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# Levels of Fibrinogen Variants Are Altered in Severe COVID-19

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## Abstract

**Background** Fibrinogen variants as a result of alternative messenger RNA splicing or protein degradation can affect fibrin(ogen) functions. The levels of these variants might be altered during coronavirus disease 2019 (COVID-19), potentially affecting disease severity or the thrombosis risk.

**Aim** To investigate the levels of fibrinogen variants in plasma of patients with COVID-19.

**Methods** In this case-control study, we measured levels of functional fibrinogen using the Clauss assay. Enzyme-linked immunosorbent assays were used to measure antigen levels of total, intact (nondegraded Aα chain), extended Aα chain ($\alpha_E$), and γ´ fibrinogen in healthy controls, patients with pneumococcal infection in the intensive care unit (ICU), ward patients with COVID-19, and ICU patients with COVID-19 (with and without thrombosis, two time points).

**Results** Healthy controls and ward patients with COVID-19 ($n = 10$) showed similar fibrinogen (variant) levels. ICU patients with COVID-19 who later did ($n = 19$) or did not develop thrombosis ($n = 18$) and ICU patients with pneumococcal infection ($n = 6$) had higher absolute levels of functional, total, intact, and $\alpha_E$ fibrinogen than healthy controls ($n = 7$). The relative $\alpha_E$ fibrinogen levels were higher in ICU patients with COVID-19 than in healthy controls, while relative γ´ fibrinogen levels were lower. After diagnosis of thrombosis, only the functional fibrinogen levels were higher in ICU patients with COVID-19 and thrombosis than in those without, while no differences were observed in the other fibrinogen variants.

**Conclusion** Our results show that severe COVID-19 is associated with increased levels of $\alpha_E$ fibrinogen and decreased relative levels of γ´ fibrinogen, which may be a cause or consequence of severe disease, but this is not associated with the development of thrombosis.

**Keywords**
- splicing
- COVID-19
- fibrinogen
- fibrin
- inflammation

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## Introduction

Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) is the cause of coronavirus disease 2019 (COVID-19), mainly targeting the respiratory tract, leading to coughing, fever, and in severe cases, pneumonia. In these severe cases, an increased incidence of thrombotic complications has been reported.[1] The disease burden and mortality of thrombotic diseases are influenced by the architecture and stability of a thrombus.[2] Upon cleavage of fibrinogen by thrombin, fibrin monomers form. These fibrin monomers start polymerizing, finally forming fibrin fibers that are cross-linked by factor (F)XIII resulting in a stable fibrin network, one of the main components in a thrombus.[3] Fibrinogen is a glycoprotein of 340 kDa produced in the liver and consists of two sets of three different polypeptide chains: Aα, Bβ, and γ.[4] Variation in the fibrinogen molecule occurs due to genetic polymorphisms, alternative messenger RNA (mRNA) processing, proteolytic cleavage, and posttranslational modifications.[5,6] The structure of the fibrin network is affected by these fibrinogen variants.

Proteolytic cleavage of the C-terminus of one or two of the Aα chains leads to low-molecular-weight (LMW, 305 kDa) and low-molecular-weight prime (LMW′, 270 kDa) fibrinogen, respectively.[7] The part of the Aα chain removed during this cleavage contains functional domains affecting polymerization and lateral aggregation of protofibrils, thereby influencing the thickness of the fibrin fibers and the fibrin network structure.[8,9] Fibrin fibers formed from LMW fibrinogen are indeed thinner than fibrin fibers formed from high-molecular-weight fibrinogen,[10] resulting in a denser fibrin network.[11] In addition, the C-terminus of the Aα chain contains binding sites for endothelial cells, plasminogen, and factor XIII, thereby also affecting other processes in which fibrinogen or fibrin is involved.[11]

Other common variants of fibrinogen occur as a result of alternative mRNA splicing, such as an extension of the Aα chain ($\alpha_E$ fibrinogen). $\alpha_E$ fibrinogen represents typically 1 to 2% of the total fibrinogen molecules (as measured by quantitative western blot) and is only present as a homodimer of two extended Aα-chains.[12] It is produced upon splicing an extra exon into the Aα-chain mRNA, leading to an additional globular domain at the C-terminus.[12,13] This extension contains a binding site for β2-integrins, possibly enabling leukocytes to bind to fibrinogen. This additional domain also affects fibrin polymerization, resulting in thinner fibers, increased branching, and an increased stiffness of clots prepared from purified $\alpha_E$ fibrinogen.[13]

