

1 **Serum histone H3 levels and platelet counts are potential markers for coagulopathy**  
2 **with high risk of death in septic patients: a single-center observational study**

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4 Takashi Ito<sup>\*1,2</sup>, Takaaki Totoki<sup>\*1</sup>, Yayoi Yokoyama<sup>1</sup>, Tomotsugu Yasuda<sup>1</sup>, Hiroaki Furubeppu<sup>1</sup>,  
5 Shingo Yamada<sup>3</sup>, Ikuro Maruyama<sup>2</sup>, Yasuyuki Kakihana<sup>1</sup>

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7 \* These authors contributed equally to this work.

8 <sup>1</sup>Department of Emergency and Intensive Care Medicine and <sup>2</sup>Department of Systems Biology  
9 in Thromboregulation, Kagoshima University Graduate School of Medical and Dental  
10 Sciences, Kagoshima, Japan; <sup>3</sup>R&D Center, Shino-Test Corporation, Sagamihara, Japan

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12 Corresponding author: Takashi Ito, Department of Systems Biology in Thromboregulation,  
13 Kagoshima University Graduate School of Medical and Dental Sciences, 8-35-1 Sakuragaoka,  
14 Kagoshima 890-8544, Japan.

15 E-mail: takashi@m3.kufm.kagoshima-u.ac.jp

16 Tel: +81-99-275-6472; Fax: +81-99-275-6463

17

18 E-mail addresses:

19 Takashi Ito takashi@m3.kufm.kagoshima-u.ac.jp

20 Takaaki Totoki ttktkak@gmail.com

21 Yayoi Yokoyama yokoyama@m.kufm.kagoshima-u.ac.jp

22 Tomotsugu Yasuda yasutomo@m.kufm.kagoshima-u.ac.jp

23 Hiroaki Furubeppu m00082hf@jichi.ac.jp

24 Shingo Yamada shingo.yamada@shino-test.co.jp

25 Ikuro Maruyama maruyama@m2.kufm.kagoshima-u.ac.jp

26 Yasuyuki Kakihana kakihana@m3.kufm.kagoshima-u.ac.jp

27 **Abstract**

1 **Background:** Recent studies have suggested that anticoagulant therapy does not confer a  
2 survival benefit overall in sepsis, but might be beneficial in sepsis-associated disseminated  
3 intravascular coagulation (DIC). In particular, those with high Sequential Organ Failure  
4 Assessment (SOFA) scores might be the optimal target for anticoagulant therapy. However,  
5 both DIC and SOFA scores require the measurement of multiple markers. The purpose of this  
6 study was to explore a minimal marker set for determining coagulopathy at high risk of death  
7 in septic patients, wherein histone H3 levels were evaluated as indicators of both organ  
8 failure and coagulation activation.

9 **Methods:** We analyzed correlations among levels of serum histone H3 and other coagulation  
10 markers in 85 cases of sepsis using Spearman's rank correlation test. We then compared the  
11 utility of histone H3 to that of other coagulation markers in predicting the traditional DIC  
12 state or 28-day mortality by receiver-operating characteristics analysis. Finally, we suggested  
13 cut-off values for determining coagulopathy with high risk of death, and evaluated their  
14 prognostic utility.

15 **Results:** Serum histone H3 levels significantly correlated with thrombin-antithrombin  
16 complex (TAT) levels (Spearman's  $\rho = 0.46$ ,  $p < 0.001$ ), and weakly correlated with platelet  
17 counts (Spearman's  $\rho = -0.26$ ,  $p < 0.05$ ). Compared to other coagulation markers, histone H3  
18 levels showed better performance in predicting 28-day mortality. When combining serum  
19 histone H3 levels with platelet counts, our new scoring system showed a concordance rate of  
20 69% with the traditional four-factor criteria of DIC established by the Japanese Association  
21 for Acute Medicine. The 28-day mortality rates of the new and the traditional criteria-positive  
22 patients were 43% and 21%, respectively. Those of the new and the traditional  
23 criteria-negative patients were 5.7% and 9.4%, respectively.

