A pathological clarification of sepsis-associated disseminated intravascular coagulation based on comprehensive coagulation and fibrinolysis function

Tomoko Onishi,[1] Keiji Nogami,[1] Takashi Ishihara,[1] Satoki Inoue,[2] Masahiko Kawaguchi,[2] Kenji Nishio,[3] Hidetada Fukushima,[4] Hiroshi Kobayashi,[5] Itsuto Amano,[6] Toshiya Nishikubo,[7] Masaharu Yamasaki,[8] Masato Kasahara,[9] Midori Shima[1]

Department of [1]Pediatrics, [2]Anesthesiology, [3]General Medicine, [4]Emergency and Critical Care Medicine, [5]Obstetrics and Gynecology, [6]Second Department of Internal Medicine, [7]Neonatal Intensive Care Unit, [8]Division of Clinical Laboratory, [9] Clinical Research Center, Nara Medical University, Kashihara, Nara, Japan

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Corresponding Author:
Keiji Nogami, M.D., Ph.D.
Department of Pediatrics, Nara Medical University,
840 Shijo-cho, Kashihara, Nara 634-8522, Japan.
Tel.: +81-744-29-8881; Fax.: +81-744-24-9222
E-mail: roc-noga@naramed-u.ac.jp

Abstract

Introduction*:** The functional dynamics of coagulation and fibrinolysis in patients with disseminated intravascular coagulation (DIC) vary due to the pathology and severity of various underlying diseases. Conventional measurements of hemostasis such as thrombin-antithrombin complex, plasmin-α2-plasmin-inhibitor complex, and fibrinogen-fibrin degradation products may not always reflect critical pathophysiologic mechanisms in DIC. ***Aim: To clarify the pathology of sepsis-associated DIC using assessment of comprehensive coagulation and fibrinolysis. ***Method*:** Plasma samples were obtained from 57 patients with sepsis-associated DIC at the time of initial diagnosis. Hemostasis parameters were quantified by clot-fibrinolysis waveform analysis (CFWA) and thrombin/plasmin generation assays (T/P-GA). The results were expressed as ratios relative to normal plasma. ***Results:*** CFWA demonstrated that the maximum coagulation velocity (|min1|) ratio modestly increased to median 1.40 [min–max; 0.10–2.60] but the maximum fibrinolytic velocity (|FL-min1|) ratio decreased to 0.61 [0–1.19]. T/P-GA indicated that the peak thrombin (Th-Peak) ratio moderately decreased to 0.71 [0.22–1.20], whereas the peak plasmin (Plm-Peak) ratio substantially decreased to 0.35 [0.02–1.43]. Statistical comparisons identified a correlation between |min1| and Th-Peak ratios (ρ=0.55, P<0.001), together with a strong correlation between |FL-min1| and Plm-Peak ratios (ρ=0.71, P<0.001), suggesting that CFWA reflected the balance between thrombin and plasmin generation. With |min1| and |FL-min1| ratios, DIC was classified as follows; coagulation-predominant, coagulation/fibrinolysis-balanced, fibrinolysis-predominant, and consumption-impaired coagulation. The majority of patients in our cohort (80.7%) were coagulation-predominant. ***Conclusion*:** A pathological clarification of sepsis-associated DIC based on the assessment of coagulation and fibrinolysis dynamics may be useful for the hemostatic monitoring and management of optimal treatment in these individuals.

***Keywords*:** disseminated intravascular coagulation, sepsis, coagulation, fibrinolysis, monitoring

What is known on this topic?

- The functional dynamics of coagulation and fibrinolysis in patients with disseminated intravascular coagulation (DIC) vary due to the pathology and severity of underlying disease.
- Conventional measurements of hemostasis may not always reflect the precise pathogenesis of DIC.

What this paper adds?

- Comprehensive, functional coagulation and fibrinolysis was assessed in patients with sepsis-associated DIC using clot-fibrinolysis waveform analyses and thrombin/plasmin generation assays.
- Coagulation and fibrinolysis potentials correlated with both assays in patients with sepsis-associated DIC at onset.
- Most patients exhibited a coagulation-predominant state.
- A pathological clarification of sepsis-DIC based on comprehensive assessment of coagulation and fibrinolysis may be useful as a clinical aid in the clinical practice.

Introduction

Disseminated intravascular coagulation (DIC) is a grave acquired coagulopathy characterized by systemic and persistent activation of coagulation together with varying degrees of fibrinolytic activation induced by the primary coagulopathy. The pathogenesis of DIC is governed by underlying clinical disorders including sepsis, malignancy, aortic aneurysm, and acute pancreatitis.[1] The persistent activation of coagulation promotes the development of microthrombi in the systemic microvasculature, resulting in disordered hemodynamics, and if left unchecked can lead to multiple organ failure.[2,3] DIC is characterized by a consumption coagulopathy and progressive clinical deterioration, mediated by an acute loss of circulating hemostatic components including platelets and clotting factors.[4] The overall prognosis of DIC is poor. Mortality rates of 42.4% and 35.6% have been reported in prospective clinical studies utilizing the diagnostic criteria defined by the Japanese Ministry of Health and Labor and Welfare (JMHLW) and Japanese Association for Acute Medicine (JAAM), respectively.[5] Early diagnosis and therapeutic intervention are essential, therefore, but the variable rates and intensities of acceleration and deceleration of both coagulation and fibrinolytic mechanisms hamper the reliable assessment of coagulation profiles using conventionally available laboratory methods.

Sepsis is the most common underlying disease associated with DIC. Sepsis-associated DIC is characterized by fibrinolytic suppression and decreased solubility of microthrombi in the vasculature potentiating the development of multiple organ failure.[2,3] The persistent activation of coagulation is promoted by an aberrant expression of tissue factor (TF) synthesized and released by monocytes and damaged endothelial cells.[6-8] Simultaneously, the fibrinolytic inhibitory factor, plasminogen activator inhibitor-1 (PAI-1), is excessively produced by cytokine-activated endothelial cells, and consequently, fibrinolysis is profoundly suppressed.[9] However, the degree of fibrinolysis can vary, depending on the progression and/or complications associated with the underlying conditions including malignancy or aortic aneurysm. Moreover, this balance between coagulation and fibrinolysis is critical for preventing excessive thrombosis or hemorrhage, and an accurate and comprehensive assessment of coagulation and fibrinolytic potential is required to enable a clear understanding of the heterogeneity and variability that contributes to the pathogenesis of DIC. The selection of supportive anticoagulation or antifibrinolytic treatment could depend critically on the balance in each individual patient.[1,10-12] Current techniques used to examine these inter-dependent mechanisms are likely to be inadequate, especially those based on specific measurements of the thrombin–antithrombin complex (TAT) or the plasmin-α_2-plasmin inhibitor complex (PIC) levels

that reflect only a part of the entire spectrum of coagulation and fibrinolysis.[13-15]

We have recently established two global hemostatic assays that facilitate the simultaneous assessment of coagulation and fibrinolytic potential. The clot-fibrinolysis waveform analysis (CFWA) is a novel assay designed to concurrently monitor the potential for both clot formation and fibrinolysis.[16] The principle of this assay is modified from the original clot waveform analysis (CWA) technique, and centers on the use of a mixture of an activated partial thromboplastin time (APTT) reagent along with an optimal concentration of recombinant tissue-type plasminogen activator (r-tPA) to initiate a series of reactions that are completed in <500 sec (<8.33 min). Similarly, the thrombin/plasmin generation assay (T/P-GA) evaluates of both coagulation and fibrinolysis in patient plasma with a reagent that contains a specific mixture of TF at low concentration and r-tPA.[17] Thrombin and plasmin are end-product enzymes of the coagulation and fibrinolysis cascades, respectively, and an increase in thrombin levels indicates a hypercoagulable state, whereas an increase in plasmin levels reflects enhanced fibrinolysis. Consequently, physiologic homeostasis is achieved by an appropriate balance of thrombin and plasmin generation within the peripheral circulation. Both CFWA and T/P-GA provide relevant data for determining this critical balance of coagulation and fibrinolysis, although there are advantages and/or disadvantages with respect to clinical applicability with each technique. CFWA is an easy-to-use assay and is based on the widely used APTT, whereas T/P-GA requires technical expertise and is typically performed in specialized laboratories.[16,17] In addition, compared with the T/P-GA, the results of CFWA, may depend more on plasma fibrinogen concentrations.[16-18] The present study was designed, therefore, to assess these methods for measuring global coagulation and fibrinolysis in clinical practice, and to clarify the pathology of sepsis-associated DIC at initial diagnosis using these two hemostatic assays.

Materials and Methods

The study was approved by the Medical Research Ethics Committee of Nara Medical University (No.1359-2).