The mRNA splice variant γ′ derives from the replacement of the last four amino acids of the γ chain by 20 other amino acids, leading to an extended γ chain.[14] Between 5 and 15% of fibrinogen molecules are heterodimers of γ′ with the normal γ chain (γA/γ′) and less than 1% are homodimers of γ′.[15] The variation occurs in the D-region of the fibrinogen molecule, thereby affecting fibrin polymerization, decreasing platelet binding and increasing binding of thrombin and FXIII.[16–18] Studies have reported thinner fibers and a more branched network in clots made with γA/γ′ fibrinogen compared to clots prepared from γA/γA fibrinogen.[19–21] γ′ fibrinogen levels can vary largely between individuals and are associated with various diseases.[22–27]

Since fibrinogen variants were previously associated with various thrombotic diseases and an altered fibrin network structure, we hypothesized that these fibrinogen variants would be increased in patients with severe COVID-19 and thrombosis. Therefore, we investigated whether levels of functional fibrinogen, total fibrinogen, intact fibrinogen, γ′ fibrinogen, and $\alpha_E$ fibrinogen are altered in COVID-19 and whether this can explain why some patients with COVID-19 develop thrombosis and others do not.

## Methods

### Study Design and Patient Population

This study was a case-control study conducted in the Erasmus Medical Center in Rotterdam, the Netherlands, as part of the Dutch COVID and Thrombosis Coalition.[28] The patients and laboratory measurements are described previously.[29] Briefly, we collected citrated platelet-poor plasma samples between April and December 2020. Samples were collected from patients with COVID-19 admitted to the intensive care unit (ICU) who did and did not develop thrombosis during their stay at the ICU as confirmed by positive or negative computed tomography pulmonary angiograms (performed for all patients with COVID-19) and compression ultrasound of the extremities (only performed if symptoms compatible with venous thrombosis were present). Samples were collected before and after diagnosis of thrombosis or at similar time points in ICU patients without confirmed thrombosis. Additionally, we collected plasma from patients with COVID-19 admitted to general wards who did not have thrombosis, SARS-CoV-2-negative ICU patients with pneumococcal infection, and healthy controls.[30] Study protocols were in accordance with the Declaration of Helsinki and were approved by the Medical Ethics Committee of Erasmus Medical Center (healthy controls: MEC-2004-251; pneumococcal ICU patients: MEC-2017-417; COVID-19 patients: METC-2020-0758). We obtained written informed consent from each healthy control and ICU patient with pneumococcal infection. An opt-out procedure was in place for the patients with COVID-19. Functional fibrinogen levels were measured using the Clauss assay (Thrombin Reagent, Siemens Healthineers, Erlangen, Germany) on the Sysmex CS5100 coagulation analyzer (Siemens Healthcare Diagnostics B.V., Newark, Delaware, United States).

### Fibrinogen Variant ELISAs

We used enzyme-linked immunosorbent assays (ELISAs) based on monoclonal antibodies to measure antigen levels of total, intact, γ′ and $\alpha_E$ fibrinogen. First, 96-well MaxiSorp plates (439454, Thermo Fisher Scientific, Waltham, Massachusetts, United States) were coated overnight at 37°C with 120 µL coating antibody in phosphate-buffered saline (PBS). A fibrinogen polyclonal antibody (GaHu/Fbg/7S, Thermo Fisher Scientific) (10 µg/mL) and the G8 monoclonal antibody targeting the C-terminus of the Aα chain (FB-G8-1-2,

Quickzyme, Leiden, the Netherlands) (10 µg/mL) were used as coating antibodies for total and intact fibrinogen, respectively. For both ELISAs, reference lines were prepared using purified human fibrinogen (FIB3, Enzyme Research Laboratories, South Bend, Indiana, United States). The 2.G2.H9 antibody (1 µg/mL) (sc-81620, Santa Cruz, Dallas, Texas, United States)[27] and $\alpha_E$ antibody (1 µg/mL) (ab247586, Abcam, Cambridge, United Kingdom) were used as coating antibodies for γ′ fibrinogen and $\alpha_E$ fibrinogen, respectively. Reference lines were prepared with Peak 2 (P2 FIB, Enzyme Research Laboratories) and rhFib $\alpha_E$ (kind gift of Fibriant BV). After incubation of 100 µL diluted plasma (independent triplicates per sample) for 1 hour at 37°C, plates were washed using PBS with 0.05% Tween 20 (524653, Merck Millipore, Burlington, Massachusetts, United States) and incubated with Y18/PO conjugate (FB-Y18-4, Quickzyme) (1:10.000 ×) for 1 hour at 37°C. After thorough washing, each well was incubated with 100 µL 3,3′-5,5′-tetramethylbenzidine (TMB) (TMB Ultra, WD3243711, 34029, Thermo Fisher Scientific). To stop the substrate reaction, 100 µL of 2 M sulfuric acid was added to each well, after which the absorbance was measured at 450 nm using the Multiskan GO Microplate Spectrophotometer (Thermo Fisher Scientific). Results were calculated based on the four-parameter logistic fit using the SkanIt software (Thermo Fisher Scientific). Relative levels of $\alpha_E$ and γ′ fibrinogen were calculated as percentage of total fibrinogen measured using the GaHu/Fbg/7S antibody.

