Screening of the protein C pathway abnormality-related thrombophilia by using thrombomodulin-mediated tissue factor-triggered clot waveform analysis

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What is the NEW aspect of your work?; In order to detect protein C pathway abnormalities, we utilized a parameter of coagulation velocity in clot waveform analysis (fibrin formation) combined with addition of recombinant thrombomodulin.
What is the CENTRAL finding of your work?; We established a clot waveform analysis-based screening assay to distinguish protein C pathway abnormality-related thrombophilia.
What is (or could be) the SPECIFIC clinical relevance of your work?; Our new assay has a potential for screening protein C pathway abnormalities in more physiological settings.

Data Availability Statement
The full data that support the findings of this study are available on request from the corresponding

author. The data are not publicly available due to privacy or ethical restrictions.

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Abstract

Objectives: Absolute or relative protein (P)C pathway abnormalities (PC-deficiency, PS-deficiency, antiphospholipid syndrome (APS), factor (F)V-abnormality, and high FVIII level) cause thrombophilia. Although screening assays for these thrombophilias are available, one utilizing clot waveform analysis (CWA) remains unknown. We aimed to establish a CWA-based screening assay to distinguish PC pathway abnormality-related thrombophilia.

Methods: Samples were reacted with tissue factor (TF)/phospholipids and recombinant-thrombomodulin (rTM; optimal 20 nM), followed by CWA measurement. The peak-ratio (with/without rTM) of the first derivative curve of clot waveform was calculated.

Results: The peak-ratio in healthy plasmas (n=35) was 0.36±0.13; hence, the cut-off value was set to 0.49. The peak-ratios in plasmas with PC-deficiency, PS-deficiency, high-FVIII (spiked 300 IU/dL), and APS were higher than the cut-off values (0.79/0.97/0.50/0.93, respectively). PC-deficient plasma or PS-deficient plasma mixed with normal plasma (25/50/75/100% PC or PS level) showed dose-dependent decreases in the peak-ratios (PC-deficient: 0.85/0.64/0.44/0.28; PS-deficient: 0.69/0.53/0.40/0.25), suggesting that the peak-ratio at ≤50% of PC or PS level exceeded the cut-off value. The peak-ratio in FV-deficiency with FV ≤25% was higher than the cut-off value. FV-deficient plasma spiked with 40 IU/dL rFV-R506Q (FV_{Leiden}) or rFV-W1920R (FV_{Nara}) showed >90% peak-ratios.

Conclusions: rTM-mediated TF-triggered CWA might be useful for screening PC pathway abnormality-related thrombophilia.

Key Words: thrombophilia, protein C pathway, thrombomodulin, clot waveform analysis, factor V

Introduction

Protein C (PC) and protein S (PS) are major vitamin K-dependent blood coagulation regulatory proteins. Activated PC (APC), which is generated by thrombin and thrombomodulin (TM) complex, exhibits anticoagulant activity by inactivating activated factor (F)V (FVa) and FVIIIa together with PS as a cofactor on the phospholipid membrane [1-3]. This APC-mediated anticoagulant reaction, known as the "PC pathway regulation", is one of major disturbed anticoagulant mechanisms on thrombophilia [4,5].

In patients with a suspected thrombophilia, each measurement of procoagulant and anticoagulant factors is essential but time-consuming to obtain the results. Considering the frequency of PC pathway abnormalities in thrombophilia patients, it is necessary to develop an easy-to-use assay to screen for these abnormalities in patients with suspected thrombophilia by assessing coagulation and anticoagulant regulatory function(s). The different assays for the detection of PC pathway abnormalities have been developed; representative assays include an activated partial thromboplastin time (aPTT)-based clotting time assay and a thrombin generation assay (TGA). ProC®Global (Dade-Behring, Marburg, Germany), one of the aPTT-based clotting time assay, can evaluate the ratio of clot time before and after the addition of snake venom (Protac), which directly activates endogenous PC, to the plasma samples [6,7]. However, the clotting trigger is not a tissue factor (TF), and the measurement sensitivity seems to be lower than that of HemosIL Thrombopath® (Instrumentation Laboratory, Bedford, MA) [8,9]. Thrombopath®, one of a TF-triggered TGA, is capable of assessing the endogenous function of APC induced by Protac, similar to the principle of ProC®Global [8]. This assay is costly, however, because of the use of chromogenic substrates, and has recently been discontinued. Common to both assays, the addition of Protac would not be able to detect upstream abnormalities of the PC pathway, such as TM or prothrombin molecular abnormalities, showing the defective PC pathway activation [10,11]. It is necessary, therefore, to establish a novel and advanced easy-to-use assay.

Clot waveform analysis (CWA) is a recently developed global coagulation technique based on the continuous observation of changes in light transmittance that occur as fibrin is formed in plasma during the performance of routine clotting tests including aPTT and prothrombin time (PT) [12]. Recommendations on the standardization and clinical application of CWA from the Scientific and Standardization Committee of the International Society on Thrombosis and Haemostasis have been already published [12]. We have intensively analysed many congenital and acquired

coagulopathy-related disorders, such as haemophilia [13,14], coagulation factor inhibitors [15,16], anti-phospholipid antibody syndrome (APS) [16], disseminated intravascular coagulation [17], and Kawasaki disease [18].

In addition, modified versions of the CWA such as the soluble TF-triggered CWA [19], aPTT/PT mixture-triggered CWA [14] and clot-fibrinolysis waveform analysis (CFWA) [20], have been developed. The aPTT/PT-CWA has been reported to be useful for monitoring hemophilia patients with FVIII inhibitor receiving emicizumab prophylaxis [14], and the results of CFWA have suggested that hyper-fibrinolysis could be evaluated by a modified CWA that incorporates tissue type plasminogen activator [20].

In the present study, we performed CWA by triggering TF and phospholipid vesicles (PL) instead of aPTT. Furthermore, the addition of recombinant TM (rTM) to the sample plasmas drives the APC-mediated PC pathway function via the thrombin-TM complex [21,22], resulting in a marked suppression of the coagulation reaction. In contrast, if the PC pathway function does not function approximately, even the addition of rTM does not suppress the coagulation reaction. Using this principle, we attempted to establish a novel assay to evaluate PC pathway abnormality-related thrombophilia by comparing the clot waveforms of TF-triggered CWA in the absence and presence of rTM.

Materials and Methods

This study was approved by the Medical Research Ethics Committee of Nara Medical University (No. 2503), and blood samples were collected after obtaining informed consent in accordance with the ethical guidelines of our university.

Reagents - Recombinant thrombomodulin (rTM, Recomodulin®, Asahi-Kasei Pharma Corp., Tokyo, Japan), recombinant human tissue factor (rTF; Innovin®, Dade), rFVIII preparation (Advate®, Takeda Pharmaceutical Company Limited, Tokyo), Thrombocheck PT-SLA® (Sysmex Cop. Kobe, Japan), PS-deficiency (-def) and PC-def plasma (immune-depleted, activity <1%, Affinity Biologicals Inc. ON, Canada), APS, FV-def, and FVIII-def plasma (patient-derived, activity <1%, George King Inc. Overland Park, KS), and human plasma-derived FV (Haematologic Technologies Inc., Essex Junction, VT) were obtained from the indicated vendors. Phospholipid (PL) vesicles containing 10% phosphatidylserine, 60% phosphatidylcholine, and 30% phosphatidylethanolamine

were prepared as previously described [23].

