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Full Length Article

Clot waveform analysis in Clauss fibrinogen assay contributes to classification of fibrinogen disorders

Atsuo Suzuki[a,*], Nobuaki Suzuki[b], Takeshi Kanematsu[c], Sho Shinohara[d], Nobuo Arai[d], Ryosuke Kikuchi[a], Tadashi Matsushita[b,c]

[a] Department of Medical Technique, Nagoya University Hospital, Japan
[b] Department of Transfusion Medicine, Nagoya University Hospital, Japan
[c] Department of Laboratory Medicine, Nagoya University Hospital, Japan
[d] Sysmex Corporation, Japan

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ABSTRACT

Background: Clauss fibrinogen assay (CFA) is widely used as a screening test to detect fibrinogen disorders. However, CFA alone cannot distinguish quantitative and qualitative defects because it depends on functional fibrinogen activity (Ac), and fibrinogen antigen (Ag) determination is required to classify fibrinogen disorders.
Objectives: To establish a novel approach to classify fibrinogen disorders, we investigated the potential of clot waveform analysis (CWA) of CFA and searched for a surrogate marker for fibrinogen Ag.
Materials and methods: We analyzed CWA parameters obtained from CFA using plasma from normal patients (n = 91) and those with fibrinogen disorders (n = 27, including 15 hypofibrinogenemia, 6 dysfibrinogenemia and 6 hypodysfibrinogenemia) with a CS-5100 autoanalyzer.
Results: We found that maximum coagulation velocity (Min1) levels were most strongly correlated with fibrinogen Ag in both normal and fibrinogen disorders. Hence, Min1 appeared to function as a surrogate for fibrinogen Ag. Although the Ac/Min1 ratio did not simply reflect the measured Ac/Ag ratio, we found that the Ac/Min1 ratio was significantly higher than normal in hypofibrinogenemia and hypodysfibrinogenemia, but not in dysfibrinogenemia. On the other hand, we could distinguish type II deficiency from type I using estimated fibrinogen Ag (eAg) predicted from Min1. The Ac/eAg ratios of dysfibrinogenemia and hypodysfibrinogenemia were significantly lower than those of normal and hypofibrinogenemia.
Conclusion: The CWA of CFA could distinguish fibrinogen disorders using a combination of Ac/Min1 and Ac/eAg values. This analysis allows the qualitative detection of fibrinogen disorder easily and represents a novel screening test for fibrinogen disorders.

1. Introduction

Fibrinogen disorders are usually classified as quantitative (type I, hypo- or afibrinogenemia) or qualitative deficiencies (type II, dys- or hypodysfibrinogenemia). Type I quantitative fibrinogen deficiencies are characterized by absent or low levels of plasma fibrinogen antigen, and type II qualitative deficiencies are characterized by normal or reduced antigen levels associated with disproportionately low functional activity [1]. Type II deficiencies in particular show various symptoms such as bleeding or thrombosis [1,2], making it necessary to distinguish these fibrinogen abnormalities.

In routine laboratory analyses, plasma fibrinogen could be examined by several assays, such as Clauss fibrinogen assay (CFA) and prothrombin-time- (PT-) derived methods. CFA is generally used for primary examinations and is well-optimized for a large population of automated coagulation analyzers. Type II deficiencies, dysfibrinogenemia or hypodysfibrinogenemia, are usually suspected if there is a discrepancy between functional and immunologic fibrinogen levels [2]. Although CFA is widely used as a screening test, it provides information regarding only functional fibrinogen [3]. It is currently not possible to discriminate between low levels of functional fibrinogen and low levels of fibrinogen antigen using CFA alone. Immunological fibrinogen antigen determination is needed to evaluate the amount of fibrinogen and the discrepancy in the two kinds of fibrinogen levels. Hence, the current diagnostic algorithm for fibrinogen disorders requires the measurement of both functional fibrinogen levels and

* Corresponding author at: Department of Medical Technique, Nagoya University Hospital, 65 Tsurumai-cho, Showa-ku, Nagoya 466-8560, Japan.
E-mail address: asuzuki@med.nagoya-u.ac.jp (A. Suzuki).

