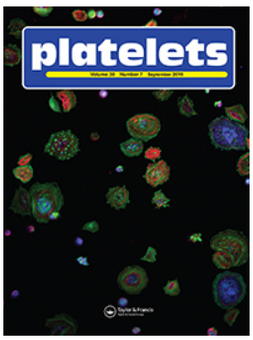

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Evaluation of the Total Thrombus-Formation System (T-TAS): application to human and mouse blood analysis

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Abstract

The Total Thrombus-formation Analyser System (T-TAS) is a whole blood flow chamber system for the measurement of *in vitro* thrombus formation under variable shear stress conditions. Our current study sought to evaluate the potential utility of the T-TAS for the measurement of thrombus formation within human and mouse whole blood. T-TAS microchips (collagen, PL chip; collagen/tissue thromboplastin, AR chip) were used to analyze platelet (PL) or fibrin-rich thrombus formation, respectively. Blood samples from humans (healthy and patients with mild bleeding disorders) and wild-type (WT), mice were tested. Light transmission lumi-aggregometer (lumi-LTA) was performed in PRP using several concentrations of ADP, adrenaline, arachidonic acid, collagen, PAR-1 peptide and ristocetin. Thrombus growth (N = 22) increased with shear within PL (4:40 ± 1.11, 3:25 ± 0.43 and 3:12 ± 0.48 mins [1000, 1500 and 2000s^{-1}]) and AR chips (3:55 ± 0.42 and 1:49 ± 0.19 [240s^{-1} and 600s^{-1}]). The area under the curve (AUC) on the PL chip was also reduced at 1000s^{-1} compared to 1500/2000s^{-1} (260 ± 51.7, 317 ± 55.4 and 301 ± 66.2, respectively). In contrast, no differences in the AUC between 240s^{-1} and 600s^{-1} were observed in the AR chip (1593 ± 122 and 1591 ± 158). The intra-assay coefficient of variation (CV) (n = 10) in the PL chip (1000s^{-1}) and AR chip (240s^{-1}) were T_{10}14.1%, T_{60}16.7%, $T_{10\text{-}60}$22.8% and AUC_{10}24.4% or T_{10} 9.03%, T_{80}8.64%, $T_{10\text{-}80}$23.8% and AUC_{30}5.1%. AR chip thrombus formation was inhibited by rivaroxaban (1 μM), but not with ticagrelor (10 μM). In contrast, PL chip thrombus formation was totally inhibited by ticagrelor. T-TAS shows an overall agreement with lumi-LTA in 87% of patients (n = 30) with normal PL counts recruited into the genotyping and phenotyping of platelet (GAPP) study and suspected to have a PL function defect. The onset (T_{10}) of thrombus formation in WT mice (N = 4) was shorter when compared to humans e.g. PL chip (1000s^{-1}) T_{10} were 02:02 ± 00:23 and 03:30 ± 0:45, respectively). T-TAS measures *in vitro* thrombus formation and can be used for monitoring antithrombotic therapy, investigating patients with suspected PL function defects and monitoring PL function within mice.

Keywords

Light transmission lumi-aggregometry, mild bleeding disorders, platelet aggregation, platelet function defects, total thrombus-formation system, WT mice

History

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Introduction

Hemostasis is an important process that maintains the integrity of the circulatory system and minimizes blood loss upon vascular damage. When a blood vessel-wall is injured a number of concomitant events occur. Initially, circulating platelets (PLs) are recruited to the site of injury, where they are activated to become a PL plug. Simultaneously blood coagulation is triggered by tissue factor resulting in thrombin generation and fibrin formation. The formation of fibrin, together with the PL plug become major components of the developing thrombus at the sight of injury to prevent bleeding [1].

A number of instruments have now become available to investigate bleeding disorders by accessing PL function defects. However, the most common used techniques such LTA are aggregation based and so do not access PL in their physiological condition. The more recent developed methods such as the impact cone and plate(let) analyser (CPA), PFA-200 and the Total Thrombus-formation Analysis System (T-TAS) are flow-based methods using whole blood and therefore measure PL function under more physiological conditions. Although both systems measure thrombus formation under high shear, CPA monitors PL adhesion to a polystyrene plate and the PFA-200 measures thrombus formation within a small aperture [2]. Therefore, both are sensitive to VWF levels [3]. In contrast, the T-TAS has the advantage of evaluating thrombus formation in more physiological and variable flow conditions with varying effects of VWF levels [4].

Institutions participating in study
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Color versions of one or more of the figures in the article can be found online at www.tandfonline.com/iplt.

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The T-TAS device is provided with two types of disposable ready-to-use microchips: (1) a PL chip coated with collagen for assessing PL thrombus formation and (2) an atherome (AR) chip coated with collagen and tissue factor for assessing white thrombus formation mediated by the activation of coagulation and PLs. The device therefore offers a potential advantage of simultaneous assessment of both PL and coagulation defects [5,6].

The T-TAS was initially designed to monitor the effectiveness of antithrombotic agents. A number of studies have reported its potential utility in assessment of anti-PL drugs e.g. aspirin, clopidogrel, PAR-1 and PAR-4 antagonists [6–8] and anti-thrombotic agents e.g. direct thrombin and factor Xa inhibitors [9]. T-TAS has also demonstrated high sensitivity in detecting coagulation disorders such as haemophilia [10] and von Willebrand disease [11], as well as PL function defects for example, storage pool disease (SPD) [12]. In assessing acquired haemorrhagic conditions, the T-TAS was able to predict the risk of bleeding in atrial fibrillation patients undergoing catheter ablation [13]. Furthermore, T-TAS has been demonstrated to be a useful tool in the study of thrombus formation in blood taken from animal models such as miniature pigs [14] and mice [10,15].

