Thrombin formation via the intrinsic coagulation pathway and von Willebrand factor reflect disease severity in COVID-19

Severe coronavirus disease 2019 (COVID-19) has been characterized by hyperinflammation, vascular damage, and thrombosis.[1,2] Hyperinflammation causes a vasculopathy, particularly in the lungs. Von Willebrand factor (vWF), a marker of the vasculopathy, indeed, is associated with mortality.[3,4] Vascular damage triggers activation of the extrinsic pathway of coagulation via exposure of tissue factor (TF). Moreover, activation of the intrinsic pathway of coagulation on the background of neutrophils and neutrophil extracellular trap (NET) formation appears to be key for COVID-19's hypercoagulability to occur.[1,5,6] Yet, the relative contribution of the extrinsic pathway has not been studied in relation to the intrinsic pathway in COVID-19's hypercoagulability and thus, firm conclusions cannot be drawn. Here, we assessed markers of the extrinsic and intrinsic pathway in a large and well-defined prospective cohort of patients with COVID-19. The dynamics of coagulation were studied during the first wave of the pandemic using a longitudinal design. Also, associations between activation markers of coagulation, vascular damage, and disease severity (i.e., intensive-care unit [ICU] admission, thrombosis, and mortality) were studied.

Consecutive patients with COVID-19 who presented at the Maastricht University Medical Center, Maastricht, the Netherlands, between March 21, 2020, and April 28, 2020, were included. Disease severity was classified as mild (patients not admitted to hospital), moderate (patients admitted to the general ward requiring supplemental oxygen via nasal cannula [≤5 L/min]), and severe (patients requiring supplemental oxygen via a face mask, admitted to the ICU for mechanical ventilation, and/or those who died due to COVID-19). At presentation and at fixed time points during follow-up (i.e., every 5 [±2] days), blood samples were obtained using vacutainer tubes containing 3.2% trisodium citrate and serum tubes; citrated blood was processed immediately and centrifuged at 2,000 g for 10 minutes (min) at room temperature (RT), while serum tubes were allowed to clot for 30 min and centrifuged at 1,885 g for 10 min at RT. Plasma and serum samples were aliquoted and stored at −80°C until testing. Follow-up samples were used when available. Activated coagulation factors in complex with their natural inhibitors (i.e., activated FVII:antithrombin [FVIIa:AT], plasma kallikrein:C1 esterase inhibitor [PKa:C1Inh], FXIa:AT, FXIa:α1-antitrypsin [α1AT], FIXa:AT, and thrombin:antithrombin [T:AT]), desarginated complement 5a (C5a), and vWF:antigen (vWF:Ag), were quantified as described.[1,7] Free FVIIa (Staclot VIIa-rTF; Stago, Asniéres-sur-Seine, France) was quantified according to the manufacturer's instructions. This study was approved by the appropriate ethics committee (2020-1315), with a waiver of informed consent.

The demographics of the 220 included patients with COVID-19 are depicted in Table 1. Forty-six (21%) patients had mild, 68 (31%) had moderate, and 106 (48%) had severe COVID-19. Thus, 174 patients were admitted, most of whom were treated with antibiotics (n/N=157/174, 90%), chloroquine (n/N=131/174, 75%), and anticoagulation (n/N=154/174, 88%; either prophylactic [n=126] or therapeutic [n=28]) besides oxygen support. At that time, steroids (n/N=6/174, 3%) were not routinely prescribed.

In line with previous studies,[8] most patients with severe COVID-19 had elevated levels of D-dimer (n/N=62/64, 97%) and fibrinogen (n/N=55/62, 89%), whereas the activated partial thromboplastin time (n/N=52/71, 73%) and prothrombin time (n/N=56/70, 80%) were often normal (Table 1). Routine coagulation tests were not measured in patients with mild or moderate COVID-19.

