

## Altered fibrin clot structure contributes to thrombosis risk in severe COVID-19

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43 **Abstract**

44 The high incidence of thrombotic events suggests a possible role of the contact system  
45 pathway in COVID-19 pathology. Here, we demonstrate altered levels of factor XII  
46 (FXII) and its activation products in two independent cohorts of critically ill COVID-19  
47 patients in comparison to patients suffering from severe acute respiratory distress  
48 syndrome due to influenza virus (ARDS-influenza). Compatible with this data, we  
49 report rapid consumption of FXII in COVID-19, but not in ARDS-influenza, plasma.  
50 Interestingly, the kaolin clotting time was not prolonged in COVID-19 as compared to  
51 ARDS-influenza. Using confocal and electron microscopy, we show that increased FXII  
52 activation rate, in conjunction with elevated fibrinogen levels, triggers formation of  
53 fibrinolysis-resistant, compact clots with thin fibers and small pores in COVID-19.  
54 Accordingly, we observed clot lysis in 30% of COVID-19 patients and 84% of ARDS-  
55 influenza subjects. Analysis of lung tissue sections revealed wide-spread extra- and  
56 intra-vascular compact fibrin deposits in COVID-19. Together, our results indicate that  
57 elevated fibrinogen levels and increased FXII activation rate promote thrombosis and  
58 thrombolysis resistance *via* enhanced thrombus formation and stability in COVID-19.

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## 75 Introduction

76 Severe acute respiratory syndrome coronavirus 2 (SARS-CoV2) is a corona virus that  
77 causes a multisystem disease emanating from the respiratory tract designated as a  
78 coronavirus disease (COVID)-19<sup>1-3</sup>. Rapidly accumulating data suggests that a major  
79 underlying molecular mechanism in COVID-19-related morbidity and mortality is  
80 widespread endothelial injury associated with hyperactivation of the immune system,  
81 consequently leading to numerous haemostasis abnormalities<sup>4-6</sup>. Accordingly, next to  
82 markedly elevated levels of pro- and anti-inflammatory mediators such as interleukin  
83 (IL)-6, IL-2R, IL-10, and tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ), elevated levels of D-dimer,  
84 fibrinogen, and prolonged prothrombin time (PT) have been reported in severely ill  
85 COVID-19 patients<sup>7-10</sup>. The clinical relevance of these processes is highlighted by the  
86 association between abnormal levels of D-dimer and the 28-day mortality in patients  
87 with COVID-19<sup>11-15</sup>, and post-mortem studies stressing the presence of micro-thrombi  
88 and capillarostasis in the lungs of affected subjects<sup>16,17</sup>.

89 The high incidence of thrombotic events, in particular deep vein thrombosis and  
90 pulmonary embolism, in conjunction with mildly prolonged activated partial  
91 thromboplastin time (APTT)<sup>18,19</sup>, suggests a possible role of coagulation factor XII  
92 (FXII) in COVID-19 coagulopathy. FXII is a serine protease of the contact-phase  
93 system of blood coagulation and circulates in plasma as a single-chain zymogen<sup>20</sup>.  
94 Following contact with anionic surfaces such as kaolin, but also extracellular RNA  
95 (eRNA) released from damaged cells<sup>21</sup>, neutrophil extracellular traps (NETs)<sup>22</sup>, or  
96 polyphosphates secreted from activated platelets<sup>23</sup>, FXII undergoes autoactivation to  
97  $\alpha$ FXIIa (herein referred to as FXIIa)<sup>24</sup>. FXIIa cleaves plasma prekallikrein (PK) to  
98 kallikrein (PKa), which in turn reciprocally activates FXII and amplifies FXIIa  
99 generation<sup>25</sup>. As a consequence, the plasma kallikrein-kinin system is activated,  
100 leading to the release of the vasodilatory and vascular barrier disrupting peptide  
101 bradykinin (BK) from high molecular weight kininogen (HK)<sup>26,27</sup>. Overall, activation of  
102 the contact-phase system contributes to an increased production of thrombin and  
103 fibrin, although FXIIa/PKa-mediated conversion of plasminogen to plasmin may have  
104 a minor effect on fibrinolysis.

105 A congenital deficiency of FXII in humans does not cause any bleeding complications,  
106 suggesting that FXII is dispensable for physiological haemostasis and fibrin  
107 formation<sup>28</sup>. However, the contact phase pathway may play an important role in  
108 thrombosis development when contact surfaces are exposed in scenarios such as

109 trauma injury or bacterial and viral infections<sup>29,30</sup>. Indeed, numerous *in vivo* studies  
110 have confirmed a critical function of FXII in thrombus growth and stabilization under  
111 the mentioned conditions and provided the rationale for the development of new FXIIa  
112 inhibitors, which ensure thrombo-protection in patients without causing a bleeding  
113 complications<sup>29,31,32</sup>.  
114 Given the high incidence of thromboembolic complications in severely ill COVID-19  
115 patients<sup>18,19</sup>, we investigated the contribution of FXII to fibrin formation and fibrinolysis  
116 in this patient cohort in comparison to patients infected with the influenza virus.

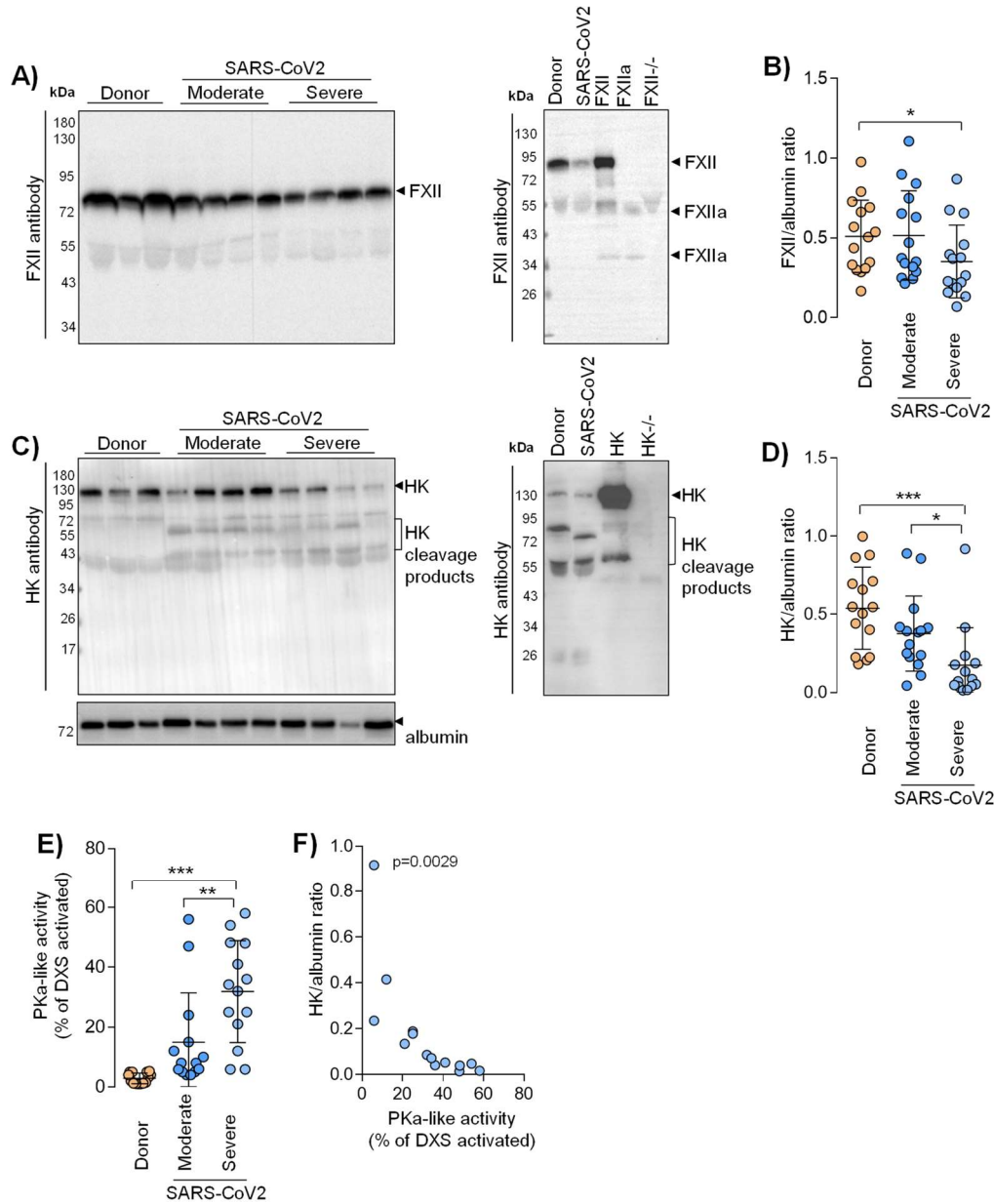
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## 118 **Results**

