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PLATELETS AND THROMBOPOIESIS

Characterization of multiple platelet activation pathways in patients with bleeding as a high-throughput screening option: use of 96-well *Optimul* assay

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Key Points

- The *Optimul* 96-well platelet aggregation assay has high levels of sensitivity and specificity for detecting platelet defects.
- The requirement for a small volume of blood, straightforward nature, and speed make *Optimul* a promising screening test in bleeding patients.

Up to 1% of the population have mild bleeding disorders, but these remain poorly characterized, particularly with regard to the roles of platelets. We have compared the usefulness of *Optimul*, a 96-well plate-based assay of 7 distinct pathways of platelet activation to characterize inherited platelet defects in comparison with light transmission aggregometry (LTA). Using *Optimul* and LTA, concentration-response curves were generated for arachidonic acid, ADP, collagen, epinephrine, Thrombin receptor activating-peptide, U46619, and ristocetin in samples from (1) healthy volunteers (n = 50), (2) healthy volunteers treated with antiplatelet agents in vitro (n = 10), and (3) patients with bleeding of unknown origin (n = 65). The assays gave concordant results in 82% of cases ($\kappa = 0.62$, $P < .0001$). Normal platelet function results were particularly predictive (sensitivity, 94%; negative predictive value, 91%), whereas a positive result was not always substantiated by LTA (specificity, 67%; positive predictive value, 77%). The *Optimul* assay was significantly more sensitive at characterizing defects in the thromboxane pathway, which presented with normal responses with LTA. The *Optimul* assay is sensitive to mild platelet defects, could be used as a rapid screening assay in patients presenting with bleeding symptoms, and detects changes in platelet function more readily than LTA. This trial was registered at www.isrctn.org as #ISRCTN 77951167. (*Blood*. 2014;123(8):e11-e22)

Introduction

Mild bleeding disorders are prevalent in the general population with a frequency of up to 1%.[1] Nonetheless, they often remain poorly characterized, both in terms of clinical and laboratory-based diagnosis.[1] This stems in part from the fact that excessive bleeding only manifests in some patients in response to an appropriate challenge such as surgery, trauma, menstruation, and childbirth, and in part because the laboratory measures of platelet dysfunction, including the current gold standard light transmission aggregometry (LTA), require specialized expertise and are time-consuming.[2] The recognition that bleeding is a multifactorial and complex process has fueled the search for platelet function assays that have the ability to assess a whole spectrum of platelet responses, require a small volume of blood, and require minimal technical expertise.[2-4]

Several attempts at developing a high-throughput platelet function assay are currently being investigated. These include devices that measure global platelet reactivity in response to shear alone,[5] microfluidic devices with precoated adhesion or activation molecules,[6-8] assays that measure calcium flux in platelets by fluorescent imaging,[9] enzyme-linked immunosorbent assay–type assays to capture platelets on agonist-coated surfaces,[10] luminometric assays of platelet secretion in response to various platelet agonists,[11] and flow cytometric counting techniques with platelet immunostaining.[12] Most of these techniques require specialized laboratory instruments (flow cytometers, osmotic pumps, microscopes, imaging devices) or can be expensive to perform (single use precoated cartridges or capillaries, fluorescent antibodies), and not all are sensitive to mild platelet inhibition,[10] making them unsuitable for use in nonspecialized centers.

The development of the *Optimul* assay with lyophilized reagents on a standard half-area 96-well microtiter plate may provide a more

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cost-effective alternative for high-throughput platelet function testing.[13] The assay requires a plate heater/shaker and an absorbance plate reader, which are available in most clinical and research laboratories.[13] The *Optimul* assay is carried out on 2.5 mL of platelet-rich plasma (PRP), takes 10 minutes to perform and analyze, and provides full dose-response curves to 7 commonly used platelet agonists.[13,14]

We sought to investigate how the *Optimul* assay compares with the gold standard in platelet function testing, lumi-aggregomery, and whether it could be used to diagnose platelet defects in patients presenting with bleeding symptoms suggestive of inherited platelet function defects.

Material and methods

Participant selection

Healthy volunteers. Participants were considered healthy if they were ≥18 years of age, did not require long-term medical therapy, had refrained from drugs known to influence platelet function in the previous 2 weeks, and did not have a history of bleeding symptoms.

Patients suffering from bleeding diathesis. Participants with suspected inherited platelet function defects were recruited to the Genotyping and Phenotyping of Platelets study (GAPP; ISRCTN 77951167) from November 2011 to December 2012 from UK Comprehensive Care Haemophilia Centers and were invited to participate in this study if they satisfied all the following inclusion criteria: (1) abnormal bleeding symptoms compatible with a platelet function defect (spontaneous mucocutaneous bleeding or abnormal bleeding at other sites following trauma or invasive procedures); (2) results from coagulation factor tests within local laboratory reference intervals (minimum panel of prothrombin time, activated thromboplastin time, Clauss fibrinogen activity, von Willebrand profile); and (3) absence of demonstrable acquired platelet dysfunction. Patients with existing diagnoses of Glanzmann's thrombasthenia, Bernard-Soulier syndrome, Hermansky Pudlak syndrome, or MYH9-related disorder were excluded. Participants with platelet counts outside the 150 to 450 $\times$ 10^9/L range were excluded. Laboratory testing was deferred in participants exposed within 2 weeks to drugs known to affect platelet function.

This study was approved by the National Research Ethics Service Committee West Midlands–Edgbaston (REC reference: 06/MRE07/36), and all participants gave written informed consent. This study was conducted in accordance with the Declaration of Helsinki.

Assessment of platelet function

Blood was drawn by venipuncture into evacuated tubes containing 3.1% trisodium citrate (*S*-Monovette 0.106 M; Sarstedt, Leicester, United Kingdom [UK], in line with the latest expert consensus documents.[15] PRP was prepared by centrifugation of whole blood at 200*g* for 20 minutes. Platelet-poor plasma (PPP) was prepared by centrifugation of the remaining blood at 1000*g* for 10 minutes.

To assess the effect of antiplatelet drugs in vitro, in some experiments, samples were pretreated with 100 μM aspirin (Sigma-Aldrich, Dorset, UK) or 1 μM cangrelor (The Medicines Company, Abingdon, UK) prior to platelet function testing.

LTA. Platelet function was assessed using LTA as described previously.[16] Platelet aggregation was measured in PRP using a dual Chronolog lumiaggregometer (Model 460 VS; Havertown, PA) in response to ADP (Sigma-Aldrich, Poole, UK); epinephrine (Sigma-Aldrich, Poole, UK); arachidonic acid (AA; Cayman Chemical, Cambridge Bioscience Ltd.); PAR-1 receptor-specific peptide (SFLLRN; Alta Biosciences, Birmingham, UK), collagen (Nycomed, Linz, Austria); and ristocetin (Helena Biosciences, Sunderland, UK). ATP secretion was assessed using the Luciferin-Luciferase reagent (Chronolume) and an ATP standard solution. Results were classified as abnormal by reference to a bank of local healthy volunteers (presented in supplemental Figure 1 and supplemental Table 1, available on the *Blood* Web site).