We previously demonstrated that T:AT is elevated and linked to disease severity in COVID-19. Neutrophils, NET formation, and activation of the intrinsic pathway drive T:AT levels and COVID-19's hypercoagulability.[1] NET formation, with release of TF, may also activate the extrinsic pathway.[9] In order to better understand the interplay and balance between the intrinsic and extrinsic pathways, we assessed markers of the extrinsic pathway. FVIIa:AT, a marker of circulating FVIIa-TF complexes, and free FVIIa did not differ between patients with mild, moderate, or severe COVID-19 and remained stable over time (Table 1; *Online Supplementary Table S1*). Spearman's ρ indicated a strong positive correlation between T:AT and FXIa:AT (r=0.64; *P*<0.001) as well as FIXa:AT (r=0.74; *P*<0.001), but not FVIIa:AT (r=0.14; *P*=0.097) or free FVIIa (r=0.16; *P*=0.021; *Online Supplementary Figure S1*). The role of FVIIa:AT and free FVIIa decreased even more whereas the association of FXI:AT, FIX:AT and T:AT persisted with increasing disease severity, suggesting that FXI activation is mainly caused by FXIIa.[10]

Activation of the intrinsic pathway may occur on the background of hyperinflammation in COVID-19. FXIa:α1AT (r=0.38; *P*<0.001), FIX:AT (r=0.41; *P*<0.001), T:AT (*r*=0.35, *P*<0.001), and vWF:Ag (r=0.39; *P*<0.001) correlated with CRP (*Online Supplementary Table S2*). We previously

Table 1. Baseline characteristics of 220 patients with COVID-19.

	Normal range	Mild (N=46)	Moderate (N=68)	Severe (N=106)	Overall *P*
M/F		26/20	42/26	78/28	0.077
Age in years, median (IQR)		64 (52-74)	73 (60-79)‡	73 (60-77)†	0.014
Days from illness onset, median (IQR)		7 (5-11)	7 (5-14)	7 (5-14)	0.975
SBP mmHg, mean (SD)		129 (18)	138 (22)	138 (25)	0.063
DBP mmHg, median (IQR)		80 (70-86)	83 (74-87)	80 (70-88)	0.714
Heart rate bpm, median (IQR)		88 (75-100)	90 (80-100)	95 (80-110)*, †	0.013
Body temperature °C, mean (SD)		37.6 (0.9)	38.1 (1.0)‡	38.1 (1.0)†	0.006
Fever, N (%)	>37.9	12 (27)	39 (58)‡	55 (62)†	<0.001
Medical history Hypertension, N (%) Diabetes, N (%) CVA, N (%) Cardiac disease, N (%) COPD/asthma, N (%) None, N (%)		 13 (28) 9 (20) 5 (11) 11 (24) 6 (13) 12 (26)	 27 (40) 11 (16) 9 (13) 23 (34) 15 (22) 16 (24)	 35 (33) 24 (23) 14 (13) 32 (30) 11 (10) 28 (26)	 0.452 0.603 0.931 0.560 0.110 0.918
Platelets $\times 10^9$/L, median (IQR)	130-350	187 (154-292)	214 (147-260)	211 (168-247)	0.985
Leukocytes $\times 10^9$/L, median (IQR)	3.5-11.0	6.0 (4.8-6.5)	6.6 (4.7-9.0)	7.4 (5.8-10)*, †	0.016
Neutrophils $\times 10^9$/L, median (IQR)	1.4-7.7	4.8 (3.4-6.5)	5.0 (3.4-7.4)	5.9 (4.7-8.1)†	0.023
Lymphocytes $\times 10^9$/L, median (IQR)	1.1-4.0	1.1 (0.7-1.5)	0.8 (0.6-1.2)	0.7 (0.5-1.1)†	0.013
NLR, median, (IQR)		4.7 (2.9-6.9)	6.0 (4.1-9.0)‡	8.6 (5.2-12.5)*, †	<0.001
AST U/L, median, (IQR)	<35	36 (26-58)	49 (37-64)‡	55 (40-80)†	<0.001
LDH U/L (IQR)	<250	253 (202-344)	328 (266-451)‡	451 (358-595)*, †	<0.001
Serum creatinine μmol/L, median (IQR)	60-115	83 (62-113)	88 (71-119)	91 (71-120)	0.369
Albumin g/L, median (IQR)	32.0-47.0	34 (31-38)	33 (30-36)	29 (26-32)*, †	<0.001
CRP mg/L, median (IQR)	<10	57 (17-95)	69 (39-130)‡	103 (56-178)*, †	<0.001
C5a ng/mL, median (IQR) High C5a, N (%)	≤21.1	15.4 (9.0-25.4) 27 (61)	21.8 (16.8-28.7)‡ 51 (90)‡	22.1 (11.0-31.5)† 73 (75)*	0.024 0.004
D-dimer μg/L, median (IQR)	<500	-	-	2,774 (1,167-10,000)	-
Fibrinogen g/L, median (IQR)	1.7-4.0	-	-	6.6 (5.3-8.0)	-
aPPT sec, median (IQR)	23-32	-	-	29 (27-33)	-
PT sec, median (IQR)	9.9-12.4	-	-	11.8 (11.0-12.0)	-
FVIIa mIU/mL, median (IQR) High FVIIa N (%)	-	33.1 (21.0-50.8) -	31.7 (20.0-47.6) -	39.8 (20.5-55.3) -	0.360 -
FVIIa:AT pM, median (IQR) High FVIIa:AT, N (%)	≤910	599 (485-719) 3 (7)	556 (476-741) 3 (4)	607 (458-784) 15 (14)	0.790 0.282
PKa:C1Inh nM, median (IQR) High Pka:C1Inh, N (%)	≤0.3	1.6 (0.7-4.1) 40 (89)	2.5 (0.9-4.8) 60 (90)	1.9 (1.0-3.5) 94 (90)	0.957
FXIa:AT pM, median (IQR) High FXIa:AT, N (%)	≤42	23.4 (17.3-35.9) 7 (16)	25.7 (19.8-38.0) 13 (19)	30.0 (22.3-65.2)*, † 40 (39)*, †	0.001 0.003
FXIa:α1AT pM, median (IQR) High FXIa:α1AT (%)	≤248	377 (308-597) 38 (84)	515 (396-751)‡ 64 (96)‡	545 (402-806)† 103 (99)†	0.003 0.002
FIXa:AT pM, median (IQR) High FIXa:AT N (%)	≤56	53.7 (33.0-71.8) 18 (39)	64.2 (43.4-91.0) 43 (64)‡	86.8 (62.0-125.6)*, † 84 (81)*, †	<0.001 <0.001
T:AT ng/mL, median (IQR) High T:AT N (%)	≤5	4.2 (3.0-5.7) 17 (38)	5.5 (3.7-10.1)‡ 36 (54)	8.7 (4.9-21.2)*, † 78 (75)*, †	<0.001 <0.001
vWF:Ag (%) median (IQR) High vWF:Ag, N (%)	≤160	323 (214-422) 40 (89)	361 (263-488) 65 (97)	438 (343-557)*, † 101 (97)	<0.001 0.084