### 119 **FXII is activated in severely ill COVID-19 patients**

120 In the discovery cohort, the plasma levels of FXII were decreased in severe COVID-  
121 19 patients as compared to controls (Figure 1A, B; moderate is defined by WHO  
122 severity score: 3–4, hospitalized, no invasive ventilation; severe is defined by WHO  
123 severity score: 5–7, high flow O<sub>2</sub> or intubated and mechanically ventilated).  
124 Disappearance of FXII in plasma typically corresponds to its activation and conversion  
125 into two chain FXIIa protein composed of the 50 kDa-heavy chain and 30-kDa light  
126 chain. Detection of FXIIa plasma is, however, hindered by its rapid inactivation and  
127 complex formation with C1 esterase inhibitor (C1INH). Thus to better monitor the  
128 presence of FXIIa in COVID-19 plasma, we measured products of its activation, such  
129 as HK and PKa. As expected, disappearance of FXII in plasma was accompanied by  
130 HK cleavage, seen as diminished signal intensity of intact HK band at 130 kDa (Figure  
131 1C, D). A decrease in intact HK levels was associated with the appearance of cleaved  
132 HK fragments: the cleaved HK light chain band migrating at 55 kDa and an additional  
133 45-kDa band representing a degradation product of 55-kDa cleaved HK light. To further  
134 examine whether the reduction in intact levels of FXII and HK is a result of contact  
135 system activation, we measured the activity of plasma PKa. PKa-like activity was  
136 markedly elevated in severe COVID-19 patients in comparison to donors and patients  
137 suffering from moderate SARS-CoV2 infection (Figure 1E). Furthermore, a strong  
138 negative correlation between the levels of intact HK and PKa-like activity in plasma of  
139 severe COVID-19 patients was observed (Figure 1F). Purified plasma proteins and  
140 deficient plasma samples were used to prove the specificity of the bands shown in  
141 western blots (Figure 1A, C; right panels).

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144 **Figure 1. Activation of the contact phase system in plasma of critically ill COVID-19 patients.** A,C)  
 145 Western blot analysis (left panels) of factor XII (FXII) A) and high molecular weight kininogen (HK) C)  
 146 in plasma from moderate and severe COVID-19 patients (infected with SARS-CoV2) and donors. Four out  
 147 of 15 moderate and severe COVID-19 patients and 3 out of 15 donors are demonstrated. Rights panels  
 148 show the specificity of the antibodies used. B, D) Densitometric analysis of A) and C), respectively.  
 149 COVID-19 moderate/severe n=15, donor n=15. E) PKa-like activity in plasma from moderate (n=14) and  
 150 severe (n=14) COVID-19 patients and donors (n=15). F) Correlation between the levels of intact HK and  
 151 PKa-like activity in plasma of severe Covid-19 patients. n=14. Correlation is performed using  
 152 Spearman's rank correlation coefficient. \*p<0.05, \*\*p<0.01, \*\*\*p<0.001. Data in B), D), and E) are shown  
 153 as mean±SD.

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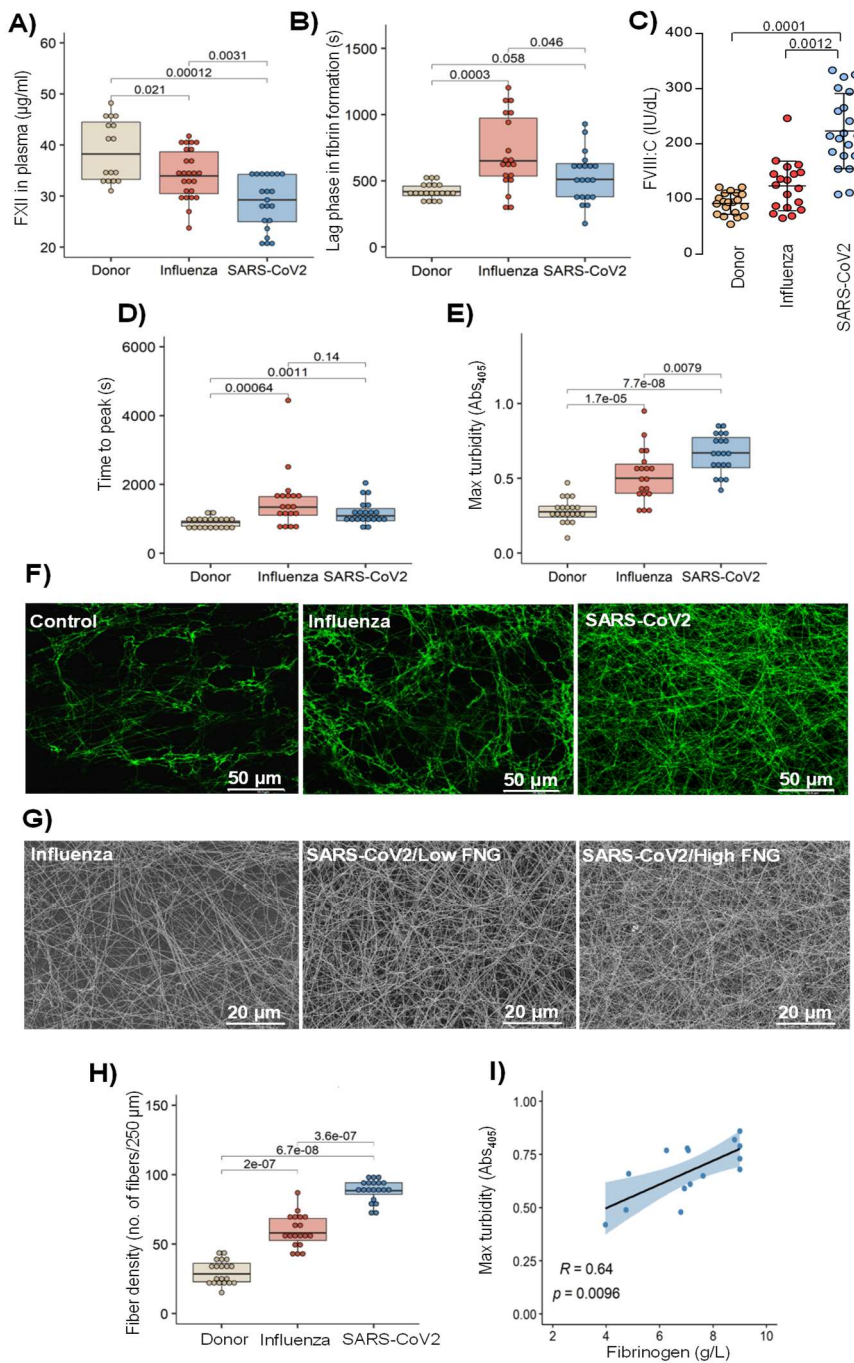
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## 158 **Fibrinogen and FXIIa regulate fibrin network density in COVID-19**