Plasma samples - Whole blood samples from patients with PS-def (n=2), PC-def (n=1), and APS (n=2, LA positive), and healthy individuals (n=35) in our hospital were collected into plastic tubes containing 3.2% sodium citrate at a ratio of 9:1 (Fuso Pharmaceutical Industries, Osaka, Japan) or into tubes containing hirudin (final concentration: f.c. 25 μg/mL, Roche Diagnostics, Rotkreuz, Switzerland). Inclusion criteria was defined as follows; i) patients who had been diagnosed already in our hospital, ii) patients with no other complicated disease, iii) patients who had taken no medication that might have influenced platelets or coagulation function two weeks prior to blood sampling. Platelet-poor plasma was separated by centrifugation of the citrated whole blood for 10 minutes at 1,500 *g*. All plasma samples were stored at -80°C and thawed at 37°C immediately prior to assays. Pooled normal plasma (PNP) samples were mixed with individual plasma samples (n=20).

Construction, expression, and purification of rFV mutants - As the PC pathway-related thrombosis, FV abnormality is one of the famous congenital thrombophilia [24]. FV_{Leiden} (R506Q) is a well-known congenital thrombophilia in Caucasian [24], and FV_{Nara} (R1920W) is a recently reported congenital thrombophilia in Japan [25]. These rFV mutants needed to be created, since the untreated plasma samples could not be obtained from patients carrying these mutations. To solve this, the piggyBac transposon system was used to express FV wild-type (WT) and mutants, as described previously [26]. rFV-WT and mutated FV-R506Q and FV-W1920R were stably expressed in HEK293T cells and purified [26]. The resultant FV forms were typically >90% in purity as determined by SDS polyacrylamide gel electrophoresis with albumin being the major contaminant (data not shown). The FV abnormality model plasma was prepared by adding each of rFV preparations (WT, R506Q, and R1920W mutations) *in vitro* to the commercial FV-deficient plasma.

TM-mediated clot waveform analysis (CWA) - CWA [12,13] was performed on an ACL-TOP® instrument (Instrumental Laboratory) using an rTF-trigger reagent together with rTM. For the procedure, 90 μL plasma sample was mixed with 10 μL rTM or buffer. This mixture sample was preincubated for 3 min with 30 μL of trigger reagent of rTF and PL (f.c. 1 pM and 12 μM, respectively), prior to the addition of 30 μL $CaCl_2$ (10 mM) to initiate coagulation. The automated coagulation analyser detected the intensity of absorbance, and the resulting clot reaction curves were computer-processed using the commercial kinetic algorithm provided by the manufacturer.

The first derivative of the clot reaction curve identified the coagulation velocity at each time point. The 'peak value' of the first derivative curve was calculated as an indicator of coagulation potential achieved. The ratio (with rTM/without rTM) of the peak parameter with and without the addition of rTM (termed as the peak ratio) was expressed as an evaluation of the PC pathway potential.

Data analyses - Experiments were performed at least three times, and the mean, median, and standard deviation (SD) are shown. Since the distribution of test results in some plasma samples was asymmetrical and did not show a normal distribution, Mann-Whitney *U*-test or Kruskal-Wallis test was used to compare the results between different plasma samples. Statistical significance was set at $P < 0.05$. The statistical software 'EZR' (Easy R) was used for statistical calculations.

Results

rTM-mediated CWA for detecting the PC pathway abnormality and the cut-off value of peak ratio

We first performed the rTM-mediated TF-triggered CWA using PNP, and the clot reaction curves and first derivative curves with and without rTM are illustrated in **Figure 1**. The optimal concentration of rTF and PL were determined as described in Methods, based on the results by the addition of various concentrations of rTF (0.5-5 pM) or PL (2-40 μM) (**Supplemental Figure 1**), together with earlier reports on the measurement conditions on TGA [17,18] and CWA [27], to be the maximum effect of rTM (as described below; peak ratio ~0.35). The addition of rTM significantly decreased the maximum absorbance of the clot reaction curve and the peak height (peak) of the first derivative curve. Under these conditions, fibrin formation was markedly suppressed when rTM was added, resulting in a marked decrease in the maximum absorbance and the peak value of the second derivative curve (data not shown). As a result, since the reaction curve was not sufficiently drawn, the evaluation of second derivative curve was precluded in the present study.

To examine the optimal concentration of rTM in rTM-mediated TF-triggered CWA, the peak values with various concentrations of rTM, and the "peak ratio" as (the peak with rTM)/(peak without rTM) were used (**Figure 2A**). The peak value decreased in a rTM dose-dependent manner. Likewise, the peak ratio decreased with a decline in the peak values. From the obtained result, since the peak ratio with rTM at 20 nM (~0.35) in the PNP sample was low enough to detect abnormal PC pathway function, by referring to the cut-off values of other conventional evaluation methods [8], 20 nM

rTM was used in subsequent studies.

The intra-assay coefficient of variation (CV) was calculated using five replicate PNP samples. All parameters of peak value (−rTM), peak value (+rTM), and peak ratio had good intra-assay %CVs of <10% (2.1%, 8.2%, and 8.6%, respectively). In addition, inter-assay %CVs were calculated using a single sample measured six times on different days, resulting in good inter-assay %CVs of <15% (2.6%, 11.6%, and 10.8%, respectively).

We performed rTM-mediated CWA using individual plasma from 35 healthy controls and confirmed the normal range of peak value and peak ratio (**Figure 2B**). The peak values without and with rTM, and the peak ratio in healthy plasma samples (n=35) was 109.2±21.6 mAbs/s, 40.4±19.5 mAbs/s, and 0.36±0.13, respectively. In the Thrombopath® assay using the ACL-TOP™, Toulon et al.[9] determined the mean±SD value obtained from 30 healthy control samples as a cut-off value for the parameter. The instruction manual of Thrombopath® describes that the mean±SD of approximately 20 cases of normal plasma could be set as the cut-off value. Therefore, we determined the cut-off value of the peak ratio to 0.49, based on the mean+SD of healthy plasma samples. Therefore, in the rTM-mediated CWA, the plasma sample with a peak ratio >0.49 was considered to possess the possibility of a PC pathway abnormality because of the lack of anticoagulant effect by the presence of rTM.

rTM-mediated CWA on various commercial plasmas with the PC pathway abnormality

First, commercial plasma samples with PS-def (n=5), PC-def (n=4), FVIII-def spiked by high rFVIII level (n=4), and APS (n=2), which are the representative thrombophilia samples that could exhibit PC pathway abnormalities, were assessed by rTM-mediated CWA. The median peak values prior to the addition of rTM were significantly higher in PS-def (185.0 mAbs/s, p=0.005) and PC-def (174.0 mAbs/s, p=0.02) than in the healthy controls (109.2 mAbs/s). The median peak value (141.0 mAbs/s) in high-FVIII plasma that was prepared by the addition of rFVIII (300 IU/dL) to FVIII-def plasma was higher than that in FVIII-def plasma (92.6 mAbs/s), but not significantly different from that in healthy controls. The median peak value in APS (91.4 mAbs/s) was similar to that in the healthy controls (**Figure 3**).

The addition of rTM resulted in a slight decrease in the peak value in plasmas with PS-def or PC-def. The peak ratios in all samples by the addition of rTM (0.98±0.02; p=0.005 and 0.79±0.08;

p=0.02, respectively) were significantly higher than the cut-off value. The peak ratio in FVIII-def plasma was 0.32±0.05, suggesting no significant difference from that in healthy controls. In those reconstituted with high levels of FVIII, however, some samples (highest value: 0.51) exceeded the cut-off value, but the median value was lower than the cut-off value. APS plasma has also been reported to exhibit PC pathway abnormalities [28-31], and the peak ratio in APS was high (0.98±0.07) and exceeded the cut-off value in all samples (**Figure 3**).