Reagents - Thrombocheck APTT-SLA® (Sysmex Corporation, Kobe, Japan), recombinant human TF (rTF; Innovin®, Dade, Marburg, Germany), r-tPA (Activacin®, American Diagnostica Inc., Stamford, CT), thrombin-specific fluorogenic substrate (Z-Gly-Gly-Arg-AMC; Bachem, Bubendorf, Switzerland), and plasmin-specific fluorogenic substrate (Boc-Glu-Lys-Lys-MAC; Peptide Institute Inc., Osaka, Japan) were purchased, and phospholipid (PL) vesicles containing 10 %

phosphatidylserine, 60 % phosphatidylcholine, and 30 % phosphatidylethanolamine (Sigma-Aldrich, St Louis, MO) were prepared as previously described.[19]

Patient selection - Patients included in the present study were initially diagnosed with sepsis-associated DIC at Nara Medical University Hospital in Japan between September 2016 and July 2019. The diagnosis of DIC was based on the JAAM, JMHLW and JSTH (Japanese Society on Thrombosis and Hemostasis) criteria.[20] Patients with non-sepsis-associated DIC (including DIC secondary to leukemia or aneurysm) were excluded from the present study. Since there was no study of DIC using CFWA or T/P-GA, it was difficult to calculate the required number of samples before the present study. The sample size of the present study was determined by the number of all patients who met the criteria from 2016 to 2019.

Blood samples - Blood samples were obtained after written informed consent from the patient or patient's family. Blood was obtained by venipuncture at the time of initial diagnosis and before the start of treatment. Whole blood samples were taken into plastic tubes containing 3.2 % sodium citrate at a 9:1 ratio, and platelet-poor plasma was recovered after centrifugation for 15 min at 1,500 $\times g$. Pooled normal plasma was prepared using samples from 20 healthy volunteers. All plasma samples were stored at −80 °C and thawed at 37 °C immediately prior to use.

Conventional laboratory parameters for DIC-related coagulation and fibrinolysis - The plasma levels of fibrinogen, plasminogen, α2-plasminogen inhibitor (α2PI), fibrinogen degradation products (FDP), D-dimer, thrombin–antithrombin complex (TAT; HISCLTM™ TAT Assay Kit; Sysmex); plasmin-α_2-plasmin inhibitor complex (PIC; HISCLTM™ PIC Assay Kit; Sysmex) and plasminogen activator inhibitor (PAI-1; Nanopia® PAI-1 Kit; Sekisui Medical, Tokyo, Japan) were measured using commercially available reagents.

Simultaneous clot-fibrinolysis waveform analysis (CFWA) - CFWA was performed on a CS-2000i™ instrument (Sysmex Corporation), to monitor the dynamic processes of fibrin formation and clot lysis as previously described.[16] Briefly, plasma samples (50 μL) were mixed with the APTT-systemic lupus anticoagulant reagent (APTT-SLA; 50 μL) and activated with 50 μL of a solution containing $CaCl_2$ together with optimized concentrations of r-tPA [final concentration (f.c.) 0.63 μg/mL]. A representative clot-fibrinolysis waveform is shown in **Figure 1A-a**. The waveform was computer-analyzed using a commercial kinetic algorithm. The reaction time (sec) is illustrated on

the horizontal axis, and the transmittance (T), which is defined as the percent (%) light intensity transmitted from the pre- to post-coagulation states is shown on the vertical axis. The clot time (CT) was determined as the time to the point at which T was reduced to a predefined level. The first derivative of transmittance (dT/dt) reflected the coagulation and fibrinolysis velocities (**Figure 1A-b**). The minimum value of the first derivative (min1) was used to indicate the maximum velocity of coagulation. Because min1 was derived from negative values, the data were expressed as absolute values |min1|. The fibrinolysis time (FLT) was recorded as the interval between CT and the start of fibrinolysis. In this later phase (Figure 1A-b), the data generated an inverted curve, and the maximum velocity of fibrinolysis (|FL-min1|) was determined from this curve (**Figure 1A-c**). Calculations were performed using Excel software.

Simultaneous thrombin and plasmin generation assay (T/P-GA) - T/P-GA was performed using our previously established protocol.[17] Briefly, the trigger reagent (20 µL; 1 pM rTF, 4 µM PL vesicles, and 3.3 nM r-tPA) was mixed with plasma samples (80 µL) and transferred into 96-well plates (Nunc; Thermo Scientific, Waltham, MA). Thrombin generation (TG) and plasmin generation (PG) were determined with two specific fluorometric substrates, Z-Gly-Gly-Arg-AMC and Boc-Glu-Lys-Lys-MCA, respectively. Assays were performed using a Fluoroskan AscentTM™ microplate reader (Thermo Electron Co., Waltham, MA) with excitation at 390 nm and emission at 460 nm. Standard curves were prepared using serial dilutions of purified α-thrombin and plasmin as previously described.[21] Data analyses were performed using Excel software. Representative TG and PG curves obtained with control plasma are shown in **Figure 2A** and **2B**. Thrombin and plasmin lag time (Th-LT and Plm-LT, respectively), which reflect the time until initiation, were determined from the first derivatives of TG and PG, respectively. Peak thrombin (Th-Peak) and peak plasmin (Plm-Peak), which reflect the maximum levels of each parameter, were also recorded.

Statistical analyses - All results were expressed as median [min–max]. Statistical analyses were performed using JMP 10.0.2 (SAS Institute Inc., Cary, NC). The correlations between parameters were analyzed using the Spearman correlation coefficient (ρ).

Two-tailed tests were performed for all analyses and *P*-values of <0.05 were considered as statistically significant. A logistic-regression model was developed to explore parameters associated with the outcome (defined as the mortality rate after 28 days of diagnosis).

Results

Patient demographics - A total of 84 patients were initially screened. Of these, 27 patients with non-sepsis related DIC (for example, leukemia, aneurysm, and liver failure) were excluded from the present study. Overall therefore, 57 patients with sepsis-associated DIC (36 males, 21 females) were enrolled in this study. Clinical characteristics of the patients are summarized in **Table 1**. The age at onset ranged from 0 to 91 (median 74) years. The median [min–max] of DIC scores determined using the JAAM, JMWLW, and Japanese Society on Thrombosis and Hemostasis (JSTH) criteria were 5 [4-8], 6 [2-12], and 6 [3-10] points, respectively, and the mean Sequential Organ Failure Assessment (SOFA) score[22] was 9 [2-17] points. Laboratory findings revealed decreased platelet counts and markedly elevated levels of FDP, D-dimer, TAT, and PIC as well as fibrinogen and total PAI-1.

Dynamics of coagulation and fibrinolysis evaluated using CFWA and T/P-GA in sepsis-associated DIC - The global effects of coagulation and fibrinolytic mechanisms in patients with sepsis-associated DIC were first assessed using CFWA. Representative clot-fibrinolysis waveforms in patient plasma samples are shown in **Figure 1B**. Case 1, illustrates that the initiation of clot formation was slightly delayed and the percent light transmitted was markedly diminished compared with the normal control; and that this curve approached but did not return to baseline (**Figure 1B-a**). The calculated |min1| value was increased (**Figure 1B-b**) and |FL-min1| value was markedly decreased (**Figure 1B-c**) compared with the values obtained with control samples. In Case 2, the initiation of clot formation was significantly delayed and the clot-fibrinolysis waveform indicated no recovery to baseline (**Figure 1B-a**). Both |min1| and |FL-min1| values were substantially impaired compared with those of the control samples (**Figure 1B-b** and **-c**, respectively).

Next, T/P-GA was utilized to further evaluate this pivotal balance between coagulation and fibrinolysis in these patients. Representative TG and PG curves from two cases are presented in **Figure 2B**. In Case 1, Th-Peak was comparable to that determined in plasma from healthy controls (**Figure 2B-a**), and simultaneous measurements of PG revealed a prolonged Plm-LT and a moderately decreased Plm-Peak compared with those in the control samples (**Figure 2B-b**). In Case 2, both Th-Peak and Plm-Peak were markedly diminished compared with those observed in the control samples, and Plm-LT was substantially prolonged (**Figure 2B-a** and **-b**, respectively).