### Fibrin Network Characteristics

To study the characteristics of the fibrin network, clots were prepared from the citrated platelet-poor plasma and imaged as described previously.[29] Plasma clot lysis time was measured to investigate the susceptibility of plasma clots to fibrinolysis, as described previously.[29]

### Statistical Analysis

Normally distributed data are shown as mean ± standard deviation, not-normally distributed data as median [25th–-75th percentile], and categorical data as *n* (%). To test for differences between multiple groups, one-way ANOVA (normally distributed data), Kruskal–Wallis test (not-normally distributed data), or Chi-square test (categorical data) was used with post-hoc Tukey's tests. Changes in variables between the two time points were evaluated using the paired students' *t*-test (normally distributed data) or Wilcoxon signed-rank test (not-normally distributed data). Correlations were assessed using Spearman's rank correlation. We used pairwise deletion in case of missing data. Statistical analyses were performed using IBM SPSS Statistics v25 (IBM, Armonk, New York, United States) and GraphPad Prism version 8.2.1 (GraphPad Software, San Diego, California, United States).

## Results

### Baseline Patient Characteristics

Patient characteristics at the first time point are shown in ►**Table 1**. Of the 19 ICU patients with COVID-19 and confirmed thrombosis, 16 had pulmonary thrombosis, 1 deep venous thrombosis, 1 pulmonary thrombosis in combination with deep venous thrombosis, and 1 jugular vein thrombosis. The diagnosis of thrombosis in the ICU patients with COVID-19 and thrombosis was made after a median of 10 [6–17] days in the ICU. Furthermore, we had plasma samples from 18 ICU patients with COVID-19 without confirmed thrombosis, 10 ward patients with COVID-19 without confirmed thrombosis, 6 ICU patients with pneumococcal infection, and 7 healthy controls. Mean age and sex were comparable, while body mass index was slightly higher in ward and ICU patients with COVID-19 than in healthy controls (►**Table 1**). Results from laboratory measurements can be found in ►**Table 1**.

### Levels of Fibrinogen Variants

First, we analyzed plasma samples from healthy volunteers and from all patients collected at the first available time point after admission to the hospital (►**Fig. 1** and ►**Supplementary Table S1**). Levels of fibrinogen and fibrinogen variants were not significantly different in ward patients with COVID-19 compared to healthy controls. In ICU patients with COVID-19 with and without thrombosis and in ICU patients with pneumococcal infection, we observed significantly higher absolute levels of functional fibrinogen, total fibrinogen, intact fibrinogen, and $\alpha_E$ fibrinogen than in healthy controls. Levels of functional fibrinogen, intact fibrinogen, and $\alpha_E$ fibrinogen were also significantly higher in all ICU patients than in ward patients with COVID-19. Relative levels of $\alpha_E$ fibrinogen were significantly higher in ICU patients with COVID-19 with and without thrombosis than in healthy controls. Finally, the absolute levels of γ′ fibrinogen were not different among the different groups. The relative levels of γ′ fibrinogen showed a trend toward lower levels in patients with COVID-19, which only reached statistical significance in ICU patients with COVID-19 without thrombosis compared to healthy controls. No differences in fibrinogen (variant) levels were observed between ICU patients with COVID-19 who did and did not develop thrombosis.

From ICU patients with COVID-19, plasma samples were collected at a second time point as well, namely the first available sample after the diagnosis of thrombosis (median of 11 [7–18] days since ICU admission) or at a similar time point for patients without thrombosis (median of 12 [9–15] days since ICU admission) (►**Fig. 2** and ►**Supplementary Table S1**). In these plasma samples, we observed significantly higher functional fibrinogen, total fibrinogen, intact fibrinogen, and relative and absolute levels of $\alpha_E$ fibrinogen in both ICU patients with COVID-19 with and without thrombosis than in the healthy controls. Absolute levels of γ′ fibrinogen were similar among the groups. The decrease in relative levels of γ′ fibrinogen was more pronounced in the samples taken on the second time point and now reached significance in all ICU patients with COVID-19 (with or without thrombosis) compared to healthy controls. No differences were observed in the absolute or relative levels of fibrinogen variants between ICU patients with COVID-19 with and

