Manuscript: 26 pages (excluding title page)
Illustrations: 3

Screening method for congenital dysfibrinogenemia using clot waveform analysis with the Clauss method

Shinpei Arai[a], Tomu Kamijo[b], Fumiaki Hayashi[c], Sho Shinohara[c], Nobuo Arai[c], Mitsutoshi Sugano[b], Takeshi Uehara[b], Takayuki Honda[b], Nobuo Okumura[d,]

[a] Department of Biomedical Laboratory Sciences, School of Health Sciences, Shinshu University, Matsumoto, Japan
[b] Department of Laboratory Medicine, Shinshu University Hospital, Matsumoto, Japan
[c] Sysmex Corporation, Japan
[d] Department of Clinical Laboratory Investigation, Graduate School of Medicine, Shinshu University, Matsumoto, Japan

Address correspondence to:
Shinpei Arai, Ph.D.
Department of Biomedical Laboratory Sciences, School of Health Sciences,
Shinshu University.
3-1-1 Asahi, Matsumoto, 390-8621 Japan
TEL: +81-263-37-2387
FAX: +81-263-37-2370
e-mail: arais@shinshu-u.ac.jp

Article type: Original Articles

Running title: Screening method for dysfibrinogenemia using clot waveform analysis

Word count:
Abstract; 242
Manuscript; 3,336 (excluding Title page, Abstract, Key Words and Reference list)
Table; 2, Figures; 3
References; 31

Abstract

Introduction: Congenital fibrinogen disorders (CFD) are classified as afibrinogenemia or hypofibrinogenemia (Hypo), dysfibrinogenemia (Dys), or hypodysfibrinogenemia (Hypodys), according to functional and antigenic fibrinogen concentrations. However, in routine laboratory tests, plasma fibrinogen levels are mostly measured using the functional Clauss method and not as an antigenic level. Therefore, it is difficult to discriminate CFD from acquired hypofibrinogenemia (aHypo). To establish a screening method for CFD, we investigated the parameters of clot waveform analysis (CWA) from the Clauss method.

Methods: We compared fibrinogen concentrations determined using Clauss and prothrombin time (PT)-derived methods for 67 aHypo and CFD cases (19 Dys, 4 Hypodys, and 1 Hypo determined using antigen levels and DNA sequence analysis) with a CS-2400 instrument, and the CWA parameters, dH and Min1, were analyzed automatically with an on-board algorithm. dH and Min1 are the maximum change in transmittance at the end of coagulation and the maximum velocity of transmittance change during coagulation, respectively.

Results: Clauss/PT-derived ratios detected 18 cases of Dys and Hypodys but no Hypo cases, whereas Clauss/dH plus Clauss/Min1 ratios calculated from fibrinogen

concentration using the Clauss method and CWA parameters detected 21 cases of Dys and Hypodys and one Hypo cases. Moreover, the Clauss/PT-derived ratio and Clauss/dH plus Clauss/Min1 ratio detected 22 cases of Dys and Hypodys cases and one Hypo cases. *Conclusion:* This report demonstrates that CWA parameters of the Clauss method, Clauss/dH plus Clauss/Min1 ratio, screened Dys patients with a higher rate, whereas, Clauss/PT-derived ratios did not.

Key Words:

Fibrinogen, congenital fibrinogen disorders, Clauss method, clot waveform analysis.

1. Introduction

Fibrinogen is a 340 kDa hexametric plasma glycoprotein that is synthesized by hepatocytes. Fibrinogen consists of two sets of three polypeptide chains, Aα, Bβ, and γ, which are encoded by *FGA, FGB*, and *FGG*, respectively[1]. Circulating fibrinogen concentrations range between 1.8 and 3.5 g/L (reference range of Shinshu University Hospital), and congenital fibrinogen disorders (CFD) are characterized by functional and antigenic fibrinogen concentrations. Patients with lower fibrinogen levels or who are below the detection limit of functional and antigenic fibrinogen are fibrinogen-deficient (type I; afibrinogenemia as a homozygote and hypofibrinogenemia [Hypo] as a heterozygote). Patients with lower levels of functional fibrinogen and a normal level of antigenic fibrinogen have dysfunctional fibrinogenemia (type II; dysfibrinogenemia [Dys] or hypodysfibrinogenemia [Hypodys] as a homozygote and heterozygote).[2] There is significant heterogeneity in the clinical manifestations of CFD, with most patients being asymptomatic. Approximately 25% of patients experience bleeding, 20% have thrombosis, and some have both a bleeding tendency and thrombosis.[3,4] Because of the implications for patient management, it is important to identify whether fibrinogen levels are low due to congenital or acquired factors and provide an accurate CFD diagnosis.

In routine laboratory tests, plasma fibrinogen levels are measured using a method based on the detection of fibrin polymerization and clot formation, most commonly the Clauss method.[5] This method has been adopted in well-optimization automated coagulation analyzers. Although the Clauss method is widely used as a coagulation screening test, it is currently difficult to discriminate between low levels of functional fibrinogen and low levels of fibrinogen antigen. We have occasionally found suspected cases of CFD in routine laboratory testing, and the measurement of fibrinogen antigen levels is necessary to evaluate the phenotype of quantitative and qualitative abnormality. Unfortunately, fibrinogen antigen measurement and fibrinogen DNA analysis are carried out only in specialized laboratories, so it is difficult to accurately distinguish between Type II deficiencies and acquired hypofibrinogenemia (aHypo) in most laboratories.