Effect of PC, PS, and FVIII on the peak ratio in rTM-mediated CWA

Next, we examined the impact of PC, PS, and FVIII on the peak ratio in an rTM-mediated CWA. PS-def or PC-def plasmas were mixed with PNP, and the PS or PC concentrations were adjusted to 0, 25, 50, 75, and 100% (PNP), followed by the rTM-mediated CWA measurement. Plasmas with PS or PC concentrations of 50% or less were beyond the cut-off value in the peak ratio (**Figure 4A, 4B**), suggesting that rTM-mediated CWA under the present conditions was able to distinguish the plasma samples with PS or PC concentrations below 50% and normal controls. In contrast, in FVIII-def plasma added rFVIII (0, 37.5, 75, 150, and 300 IU/dL), the peak ratio tended to increase in a FVIII dose-dependent manner (0.38±0.07, 0.36±0.05, 0.38±0.07, and 0.46±0.05, respectively), but none of the median values exceeded the cut-off value (**Figure 4C**).

rTM-mediated CWA in our patients with PS-def, PC-def, and APS

To further evaluate this screening assay, rTM-mediated CWA using plasma samples (PS-def, PC-def, and APS) from untreated patients with thrombophilia who visited our hospital was performed (**Figure 5**). We did not examine high-FVIII samples because they were not available in clinical practice. The peak ratios in the plasmas with two PS-def (PS activity <10%, 30%), PC-def (PC activity 60%), and two APS were 0.95, 0.73, 0.31, 0.81, and 0.74, respectively, which supports the validity of this assay.

rTM-mediated CWA in FV abnormality

FV abnormality is a representative coagulation disorder that exhibits PC pathway abnormalities. FV-R506Q (FV_{Leiden}), which shows APC resistance, is one of the most common types of thrombophilia in the USA and Europe [24]. FV-W1920R (FV_{Nara}) is also a FV abnormality reported by our group in Japan [25]. Therefore, we examined whether rTM-mediated CWA could detect these FV abnormalities.

We performed rTM-mediated CWA using FV-deficient plasma samples containing 6.25, 25, 100, and 400 IU/dL of commercial plasma-derived (pd-)FV preparations (**Figure 6A**). Samples with 0% FV activity showed coagulation potential because of a complete lack of FV procoagulant function. In rTM-mediated CWA, coagulation potential was obtained at FV 6.25% and above, and FV concentration-dependent decrease in peak value because of an anticoagulant effect of FV was observed, resulting in dose-dependent decreases in the peak ratio (0.67, 0.56, 0.39, and 0.34, respectively).

Next, we performed rTM-mediated CWA by adding rFV (WT, R506Q, and W1920R) expressed in HEK-293 cells to FV-def plasma [26]. Preliminary experiments confirmed that the peak values and peak ratio values in FV-def plasma with 0.5 IU/mL of pd-FV and FV-WT were similar (peak without rTM; 130.0 and 128.3 mAbs/s, peak ratio; 0.43 and 0.45, respectively). The peak values and peak ratios in FV-def with 0.4 IU/ml, FV-WT, FV-R506Q, and FV-W1920R were 132.3, 126.0, and 140.7 mAbs/s, respectively, and 0.47, 0.91, and 0.92, respectively. The peak ratios in FV-R506Q and FV-W1920R were much higher than the cut-off value, and both FV mutants suggested a similar level of rTM-induced APC resistance (**Figure 6B**).

Discussion

Screening assays to detect PC pathway abnormalities, a major cause of thrombophilia, have been developed. An aPTT-based clotting time assay, such as ProC®Global, reflects only a single parameter such as the clotting time [6,7], whereas TGA and CWA reflect the entire process of thrombin and clot formation and obtain many parameters in addition to clotting time [32]. TGA, a widely used assay to evaluate PC pathway impairment, evaluates global coagulation potential based on thrombin generation but not total fibrin formation [8,9]. Thrombopath® was used to assess the endogenous function of APC induced by protac but was discontinued. This assay is technically demanding and is generally restricted to expert laboratories, and thrombin substrate for thrombin generation is needed. While, CWA is developed as an automated, global coagulation technique for use in routine clinical laboratories during aPTT/PT measurement, thereby being an easy-to-use assay without the substrate use [32]. The clinical applications of CWA for patients with coagulation disorders are available. In the present study, we successfully established a novel assay to evaluate PC pathway abnormalities using rTM-mediated CWA by the TF/PL-trigger. This was based on the physiological activation of endogenous PC by adding rTM [21] and on the comprehensive coagulation function potentials, not clotting time, by analysing real-time clot waveforms [12,13,32].

Human soluble rTM (Recomodulin®) was developed as an anticoagulant agent and is clinically used as an anti-thrombotic and/or anti-inflammatory drug [22]. Similar to thrombomodulin, rTM has two modes of thrombin-inhibitory action, direct or indirect, and the latter is via PC activation [22]. However, rTM concentration that is required for direct thrombin inhibition is fifty-fold higher than that for PC activation [22], and the plasma concentration of rTM in clinical settings (4.7 to 22 nM) [33], does not reach the level of the direct function. Therefore, we assumed that the main anticoagulation effects of rTM in this assay were achieved by PC activation. In rTM-mediated CWA, fibrin formation was depressed in a rTM dose-dependent manner and the peak value was decreased, but the peak ratio was higher in thrombophilia related to the PC pathway abnormality.

Patients with PC-def and PS-def exhibited absolute APC resistance. An earlier report using Thrombopath® [8] demonstrated that the Protac-induced coagulation inhibitor percentages (PICI%) in plasmas with PS-def or PC-def were significantly lower than those of the healthy controls, suggesting the usefulness of Thrombopath® to identify PC pathway abnormalities [8]. The rTM-mediated CWA similarly revealed that the peak values in plasmas with PS-def and PC-def were higher than those in normal controls, and the peak ratios were significantly higher, reflecting the abnormal PC pathway, and exceeding the cut-off value in all samples with PC-def and PS-def.

High levels of FVIII:C (>150 IU/dL) are reported to be risk factors for thrombosis [34,35]. Toulon et al. [8] reported that the PICI% value in plasmas with high levels of FVIII:C (100 to 300 IU/dL, especially >250 IU/dL) was decreased by Thrombopath®, indicative of the relative PC pathway abnormality. In our results, the rTM-mediated CWA revealed no significant difference from FVIII-def plasma spiked with FVIII:C 300 IU/dL compared to the controls, but there were some samples that exceeded the cut-off value, suggesting that high FVIII levels appear to be assessed as a relatively PC pathway abnormality.

There are increasing reports of APS patients exhibiting APC resistance, and APC resistance seems to be associated with thrombogenicity in APS patients [30,31]. The effect of antiphospholipid antibodies (aPL antibodies) found in APS patients on the PC pathway involves differences in aPL subtypes [31]. An anti-β2GPI, which exhibits LA, competes with APC for phospholipid binding, thereby conferring APC resistance [36]. Antibodies against PC and PS exhibit APC resistance by reducing plasma PC and PS levels [37,38], and anti-prothrombin antibodies also exhibit APC

resistance by reducing plasma prothrombin levels [39]. Katayama et al. [40] reported that the peak value of the velocity curve decreased and the time to peak velocity curve was prolonged in LA-positive plasma in the aPTT-based assay using ACL-TOP®. In the present study, the peak value in APS was lower and the time to reach peak value was prolonged (data not shown), although no significant difference was observed, possibly because of the small number of samples. However, the peak ratio exceeded the cut-off value in all APS samples, showing trends similar to those of previous reports [8,28,29].

Activated FV (FVa) and FXa promote thrombin generation (procoagulant function). FVa is proteolytically cleaved by APC at three sites, including R506, to form inactivated FVa, which loses its procoagulant activity [41]. In contrast, when FV is cleaved at R506 by APC, it shows anticoagulant activity as a cofactor of APC (anticoagulant function) [42]. FV_{Leiden} (R506Q) shows a thrombotic tendency because of APC resistance [43]. Some FV abnormalities with other mutations showing APC resistance have been recently reported [25,44]. The rTM-mediated CWAs could observe the balancing of procoagulant and anticoagulant potential that was affected by FV:C levels. In addition, a representative FV-R506Q and FV-W1920R showed high peak ratios, again confirming the APC resistance. The rTM-mediated CWA is very useful for detecting FV abnormalities with APC resistance.