Optimul assay. *Optimul* 96-well plates were prepared as previously described.[13] Briefly, flat-bottom half-area microtiter plates (Greiner Bio-One; Stonehouse, Gloucestershire, UK) were precoated with hydrogenated gelatin (0.75% weight/volume, Sigma-Aldrich, Poole, UK) in phosphate-buffered saline (Sigma-Aldrich, Poole, UK) to block the surface activation of platelets before the addition of platelet agonists. The agonists used were AA (0.03-1 mM; Sigma-Aldrich, Poole, UK), ADP (0.005-40 μM; Sigma-Aldrich, Poole, UK), epinephrine (0.0004-10 μM; Labmedics, Stockport, UK), collagen (0.01-40 μg/mL; Nycomed, Linz, Austria), TRAP-6 amide (SFLLRN; 0.03-40 μM, Bachem, St. Helens, UK), U46619 (0.005-40 μM; Labmedics, Stockport, UK), and ristocetin (0.14-4 mg/mL; Helena Biosciences, Tyne and Wear, UK). The agonists were lyophilized by placing the plates in a −80°C freezer for 1 hour and transferring into a freeze-dryer overnight at −40°C. The plates were then removed from the freeze-dryer, vacuum-sealed, foil-packed, and kept at room temperature until use within 12 weeks of manufacture.

PRP or PPP (40 μL) was added into the appropriate agonist-free control wells of the 96-well plate. PRP was then added to the agonist-coated wells with a multi-channel pipette. The plate was sealed with film and placed on a heater/shaker (BioShake iQ; Q Instruments, Jena, Germany) at 37°C to mix at 1200 rpm for 5 minutes. Absorbance was then measured at 595 nm on a 96-well plate reader (VersaMax Microplate reader; Associates of Cape Cod Inc., East Falmouth, MA). Platelet aggregation was expressed as the maximal percent change in light transmittance from PRP wells in response to agonists, using PPP wells as reference. Optimization and characterization of the *Optimul* assay have been previously reported.[13,14,17]

An *Optimul* panel was defined as abnormal if the dose-response curve was below the lower limit of the 95% reference interval for >1 agonist.

Plasma thromboxane B_2. After aggregation, indomethacin (30 μM) was added in relevant wells to stop cyclooxygenase (COX) activity. The whole plate was then centrifuged (1300*g* for 10 minutes) at room temperature, and the supernatants were collected and frozen at −80°C. Plasma thromboxane ($Tx)B_2$ levels, a measure of TxA_2 formation, were determined by a competitive immunoassay (in-house homogeneous time resolved fluorescence assay, developed with assistance of Cisbio Bioassays, Codolet, France).

Statistical analyses

Normally distributed continuous variables are presented as mean ± standard deviation, non-normally distributed continuous variables as median (interquartile range), and categorical variables as frequencies (percentages). Variables were analyzed for a normal distribution with the Kolmogorov-Smirnov test. Concentration-response curves were generated using a 4-parameter log-linear function in GraphPad Prism Software 5 for Windows (GraphPad Software, San Diego, CA). The area under the curve (AUC) analysis was carried out for each individual curve generated. For comparison of assays where the tests were performed by both assays on the same blood samples, paired analyses were used. Agreement in the classification of platelet defects between the 2 assays was assessed by the κ statistic. Analyses were performed with Statistical Package for the Social Sciences (SPSS) 14.0 for Windows (SPSS Institute).

Results

Optimul performance characteristics in healthy volunteers

Platelet function was assessed in 50 healthy volunteers (aged 33 ± 7 years, 38% men); in 10 of these, platelet function testing was repeated 2 weeks later to assess intraindividual variability. As shown in Figure 1 and supplemental Table 2, with all agonists used in the *Optimul* assay, platelets displayed a dose-response relationship that was similar to that observed with LTA and was reproducible within subjects with repeat testing of blood sample obtained at different venepunctures. There was no association between the extent of platelet aggregation and either platelet count in PRP or mean platelet volume (supplemental Figure 2). We observed greater variability between healthy donor subjects in the

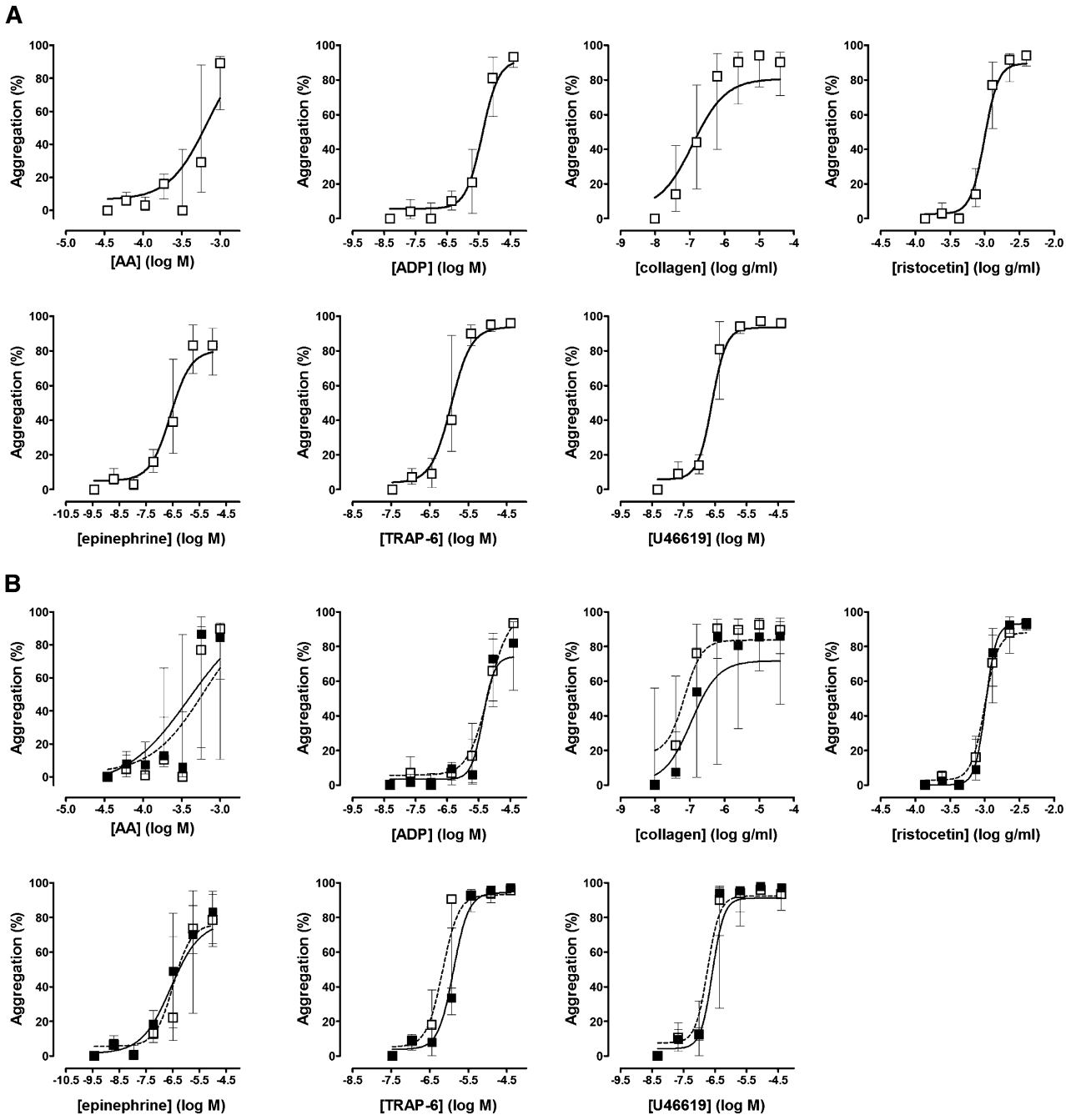

Figure 1. Reference intervals for the *Optimul* assay. (A) Dose-response curves in 50 healthy volunteers, presented as median and interquartile range. (B) Repeat testing in 10 healthy volunteers, presented as median and interquartile range. Open squares represent the first test, and closed squares represent repeat testing in the same individuals.