Table 2 illustrates these parameters as ratios relative to those obtained from normal plasma samples. In most cases the CFWA |min1| ratio was modestly elevated (median 1.40 [min–max; 0.10–2.60]),

the |FL-min1| ratio was moderately diminished (0.61 [0–1.19]), and the |FL-T| ratio was prolonged (1.75 [1.04–4.20]). These findings indicated that the fibrinolytic potential was diminished and coagulation capacity was slightly elevated at an early stage of sepsis-associated DIC. The T/P-GA results in all cases demonstrated that both Th-Peak and Plm-Peak ratios were decreased to 0.71 [0.22–1.20] and 0.35 [0.02–1.43], respectively, and that the Plm-LT ratio was prolonged (2.46 [0.83–17.78]). These findings suggested that the coagulation and fibrinolysis potentials were decreased, indicating that thrombin levels in patients with sepsis-associated DIC were not always elevated compared with these levels in controls. Further statistical analysis demonstrated a high correlation between the different parameters ($P < 0.001$), suggesting that the CFWA patterns reflected the thrombin/plasmin relationship. These findings led to the conclusion that the overall hemostatic balance in these individuals could be described as representing a 'coagulation-predominant' phenotype (|min1| ratio > |FL-min1| ratio, Th-Peak ratio > Plm-Peak ratio).

Relationship between CFWA parameters and DIC-related laboratory parameters - The CFWA technique is a straightforward procedure that provides a direct and comprehensive evaluation of fibrin clot formation and fibrinolytic potential. The method is being adopted on a wider scale, and we further focused, therefore, on a comparison between CFWA parameters and the commonly accepted and conventional laboratory parameters used to measure DIC-related coagulation and fibrinolysis. The correlations between CFWA parameters and routine laboratory and DIC score measurements are summarized in **Table 3A**. The |min1| ratio correlated with the prothrombin time-international normalized ratio (PT-INR) and with fibrinogen levels, but there was no correlation between |min1| ratio and liver function (AST/ALT), TAT or total PAI-1 level. Moreover, |FL-min1| ratio significantly correlated with the PIC level and SOFA score but did not correlate with liver function, α_2-plasmin inhibitor (α_2PI), FDPs, and total PAI-1 level. These results confirmed that the conventional laboratory parameters did not adequately reflect dynamic relationship between coagulation and fibrinolysis. While, both parameters of CFWA correlated with some DIC scores (JAAM, JMHLW, and JSTH scores), in particular well-correlated with JSTH.

Regards the correlation with T/P-GA parameters, Th-Peak ratio correlated with PT-INR and fibrinogen levels, but did not correlate with liver function (ALT/γGTP), TAT, and total PAI-1 levels. Plm-Peak ratio also correlated with PIC, total PAI-1, and SOFA score whilst did not correlate with liver function, α_2PI, and FDP levels. Both parameters correlated with the DIC scores (JAAM and JSTH score) (**Table 3B**).

Assessment of DIC based on the dynamics of comprehensive coagulation and fibrinolysis - In contrast to the conventional assays discussed above, CFWA and T/P-GA appeared to provide data to determine the balance between coagulation and fibrinolysis in sepsis-associated DIC. The results of these findings in all patients are shown in **Figures 3A** and **3B**, respectively. Increased values on the vertical axis indicated a tendency toward a hypercoagulable state, whereas similar increases along the horizontal axis indicated a tendency toward excessive fibrinolysis. The pattern observed at initial diagnosis clearly supported the presence of a coagulation-predominant state. Consequently, the CFWA parameters were utilized to categorize coagulation and fibrinolytic potential in sepsis-associated DIC into four classes, with Type 1 defined as a coagulation-predominant pattern (|min1| ratio > |FL-min1| ratio); Type 2 as a coagulation/fibrinolysis-balanced pattern (|min1| ratio ≈ |FL-min1| ratio); Type 3 as a fibrinolysis-predominant pattern (|min1| ratio < |FL-min1| ratio); and Type 4 as a consumption-impaired coagulation pattern or consumptive coagulopathy (|min1| and |FL-min1| ratios both < 0.5). This classification is schematically illustrated in **Figure 4**.

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Similar categories were identified using the T/P-GA data (Th-Peak and Plm-Peak ratios). A comparison between the CFWA and T/P-GA findings is illustrated in **Figure 5.** Most cases (80.7 %) assessed using CFWA were classified as having the Type 1 coagulation-predominant condition, and 69.6 % (n = 46) were similarly categorized using T/P-GA (**Figure 5A**). Moreover, all cases classified as Type 3 (n = 2) or Type 4 (n = 5) using CFWA data were placed into the same categories with the T/P-GA results (**Figure 5C** and **5D**). However, patients classified using Type 2 (n = 4) by CFWA were categorized differently by T/P-GA (**Figure 5B**).

The CFWA-based findings in these patients were also compared with conventional laboratory diagnostic criteria (**Table 4**). The coagulation markers, platelet counts, PT-INR, and fibrinogen levels, were markedly decreased among individuals categorized as Type 4, suggesting that this classification represented an advanced stage of sepsis-associated DIC. In contrast, the TAT and total PAI-1 levels did not differentiate patients categorized as Type 4 from any of the other types. Although the |FL-min1| ratio was comparatively high in the two Type 3 cases, the conventional fibrinolysis markers, FDP, D-dimer, PIC, and α_2PI levels were not informative in these individuals.

Association of patients' outcomes and parameters in CFWA and T/P-GA - We further investigated the relationship between patients' outcomes and parameters in CFWA and T/P-GA. In the present

study, we utilized the mortality rate after 28 days of diagnosis as the outcome of DIC. A logistic regression analysis demonstrated that Plm-peak ratio alone significantly correlated with patients' mortality rare (odds ratio: 0.09, $P = 0.04$), but the other parameters did not correlate at all (**Table 5**).

Discussion

Quantitative assessment of coagulation and fibrinolysis potential at the time of diagnosis of sepsis-associated DIC - The typical biphasic CWA waveform has been studied extensively in association with the different causes of DIC, including sepsis,[23-27] and has been considered a predictor of poor clinical outcome.[26,27] Suzuki *et al.*[28] reported that parameters of CWA was useful for the diagnosis of DIC and prediction of bleeding risk or outcome. The precise balance between coagulation and fibrinolysis may vary in relation to the underlying disease and associated complications, however,[1,10-12] and quantifying this balance could be essential for the clarification DIC-related pathogenetic mechanisms and the selection of appropriate treatment protocols. Asakura *et al.*[1] were among the first to demonstrate the importance of the interactions between coagulation and fibrinolysis by analyzing a combination of DIC-related laboratory parameters. More modern studies regarding the analysis of global coagulation and fibrinolytic potential are lacking, however. To the best of our knowledge, the present study is the first to examine simultaneous coagulation and fibrinolysis at the initial diagnosis of sepsis-associated DIC using the established parameters derived from CFWA and T/P-GA.[16,17]

The successful quantification of synchronized coagulation and fibrinolysis is described together with a classification system based on comparisons of specific parameters. We have previously presented similar comprehensive analyses of coagulation and fibrinolysis using these techniques in pediatric patients with DIC secondary to several types of acute leukemia[29-31] and Kawasaki disease.[18]

Use of CFWA and T/P-GA in for quantitative evaluation of sepsis-associated DIC - The principles of the two assays employed in the present study are clearly distinguishable from one another. CFWA depends on fibrin-mediated blood clotting,[16,32,33] whereas T/P-GA firstly monitors TG, an enzyme that ultimately modulates fibrin formation.[17,33,34] Analysis of coagulation potential using these alternative procedures demonstrated mild discrepancies, possibly related to the different stages of coagulation (TG *vs.* fibrin formation) or fibrinogen levels[16,35] in our patients with sepsis-associated DIC. Notably, however, these parameters of global coagulation based on the kinetics of fibrin formation were higher than those determined in healthy controls, and in contrast, fibrinolysis

initiated by the formation of fibrin appeared to be diminished. Hence, both CFWA and T/P-GA suggested that an overall coagulation-predominant state was prevalent in these patients.

Results from CFWA and DIC-related laboratory parameters - The |min1| ratio, which was used to compare the relationship between patients with sepsis-associated DIC and healthy controls, is a principal coagulation parameter in APTT-based CFWA, and the results correlated with both the PT-INR and fibrinogen levels. Fibrinogen is universally known as the soluble precursor of fibrin, the fundamental component of clot formation and stability. In addition, elevated levels of fibrinogen are procoagulant[36] and influence blood viscosity. Moreover, fibrinogen is synthesized and released in response to inflammatory cytokines and acute tissue destruction, and regulates pivotal aspects of inflammation, promoting endothelial cell activation, platelet aggregation/activation, and immune cell recruitment.[37] Consequently, elevated fibrinogen levels are common in sepsis, and in view of these multi-functional roles for fibrinogen in hemostasis and systemic inflammation, the association between increased fibrinogen concentrations and high |min1| ratios was expected. Only a weak correlation was observed, however, between |min1| ratios and TAT, another important conventional marker of DIC. Circulating TAT complexes are critically dependent on physiologic interactions between thrombin and antithrombin, and high TAT levels are known to be associated with a hypercoagulable state. Our data suggested that a tendency to enhanced coagulation should be considered even in the absence of elevated TAT assays.