There are some limitations to the present study. Similar to a previous report in the ProC®Global study [7], the high peak ratio in this assay may reflect not only the absolute defective PC pathway function, but also the relative abnormalities of the PC pathway because of the increased coagulation potential between the balancing of coagulation-anticoagulation system. For example, a high fibrinogen level (>5 g/L) might be associated with a 4-fold risk of thrombosis [45], and hyperprothrombinemia is a potential APC inhibitor [46]. In the present study, we did not measure fibrinogen or prothrombin concentrations in these plasma samples. The addition of rTM instead of Protac has the potential to detect thrombophilia because of PC or prothrombin molecular abnormality showing TM resistance [11], but it would be difficult to detect abnormalities in endogenous TM itself [10].

In summary, rTM-mediated CWA, based on global coagulation function by conventional PT clotting assay, could be a candidate for an easy and quick-to-use assay to screen for representative thrombophilia related to PC pathway abnormalities in clinical practices, instead of the measurement

of APC resistance and PC/PS activity with coagulometric and/or chromogenic methods. Further investigation with a large number of patients with thrombophilia is needed to clarify the sensitivity and specificity of the current assay.

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Authorship

N.H. performed all experiments, analysed the data, interpreted the data, made the figures, and wrote the manuscript. **K.O.** designed the experiments, supported clinically, analysed the data, interpreted the data, edited the manuscript, and approved the submission of the first version. **N.S.**, **T.N.** and **Y.N.** supported technically, and interpreted the data. **S.F.** and **M.T.** supported clinically, interpreted the data. **K.N.** designed the experiments, supported clinically, interpreted the data, made figures, wrote the manuscript and edited the manuscript.

Conflicts of interests

The authors declare that they have no conflicts of interest.

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Figure Legends

Figure 1. Recombinant thrombomodulin-mediated tissue factor-triggered clot waveform analysis

Pooled normal plasma was incubated with recombinant tissue factor (rTF) and phospholipid (PL) vesicles, followed by the addition of $CaCl_2$ together with recombinant thrombomodulin (rTM) as described in the Methods. Changes in absorbance were recorded over time during the clot reaction (clot reaction curve). The curve from the first derivative (dT/dt) of the obtained clot reaction curve shows the coagulation velocity. The peak values of the first derivative curve with and without rTM are shown, and the peak ratio was calculated as the peak value with rTM/peak value without rTM.

Figure 2. rTM dose-dependent reaction and set of cut-off value on the rTM-mediated CWA

(A) rTM dose-dependent reaction. Pooled normal plasma (PNP) was incubated with rTF (1 pM) and PL vesicles (12 μM), followed by the addition of $CaCl_2$ together with rTM (0-40 nM). The peak value without (*open square*) and with (*closed square*) rTM was observed and the peak ratio (*closed circle*) was calculated as described in Methods.

(B) Cut-off value of peak ratio in healthy control plasmas. PNP and healthy individual plasmas (n=35) were incubated with rTF (1 pM) and PL vesicles (12 μM), followed by the addition of $CaCl_2$ without or with rTM (20 nM). The peak value without (*white column*) and with (*gray column*) rTM was shown and the peak ratio (*closed circle*) was calculated. A cut-off value (0.49) was set to the +1SD of mean value in healthy controls.

Figure 3. rTM-mediated CWA using various types of commercial plasmas with PC pathway abnormality

Commercial plasma with PS deficiency (PS-def), PC-def, FVIII-def, FVIII-def spiked with FVIII (300 IU/dL), and APS were incubated with rTF (1 pM) and PL vesicles (12 μM), followed by the addition of $CaCl_2$ with or without rTM (20 nM). The peak value without (*white column*) and with (*gray column*) rTM is shown, and the peak ratio (*closed circle*) was calculated. A cut-off value of 0.49 was set to +1SD of the mean value in healthy controls.

Figure 4. Impacts of PS, PC, and FVIII concentrations on rTM-mediated CWA

PS-def plasma (**A**) and PC-def plasma (**B**) were mixed with pooled normal plasma (PNP) to adjust to the final concentrations of 0, 25, 50, 75, and 100% of PS and PC levels, respectively. FVIII-def plasma (**C**) was mixed with rFVIII (0-300 IU/dL). The mixture samples were incubated with rTF (1

pM) and PL vesicles (12 μM), followed by assessment of rTM-mediated CWA. The peak values without (*white column*) and with (*gray column*) rTM (20 nM) were obtained, and the peak ratio (*closed circle*) was calculated. The *dotted line* shows the cut-off value (0.49) for the normal controls.

Figure 5. rTM-mediated CWA on the patient-derived plasmas with PC pathway abnormality

Two patient-derived PS-def plasmas (PS activity <10% and 30%), patient-derived PC-def plasma (PC activity 60%), and plasma samples from two patients with APS (LA positive) were incubated with rTF (1 pM) and PL vesicles (12 μM), followed by assessment of rTM-mediated CWA. The peak values without (*white column*) and with (*gray column*) of rTM (20 nM) were obtained, and the peak ratio (*closed circle*) was calculated. The dotted line shows the cut-off value (0.49) for the normal controls.

Figure 6. rTM-mediated CWA on FV-def plasma with various amounts of FV or with rFV mutants with APC resistance

FV-def plasma mixed with (**A**) plasma-derived FV (0-400 IU/dL) or with (**B**) rFV (FV-WT, FV-R506Q, and FV-W1920R; 40 IU/dL) was incubated with rTF (1 pM) and PL vesicles (12 μM), followed by assessment of rTM-mediated CWA. The peak values without (*white column*) and with (*gray column*) rTM (20 nM) were obtained, and the peak ratio (*closed circle*) was calculated. The dotted line shows the cut-off value (0.49) for the normal controls.

Supplemental Figure 1. rTF or PL dose-dependent reaction on the rTM-mediated CWA

(A) rTF dose-dependent reaction. Pooled normal plasma (PNP) was incubated with rTF (0.5-5 pM), PL vesicles (4 μM), followed by the addition of $CaCl_2$ together with rTM (20 nM).

(B) PL dose-dependent reaction. PNP was incubated with rTF (1 pM), PL vesicles (2-40 μM), followed by the addition of $CaCl_2$ together with rTM (20 nM). The peak value without (*open squares*) and with (*closed squares*) rTM was observed and the peak ratio (*closed circles*) was calculated as described in Methods.