Optimul test results compared with LTA test results at intermediate concentrations of AA and collagen (Figure 2). Because platelet aggregation responses to both collagen and AA are sensitive to defective metabolism of AA into TxA_2, we investigated this difference further by measuring concentrations of TxB_2 (a marker of TxA_2 synthesis) in a subset of healthy volunteers (n = 25). This demonstrated that most healthy controls who displayed reduced aggregation responses by *Optimul* to AA and collagen also showed reduced TxB_2 production (Figure 3), indicating that this group had impaired AA metabolism, most likely as a result of inadvertent nonsteroidal anti-inflammatory drug ingestion. Our observation that this group displayed normal LTA results with AA and collagen suggests that the *Optimul* offers greater sensitivity to defects in AA metabolism than LTA.

Effect of antiplatelet agents

To assess the performance of the *Optimul* assay in detecting the effect of antiplatelet therapy, PRP samples obtained from 10 healthy volunteers were treated in vitro with 100 μM aspirin to assess COX inhibition or 1 μM cangrelor to assess $P2Y_{12}$ receptor inhibition. As shown in Figure 4, both LTA and *Optimul* detected inhibition of platelet function by in vitro addition of antiplatelet agents, with *Optimul* being significantly more sensitive than LTA and displaying marked

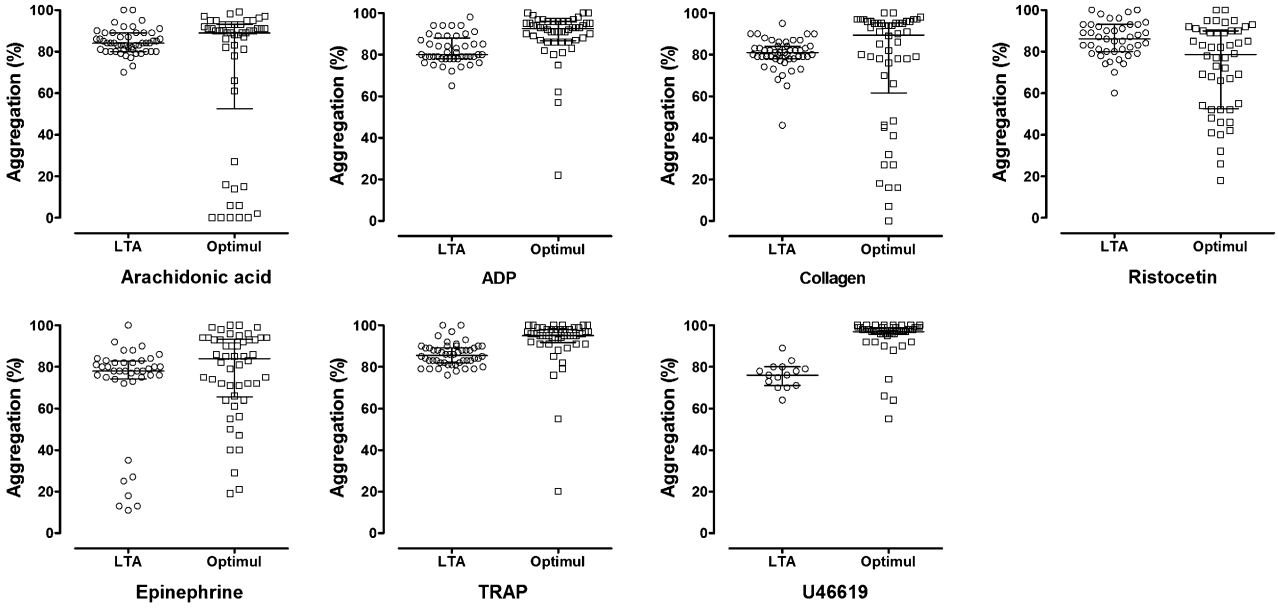

Figure 2. Agreement between LTA and the *Optimul* assay in healthy volunteers. Individual data for top concentrations of the agonists studied with LTA and within a similar range on *Optimul*: AA, 1 mM (LTA) to 1 mM (Optimul); ADP, 30-40 μM; collagen, 3 μg/mL to 2.5 μg/mL; epinephrine, 10 μM to 10 μM; TRAP, 100 μM peptide to 40 μM amide; U46619, 10 μM to 8.9 μM; ristocetin, 1.5 mg/mL to 1.3 mg/mL.

impairment in multiple platelet activation pathways, consistent with the role of TxA_2 and ADP as amplifying signals for other agonists.[18,19] Receiver-operator characteristic (ROC) curve analysis is presented in supplemental Figure 3, showing how the distribution of healthy donor response overlaps with healthy donor response treated with anti-platelet agents. Both lumi-aggregometry and the *Optimul* assay had high levels of discrimination, with *Optimul* achieving higher levels of AUC for multiple agonists (supplemental Figure 3). Most notably, inhibition of the $P2Y_{12}$ receptor resulted in marked inhibition of AA- and collagen-induced platelet responses on *Optimul* that was not seen with LTA, highlighting the reliance of the *Optimul* assay on adequate ADP receptor activity as an amplifying signal leading to sustained platelet aggregation to these 2 agonists (Figure 4). Inhibition of COX by aspirin induced a less severe phenotype of platelet responses, with only AA- and collagen-induced aggregation being significantly affected (Figure 4).

Investigation of patients with a suspected impairment in platelet function

We sought to investigate whether the *Optimul* assay could be used to detect platelet defects in patients with a suspected inherited impairment in platelet function. Patients with suspected inherited platelet function defects (n = 65, aged 38 ± 19 years, 25% male) were recruited to the GAPP study from UK Comprehensive Care Haemophilia Centers. Participants had a lifelong bleeding history as demonstrated by their elevated ISTH Bleeding Assessment Tool score (median, 10; interquartile range, 7-15; 95th percentile in healthy volunteers was 4).[20,21]