PIC is a similar molecular complex that is currently used as an important marker of fibrinolytic potential and classifying DIC.[1] PIC measurements are elevated in association with activated fibrinolysis, and although PIC alone provides only limited information on suppressed fibrinolysis, raised PIC levels are highly informative for the diagnosis and treatment of sepsis-associated DIC. Normal PIC levels are typically near zero, however, (normal range < 0.8 μg/mL) and therefore measurements of PIC alone cannot distinguish between normal and suppressed fibrinolysis. In contrast, our data identified a correlation between PIC and the |FL-min1| ratio, indicating that this CFWA parameter of fibrinolysis could help to improve understanding of both activation and suppression of fibrinolysis in patients with sepsis-associated DIC. In addition, our |FL-min1| ratios correlated with SOFA scores that are believed to provide an index of microthrombus formation often resulting in organ failure in patients with DIC. Our results were in keeping with a dynamic relationship between fibrinolytic status and fibrin clot formation, and clearly suggested that the |FL-min1| ratio may be considered a predictor of the risk of organ failure.

In addition, it was noteworthy that |min1| and |FL-min1| in CFWA parameters correlated with the DIC scores (JAAM, JSTH score, and/or JMHLW), especially appeared to well correlate with JSTH score. These results may support that CFWA may be a good predictor of DIC and contribute to a diagnosis of DIC.

The JSTH criteria is the high sensitivity and specificity to diagnose the DIC compared to the other criteria used in the present study.[38] We repeatedly analyzed the parameters of CFWA and T/P-GA for the subjects who met the JSTH criteria ($\geq$ 5 for infection type) and demonstrated a high correlation ($P < 0.001$) between parameters in both assays, supporting that the CFWA patterns well reflected the thrombin and plasmin relationship (Supplemental Table 1). Furthermore, the relationship between CFWA or T/P-GA parameters and DIC-related parameters were also analyzed. The correlation of JAAM, JMHLW, |min1| ratio, and |FL-min1| ratio became low, but other correlation remained high (|min1| ratio *vs.* PT-INR, fibrinogen, or JSTH criteria; |FL-min1| ratio *vs.* PIC, SOFA score, or JSTH criteria) (**Supplemental Table 2A and B, respectively**).

By the way, regards as patients' outcomes, Plm-peak ratio alone in T/P-GA significantly correlated with the mortality rate after 28 days of diagnosis in all patients enrolled. This result may support that this parameter provides some help on the management of sepsis-associated DIC in the clinical setting. However, since there was small sample size in this study, this limitation might have influenced on our results. Further investigation is required to clarify whether the comprehensive assays could assess the prognosis of septic-associated DIC.

Four types of sepsis-associated DIC, based on a balance between coagulation and fibrinolysis - Marked activation of coagulation is a major, common factor promoting DIC pathology and is dependent on disease stage.[1,2] Moreover, the degree of fibrinolytic activation depends on the nature and extent of the underlying disease.[1,11,39] Therefore, the critical balance between coagulation and fibrinolysis requires careful and individualized analysis. An earlier report classified DIC into three types based on the degree of fibrinolysis,[1] but that study included assays of PIC, PAI-1, and D-dimer that only partially reflect existing fibrinolytic activity. Our results suggested that this classification may be inadequate for evaluating global fibrinolysis, and we devised a classification system, therefore, that could improve understanding of the pathogenesis of sepsis-associated DIC, and thereby aid the selection of appropriate treatment. In the present study, four distinct categories of

sepsis-associated DIC appeared to be distinguishable based on the CFWA parameters, |min1| ratio and |FL-min1| ratio, used to assess comprehensive coagulation and fibrinolysis.

- **Type 1; Coagulation-predominant (|min1| ratio > |FL-min1| ratio) sepsis-associated DIC**

In previous studies, patients with non-DIC related bacterial infection demonstrated markedly higher CWA parameters compared with control individuals.[40] These results supported our hypothesis that most patients with pre-DIC or early stage DIC have features consistent with a coagulation-predominant disorder. It was not surprising, therefore, that Type 1 characteristics were observed in most patients (80.7%) in the present study immediately on diagnosis. Among those patients in this group, PAI-1 was markedly elevated and fibrinolysis was substantially decreased. In these circumstances, microthrombi could become more resistant to dissolution and the microvascular circulation would be impaired. These events could lead to multiple organ dysfunctions, albeit without any significant risk of bleeding.

- **Type 2; Coagulation/fibrinolysis-balanced (|min1| ratio ≈ |FL-min1| ratio)**

A controlled balance between coagulation and fibrinolysis was evident in four (7%) of our patients with sepsis-associated DIC. These individuals were classed as having the Type 2 disorder. This type of DIC may be common among patients with sepsis related to solid tumors, particularly in those with prostate cancer. Hemorrhagic symptoms or vital organ dysfunction are thought to be relatively rare. Type 2 DIC might be diagnosed in patients from within another category during recovery from the consumption of clotting factors or active fibrinolysis.

- **Type 3; Fibrinolysis-predominant (|min1| ratio < |FL-min1| ratio)**

Patients with DIC in which fibrinolysis activation exceeds coagulation activation were grouped as Type 3 (fibrinolysis-predominant). Two (3%) of our patients were recorded in this category. This type is identified occasionally in sepsis with cancer or abdominal aortic aneurysm, and may be seen as complication of esophageal or liver cancer like our cases. Patients with Type 3 DIC tend to develop a bleeding tendency.

- **Type 4; Consumption-impaired coagulation (|min1| < 0.5 and |FL-min1| < 0.5)**

Severely impaired coagulation and fibrinolytic activation is frequently referred to as a consumptive coagulopathy, and is typically diagnosed in advanced DIC. In general, patients with Type 4 DIC present with severe bleeding and develop organ failure. Intensive treatment may be required for the

management of uncontrolled hemorrhage.

Advantages of CFWA in the clinical practice - Our classification focused on the principle that in clinical practice treatment of the underlying pathogenesis of DIC is critical. In acute circumstances it may be particularly important to have a clear understanding of the physiologic balance between coagulation and fibrinolysis in order to provide supportive therapy that addresses the precise nature of this relationship. The T/P-GA technique has been shown to provide an accurate reflection of the processes of coagulation and/or fibrinolysis. Reliable results are dependent on trained personnel and meticulous technical expertise, however, and this limits widespread clinical application. In contrast, CFWA is a straightforward modified version of the more widely used CWA, and does not require special training or techniques. This method offers an attractive option for routine diagnostic laboratories, especially when a rapid diagnosis is required at the challenging onset of DIC.

Limitation - There is limitation in the present study, however. The sample size was small. Fifty-seven patients were enrolled and the majority of them were the Type 1 pattern. Since only very few patients were included in the groups not being "Type 1", we might not possibly capture the overall feature of them sufficiently. The increase of the number of samples could demonstrate the precious features of each type of sepsis-DIC. In addition, further investigation would be needed to achieve the classification of these types that reflects the clinical data.

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Disclosures

Author contributions: TO designed the research, collected the samples, performed experiments, interpreted the data, and drafted the manuscript; KN designed the research, interpreted the data, drafted the manuscript, edited the manuscript, and approved the final version for publication; TI designed the research, interpreted the data, and supported the experimental studies; MK, KN, HF,

HK, IA, and NT all provided clinical support for this study; MY performed the laboratory examinations; and MK and MS supervised the study.

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Figure Legends

Figure 1. Simultaneous clot-fibrinolysis waveform analysis (CFWA)

(A) *Clot-fibrinolysis waveforms and parameters in normal plasma*: Normal plasma was incubated with the APTT reagent followed by the addition of $CaCl_2$ with or without r-tPA (0.63 μg/mL) as described in Methods. *Panel* (a) documents the changes in light transmittance (%T) observed over time during the performance of the APTT. *Panel* (b) shows the curves from first derivative over time (dT/dt) of the waveform in (a). *Panel* (c) shows the inverted curve during the fibrinolysis phase (enclosed by the dotted circle) in (b). APTT, activated partial thromboplastin time; CT, clot time; |min1|, maximum coagulation velocity; FLT, fibrinolysis time; |FL-min1|, maximal fibrinolysis velocity; r-tPA, recombinant tissue-type plasminogen activator

(B) *Representative waveforms of CFWA in sepsis-associated DIC*: Clot formation and fibrinolysis waveforms in plasma samples from patients with sepsis-associated DIC are illustrated. *Panel* (a) shows the clot waveform (%T) observed during APTT. *Panel* (b) shows the curves from first derivative (dT/dt) over time of the waveforms in (a). *Panel* (c) includes the inverted curve observed during the fibrinolysis phase in (b). Case 1, *solid gray line*; Case 2, *dotted line*; control, *solid black line*. DIC, disseminated intravascular coagulation

Figure 2. Simultaneous thrombin/plasmin generation assay (T/P-GA)

(A) *Thrombin and plasmin generation curves and their parameters in normal plasma*: TF, tPA, and PL (final concentrations, 1 pM, 3.2 nM, and 4 μM, respectively) were added to control plasma as described in the Methods. *Panel* (a) illustrates thrombin generation (TG) and the associated parameters. *Panel* (b) illustrates plasmin generation (PG) and the associated parameters. Th-LT and Plm-LT indicate the lag time of TG and PG, respectively. Th-Peak and Plm-Peak identify maximum TG and PG, respectively.