Prothrombin time (PT)-derived method is also widely used, which estimates fibrinogen concentrations based upon the change in transmittance during a PT assay. This has been used as an alternative fibrinogen assay, and PT-derived fibrinogen concentrations show a good correlation with fibrinogen antigen levels.[6] However, this can only be measured using automated coagulation analyzers with a customized algorithm, and such algorithms are not as standardized as the Clauss method because the fibrinogen value by the PT-derived method changes depending on the type of PT reagent.[7]

Clot waveform analysis (CWA), using an automated coagulation analyzer, is a recently developed algorithm for monitoring fibrin formation.[8-10] During clot formation in routine coagulation assays, it is possible to obtain a continuous measurement of changes in light transmittance. CWA can be performed at the same time as measuring for the end point of clotting, and we can obtain useful information concerning the coagulation process using specialized algorithms. Numerous studies have reported on the development of CWA and on the usefulness of CWA for hemophilia and other coagulation factor disorders.[11-14] Several studies using CWA in fibrinogen measurement have been reported. Jacquemin et al. reported that the amplitude and velocity of the clot waveform from the thrombin time test of a Dys variant, γR275C, were higher than those of a healthy donor and aHypo cases.[15] Furthermore, Suzuki et al. reported a new approach that can classify fibrinogen disorders using the estimated fibrinogen antigen predicted from the CWA parameters using the Clauss method.[16]

In this study, we measured plasma fibrinogen concentrations in 24 cases with CFD using the Clauss and the PT-derived method. We investigated whether the shape of the coagulation curve and CWA parameters from the Clauss method carried out on using an automated coagulation analyzer can provide further information for the screening of CFD and compared the usefulness of the PT-derived method for CFD screening.

2. Materials and methods

This study was approved by the Ethics Review Board of Shinshu University School of Medicine (Identification number: 4153).

2.1. Plasma samples

Blood samples were collected from 67 patients with lower fibrinogen levels than the reference interval (<1.8 g/L) using the Clauss method and these were described as aHypo. Blood samples were drawn into evacuated anticoagulant tubes with a blood to 0.109 mol/L sodium citrate ratio of 9:1. Citrated blood samples were centrifuged at 2000×g for 10 min. Plasma samples were separated into polypropylene tubes, stored at −80°C, and then thawed at 37°C immediately prior to performing assays. Normal pooled plasma was prepared from patients (n=10) who had no abnormal values in the coagulation tests and no liver disease. Plasma samples from patients with CFD were collected from 24 patients with Dys (n=19), Hypodys (n=4), or Hypo (n=1). These phenotypes had been classified based on coagulation test results including Clauss and immunological methods for fibrinogen concentration and genetic variants determined by PCR-amplified DNA sequence analysis **(Table 1)**.[17]

2.2. Measurement of plasma specimens

Coagulation tests for sodium citrate plasma were measured using an automated coagulation analyzer CS-2400 (Sysmex, Kobe, Japan) and the required reagents. Fibrinogen concentrations were determined using both the Clauss method with Thrombocheck Fib (L) (Sysmex) and a PT-derived method with Thromborel S (Sysmex) (Figure 1A), in accordance with the manufacturer's instructions. A calibration curve was generated by serial dilution of Coagtrol N (Sysmex). Plasma fibrinogen antigen (Ag) levels were analyzed using a latex agglutination immunoassay with FactorAuto Fibrinogen (Q-may, Ohita, Japan) and an automated coagulation analyzer, Coapresta2000 (Sekisui Medical Co., Tokyo, Japan). A calibration curve was generated using FactorAuto fibrinogen standard plasma (Q-may).

2.3. DNA sequence analysis

Genomic DNA purification, PCR-amplification, and DNA sequencing were performed as described previously.[18]

2.4. Clot waveform analysis

CWA with the Clauss method was monitored during clot formation as the amount of transmitted light and analyzed using algorithms installed in the CS-2400 coagulation analyzer. Transmittance decreased depending on fibrin formation, and the total difference in the transmittance level is shown as delta H (dH). The minimum value of the first derivative curve (Min1) of waveform was calculated as an indicator of the maximum velocity of coagulation. Interpretation of the CWA parameters was performed in accordance with official recommendations[10]; we analyzed dH and Min1 as CWA parameters in this study. Furthermore, we calculated the ratio determined using the Clauss method to dH and Min1 (Clauss/dH and Clauss/Min1, respectively).

2.5. Statistical analysis

Pearson and Spearman rank tests were performed to define the correlation of fibrinogen levels determined using the Clauss method with the CWA parameters. The significance of differences in the Clauss/PT-derived ratio and CWA parameters among acquired Hypo cases and CFD were determined using one-way ANOVA with Dunnett post hoc test. A difference was considered significant when the p value was lower than 0.05.

3. Results

3.1 Comparison of fibrinogen concentrations quantitated using the Clauss and PT-derived methods

Plasma samples from four groups of patients: 67 with aHypo, 19 Dys, 4 Hypodys, and 1 Hypo had their fibrinogen levels measured using the Clauss and PT-derived methods. In patients in the aHypo group, fibrinogen levels determined using the Clauss and PT-derived methods were 0.48–1.78 and 0.59–2.18 g/L, respectively, with the Clauss/PT-derived ratios ranging from 0.48 to 1.39 (median, 0.95). There was a good correlation between fibrinogen concentration determined using the Clauss and PT-derived methods in patients with aHypo ($r = 0.788$). For samples from 19 patients in the Dys group, fibrinogen levels determined using the Clauss method ranged from 0.10 to 1.74 g/L, whereas those in the PT-derived method were significantly higher (0.89–5.39 g/L). The Clauss/PT-derived ratios in the Dys group were markedly lower (median, 0.29) than those in the aHypo group. On the other hand, for patients in the Hypodys and Hypo groups, fibrinogen levels of the Clauss method and PT-derived method were similar, and median Clauss/PT-derived ratio of the Hypodys and Hypo groups were 0.79 and 1.32, respectively, and neither showed a significant difference from the aHypo group.

