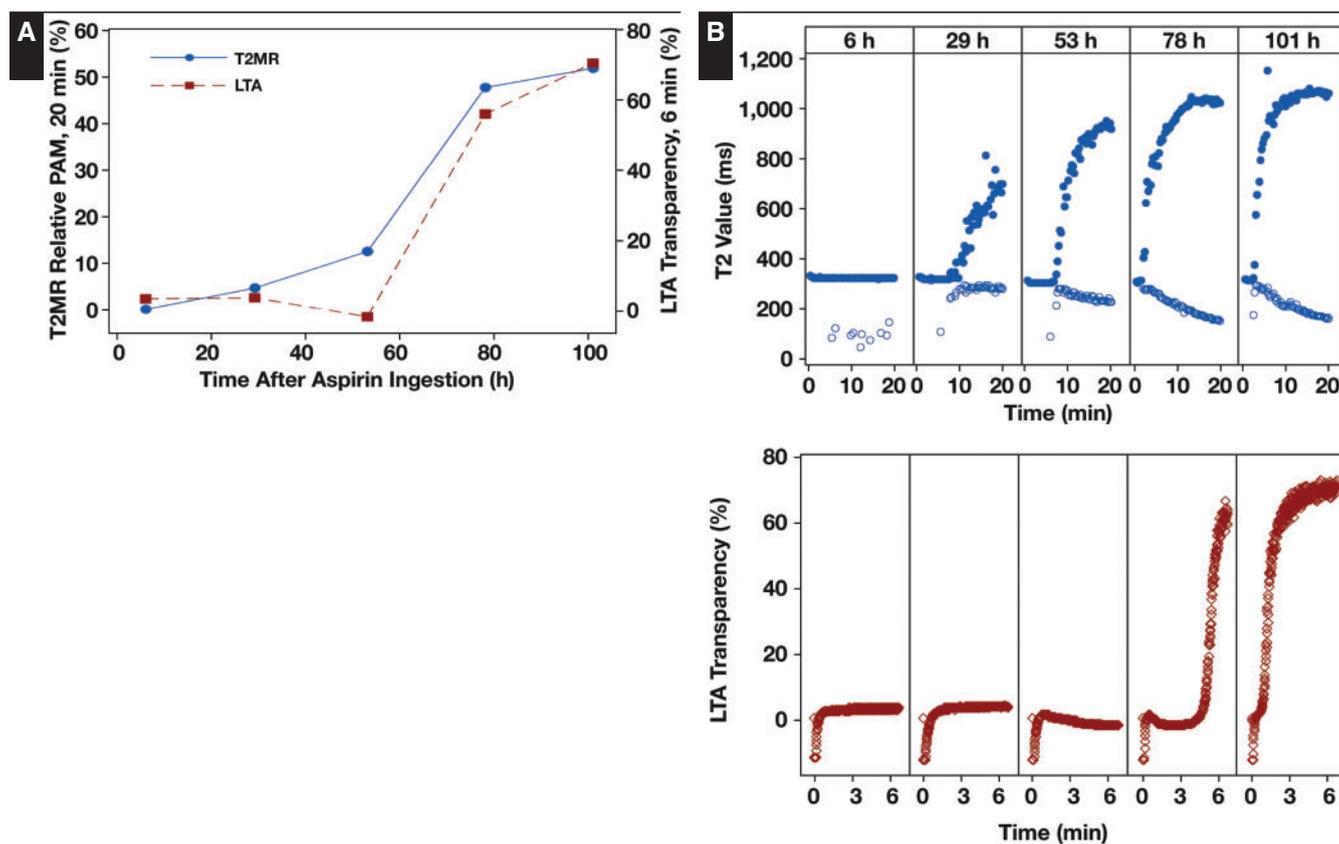


Figure 3 Recovery of platelet function in vivo in response to aspirin ingestion using arachidonic acid activation on T2 magnetic resonance (T2MR) and light transmission aggregometry (LTA). A donor ingested 325 mg of aspirin at time zero and returned for periodic blood draws over 5 days. **A**, Comparison of platelet function measured by each platform, where T2MR detects the return of weak platelet function earlier than LTA. **B**, Raw data curves demonstrating T2MR detects partial platelet function recovery at 29 hours compared with 78 hours using LTA. LTA curves begin with negative transparency due to addition of oil-based emulsion of arachidonic acid to platelet-rich plasma. PAM, platelet activity metric.

relative PAM scale, where 100% represents the maximum PAM value measured across 21 healthy volunteers, the relative PAM thresholds were as follows: arachidonic acid (AA), 9.6%; adenosine diphosphate (ADP), 21.0%; epinephrine, 1.0%; and TRAP, 15.8%. These values were generated by the threshold equations described in the Materials and Methods.

The specificity of T2MR PAM measurements to platelet activation through ADP receptors was evaluated using inhibitors of the P2Y1 (MRS2279) and P2Y12 (MeSAMP) receptors. MeSAMP alone blocked ADP-mediated platelet aggregation as measured by LTA (data not shown). However, when either MeSAMP or MRS2279 alone was added to whole blood, only partial inhibition of ADP-mediated platelet function was detected by T2MR. In contrast, simultaneous addition of both inhibitors abolished platelet function on T2MR, indicating that both P2Y1 and P2Y12 receptor pathways contribute to the T2MR PAM signal when platelets are activated by ADP in whole blood

Figure 2B.