1 **Conclusions:** Serum histone H3 levels and platelet counts are potential markers for  
2 determining coagulopathy with high risk of death in septic patients. Further studies are  
3 needed to clarify the utility of serum histone H3 levels in the diagnostic of coagulopathy/  
4 DIC.

5

6 **Keywords:** sepsis, disseminated intravascular coagulation (DIC), diagnosis, histone

7

## 1 **Background**

2 Sepsis is a life-threatening disorder resulting from dysregulated host responses to infection  
3 [1]. Although the details of dysregulated host responses are not defined, insufficiency of  
4 microcirculation associated with excessive activation of the complement system, the  
5 coagulation system, and the innate immune system might be involved [2]. These exaggerated  
6 proinflammatory and procoagulant responses have been the predominant target of clinical  
7 intervention; however, none has proven effective to date [3]. Considering that sepsis is a  
8 particularly heterogeneous syndrome, the selection of patients who may benefit from a  
9 certain intervention is required [2].

10         Recent studies have suggested that anticoagulation therapy does not confer a  
11 survival benefit overall in sepsis, but might be beneficial in the specific subpopulation with  
12 sepsis-associated disseminated intravascular coagulation (DIC) [4-6], with those with high  
13 disease severity as the optimal target for anticoagulation [7]. Several organizations have  
14 proposed diagnostic criteria for DIC, including the International Society on Thrombosis and  
15 Haemostasis (ISTH) [8], the Japanese Association for Acute Medicine (JAAM) [9], and the  
16 Japanese Society on Thrombosis and Hemostasis (JSTH) [10]. However, DIC is not routinely  
17 diagnosed in most institutions in part because diagnostic criteria require the measurement of  
18 multiple coagulation markers, such as prothrombin time (PT), fibrin(ogen) degradation  
19 products (FDP), and thrombin-antithrombin complex (TAT), some of which are not  
20 commonly available.

21         Histone H3 is one of the nucleosome core proteins, acting as a spool around which  
22 DNA winds. In septic conditions, histone H3 and other histones can be released from  
23 activated neutrophils and severely injured cells into the extracellular space, where they

1 induce platelet aggregation [11, 12], coagulation activation [13], endothelial damage [14, 15],  
2 and cardiac injury [16]. Recently, we developed an enzyme-linked immunosorbent assay  
3 (ELISA) which can measure serum or plasma histone H3 levels with high sensitivity and  
4 specificity [17]. Using this ELISA, we found that circulating histone H3 levels in septic  
5 patients were associated with coagulopathy, multiple organ failure, and death [18]. These  
6 findings led us to speculate that serum histone H3 levels might be a simple biomarker for  
7 selecting coagulopathy patients at high risk of death who may benefit from anticoagulation  
8 therapy [5, 7]. In this study, we explore a minimal marker set for determining coagulopathy at  
9 high risk of death in septic patients, wherein histone H3 levels were evaluated as indicators of  
10 both organ failure and coagulation activation.

11

## 12 **Methods**

### 13 **Study population**

14 This investigation was performed using a data set from a single-center observational study  
15 conducted in Kagoshima University Hospital between July 2015 and June 2016 [18]. Among  
16 85 cases of sepsis enrolled in this study, serum samples were obtained in 81 patients within  
17 24 hours after intensive care unit admission (ICU day 1) and analyzed for histone H3 levels.  
18 Diagnosis of DIC was made according to the JAAM DIC criteria on day 1, 3, 5 and 7. The  
19 Acute Physiology and Chronic Health Evaluation II (APACHE II) scores and the Sequential  
20 Organ Failure Assessment (SOFA) scores were calculated with reference to the medical  
21 records although the APACHE II scores and the SOFA scores in this study did not include the  
22 Glasgow Coma Scale scores due to invalid assessment of the scores under sedation. The  
23 study was compliant with the Declaration of Helsinki and approved by the Ethics Committee

1 of Kagoshima University.