159 To assess whether enhanced activation of FXII in critically ill COVID-19 patients  
160 represents a characteristic feature of SARS-CoV-2 infection, we analyzed plasma  
161 samples of patients suffering from acute respiratory distress syndrome (ARDS) due to  
162 influenza virus infection. The decrease in FXII plasma levels in severe COVID-19 was  
163 confirmed in the validation cohort of the patients. Furthermore, the levels of FXII in  
164 COVID-19 were significantly lower than those in ARDS-influenza (Figure 2A).  
165 Surprisingly yet, the lag phase in fibrin formation, triggered by the FXII activator kaolin,  
166 was shorter in plasma of critically ill COVID-19 patients as compared to patients  
167 suffering from ARDS-influenza (Figure 2B). The phenomenon, which might be  
168 explained by markedly higher plasma levels of FVIII:C in COVID-19 than ARDS-  
169 influenza (Figure 2C). Notably, both COVID-19 and ARDS-influenza patients received  
170 the same daily dose of unfractionated heparin, excluding iatrogenic anticoagulation as  
171 a cause of prolonged kaolin-triggered clotting time in ARDS-influenza. In addition, we  
172 excluded lupus anticoagulant and the presence of anti-FXII antibodies as a cause of  
173 FXII deficiency in critically ill COVID-19 patients in our cohort (data not shown).  
174 Further analysis of kaolin-triggered plasma clotting time revealed an increase in the  
175 time to reach the turbidity peak in both patient groups as compared to control, but no  
176 difference between ARDS-influenza and severe COVID-19 (Figure 2D). The density of  
177 the clot (indicated by the maximum turbidity measurement) was higher in both patient  
178 groups as opposed to control. A direct comparison between clots of ARDS-influenza  
179 and severe COVID-19 showed significantly higher maximal turbidity values in the latter  
180 group (Figure 2E). Visualization of fibrin clots by laser scanning confocal microscopy  
181 and scanning electron microscopy revealed an increase in fibrin structure  
182 compactness with thinner fibers and smaller pores in clots from COVID-19 plasma, as  
183 compared to clots generated in plasma obtained from ARDS-influenza patients (Figure  
184 2F-H). A detailed analysis of the clots generated from plasma of severe COVID-19  
185 patients demonstrated association between packing density of fibrin fibers and plasma  
186 fibrinogen concentration, with dense fibrin network in clots formed in plasma of patients  
187 exhibiting high fibrinogen levels (Figure 2G). Accordingly, a strong positive correlation  
188 between maximum turbidity values and fibrinogen concentration in plasma of critically  
189 ill COVID-19 patients was noted (Figure 2I).

190



191 **Figure 2. Dense fibrin clots are formed in severe COVID-19 plasma.** A) Factor XII (FXII) levels in  
 192 plasma of ARDS-influenza (Influenza; n=25) and severe COVID-19 (SARS-CoV2; n=21) patients as  
 193 well as donors (n=16). B) Lag phase in fibrin formation-triggered by kaolin. Influenza, n=19; SARS-  
 194 CoV2, n=20; donor, n=20. C) FVIII activity (FVIII:C) in patient and donor plasma. Influenza, n=19; SARS-  
 195 CoV2, n=20; donor, n=20. Mean±SD is shown. D, E) Time to reach the turbidity peak D) and maximum  
 196 (Max) turbidity E) values for Influenza (n=19), SARS-CoV2 (n=20) and donor (n=20) plasma. Clot  
 197 formation was induced by the addition of kaolin to plasma. F) Laser scanning confocal microscopy  
 198 images of fibrin fibers in clots formed from Influenza (n=19), SARS-CoV2 (n=20), and donor (n=20)  
 199 plasma. Representative pictures are demonstrated. G) Scanning electron microscopy images of fibrin  
 200 network in clots generated from Influenza as well as low- and high-fibrinogen (FNG) SARS-CoV2  
 201 plasma. Representative pictures are demonstrated. H) Fibrin fiber density in donor (n=20), ARDS-  
 202 Influenza (n=19) and COVID-19 (n=20) clots. Per patient 3 separate clots were prepared, 5 pictures  
 203 were taken in different areas of the clots and fibril density was determined in all pictures. I) Correlation  
 204 between Max turbidity values and FNG levels in plasma of COVID-19 patients. SARS-CoV2-infected

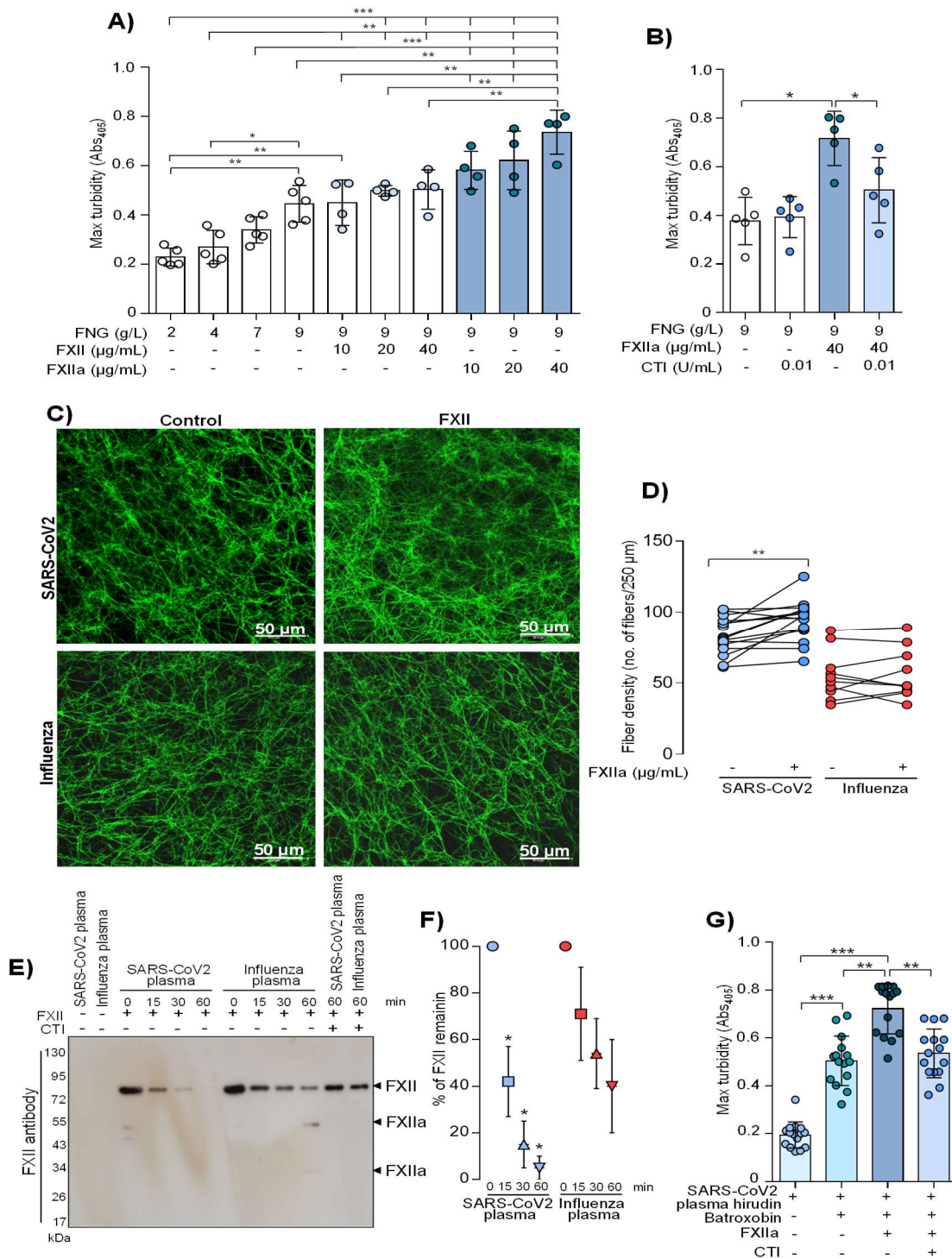
205 patients with available FNG levels are included into the analysis (n=15). Correlation is performed using  
206 Spearman's rank correlation coefficient. Data in A), B), D), E), and H) are shown as single data points  
207 with boxplot overlay indicating median and interquartile range.  
208

209 As the architecture of fibrin clots may be influenced not only by fibrinogen (FNG) but  
210 also FXIIa<sup>33,34</sup>, we next analyzed the impact of these two proteins on the clot structure  
211 in a purified system. As depicted in figure 3A high concentrations of fibrinogen  
212 increased peak turbidity values and this effect was potentiated by the addition of FXIIa.  
213 Accordingly, corn trypsin inhibitor (CTI), the inhibitor of FXIIa, reduced maximum  
214 turbidity of the clot generated by mixing fibrinogen and FXIIa (Figure 3B).