Agreement Between T2MR and LTA in Samples From Healthy Volunteers

To assess agreement between T2MR and LTA, platelet aggregation and T2MR were measured in parallel using samples from 21 healthy volunteers. Samples that were classified on LTA as activated are shown in green and those that were classified as inhibited are shown in red (Figure 2A). Both positive percent agreement and negative percent agreement were 100% between T2MR and LTA for each of the four platelet agonists tested, as shown by the complete separation of the green (LTA active) and red (LTA inhibited) data points (Figure 2A).

Aspirin Fall-Off Study

To determine the feasibility of T2MR to evaluate platelet activity and inhibition *ex vivo*, recovery of platelet function after a 325-mg dose of aspirin was monitored with T2MR and LTA over 5 days in samples from one healthy donor. Using AA as the activator, T2MR showed partial

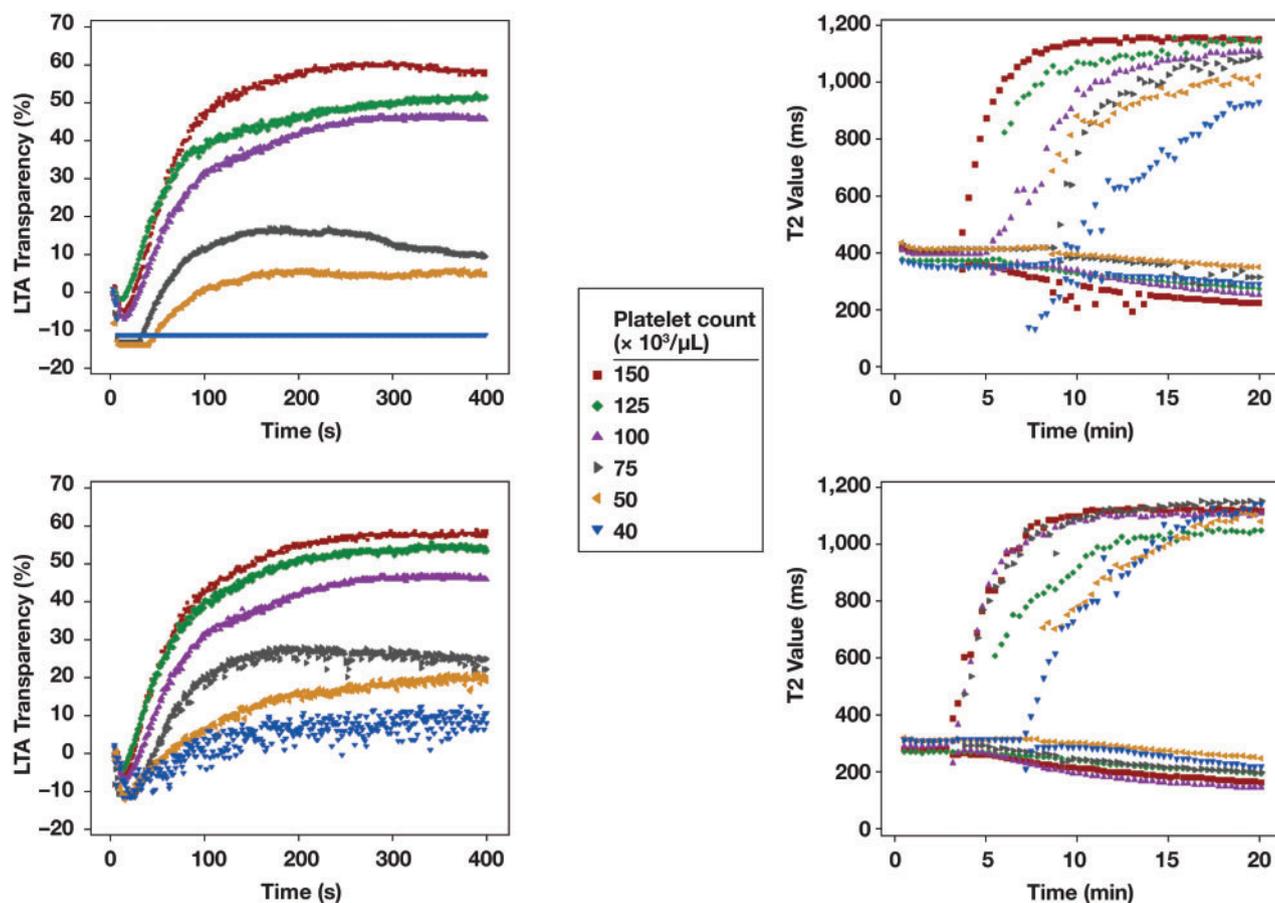


Figure 4 Responses to low platelet counts on light transmission aggregometry (LTA, left) and T2 magnetic resonance (T2MR, right) using 10 $\mu\text{mol/L}$ adenosine diphosphate activation with two donor samples. LTA was measured in platelet-rich plasma diluted with autologous plasma and T2MR in reconstructed whole blood.

recovery of platelet function at 29 hours after aspirin ingestion, whereas recovery with LTA was first evident at 78 hours **Figure 3**. This result suggests that T2MR may be more sensitive to recovery of platelet function after ingestion of aspirin and perhaps may be able to detect a smaller number of functional platelets as nonaspirinated platelets enter the circulation.