Materials and Methods

Participants

A total of 59 individuals (37 patients and 22 controls) were evaluated and recruited into the GAPP study (www.birmingham.ac.uk/plateletgapp) from 9 UK Hemophilia comprehensive care centers. All patients or their parents gave written informed consent in accordance with the GAPP project ethical approval (REC reference: 06/MRE07/36).

Reagents

ADP, ristocetin and adrenaline were purchased from Sigma (Poole, UK). Arachidonic acid and U46619 were purchased from Cayman Chemical Company (Michigan, USA). The PAR-1 peptide (SFLLRN) was purchased from Severn Biotech (Kidderminster, UK). Collagen was purchased from Takeda (Austria) and luciferin luciferase reagent (Chrono-lume) was purchased from Chrono-log Corporation (Havertown, PA, USA). The reagents were dissolved in phosphate-buffered saline (PBS) at pH 7.4 and stored as frozen aliquots, thawed and diluted in PBS when required and kept on ice. Collagen was stored as a concentrated stock at 1 mg/ml as supplied by the manufacturer at 4°C and diluted with the buffer provided. AR chip, PL chip, $CaCl_2$ containing 1.25 mg/ml of Corn Trypsin Inhibitor (CaCTI) and 3.2% sodium citrate were provided by Quadratech Diagnostics Limited (Epsom, UK). 25 μg/ml hirudin blood tubes were purchased from Roche Diagnostics (Munich, Germany). Ticagrelor (10 mM) and Rivaroxaban (10 mM) stock solutions were provided by Leeds Institute of Cardiovascular and metabolic medicine, University of Leeds (Leeds, UK).

Blood Sampling, PL Preparation and PL Count

Whole blood (40 ml) was obtained and anticoagulated with one tenth volume of 0.109 M/L buffered trisodium citrate in vacutainer plastic tubes (Becton Dickinson, Plymouth, UK). An additional (5 ml) blood was collected, 2 ml was anticoagulated with EDTA in vacutainer plastic tubes (Becton Dickinson, Plymouth, UK) and 3 ml was anticoagulated with hirudin in vacutainer plastic tubes (Roche Diagnostics, Munich, Germany), (final concentration, 25 μg/ml). The same volume of blood was collected from a healthy control at the same time in each Center. Blood samples were immediately transported to the testing laboratory where they remained capped and stored upright at room and assayed within 4 hours (by T-TAS) and within 6 hours (by lumi-LTA) from collection. Platelet-rich plasma (PRP) was prepared by centrifuging the citrated blood samples at 200 g for 20 min. Platelet-poor plasma (PPP) was prepared by further centrifugation at 1,000 g for 10 min. Both samples were transferred into 15 ml Falcon tubes, capped and stored upright at room temperature. Whole blood PL parameters were analyzed from EDTA blood samples from patients and controls using the XN-1000 whole blood counter (Sysmex UK, Milton Keynes). An impedance analyser (Coulter Z2 Beckman Coulter, High Wycombe, Bucks, UK) was used to count the PLs within the PRP by diluting 5 μl of PRP into 10 ml of fresh Isoton II diluent (Beckman Coulter, Bucks, UK).

PL Aggregation in PL-Rich Plasma

Tests were carried out as described previously [16]. Aggregation studies were performed by using a dual Chrono-log lumiaggregometer (model ChronoLog 460 VS aggregometer, LabMedics, UK) in 400 μl mini cuvettes and stirred at 1200 rpm at 37°C. The 100% line was set using autologous PPP and the 0% baseline established with native undiluted PRP. After one min PLs were stimulated with ADP (10, 30, and 100 μM), adrenaline (10, 30, and 100 μM), arachidonic acid (0.5, 1, and 1.5 mM), U46619 (1 and 3 μM), collagen (1 and 3 μg/ml), collagen related peptide CRP (1, 3, and 10 μg/ml), PAR-1 peptide (10, 30 and 100 μM), and ristocetin (1.5 and 2 mg/ml). PL aggregation was monitored by measuring the change in optical density over 5 min after addition of the agonists and the maximal percentage of aggregation was recorded. ATP secretion from PL dense granules was assessed simultaneously by lumi-LTA using the luciferase reagent (Chronolume) as described previously [16].

Evaluation of Thrombus Formation Under Flow Conditions on Human Blood

In vitro thrombus formation was analyzed on the T-TAS instrument (Zacros, Fujimori Kogyo Co. Ltd., Tokyo, Japan) using two types of microchips, the PL chip (width 40 μm × depth 40 μm) (containing 25 capillary channels coated with type 1 collagen) and the AR chip (width 300 μm, depth 60 or 120 μm, length 15 mm) (consisting of a single capillary channel coated with collagen and thromboplastin). The PL chip was used to analyze platelet thrombus formation (PTF) under three different shear rates (1000, 1500, and 2000s^{-1}). Briefly, hirudin anticoagulated whole blood (320 μl) was pipetted in the reservoir then perfused at 37°C through the PL chip by a pneumatic pump. After the perfusion of blood was initiated, PLs were then activated by the collagen coated on the capillary. The AR chip was used to analyze white thrombus formation (WTF) under two different shear rates (240s^{-1} and 600s^{-1}). Briefly, citrated whole blood (480 μl) was re-calcified by mixing with 20 μl of 0.3 M CaCTI immediately before it was pipetted in the reservoir. The re-calcified blood was then perfused at 37°C through the AR chip by the pneumatic pump. After the perfusion of blood through the capillary was initiated, PLs and the extrinsic coagulation pathway were simultaneously activated by collagen and tissue thromboplastin. To prevent the outlet port from clotting, the blood is mixed with 25 mM EDTA (pH 10.5). The process of thrombus formation in both chips was monitored by flow pressure changes in the capillary using the pressure sensor located between the pump and the reservoir. As thrombus formation proceeded on the coated surface, the capillary is gradually occluded, increasing the flow pressure. Based on the flow pressure pattern, the following four parameters are used to analyze the results (i) T10 (time to reach