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antigen levels. On the other hand, the measurement of fibrinogen antigen is typically carried out only in specialized laboratories and it is difficult to perform fibrinogen Ag determinations in every laboratory.

By developing automated coagulation analyzers that use optical methods, we could monitor fibrin formation during coagulation assays and obtain more information about the coagulation process [4,5]. Recent studies reported on the development of clot waveform analysis (CWA) and the usefulness of CWA [6,7].

In this study, we aimed to investigate whether CWA could provide a surrogate marker for fibrinogen Ag and/or an indicator to classify fibrinogen disorders without the need for additional fibrinogen Ag determination. We performed CWA in CFA and analyzed the parameters derived from the clot formation curve by comparing plasma samples from healthy donors to those with fibrinogen defects, and evaluated whether CWA could be applied to distinguish between quantitative and qualitative defects.

2. Materials and methods

2.1. Sample collection

This study was approved by the Nagoya University Hospital Ethics Committee (Identification number: 2010-1038). Blood was collected in polypropylene tubes with 0.109 mol/L sodium citrate. Citrated blood was centrifuged at 2500 ×*g* for 5 min and the separated plasma was placed in new polypropylene tubes. Plasma samples were kept at −30 °C for < 2 weeks before storing them at −80 °C. Normal plasma (n = 91) was collected from patients with no liver diseases according to the following criteria: 1) prothrombin time (PT) and activated partial thromboplastin time (aPTT) were normal in our hospital, and 2) D-dimer and fibrin/fibrinogen degradation products (FDPs) were < 0.5 μg/mL and < 2.5 μg/mL, respectively. Plasma was also collected from patients with fibrinogen disorders after obtaining written informed consent. Fibrinogen disorders showing plasma fibrinogen Ag levels below lower limit of normal value (1.5 g/L) were classified as hypofibrinogenemia or hypodysfibrinogenemia according to the recommendation [1,8].

2.2. Measurement of plasma functional fibrinogen (Ac)

We examined functional fibrinogen levels by CFA using Thrombocheck Fib (L) reagent and the automated coagulation analyzer CS-5100 (Sysmex, Kobe, Japan), according to manufacturer's instructions. Ten microliters of sample plasma were diluted with 90 μL of Owren's veronal buffer (Sysmex). The diluted plasma was incubated at 37 °C for 190 s, and then mixed with 50 μL of thrombin reagent. The reaction mixture was incubated at 37 °C and the absorbance was measured at 405 nm for 200 s. A calibration curve was generated by a serial dilution of Coagtrol N (Sysmex) and Fibrinogen Calibrator Kit (Siemens, Berlin, Germany). Functional fibrinogen determined by CFA are here termed fibrinogen activity (Ac).

2.3. Measurement of plasma fibrinogen antigen (Ag)

We determined plasma fibrinogen antigen (Ag) levels by latex immunoagglutination assay (LIA). LIA was performed using FactorAuto Fibrinogen (Q-may, Ohita, Japan). A calibration curve was generated using FactorAuto fibrinogen standard plasma (Q-may). The measurement assay protocol was newly established as follows: 5 μL of sample plasma was diluted with 95 μL of dilution buffer, and 10 μL of the diluted plasma was further diluted with an equal volume of dilution buffer. Then, 150 μL of reaction buffer was added to the 20 μL of diluted plasma and incubated at 37 °C for 40 s. Next, 50 μL of latex particles conjugated with anti-fibrinogen antibody was added to the sample and incubated at 37 °C for 190 s. Then, the absorbance of the reaction mixture was measured at 575 nm for 200 s, and the data was analyzed from 13 to 180 s. The measurement values were validated using the control plasma (Q-may) and the WHO International Standard for plasma fibrinogen (09/264; NIBSC, London, UK).