3.2 Clot waveform of congenital fibrinogen disorders

We investigated whether clot waveforms differed among patients in the Dys group that showed the same fibrinogen variants. Representative coagulation curves of reference plasma (0.61, 1.00, and 2.20 g/dL) from aHypo or normal control and plasma from CFD are shown in Figure 1. In reference plasma from the normal control and aHypo groups, the pre-coagulation phase was shorter, and the dH increased depending on the fibrinogen concentration. These trends were similar in all of five groups of Dys variants. Conversely, the dH of plasma from patients in the Dys group was unexpectedly larger than that of aHypo or normal control groups, even though fibrinogen values from the Clauss method were lower than 1.00 g/dL [cases 2 to 4 (Figure 1A), cases 7 and 8 (Figure 1B), case 9 (Figure 1C), cases 11 to 13 (Figure 1D), and cases 14 to 17 (Figure 1E)]. However, in case 10 with a BβA68T heterozygous variant, the fibrinogen value was 1.74 g/L, which was close to the normal value, and the waveform was not different from that of the normal control (Figure 1C).

Subsequently, the waveforms from the Clauss method were compared for four cases with Dys, four cases with Hypodys, and one case with Hypo variants (Figure 2). Among plasma samples from the four cases with Dys, case 1 had a similar waveform and dH as the cases presented in Figure 1; however, all the cases with Hypodys or Hypo were not

different in waveform or dH from the aHypo cases.

3.3 Comparison of CWA parameters, dH and Min1, in CFD

We investigated which CWA parameter, dH or Min1, can express the coagulation waveform objectively rather than visually, and, therefore, may be useful in differentiating aHypo and CFD. As shown in Figure 3, the values of dH and Min1 obtained from the aHypo and CFD groups were compared. The median dH values of aHypo, Hypodys, and Hypo cases were 119, 34.5, and 45, respectively, with no significant difference observed among the groups (Figure 3A). On the other hand, the dH of the Dys group (median, 246) was significantly higher (p<0.01) than that of the aHypo and other groups. Whereas, the median of Min1 for the Dys, Hypodys, and Hypo groups were 0.301, 0.101, and 0.104, respectively, and these were not significant different from that of the aHypo group (median, 0.241) (Figure 3B). As well as the visual coagulation waveform amplitude, these results indicated that dH can be used to distinguish Dys from aHypo, whereas Hypodys and Hypo were not distinguishable.

In the aHypo group, dH and Min1 showed good correlations with the Clauss value (r = 0.773 and 0.963, respectively). Individual Clauss values were divided by the dH and Min1 values, and the resultant ratios, Clauss/dH and Clauss/Min, for the aHypo and CFD groups were compared. The median of Clauss/dH for the aHypo group was 0.0099, with

a range of 0.0049–0.0161 (whisker range, Figure 3C). The Clauss/dH for the Dys group (median, 0.0018) and Hypodys group (median, 0.0124) were significantly different (p<0.01 and p<0.05, respectively) from that of the aHypo group. On the other hand, the median Clauss/dH for the Hypo case was 0.0164 with no significant difference (Figure 3C). Whereas, the median Clauss/Min1 of the aHypo group was 4.638, with a range of 3.170–7.468 (whisker range, Figure 3D). The median of Clauss/Min1 for the Dys, Hypodys, and Hypo groups were 1.833, 7.494, and 7.115, respectively, and that of Clauss/Min1 for the Dys and Hypodys groups were significantly different (p<0.01 and p<0.05, respectively) from that of the aHypo group. These results also indicated that Clauss/dH and Clauss/Min1 can be used to distinguish the Dys and Hypodys groups from the aHypo group, whereas the Hypo case was not distinguishable.

3.4 CFD positive rates using the Clauss/PT-derived, Clauss/dH, and Clauss/Min1 ratios

In order to investigate the positive rate for the diagnosis of CFD using the Clauss/PT-derived, Clauss/dH, and Clauss/Min1 ratios, we calculated the cut-off value for these parameters using the minimum and maximum values of the aHypo group; less than 0.48 or more than 1.39 (Clauss/PT-derived), less than 0.0049 or more than 0.0161 (Clauss/dH), and less than 3.170 or more than 7.468 (Clauss/Min1), respectively. As shown in Table 2,

the detection number and undetection cases are listed. When cut-off values of the Clauss/PT-derived ratio were used, we detected 18 of 23 cases as CFD and five cases could not be distinguished from the aHypo group (Table 2 [1]). On the other hand, when the Clauss/dH and Clauss/Min1 ratios were used individually or together, the number of undetected cases was four, three, and two, respectively, and it was found that using these parameters detected more CFD than the Clauss/PT-derived ratio (Table 2 [2], [3] and [4]). Furthermore, when all parameters were used, only one case of Hypodys could not be detected as a CFD (Table 2 [5]).

4. Discussion

In order to establish a screening method for CFD patients, we measured the plasma fibrinogen concentrations for aHypo cases, which were determined by routine coagulation screening tests and previously diagnosed samples from CFD using a CS-2400 automated coagulation analyzer, which is able to monitor and analyze the clot waveform. Because Hypodys and Hypo were investigated in only four and one cases, respectively, our findings for these groups are limited by the small case numbers, and we discuss here our analyses of the Dys group only, as follows.

First, we compared the Clauss/PT-derived ratio between aHypo and CFD cases. As

many reports have already demonstrated,[6,19,20] a lower Clauss/PT-derived ratio was a useful parameter to differentiate Dys from aHypo cases. Two cases were negative in screening for Dys, one was BβG15C (case 6) and the other was a heterozygous variant of BβA68T (case 10). The former variant fibrinogen showed markedly impaired thrombin catalyzed fibrin polymerization, as described previously.[21] This variant showed a fibrinogen value obtained using the PT-derived method that was smaller than the fibrinogen Ag value, just as dH in the Clauss method was small, so the Clauss/PT-derived ratio did not differ from that of the aHypo group. For the heterozygous variant of BβA68T, it was reported that fibrin polymerization was almost the same as the normal control,[22] there was no large discrepancy between the fibrinogen values measured using the Clauss and PT-derived methods.