**Table 1** Patient characteristics at the first time point

| | Healthy controls ($n=7$) | Ward patients with COVID-19 ($n=10$) | ICU patients with COVID-19 without thrombosis ($n=18$) | ICU patients with COVID-19 with thrombosis ($n=19$) | ICU patients with pneumococcal infection ($n=6$) | *p*-Value |
|---|---|---|---|---|---|---|
| Age (y) | 57.0 ± 4.7 | 60.2 ± 10.6 | 56.5 ± 15.8 | 57.8 ± 14.9 | 61.0 ± 8.3 | 0.93 |
| Male | 2 (29%) | 5 (50%) | 12 (67%) | 13 (68%) | 3 (50%) | 0.37 |
| Body mass index | 23.2 ± 2.1 | 30.8 ± 6.5 | 30.9 ± 8.0[a] | 29.8 ± 4.8 | 27.4 ± 6.4 | 0.07 |
| Days since (ICU) admission | – | 3 [2–6] | 5 [3–8][b] | 2 [1–6][b] | 0 [0–0] | **<0.01** |
| Anticoagulation | | | | | | **<0.01** |
| None | 7 (100%) | 0 (0%) | 0 (0%) | 0 (0%) | 0 (0%) | |
| Standard prophylaxis | 0 (0%) | 10 (100%) | 6 (33%) | 2 (11%) | 5 (84%) | |
| Intermediate prophylaxis | 0 (0%) | 0 (0%) | 10 (56%) | 14 (74%) | 0 (0%) | |
| Therapeutic | 0 (0%) | 0 (0%) | 2 (11%) | 3 (16%) | 1 (17%) | |
| Anti-Xa (U/mL) | <0.10 | 0.17 ± 0.12 | 0.37 ± 0.22[a] | 0.54 ± 0.28[a,b,c] | 0.29 ± 0.25 | **<0.01** |
| Corticosteroids | – | 7 (70%) | 11 (61%) | 10 (53%) | 2 (33%) | **0.04** |
| Mortality | 0 (0%) | 0 (0%) | 1 (6%) | 4 (21%) | 0 (0%) | 0.37 |
| Laboratory measurements | | | | | | |
| C-reactive protein (mg/L) | NA | 15 [12–45] | 91 [68–157][b,c] | 167 [88–240][c] | 305 [207–349][c] | **<0.01** |
| Interleukin-6 (pg/mL) | NA | NA | 59 [20–110] | 32 [14–134] | NA | 0.74 |
| Procalcitonin (ng/mL) | NA | NA | 0.37 [0.24–0.57] | 1.26 [0.30–12.48] | NA | 0.07 |
| FVIII (U/mL) | 0.81 ± 0.27 | 2.61 ± 1.12[a] | 3.39 ± 1.20[a] | 3.07 ± 1.17[a] | 2.37 ± 1.24 | **<0.01** |
| FXIII (U/mL) | 1.32 ± 0.19 | 1.36 ± 0.24 | 0.85 ± 0.25[a,c] | 0.95 ± 0.27[a,c] | 0.81 ± 0.58[a,c] | **<0.01** |
| D-dimer (mg/L) | 0.21 [0.19–0.28] | 0.41 [0.26–0.75] | 1.02 [0.75–2.07][a] | 1.35 [0.85–3.26][a,c] | 1.30 [0.98–7.18][a] | **<0.01** |
| Plasminogen activator inhibitor 1 (ng/mL) | <0.3 | 3.5 [2.7–4.8] | 6.5 [4.5–8.0][a] | 10.2 [4.5–32.3][a,c] | 13.3 [3.3–32.2][a] | **<0.01** |

Abbreviations: COVID-19, coronavirus disease 2019; FXIII, factor XIII; ICU, intensive care unit; NA, not available.
Note: Mean ± SD, median [25th–75th percentile], or *n* (%) is given. Statistically significant *p*-values are indicated in bold.
[a]Significantly different from healthy controls.
[b]Significantly different from ICU patients with pneumococcal infection.
[c]Significantly different from ward patients with COVID-19.