Continuous variables were presented as mean (± standard deviation, SD) or median (interquartile range, IQR) as appropriate. Differences between groups were analyzed by unpaired sample *t* test, Mann Whitney U test, one-way ANOVA, or Kruskal Wallis. Differences in categorical variables were analyzed by chi square test or Fisher's exact test when appropriate; significant differences between patients groups: severe *vs.* *moderate or †mild disease; moderate *vs.* ‡mild disease. M: male; F: female; NLR: neutrophil-lymphocyte ratio; AST: aspartate transaminase; COPD: chronic obstructive pulmonary disease; CRP: C-reactive protein; CVA: cerebrovascular accident; SBP: systolic blood pressure; DBP: diastolic blood pressure; LDH: lactate dehydrogenase; APTT: activated partial thromboplastin time; PT: prothrombin time; FVIIa: activated FVII; AT: antithrombin; Pka: plasma kallikrein; C1INH: C1 esterase inhibitor; FXIa: activated factor XI; α1AT: α1-antitrypsin; FIXa: activated factor IX; T:AT: thrombin in complex with antithrombin; vWF:Ag: von Willebrand factor antigen.

showed the intricate link between the intrinsic pathway, neutrophils, and NET formation.[1] NET formation, with the release of RNA and DNA, activates the intrinsic pathway.[11-13] NET formation, indeed, has been localized to the site of (micro) thrombosis.[14] Moreover, NET colocalized with FXII and caused *in vitro* activation of FXII.[5]