10 kPa) was defined as the onset of thrombus formation and represents the duration (min) for the flow pressure to increase to 10 kPa from baseline due to partial occlusion of microcapillaries. (ii) OT (occlusion time) was defined as the complete occlusion of the capillary, which coincides with a pressure of 60 and 80 kPa for the PL and AR chips respectively. (iii) T10–60 for PL chip and T10–80 for AR chip were defined as the interval between T10 and OT, representing the rate of thrombus growth; (iv) AUC (area under curve) is an area under the flow pressure curve (under 60/80 kPa) for 10/30 min after the start of assay for the PL and AR chips respectively. AUC is used to quantify a decrease in WTF when OT is not achieved during the time period of assay.

Antithrombotic Effects of Ticagrelor and Rivaroxaban Under Flow Conditions

Ticagrelor (final concentration, 10 μM) was incubated either in hirudin or citrate anticoagulated blood for 10 min at room temperature. Rivaroxaban (final concentration, 1 μM) was incubated in citrate anticoagulated blood for 3–5 min at room temperature. After incubation blood were perfused through either the PL chip and/or AR chip within the T-TAS instrument as described above.

Statistical Analysis

All control results were run on Graph Pad Prism version 7.0 software to demonstrate the normal distribution and to obtain the median and standard deviation values, which were then used to determine the cut-off values. Abnormal results by lumi-LTA were determined based on our previously published methodology that is based upon both the magnitude and time course of response[16,17]. The time course, including reversibility is of particular importance. In contrast, abnormal thrombus formation by T-TAS was determined based on the cut off values of AUC_{10} and the T_{10} parameters calculated as the 5^{th} percentile from healthy controls.

Results

Measurement of T-TAS in Healthy Subjects

22 healthy subjects (10 males and 12 females; median age 31 ± 7 years) were investigated. Overall, the T-TAS shows some variation in thrombus formation among healthy subjects on both (PL and AR) chips (Figure 1A and 1C). Within the PL chip (Figure 1B), the onset (T_{10}) value (median) and occlusion time (T_{60}) at a shear rate of 1000 s^{-1} were greater than at 1500/2000 s^{-1} (T_{10} = 03:30, 03:02, 03:29 and T_{60} = 08:10, 06:27, 06:42, respectively). In contrast, the AUC_{10} value (median) was reduced at a shear rate of 1000 s^{-1} than at 1500/2000 s^{-1} (260, 317, and 301, respectively). Furthermore, the thrombus growth rates ($T_{10–60}$) in the PL chip (median) decreased as the shear rate increased (04:40, 03:25, and 03:12, respectively) (Figure 1B). A shear dependent decrease was also observed on the occlusion times (T_{80}) and thrombus growth rates (T_{10-80}) on the AR chip (T_{80} = 12:1 and 11:30; T_{10-80} = 4:36 and 2:21 respectively) (Figure 1D). However, no significant different of the AUC_{30} values (median) was observed between low and high shear values (1593 and 1591 respectively). Surprisingly, the onset (T_{10}) value (median) on AR chip was greater at high shear (09:19) than at the low shear (08:16) (Figure 1D).

Intra-assay coefficients of variation (CV) were calculated from 10 sequential measurements of a single sample obtained from one healthy subject (Table I). Overall, the difference of CV between shear rates within the PL chip was more significant compared to the AR chip. On PL chip the CVs of T_{60}, $T_{10–60}$ and AUC_{10} were smaller at 2000 s^{-1} than at 1000/1500 s^{-1} whereas the CVs of T_{10} were almost identical at all three shear rates. In AR chip however, the CV of all four parameters were low at 600 s^{-1} than that at 240 s^{-1} (Table I).

Measurement of Blood from Healthy Subjects Spiked with Antithrombotic Drugs

No thrombus formation was observed in the PL chip when blood was pre-treated with ticagrelor (10 μM) (Figure 1E). In contrast, thrombus formation in the AR chip was not affected by addition of ticagrelor (Figure 1F). However, thrombus formation in the PL chip was observed with blood pre-treated with rivaroxaban (1 μM) but varies between subjects when perfused through the AR chip at lower shear rates (Figure 1G).

Measurement of T-TAS on Samples from Patients Recruited into the GAPP Study with Suspected PL Function Defects

We analyzed 30 patients (8 males and 22 females) with a median age 35 (range 7–73) recruited into the GAPP study and compared T-TAS (PL chip) results with lumi-LTA. All patients had normal PL counts (261 ± 68 × 10^9/L). Of 30 patients tested (Figure 2C), 10 (33.3%) patients gave abnormal responses by lumi-LTA to one or more agonists as defined using previous criteria [17]. In contrast, 8 (27%) patients were detected to have abnormal thrombus formation by T-TAS, as defined using the cut off values obtained from healthy controls. T-TAS detected an abnormality in 1 patient that was normal by lumi-LTA, whereas 3 samples were normal by T-TAS but abnormal by lumi-LTA (Figure 2A and 2B, respectively). The overall agreement between the two instruments is shown in Table II. 26/30 samples gave identical results by both tests, with the majority (19) giving normal responses, whereas 7 samples gave abnormal results by both tests. In contrast, there was disagreement in 4/30 samples. In this study we used AUC_{10} and the T_{10} parameters to identify a defect on T-TAS. 7/8 (88%) samples with an abnormality detected by T-TAS have low values of AUC_{10}. One patient however, had normal AUC_{10} value (AUC_{10} = 177.8s) but a delayed T_{10} parameter (T_{10} = 5.44s).