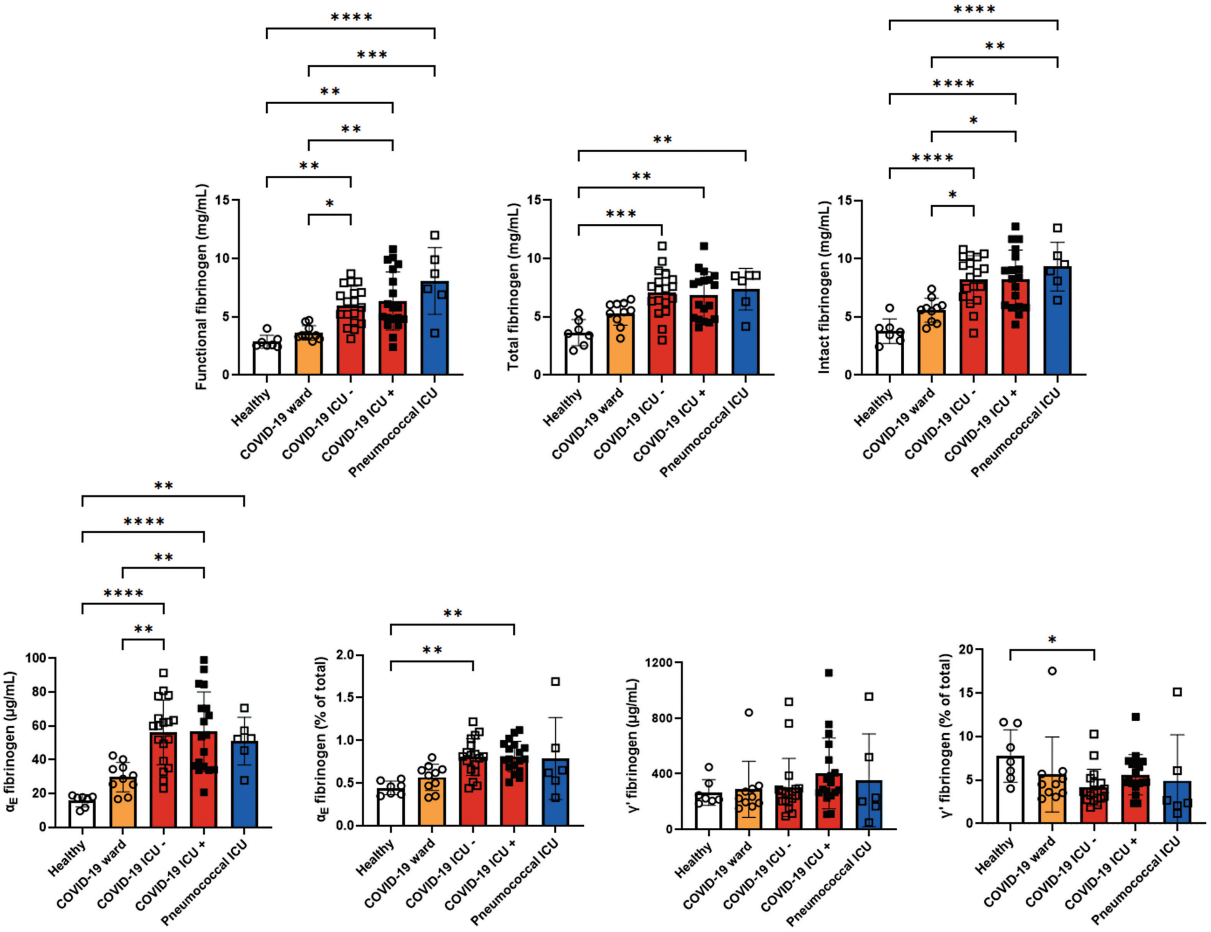


**Fig. 1** Levels of fibrinogen (variants) in plasma collected from healthy controls, COVID-19 ward patients with COVID-19, pneumococcal ICU patients, ICU patients with COVID-19 without thrombosis (COVID-19 ICU −), and ICU patients with COVID-19 and thrombosis before their diagnosis of thrombosis (COVID-19 ICU +). $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$. COVID-19, coronavirus disease 2019; ICU, intensive care unit.

without thrombosis, except for a small significant difference in functional fibrinogen levels.

The relative levels of γ′ fibrinogen significantly decreased in both ICU patients with COVID-19 with and without thrombosis between the first and second time point (►**Fig. 3**), while levels of functional, total, intact, and $\alpha_E$ fibrinogen did not change (data not shown). The decrease in the relative level of γ′ fibrinogen was not correlated with the number of days between the two plasma samples (data not shown).

### Correlations of Fibrinogen Variant Levels with Other Factors, Fibrin Network Structure, and Fibrinolysis

Functional fibrinogen levels correlated strongly with antigen levels of total and intact fibrinogen (►**Supplementary Table S2**). These fibrinogen levels showed correlations with C-reactive protein, interleukin-6, procalcitonin, plasminogen activator inhibitor 1, FVIII, FXIII, fibrin network density, turbidity change, and clot lysis time. The relative levels of $\alpha_E$ fibrinogen were positively correlated with Clauss and intact fibrinogen levels, while the relative levels of γ′ fibrinogen were not correlated to fibrinogen levels. The relative levels of $\alpha_E$ fibrinogen showed weak correlations with the turbidity change and clot lysis time, while the relative levels of γ′ fibrinogen were weakly correlated with fiber diameter.