Measurement of Platelet Function in Simulated Thrombocytopenic Samples

To evaluate the effect of platelet count on T2MR and LTA, “reconstructed” blood⁷ and PRP from two healthy volunteers were adjusted to platelet counts from 40 to 150 $\times 10^9/\text{L}$ and activated with 10 $\mu\text{mol/L}$ ADP. Whereas LTA showed abnormal attenuated responses at platelet counts below 100 $\times 10^9/\text{L}$, platelet function was reliably detected at platelet counts as low as 40 $\times 10^9/\text{L}$ using T2MR **Figure 4**.

Specificity and Cross-Inhibition of Platelet Antagonists Using T2MR

To investigate the specificity of platelet antagonists and their cross-inhibitory effects, a “percent inhibited” metric was defined where 100% indicates complete inhibition and 0% indicates the inhibitor produced a PAM value equal to or greater than the agonist alone. Therefore, the inhibitor response is normalized to the agonist-alone control for each donor sample. As expected, 100% inhibition was found with each cognate agonist-antagonist pair across four to 19 healthy donors depending on the agonist **Figure 5**. However, partial cross-inhibition was also detected for all agonist-antagonist pairs. For example, AA-mediated platelet activation was partially inhibited in most donor samples by MeSAMP, yohimbine, and vorapaxar **Figure 5A**. Within this donor population, T2MR again detected partial inhibition of the ADP signal with MeSAMP alone but complete inhibition with MeSAMP and MRS2779 combined **Figure 5B**. In addition, the PAR-1 inhibitor vorapaxar

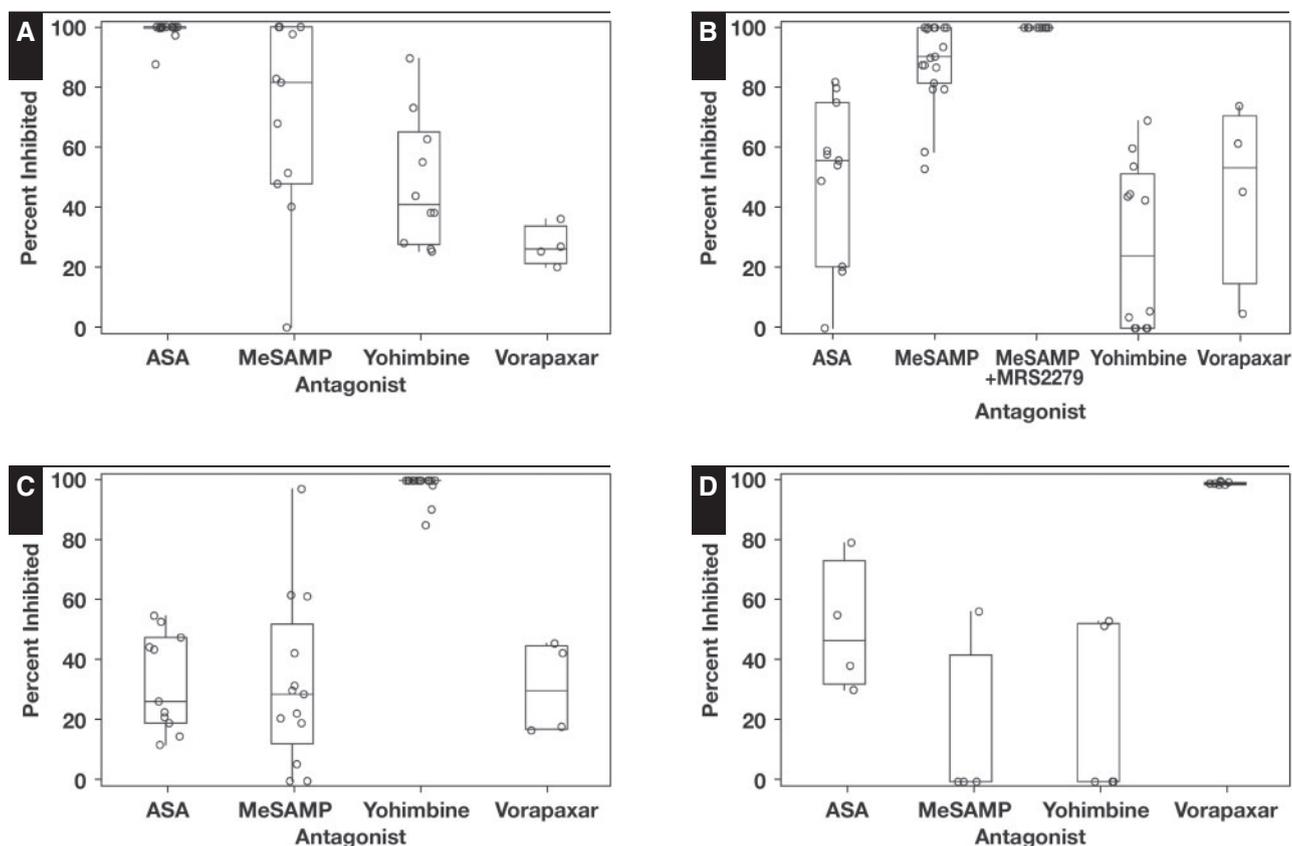


Figure 5 Cross-inhibition of multiple antagonists on T2 magnetic resonance after activation with (A) arachidonic acid, (B) adenosine diphosphate, (C) epinephrine, or (D) thrombin receptor activator peptide. “Percent inhibited” is the normalized platelet activity metric (PAM) value on a linear scale compared with activation with the agonist only for each donor (control), where 100% indicates complete inhibition and 0% indicates the PAM value from antagonist is equal to or greater than agonist alone. Data points represent individual normalized PAM values, with boxes representing the median and interquartile range ($n = 4-19$). ASA, acetylsalicylic acid.