(B) *A representative curves of TG and PG in patients with sepsis-associated DIC*: Values of TG and PG in plasma samples from patients with sepsis-associated DIC are shown. Case 1, *solid gray line*; Case 2, *dotted line*; control plasma, *solid black line*. DIC, disseminated intravascular coagulation

Figure 3. Distribution of coagulation and fibrinolytic potentials from parameters obtained using CFWA and T/P-GA in sepsis-associated DIC cases

(**A**) The vertical axis shows the values calculated for |min1| ratio. The horizontal axis shows the analogous values for |FL-min1| ratio. The diagonal line depicting unity (|min1| ratio = |FL-min1| ratio) is shown (*dashed line*). (**B**) Th-peak ratio is on the vertical axis and, and Plm-Peak ratio is on the horizontal axis. The dotted line denotes Th-Peak ratio = Plm-Peak ratio.

Figure 4. A classification of sepsis-associated DIC based on the balance of coagulation/fibrinolysis dynamics as determined using CFWA and T/P-GA

Type 1, coagulation-predominant pattern; Type 2, coagulation/fibrinolysis-balanced pattern; Type 3, fibrinolysis-predominant pattern; Type 4, Consumption-impaired coagulation. Relationships among the four categories and factors contributing to differential diagnosis are as described in the text.

Figure 5. Comparison of the classifications based on CFWA with those predicted using T/P-GA in patients with sepsis-associated DIC

The coagulation and fibrinolysis parameters obtained by CFWA and used to generate the four categories were compared with those generated using T/P-GA (**A**) Type 1, (**B**) Type 2, (**C**) Type 3, and (**D**) Type 4.

Table 1. Clinical characteristics at diagnosis of patients with sepsis-associated DIC

	Patient (n = 57)	Normal range
Male / Female (n)	36 / 21	
Age (years)	74 [0–91]	
DIC score (defined as DIC)		
JAAM criteria (≥ 4)	5 [4–8]	
JMHLW criteria (≥ 7)	6 [2–12]	
JSTH criteria (≥ 5 for infection type)	6 [3–10]	
SOFA score	9 [2–17]	
Laboratory data		
PLT ($\times 10^4/\mu L$)	8.9 [0.40–24.6]	13.1–36.5
PT-INR	1.38 [0.95–3.44]	0.85–1.15
Fibrinogen (mg/dL)	413 [117–755]	150–400
Plasminogen (%)	43.6 [24.9–110.5]	75–125
α2PI (%)	77.4 [30.6–112.6]	85–115
FDP (μg/mL)	29.3 [4.8–211.6]	< 4.0
D-dimer (μg/mL)	13.3 [1.6–79.6]	< 1.0
TAT (ng/mL)	16.3 [3.7–149.3]	< 3.0
PIC (μg/mL)	1.1 [0.4–30.0]	< 0.8
Total PAI-1 (ng/mL)	83 [25–2,961]	< 50

Values are presented as median [min–max]. JAAM, Japanese Association for Acute Medicine; JMHLW, Japanese Ministry of Health, Labor and Welfare; JSTH, Japanese Society on Thrombosis and Hemostasis; PLT, platelet; PT-INR, prothrombin time-international normalized ratio; α2PI, α2 plasminogen inhibitor; FDP, fibrinogen/fibrin degradation products; TAT, thrombin-antithrombin complex; PIC, plasmin-α_2-plasmin inhibitor complex; PAI-1, plasminogen activator inhibitor

Table 2. Comprehensive coagulation and fibrinolysis assays in patients with sepsis-associated DIC

CFWA		T/P-GA		Correlation coefficient (ρ)†	*P* value‡
	*Ratio**		*Ratio**		
|min1|	1.40 [0.10–2.60]	Th-Peak	0.71 [0.22–1.20]	0.55	< 0.001
|FL-min1|	0.61 [0–1.19]	Plm-Peak	0.35 [0.02–1.43]	0.71	< 0.001
|FL-T|	1.75 [1.04–4.20]	Plm-LT	2.46 [0.83–17.78]	0.39	0.007

*; The value shows the ratio relative to control.

† ; Spearman's rank-order correlation coefficient

‡ ; The correlations between parameters were analyzed using Spearman correlation coefficient.

Values are presented as median [min–max]. Significant differences were expressed as $P < 0.05$.

Table 3A. Comparison between CFWA parameters and DIC-related parameters

Parameters	Laboratory parameters	Correlation coefficient *(ρ)*	*P* value
|min1| ratio	AST/ALT/ γGTP	-0.16/0.05/0.27	0.23/0.70/0.04
	PT-INR	-0.50	<0.001
	Fibrinogen (mg/dl)	0.77	<0.001
	TAT (ng/ml)	-0.12	0.36
	Total PAI-1 (ng/ml)	-0.12	0.37
	JAAM/JMHLW/JSTH criteria	-0.29/-0.24/-0.44	0.02/0.07/<0.001
|FL-min1| ratio	AST/ALT/γGTP	-0.24/-0.11/0.11	0.07/0.40/0.43
	α2PI (%)	0.10	0.47
	PIC (μg/ml)	0.44	<0.001
	FDP (μg/ml)	0.11	0.43
	Total PAI-1 (ng/ml)	-0.25	0.07
	SOFA score	-0.35	0.007
	JAAM/JMHLW/JSTH criteria	-0.25/-0.28/-0.42	0.05/0.03/0.001

Table 3B. Comparison between T/P-GA parameters and DIC-related parameters

Parameters	Laboratory parameters	Correlation coefficient *(ρ)*	*P* value
Th-Peak	AST/ALT/γGTP	-0.28/-0.004/0.05	0.04/0.97/0.70
	PT-INR	-0.55	< 0.001
	Fibrinogen (mg/dl)	0.42	0.0015
	TAT (ng/ml)	-0.20	0.15
	Total PAI-1 (ng/ml)	-0.23	0.09
	JAAM/JMHLW/JSTH criteria	-0.29/-0.27/-0.45	0.02/0.04/<0.001
Plm-Peak	AST/ALT/ γGTP	-0.29/-0.08/0.06	0.03/0.57/0.65
	α2PI (%)	0.21	0.13
	PIC (μg/ml)	0.31	0.02
	FDP (μg/ml)	0.05	0.72
	Total PAI-1 (ng/ml)	-0.57	< 0.001
	SOFA score	-0.45	< 0.001

JAAM/JMHLW/JSTH criteria	-0.30/-0.21/-0.33	0.02/0.11/0.01

Significant differences were expressed as $P < 0.05$. PT-INR, prothrombin time-international normalized ratio; AST, aspartate aminotransferase; ALT, alanine aminotransferase; γGTP, γ-glutamyl transpeptidase; α_2PI, α_2-plasminogen inhibitor; FDP, fibrinogen/fibrin degradation products; TAT, thrombin–antithrombin complex; PIC, plasmin-α_2-plasmin inhibitor complex; PAI-1, plasminogen activator inhibitor; SOFA, sequential organ failure assessment; JAAM, Japanese Association for Acute Medicine; JMHLW, Japanese Ministry of Health, Labor and Welfare; JSTH, Japanese Society on Thrombosis and Hemostasis