## Discussion

Besides strongly elevated absolute levels of functional, total, and intact fibrinogen in ICU patients with COVID-19, we also showed that ICU patients with COVID-19 had significantly increased absolute and relative levels of $\alpha_E$ fibrinogen compared to healthy controls. Furthermore, fibrinogen (variant) levels were similar in ICU patients with pneumococcal infection and ICU patients with COVID-19, suggesting these increases in fibrinogen (variant) levels may be a more general observation in severe disease. Between ICU patients with COVID-19 with and without thrombosis, we did not observe differences in levels of $\alpha_E$ fibrinogen and γ′ fibrinogen, but we did observe a small significant difference in the functional fibrinogen level. Finally, the relative levels of $\alpha_E$ fibrinogen and γ′ fibrinogen were only weakly associated with fibrin network characteristics. To our knowledge, no other studies exist that measured $\alpha_E$ fibrinogen levels in patients. It has only been shown that the percentage of $\alpha_E$ fibrinogen is around 3.3% in newborns, which is higher than

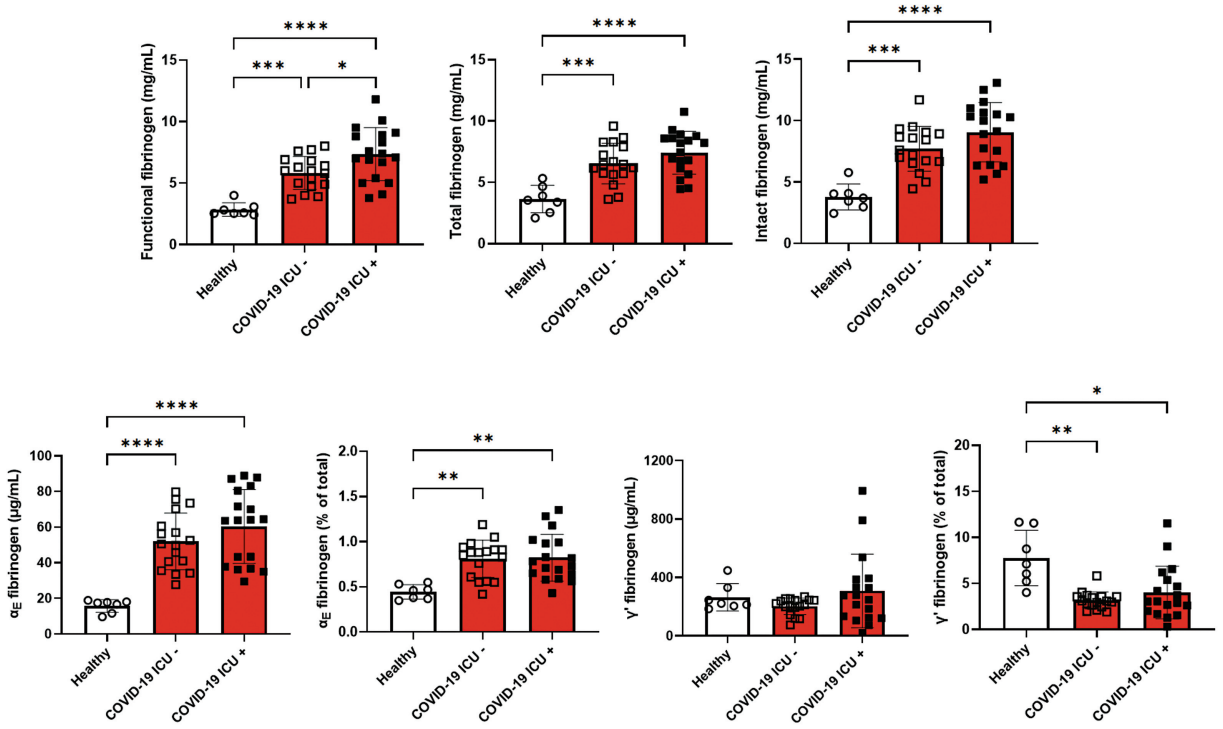


**Fig. 2** Levels of fibrinogen (variants) in plasma samples collected from healthy controls, patients with COVID-19 without thrombosis (COVID-19 ICU −), and ICU patients with COVID-19 and thrombosis after their diagnosis of thrombosis (COVID-19 +). $^{*}p < 0.05$; $^{**}p < 0.01$; $^{**}p < 0.001$; $^{****}p < 0.0001$. COVID-19, coronavirus disease 2019; ICU, intensive care unit.

the 1 to 2% found in adults assessed by quantitative western blot.[31] The mechanism of the increased relative levels of $\alpha_E$ fibrinogen in ICU patients with COVID-19 remains speculative. It may be increased synthesis due to an altered alternative mRNA splicing in severe COVID-19. In addition, $\alpha_E$ fibrinogen is suggested to be less susceptible to proteolytic degradation than the normal Aα chain, possibly leading to increased relative levels in situations with upregulated synthesis of fibrinogen.[32] Finally, since we did not see a difference in relative and absolute levels of $\alpha_E$ fibrinogen between patients with and without thrombosis, we hypothesize that there is no causal relation between $\alpha_E$ fibrinogen and the risk of thrombosis.