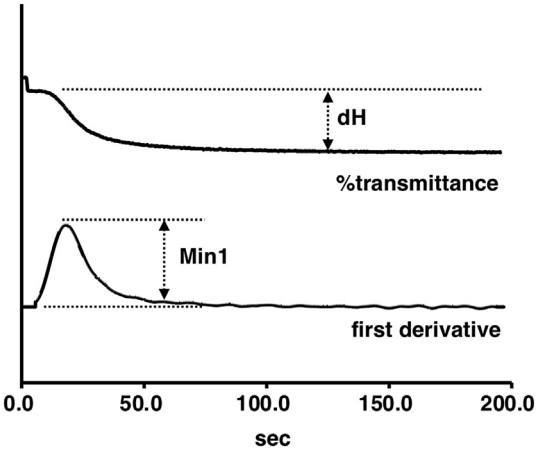

Fig. 1. Clot waveform analysis of Clauss fibrinogen assays in a CS-5100 instrument.
Clot waveform of normal plasma in a Clauss fibrinogen assay. The upper trace shows the changes in light transmittance during the monitoring of fibrin generation for 200 s. The lower trace indicates the first derivative curve, the direction of which was reversed in the CS-5100 autoanalyzer.

2.4. Clot waveform analysis (CWA)

Clot waveform analysis (CWA) of CFA was performed using the CS-5100. The clot waveforms were recorded as the amount of transmitted light to monitor the coagulation process. Representative data of CWA in normal pooled plasma is shown in Fig. 1. The upper curve is a reaction curve representing changes in light transmittance (% transmittance). Transmittance decreased depending on fibrin formation, and the total difference in the transmittance level was shown as delta H (dH). The first derivative curve (lower curve) was generated from a reaction curve by considering fibrin formation as “positive” (upward) in CS-5100. The interpretation of CWA parameters was according to the official recommendations [9]; we analyzed maximum coagulation velocity (Min1) from the first derivative curve in this study.

2.5. Statistical analysis

Pearson and Spearman rank tests were performed to define the correlation of the levels of fibrinogen Ag with CWA parameters. A comparison between two groups was performed using the Student's *t*-test for normally distributed variables and the Mann-Whitney *U* test for non-normally distributed variables. We evaluated differences among groups using a one-way ANOVA. Regression analysis was performed and calculated r-square values from the standard curve. A probability of < 5% was considered to indicate a statistically significant difference.

3. Results

3.1. Relationships between fibrinogen antigen and CWA parameters in normal plasma

Normal plasma, which displayed a wide range of fibrinogen levels, was utilized in this study, and the respective fibrinogen Ac and Ag levels were measured by CFA and LIA. The levels of fibrinogen Ac and Ag were 1.40–6.60 and 1.65–6.95 g/L, respectively, and specific activity (Ac/Ag ratio) were 0.85–1.26. Light transmittance changes in CFA (dH) and Min1 values were calculated by CWA, and the relationship between fibrinogen Ag and CWA parameters were analyzed. The dH and Min1