In order to perform screening of patients with CFD using an automated coagulation analyzer without the PT-derived method, the Clauss/dH and Clauss/Min1 ratios were examined. Screening for Dys cases using the Clauss/PT-derived ratio detected 16 cases, whereas, using CWA with the Clauss/dH and Clauss/Min1 ratios detected 18 and 19 cases, respectively. These results indicated that CWA parameters from the Clauss method are useful for screening Dys from aHypo cases before measuring fibrinogen antigen and DNA analysis. Recently, Suzuki et al. reported that, 1) Min1 values were strongly associated

with fibrinogen antigen in CFD and 2) the Clauss/Min1 ratio may be an indicator to distinguish Dys cases from Hypodys and Hypo cases.[16] For the former finding, our results showed a poor correlation between Min1 and fibrinogen antigen in Dys cases ($r = 0.124$). We presume that our Dys variants AαC472S (case 5), BβG15C and γD364H (case 19), whose polymerizations were markedly affected, and whose Min1 values were small, resulted in a poor correlation between fibrinogen antigen and Min1. On the other hand, the latter finding was coincident with our data.

In this study, we showed for the first time that the clot waveform and values of CWA parameters in the Clauss method, especially for dH and Min1, using samples from multiple CFD cases. We can easily distinguish Dys from aHypo cases by confirming large changes in transmittance of the clot waveform, that is, whether the dH value is higher, or the values of the Clauss/dH and/or the Clauss/Min1 ratios are lower. However, this study has some limitations. Since measuring time of 100 seconds was inappropriate for some CFD cases, analyzer automatedly remeasured using longer measuring time and/or increasing sample volume. Although CWA parameters change depending on the measuring time and sample volume, we could not compare the CWA parameters between aHypo and remeasured CFD cases under the same measuring conditions. Furthermore, the positive rate for Hypodys cases was lower and because only one Hypo case was

included, it was not possible to perform estimates for these groups. Therefore, further studies are required to establish new screening methods for CFD, especially for Hypodys and Hypo cases, using CWA parameters.

In spite of having an aberrant fibrinogen function, why was the dH of the Dys groups larger than that of the aHypo group? The process of fibrin clot formation (fibrin polymerization) is commonly described as consisting of at least three steps: 1) the cleavage of fibrinopeptide A (FpA) by thrombin and fibrin monomer production; 2) formation of two-stranded protofibrils; and 3) so-called lateral aggregation of protofibrils to produce fibrin fibers. Fibrin polymerization using fibrinogen derived from patients with dysfibrinogenemia[23-27] and computer modeling[28] demonstrated that affected FpA release (step 1) and/or protofibril formation (step 2) had a long lag period, then minor changes in transmittance (Min1), large final transmittance (dH), and finally formation of thicker fibrin fibers. Whereas, a reduced ability in the lateral aggregation (step 3) led to a long lag period, minor changes in transmittance (Min1), small final transmittance (dH), and finally the formation of thinner fibrin fibers.[29,30] AαC472S and γD364H variants in the Dys group were affected in the lateral aggregation site of the αC domain and γD module, respectively;[30,31] therefore, Min1 and dH were markedly reduced. Moreover, BβG15C variant in the group was affected at the "B:b" interaction site, which is one of

the lateral aggregation sites, resulting in markedly reduced dH.[28,29]

In conclusion, this study revealed that the CWA using the Clauss method, with the Clauss/dH and Clauss/Min1 ratios, were better parameters for the screening of CFD, especially patients with Dys than the Clauss/PT-derived ratio. Furthermore, laboratories that do not have an automated coagulation analyzer with CWA can suspect CFD using careful monitoring of the clot waveform, namely unexpectedly large changes in transmittance, even though the fibrinogen values from the Clauss method were lower than 1.00 g/dL.

Acknowledgments

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

Conflict of interests

F. Hayashi, S. Shinohara, and N. Arai are employees of Sysmex Corporation.

Author contributions

S. Arai, T. Kamijo, F. Hayashi, and S. Shinohara performed the research, analyzed the date. S. Arai wrote the manuscript. F. Hayashi, S. Shinohara, N. Arai, M. Sugano, T. Uehara, T. Honda, and N. Okumura designed the research and discussed the data, and N. Okumura reviewed the manuscript

References

[1] Mosesson MW. Fibrinogen and fibrin structure and functions. J Thromb Haemost. 2005; 3 (8):1894–1904.

[2] Lord ST. Fibrinogen and fibrin: scaffold proteins in hemostasis. Curr Opin Hematol. 2007; 14 (3):236–241.

[3] de Moerloose P, Casini A, Neerman-Arbez M. Congenital fibrinogen disorders: an update. Semin. Thromb Hemost. 2013; 39 (6):585–595.

[4] Casini A, Neerman-Arbez M, Ariëns RA, de Moerloose P. Dysfibrinogenemia: from molecular anomalies to clinical manifestations and management. J Thromb Haemost. 2015; 13 (6):909–919.

[5] Clauss A. Rapid physiological coagulation method in determination of fibrinogen. Acta Haematol. 1957; 17 (4):237-246.

[6] Miesbach W, Schenk J, Alesci S, Lindhoff-Last E. Comparison of the fibrinogen Clauss assay and the fibrinogen PT derived method in patients with dysfibrinogenemia. Thromb Res. 2010; 126 (6):428-433.

[7] Chitolie A, Mackie I.J, Machin S.J. The type of thromboplastin reagent has important effects on the PT-derived fibrinogen potency. Laboratory Hematology. 1998; 4:149-155.

[8] Chantarangkul V, Tripodi A, Mannucci P.M. Evaluation of a fully automated

centrifugal analyzer for performance of hemostasis tests. Clin Chem. 1987; 33 (10):1888-1890.

[9] Rossi E, Mondonico P, Lombardi A, Preda L. Method for the determination of functional (clottable) fibrinogen by the new family of ACL coagulometers. Thromb Res. 1988; 52 (5):453-468.