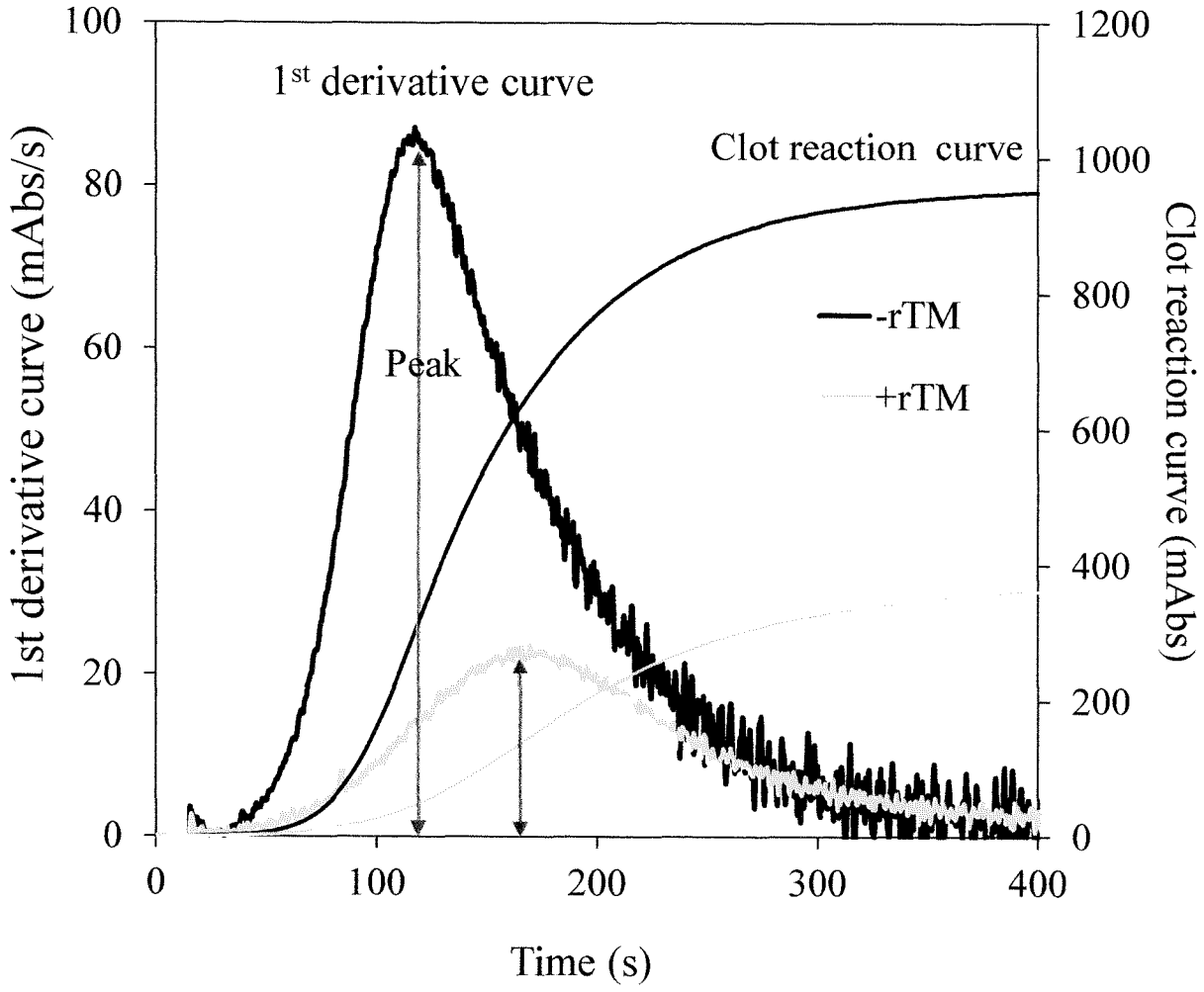

1st derivative curve
Clot reaction curve
Peak
-rTM
+rTM
1st derivative curve (mAbs/s)
Clot reaction curve (mAbs)
Time (s)
100
80
60
40
20
0
1200
1000
800
600
400
200
0
0
100
200
300
400

Figure 1

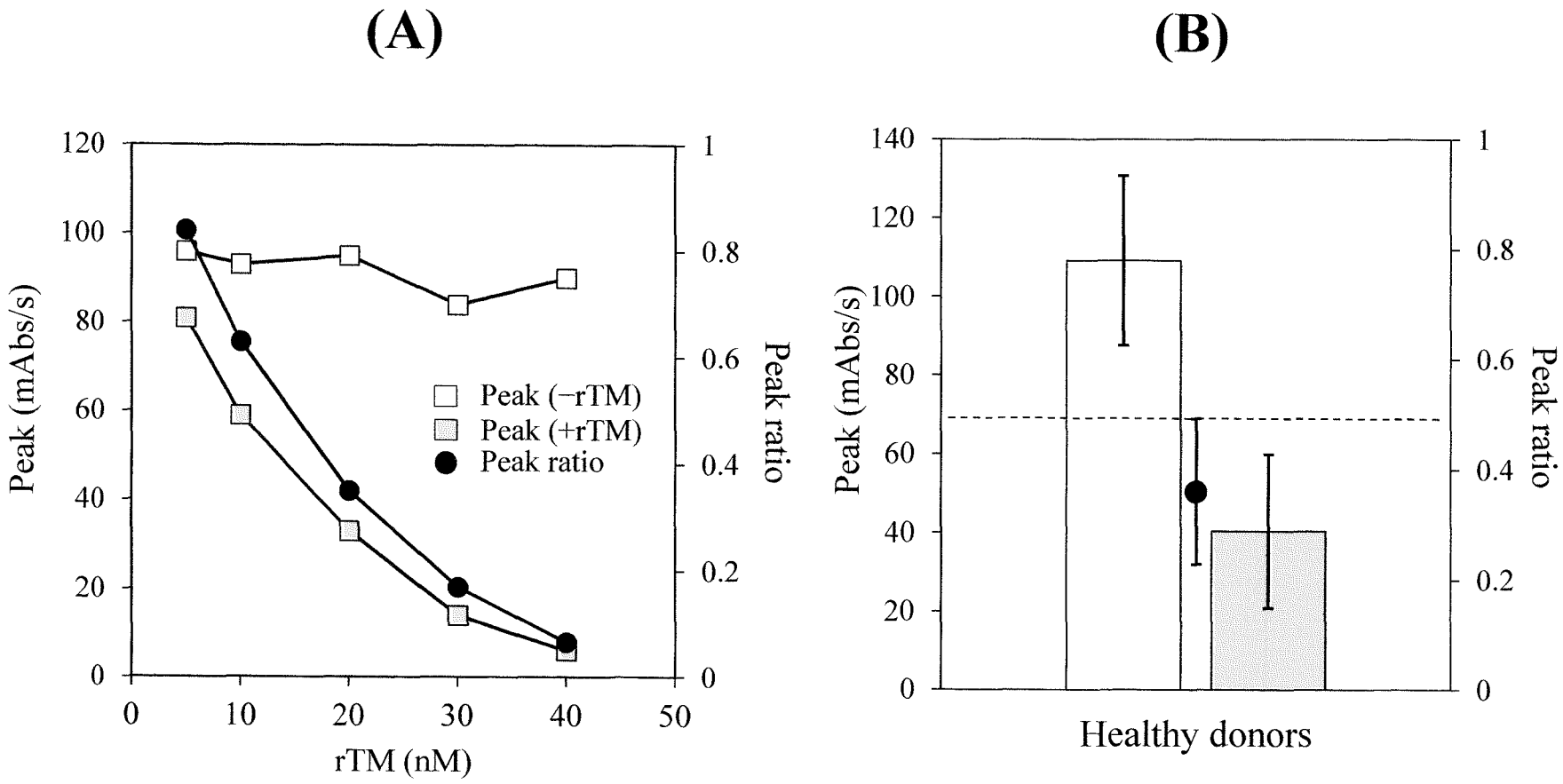

(A)
(B)
120
100
80
60
40
20
0
Peak (mAbs/s)
1
0.8
0.6
0.4
0.2
0
Peak ratio
Peak (−rTM)
Peak (+rTM)
Peak ratio
0
10
20
30
40
50
rTM (nM)
140
120
100
80
60
40
20
0
Peak (mAbs/s)
1
0.8
0.6
0.4
0.2
0
Peak ratio
Healthy donors