Analysis and Identification of Patients with PL Function Defects

Functional defects identified by lumi-LTA were classified into four main groups (Gi defects, secretion defects, COX-like defect and multiple defects) according to their pattern of responses to specific agonists as previously described[17]. As shown in Figure 2D, T-TAS detected all patients classified by lumi-LTA with COX-like defect or secretion defects (1 and 2 patients, respectively). In contrast, T-TAS only detected 1/3 patients with Gi defects but 3 out of 4 with multiple defects.

Measurement of T-TAS in Sample from GAPP Patients with Thrombocytopenia Recruited with Suspected PL Function Defects

We additionally analyzed seven patients (2 male and 5 female) found to have mild to marked thrombocytopenia as characterized by low whole blood PL counts (< 150 × 10^9/L), but normal PL counts in PRP when tested by lumi-LTA. The whole blood PL counts (mean ± SD) were 77 ± 34 (×10^9/L). The waveform of each individual patient is presented in Figure 2A. AUC_{10} parameters (Figure 2B) showed significant differences between healthy controls (n = 22) and patients (n = 7). All seven patients tested exhibited abnormal thrombus formation within the T-TAS (Figure 2A). Interestingly, 4/7 patients were also detected by lumi-LTA to have PL function defects (3 secretion and 1 Cox-like defects) in additional to their thrombocytopenia.

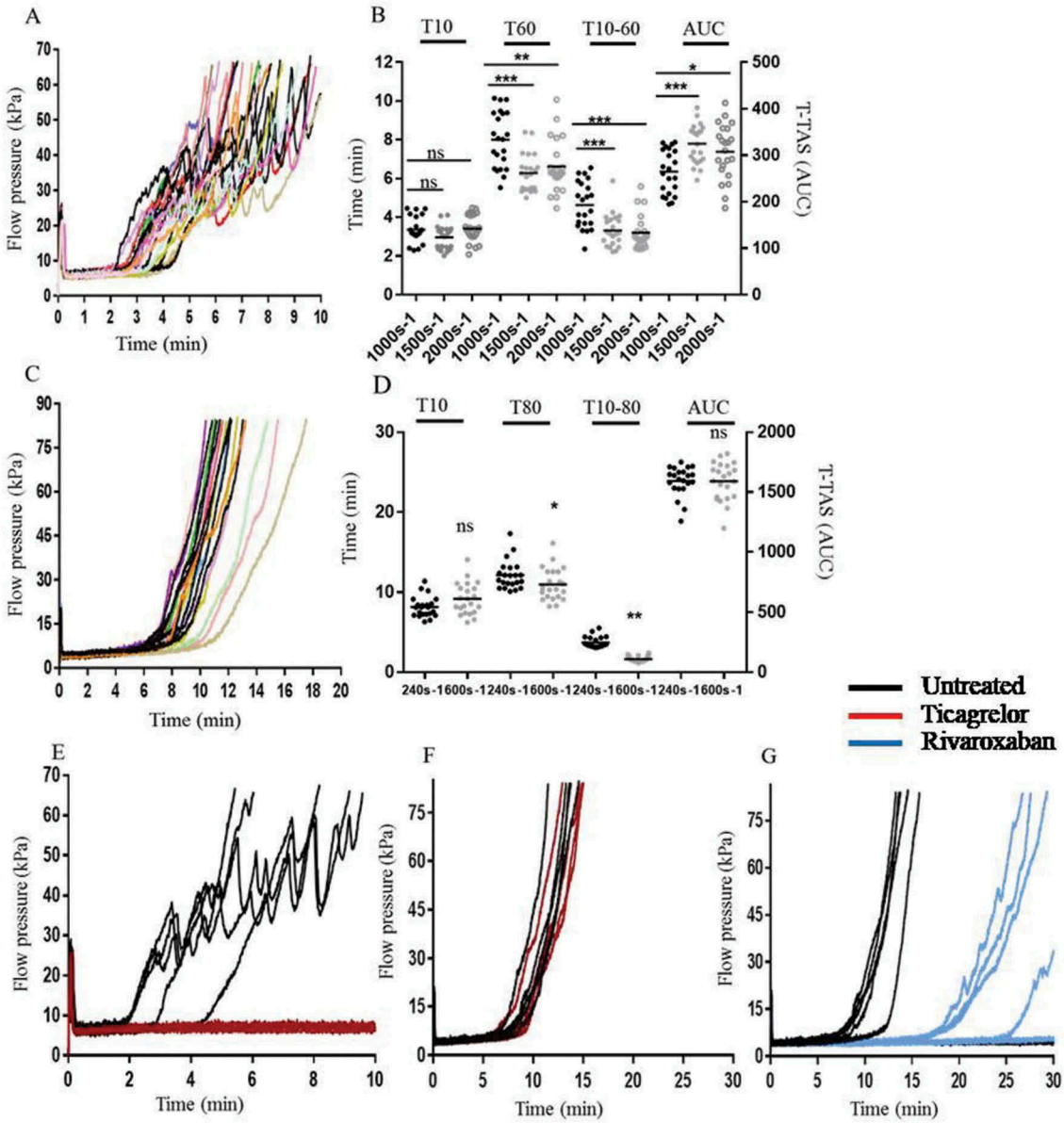

Figure 1. Measurements of blood from healthy controls (n = 22) using the PL chip and AR chip. For panel A and C, flow pressure curves of all individuals measured at low shear rates on PL chip (1000 s^{-1}) and AR chip (240 s^{-1}), respectively. For panel B and D, distribution of the T-TAS measurements from different shear rates using PL chip and AR chip, respectively. For panel E and F, effects of the ticagrelor (10μM) on blood from healthy controls (n = 4) measured on PL chip and AR chip, respectively. For panel G, effects of the rivaroxaban (1μM) on blood from healthy controls (n = 5) measured on AR chip (bars represent median values). Statistical analysis performed by the Mann Whitney U test and the one-way ANOVA test with Sidak's adjustment for multiple comparisons (***p < 0.001, **p < 0.01 and *p < 0.05).