Table 4. DIC-related laboratory data related to CFWA in sepsis-associated DIC

Potential	Type 1 *Coagulation predominant* (n = 46)	Type 2 *Both Balanced* (n = 4)	Type 3 *Fibrinolysis predominant* (n = 2)	Type 4 *Consumptive impaired coagulation* (n = 5)
Laboratory				
PLT ($\times 10^4/\mu l$)	9.1 [0.4–24.6]	10.1 [9.7–10.3]	6.5, 3.0	4.6 [0.5–10.6]
PT-INR	1.32 [0.95–1.80]	1.33 [1.3–2.02]	1.3, 2.7	1.93 [1.41–3.44]
Fibrinogen (mg/dl)	464 [250–755]	220 [117–330]	269, 139	154 [120–237]
FDP (μg/ml)	23.5 [4.8–211]	35.3 [15–47.5]	12.3, 32.4	32.5 [11.5–78.5]
D-dimer (μg/ml)	10.2 [1.6–79.6]	20.1 [6.5–23.8]	4.7, ND	16 [6.5–33.1]
TAT (ng/ml)	13.3 [3.7–41]	25.1 [8.6–41.5]	5.5, 36.5	21.5 [10.2–149.3]
PIC (μg/ml)	1.1 [0.4–30.0]	1.6 [1.1–2.0]	1.5, 1.8	1.0 [0.6–1.8]
α2PI (%)	86.9 [56.1–112.6]	55.8 [47.4–64.2]	65.4, 57.6	47.8 [30.6–68.8]
Plasminogen (%)	44.8 [26.8–110.5]	40.6 [37.2–44]	58.2, 51.3	28.2 [24.9–36.0]
Total PAI-1 (ng/ml)	77.1 [24.5–296]	147.2 [39.3–255]	15.5, 260	142.7 [44.8–2960]
CFWA				
|min1| ratio	1.5 [0.61–2.60]	0.95 [0.56–1.1]	0.89, 0.25	0.34 [0.10–0.46]
|FL-min1| ratio	0.64 [0.12–1.19]	0.96 [0.62–1.1]	1.22, 0.52	0.37 [0–0.45]
|FL-T| ratio	1.79 [1.18–4.2]	1.28 [1.05–1.44]	1.18. 0.96	1.45 [1.04–2.82]

PLT, platelet; α2PI, α2 plasminogen inhibitor; FDP, fibrinogen/fibrin degradation products; TAT, thrombin–antithrombin complex; PIC, plasmin-α_2-plasmin inhibitor complex; PAI-1, plasminogen activator inhibitor-1. Values are presented as median [min–max].

Table 5. Association of parameters in CFWA and T/P-GA with patients' outcomes

Regression Variable	Mortality rate*, Odds Ratio (95% CI)	*P* value
Parameters		
CFWA		
|min1| ratio	0.83 (0.27-2.42)	0.74
|FL-min1| ratio	0.23 (0.02-1.90)	0.17
T/P-GA		
Th-peak ratio	0.12 (0.01-1.62)	0.12
Plm-peak ratio	0.09 (0.004-0.91)	0.04

*Mortality rate was evaluated after 28 days of diagnosis of DIC.

Significant differences were expressed as $P < 0.05$.

Supplemental Table 1. Comprehensive coagulation and fibrinolysis assays in patients with sepsis-associated DIC for patients who met the JSTH criteria

CFWA		T/P-GA		Correlation coefficient (ρ)†	*P* value‡
	*Ratio**		*Ratio**		
|min1|	1.20 [0.10–2.60]	Th-Peak	0.67 [0.22–1.17]	0.53	< 0.001
|FL-min1|	0.59 [0–1.10]	Plm-Peak	0.35 [0.02–1.43]	0.73	< 0.001
|FL-T|	1.75 [1.04–4.20]	Plm-LT	2.46 [0.83–17.78]	0.32	0.06

*; The value shows the ratio relative to control.

†; Spearman's rank-order correlation coefficient

‡; The correlations between parameters were analyzed using Spearman correlation coefficient.

Values are presented as median [min–max]. Significant differences were expressed as $P < 0.05$.

Supplemental Table 2. Comparison between CFWA or T/P-GA parameters and DIC-related parameters for patients who met the JSTH criteria

(A) CFWA

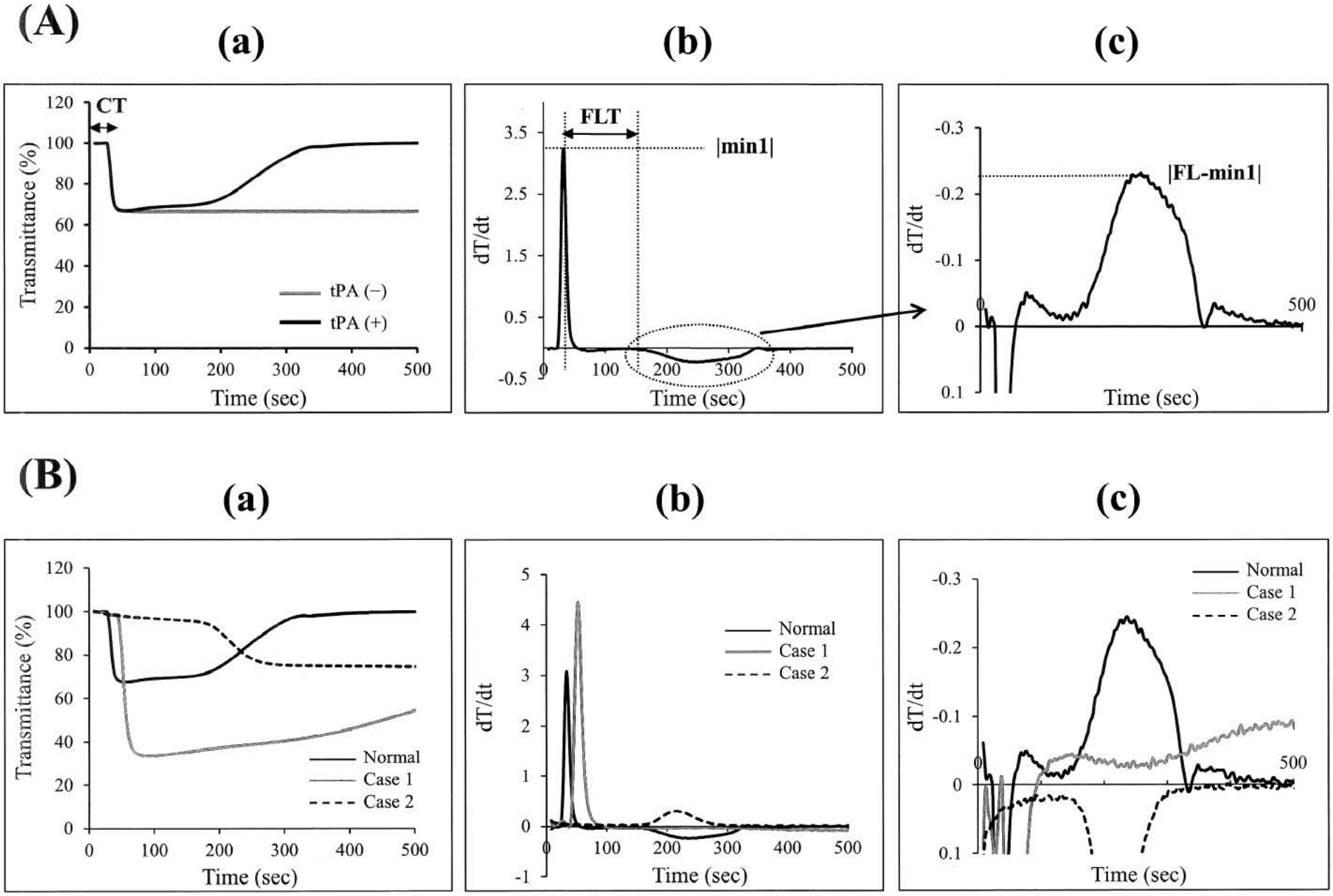
Parameters	Laboratory parameters	Correlation coefficient *(ρ)*	*P* value
|min1| ratio	AST/ALT/ γGTP	0.07/0.17/0.30	0.63/0.23/0.04
	PT-INR	-0.45	0.001
	Fibrinogen (mg/dl)	0.72	<0.001
	TAT (ng/ml)	-0.09	0.53
	Total PAI-1 (ng/ml)	-0.08	0.60
	JAAM/JMHLW/JSTH criteria	-0.11/-0.02/-0.29	0.45/0.88/0.04
|FL-min1| ratio	AST/ALT/γGTP	-0.17/-0.06/0.14	0.25/0.67/0.33
	α2PI (%)	0.10	0.49
	PIC (μg/ml)	0.42	0.003
	FDP (μg/ml)	0.13	0.36
	Total PAI-1 (ng/ml)	-0.24	0.11
	SOFA score	-0.44	0.002
	JAAM/JMHLW/JSTH criteria	-0.20/-0.25/-0.45	0.16/0.07/0.001

(B) T/P-GA

Parameters	Laboratory parameters	Correlation coefficient *(ρ)*	*P* value
Th-Peak	AST/ALT/γGTP	-0.17/-0.05/0.07	0.24/0.70/0.64
	PT-INR	-0.58	< 0.001
	Fibrinogen (mg/dl)	0.36	0.02
	TAT (ng/ml)	-0.17	0.26
	Total PAI-1 (ng/ml)	-0.18	0.23
	JAAM/JMHLW/JSTH criteria	-0.20/-0.17/-0.38	0.17/0.26/0.007
Plm-Peak	AST/ALT/ γGTP	-0.34/-0.09/0.02	0.02/0.51/0.90
	α2PI (%)	0.18	0.23
	PIC (μg/ml)	0.21	0.16
	FDP (μg/ml)	-0.02	0.91