215 As sustained activation of FXII was described in COVID-19<sup>35</sup>, we next investigated the  
216 potential contribution of FXIIa to the regulation of fibrin clot structure in severe COVID-  
217 19. Supplementation of COVID-19 plasma with FXII to the levels observed in healthy  
218 subjects increased fibrin network density but not fibrin fiber diameter. No apparent  
219 effect of FXII addition on fibrin clot architecture was seen in ARDS-influenza samples  
220 (Figure 3C, D). Furthermore, rapid decay of exogenous, biotinylated FXII in COVID-  
221 19, but not in ARDS-influenza, plasma, implying an accelerated rate of FXII activation  
222 in the former group of the patients was observed (Figure 3E, F). The addition of CTI to  
223 plasma samples prevented the conversion of FXII into FXIIa (Figure 3F). To  
224 demonstrate a direct effect of FXIIa on fibrin structure, we clotted hirudin-preincubated  
225 COVID-19 plasma with batroxobin in the presence of FXIIa and/or CTI and measured  
226 the maximum turbidity. As shown in figure 3G, FXIIa increased fibrin density and this  
227 effect was diminished by CTI thus ensuring direct, thrombin independent, role of  
228 proteolytically active FXII in the modulation of fibrin architecture. The elevated levels  
229 of factor XIIIa were not detected in plasma of critically ill COVID-19 patients (data not  
230 shown).

231 Together, these results suggest that high levels of fibrinogen along with markedly  
232 increased rates of FXII activation in COVID-19 plasma create a specific pro-  
233 coagulatory microenvironment, which promotes the formation of particularly dense  
234 fibrin networks.





235 **Figure 3. Fibrinogen and FXIIa contribute to dense fibrin network in severe COVID-19.** A, B) Max  
 236 turbidity values of fibrin clots generated in the purified system from increasing concentrations of FNG  
 237 and/or FXII/FXIIa in the absence or presence of CTI. Clot formation was induced by thrombin. n=4-5.  
 238 C) Laser scanning confocal microscopy images of fibrin fibers in clots formed from SARS-CoV2 or  
 239 Influenza plasma supplemented with factor XII (FXII). Representative pictures are demonstrated. D)  
 240 Fibrin fiber density in ARDS-Influenza (n=10) and COVID-19 (n=10) clots generated in C). Per patient 3  
 241 separate clots were prepared, 5 pictures were taken in different areas of the clots and fibril density was  
 242 determined in all pictures. Paired data is shown interconnected. E) Rate of FXII activation in ARDS-  
 243 Influenza and SARS-CoV2 plasma. Biotin-labeled FXII was added to plasma and its decay was  
 244 monitored by western blotting using horseradish peroxidase-labeled streptavidine (upper panel).  
 245 Representative blot is shown. F) Quantification of FXII decay in ARDS-Influenza and SARS-CoV2

246 plasma. FXII signal at time point 0 was considered as 100%. n=20/group. G) Maximum (Max) turbidity  
247 values of fibrin clots generated by the addition of batroxobin to hirudin-preincubated plasma in the  
248 presence of active FXII (FXIIa) and/or corn trypsin inhibitor (CTI). n=15 biological replicates. Data in A,  
249 B), F) and (G) indicate mean  $\pm$  SD. \*p<0.05, \*\*p<0.01, \*\*\*p<0.001.  
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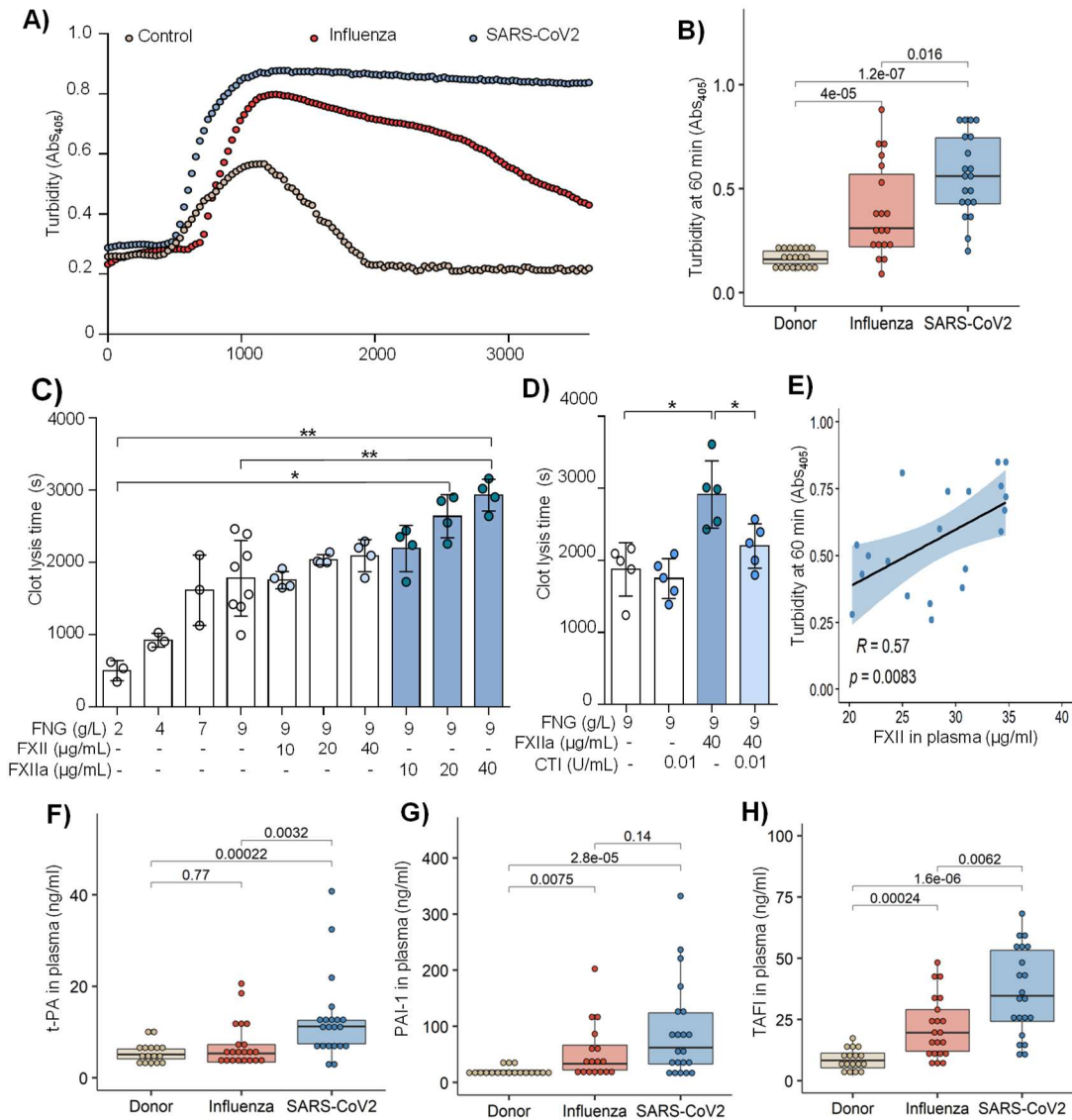
### 251 **Elevated fibrin network density increases clot resistance to fibrinolysis**

252 Fibrin network density was previously found to determine clot resistance to  
253 fibrinolysis<sup>37</sup>. Accordingly, we next evaluated the lysis resistance of fibrin clots in  
254 patient plasma, using an *in vitro* turbidimetric clot-lysis assay. Here, kaolin together  
255 with tissue-plasminogen activator (t-PA) were added to plasma to initiate the intrinsic  
256 pathway of coagulation, followed by fibrin-dependent plasmin generation *via* t-PA-  
257 mediated activation of plasminogen in the same sample. While in normal plasma, the  
258 characteristic bell-shaped clot-lysis curve, representing the complete fibrin clot  
259 dissolution, was observed, only partial clot-lysis was detected in ARDS-influenza  
260 samples, and clot-lysis was completely absent in COVID-19 samples over the entire  
261 time period of the experiment (Figure 4A). This observation is supported by the highest  
262 turbidity values at 60 min in severe COVID-19 samples (Figure 4B). Overall, clot lysis  
263 was observed in 84% of ARDS-influenza patients and only 30% of COVID-19 patients  
264 suggesting fibrinolysis shutdown in the vast majority of SARS-CoV2-infected patients  
265 in our cohort.