produced partial cross-inhibition with AA, ADP, and epinephrine as agonists, consistent with studies demonstrating crosstalk between the PAR-1 and P2Y₁₂ ADP receptors,^{13,14} as well as PAR-1 and the α 2A-adrenergic pathway.¹⁵ These data suggest that T2MR measurements may help to identify the activity of various components in the intracellular signaling pathways involved in platelet activation in healthy and dysfunctional platelets.

Detection of Contraction by T2MR With Abnormal Aggregation on LTA

We evaluated whether it is possible for T2MR to produce a normal PAM result despite an abnormal LTA response in the cross-inhibition study. We found some cross-inhibited samples where LTA showed a maximum aggregation of 20% to 40% (below the 60% threshold), while T2MR produced a PAM result above the normal threshold defined in this study **Figure 6**. In addition, some

AA and ADP samples showed a primary but not a secondary wave of aggregation but still generated a normal PAM result (Figure 6). However, most samples with an abnormal aggregation response had a concomitant abnormal PAM result in the cross-inhibition study (70% across all agonists).

To investigate these differences further, the performance of platelet antagonists and their cross-inhibitory effects on platelet function was evaluated by comparing the percentage of samples inhibited by noncognate (ie, off-target) inhibitors on T2MR and LTA **Figure 7**. A lower proportion of samples was classified as inhibited by off-target inhibitors on T2MR compared with LTA ($P < .05$ for AA and epinephrine, Fisher exact test).

Agreement of T2MR and LTA in Patients With Known Platelet Function Defects

We measured platelet function using LTA and T2MR in seven patients with a confirmed hereditary or acquired