Figure 2A,B

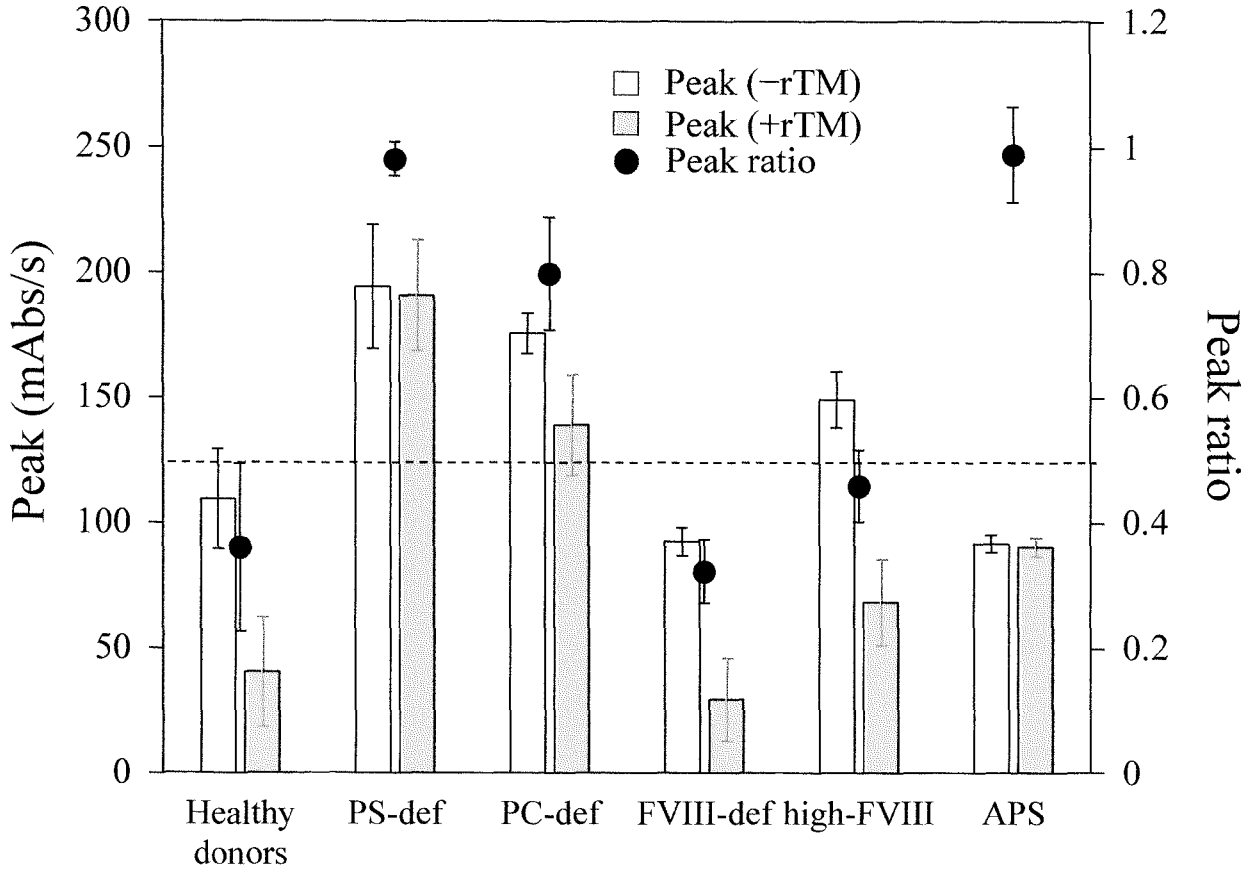

300
250
200
150
100
50
0
Peak (mAbs/s)
1.2
1
0.8
0.6
0.4
0.2
0
Peak ratio
Peak (−rTM)
Peak (+rTM)
Peak ratio
Healthy donors
PS-def
PC-def
FVIII-def
high-FVIII
APS

Figure 3

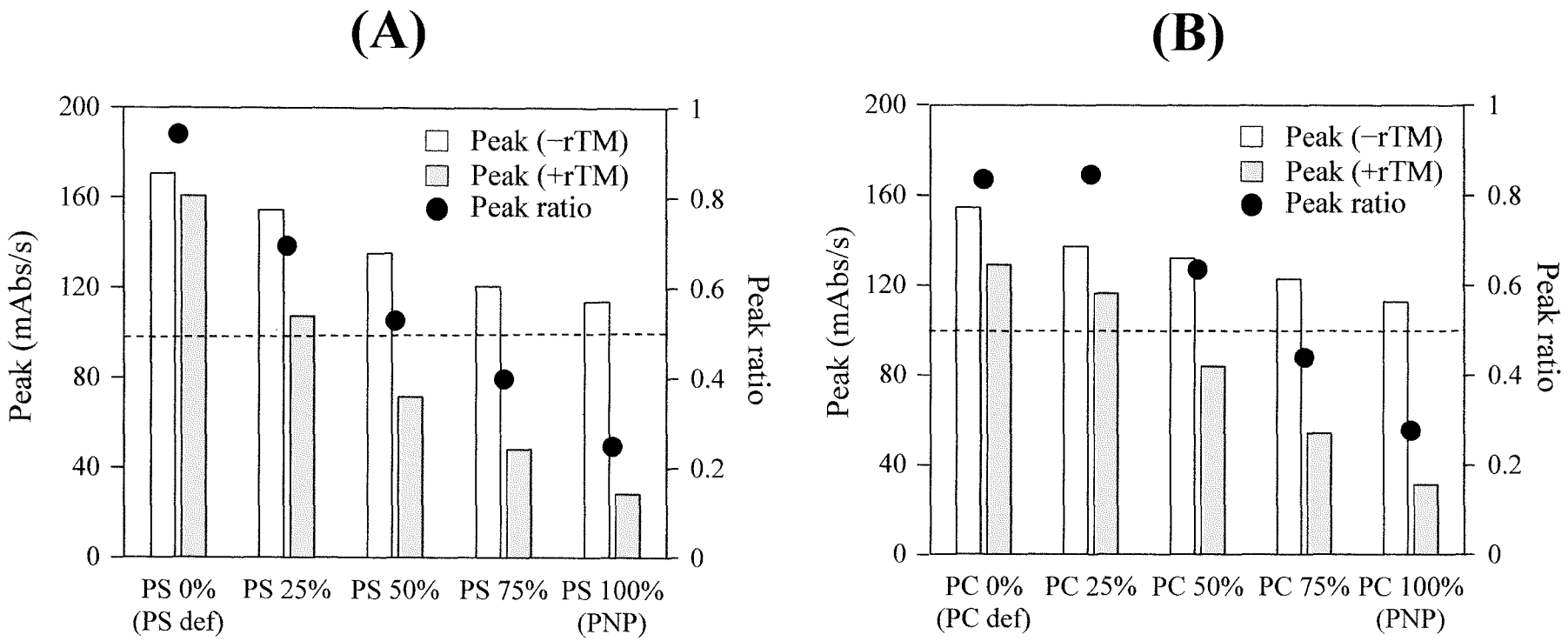

(A)
(B)
Peak (mAbs/s)
Peak ratio
Peak (−rTM)
Peak (+rTM)
Peak ratio
200
160
120
80
40
0
1
0.8
0.6
0.4
0.2
0
PS 0% (PS def)
PS 25%
PS 50%
PS 75%
PS 100% (PNP)
PC 0% (PC def)
PC 25%
PC 50%
PC 75%
PC 100% (PNP)