Table I. Intra-assay CV in PL and AR chips measurements in a single sample obtained from a healthy control (n = 10).

PL chip	1000 s^{-1}	1500 s^{-1}	2000 s^{-1}	AR chip	240s^{-1}	600s^{-1}
T_{10} (CV%)	14.4%	15.3%	14.7%	T_{10} (CV%)	8.9%	7.9%
T_{60} (CV%)	17.9%	14.3%	11.4%	T_{80} (CV%)	8.6%	6.8%
$T_{10\text{-}60}$ (CV%)	24.4%	26.4%	19.4%	$T_{10\text{-}80}$ (CV%)	22.9%	20.2%
AUC_{10} (CV%)	24.4%	26.9%	8.6%	AUC_{30} (CV%)	5.1%	5.0%

Measurement of T-TAS in Samples from WT Mice

Blood samples from WT (n = 5) were tested using PL chips (1000 s^{-1} and 2000 s^{-1}) and AR chips (240 s^{-1}). Within the PL chip, shear enhanced thrombus formation was observed with all parameters; however, any differences in measurements between shear rates was only significant with the AUC_{10} parameter. For example, the values (median) at 1000 s^{-1} (T_{10} = 02:02, T_{60} = 04:40 and $T_{10\text{-}60}$ = 02:38) were greater than at 2000 s^{-1} (T_{10} = 01:16, T_{60} = 03:26 and $T_{10\text{-}60}$ = 02:09). Consequently, the AUC_{10} at 1000 s^{-1} was shorter than at 2000 s^{-1} (405.5 and 463.6, respectively) (Table III). The values (median) of thrombus formation within the AR chip (240s^{-1}) are shown in Table III. For example, the median values of T_{10}, T_{80}, $T_{10\text{-}80}$ and AUC_{30} were 02:52, 06:41, 03:48, and 2048.98, respectively.

The difference in thrombus formation between WT mice (n = 5) and healthy humans (n = 22) was then compared. Overall, thrombus formation in WT mice was more rapid on both the PL and AR chips (Figure 3A) than in humans. With PL chip measurements, the differences between two models were significant with all parameters. For example, WT mice values (median) at 1000 s^{-1} were T_{10} = 02:02, T_{60} = 04:40, $T_{10\text{–}60}$ = 02:38 and AUC_{10} = 405.5 (Table III) whereas the values in humans (median) at the same shear were T_{10} = 03:30, T_{60} = 08:10, $T_{10\text{–}60}$ = 04:40 and AUC_{10} = 260