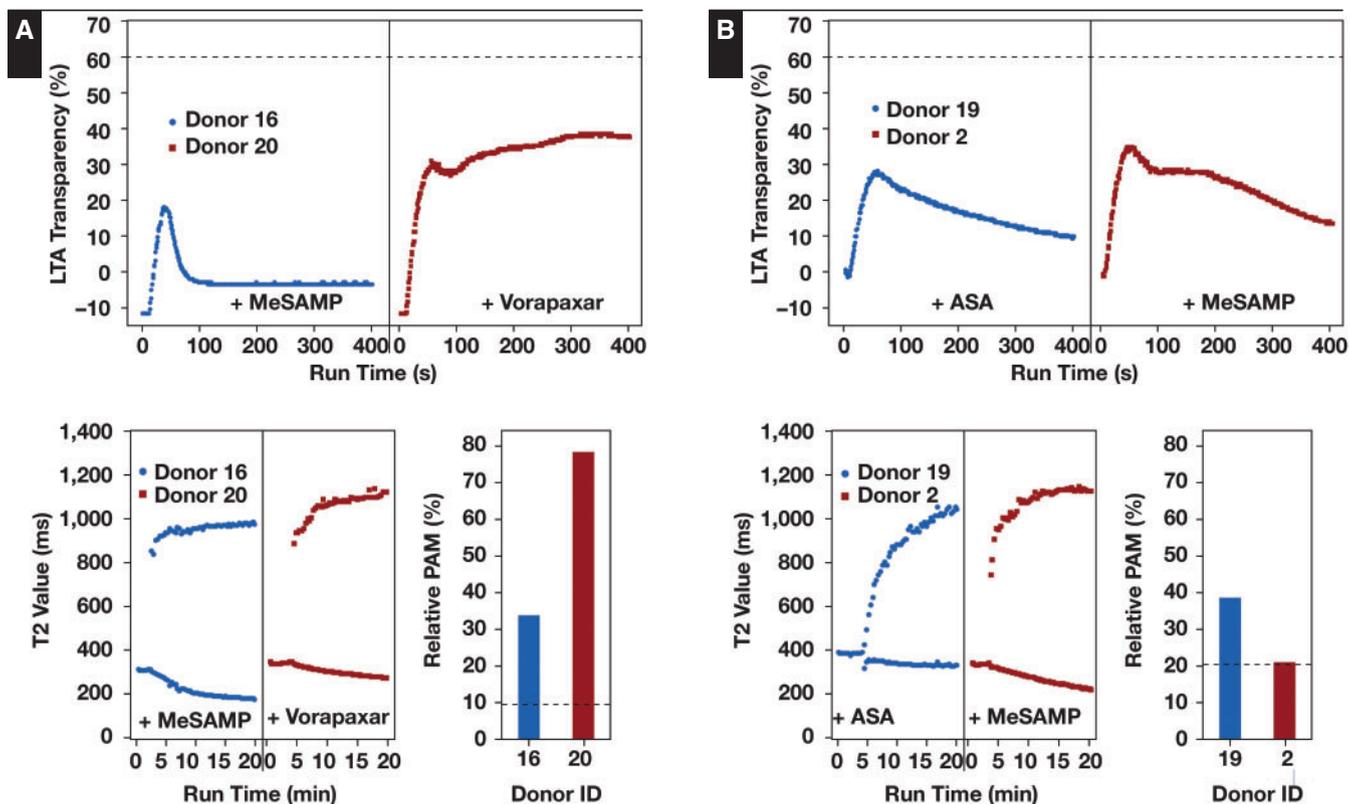


Figure 6 T2 magnetic resonance (T2MR) can produce a contraction signal in the normal range when light transmission aggregometry (LTA) shows only a primary wave of aggregation or weak aggregation. Example LTA vs T2MR comparisons from the cross-inhibition study with (A) arachidonic acid or (B) adenosine diphosphate activation, with the added antagonist shown as “+ antagonist” in the panel. While LTA signal only reached 20% to 40% maximum transparency, well below the 60% threshold for activation, T2MR showed a platelet activity metric (PAM) signal above the normal threshold for each agonist with these donors. The hatched lines represent the normal thresholds. ASA, acetylsalicylic acid.

QPD **Table 2**. Of these, five patients had a hereditary disorder and two patients without known hereditary disorders were taking nonsteroidal anti-inflammatory drugs (NSAIDs) during testing and were therefore classified as having an acquired QPD.

Six of the seven patients with QPDs showed abnormalities by both T2MR and LTA, and there was good agreement at the individual agonist level. For example, the patient with Glanzmann thrombasthenia showed abnormalities with AA, ADP, and epinephrine with T2MR and LTA. However, results in one patient with Hermansky-Pudlak syndrome (HPS) showed abnormal aggregation with all three agonists by LTA but normal clot contraction by T2MR. This patient was subsequently diagnosed with a novel *HPS4* mutation, but the nature of the defect has not been further characterized. These LTA results differed notably from those of the other patient with HPS (type 1), in which we observed normal responses with ADP and AA and abnormal responses with epinephrine by both methods. For the two patients taking

NSAIDs, both modalities detected abnormal responses with AA. However, for ADP and epinephrine, T2MR showed a normal response, while LTA was abnormal. These observations from patients taking NSAIDs are consistent with the results of the cross-inhibition study (Figure 5).

Discussion

We describe a novel method to measure platelet function in whole blood by quantifying the capacity of activated platelets to contract fibrin clots and exude serum.⁷ The changes in the T2MR tracings are generated by the greater freedom of water molecules in the evolving serum compartment relative to whole blood and fibrin. Results are quantified using a PAM, which reflects the onset, duration, and magnitude of serum formation. The force generated by platelets in clot contraction, as reflected by the PAM, is evident from the inhibitory effects of systematically lowering the platelet count in samples, by inhibiting platelet contractility

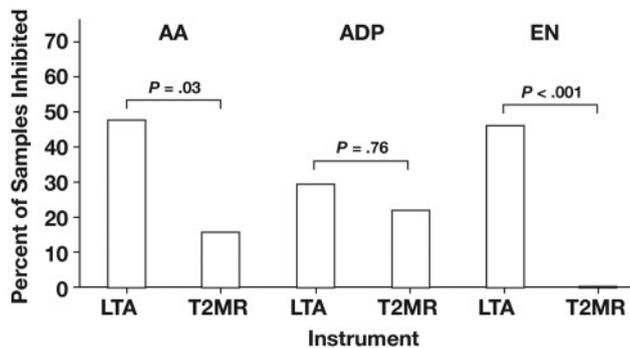


Figure 7 Analytical specificity of light transmission aggregometry and T2 magnetic resonance to noncognate (off-target) inhibitors from the cross-inhibition study: arachidonic acid (AA, $n = 25$), adenosine diphosphate (ADP, $n = 27$), and epinephrine (EN, $n = 28$). With each agonist and instrument pair, samples with noncognate inhibitors were evaluated as “normal” or “abnormal,” and the ratio “% of samples inhibited” was defined as “abnormal,”/ (“normal” + “abnormal,”). These ratios were then evaluated statistically using a two-proportion test with Fisher’s exact method. Noncognate inhibitors included AA (MeSAMP, vorapaxar, yohimbine), ADP (acetylsalicylic acid [ASA], vorapaxar, yohimbine), and EN (ASA, MeSAMP, vorapaxar).

using cytochalasin D,⁷ from the use of specific inhibitors of platelet-activating ligands, and by the absence of clot contraction in patients with Glanzmann thrombasthenia (Figure 1) or a MYH9 cellular myosin gene mutation.¹⁶

We defined analytic thresholds for platelet activation by measuring the difference in PAM values generated by platelet agonists in the absence and presence of their cognate antagonists. Among the 21 healthy donors, there was complete separation between PAM values generated by agonists alone and agonists with antagonists, although there was near overlap with epinephrine, presumably attributable to known variation in α -adrenergic receptor density^{11,12} (Figure 2A). Complete inhibition was found with each cognate agonist-antagonist pair (Figure 2A), affirming the specificity of the assay. Of interest, complete inhibition of ADP-induced platelet activation in whole blood required combined P2Y1 and P2Y12 blockade (Figure 2B), in contrast to the full inhibition in LTA achieved by blocking P2Y12 alone. Additional study is needed to determine whether this reflects the contribution of RBCs to ADP levels and if the extent of residual P2Y1 function identified in whole blood correlates with clinical variability in response to P2Y12 inhibitors.^{17,18} In addition, partial cross-inhibition among agonist-antagonist pairs was also observed, likely because of overlapping intracellular activation pathways¹⁹ (Figure 5).