2

### 3 **Measurement of histone H3 and coagulation markers**

4 Serum histone H3 levels were measured by ELISA using antibodies against histone H3  
5 (Shino-Test Corporation, Sagami-hara, Japan) as described previously [17, 18]. The lower  
6 detection limit of the ELISA was 2 ng/mL, and the linearity was observed in the range up to  
7 250 ng/mL. The ELISA specifically detected histone H3, and did not react with other  
8 histones, including histone H2A, H2B, and H4 [17, 18].

9 Plasma FDP and TAT levels were determined by LPIA FDP (LSI Medience, Tokyo,  
10 Japan) and STACIA CLEIA TAT (LSI Medience, Tokyo, Japan), respectively, according to  
11 the manufacturer's instructions. Plasma PT and antithrombin (AT) activity were analyzed  
12 using HemosIL RecombiPlasTin (Instrumentation Laboratory Company, Bedford, MA, USA)  
13 and HemosIL Liquid Antithrombin (Instrumentation Laboratory Company), respectively,  
14 according to the manufacturer's instructions. Platelet counts were analyzed using an  
15 automated counting device XE-5000 (Sysmex Corporation, Kobe, Japan).

16

### 17 **Statistical analysis**

18 Statistical analysis was performed using commercial software (SPSS version 23 IBM, Inc.,  
19 Armonk NY, USA). The relationship between serum histone H3 levels and other coagulation  
20 markers was analyzed by Spearman's rank correlation test. The predictive ability of each  
21 parameter for the traditional DIC state established by JAAM or 28-day mortality was  
22 compared by receiver-operating characteristics (ROC) analysis. A  $p < 0.05$  was considered  
23 statistically significant.

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## Results

First, we assessed the characteristics of histone H3 levels in the setting of sepsis-associated DIC by analyzing its correlation with other coagulation markers. The basic characteristics of the study population were shown previously [18]. As shown in Fig. 1, serum histone H3 levels were significantly correlated with TAT levels (Spearman's  $\rho = 0.46$ ,  $P < 0.001$ ) and FDP levels (Spearman's  $\rho = 0.46$ ,  $P < 0.001$ ), and weakly correlated with platelet counts (Spearman's  $\rho = -0.26$ ,  $P < 0.05$ ).

We then compared the diagnostic utility of histone H3 to that of other coagulation markers for predicting the traditional DIC state or 28-day mortality. The area under the ROC curve (AUC) of serum histone H3 levels for predicting the traditional DIC state was 0.75, approximately equivalent to the value of constituent makers for DIC, such as platelets (0.79), PT (0.60), FDP (0.76), and TAT (0.70), and better than that of high mobility group box 1, the prototypical damage-associated molecular pattern molecule. The AUC of serum histone H3 levels for predicting 28-day mortality was 0.73, better than that of other coagulation markers, such as platelets (0.72), PT (0.68), FDP (0.57), and TAT (0.58).

Subsequently, we assessed whether serum histone H3 levels could be a good substitute for FDP, PT, and the disease severity markers for selecting coagulopathy patients at high risk of death. To this end, we proposed a new scoring system, consisting of platelet counts and serum histone H3 levels (Table 1). The cut-off values of serum histone H3 levels and platelet counts were determined by ROC analysis (Supplementary Fig. 1, see Additional file 1). A sum score  $\geq 3$  was considered to be coagulopathy with high risk of death in the new scoring system. As shown in Table 2, the diagnostic concordance rate was 69% (51/74)

1 between the traditional JAAM criteria and the new two-factor scoring system. The 28-day  
2 mortality rates of the new and traditional criteria-positive patients were 43% and 21%,  
3 respectively. The 28-day mortality rates of the new and traditional criteria-negative patients  
4 were 5.7% and 9.4%, respectively. Consistent with these findings, the new scoring system  
5 could clearly discriminate patients at high risk of death from those at low risk (Fig. 2). The  
6 AUC of the new scoring system for predicting 28-day mortality was 0.80, better than that of  
7 other prognostic scores in the ICU setting, such as the SOFA score (0.78) and the APACHE II  
8 score (0.71). Thus, the new scoring system could determine coagulopathy at high risk of  
9 death in septic patients in a simple manner.