Figure 4A,B

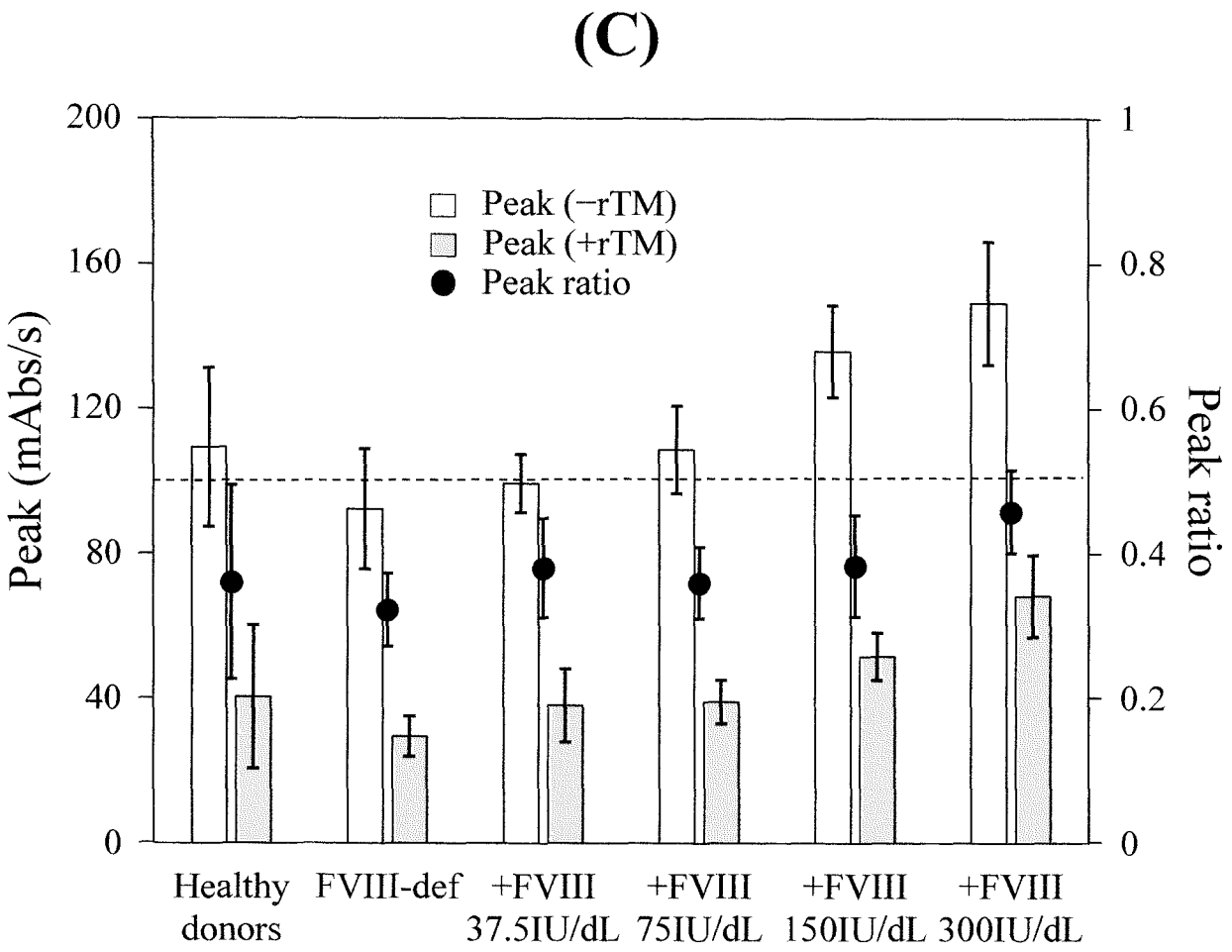

(C)
200
160
120
80
40
0
1
0.8
0.6
0.4
0.2
0
Peak (mAbs/s)
Peak ratio
Peak (−rTM)
Peak (+rTM)
Peak ratio
Healthy donors
FVIII-def
+FVIII 37.5IU/dL
+FVIII 75IU/dL
+FVIII 150IU/dL
+FVIII 300IU/dL

Figure 4C

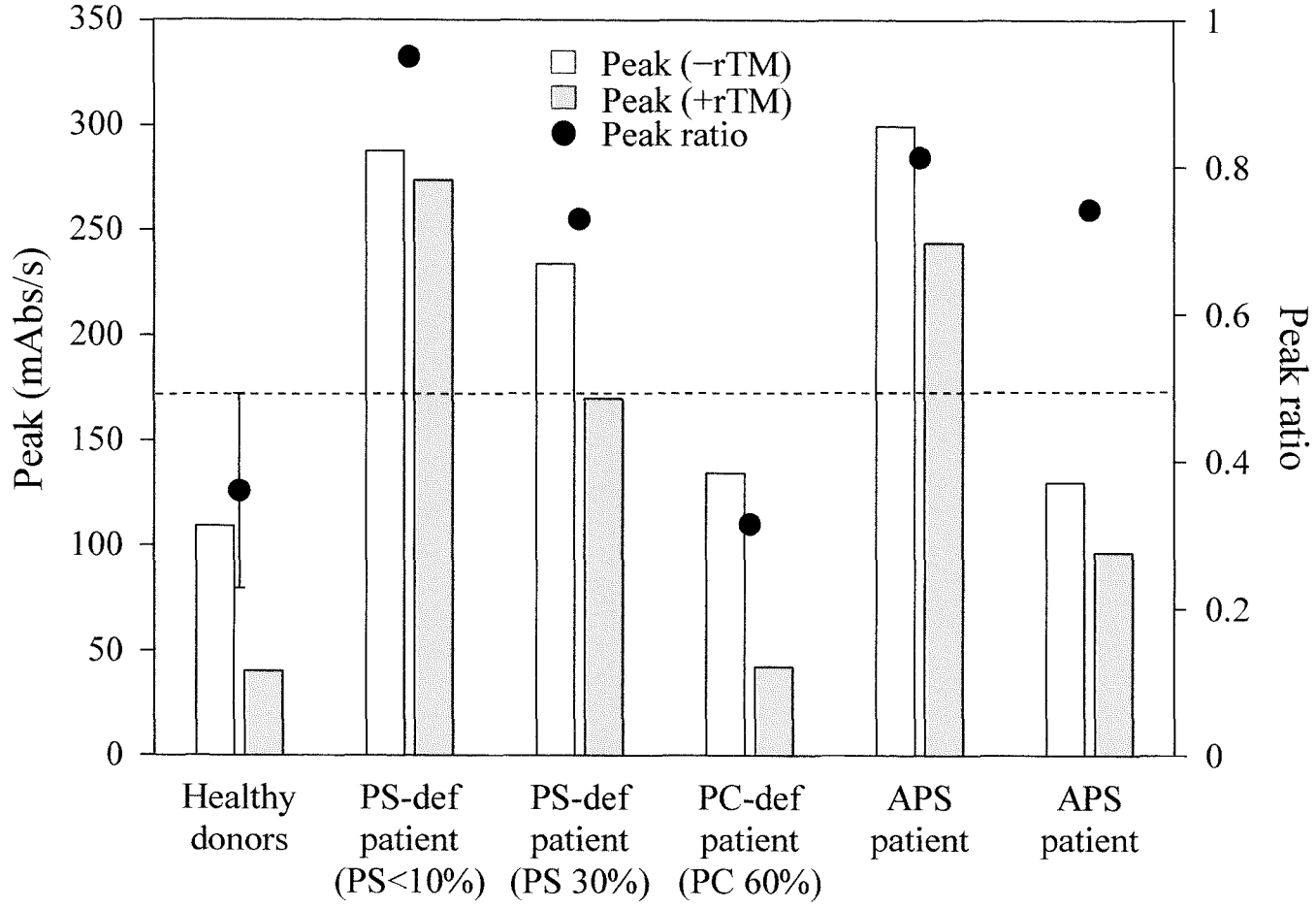

Peak (−rTM)
Peak (+rTM)
Peak ratio
Peak (mAbs/s)
Peak ratio
350
300
250
200
150
100
50
0
1
0.8
0.6
0.4
0.2
0
Healthy donors
PS-def patient (PS<10%)
PS-def patient (PS 30%)
PC-def patient (PC 60%)
APS patient
APS patient