266 To probe for high fibrinogen levels and increased rate of FXII activation as potential  
267 cause of fibrinolysis shutdown in plasma from critically ill COVID-19 patients, clot-lysis  
268 assays were performed in a purified system. As expected, increasing amounts of  
269 fibrinogen and FXIIa prolonged clot lysis time, with an additive effect being observed  
270 at the highest concentrations of both proteins (Figure 4C). The addition of CTI to the  
271 assay shortened clot lysis time supporting the requirement of FXII proteolytic activity  
272 for this effect (Figure 4D). In addition, a strong positive correlation between the turbidity  
273 values at 60 min and FXII levels in COVID-19 plasma was seen (Figure 4E).

274 To test whether other components of the fibrinolytic system, such as t-PA, plasminogen  
275 activator inhibitor-1 (PAI-1) and thrombin-activatable fibrinolysis inhibitor (TAFI, also  
276 designated plasma carboxypeptidase B2) may be dysregulated in critically ill COVID-  
277 19 patients, we measured their levels by means of ELISA. The concentration of t-PA  
278 was elevated in severe COVID-19 as compared to control and ARDS-influenza  
279 patients (Figure 4F). An increase of PAI-1 was also noted in plasma of ARDS-influenza  
280 and severe COVID-19 patients as opposed to control, yet, a significant difference  
281 between both patient groups was not detected (Figure 4G). Interestingly, TAFI was not

282 only markedly elevated in both patient groups as compared to control, but exhibited  
 283 also significantly higher plasma values in severely ill patients with COVID-19 as  
 284 compared to ARDS-influenza (Figure 4H).  
 285



286 **Figure 4. Fibrinolysis shutdown in severe COVID-19.** A) Turbidimetric analysis of clot lysis in severe  
 287 COVID-19 (SARS-CoV2), ARDS-influenza and control plasma. Representative clot lysis curves are  
 288 shown. SARS-CoV2, n=20; ARDS-Influenza; n=19, control, n=20. B) Turbidity values ( $A_{405}$ ) of the fibrin clots at 60 min. SARS-CoV2, n=20; ARDS-influenza, n=19; control, n=20. C, D) Clot lysis time. Clots  
 289 were generated in purified system with increasing concentrations of fibrinogen (FNG) and/or factor XII  
 290 (FXII)/active FXII (FXIIa). Clot formation was induced by thrombin and clot lysis by plasmin generated  
 291 from plasminogen by t-PA. In some experiments FXII was preincubated with corn trypsin inhibitor (CTI).  
 292 Clot formation and lysis were monitored via turbidimetry. n=3-5. Mean $\pm$ -SD is shown. \* $p$ <0.05,  
 293 \*\* $p$ <0.01, \*\*\* $p$ <0.001. E) Correlation between turbidity values at 60 min and FXII plasma levels in severe  
 294 COVID-19. n=20. Correlation is performed using Spearman's rank correlation coefficient. F-H) t-PA (F),  
 295 plasminogen activator inhibitor-1 (PAI-1; G), and thrombin-activatable fibrinolysis inhibitor (TAFI, H)  
 296 levels in plasma of severe COVID-19 (n=21), ARDS-influenza (n=21) and control (n=17) as assessed  
 297

298 by ELISA. Data in B) and F)-H) are shown as single data points with boxplot overlay indicating median  
299 and interquartile range.  
300

### 301 **Dense fibrin clots are observed in the lungs of severe COVID-19 patients**

302 To demonstrate the *in vivo* relevance of our findings, we stained autopsy lung tissue  
303 sections from SARS-CoV2- and influenza-infected ARDS patients as well as subjects  
304 who died due to no respiratory causes for fibrin. Notably, time from death to autopsy  
305 was matched for all groups examined. As demonstrated in figure 5A, intra- and extra-  
306 vascular fibrin aggregates were observed in both severe COVID-19 and ARDS-  
307 influenza patients. However, in contrast to ARDS-influenza subjects, in the lungs of  
308 COVID-19 patients the deposits of fibrin appeared to be more widespread and evenly  
309 present not only in alveolar spaces but also around alveolar septae over the whole  
310 lung examined. In ARDS-influenza patients, fibrin deposit were predominantly  
311 observed in alveolar spaces and present in selected regions of the lung (Figure 5A).  
312 Overall, in COVID-19 lungs fibrin clots were more compact and homogeneous whereas  
313 in ARDS-influenza lungs they were widespread and characterized by regions of high  
314 and low fibrin fiber density (Figure 5B).

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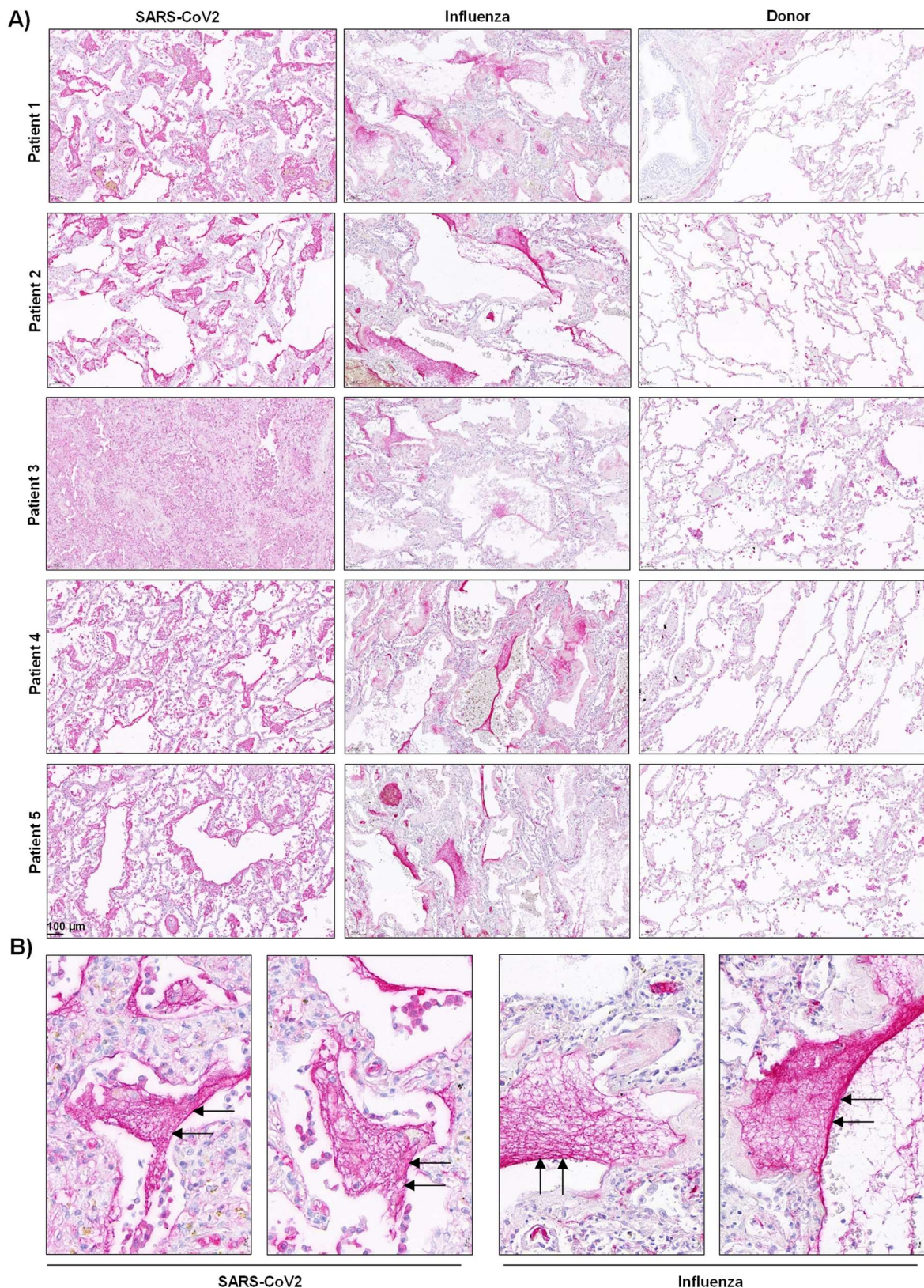
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322 **Figure 5. Fibrin deposits in the lungs of severe COVID-19 and ARDS-influenza patients.** A, B)  
323 Fibrin (red) accumulation in postmortem lung tissue sections of severe COVID-19 (n=5), ARDS-  
324 influenza (n=5) and donors (n=5). Time from death to autopsy was matched for all groups examined.  
325 Arrows indicate fibrin deposits in the lung. Magnification bar 100 µm.