10

## 11 **Discussion**

12 In this study, we propose a simple scoring system for determining coagulopathy at high risk  
13 of death in septic patients. The new scoring system is designed to be (i) simple, (ii)  
14 concordant with traditional DIC diagnosis, and (iii) prognostically relevant. A set of two  
15 parameters, platelet counts and serum histone H3 levels, showed excellent performance for  
16 this purpose.

17         Recent studies have suggested that the optimal target for anticoagulation therapy in  
18 septic conditions might be DIC patients with high disease severity [5, 7]. The JAAM DIC  
19 criteria are widely used in Japan and are recommended for the diagnosis of sepsis-associated  
20 DIC [19]. They consist of four parameters, namely platelet count, FDP, PT, and systemic  
21 inflammatory response syndrome (SIRS) [9]. Allowing that no single biomarker has yet been  
22 reported by which DIC can be specifically diagnosed, combinational evaluation of four  
23 parameters might become a bottleneck in the diagnosis process. In the setting of sepsis, a



1 decrease in platelet count is associated with impairment of vascular integrity [20]. An  
2 elevation of FDP is associated with activation of the coagulation and fibrinolysis pathways  
3 [21]. A prolongation of PT is generally associated with liver dysfunction, including shock  
4 liver, in the setting of sepsis [22]. SIRS is associated with an increased risk of coagulopathy  
5 and organ dysfunction [23]. Overall disease severity can be assessed by the SOFA score [5, 7,  
6 24]. Circulating histone H3 levels are associated with multiple organ failure and  
7 coagulopathy [18] and significantly correlated with FDP levels (Fig. 1). Thus, circulating  
8 histone H3 levels can be a good substitute for FDP, PT, SIRS, and the disease severity marker.  
9 Indeed, when combining circulating histone H3 levels with platelet counts, our simple  
10 two-factor scoring system could identify 70% of patients with DIC and high disease severity  
11 and 83% of those without (Supplementary Table 1, see Additional file 1). Furthermore, with  
12 respect to the remaining 20% unmatched cohort, our two-factor scoring system showed good  
13 prognostic performance. These findings indicate that the new scoring system could identify  
14 coagulopathy patients with high disease severity in a simple manner.

15         The present study has several limitations. First, this is a retrospective observational  
16 study, and the cut-off values of our new scoring system were set to optimize diagnostic  
17 performance. Thus, the utility of the new scoring system could not be validated in this study  
18 and should be validated in future studies. Second, most of the JAAM DIC-positive and new  
19 scoring system-negative patients survived 28 days, but it is not clear whether anticoagulant  
20 therapy was dispensable in these patients. Anticoagulant therapy was conducted in 52% of  
21 enrolled patients based on the JAAM DIC scores and clinical features, and might have  
22 contributed to the survival of the JAAM DIC-positive and new scoring system-negative  
23 patients. Prospective intervention studies are needed for rigorous comparison of JAAM DIC

1 criteria and our new scoring system. Third, DIC scores other than the JAAM DIC score, such  
2 as the ISTH DIC score, were not available in this study because of the retrospective nature.  
3 Fourth, applicability of the new scoring system to patients with hematologic disorders is not  
4 clear because thrombocytopenia can be observed in these patients independently of DIC and  
5 circulating histone H3 levels may not be elevated in leukopenic patients [17]. Fifth, histone  
6 H3 levels can be measured at present for research purposes only and cannot be measured in  
7 clinical settings. This is closely related to the novelty of this study, but is simultaneously  
8 related to the disadvantage. Further studies are required for clinical application of histone H3  
9 measurement. Finally, it is not clear in this study whether the elevation of histone H3 levels is  
10 a cause or consequence of DIC, although some studies have demonstrated that extracellular  
11 histones activate platelets and coagulation pathways [11-13, 25]. This may be important when  
12 considering a therapeutic strategy for DIC.