[10] Shima M, Thachil J, Nair S.C, Srivastava A. Scientific and Standardization Committee, Towards standardization of clot waveform analysis and recommendations for its clinical applications. J Thromb Haemost. 2013; 11 (7):1417-1420.

[11] Shima M, Matsumoto T, Fukuda K, et al. The utility of activated partial thromboplastin time (aPTT) clot waveform analysis in the investigation of hemophilia A patients with very low levels of factor VIII activity (FVIII:C). Thromb Haemost. 2002; 87 (3):436-441.

[12] Matsumoto T, Shima M, Takeyama M, et al. The measurement of low levels of factor VIII or factor IX in hemophilia A and hemophilia B plasma by clot waveform analysis and thrombin generation assay. J Thromb Haemost. 2006; 4 (2):377-384.

[13] Shima M, Matsumoto T, Ogiwara K. New Assays for Monitoring Haemophilia Treatment. Haemophilia. 2008; 14 (3):83-92.

[14] Braun P.J, Givens T.B, Stead A.G, et al. Properties of Optical Data from Activated

Partial Thromboplastin Time and Prothrombin Time Assays. Thromb Haemost. 1997; 78 (3):1079-1087.

[15] Jacquemin M, Vanlinthout I, Van Horenbeeck I, et al. The amplitude of coagulation curves from thrombin time tests allows dysfibrinogenemia caused by the common mutation FGG-Arg301 to be distinguished from hypofibrinogenemia. Int J Lab Hematol . 2017; 39(3):301-307.

[16] Suzuki A, Suzuki N, Kanematsu T, et al. Clot waveform analysis in Clauss fibrinogen assay contributes to classification of fibrinogen disorders. Thromb Res. 2019; 174:98-103.

[17] Casini A, Undas A, Palla R, Thachil J, de Moerloose P. Subcommittee on Factor XIII and Fibrinogen, Diagnosis and classification of congenital fibrinogen disorders: communication from the SSC of the ISTH. J Thromb Haemost. 2018; 16 (9):1887-1890.

[18] Terasawa F, Okumura N, Kitano K, et al. Hypofibrinogenemia associated with a heterozygous missense mutation gamma153Cys to Arg (Matsumoto IV): in vitro expression demonstrates defective secretion of the variant fibrinogen. Blood. 1999; 94 (12):4122–4131.

[19] Mackie J, Lawrie AS, Kitchen S, et al. A performance evaluation of commercial fibrinogen reference preparations and assays for Clauss and PT-derived fibrinogen.

Thromb Haemost. 2002; 87 (6):997-1005.

[20] Xiang L, Luo M, Yan J, et al. Combined use of Clauss and prothrombin time-derived methods for determining fibrinogen concentrations: Screening for congenital dysfibrinogenemia. J Clin Lab Anal. 2018; 32 (4):e22322.

[21] Hirota-Kawadobora M, Terasawa F, Yonekawa O, et al. Fibrinogens Kosai and Ogasa: Bbeta15Gly→Cys (GGT→TGT) substitution associated with impairment of fibrinopeptide B release and lateral aggregation. J Thromb Haemost. 2003; 1 (2):275-283.

[22] Kamijo T, Nagata K, Taira C, Higuchi Y, Arai S, Okumura N. Fibrin monomers derived from thrombogenic dysfibrinogenemia, Naples-type variant (BβAla68Thr), showed almost entirely normal polymerization. Thromb Res. 2018; 172:1-3.

[23] Terasawa F, Okumura N, Higuchi Y, et al. Fibrinogen Matsumoto III: a variant with gamma275 Arg→Cys (CGC→TGC) comparison of fibrin polymerization properties with those of Matsumoto I (gamma364 Asp→His) and Matsumoto II (gamma308 Asn→Lys). Thromb Haemost. 1999; 81 (5):763-766.

[24] Hirota-Kawadobora M, Terasawa F, Suzuki T, Tozuka M, Sano K, Okumura N. Comparison of thrombin-catalyzed fibrin polymerization and factor XIIIa-catalyzed cross-linking of fibrin among three recombinant variant fibrinogens, gamma 275C, gamma 275H, and gamma 275A. J Thromb Haemost. 2004; 2 (8):1359-1367.

[25] Kamijyo Y, Hirota-Kawadobora M, Fujihara N, et al. Functional analysis of heterozygous plasma dysfibrinogens derived from two families of gammaArg275Cys and three families of gammaArg275His, and haplotype analysis for these families. Rinsho Byori. 2009; 57 (7):651-658.

[26] Okumura N, Furihata K, Terasawa F, Ishikawa S, Ueno I, Katsuyama T. Fibrinogen Matsumoto II: gamma 308 Asn→Lys (AAT→AAG) mutation associated with bleeding tendency. Br J Haematol. 1996; 94 (3):526-528.

[27] Okumura N, Terasawa F, Fujita K, Fujihara N, Tozuka M, Koh C.S. Evidence that heterodimers exist in the fibrinogen Matsumoto II (gamma308N-->K) proband and participate in fibrin fiber formation. Thromb Res. 2002; 107 (3-4):157-162.

[28] Weisel J.W, Nagaswami C. Computer modeling of fibrin polymerization kinetics correlated with electron microscope and turbidity observations: clot structure and assembly are kinetically controlled. Biophys J. 1992; 63 (1):111-128.

[29] Hirota-Kawadobora M, Kani S, Terasawa F, et al. Functional analysis of recombinant Bbeta15C and Bbeta15A fibrinogens demonstrates that Bbeta15G residue plays important roles in FPB release and in lateral aggregation of protofibrils. J Thromb Haemost. 2005; 3 (5):983-990.

[30] Ikeda M, Arai S, Mukai S, Takezawa Y, Terasawa F, Okumura N. Novel heterozygous

dysfibrinogenemia, Sumida (AαC472S), showed markedly impaired lateral aggregation of protofibrils and mildly lower functional fibrinogen levels. Thromb Res. 2015; 135 (4):710-717.