Reliable platelet activity metrics were generated using T2MR at platelet counts as low as $40 \times 10^9/L$, whereas

platelet counts of $40 \times 10^9/L$ produced zero or near-zero response when assessed by LTA (Figure 4). The sensitivity of T2MR to low platelet counts implies that, although the extent of contraction is greatly reduced,¹⁵ T2MR can still distinguish platelet function from platelet number at low platelet counts. Second, platelet activity after ingestion of aspirin was detected 2 days earlier by T2MR than by LTA (Figure 3). Since this aspirin study was performed only with a single individual, future work will need to test this finding in a broader context. Third, T2MR responses appeared to be less affected by off-target inhibitors, as seen in patients taking NSAIDs and the cross-inhibition studies. For two patients taking NSAIDs, both T2MR and LTA identified abnormal agonist responses to AA, which was expected since the AA signaling pathway is inhibited by NSAIDs. However, platelet aggregation in response to ADP and epinephrine was abnormal as assessed by LTA, while normal clot contraction was detected by T2MR (Table 2). In addition, in the cross-inhibition study, T2MR showed a significantly lower rate of inhibition from off-target inhibitors after activation with AA and epinephrine (Figure 7). Taken together, these findings suggest that T2MR is more sensitive to residual platelet function than LTA. The clinical significance of this finding and determination of which method better reflects in vivo platelet function will require further study.

These results demonstrate that measurement of platelet-mediated clot contraction interrogates multiple aspects of platelet activation that only partially overlap with results generated by LTA. On one hand, platelet aggregation by LTA is enhanced by the dissemination of exogenous and secreted agonists among target platelets, a process that is fostered by continuous stirring of samples that is not a feature of the current T2MR platform where diffusion may be impeded by the contracted clot. Therefore, LTA is especially poised to detect defects in platelet secretion.

T2MR, by contrast, detects platelet-mediated clot contraction, a process that reduces clot volume, prevents blood loss, and helps restore blood flow past obstructive thrombi.²⁰⁻²² Previous studies have demonstrated several differences in platelet interactions with fibrin, present in T2MR, compared with fibrinogen, present in LTA, which may provide a biological basis for our observations. First, the affinity of platelet glycoprotein $\alpha_{IIb}\beta_3$ for fibrin exceeds the affinity for fibrinogen⁴; thus, platelets will be more tightly bound to each other by fibrin compared with fibrinogen. Second, fibrin, but not fibrinogen, promotes fibronectin assembly and expression of phosphatidylserine on the platelet surface,²³⁻²⁵ which amplifies thrombin generation and fosters recruitment of additional platelets to sites of fibrin

Table 2
Characteristics of Patients With Known Congenital or Acquired Platelet Function Defects

Age, y	Sex	ISTH-BAT	Defect	Bleeding Symptoms	Any Agonist ^a		ADP		EN		AA	
					LTA	T2MR	LTA	T2MR	LTA	T2MR	LTA	T2MR
Congenital platelet function disorders												
22	F	9	<i>MYH9</i> mutation, May-Heggelin anomaly	Menorrhagia, extensive bruising	A	A	A	A	N	N	N	N
38	F	17	Glanzmann thrombasthenia	Epistaxis, oral cavity bleeding, extensive bruising, menorrhagia, postpartum hemorrhage, postsurgical bleeding	A	A	A	A	A	A	A	A
54	F	19	Hermansky-Pudlak syndrome, type 1	Easy bruising, epistaxis, major bleeding after minor procedure and tooth extraction, postpartum hemorrhage requiring blood transfusion	A	A	N	N	A	A	N	N
			Familial <i>RUNX1</i> mutation ^b	Unknown	A	A	A	N	A	A	A	A
15	M	9	Hermansky-Pudlak syndrome, type 4 (novel mutation)	Daily gingival bleeding, prolonged epistaxis, GI bleed after endoscopy with biopsy requiring platelets and rVIIa	A	N	A	N	A	N	A	N
Acquired platelet function disorder (medication effect)												
71	M	6	NSAID (ibuprofen)	Postsurgical bleeding after arthroscopic shoulder surgery	A	A	A	N	A	N	A	A
45	F	3	NSAID (naproxen)	Menorrhagia	A	A	A	N	A	N	A	A

A, abnormal response, where LTA transparency or T2MR PAM values are below their thresholds; AA, arachidonic acid; ADP, adenosine diphosphate; EN, epinephrine; GI, gastrointestinal; ISTH-BAT, International Society on Thrombosis and Haemostasis Bleeding Assessment Tool; LTA, light transmission aggregometry; N, normal response, where LTA transparency or T2MR PAM values are above their thresholds; NSAID, nonsteroidal anti-inflammatory drug; T2MR, T2 magnetic resonance.