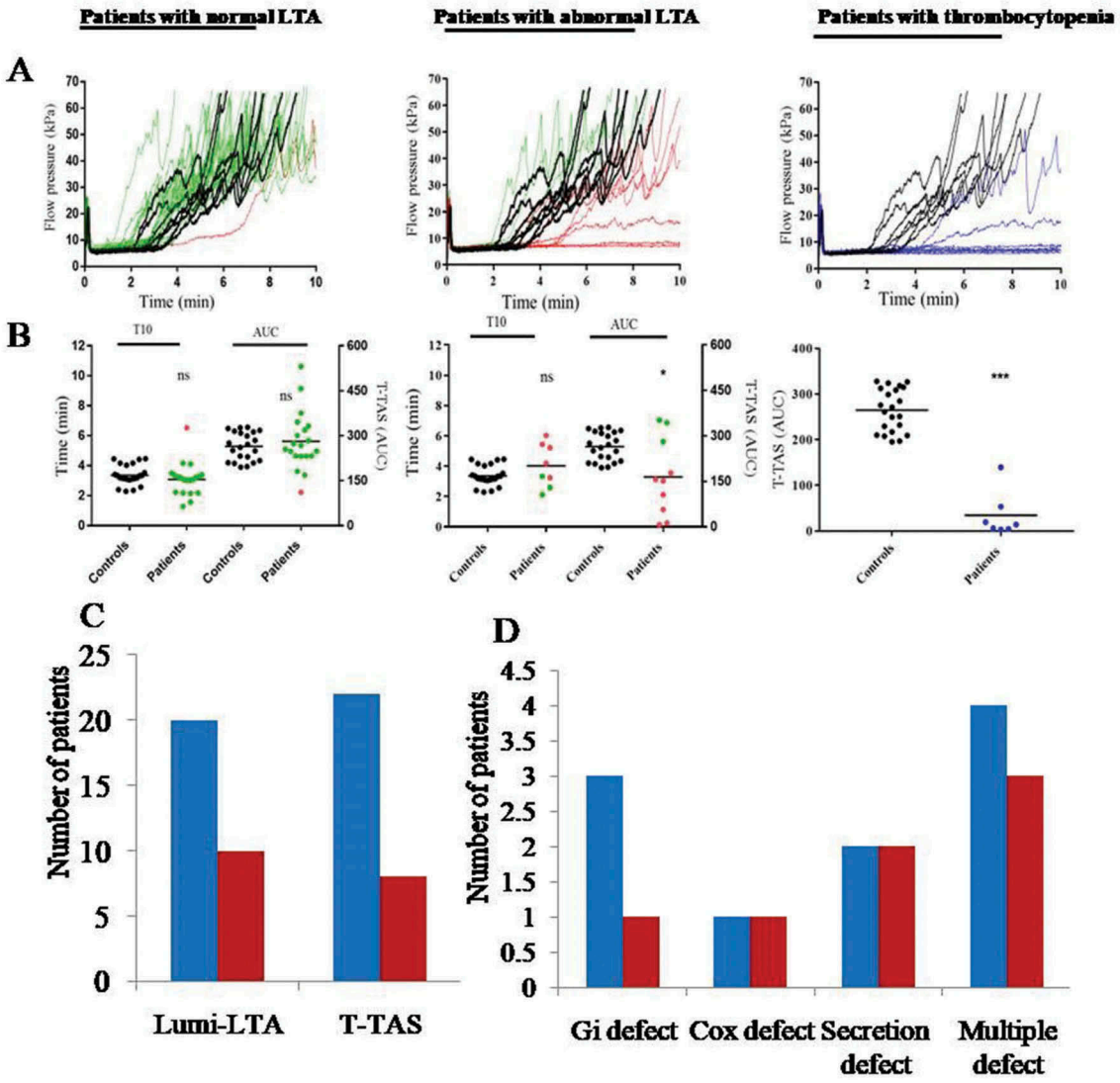

Figure 2. Measurements of thrombus formation in the PL-chip (1000 s^{-1}) with whole blood samples from healthy controls (n = 22), patients (n = 20) no detectable platelet defects by Lumi-LTA, patients (n = 10) with platelet defects as defined by lumi-LTA and patients (n = 7) with thrombocytopenia. Panel A, flow pressure curves display control (black), normal T-TAS (green), abnormal T-TAS (red) and patients with thrombocytopenia (blue). Panel B, distribution of the T-TAS measurements display control (black circles), normal T-TAS (green circles), abnormal T-TAS (red circles) and patients with thrombocytopenia (blue circles). Panel C, summary of overall patients results (n = 30) analysed on lumi-LTA and T-TAS comparing between normal (blue) and abnormal (red). Panel D, classification of platelet function defects among patients detected by lumi-LTA (blue) and T-TAS (red). The results were considered to be abnormal (by lumi-LTA) as previously established in the GAPP program using samples from healthy subjects (Dawood et al 2012) and (by T-TAS) when they fall below the cut-off values (5^{th} percentile) calculated from healthy individuals. Statistical analysis performed by the Mann Whitney U test (bars represent the median values). Significance as compared to control in each parameter (***p < 0.001 and *p < 0.05).

Table II. Analysis of the agreement between the T-TAS and lumi-LTA in patient samples (n = 30) displaying sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV).

	Lumi-LTA (Abnormal)	Lumi-LTA (Normal)	
T-TAS (Abnormal)	7	1	PPV = 88%
T-TAS(Normal)	3	9	NPV = 86%
	Sensitivity = 70%	Specificity = 95%	

Table III. T-TAS measurements in samples from WT mice (n = 5) performed on both PL and AR chips using different shear rates. Data presented as median and SD.

PL chip	1000 s^{-1} (median ± SD)	2000 s^{-1} (median ± SD)	AR chip	240 s^{-1} (median ± SD)
$T_{10(min)}$	02:02 ± 00:23	01:16 ± 00:18	T_{10}min	02:52 ± 00:41
$T_{60(min)}$	04:40 ± 00:43	03:26 ± 00:37	T_{80}min	06:41 ± 02:37
$T_{10-60(min)}$	02:38 ± 00:38	02:09 ± 00:28	T_{10-80}	03:48 ± 02:03
AUC_{10}(AUC)	405.5 ± 22.17	463.6 ± 26.34	AUC_{30}(AUC)	2048.98 ± 105.4

(results not shown). In contrast, with AR chip measurements the different between two models were only significant with T_{10}, T_{80} and AUC_{30} but not with T_{10-80} (Figure 3C).

Discussion

The T-TAS is a flow chamber system that evaluates thrombus formation using whole blood samples that flow on different thrombogenic surfaces under arterial or venous shear conditions. This approach offers an advantage for rapidly assessing thrombus formation in more physiological conditions using whole blood under flow. Furthermore, this method requires comparatively small blood volumes (350 µl) making it potentially practical for clinical application [4]. Importantly, T-TAS has all the five requirements of flow assays methods as described by Roest *et al*. [18]. We therefore sought to evaluate the potential utility