In order to test the prognostic value of activated coagulation factors in complex with their natural inhibitions and vWF:Ag on ICU admission, thrombosis, and mortality, we performed logistic regression analyses (Table 2). Sixty-four (29%) of 220 patients with COVID-19 were admitted to the ICU. FXIa:α1AT, T:AT, and vWF:Ag were associated with an increased risk of ICU admission. This observation remained significant for FXIa:α1AT and T:AT in a multivariable model adjusting for age, CRP, and cardiovascular disease. Henderson *et al*. corroborated our observations and found that FXIa:AT was associated with progression of lung disease on computed tomography; no data on thrombosis were reported.[6] We found thrombosis in 29 (13%; 22 pulmonary embolisms, 3 cerebrovascular accidents, 3 peripheral arterial occlusions, and 1 acute coronary syndrome) of 220 patients with COVID-19, with the highest incidence in patients with severe disease (n=23). Logistic regression linked FXIa:AT and T:AT to thrombosis; T:AT remained significant after adjusting for sex. Fifty-eight (33%) of 174 admitted patients with COVID-19 died in the hospital within 28 days; two patients with moderate disease died because of traumatic brain injury rather than COVID-19 and were excluded from the analysis. Univariable but not multivariable logistic regression linked T:AT and vWF:Ag to in-hospital mortality at 28 days, corroborating previous observations.[3,15] Most of these studies, however, were limited because of a cross-sectional design and small sample size. Neither FVIIa:AT nor free FVIIa were associated with clinical outcomes.

Next, we assessed the prognostic value of activated coagulation factors in complex with their natural inhibitors and vWF:Ag over time in admitted patients using linear mixed models (Figure 1). The dynamics of vWF:Ag were associated with clinical outcomes, whereas none of the activated coagulation markers (i.e., FVIIa:AT, free FVIIa, PKa:C1Inh, FXIa:AT, FXIa:α1AT, FIXa:AT and T:AT) was (*data not shown*); at presentation, however, FXIa:AT (+85.2; 95% confidence interval [CI]: 13.5-156.8; *P*=0.020) and T:AT (+9.6 (95% CI: 1.4-18; *P*=0.023) were higher in patients with thrombotic events. vWF:Ag increased over time, particularly in patients admitted to the ICU and in patients who died. The increase in vWF:Ag was steeper in patients who died as compared to those who survived. Of note, elevated levels of PKa:C1Inh, FXIa:α1AT, FIXa:AT and T:AT remained stable during hospital admission (*data not shown*). Taken together, ongoing vascular damage, as reflected by vWF:Ag, is associated with poor outcomes, whereas activation of the intrinsic pathway relates to COVID-19's hy-