	Total PAI-1 (ng/ml)	-0.54	< 0.001
	SOFA score	-0.47	< 0.001
	JAAM/JMHLW/JSTH criteria	-0.33/-0.25/-0.38	0.02/0.09/0.01

Significant differences were expressed as $P < 0.05$. PT-INR, prothrombin time-international normalized ratio; AST, aspartate aminotransferase; ALT, alanine aminotransferase; γGTP, γ-glutamyl transpeptidase; α_2PI, α_2-plasminogen inhibitor; FDP, fibrinogen/fibrin degradation products; TAT, thrombin–antithrombin complex; PIC, plasmin-α_2-plasmin inhibitor complex; PAI-1, plasminogen activator inhibitor; SOFA, sequential organ failure assessment; JAAM, Japanese Association for Acute Medicine; JMHLW, Japanese Ministry of Health, Labor and Welfare; JSTH, Japanese Society on Thrombosis and Hemostasis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(a)
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|min1|
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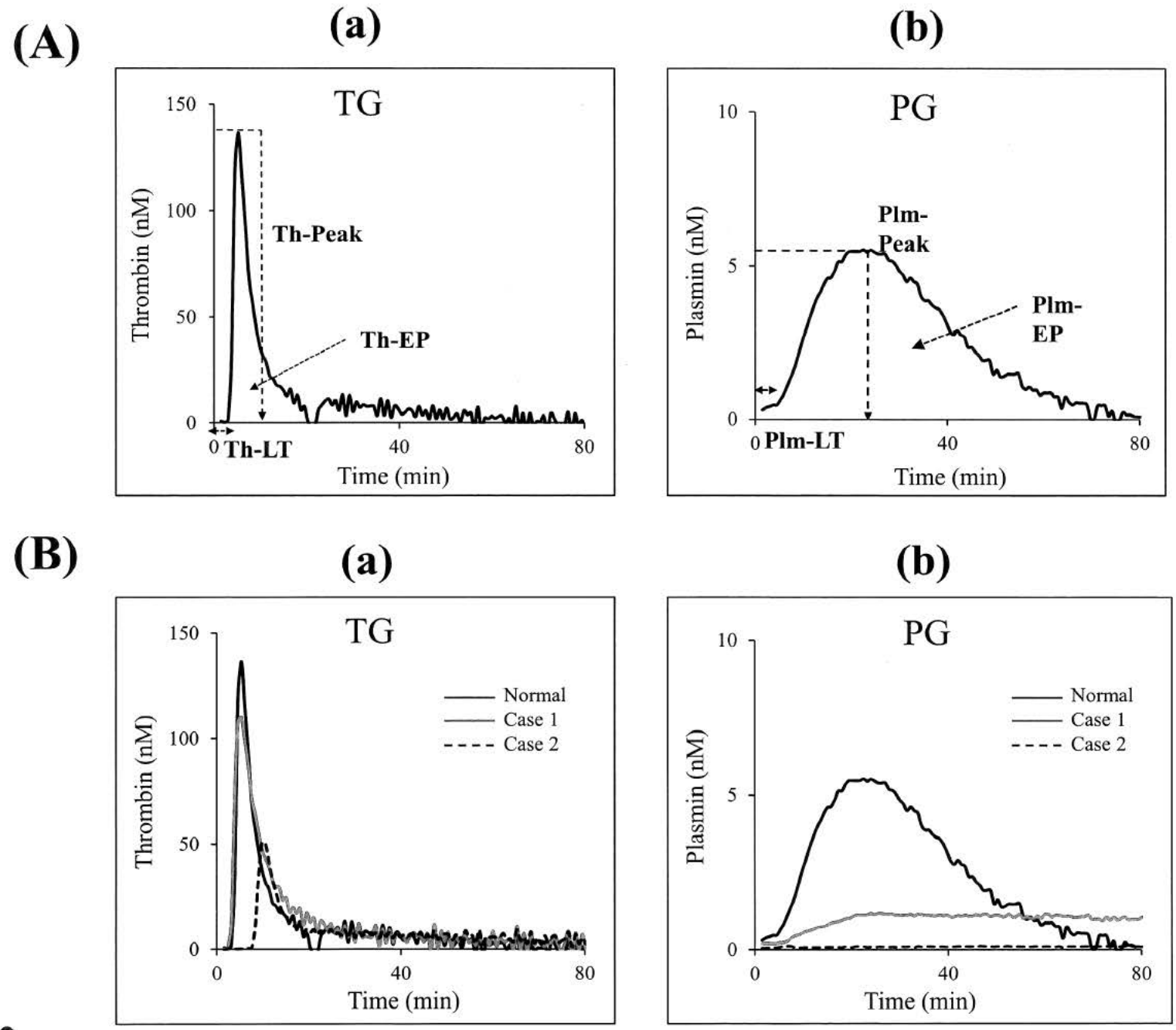
Time (sec)
(B)
(a)
(b)
(c)
Transmittance (%)
Normal
Case 1
Case 2
Time (sec)
dT/dt
Normal
Case 1
Case 2
Time (sec)
dT/dt
Normal
Case 1
Case 2
Time (sec)

Figure 1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40
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Time (min)
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Plasmin (nM)
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TG
Thrombin (nM)
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Normal
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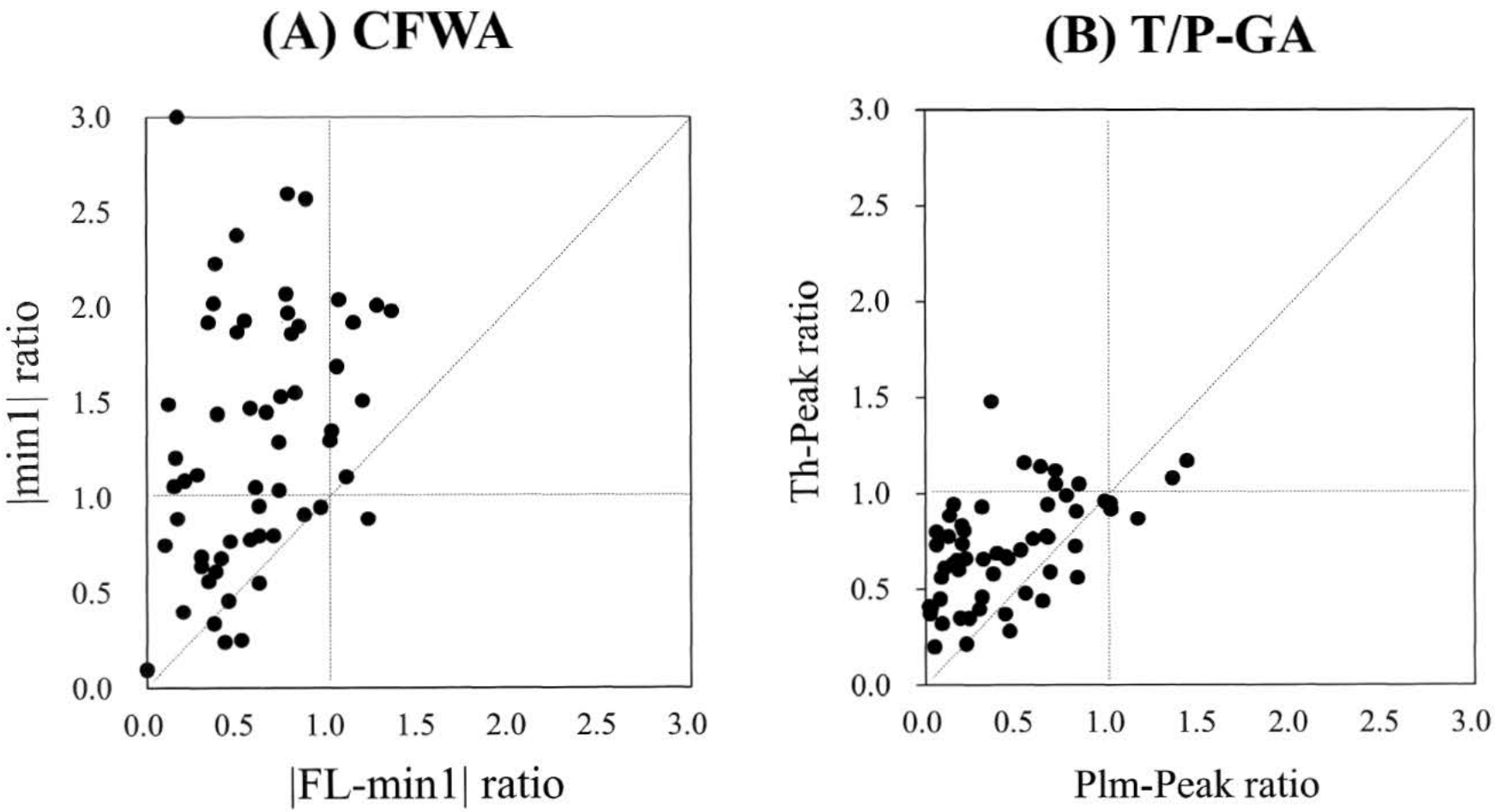
Time (min)