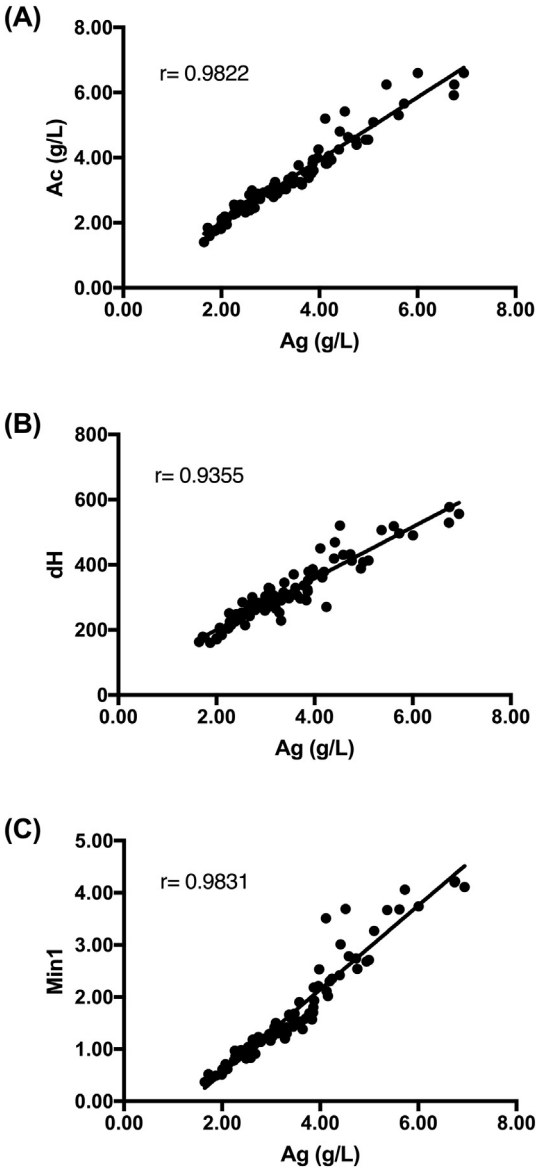

Fig. 2. Correlation between fibrinogen antigen and CWA parameters in normal plasma samples.
The graphs show the correlation between fibrinogen Ag and fibrinogen Ac (A), dH (B) and Min1 (C). The respective correlation coefficients (r) are shown in each plot.

values were 160–577 and 0.37–4.22, respectively. As shown in Fig. 2, fibrinogen Ag was obviously correlated with Ac (Fig. 2A), as expected. The CWA parameters dH and Min1 (Fig. 2B and C, respectively) also showed a good correlation. Particularly, we found the strongest correlation between Min1 and Ag (r = 0.9831).

3.2. Min1 correlates with fibrinogen antigen in fibrinogen disorders

Next, we analyzed plasma from patients with fibrinogen disorders. The fibrinogen Ac and Ag levels were measured by CFA and LIA and further CWA in CFA was also performed Fifteen hypofibrinogenemia patients participated in this study, with all showing almost no complications. Twelve patients with dys- and hypodysfibrinogenemia were analyzed and the results and their phenotypes were shown in Table 1. Each of the 6 patients with dysfibrinogenemia or hypodysfibrinogenemia all showed diminished fibrinogen Ac and Ac/Ag ratios, which indicated abnormal fibrinogen function. There were no significant differences in Ac levels or Ac/Ag ratios. In contrast, we observed significant differences in dH and fibrinogen Ag levels between dysfibrinogenemia and hypodysfibrinogenemia.

We further investigated whether Min1 levels correlated with fibrinogen Ag in fibrinogen disorders. As illustrated in Fig. 3, Min1 levels were highly correlated with fibrinogen Ag levels in hypofibrinogenemia (Fig. 3A), dysfibrinogenemia (Fig. 3B) and hypodysfibrinogenemia (Fig. 3C). The relationships between fibrinogen Ag and dH levels were also analyzed, and we found a positive correlation only in the hypodysfibrinogenemia group (r = 0.8117, Supplementary Fig. 1C). In contrast, dH levels showed a poor correlation with fibrinogen Ag levels in hypofibrinogenemia (r = 0.3087, Supplementary Fig. 1A) and dysfibrinogenemia (r = 0.5429, Supplementary Fig. 1B). As some plasma samples from hypofibrinogenemia patients were obtained from icteric patients, we investigated the potential interference of several substances, e.g., hemoglobin, bilirubin and chyle. The results demonstrated that dH values were highly prone to interference by these substances, whereas Min1 levels were not subject to such interference (Supplementary Fig. 2). Thus, dH would be unsuitable for estimation of fibrinogen Ag, while Min1 showed the ability to calculate eAg regardless of sample quality. These results suggested Min1 could function as a surrogate marker that reflects fibrinogen Ag levels in fibrinogen disorders as well as normal plasma.