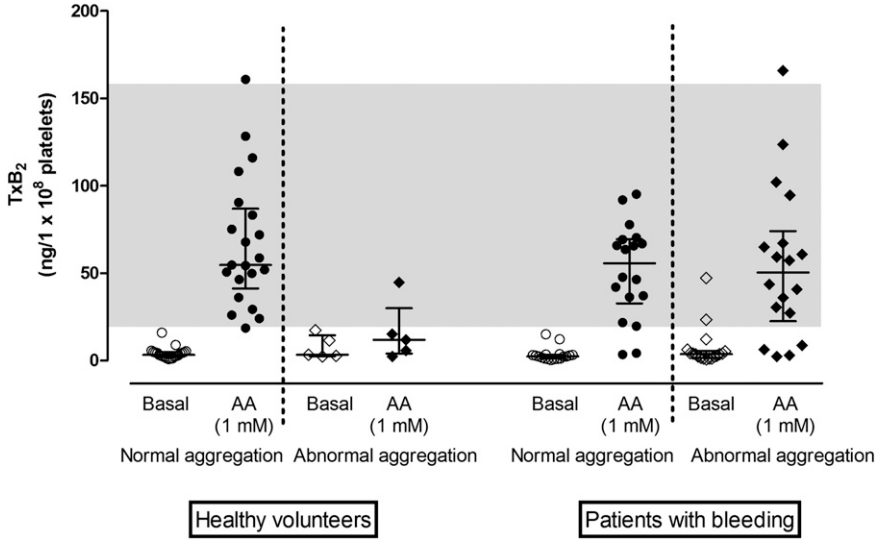

Figure 3. TxB_2 concentration in plasma supernatants from healthy volunteers and patients with bleeding symptoms. TxB_2 concentration adjusted for platelet count in healthy volunteers with normal (n = 20) or impaired (n = 5) AA response on *Optimul* and in patients with bleeding disorders with normal (n = 18) or impaired (n = 17) AA response on *Optimul*. Line and whiskers represent median and interquartile range; the gray band represents the normal range derived from bank of healthy volunteers.

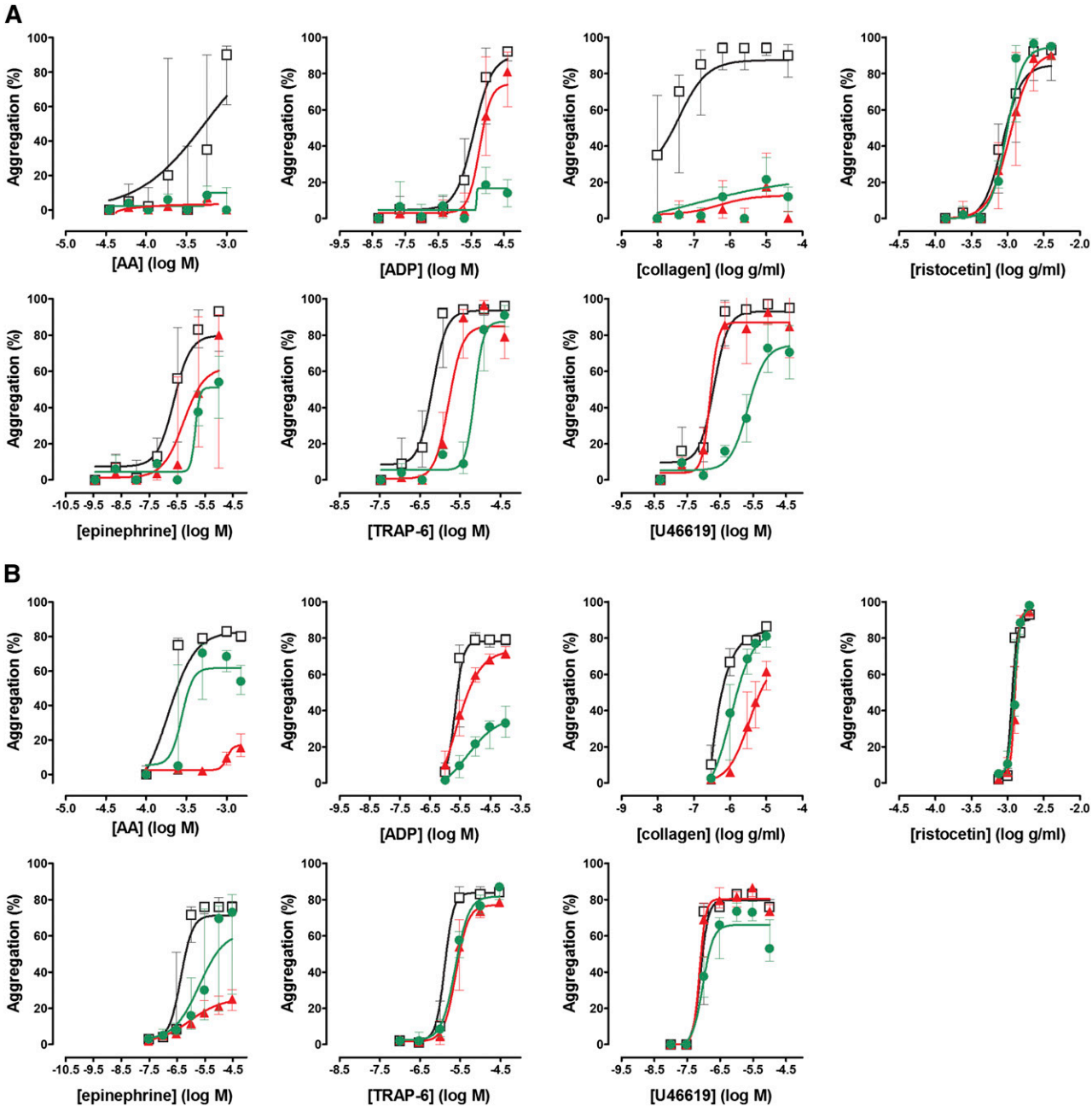

Figure 4. Effect of antiplatelet agents added in vitro to samples from healthy volunteers on maximal platelet aggregation. (A) Dose-response curves obtained with *Optimul* in 10 healthy volunteers, presented as median and interquartile range. Open squares represent nontreated samples, the red triangles represent aspirin 100 μM–treated samples, and the green circles represent cangrelor 1 μM–treated samples in the same individuals. (B) Dose-response curves obtained with LTA in 10 healthy volunteers, presented as median and interquartile range. Open squares represent nontreated samples, the red triangles represent aspirin 100 μM–treated samples, and the green circles represent cangrelor 1 μM–treated samples in the same individuals.

A platelet defect was found in 54% of participants with bleeding symptoms on LTA and in 66% with the *Optimul* assay (Table 1). The participants' bleeding history was not predictive of a platelet function defect identified by either LTA or the *Optimul* assay (Figure 5).[21] The assays gave concordant results in 82% of cases ($\kappa = 0.62$, $P < .0001$), suggesting substantial agreement between assays. A normal platelet function result detected by *Optimul* testing was particularly predictive (sensitivity, 94%; negative predictive value, 91%) of a normal result by reference standard LTA testing. Strikingly, the dose-response curves of patients with bleeding symptoms but in whom no platelet defect was found by lumi-aggregometry were superimposable on the dose-response curves obtained from healthy volunteers (Figure 6), further supporting the high negative predictive value of this assay. This was in line with ROC curve analyses with AUCs not significantly different from 0.5 (supplemental Figure 4A). By contrast, an abnormal platelet function test was detected by *Optimul* testing in 10 of 65 subjects with suspected platelet function disorders who displayed normal LTA test results. Of the 10 patients with discordant results on *Optimul*, 7 had impaired collagen responses either in isolation or associated with impairments in other pathways; 1 had a G_i-type presentation; 1 had isolated impairment in AA responses; and 1 had isolated impairment in TRAP responses.