13

#### 14 **Conclusions**

15 Serum histone H3 levels correlated with FDP and TAT levels in septic patients. When  
16 combining serum histone H3 levels with platelet counts, the new scoring system indicated  
17 coagulopathy with high risk of death in a simple manner. Further studies are needed to clarify  
18 the utility of serum histone H3 levels in the diagnosis of coagulopathy/DIC.

19

#### 20 **Abbreviations**

21 APACHE II: Acute Physiology and Chronic Health Evaluation II

22 AT: antithrombin

23 AUC: area under the ROC curve

- 1 DIC: disseminated intravascular coagulation
- 2 ELISA: enzyme-linked immunosorbent assay
- 3 FDP: fibrin(ogen) degradation products
- 4 ICU: intensive care unit
- 5 ISTH: International Society on Thrombosis and Haemostasis
- 6 JAAM: Japanese Association for Acute Medicine
- 7 JSTH: Japanese Society on Thrombosis and Hemostasis
- 8 PT: prothrombin time
- 9 ROC: receiver-operating characteristics
- 10 SIRS: systemic inflammatory response syndrome
- 11 SOFA: Sequential Organ Failure Assessment
- 12 TAT: thrombin-antithrombin complex

13

#### 14 **Declarations**

#### 15 **Ethics approval and consent to participate**

16 This single-center observational study was approved by the Ethics Committee of Kagoshima  
17 University.

18

#### 19 **Consent for publication**

20 Not applicable.

21

#### 22 **Availability of data and material**

23 The datasets used and/or analyzed during the current study are available from the

1 corresponding author on reasonable request.

2

### 3 **Competing interests**

4 The ELISA for histone H3 is a product in development of Shino-Test Corporation, where SY  
5 is an employee. IM holds an endowed chair at Kagoshima University and receives research  
6 funding from Shino-Test Corporation. The funding is for academic promotion and is not  
7 directly related to the present study. The remaining authors declare that they have no  
8 competing interests.

9

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12 Science.

13

### 14 **Authors' contributions**

15 TI designed the experimental protocol and wrote the manuscript. TI, TT, YY, TY, and HF  
16 analyzed clinical data. SY participated in the laboratory experiments. IM and YK critically  
17 appraised the manuscript. All authors read and approved the final manuscript.

18

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**Fig. 1. Serum histone H3 levels are correlated with coagulation markers.** Correlations between serum histone H3 levels (ng/mL) and coagulation marker levels, including platelet counts (PLT,  $\times 10^3/\mu\text{L}$ ), fibrin(ogen) degradation products levels (FDP,  $\mu\text{g/mL}$ ), thrombin-antithrombin complex levels (TAT, ng/mL), SIRS score, prothrombin time (PT) ratio, and antithrombin activity (AT, %), on ICU day 1 are shown. Spearman's  $\rho$ , and  $p$  values, are also shown. Logarithmic scales are used, except for SIRS score.

**Fig. 2. The new scoring system clearly detect patients at high risk of death.** Mortality rates of patients with each JAAM DIC score and the new score on ICU day 1 are shown. Dotted lines represent cut-off lines for JAAM DIC criteria and the new scoring system. The numerator and denominator of a fraction represent the number of non-survivors divided by the sum of the survivors + non-survivors in each score.