## 327 Discussion

328 Many patients with severe COVID-19 exhibit coagulation abnormalities that mimic  
329 other systemic coagulopathies associated with severe infections, such as  
330 disseminated intravascular coagulation (DIC) or thrombotic microangiopathy<sup>36</sup>. A high  
331 incidence of venous thromboembolism, pulmonary embolism, deep vein thrombosis,  
332 and multiple organ failure with a poor prognosis and outcome appears to be causally  
333 related to dysregulation of blood coagulation in critically ill COVID-19 patients. Besides  
334 an elevated inflammatory status (e.g. increased cytokine levels) that might induce  
335 monocyte-related coagulation and suppression of anticoagulant pathways, typical  
336 laboratory findings in COVID-19 patients with coagulopathy are increased D-dimer  
337 levels and elevated fibrinogen concentrations<sup>36</sup>. Moreover, inflammation-induced  
338 endothelial cell injury in different vascular beds may contribute to a hypercoagulable  
339 state and the risk of thromboembolic complications<sup>37,38</sup>.

340 In order to provide mechanistic insights into the reported hypercoagulable state of  
341 severe COVID-19 patients, we compared changes in the contact phase system  
342 activation and fibrinolysis between COVID-19 patients, individuals suffering from  
343 ARDS-influenza, and donors. While some critical parameters such as fibrinogen, PAI-  
344 1, and TAFI were significantly increased, FXII levels were reduced in severe COVID-  
345 19, and the process of fibrin formation and the resulting fibrin clot structure and lysis  
346 were substantially different between patient cohorts. Histological data provided  
347 evidence for widespread, compact fibrin deposition in the lungs of patients with COVID-  
348 19 as opposed to those with ARDS-influenza.

349 In particular, although the levels of FXII were significantly decreased in severe COVID-  
350 19 patients as compared to ARDS-influenza and donors, FXII-activation products were  
351 markedly altered in patients with SARS-CoV2 infection. This scenario very likely  
352 reflects FXII consumption due to its increased binding to and auto-activation on  
353 negatively charged surfaces. Decreased FXII levels in COVID-19 plasma are also in  
354 accordance with moderately elevated APPT reported in other studies<sup>39,40</sup>. The  
355 exacerbated consumption of FXII in severe COVID-19 is further supported by our *in*  
356 *vitro* studies, in which the supplementation of COVID-19 plasma with exogenous FXII  
357 resulted in its rapid activation, presumably due to the presence of FXII auto-activation  
358 cofactors. Indeed, common pathological events observed in COVID-19 such as  
359 increased tissue cell stress together with virus-mediated necrosis, endothelial

360 dysfunction, and excessive neutrophil activation, lead to the release/exposure of large  
361 amounts of negatively charged molecules including NETs. NETs not only bind FXII but  
362 also serve as a potent endogenous inflammation-dependent inducer of FXII auto-  
363 activation, eventually propagating thrombosis<sup>21,41</sup>. Enhanced vascular NETosis along  
364 with impaired NET clearance were described in COVID-19 patients<sup>35,42</sup>. In line with  
365 these findings, several studies found an increase in NET components in COVID-19  
366 plasma including cell-free DNA, myeloperoxidase-DNA complexes, neutrophil  
367 elastase-DNA complexes, and citrullinated histone H3<sup>43,44</sup>. In addition, active FXII was  
368 described to colocalized with NETs in the lungs of COVID-19 patients and NET positive  
369 pulmonary vessels were reported to be frequently clogged<sup>38,45</sup>. Together with these  
370 findings, our results speak for NET-induced, accelerated, and constant activation of  
371 FXII in COVID-19 and thus for its role in immunothrombotic processes in this  
372 pathology. In fact, FXII auto-activation cofactors were found to be relevant for the  
373 initiation and progression of sepsis and DIC<sup>46</sup>.

374 Interestingly enough, low plasma levels of FXII in severe COVID-19 patients did not  
375 result in markedly prolonged kaolin clotting time (KCT) suggesting that other  
376 hemostatic abnormalities/factors compensate for low amounts of FXII in critically ill  
377 COVID-19 subjects. As previous studies reported that high plasma levels of FVIII:C  
378 may associate with a short KCT and an increased risk of thromboembolism<sup>47</sup>, it is  
379 plausible to assume that the excessive amounts of FVIII:C in COVID-19, as opposed  
380 to ARDS-influenza, plasma induced shortening of KCT in our cohort of patients. These  
381 results, together with previously described high levels of fibrinogen, mild  
382 thrombocytopenia, and slightly altered plasma concentrations of coagulation factors  
383 and physiological anticoagulants<sup>48</sup> argue for a specific form of intravascular  
384 coagulation in severe COVID-19 that is distinguishable from classical DIC. The  
385 prominent increase in vascular complications<sup>41</sup> points to strong involvement of  
386 endothelial cells in hemostatic abnormalities seen in COVID-19. Injured endothelial  
387 cells may provide a scaffold for thrombus generation and elevated levels of von  
388 Willebrand factor multimers (recently described in COVID-19 plasma<sup>49</sup>) may facilitate  
389 platelet-vessel wall interactions ultimately leading to the formation of platelet-rich  
390 thrombotic deposits in microvasculature. Such platelet-rich thrombotic aggregates  
391 have been observed in alveolar capillaries of critically ill COVID-19 patients<sup>16,17</sup>.  
392 Altogether haemostatic alterations seen in COVID-19 subjects reflect widespread

393 occlusive thrombotic microangiopathy with destruction of alveoli that supports  
394 persistence of microthrombi.

395 Elevated levels of fibrinogen were reported to contribute to the faster fibrin formation  
396 and increased fibrin network density, strength, and stability<sup>34</sup>. In line with this  
397 assumption, clots generated from COVID-19 plasma exhibited much higher packing  
398 density as compared to those formed from ARDS-influenza plasma. Further  
399 experiments with COVID-19 plasma and in a purified system revealed that next to  
400 fibrinogen also FXIIa may regulate clot compactness. Indeed, higher levels of  
401 fibrinogen and increased rate of FXII activation were associated with denser fibrin clots  
402 with smaller pores. The compact architecture of clots generated from COVID-19  
403 plasma correlated with their resistance to lysis consolidating the notion of  
404 hyperfibrinogenemia and FXII consumption coagulopathy as driving causes of an  
405 increased risk of thrombosis in critically ill COVID-19 patients. Our findings are  
406 consistent with the studies demonstrating the role of fibrinogen and FXIIa in  
407 organization of clot architecture<sup>33,34</sup> and the reports linking abnormal fibrin network  
408 structure/function with thrombotic events seen in patients with diabetes<sup>50</sup>, ischemic  
409 stroke<sup>51</sup>, pulmonary hypertension<sup>52</sup>, myocardial infarction<sup>53</sup>, or venous  
410 thromboembolism<sup>54</sup>. Although, increased fibrinogen levels independently promote  
411 thrombus formation and stability, the role of FXII in these processes seems to be more  
412 complex and dependent on environment conditions. Those include, the presence of  
413 NETs (or any other molecule being able to activate FXII) which orchestrate not only  
414 FXII but also platelets activation, activated platelets may perpetuate FXIIa generation  
415 by the release of polyphosphates and the availability of haemostatic factors.  
416 Coagulation proteases ensure FXIIa-dependent thrombin formation and a direct  
417 binding of FXII/FXIIa to fibrinogen may define aggregation of fibrin fibers<sup>33</sup>. Whether  
418 the interaction of FXII/FXIIa with fibrinogen can interfere with the binding of t-PA to  
419 fibrin and thereby inhibits fibrinolysis warrants further investigation.