Table 1. Distribution of platelet defects in patients with bleeding symptoms suggestive of a platelet defect

		LTA findings		
		No defect	Defect	
Optimul findings	No defect	20	2	**Negative predictive value = 20 / (20+2) = 91%**
	Defect	10	33	**Positive predictive value = 33 / (33+10) = 77%**
		Specificity = 20 / (20+10) = 67%	**Sensitivity = 33 / (33+2) = 94%**	

Concordant in 82% of cases; $\kappa = 0.62$, $P < .0001$, positive likelihood ratio = sensitivity/(1 − specificity) = 2.83, negative likelihood ratio = (1 − sensitivity)/specificity = 0.09.

The molecular significance of this is unknown, in view of the discrepant results with gold standard lumi-aggregometry and the positive bleeding history suggestive of a platelet defect. Accordingly, *Optimul* testing showed lower specificity (67%) and positive predictive value (77%) for platelet function disorders compared with the reference standard LTA test. It is not possible to distinguish in this analysis whether this finding indicates a true reduction in *Optimul* test specificity or whether the *Optimul* test offers greater sensitivity for the detection of platelet function disorders than LTA.

Patterns of response in patients with platelet defects

The patients in whom a platelet defect was found by LTA were further subdivided into categories, as recently described by our group.[16] The pattern of platelet responses obtained with the *Optimul* assay for each type of defect based on lumi-aggregometry is shown in Figure 6. ROC curve analyses are presented in supplemental Figure 4B-D.

A secretion defect (defined on lumi-aggregometry as dense granule secretion <5th percentile of normal response to 100 μM TRAP peptide, n = 14 [22%]) was apparent on the *Optimul* assay as a rightward shift in dose-response in response to most agonists (Figure 6A), except ADP (which adds excess ADP and is relatively insensitive to secretion defects), and ristocetin (which relies on passive agglutination of platelets and does not require platelet activation). It follows that the AUC for all agonists, with the exception of ADP and ristocetin, was significantly lower in patients with secretion defects (Figure 6D and supplemental Figure 4B).

A defect in the thromboxane pathway (defined as either impaired thromboxane generation or thromboxane receptor activity, n = 4 [6%]) appears as a lack of response to AA, accompanied by a significant shift in response to collagen, which strongly relies on thromboxane generation as a secondary mediator (Figure 6B). The thromboxane mimetic (U46619) response can be impaired if there is a defect in the thromboxane receptor, but preserved if there is a defect in the COX enzyme leading to abnormal thromboxane generation. Accordingly, the AUC for these agonists were significantly reduced in comparison with healthy volunteers or patients without a platelet defect (Figure 6D and supplemental Figure 4C).

Finally, a G_i-type defect (defined as a defect in aggregation and secretion to the 2 G_i-coupled receptor agonists ADP and epinephrine, n = 12 [18%]) was the most heterogeneous presentation on *Optimul* (Figure 6C). Whereas the dose-response curve to epinephrine was systematically impaired, the level of inhibition of the other agonists was significantly more variable with minor rightward shifts in dose-response curves, which nonetheless translated into significant differences on AUC (Figure 6D and supplemental Figure 4D).

Because most platelet defects presented as impairments in AA-induced aggregation, further investigation was undertaken into TxA_2 generation in a subset of patients with bleeding symptoms of unknown etiology (n = 35; n = 17 with abnormal AA response on *Optimul*). Interestingly, although in some participants TxB_2 concentration suggested impaired COX activity, in most participants TxB_2 concentration fell within ranges compatible with normal COX function (Figure 3), thus shifting the interpretation toward a defect in dense granule/ADP-dependent amplification pathways.

Phenotype-genotype associations

To determine whether the *Optimul* assay could detect known platelet defects in the main platelet activation pathways, analysis was undertaken in patients with platelet function defects in whom specific mutations had been identified within the GAPP study.

Lumi-aggregometry suggestive of a defect in the thromboxane receptor had led to genotyping of the *TBXA2R* gene in a participant with a severe bleeding history. A novel heterozygous mutation was found translating into an asparagine to serine change at position 42. This mutation prevents agonist activation of the receptor as a result of poor expression at the cell surface membrane.[22] The participant's

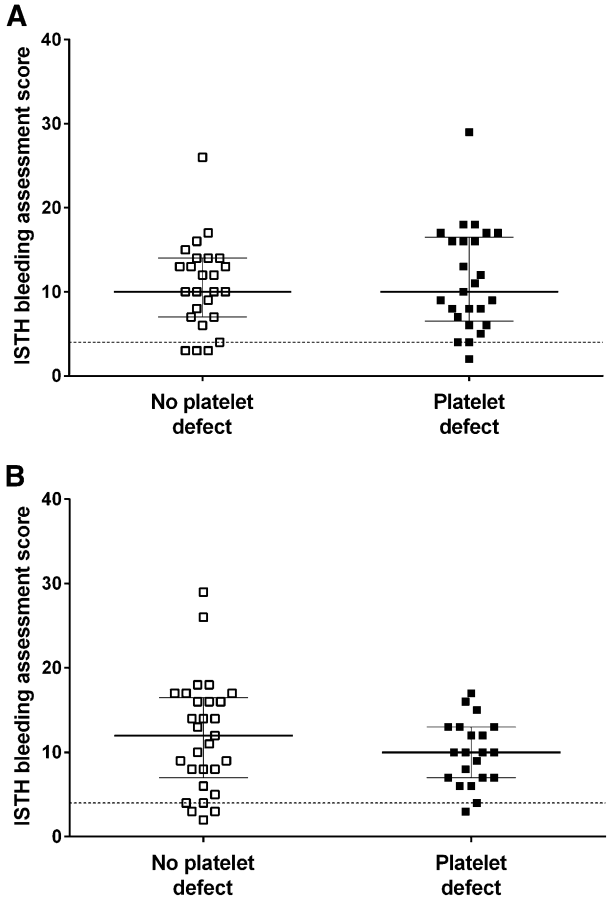

Figure 5. Association between presence of a platelet function defect and the ISTH bleeding assessment tool score. Ninety-fifth percentile (score of 4) calculated from healthy volunteers and represented by horizontal dotted line. The line represents the median and the whiskers represent the interquartile range. (A) Presence and absence of a platelet defect defined by lumi-aggregometry. (B) Presence and absence of a platelet defect defined by the *Optimul* assay.

Optimul results are shown in Figure 7A and confirmed a marked impairment of AA-induced aggregation with a profound rightward shift in response to the thromboxane mimetic U46619, whereas responses to other agonists were unaltered. Measurement of TxB_2 on the highest concentration of the AA-induced aggregation on the *Optimul* plate demonstrated normal TxA_2 generation, despite absence of an aggregation response to AA (40.7 ng/L $\times$ 10^8 platelets; normal range, 19.1-157.6 ng/L $\times$ 10^8 platelets), confirming the defect was at the level of the receptor and not in the enzymatic conversion of AA into TxA_2.