^aThe “any agonist” column is defined as abnormal for LTA or T2MR if AA, ADP, or EN is abnormal; otherwise, it is normal.

^bDemographic information, bleeding symptoms, and ISTH-BAT score are not available for one patient with familial *RUNX1* mutation.

formation. Third, platelets undergo complete spreading on fibrin but not on fibrinogen, which induces expression of procoagulant phosphatidylserine via glycoprotein VI²⁶ and enhances clot stability. Fourth, platelet interactions with fibrin in whole blood may also reflect the contribution of other blood cells to clot dynamics, including erythrocytes and leukocytes, that may affect hemostasis but are not present in PRP. Taken together, the differences in platelet interactions between fibrinogen and fibrin may explain why fewer platelets appear to be required to generate contraction measurable by T2MR than are needed for aggregation measurable by LTA. These differences in platelet function in whole blood compared with PRP may help classify existing QPDs in novel ways and provide new insights into platelet biology, although more work is needed to characterize platelet contractile responses in whole blood.

Recent data indicate that there is considerable variability in the spatiotemporal extent of platelet activation during clot formation in vivo, with more intense platelet activation, adhesion, and thrombin generation within the inner core of the clot apposed to the damaged vasculature.

In contrast, in the outer shell, there is partial platelet activation, as well as less thrombin generation and fibrin formation.²⁷ LTA might reflect the biology in this outer shell, whereas T2MR, which relies on platelet-mediated clot contraction, may provide more insight into the events that occur within the inner core.

Several limitations of our studies must be acknowledged. We have not characterized responses in whole blood to collagen and ristocetin, which are needed to detect rare disorders such as Bernard-Soulier syndrome and glycoprotein VI deficiency. In addition, we developed PAM thresholds in an adult cohort, and it cannot be assumed that these thresholds will translate directly to pediatric populations.²⁸ Furthermore, the T2MR platform does not incorporate potentially relevant effects of flow, and we did not compare analysis of whole-blood specimens with impedance aggregometry or thromboelastography.²⁹ Finally, the number of samples from patients with bleeding disorders that we studied was small. Larger studies, accompanied by extensive clinical and laboratory evaluation for other bleeding disorders, will be needed to elucidate the clinical utility of T2MR measurements.

Notwithstanding the limitations of our study, assessment of platelet function using T2MR has several practical advantages over LTA. First, samples can be assessed in patients who have low platelet counts, because the need to prepare PRP is obviated and T2MR appears to be normal even at relatively low platelet counts. Second, much smaller blood volumes are required (<0.1 mL vs >20 mL), which is especially valuable in settings where blood is precious or being used for other assays. Third, essentially no sample preparation is needed, which may expand the number of clinical care settings capable of analyzing platelet function. Fourth, the fact that blood centrifugation is unnecessary with T2MR means that turnaround time to result is faster. Fifth, the same T2MR instrument can be used to measure clot times, fibrinogen, and fibrinolysis, all directly from whole blood.⁷ Sixth, the T2MR curves are feature rich, providing opportunities to tailor metrics and assess aspects of platelet biology beyond the applications described here. Seventh, unlike LTA, which measures the interaction of platelets and fibrinogen, T2MR assesses the more physiologically and pathophysiologically relevant relationship between platelets and fibrin.⁴

Overall, our findings in this pilot study suggest that T2MR may eventually enable the identification and characterization of QPDs in native whole blood and that the results may extend to global assessments of hemostasis when including the other reported metrics. Additional basic and clinical studies will be needed to determine whether this new technology indeed provides a more biologically relevant readout of platelet dysfunction in addition to its capacity to quantify weak platelet function.

Corresponding author: Douglas B. Cines, MD, University of Pennsylvania, 422 Curie Blvd, Philadelphia, PA 19104; dcines@mail.med.upenn.edu.

This work was supported by the National Institutes of Health (P01 HL120846, P01 HL40387, U01 HL116330, R56 HL090774, and T32 HL007971). J. E. Marturano, W. Masefski, and T. Lowery are employees of T2 Biosystems, the manufacturer of the T2Plex instrument used in this study. D. B. Cines has received research support from T2 Biosystems.

Acknowledgment: We thank Koneti Rao, MD, Temple University School of Medicine, for providing a patient sample.