3.3. High levels of Ac/Min1 ratio indicates hypo- and hypodysfibrinogenemia

We hypothesized that Min1 values would correspond with plasma fibrinogen Ag levels, and therefore assumed that the Ac/Min1 ratio would be equivalent to the Ac/Ag ratio. Individual fibrinogen Ac values were divided by Min1 values and the resultant Ac/Min1 ratios from normal and fibrinogen disorders were compared. In Fig. 4, the mean Ac/Min1 ratio of normal and dysfibrinogenemia were 2.31 and 1.89, respectively, with no significant difference observed between the groups. On the other hand, the ratios of Ac/Min1 in hypofibrinogenemia and dysfibrinogenemia were significantly higher than that of normal and dysfibrinogenemia. These results indicate that the levels of Ac/Min1 can be used to distinguish hypo- and hypodysfibrinogenemia from dysfibrinogenemia, whereas dysfibrinogenemia could not be detected using this method.

3.4. Classification of fibrinogen disorders using estimated fibrinogen Ag

Since the Ac/Min1 ratio did not simply reflect the measured Ac/Ag ratio, we further investigated a method to distinguish dysfibrinogenemia from normal, hypo- and hypodysfibrinogenemia. We speculated that Min1 could not directly reflect fibrinogen Ag levels; therefore, we attempted to predict fibrinogen Ag from Min1 values using a regression curve covering a broad range of fibrinogen Ag levels. We generated a standard curve of fibrinogen Ag and Min1 values of plasma fibrinogen calibrators and then calculated estimated fibrinogen Ag (eAg) from each Min1 value. A non-linear regression analysis, second order polynomial, was used to fit the standard curve (R^2 = 0.9986, Supplementary Fig. 3). The estimated specific activity, which is represented in the ratio of Ac/eAg, was derived from fibrinogen Ac divided by eAg and compared between normal and fibrinogen disorders (Fig. 5A). Although we did not identify significant differences in Ac/eAg ratios between dysfibrinogenemia (mean: 0.38) and hypodysfibrinogenemia (mean: 0.46), both type II deficiencies exhibited significantly lower Ac/eAg levels than normal (both $P < 0.0001$). The distribution pattern of Ac/eAg ratios was quite similar and corresponded to measured Ac/Ag ratios, illustrated in Fig. 5B. These results indicate that Ac/eAg ratios are concordant with Ac/Ag ratios, but not Ac/Min1, thereby indicating that eAg is a suitable surrogate for measuring fibrinogen Ag. The summarized data of the CFA-CWA in type II deficiencies are shown in Table 1.

Table 1
Characteristics of the dys- and hypodysfibrinogenemia patients.

Case	Diagnosis	Manifestation	Type*	Ac	Ag	Ac/Ag	dH	Min1	Ac/Min1	eAg	Ac/eAg
1	Dysfibrinogenemia	Asymptomatic	3A	1.45	3.15	0.46	489	0.894	1.63	2.98	0.49
2		Asymptomatic	3A	0.14	2.15	0.06	221	0.081	1.70	0.73	0.19
3		Asymptomatic	3A	0.13	1.92	0.07	272	0.075	1.75	0.71	0.18
4		Asymptomatic	3A	0.44	1.87	0.23	265	0.210	2.08	1.12	0.39
5		Asymptomatic	3A	1.23	2.61	0.47	288	0.580	2.13	2.17	0.57
6		Asymptomatic	3A	0.77	1.96	0.39	304	0.377	2.04	1.61	0.48
7	Hypodysfibrinogenemia	Asymptomatic	4C	0.44	1.11	0.40	118	0.110	4.00	0.82	0.54
8		Asymptomatic	4C	0.49	1.11	0.44	143	0.108	4.52	0.81	0.60
9		Asymptomatic	4C	0.56	1.31	0.43	154	0.136	4.10	0.90	0.62
10		Bleeding	4C	0.59	1.46	0.41	187	0.186	3.17	1.05	0.56
11		Thrombotic	4C	0.12	1.04	0.12	35	0.022	5.45	0.55	0.22
12		Thrombotic	4C	0.15	1.22	0.12	100	0.048	3.10	0.63	0.24

Ac, activity, g/L; Ag, antigen, g/L; eAg, estimated Ag, g/L.
* Classification according to recommendation [8].