Lumi-aggregometry suggestive of a defect in the ADP $P2Y_{12}$ receptor had led to the identification of a novel heterozygous mutation predicting an arginine to histidine change at position 122. This mutation maps to the DRY motif, a highly conserved region in G-coupled receptors, which was shown in expression studies to inhibit platelet activation by ADP.[23,24] Representative *Optimul* traces from this family are shown in Figure 7B. Impairment in all platelet pathways (except for passive agglutination onto ristocetin) was consistent with a platelet activation defect and was seen in all family members. Further investigation into TxA_2 generation confirmed normal COX activity in all family members and reinforced the likelihood that the defect was due to lack of amplification by ADP, with reduced ADP responses suggestive of a defect in ADP receptors.

Discussion

The main findings of this study are as follows: (1) the *Optimul* assay was robust and reproducible, was completed in a much shorter time after preparation of PRP (10 minutes vs 2 hours) and required significantly less blood than traditional LTA testing (6 vs 50 mL); (2) both assays detected platelet inhibition induced by commonly used antiplatelet agents in samples from healthy volunteers treated with antiplatelets in vitro; (3) both assays detected platelet defects in patients presenting with bleeding symptoms suggestive of a platelet function disorder; (4) *Optimul* offers high sensitivity for platelet function disorders identified by the reference standard of LTA testing; and (5) *Optimul* showed lower specificity for platelet function disorders defined by LTA. However, the greater sensitivity of *Optimul* to platelet inhibition by antiplatelet drugs may suggest that this reflects an inability of LTA to identify some subtle platelet function disorders. The higher sensitivity of the *Optimul* assay to platelet inhibition and the more global portrait of platelet reactivity to a wider range of agonists and agonist concentrations make it particularly appealing for investigation of platelet phenotypes associated with the complex process of bleeding.

Utility of platelet function testing in mild bleeding disorders

Inherited platelet function disorders are a heterogeneous group of defects, which include defects in platelet receptor function, enzymatic activity, trafficking and granule secretion, and procoagulant activity.[1,25] Despite this variety in the molecular basis of the disorder, patients present with a common mucocutaneous bleeding phenotype, which is difficult to distinguish from other more common mild bleeding disorders, for example von Willebrand's disease.[1,25,26] The situation is further complicated by the fact that mucocutaneous bleeding symptoms are reported in a significant proportion of the healthy population, leading to difficulties in determining what level of bleeding is to be considered pathological.[27] Laboratory investigation of individuals with bleeding symptoms has thus far suffered from the absence of a test that could monitor a whole spectrum of platelet responses and require a small volume of blood and little technical expertise.[4,26] As a consequence, inherited platelet disorders remain underdiagnosed, and under-researched.

We and others have previously shown LTA combined with ATP secretion assessment to be a valuable diagnostic tool.[16,28-30] Furthermore, the latest guidelines recommend the use of lumi-aggregometry in the aid to diagnosing inherited platelet defects.[26] However, there are wide variations between laboratories using lumi-aggregometry for platelet function testing, which results in lack of standardization.[31-33] Moreover, lumi-aggregometry requires specific expertise, both in its performance and its interpretation; expensive machinery; a large volume of blood; and is time and labor intensive.[4,30] The latest expert consensus document outlines a number of preanalytical, methodological, and interpretation variables that influence platelet aggregation results.[15] Extensive expertise is required in the interpretation of aggregation traces, which should include evaluation of shape change, length of lag phase, slope of aggregation, amplitude of aggregation, deaggregation, and visual examination of each individual trace, making the exercise dependent on the experience of the observer.[15] Differences in performance of LTA and interpretation of tracings result in even the best diagnostic laboratories worldwide finding the application of lumi-aggregometry to large groups of patients challenging.[31-33] In an effort to standardize and streamline the process, we previously suggested a rationalized and