Previous studies have shown increased relative levels of γ´ fibrinogen in patients during the acute phase of ischemic stroke.[25] Farrell et al reported high absolute levels of γ´ fibrinogen in patients with COVID-19, but did not report relative levels.[33] We initially hypothesized that severe COVID-19 would also lead to higher relative levels of γ´ fibrinogen, possibly due to severe inflammation. However, we saw decreased relative levels of γ´ fibrinogen, no correlation between inflammatory markers and relative levels of γ´ fibrinogen, and no difference in absolute levels of γ´ fibrinogen between the different groups. The mechanism explaining the decreased relative levels of γ´ fibrinogen in ICU patients with COVID-19 is unknown. It is hypothesized

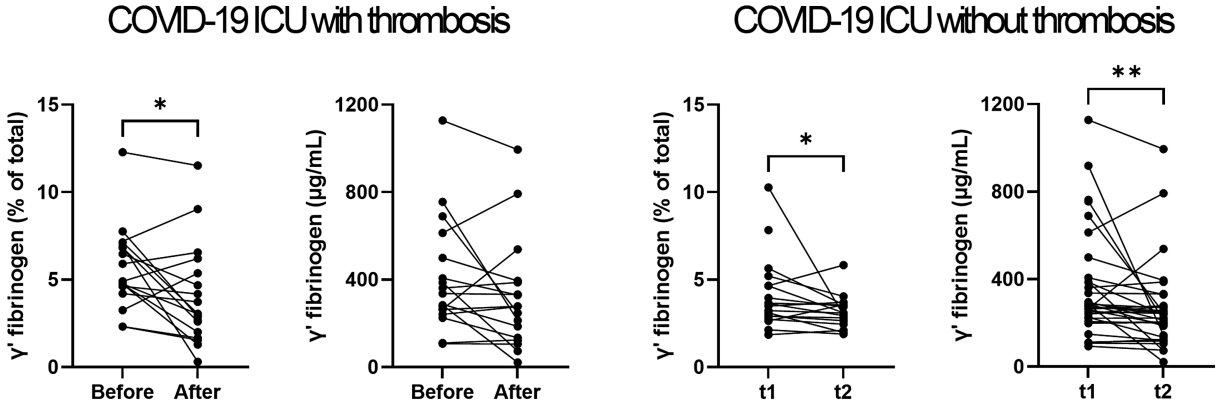


**Fig. 3** Comparison of the levels of γ´ fibrinogen in ICU patients with COVID-19 with and without thrombosis at two time points. $^{*}p < 0.05$, $^{**}p < 0.01$. COVID-19, coronavirus disease 2019; ICU, intensive care unit.

that alternative mRNA splicing resulting in γ′ fibrinogen occurs when an alternative polyadenylation site within the gene is used.[34,35] Previous studies have suggested that viral proteins in influenza can promote or interfere with polyadenylation.[36] This observation leads to the hypothesis that proteins of SARS-CoV-2 can possibly affect the process of polyadenylation in the fibrinogen genes and thereby reduce the relative level of γ′ fibrinogen. Furthermore, it is possible that there is increased consumption of γ′ fibrinogen in SARS-CoV-2 infection, for example, due to binding of γ′ fibrinogen to viral proteins or proteins involved in inflammatory responses.[37] Interestingly, $\alpha_E$ fibrinogen and γ′ fibrinogen did not correlate well in the current study. This observation suggests different mechanisms regulating the occurrence or stability of both mRNA splice variants and that these are differently affected by severe disease.

Interestingly, the relative and absolute levels of γ′ fibrinogen significantly decreased from the first to the second time point in ICU patients with COVID-19. The decrease in the relative levels occurred both in patients who did or did not develop thrombosis. Therefore, it is unlikely to be caused by the development of thrombosis.