420 Clots generated from COVID-19 plasma exhibited higher packing density, small pores  
421 and were built of thin fibers. Interestingly enough, previous studies suggested that  
422 thrombi made of thin and numerous fibers organized in tight network are resistant to  
423 fibrinolysis<sup>55</sup>. Persistent vessel occlusion seen in critically ill COVID-19 patients is  
424 reinforced by markedly increased plasma levels of TAFI and moderately elevated  
425 amounts of PAI-1<sup>56</sup>. Thus, persistent occlusion of microvessels in the lungs of COVID-



426 19 patients appears to be a result of unfortunate circumstances, starting from sustained  
427 activation/presence of thrombosis-promoting factors, going through the formation of  
428 lysis resistant thrombi, and finishing on the accumulation of fibrinolytic inhibitors<sup>57</sup>.

429 Based on current and previous findings, the scenario of defense mechanisms,  
430 including the immune and coagulation system, running out of control emerges as an  
431 underlying mechanism for severe SARS-CoV2 infection. Multiple hits from  
432 abnormalities in plasma composition, vascular cell function, and blood immune cell  
433 landscape through virus-mediated cell damage and release of intracellular debris  
434 create a milieu favoring activation of FXII. In combination with high levels of fibrinogen,  
435 FXIIa contributes to pathologic thrombus formation not only *via* thrombin generation  
436 but also through the formation of compact and lysis resistant clots. Our study thus  
437 establishes a model for future investigations on the role of altered fibrin clot structure  
438 in thrombosis and thrombolysis in severe COVID-19.

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459 **Materials and Methods**

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461 **Study population**

462 Plasma samples from COVID-19 patients were obtained from the Hannover Medical  
463 School, Hannover, Germany (validation cohort) and from the Charité-University  
464 Medicine, Berlin, Germany (discovery cohort). Plasma samples from acute respiratory  
465 distress syndrome (ARDS) due to influenza were provided from the Hannover Medical  
466 School, Hannover, Germany. All samples were taken within 6 days after onset of  
467 ARDS. All investigations were approved by the local ethics committees (Hanover  
468 samples: SEPSIS/ARDS Registry, ethic votum no.: 8146\_BO\_K\_2018; Berlin  
469 samples: ethic votum no.: EA2/066/20) and written informed consent was obtained  
470 from all participants or their next-of-kin. COVID-19 patients were classified as  
471 moderate (hospitalized, no invasive ventilation; WHO severity score: 3-4) or severe  
472 (high flow O<sub>2</sub> or intubated and mechanically ventilated; WHO severity score: 5-7) as  
473 previously described<sup>58</sup>. Control (healthy subjects) samples were provided by the  
474 Charité-University Medicine, Berlin, Germany (ethic votum no.: EA2/075/15) and from  
475 the Justus-Liebig University of Giessen, Giessen, Germany (ethic votum no.: 05/00).  
476 Baseline demographics and clinical characteristics of the patients are shown in Table  
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493 **Table 1.** Baseline demographics and clinical characteristics of COVID-19 and ARDS-influenza patients (plasma  
494 samples).  
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	Clotting			
	ARDS <sup>1</sup> -influenza	COVID <sup>2</sup> -19 (WHO 5-7)	COVID-19 (WHO 5-7)	COVID-19 (WHO 3-4)
No. of patients, n	20	21	15	15
Age, year	56; [20-86]	59; [19-82]	61; [22-84]	61; [26-80]
Sex, male (%)	87	90	69	67
BMI (kg/m <sup>2</sup> )	25; [20-36]	29; [15-62]	29; [25-36]	24; [20-36]
<sup>3</sup> CRB65 score, n				
0	0	0	NA	NA
1	0	2		
2	3	6		
3	12	9		
4	5	4		
28-day mortality (%)	30	14.3	8	0
<sup>4</sup> LOS ICU, days	19; [6-73]	27; [3-63]	30; [5-220]	NA
Ventilation, days	15; [3-66]	16; [4-50]	26; [5-220]	NA
<sup>5</sup> ECMO (%)	30	24	46	NA
<sup>6</sup> SOFA	10; [5-16]	13; [9-17]	10; [2-12]	NA
<sup>4</sup> CRP (mg/L)	264 [31-406]	151; [68-292]	85; [27-411]	29; [1-148]
Leukocytes, ×10 <sup>9</sup> /L	16; [22-90]	9; [4-36]	10; [5-27]	7; [4-22]
Platelet count, ×10 <sup>9</sup> /L	199; [70-653]	247; [99-581]	286; [129-635]	334; [173-602]
Lactate, mM	1.3; [0.7-4.8]	1.8; [0.7-5.6]	1.7; [0.4-6.6]	1.3; [1.0-2.6]
Procalcitonin, µg/L	1.7; [0.2-79.5]	0.6; [0.1-66.1]	0.6; [0.1-25]	0.1; [0-1]
D-dimer, mg/L	NA	4; [1-35]	NA	NA
Fibrinogen, g/L	4.0 [3.2-9.0]	8.0; [4.0-9.0]	NA	NA
Anticoagulant				
Heparin, n	15	14	8	7
<sup>8</sup> PTT, s	38; [25-59]	36; [26-55]	41; [30-67]	34; [30-60]

496 <sup>1</sup>ARDS, acute respiratory distress syndrome; <sup>2</sup>COVID-19, coronavirus disease 2019; <sup>3</sup>CRB 65, confusion,  
497 respiratory rate, blood pressure, age 65 score; <sup>4</sup>LOS ICU, length of intensive care unit stay, ; <sup>5</sup>ECMO,  
498 extracorporeal membrane oxygenation; <sup>6</sup>SOFA, sequential organ failure assessment; <sup>7</sup>CRP, C-reactive  
499 protein; <sup>8</sup>PTT, partial thromboplastin time.

500

501 Lung specimens were obtained from 8 ARDS patients (5 COVID-19, 3 influenza) and  
502 5 donors by autopsy. Time from death to autopsy was matched for all groups. All  
503 investigations were approved by the local ethics committees (Medical Faculty of  
504 Justus-Liebig University of Giessen, ethic votum no.: 29/01 and Medical University of  
505 Graz, ethic votum no.: 32-362 ex 19/20) and written informed consent was obtained  
506 from all participants or their next-of-kin if required. Baseline demographics and clinical  
507 characteristics of lung tissue donors are shown in Table 2.

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**Table 2.** Baseline demographics and clinical characteristics of COVID-19 and ARDS-influenza patients (lung tissue).

COVID-19						
Patient	Age, year	Sex	Background	Ventilation, days	Anticoagulant	
1	82	Male	Diffuse alveolar damage	0	Heparin	
2	77	Male	Diffuse alveolar damage	2	Heparin	
3	72	Male	Diffuse alveolar damage	6	Heparin	
4	65	Male	Diffuse alveolar damage	33	Heparin	
5	79	Female	Diffuse alveolar damage	2	Heparin	
ARDS-influenza						
1	67	Male	Community acquired pneumonia	6	Heparin	
2	72	Male	Trauma	3	Heparin	
3	77	Male	Community acquired pneumonia	5	Heparin	
4	81	Female	Community acquired pneumonia	10	Heparin	
5	80	Female	Trauma	2	Heparin	
Donor						
1	82	Female	Recurrent myocardial infarction	0	no	
2	75	Female	Heart and lung failure	0	Heparin	
3	64	Male	Myocardial infarction	0	Heparin	
4	77	Female	Dilated cardiomyopathy (right)	0	Heparin	
5	75	Male	Dilated cardiomyopathy (right)	0	Heparin	

518 <sup>1</sup>ARDS, acute respiratory distress syndrome; <sup>2</sup>COVID-19, coronavirus disease 2019.