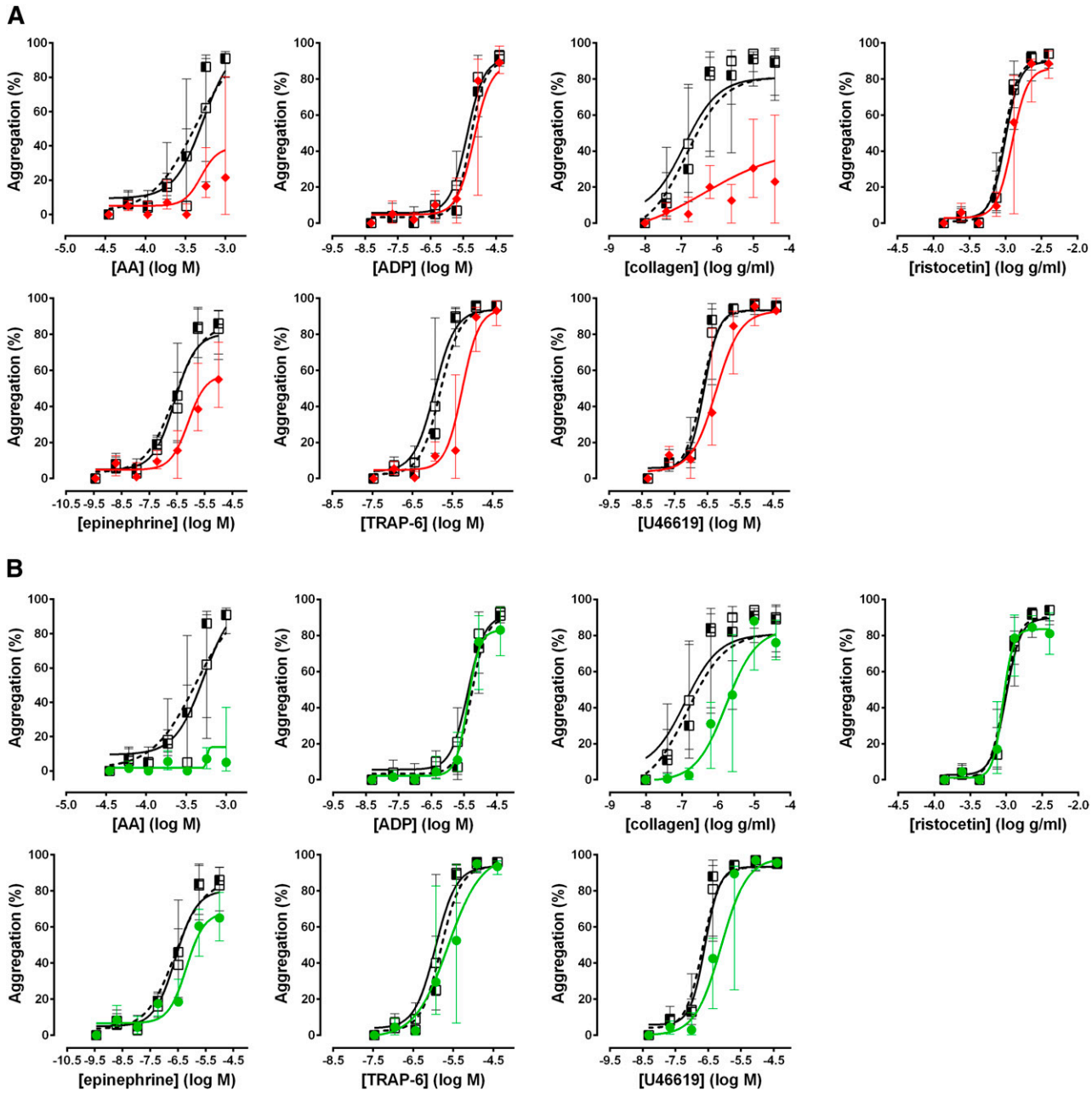

Figure 6. Pattern of platelet responses obtained with *Optimul*. (A) Dose-response curves presented as median and interquartile range. Open squares represent healthy volunteers, the striped squares represent patients with bleeding symptoms in whom no defect was found on LTA, and red lozenges represent patients in whom a secretion defect was found on LTA. (B) Dose-response curves presented as median and interquartile range. Open squares represent healthy volunteers, the striped squares represent patients with bleeding symptoms in whom no defect was found on LTA, and green circles represent patients in whom a thromboxane pathway defect was found on LTA. (C) Dose-response curves presented as median and interquartile range. Open squares represent healthy volunteers, the striped squares represent patients with bleeding symptoms in whom no defect was found on LTA, and blue triangles represent patients in whom a G_i-type defect was found on LTA. (D) Summary of finding presented as AUC for each of the types of platelet phenotypes defined by LTA. Data presented as median and interquartile range; the whiskers represent the 5th and 95th percentiles.

concise panel of agonists to be used with lumi-aggregometry and showed this to be clinically equivalent to using an extensive aggregation panel.[16] Nonetheless, the requirement for blood volume and time remains a challenge, especially in circumstances where a large blood volume is difficult to obtain, eg, in children, or in a high-throughput setting such as screening for defects in all-comers with bleeding symptoms. Thus, a high-throughput assay that could monitor a whole spectrum of platelet responses and would require a small volume of blood and little technical expertise would be clinically useful.

Because the *Optimul* assay is based on light absorbance through a stirred PRP sample, a methodology similar to that used in traditional LTA, we sought to directly compare the methodologies in their efficacy to measure platelet function. The differences in sensitivity to platelet inhibition by these 2 assays serves to stress that despite similarities in the way platelet aggregation is measured between conventional LTA and *Optimul*, the dynamic forces involved due to either the smaller volumes of PRP used or the different shaking method are sufficient to modify platelet behavior. It is also important to note that the *Optimul* assay is an end point assay; thus, the increase in

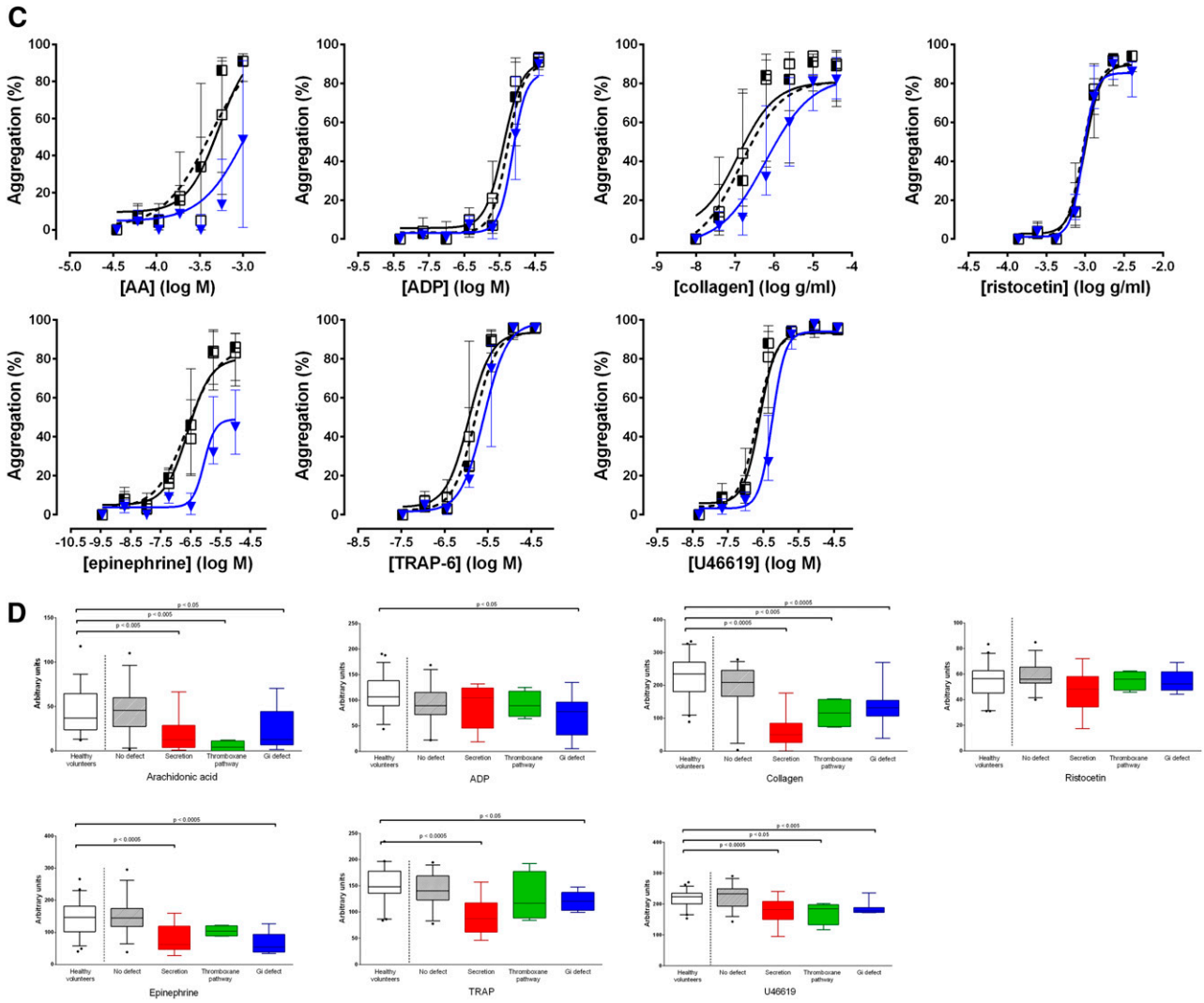

Figure 6. (Continued).

throughput is at the expense of the kinetic information available with LTA. Whereas aggregation at 5 minutes incorporates elements of lag phase, maximal amplitude, and deaggregation, visual inspection of the time-dependent nature of aggregation with shape change and primary and secondary wave of aggregation is not available with the *Optimul* assay.

Potential application of the Optimul assay for platelet function screening

There is increasing demand on physicians for complete information, which partly stems from the desire of patients to avoid unnecessary and predictable complications and to better characterize and understand their symptoms.[34] In the context of inherited bleeding disorders, availability of drugs and devices that protect at-risk patients from bleeding complications (eg, desmopressin or antifibrinolytics) renders the diagnosis of a bleeding diathesis clinically relevant.[35,36] The *Optimul* assay could be used as an initial screening assay in patients presenting with bleeding symptoms to direct further investigation, and reduce the time, blood, and labor intensity compared with standard lumi-aggregometry practices. Notwithstanding, the majority of the participants included within GAPP do not present with striking laboratory phenotypes that map to a single platelet activation pathway.[1] This finding thus opens up the field of whole-exome genotyping, with the aim of discovering novel mutations to account for the participants' bleeding and platelet phenotypes. In this setting, the negative discriminative power is crucial to select only those participants with a strong indication for a platelet defect. The *Optimul* assay used in this fashion could allow high-throughput screening.