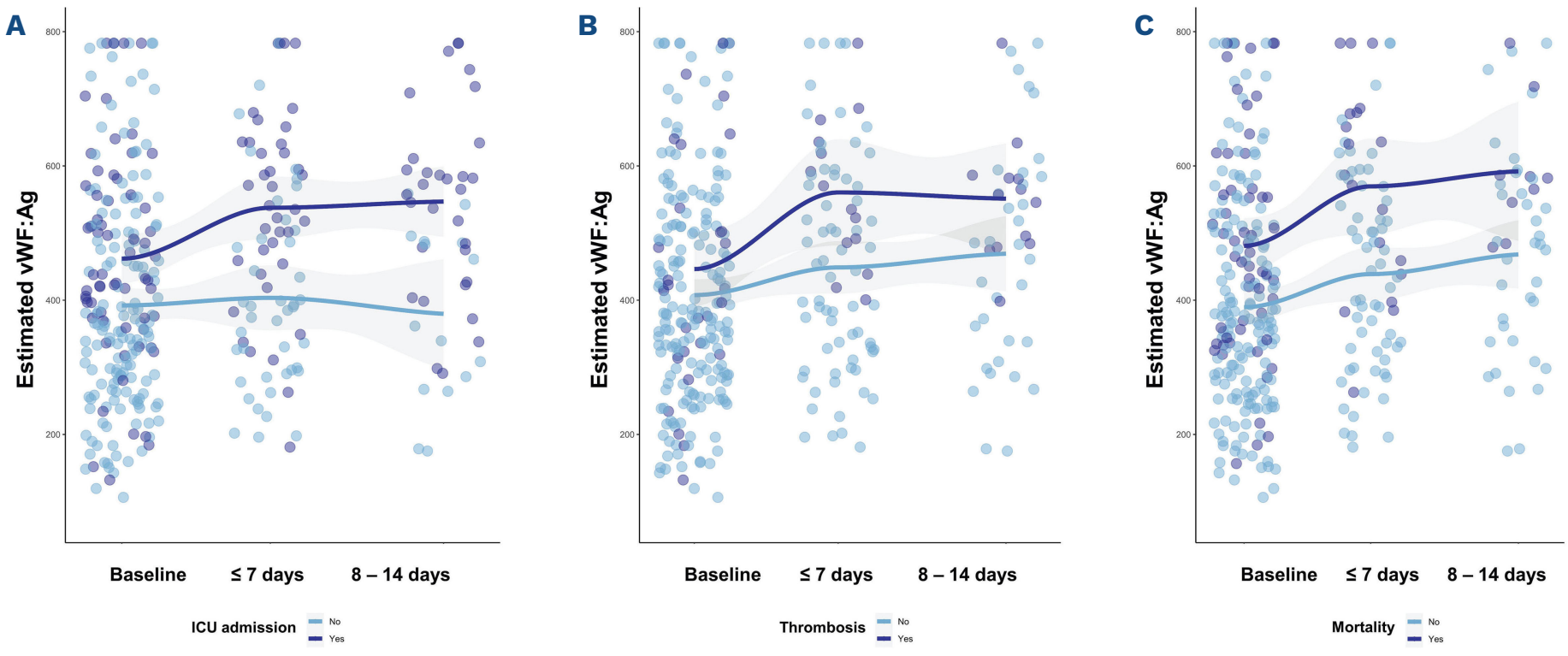

Figure 1. Predicted estimates of von Willebrand factor antigen stratified by different clinical outcomes at baseline and over time in patients with COVID-19. Linear mixed-effects models were used to illustrate the effects of von Willebrand factor antigen (vWF:Ag) on intensive-care unit (ICU) admission, thrombosis, and 28 day in-hospital mortality. (A) Estimated vWF:Ag was for ICU admitted patients at baseline +9.0 (95% confidence interval [CI]: -55 to 73; *P*=0.782) higher than non-ICU admitted patients. Over time, vWF:Ag decreased (-3.0; 95% CI: -27 to 22; *P*=0.826) overall, but increased significantly in ICU admitted patients (+61; 95% CI: 28-94; *P*<0.001). (B) Baseline vWF:Ag was comparable between patients with (+7.0; 95% CI: -80 to 93; *P*=0.879) and without thrombosis. Over time, vWF:Ag increased significantly in both groups (+28; 95% CI: 9.0-48; *P*=0.005) without a statistical significant difference for patients with thrombosis (+34; 95% CI: -7.0 to 74; *P*=0.103). (C) vWF:Ag tend to be higher in non-survivors (+45; 95% CI: -28 to 117; *P*=0.225) and increased over time (+28; 95% CI: 8-47; *P*=0.006) in both groups with a statistically significant sharper increase in non-survivor (+49; 95% CI: 7-90; *P*=0.023).

Table 2. Logistic regression was performed to ascertain the effects of the coagulation factors (per ten units) alone (univariable) and together with other predictors (multivariable) on the likelihood of intensive-care unit admission, thrombosis and 28 day mortality.