1 **Table 1. The new scoring system for identifying coagulopathy patients at high risk of**  
 2 **death.** The scoring system using serum histone H3 level and platelet counts is shown. A sum  
 3 score of  $\geq 3$  is considered to be coagulopathy with high risk of death.

4

5 Histone H3 (ng/mL)

6  $< 3$  0

7  $3 \leq < 9$  1

8  $9 \leq$  2

9 Platelet ( $\times 10^3/\mu\text{L}$ )

10  $> 120$  0

11  $80 \leq < 120$  1

12  $\leq 80$  2

13 Sum score

14  $3 \leq$  Coagulopathy with high risk of death

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16

1 **Table 2. Diagnostic concordance rates between the JAAM DIC criteria and new scoring**  
2 **system.** The diagnostic concordance rate between the JAAM DIC criteria and the new  
3 scoring system on ICU day 1 is 69%. This can be calculated by dividing the numbers of  
4 double-negative patients and double-positive patients (31 + 20) by the total number of  
5 patients (74). The mortality rates of the new and the JAAM DIC criteria-positive patients  
6 were 43% (9/21) and 21% (9/42), respectively. The mortality rates of the new and the JAAM  
7 DIC criteria-negative patients were 5.7% (3/53) and 9.4% (3/32), respectively.

8

Number of patients		JAAM DIC score		
		< 4 (negative)	4 ≤ (positive)	total
New score	< 3 (negative)	31	22	53
	3 ≤ (positive)	1	20	21
	total	32	42	74

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Mortality rate		JAAM DIC score		
		< 4 (negative)	4 ≤ (positive)	total
New score	< 3 (negative)	6.5% (2/31)	4.5% (1/22)	5.7% (3/53)
	3 ≤ (positive)	100% (1/1)	40% (8/20)	43% (9/21)
	total	9.4% (3/32)	21% (9/42)	16% (12/74)