From a clinical perspective, the treatment of patients with a platelet defect does not rely on the underlying molecular mechanisms, and thus the demonstration of a clear platelet defect is more clinically valuable than the demonstration of a defect in a specific platelet pathway.[34] The application of a high-throughput highly sensitive assay could thus allow for more widespread use of platelet function testing to aid in diagnosis of inherited platelet defects. The ease of use, speed, small blood requirement, and no need for specialized equipment for the performance of the *Optimul* assay render it particularly appealing, especially outside of large tertiary centers, and could help alleviate the burden of platelet function testing in patients presenting with bleeding. Its use in this context, however, requires large clinical studies to be carried out.

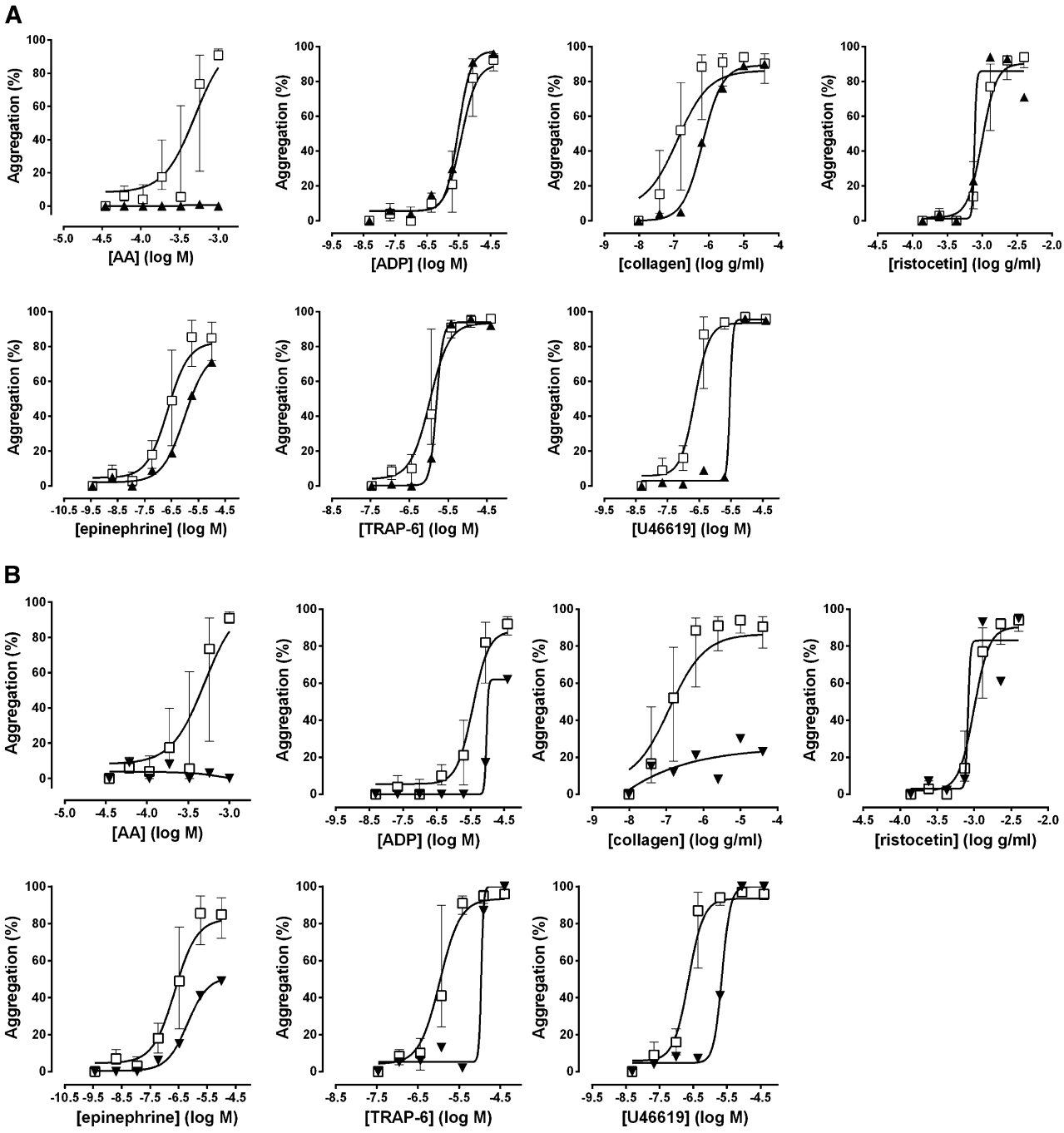

Figure 7. Platelet responses obtained with *Optimul* in patients with known mutations. (A) Patient with a mutation in the TP receptor. (B) Patient with a mutation in the $P2Y_{12}$ receptor. Dose-response curves presented as median and interquartile range. Open squares represent healthy volunteers, and the black triangles represent the participant with a thromboxane receptor mutation in A and a $P2Y_{12}$ receptor mutation in B.

Conclusion

This study demonstrates that the *Optimul* assay, which exploits the advantages of the 96-well plate format, is useful in characterizing platelet function in healthy volunteers and in patients presenting with bleeding suggestive of an inherited platelet function defect. It offers distinct advantages in terms of being faster, requiring less blood, and allowing AUCs to be determined, but the tradeoffs include greater complexity until a commercial equivalent can be devised and the loss of kinetic information. Applying this assay to the 65 participants in this study as a screening assay could have avoided detailed phenotyping with lumi-aggregometry in 22 participants (34%) and would have correctly identified 33 participants (51%) as having a platelet defect. This benefit would have occurred at the cost of 2 missed diagnoses of platelet defects and 10 extra diagnoses that were later not substantiated by lumi-aggregometry. In a clinical setting, the high negative predictive value of the *Optimul* assay makes it particularly appealing as a screening assay in nonspecialized centers. Indeed, its use could prevent extra platelet function testing in up to a third of patients

with excessive bleeding and could select the remaining patients to be referred on for more detailed phenotyping in a specialized center.

Although this assay is not quite yet a "lab on a chip," it provides a multifaceted portrait of platelet function that is frequently lacking in current platelet function assays. Future studies are needed to explore whether the *Optimul* assay used as an initial screening tool, in conjunction with a rationalized LTA panel, may be useful in patients with a clinical history of mild bleeding to identify hidden genetic hemostatic defects, which often go unnoticed until a challenge, such as surgery or addition of an antiplatelet agent, unveils the underlying defect.

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Authorship

Contribution: M.L. designed the research, performed the assays and collected data, analyzed and interpreted data, performed statistical analysis, and wrote the manuscript; G.C.L. and N.S.K. collected data, analyzed and interpreted data, and revised the manuscript; M.V.C., M.H.L., N.V.M., D.B., S.P.N., V.C.L., M.L.J., S.J.M., M.E.D., and A.D.M. analyzed and interpreted data and revised the manuscript; and T.D.W. and S.P.W. designed the research, analyzed and interpreted data, and revised the manuscript. A complete list of the members of the UK Genotyping and Phenotyping of Platelets Study Group appears in the Appendix.

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Appendix

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