Figure 5

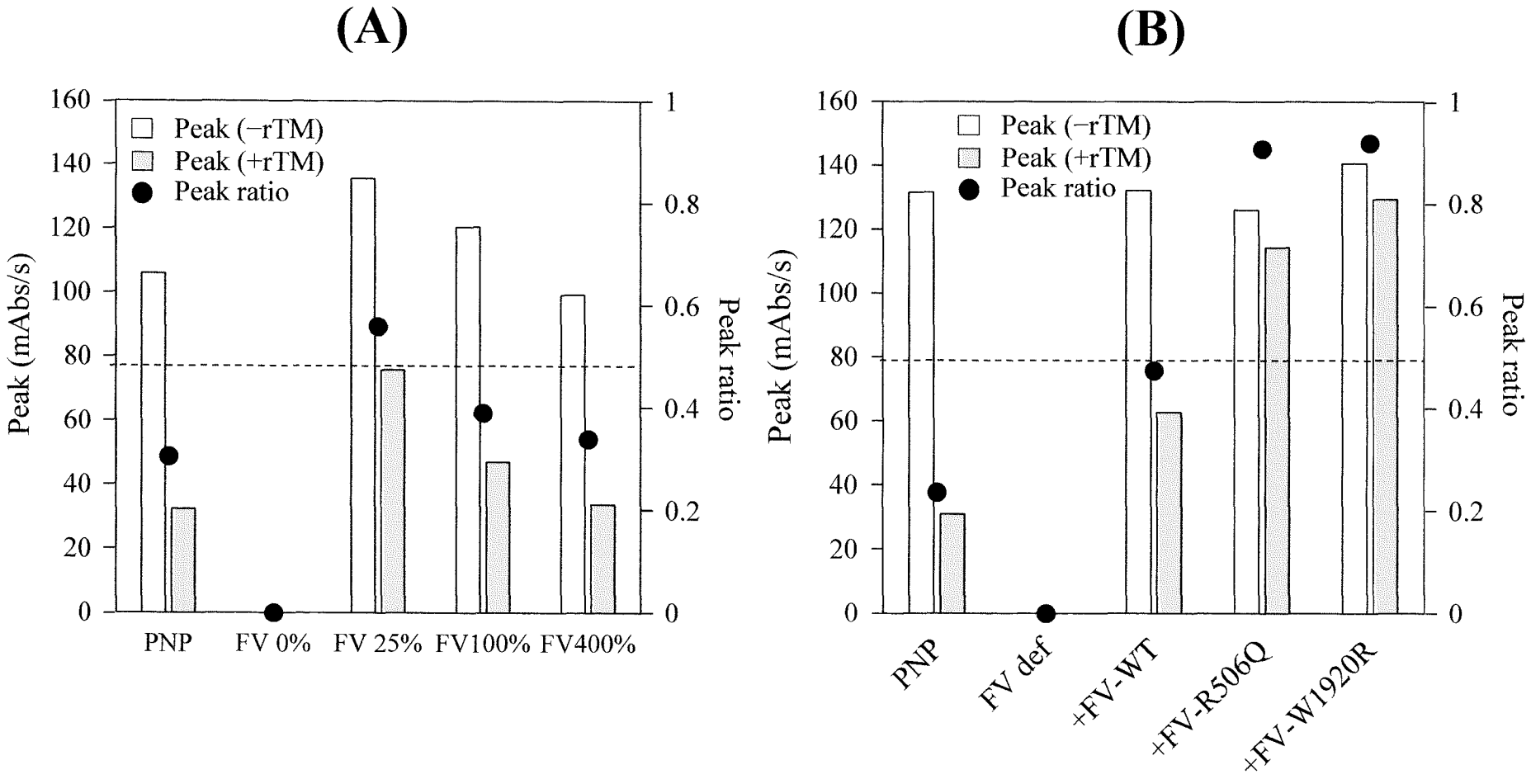
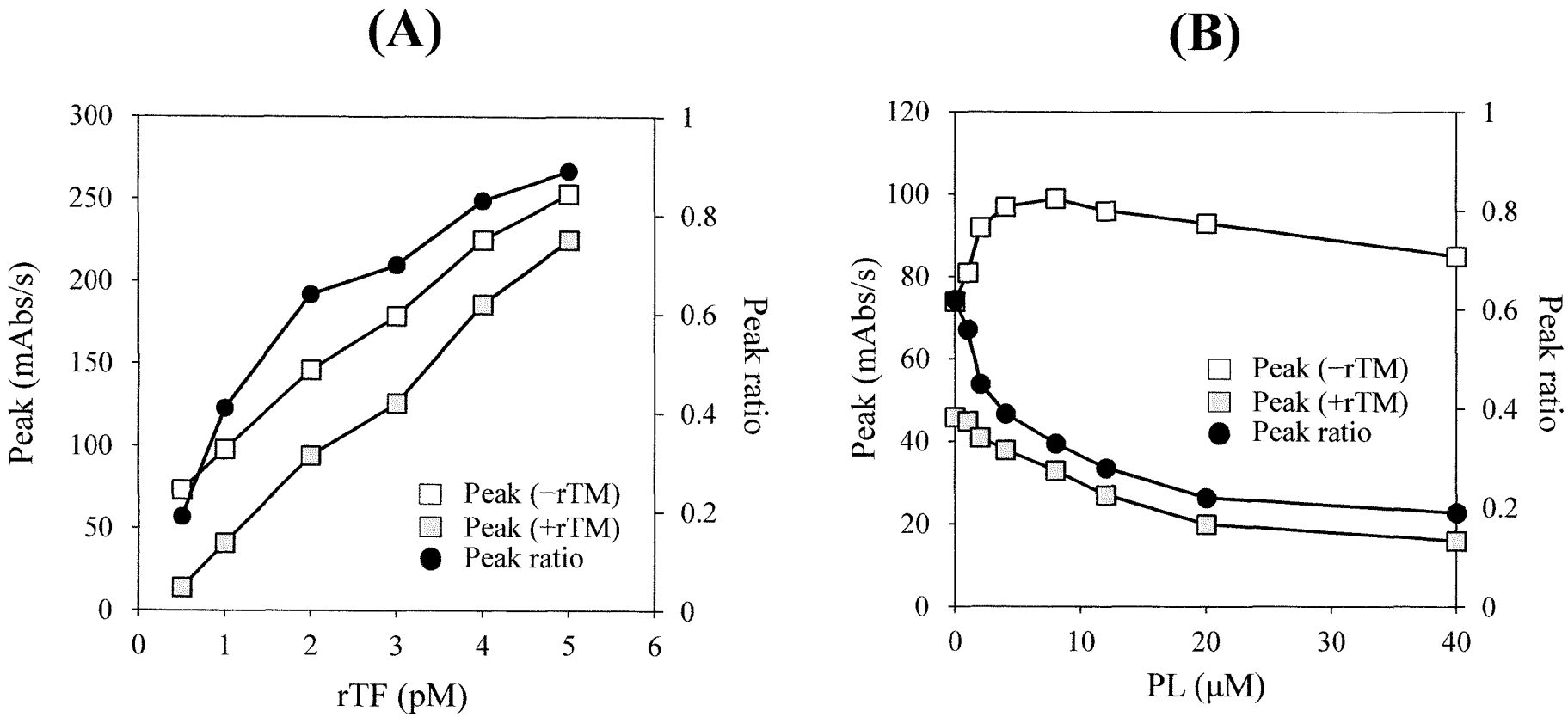

(A)
(B)
Peak (−rTM)
Peak (+rTM)
Peak ratio
Peak (mAbs/s)
Peak ratio
160
140
120
100
80
60
40
20
0
1
0.8
0.6
0.4
0.2
0
PNP
FV 0%
FV 25%
FV100%
FV400%
FV def
+FV-WT
+FV-R506Q
+FV-W1920R

Figure 6A,B



Supplemental Figure 1