[31] Okumura N, Furihata K, Terasawa F, Nakagoshi R, Ueno I, Katsuyama T. Fibrinogen Matsumoto I: a gamma 364 Asp→His (GAT→CAT) substitution associated with defective fibrin polymerization. Thromb Haemost. 1996; 75 (6):887-891.

Figure legends

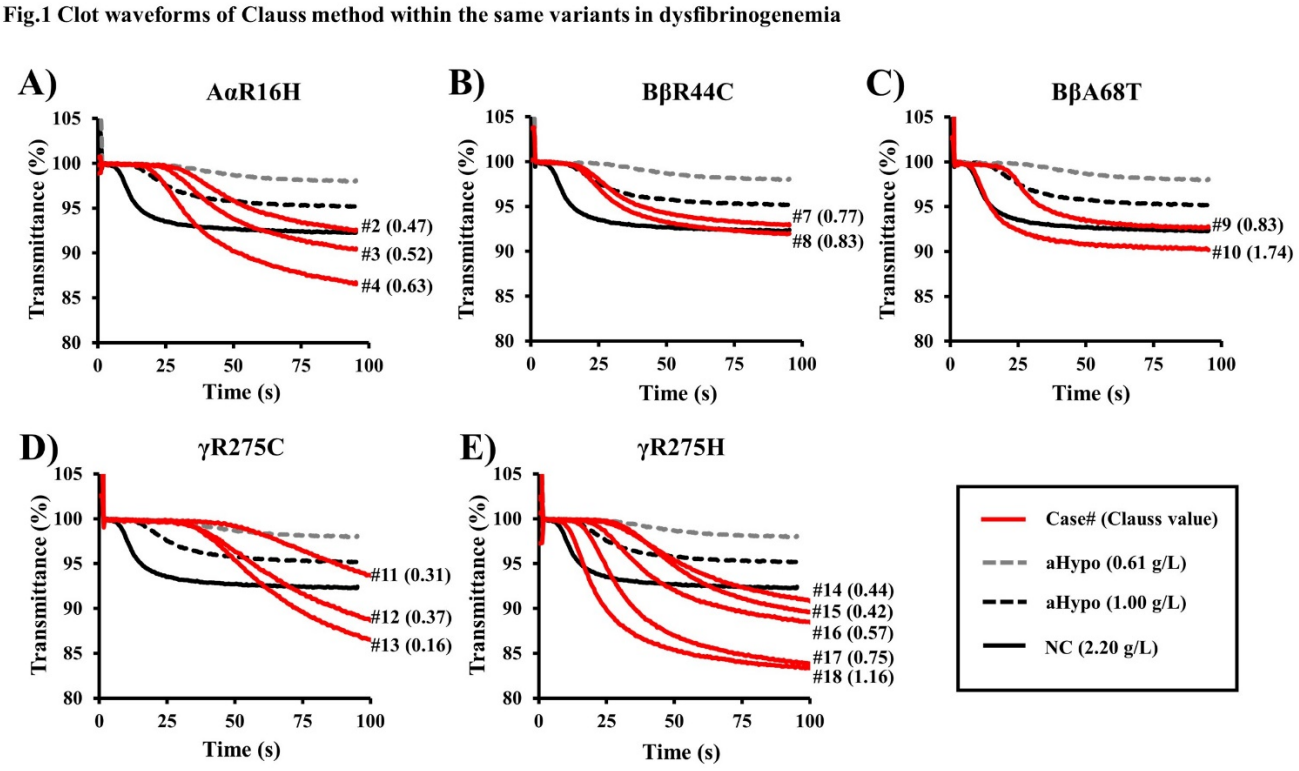

Figure 1. Clot waveforms of the Clauss method within the same variants with dysfibrinogenemia.

The clot waveforms of the Clauss method are shown for five types of dysfibrinogenemia: three with AαR16H (A), two with BβR44C (B), two with BβA68T (C), three with γR275C (D), and five with γR275H (E). aHypo; pooled plasma from patients with acquired hypofibrinogenemia, NC; pooled plasma made from normal controls.

Fig.2 Clot waveforms of the Clauss method in the congenital fibrinogen disorders.