Figure 2


(A) CFWA
|min1| ratio
|FL-min1| ratio
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1.0
1.5
2.0
2.5
3.0
(B) T/P-GA
Th-Peak ratio
Plm-Peak ratio

Figure 3

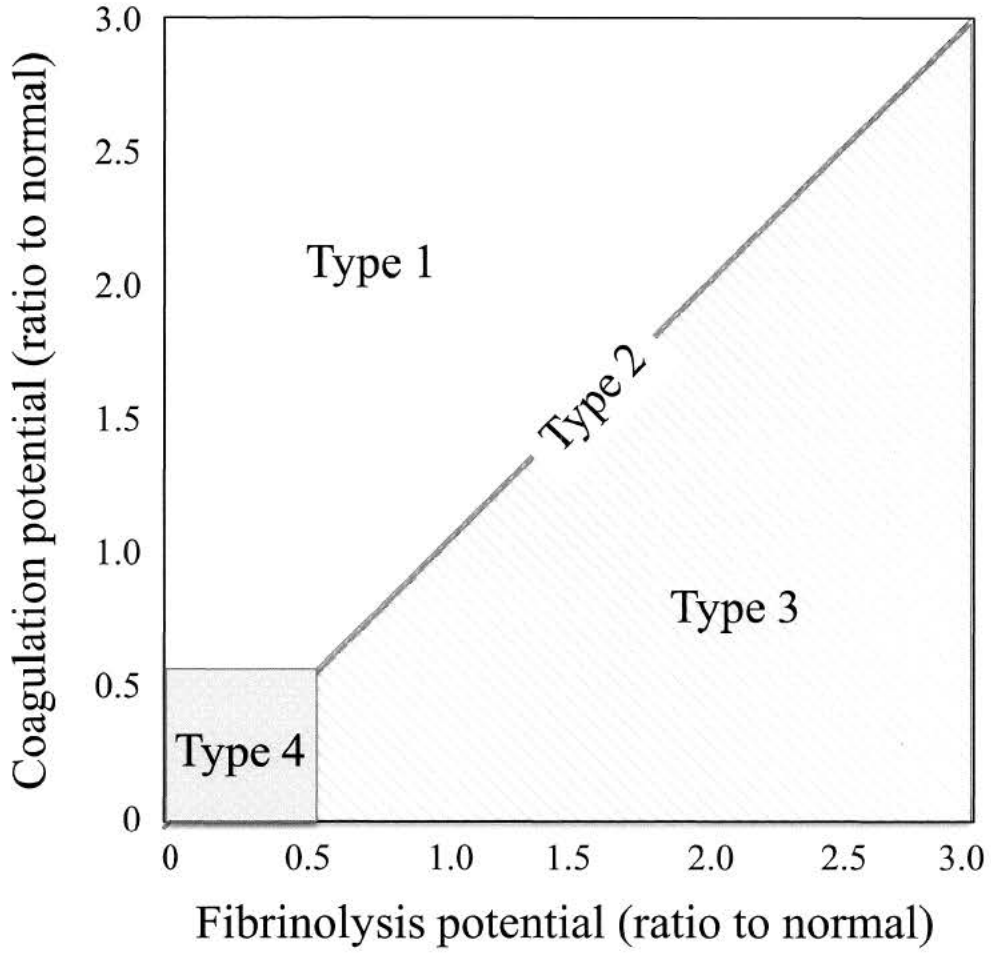
Coagulation potential (ratio to normal)
3.0
2.5
2.0
1.5
1.0
0.5
0
Type 1
Type 2
Type 3
Type 4
0
0.5
1.0
1.5
2.0
2.5
3.0
Fibrinolysis potential (ratio to normal)

Figure 4

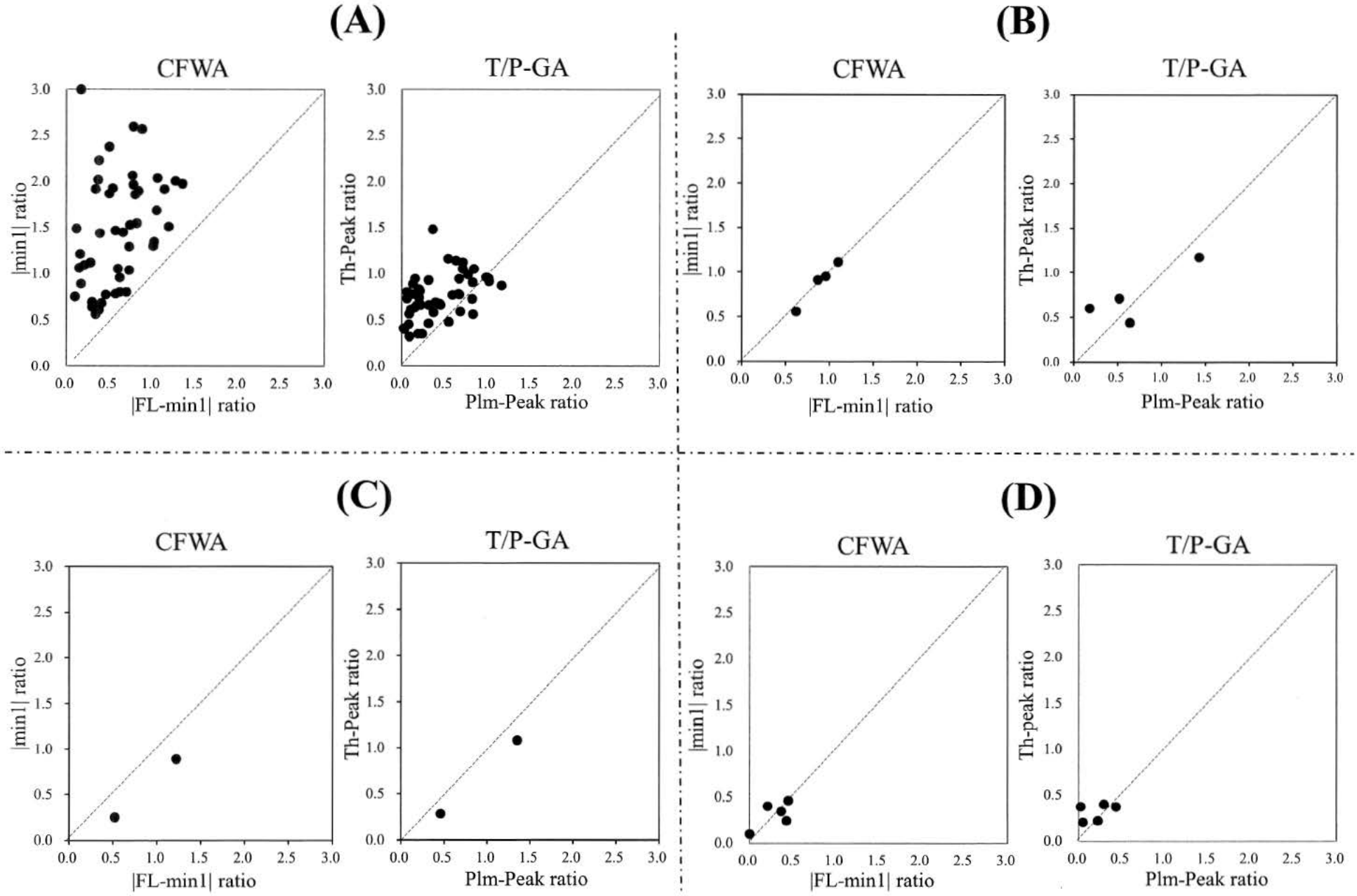
(A)
CFWA
T/P-GA
|min1| ratio
|FL-min1| ratio
Th-Peak ratio
Plm-Peak ratio
(B)
CFWA
T/P-GA
|min1| ratio
|FL-min1| ratio
Th-Peak ratio
Plm-Peak ratio
(C)
CFWA
T/P-GA
|min1| ratio
|FL-min1| ratio
Th-Peak ratio
Plm-Peak ratio
(D)
CFWA
T/P-GA
|min1| ratio
|FL-min1| ratio
Th-peak ratio
Plm-Peak ratio

Figure 5