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12 **Additional file**

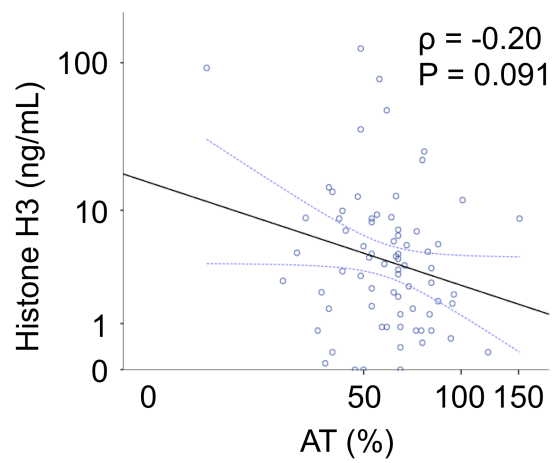
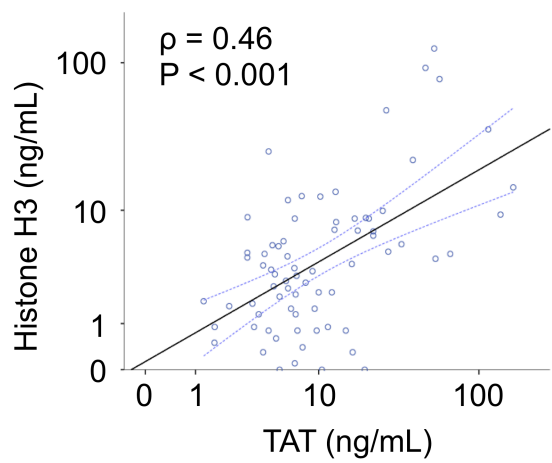
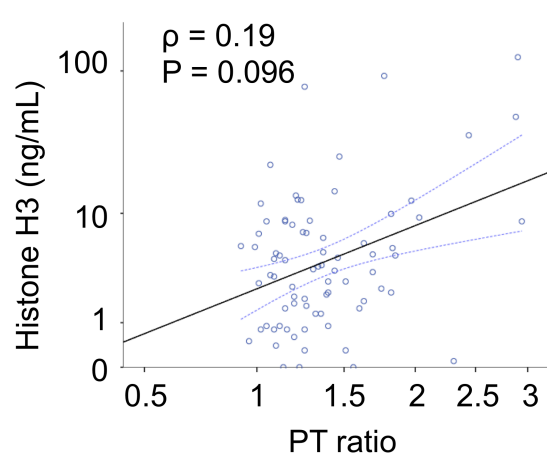
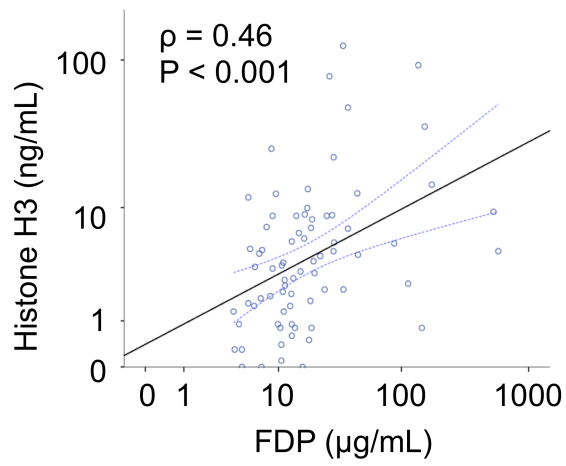
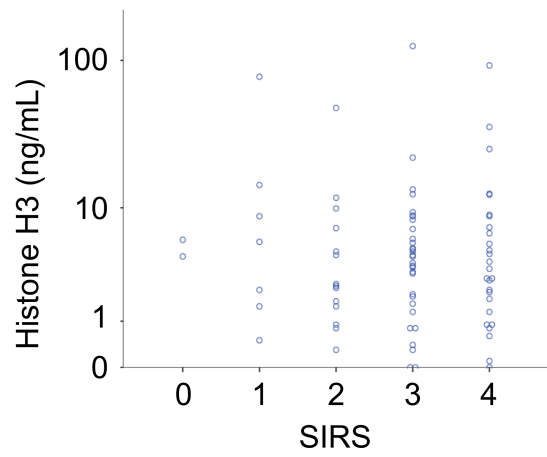
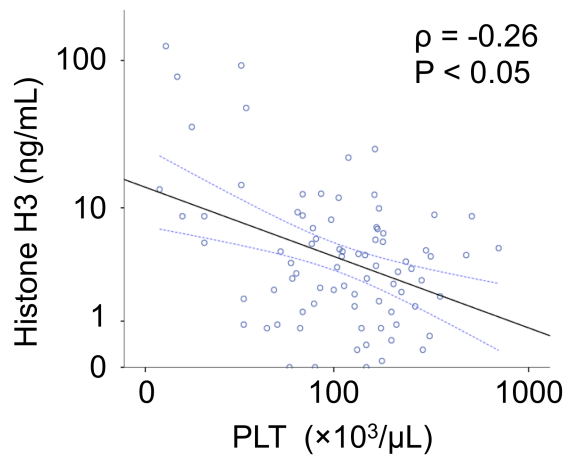
13 Titles and descriptions of data:

14 Supplementary Fig. 1. ROC analyses of platelet counts and serum histone H3 levels for  
15 predicting 28-day mortality.