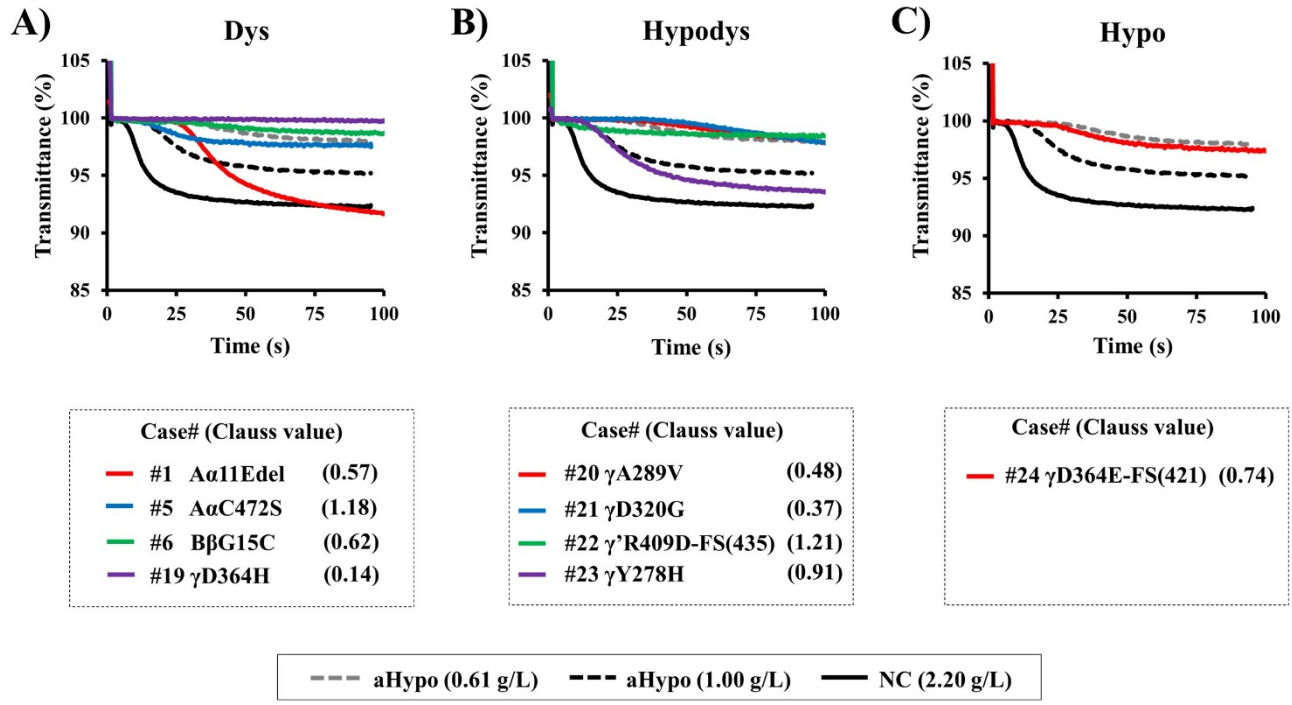

Figure 2. Clot waveforms of the Clauss method in patients with congenital fibrinogen disorders.

The clot waveforms of the Clauss method are shown for patients with congenital fibrinogen disorders: four variants with dysfibrinogenemia other than those presented in Figure 3 (A), four with hypodysfibrinogenemia (B) and one with hypofibrinogenemia (C). aHypo, pooled plasma made from patients with acquired hypofibrinogenemia; NC, pooled plasma made from normal controls. Dys, dysfibrinogenemia; Hypodys, hypodysfibrinogenemia; Hypo, hypofibrinogenemia.

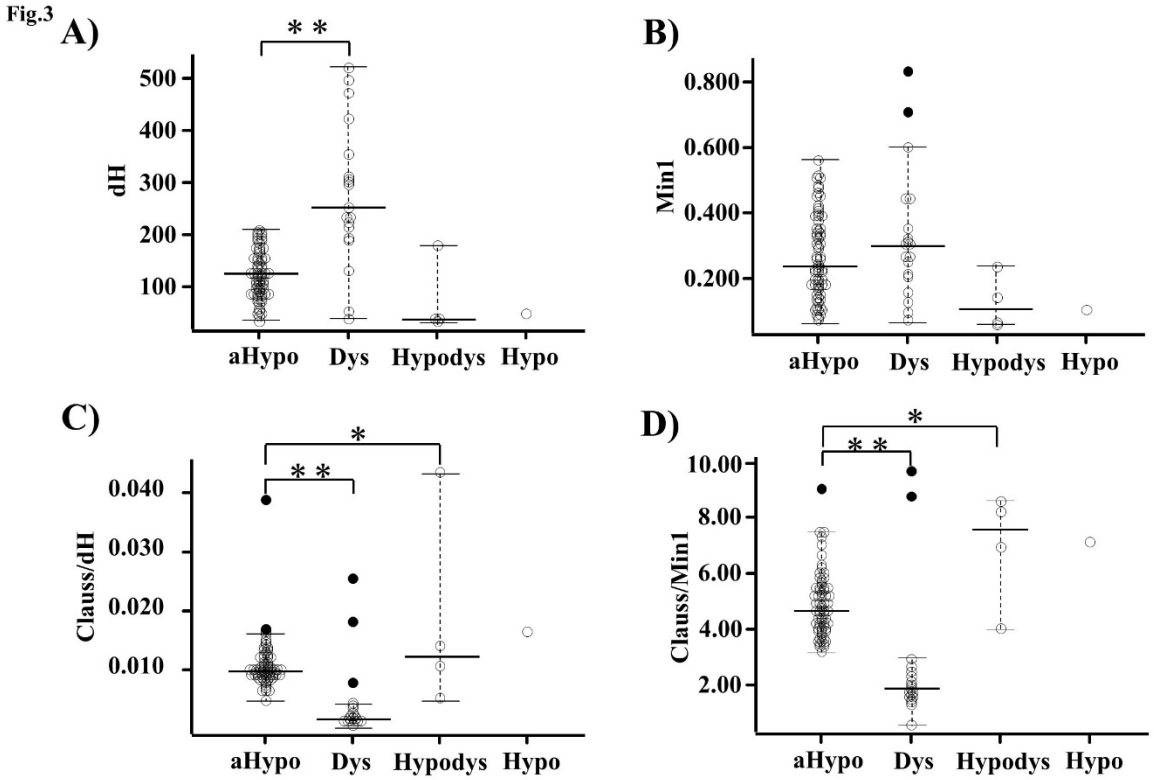

Figure 3. Distribution of clot waveform analysis parameters for patients with congenital fibrinogen disorders using dH, Min1, and Clauss/dH and Clauss/Min1 ratios.

Distribution of dH (A), Min1 (B), Clauss/dH (C) and Clauss/Min1 ratios (D) from acquired hypofibrinogenemia and congenital fibrinogen disorders were compared. The bottom and top lines indicate the first and third quartiles of the data, respectively, and the horizontal line indicates the median value. The whiskers extend out to the most extreme data point that is at most 1.5 times the interquartile range above the third quartile or below the first quartile. Closed circles indicate a result from a patient beyond this range. aHypo,

acquired hypofibrinogenemia; Dys, dysfibrinogenemia; Hypodys, hypodysfibrinogenemia; Hypo, hypofibrinogenemia. * and ** shows significant difference ($P < 0.05$ and $P < 0.01$, respectively) between acquired hypofibrinogenemia and congenital fibrinogen disorders.