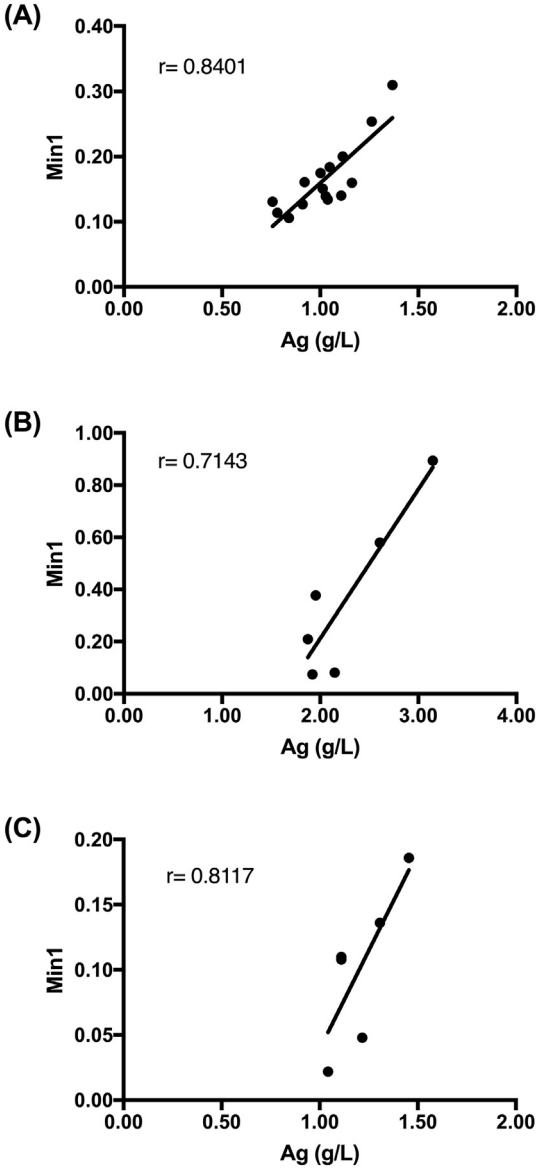

Fig. 3. Correlation between fibrinogen antigen and Min1 in fibrinogen disorders.
The graphs show the correlation of fibrinogen Ag and Min1 in hypofibrinogenemia (A), dysfibrinogenemia (B) and hypodysfibrinogenemia (C). The respective correlation coefficients (r) are shown in each plot.

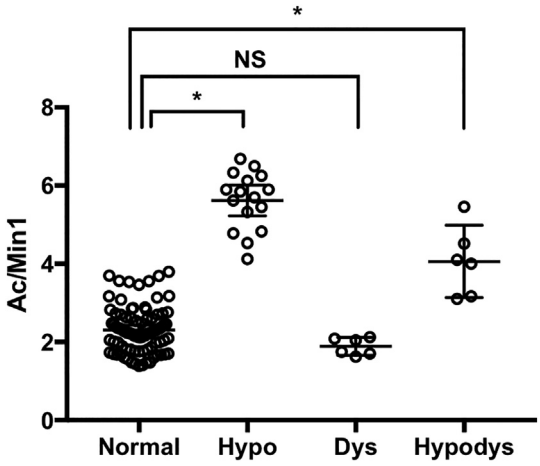

Fig. 4. Comparison of Ac/Min1 ratios.
The scatter plots show Ac/Min1 ratios in normal and fibrinogen disorders. The horizontal lines and error bars indicate mean and 95% CI, respectively. Hypo, hypofibrinogenemia; Dys, dysfibrinogenemia; Hypodys, hypodysfibrinogenemia; NS, not significant. *$P < 0.0001$ comparisons of Ac/Min1 between the groups.

4. Discussion

We have occasionally found suspected cases of fibrinogen abnormalities in routine laboratory testing, and it is clinically important to evaluate both the quantity and quality of fibrinogen. Our current study revealed that CFA-CWA could be a novel screening test to classify fibrinogen disorders without a need of fibrinogen Ag determination.