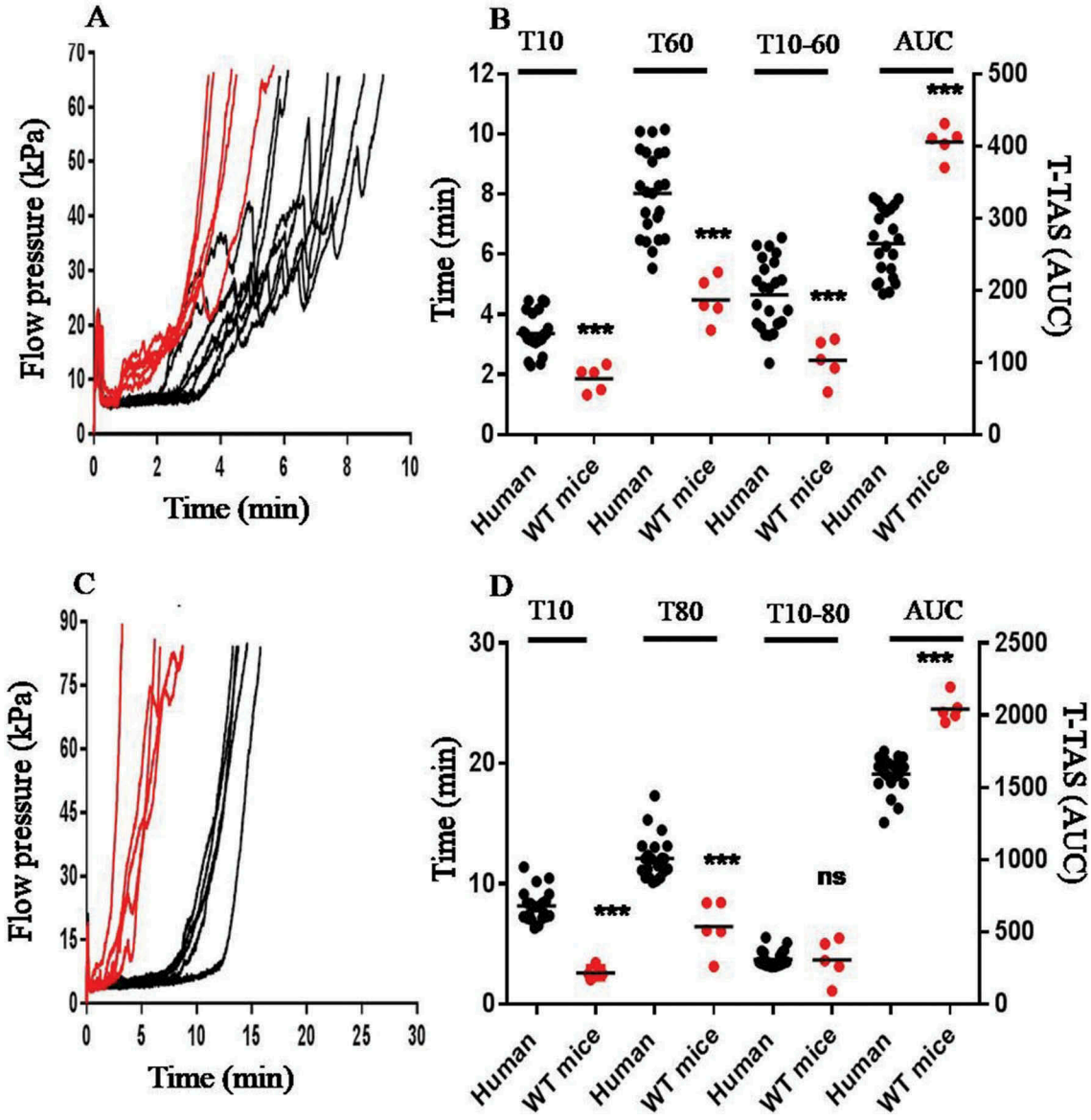

Figure 3. Measurements of thrombus formation within the PL-chip (1000 s^{-1}) and AR-chip (240 s^{-1}) with blood from human (black) and WT mice (red). Panel A, flow pressure curves observed within PL-chip (1000 s^{-1}). Panel B, distribution of the T-TAS measurements obtained from PL-chip (1000 s^{-1}). Panel C, flow pressure curves observed within AR-chip (240 s^{-1}). Panel D, distribution of the T-TAS measurements obtained from AR-chip (240 s^{-1}). Statistical analysis performed by the Mann Whitney U test (bars represent median values). Significance as compared between human and WT blood in each parameter (***$p < 0.001$).

of the T-TAS for assessing thrombus formation in blood from healthy individuals, blood spiked with antithrombotic agents, blood from GAPP study patients with mild bleeding disorders and blood from mice.

In healthy controls, both the start and end points of thrombus formation varied among individuals within both chips suggesting that measurements obtained using the T-TAS may reflect individual variability of thrombus formation. Within the PL chip, the rate of thrombus formation was shorter at higher shear rates, which was reflected in all parameters measured (Figure 1B). This supports the evidence that high-shear rates favor the GPIb-IX-V/VWF axis to induce more efficient thrombus formation [19]. In contrast, the effect of the shear rate within the AR chip was variable, in particular the onset (T_{10}) (Figure 1D) where the value at the high shear was greater than at the low shear in contrast to previous findings[4,6]. However, an increased rate of thrombus growth ($T_{10–80}$) in accordance with an increase in shear rate was observed on AR chip similar to that on PL chip, indicating that the shear rates accelerated the growth rate of the thrombus. Shear rate enhancement of T-TAS parameters were also reported by Hosokawa *et al*., and Yamaguchi *et al*. [4,6]. In our study, the T-TAS also exhibited high intra-assay CV within both chips (Table I). Typical CVs obtained were much higher than those described by Yamaguchi *et al*. although their sample size (n = 5) was smaller [4].

We also evaluated the utility of T-TAS in detecting the effect of antithrombotic therapy on hemostasis using two types of drugs. 1) The anti-PL drug Ticagrelor ($P2Y_{12}$ antagonist) and 2) The anticoagulant Rivaroxaban (factor Xa inhibitor). Ticagrelor (10 μM) completely suppressed thrombus formation within the PL chip. These findings agree with Hokosawa *et al*. who also previously demonstrated that thrombus formation within the PL chip was inhibited by $P2Y_{12}$ antagonists [5,8]. In contrast, the inhibitory effect of Ticagrelor on thrombus formation was not observed within the AR chip. As thrombus formation within the AR at low shear rate is more dependent upon fibrin formation [6,20], it is possible that $P2Y_{12}$ inhibitors are therefore expected to have no effect in this chip as PLs play little or no role in

thrombus formation at low shear rates [7,21]. Secondly, PLs within the AR chip could also be activated via the PAR-1 receptor by thrombin, which is generated from activation of the coagulation pathways [21]. This was demonstrated by Hokosawa *et al*. by demonstrating that PAR-1 antagonism reduced thrombus formation within the AR chip [7]. In contrast, Rivaroxaban (1 μM) incubation with whole blood within the AR chip displayed considerable variability on affecting thrombus formation with a delayed onset despite complete thrombus formation (Figure 1G). Hokosawa *et al*. also demonstrated that Rivaroxaban (1 μM) only moderately suppressed thrombus formation within the AR chip [9]. However, Sugihara *et al*. demonstrated that Rivaroxaban at the estimated clinical peak dose (800 nM) significantly suppressed thrombus formation[22]. Furthermore, Hosokawa *et al*., also used T-TAS to show that the anticoagulant effect of thrombus formation was intensified when anticoagulant and anti-PL drugs were used in combination [9]. These findings suggest that T-TAS could be a clinically useful tool for monitoring the antithrombotic effect on both PL and coagulation pathways.