References

- Born GV. Aggregation of blood platelets by adenosine diphosphate and its reversal. *Nature*. 1962;194:927-929.
- Cattaneo M, Cerletti C, Harrison P, et al. Recommendations for the standardization of light transmission aggregometry: a consensus of the working party from the platelet physiology subcommittee of SSC/ISTH [published online April 10, 2013]. *J Thromb Haemost*.
- Cines DB, Lebedeva T, Nagaswami C, et al. Clot contraction: compression of erythrocytes into tightly packed polyhedra and redistribution of platelets and fibrin. *Blood*. 2014;123:1596-1603.
- Litvinov RI, Farrell DH, Weisel JW, et al. The platelet integrin α IIb β 3 differentially interacts with fibrin versus fibrinogen. *J Biol Chem*. 2016;291:7858-7867.
- Karon BS, Tolan NV, Koch CD, et al. Precision and reliability of 5 platelet function tests in healthy volunteers and donors on daily antiplatelet agent therapy. *Clin Chem*. 2014;60:1524-1531.
- Lordkipanidze M, Pharand C, Nguyen TA, et al. Comparison of four tests to assess inhibition of platelet function by clopidogrel in stable coronary artery disease patients. *Eur Heart J*. 2008;29:2877-2885.
- Skewis LR, Lebedeva T, Papkov V, et al. T2 magnetic resonance: a diagnostic platform for studying integrated hemostasis in whole blood—proof of concept. *Clin Chem*. 2014;60:1174-1182.
- Rodeghiero F, Tosetto A, Abshire T, et al. ISTH/SSC bleeding assessment tool: a standardized questionnaire and a proposal for a new bleeding score for inherited bleeding disorders. *J Thromb Haemost*. 2010;8:2063-2065.
- CLSI/NCCLS. *User Protocol for Evaluation of Qualitative Test Performance; Approved Guideline*. 2nd ed. Wayne, PA: CLSI; 2008. CLSI/NCCLS document EP12-A2.
- George JN, Caen JP, Nurden AT. Glanzmann's thrombasthenia: the spectrum of clinical disease. *Blood*. 1990;75:1383-1395.
- Gaxiola B, Friedl W, Propping P. Epinephrine-induced platelet aggregation: a twin study. *Clin Genet*. 1984;26:543-548.
- Panzer S, Hocker L, Koren D. Agonists-induced platelet activation varies considerably in healthy male individuals: studies by flow cytometry. *Ann Hematol*. 2006;85:121-125.
- Shakhidzhanov SS, Shaturny VI, Pantelev MA, et al. Modulation and pre-amplification of PAR1 signaling by ADP acting via the P2Y12 receptor during platelet subpopulation formation. *Biochim Biophys Acta*. 2015;1850:2518-2529.
- Kreutz RP, Breall JA, Kreutz Y, et al. Protease activated receptor-1 (PAR-1) mediated platelet aggregation is dependent on clopidogrel response. *Thromb Res*. 2012;130:198-202.
- Grenegard M, Vretenbrant-Oberg K, Nylander M, et al. The ATP-gated P2X1 receptor plays a pivotal role in activation of aspirin-treated platelets by thrombin and epinephrine. *J Biol Chem*. 2008;283:18493-18504.
- Leon C, Eckly A, Hechler B, et al. Megakaryocyte-restricted MYH9 inactivation dramatically affects hemostasis while preserving platelet aggregation and secretion. *Blood*. 2007;110:3183-3191.
- Marcus AJ, Broekman MJ, Drosopoulos JHF, et al. The endothelial cell ecto-ADPase responsible for inhibition of platelet function is CD39. *J Clin Invest*. 1997;99:1351-1360.
- Gayle RB III, Maliszewski CR, Gimpel SD, et al. Inhibition of platelet function by recombinant soluble ecto-ADPase/CD39. *J Clin Invest*. 1998;101:1851-1859.
- Colman RW, Hirsh J, Marder VJ, et al, eds. *Hemostasis and Thrombosis: Basic Principles and Clinical Practice*. 4th ed. Philadelphia, PA: Lippincott Williams & Wilkins; 2001.
- Stalker TJ, Welsh JD, Tomaiuolo M, et al. A systems approach to hemostasis: 3. Thrombus consolidation regulates intrathrombus solute transport and local thrombin activity. *Blood*. 2014;124:1824-1831.

21. Carr ME Jr. Development of platelet contractile force as a research and clinical measure of platelet function. *Cell Biochem Biophys*. 2003;38:55-78.
22. Lam WA, Chaudhuri O, Crow A, et al. Mechanics and contraction dynamics of single platelets and implications for clot stiffening. *Nat Mater*. 2011;10:61-66.
23. Brzoska T, Suzuki Y, Mogami H, et al. Binding of thrombin-activated platelets to a fibrin scaffold through alpha(IIb)beta(3) evokes phosphatidylserine exposure on their cell surface. *PLoS One*. 2013;8:e55466.
24. Mammadova-Bach E, Ollivier V, Loyau S, et al. Platelet glycoprotein VI binds to polymerized fibrin and promotes thrombin generation. *Blood*. 2015;126:683-691.
25. Ono A, Westein E, Hsiao S, et al. Identification of a fibrin-independent platelet contractile mechanism regulating primary hemostasis and thrombus growth. *Blood*. 2008;112:90-99.
26. Alshehri OM, Hughes CE, Montague S, et al. Fibrin activates GPVI in human and mouse platelets. *Blood*. 2015;126:1601-1608.
27. Ivanciu L, Stalker TJ. Spatiotemporal regulation of coagulation and platelet activation during the hemostatic response in vivo. *J Thromb Haemost*. 2015;13:1949-1959.
28. Yip C, Linden MD, Attard C, et al. Platelets from children are hyper-responsive to activation by thrombin receptor activator peptide and adenosine diphosphate compared to platelets from adults. *Br J Haematol*. 2015;168:526-532.
29. Paniccia R, Priora R, Liotta AA, et al. Platelet function tests: a comparative review. *Vasc Health Risk Manage*. 2015;11:133-148.