Contradictory to the apparent effects of the fibrinogen variants on fibrin network structure seen in previous studies,[13,19–21] relative levels of the mRNA splice variants in our study were only weakly correlated with fibrin network characteristics. Previously, purified fibrinogen variants were studied instead of plasma samples. Plasma from the patients in the current study showed large variations in other (coagulation) factors, which can influence fibrin network characteristics and may explain why the association in our study is quite weak. The current correlations need confirmation in larger and/or other patient groups. Together with the finding that relative and absolute levels of $\alpha_E$ fibrinogen and γ′ fibrinogen were not significantly different between ICU patients with COVID-19 with and without thrombosis, these results suggest that the development of thrombosis in patients with COVID-19 cannot be explained by altered levels of $\alpha_E$ and γ′ fibrinogen. Also, the observation that ICU patients with pneumococcal infection showed similar fibrinogen (variant) levels to ICU patients with COVID-19 suggests that these levels cannot explain the increased development of thrombosis in severe COVID-19.

The higher functional fibrinogen levels as measured using the Clauss assay in ICU patients with COVID-19 and thrombosis compared to ICU patients with COVID-19 without thrombosis were only seen after the diagnosis of thrombosis and not at the first time point. In addition, no change in antigen levels of (total) fibrinogen was found between these two groups using the ELISAs. This points to the possibility that other coagulation factors than fibrinogen are increased or more active, resulting in higher results in the Clauss assay, and potentially contributing to the development of thrombosis.

Finally, we were interested in fibrinogen variants caused by the degradation of the α-chain in the circulating blood. This degradation results in LMW or LMW′ fibrinogen. Currently, it is not clear what causes this degradation and which enzymes are responsible.[11] Our study shows very similar patterns for intact and total fibrinogen in the different groups, suggesting the degree of degradation of the α chain is not altered in ICU patients with COVID-19 or pneumococcal infection.

Our study has some limitations. The ICU patients with pneumococcal infection had a bacterial instead of a viral infection. Still, this control group was homogenous and showed similar symptoms to patients with COVID-19. Therefore, we considered this as our best available control group. Another potentially important difference between the groups is medication use. Anticoagulation therapy and anti-inflammatory drugs were for example differently used in the different groups, and even within the patients with COVID-19 due to changes in treatment strategies. Therefore, these differences could have affected levels of fibrinogen (variants). In addition, even though there was no clinical suspicion of thrombosis in the ICU patients with pneumococcal infection, we cannot entirely exclude the possibility that undetected thrombosis might have developed. Furthermore, the small sample sizes are a limitation. It is possible that stronger associations or differences can be observed in larger samples, which would also make it possible to adjust for covariates in the analysis. We classified ICU patients with COVID-19 into two groups based on the diagnosis of thrombosis upon imaging. However, it is the question whether this classification is really possible. It might be that all ICU patients with COVID-19 will eventually develop microthrombi that are not always detected. Finally, patients from the first and second COVID-19 waves were used, so the question remains whether these results can be generalized to patients with different viral variants.

## Conclusion

Our results show that severe COVID-19 is associated with increased levels of functional, total, intact, and $\alpha_E$ fibrinogen and decreased relative levels of γ′ fibrinogen, which may be a cause or consequence of severe disease. Since we only find a difference in functional fibrinogen and not in fibrinogen variant levels between ICU patients with COVID-19 with and without thrombosis, alterations in levels of fibrinogen variants cannot explain or predict the development of thrombosis.

### Author Contributions

Judith J. de Vries: conceptualization, investigation, formal analysis, visualization, writing—original draft. Chantal Visser: conceptualization, investigation, writing—review and editing. Maureen van Ommen: investigation, writing—review and editing. Casper Rokx: resources, writing—review and editing. Els van Nood: resources, writing—review and editing. Eric C.M. van Gorp: conceptualization, writing—review and editing. Marco Goeijenbier: resources, writing—review and editing. Johannes P.C. van den Akker: resources, writing—review and editing. Henrik Endeman: conceptualization, resources, writing—review and editing. Dingeman C. Rijken: methodology, supervision, writing—review and editing. Marieke J.H.A. Kruip: conceptualization, resources, writing (review and editing), supervision,

funding acquisition. Miranda Weggeman: conceptualization, methodology, resources, writing (review and editing), supervision. Jaap Koopman: conceptualization, methodology, resources, writing (review and editing), supervision. Moniek P.M. de Maat: conceptualization, writing (review and editing), supervision.

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Conflicts of Interest
J.J.d.V., C.V., E.v.N., E.C.M.v.G., M.G., J.P.C.v.d.A., H.E., D.C.R., and M.P.M.d.M. declare to have no conflicts of interest. C. R. reports research grants from ViiV Healthcare, Gilead Sciences, Janssen, and Health-Holland for research outside the submitted work and participated in advisory boards for ViiV Healthcare and Gilead sciences. M.J.H.A. K. has received unrestricted grants paid to the Department for Research outside this work from Sobi, and has received a speaker's fee paid to the department from Sobi, Roche, and Bristol Myers Squibb. M.v.O., M.W., and J.K. are employees and shareholders of Fibriant BV.

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