Our major findings are as follows: First, Min1 values were strongly associated with fibrinogen Ag. Second, the Ac/Min1 ratio could be an indicator to distinguish hypo- and hypodysfibrinogenemia from dysfibrinogenemia. Third, by using the estimated fibrinogen Ag derived from a standard curve, we could distinguish between qualitative and quantitative fibrinogen disorders. We are able to classify these three kinds of fibrinogen disorder by combining measures of Ac/Min1 and Ac/eAg levels and comparing with normal control. Hence, this study clearly showed that we are able to classify type I and type II fibrinogen disorders by CWA in CFA.

It was reported that CWA was useful for monitoring hemostasis in patients with hemophilia A (PWH) [6,10] and for the early diagnosis and prognosis of sepsis and disseminated intravascular coagulation [11,12]. Fibrinogen assessment using clot waveform has also been investigated [5,13]. Recently, Jacquemin and colleagues reported the usefulness of CWA for fibrinogen analysis [14]. They concluded that the amplitude of the first derivative curve could be used as a marker to

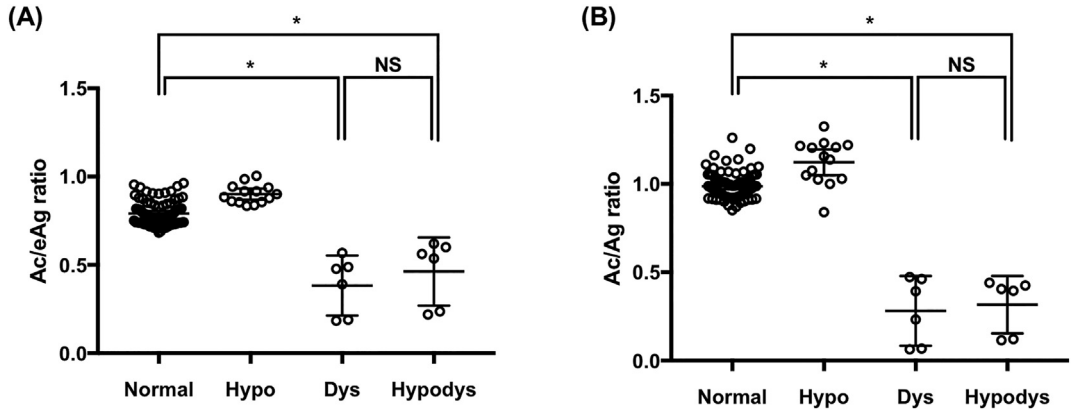

Fig. 5. Comparison of Ac/eAg and Ac/Ag ratios.
The Ac/eAg (A) and Ac/Ag (B) ratios from normal and fibrinogen disorders were compared. The scatter plot shows all samples and the horizontal line and error bars indicate the mean and 95% CI, respectively. Hypo, hypofibrinogenemia; Dys, dysfibrinogenemia. *$P < 0.0001$ comparisons of Ac/eAg or Ac/Ag between the groups.

distinguish hypofibrinogenemia from dysfibrinogenemia caused by the FGG-Arg301Cys mutation. Notably, the current CFA-CWA analysis is capable of classifying type I and type II fibrinogen deficiencies, especially dysfibrinogenemia and hypodysfibrinogenemia. Furthermore, both Ac/Min1 and Ac/eAg analyses produced an obvious discrimination between fibrinogen disorders despite the patients recruited in this study exhibiting heterogeneous phenotypes and various fibrinogen Ac levels in CFA.

While we promote the usefulness of estimated specific activity (Ac/eAg), we could not clearly define a cut-off value of the Ac/eAg ratio to distinguish fibrinogen abnormalities because of small sample numbers. Krammer and colleagues reported that a ratio of functional Ac to measured Ag lower than 0.7 was arbitrarily considered to be suggestive of congenital dysfibrinogenemia [15]. Indeed, other studies have shown that a ratio of 0.7 would permit the identification of almost all cases of congenital dysfibrinogenemia [16–18]. Although our results showed that the Ac/eAg ratio was slightly different from the measured Ac/Ag ratio, the cut off value 0.7 would be considered to be capable of distinguishing type II from type I fibrinogen deficiency since the lower 95% CI of mean normal Ac/eAg ratio was 0.78.