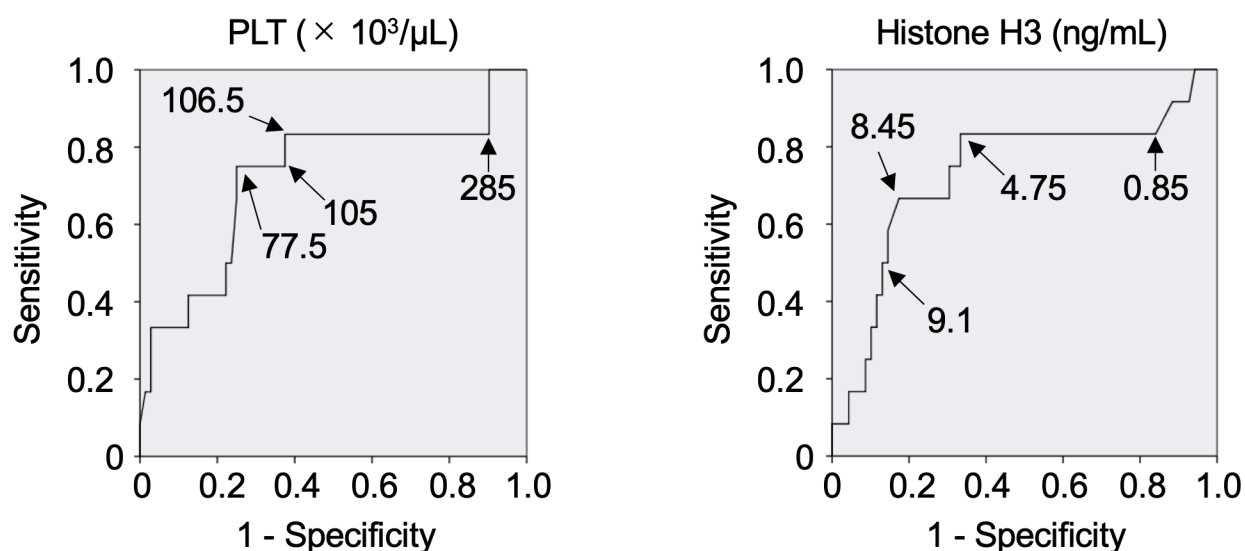
16 Supplementary Table 1. The new scoring system identified coagulopathy patients with high  
17 disease severity.

18

Fig. 1



Supplementary Fig. 1



**Supplementary Fig. 1. ROC analyses of platelet counts and serum histone H3 levels for predicting 28-day mortality.** Arrows show platelet and histone H3 values around the upper left corners of the ROC curves. In ROC analysis, values corresponding to points nearest to the upper left corner of the ROC curve are considered to be good cut-off values. For predicting 28-day mortality, platelet counts of 77.5 and  $106.5 \times 10^3/\mu\text{L}$  and histone H3 levels of 4.75 and 8.45 ng/mL can be good cut-off values. For predicting DIC, platelet counts of 91.5 and  $136.5 \times 10^3/\mu\text{L}$  and histone H3 level of 3.55 ng/mL can be good cut-off values (data not shown). Based on these findings, the cut-off values in the new criteria were determined to be 80 and  $120 \times 10^3/\mu\text{L}$  for platelet counts and 3 and 9 ng/mL for histone H3 levels.

Supplementary Table. 1

Number of non-survivors / patients		DIC and SOFA score $\geq 11$		
		-	+	total
New criteria	-	1 / 45	1 / 5	2 / 50
	+	3 / 9	6 / 12	9 / 21
	total	4 / 54	7 / 17	11 / 71

**Supplementary Table 1. The new criteria identified DIC patients with high disease severity.** The numerator and denominator of a fraction represent the number of non-survivors divided by the sum of the survivors + non-survivors, respectively, in each category. The new criteria identified 12/17 of patients with DIC and high disease severity, and 45/54 of those without. In this analysis, high disease severity was defined as a SOFA score  $\geq 11$  although previous studies suggested a SOFA score  $\geq 13$ . The difference was based on the fact that our SOFA score did not include the Glasgow Coma Scale score due to invalid assessment under sedation. Similar results were obtained even if high disease severity was defined as a SOFA score  $\geq 10$ , 11, 12, or 13. Furthermore, with respect to the remaining unmatched cohort ( $n = 14$ ), our new criteria showed better prognostic performance (3/9) than the combination of DIC and SOFA scores (1/5).