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## 521 **Plasma clot formation and lysis**

522 Twenty  $\mu\text{L}$  of plasma were preincubated for 10 min with 20  $\mu\text{L}$  of 0.1 M imidazole buffer,  
523 pH 7.4, and 20  $\mu\text{L}$  of 0.3 mg/mL kaolin in a clear, flat-bottomed 96-well plate. Clotting  
524 was initiated by the addition of 20  $\mu\text{L}$  of 20 mM  $\text{CaCl}_2$  in the absence or presence of  
525 tissue plasminogen activator (t-PA) (25 ng/mL final; Sekisui Diagnostics, Burlington,  
526 MA). Turbidity was monitored at 405 nm ( $A_{405}$ ) every 30 s for 60 min at 37°C using a  
527 SpectraMax 190 (Molecular Devices, Biberach, Germany). In some experiments,  
528 COVID-19 plasma was preincubated with hirudin (5 IE/mL final; Diapharma, West  
529 Chester, OH) and the clotting was induced by batroxobin (5U/mL final; Enzyme  
530 Research Laboratories, South Bend, IN)

531

## 532 **Fibrin formation and lysis in a purified system**

533 Thrombin (5 nM final, Sekisui Diagnostics) was mixed with fibrinogen (2-9 g/L final),  
534 pre-incubated with either FXII or FXIIa (10-40  $\mu\text{g}/\text{mL}$  final, both from Sekisui  
535 Diagnostics) in a total volume of 25  $\mu\text{L}$  of 0.1 M imidazole buffer in a clear, flat-  
536 bottomed 96-well plate. Fibrin formation was initiated by the addition of 20  $\mu\text{L}$  of 20  
537 mM  $\text{CaCl}_2$ . To measure fibrinolysis, t-PA (0.1  $\mu\text{g}/\text{mL}$  final) and plasminogen (20  $\mu\text{g}/\text{mL}$   
538 final, Enzyme Research Laboratories) were added to the clotting solution. Turbidity

539 was monitored as described above. In some experiments, FXIIa (40 µg/mL final) was  
540 incubated with corn trypsin inhibitor (CTI; 0.01 U/mL final, Sekisui Diagnostics) before  
541 mixing with fibrinogen.

542

### 543 **Western blotting**

544 Plasma (pre-diluted 1:40 into 0.9% NaCl) was separated on a SDS polyacrylamide gel,  
545 followed by electro-transfer to a PVDF membrane. After blocking with 5% non-fat dry  
546 milk in TBS buffer (25 mM Tris pH 7.5, 150 mM NaCl) supplemented with 0.1% Tween  
547 20 (TBS-T), the membrane was incubated overnight at 4°C with a goat anti-FXII (cat.  
548 no.: 206-0056; Zytomed Systems, Berlin, Germany) or rabbit-anti high molecular  
549 weight kininogen (HK; cat. no.: ab35105; Abcam, Cambridge, UK) antibody. Next,  
550 membranes were incubated with appropriate peroxidase-labelled secondary  
551 antibodies (all from Dako, Gostrup, Denmark). Final detection of proteins was  
552 performed using a Pierce™ ECL Western Blotting Substrate (Thermo-Fisher  
553 Scientific). As loading control, albumin was detected with a rabbit anti-albumin antibody  
554 (cat. no.: A001; Dako). Western blots were developed using a ChemiDoc™ Touch  
555 (BioRad Laboratories, Inc., Hercules, CA), and densitometric analysis was conducted  
556 by the ImageLab™, Version 6.0.1 (Bio-Rad Laboratories).

557

### 558 **Immunoassays**

559 Factor XII levels in plasma were quantified by the Human FXII ELISA Kit from Abnova  
560 (Taipei, Taiwan). Plasma levels of plasminogen activator inhibitor-1 (PAI-1) and t-PA,  
561 were measured using human ELISA Kits from Thermo- Fisher Scientific. Thrombin-  
562 activatable fibrinolysis inhibitor (TAFI) levels in plasma were quantified by Human  
563 CPB2/TAFI ELISA Kit from LSBio (Seattle, WA). All measurements were performed  
564 according to manufacturer's instructions.

565

### 566 **FXII decay in plasma**

567 Endogenous FXII was depleted from plasma using a goat anti-FXII antibody (cat. no.:  
568 206-0056; Zytomed Systems) covalently attached to magnetic beads (Thermo-Fisher  
569 Scientific). Afterwards, a hundred µl of plasma was supplemented with 1 nM  
570 biotinylated FXII and the sample was incubated for 1h at 37°C. Aliquots were  
571 withdrawn after the indicated time points and analyzed by western blotting. In some

572 experiments, plasma was preincubated with 12 mg/mL CTI 30 min prior to the addition  
573 of biotinylated FXII.

574

### 575 **Immunostaining of clots generated in a purified system**

576 Clots were generated from Influenza and Covid-19 plasma supplemented with 10  
577  $\mu\text{g/ml}$  exogenous FXII as described above. Next, they were fixed with 4%  
578 paraformaldehyde in PBS. Non-specific binding sites were blocked with 3% BSA in  
579 PBS for 1 h. Next, clots were incubated with a rabbit anti-fibrinogen/fibrin (cat. no.: A  
580 0082; Dako) antibody overnight at 4°C. Following extensive washing with PBS, clots  
581 were incubated with secondary antibodies labeled with Alexa Fluor™ 488 (Thermo-  
582 Fisher Scientific) for 1h at room temperature. Finally, clots were embedded in  
583 Vectashield Mounting Medium (Vector Laboratories Inc) and images were taken as  
584 described above. ImageJ was used to determine fiber density, by counting the number  
585 of fibers crossing lines of 250  $\mu\text{m}$  placed in the image using the plug-in-grid.

586

### 587 **Scanning electron microscopy**

588 Samples were fixed with 1.5% paraformaldehyde and 1.5% glutaraldehyde solution in  
589 0.15 M Hepes for 24 h at room temperature. Next, samples were washed with 0.15 M  
590 Hepes, post-fixed in 1% osmium tetroxide for 2 h, washed in distilled water, dehydrated  
591 with graded ethanol washes and critical point dried by CO<sub>2</sub> treatment using a CPD 030  
592 critical point dryer (Ecatec AG, Trübbach, Switzerland). Finally, samples were mounted  
593 with conductive adhesive tape and sputtered with gold. Images were taken with a  
594 Philips XL30 scanning electron microscope (Philips, Eindhoven, Netherlands).

595

### 596 **Activity assays**

597 The PKa-like activity assay and the activity of factor VIII were performed as described  
598 in <sup>59</sup> and <sup>60</sup>, respectively.

599

### 600 **Statistics**

601 Statistical analysis was performed in R (version 4) using the ggpubr package. Data are  
602 expressed as single data points with boxplot overlay indicating median and  
603 interquartile range, unless indicated otherwise. Multiple groups were compared by non-  
604 parametric Kruskal-Wallis test. Correlations were performed using Spearman's rank  
605 correlation coefficient.

606 **Competing interests**

607 None declared.

608

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618

619 **Contribution**

620 M.W. designed the study, performed experiments, analyzed data, and wrote the  
621 manuscript; A.B., L.M., and O.P. performed experiments and analyzed data; B.S.,  
622 S.D., T.W., J.J.S., M.C.B., S.H., F.K., L.E.S., and M.Wi. recruited patients, analyzed  
623 patient clinical data, and reviewed the manuscript; A-S.S. and F.S. analyzed patient  
624 clinical data and wrote the manuscript; M.Z. and G.G. collected autopsy tissue samples  
625 and reviewed the manuscript; N.W., R.T.S., G.B., L.S., and P.M. analyzed data and  
626 contributed to the writing of the manuscript; W.M.K., G.K., and K.T.P designed the  
627 study and wrote the manuscript.

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