A simple question remains unanswered: why does the Min1 value reflect fibrinogen Ag? This might be due to a property of differential calculus. The amount of change in light transmittance is shown as dH; thus, dH values reflect plasma turbidity due to fibrin formation, which would be dependent on the amount of fibrinogen Ag. Min1 values are strongly associated with dH values, and correlate to fibrinogen Ag. Min1 was not affected by plasma qualities because Min1 was calculated by differential calculus, which produces a velocity at a limited point (a moment) of light transmittance change. This is why Min1 showed superior correlation with Ag even in icteric samples of hypofibrinogenemia. However, Min1 could not directly reflect fibrinogen Ag. The reason for this is that the correlation between Min1 and Ag in normal samples did not include lower levels of fibrinogen. Moreover, the relationship between Min1 and Ag required a non-linear regression to fit the standard curve. Therefore, Min1 values calculated from plasma showing low levels of fibrinogen Ac did not correctly reflect the corresponding Ag levels.

This study has some limitations. The clinical manifestations of patients with dysfibrinogenemia or hypodysfibrinogenemia are heterogeneous, and patients with fibrinogen disorders are commonly identified during the clinical investigation of bleeding [19] or thrombosis [20], or following miscarriage [21]. In contrast, most individuals are asymptomatic, and are usually discovered incidentally by routine laboratory testing before surgery [16]. The current CWA of CFA could not completely distinguish these phenotype variations. In particular, it is important to detect a thrombotic risk in dysfibrinogenemia. Two proposed mechanisms may explain most of the cases of thrombosis associated with dysfibrinogenemia: (1) The abnormal fibrinogen is defective in binding thrombin, which results in elevated levels of thrombin. (2) The abnormal fibrinogen forms a fibrin polymer that is resistant to fibrinolysis [1]. Our current analysis would not be able to characterize the former mechanism because fixed amount of thrombin was added to plasma. On the other hand, although fibrinolysis state could not be evaluated by our current CWA, we should develop current CFA-CWA to address this issue. It is also necessary to analyze a larger number of fibrinogen disorders to properly elucidate the relationships between CWA parameters and phenotypes. Furthermore, we could not investigate the genotypes of the 12 patients with (hypo)dysfibrinogenemia, further studies are also required to investigate the relationships between CWA parameters and genotypes using a larger number of patients. We aim to develop laboratory testing including CWA, which would be capable of predicting clinical phenotypes without complicated analyses in the future.

5. Conclusion

Taken together, our study clearly revealed that CWA in CFA could be a novel laboratory test to classify fibrinogen disorders. The analysis could be performed not only in specialized laboratories but also non-specialized or small laboratories. Furthermore, this method does not require an additional Ag determination and the associated costs. Our current study also provided a new aspect of CWA and highlighted its potential as a diagnostic approach.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.thromres.2018.12.018.

Declarations of interest

A. Suzuki, S. Shinohara, N. Arai and T. Matsushita have a pending patent (application No. JP2017-085329). S. Shinohara and N. Arai are employees of Sysmex Corporation.

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Author contributions — Conceived and designed the experiments: A. Suzuki. Performed the experiments: A. Suzuki. Analysis and interpretation: A. Suzuki, N. Suzuki, T. Kanematsu, R. Kikuchi, and T. Matsushita. Data collection: A. Suzuki and S. Shinohara. Contributed reagents/materials/analysis tools: A. Suzuki, N. Suzuki, T. Kanematsu, S. Shinohara, and N. Arai. Writing the manuscript: A. Suzuki. Revising the manuscript: N. Suzuki, T. Kanematsu, R. Kikuchi, and T. Matsushita. All authors critically reviewed the manuscript and approved its final

version.

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