Table 1 Characteristics of patients with fibrinogen disorder

Case	Age (years)	Gender	Symptom	Variant	Status	Type	Subtype[#]	Clauss (g/L)	Ag (g/L)	Clauss /Ag	PT-derived (g/L)	dH	Min1	Clauss /PT-derived	Clauss /dH	Clauss /Min1
1	12	M	A	Aα11Edel	He	Dys	3A	0.57	1.98	0.29	1.97	246	0.311	0.29	0.0023	1.833
2	81	M	A	AαR16H	He	Dys	3A	0.47	2.70	0.17	1.50	218	0.210	0.31	0.0022	2.238
3	31	F	A	AαR16H	He	Dys	3A	0.52	2.92	0.18	1.81	296	0.301	0.29	0.0018	1.728
4	42	F	T	AαR16H	He	Dys	3A	0.63	3.43	0.18	2.37	421	0.444	0.27	0.0015	1.419
5	6	F	A	AαC472S	He	Dys	3A	1.18	2.16	0.55	**n.d**	47	0.123	**n.d**	0.0251	9.593
6	25	M	A	BβG15C	He	Dys	3A	0.62	2.44	0.25	0.89	34	0.071	0.70	0.0182	8.732
7[†]	41	M	A	BβR44C	He	Dys	3A	0.77	2.78	0.28	2.13	188	0.262	0.36	0.0041	2.939
8[†]	10	F	A	BβR44C	He	Dys	3A	0.83	3.28	0.25	2.90	229	0.320	0.29	0.0036	2.594
9[‡]	17	M	T	BβA68T	Ho	Dys	3B	0.83	4.07	0.20	2.48	210	0.439	0.33	0.0040	1.891
10[‡]	45	M	A	BβA68T	He	Dys	3A	1.74	3.31	0.53	2.36	228	0.706	0.74	0.0076	2.465
11	20	F	B	γR275C	He	Dys	3A	0.31	2.46	0.13	3.40	186	0.156	0.09	0.0017	1.987
12	40	F	A	γR275C	He	Dys	3A	0.37	3.48	0.11	4.43	291	0.245	0.08	0.0013	1.510
13	23	M	A	γR275C	He	Dys	3A	0.16 *	2.80	0.06	2.97	515 *	0.303 *	0.05	0.0003	0.528
14	2	F	A	γR275H	He	Dys	3A	0.44	2.46	0.18	3.52	300	0.262	0.13	0.0015	1.679
15	40	M	A	γR275H	He	Dys	3A	0.42	2.23	0.19	2.45	305	0.202	0.17	0.0014	2.079
16	31	F	A	γR275H	He	Dys	3A	0.57	2.67	0.21	3.02	350	0.347	0.19	0.0016	1.643
17	46	F	T	γR275H	He	Dys	3A	0.75	3.65	0.21	4.91	494	0.599	0.15	0.0015	1.252
18	32	F	A	γR275H	He	Dys	3A	1.16	2.41	0.48	5.39	469	0.829	0.22	0.0025	1.399
19	20	M	T	γD364H	He	Dys	3A	0.14	4.75	0.03	1.98	127	0.091	0.07	0.0011	1.538
20	26	F	B	γA289V	He	Hypodys	4C	0.48	1.07	0.45	0.59	34	0.059	0.81	0.0141	8.136
21	29	F	A	γD320G	He	Hypodys	4C	0.37 **	1.07	0.35	0.91	35 **	0.054 **	0.41	0.0106	6.852
22	23	M	A	γ'R409D-	He	Hypodys	4C	1.21 ***	1.81	0.67	0.64	28 ***	0.142 ***	1.89	0.0432	8.521
23	31	F	B	γY278H	He	Hypodys	4C	0.91	1.49	0.61	1.19	176	0.230	0.76	0.0052	3.957
24	0.3	M	B	γD364E-	He	Hypo	2B	0.74	0.84	0.88	0.56	45	0.104	1.32	0.0164	7.115

[#] classification according to recommendation[15], [†] family with BβR44C, [‡] family with BβA68T, * Measurement using two-fold volume of plasma during 200 sec, ** measurement during 200 sec, *** measurement using two-fold volume of plasma.

Ag; antigen, M; male, F; female, A; asymptomatic, T; thrombosis, B; bleeding, del; deletion, FS; frame shift, He; heterozygous of carrier status, Ho; homozygous of carrier status, Dys; dysfibrinogenemia, Hypodys; hypodysfibrinogenemia, Hypo; hypofibrinogenemia, n.d; not determined.

Table 2 Detection number for congenital fibrinogen disorders using three parameters

	parameters	cut-off value	type	detection number	undetection variants (case#)
[1]	Clauss/PT-derived	< 0.48 and > 1.39	Dys (n=18) Hypodys (n=4) Hypo (n=1)	16 2 0	BβG15C(#6), BβA68T(#10) γA289V(#20), γY278H(#23) γD364E-FS(422stop)(#24)
[2]	Clauss/dH	< 0.0049 and > 0.0161	Dys (n=19) Hypodys (n=4) Hypo (n=1)	18 1 1	BβA68T(#10) γA289V(#20), γD320G(#21), γY278H(#23)
[3]	Clauss/Min1	< 3.170 and > 7.468	Dys (n=19) Hypodys (n=4) Hypo (n=1)	19 2 0	γD320G(#21), γY278H(#23) γD364E-FS(422stop)(#24)
[4]	using parameters of [2] and [3]		Dys (n=19) Hypodys (n=4) Hypo (n=1)	19 2 1	γD320G(#21), γY278H(#23)
[5]	using parameters of [1], [2] and [3]		Dys (n=19) Hypodys (n=4) Hypo (n=1)	19 3 1	γY278H(#23)

Dys; dysfibrinogenemia, Hypodys; hypodysfibrinogenemia, Hypo; hypofibrinogenemia, FS; frame shift mutation.