Furthermore, we performed a comparative evaluation of the T-TAS using the PL chip with GAPP patients with suspected PL function defects and compared the results with the gold standard of lumi-LTA. Comparison of the overall results in 30 patients with normal PL counts (Table II) demonstrated a good agreement between T-TAS (PL chip) and lumi-LTA with concordance in 87% of samples tested. As 67% of samples were defined as normal by lumi-LTA, T-TAS therefore gave a NPV of 86% suggesting that the test could be a potential screening tool for the characterization of PL function disorders. Interestingly, T-TAS also detected all patients with COX-like defects and secretion defects (Figure 2D). This finding is consistent with earlier reports showing that T-TAS was able to detect all patients diagnosed with SPD[12]. T-TAS also showed high consistency in detecting abnormalities in patients with multiple defects. This finding supports the earlier report suggesting that T-TAS is reliable in detecting more severe forms of PL defects such Bernard-Soulier syndrome [12]. However, T-TAS failed to detect thrombus formation in all patients with thrombocytopenia including three patients that gave normal responses by lumi-LTA (Figure 2A and 2B). This might suggest that T-TAS is not reliable for testing samples with low PL counts and that thrombus formation is dependent on normal PL numbers. An earlier study on healthy subjects demonstrated a good correlation between normal PL counts and T-TAS parameters [4]. Further studies are required to investigate the relationship between PL counts and T-TAS parameters.

There is no single parameter that has been described to be more useful in defining hemostatic abnormalities within the T-TAS. Almost all studies evaluating bleeding disorders in the T-TAS have used $AUC_{10/30}$ and to a lesser extent the T_{10} [11–13,23]. AUC is particularly useful in quantifying a decrease in thrombus formation when occlusion time is not achieved during the time period of assay. In our study almost all samples (7/8) with abnormalities detected by T-TAS exhibited low values of AUC_{10}. One patient however, gave normal values of AUC_{10} but with delayed T_{10} parameters. Interestingly, the patient was also found to have a defect by lumi-LTA. This might therefore suggest that a combination of T_{10} and AUC parameters are sufficient to identify any hemostatic abnormalities on T-TAS.

The use of comparatively small blood volumes for T-TAS makes it practical for studying *ex-vivo* thrombus formation in small animal models. This could potentially result in significant reduction of animal usage in research laboratories. To evaluate the utility of T-TAS for testing thrombus formation in mouse blood, we first analyzed blood samples from WT mice. WT blood demonstrated more rapid total thrombus formation compared to human blood. Similar results were reported on studies of blood from miniature pigs [14], suggesting that these animals might exhibit increased thrombogenicity than humans, possibly due to higher PL counts (Table IV). As expected, all KO models displayed poor thrombus formation within the PL chip concurring with previous *in vivo* and *in vitro* findings [24]. In contrast, full thrombus formation was observed within the AR chip with all KO indicating that the coagulation pathways are normal and that PLs probably have little significant contribution to thrombus formation using this system, at a shear rate of 240 s^{-1} [23].

Table IV. Measurement of whole blood platelet count and MPV in samples from WT mice (n = 5) and human (n = 22). Data presented as mean and SD.

	Wild type mice	Human
Platelet count(Median± SD)	839 ± 166(× 10^9/L)	225 ± 65(× 10^9/L)
MPV(Median± SD)	5055 ± 0.76(fl)	11.6 ± 1.1(fl)

Our present study has several limitations. Firstly, the study was performed with only a small group of patients suspected to have PL function disorders. Therefore, for future studies it would be necessary to enroll a larger number of patients with known PL function defects as well as other hemostatic abnormalities such as coagulation defects (e.g. hemophilia and VWD). Secondly, the study of antithrombotic drugs was performed using a single concentration of either ticagrelor or rivaroxaban and were evaluated separately. It would be of interest in future studies to evaluate the T-TAS with a full range of anti-PL drugs with different molecular targets and at different concentrations as well as in the combination with anticoagulant drugs. Lastly, the mouse models were only studied within the AR chip and at low shear rates. It would be of interest to determine if higher shear rates would also have detected similar PL defects in these mice.

In summary, T-TAS has demonstrated a good agreement with Lumi-LTA suggesting that the technique could be applied to potentially screening patients with PL function defects. Moreover, the device might be a useful tool in monitoring antithrombotic therapy. Finally, we demonstrated that the T-TAS could provide valuable assistance in rapidly studying samples from animal models with the potential of reducing animal usage in research laboratories.

Authorship

Contribution: R.A. Performed research, analyzed data, and wrote the paper; J.M performed research and analyzed data; Z.N. analyzed data; L.H. analyzed data; A.M. Performed research; H.P. analyzed data; E.H. contributed to designed the research; N.V.M. designed the research and contributed to writing the paper; Y.A.S. designed the research and analyzed data; and P.H. designed the research, analyzed data and contributed to writing the paper.

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Disclosure of conflict of interest

Quadratech Diagnostics Ltd provided the T-TAS instrument and reagents for this study.

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