Univariable	OR (95% CI)	*P*	AUC (95% CI)
ICU admission			
FVIIa	0.961 (0.858-1.077)	0.497	0.507 (0.421-0.592)
FVIIa	1.005 (0.999-1.010)	0.097	0.507 (0.404-0.611)
Pka:C1Inh	1.022 (0.990-1.054)	0.178	0.515 (0.431-0.599)
FXIa:AT	1.023 (0.999-1.047)	0.064	0.630 (0.548-0.712)
FXIa:α1AT	1.001 (1.000-1.001)	0.028	0.646 (0.570-0.723)
FIXa:AT	1.007 (0.997-1.018)	0.163	0.682 (0.606-0.759)
T:AT	1.583 (1.253-1.999)	<0.001	0.680 (0.603-0.757)
vWF:Ag	1.026 (1.008-1.045)	0.005	0.644 (0.566-0.722)
Thrombotic events			
FVIIa	0.929 (0.790-1.093)	0.373	0.521 (0.424-0.618)
FVIIa	1.001 (0.999-1.004)	0.192	0.449 (0.318-0.580)
Pka:C1Inh	1.005 (0.975-1.037)	0.741	0.545 (0.435-0.655)
FXIa:AT	1.024 (1.000-1.048)	0.048	0.632 (0.523-0.741)
FXIa:α1AT	1.001 (0.997-1.005)	0.538	0.605 (0.492-0.718)
FIXa:AT	1.008 (0.997-1.019)	0.141	0.696 (0.591-0.800)
T:AT	1.402 (1.078-1.824)	0.012	0.685 (0.589-0.780)
vWF:Ag	1.014 (0.991-1.038)	0.239	0.561 (0.447-0.674)
28 day mortality			
FVIIa	1.112 (0.995-1.243)	0.061	0.593 (0.497-0.689)
FVIIa	1.001 (0.999-1.003)	0.305	0.474 (0.364-0.584)
Pka:C1Inh	0.899 (0.721-1.121)	0.344	0.559 (0.473-0.645)
FXIa:AT	1.003 (0.978-1.027)	0.837	0.588 (0.501-0.674)
FXIa:α1AT	0.998 (0.994-1.003)	0.503	0.512 (0.425-0.598)
FIXa:AT	1.003 (0.993-1.014)	0.520	0.664 (0.582-0.747)
T:AT	1.403 (1.116-1.769)	0.004	0.638 (0.553-0.722)
vWF:Ag	1.034 (1.015-1.054)	0.001	0.660 (0.579-0.740)
Multivariable	**OR (95% CI)**	***P* value**	**AUC (95% CI)**
ICU admission*			
FVIIa	0.967 (0.849-1.102)	0.614	0.734 (0.659-0.808)
FVIIa	1.007 (1.000-1.015)	0.059	0.773 (0.692-0.854)
Pka:C1Inh	1.018 (0.983-1.055)	0.306	0.744 (0.673-0.815)
FXIa:AT	1.027 (0.996-1.059)	0.085	0.734 (0.660-0.808)
FXIa:α1AT	1.005 (1.000-1.010)	0.047	0.747 (0.674-0.820)
FIXa:AT	1.008 (0,995-1.020)	0.240	0.724 (0.647-0.801)
T:AT	1.449 (1.092-1.922)	0.010	0.751 (0.677-0.825)
vWF:Ag	1.020 (0.999-1.043)	0.068	0.738 (0.662-0.814)
Thrombotic events†			
FVIIa	0.969 (0.823-1.140)	0.702	0.594 (0.500-0.687)
FVIIa	1.001 (0.999-1.004)	0.226	0.561 (0.444-0.679)
Pka:C1Inh	1.002 (0.971-1.034)	0.913	0.640 (0.542-0.739)
FXIa:AT	1.019 (0.995-1.044)	0.113	0.699 (0.592-0.806)
FXIa:α1AT	1.001 (0.997-1.005)	0.772	0.672 (0.569-0.775)
FIXa:AT	1.006 (0.995-1.017)	0.257	0.740 (0.639-0.841)
T:AT	1.336 (1.025-1.740)	0.032	0.725 (0.630-0.821)
vWF:Ag	1.010 (0.987-1.034)	0.399	0.635 (0.535-0.735)
28 day mortality‡			
FVIIa	1.101 (0.966-1.256)	0.149	0.803 (0.733-0.872)
FVIIa	1.000 (0.998-1.002)	0.910	0.817 (0.729-0.905)
Pka:C1Inh	0.904 (0.706-1.157)	0.421	0.778 (0.707-0.850)
FXIa:AT	0.992 (0.959-1.027)	0.652	0.774 (0.702-0.847)
FXIa:α1AT	0.997 (0.991-1.004)	0.379	0.777 (0.705-0.849)
FIXa:AT	0.997 (0.982-1.013)	0.725	0.774 (0.701-0.847)
T:AT	1.316 (0.990-1.750)	0.059	0.789 (0.718-0.859)
vWF:Ag	1.007 (0.984-1.031)	0.555	0.773 (0.701-0.845)

*Combined with male sex (OR 2.112; 95% CI: 1.090-4.090; *P*=0.027), a medical history of hypertension (OR 2.170; 95% CI: 1.121-4.201; *P*=0.022), cardiac disease (OR 2.139; 95% CI: 1.071-4.273; *P*=0.031), and C-reactive protein in mg/L (OR 1.008; 95% CI: 1.004-1.0011; *P*<0.001). †Combined with male sex (OR 3.616; 95% CI: 1.209-10.816; *P*=0.022). ‡Combined with age in years (OR 1.073; 95% CI: 1.040-1.107; *P*<0.001), a medical history of diabetes (OR 3.510; 95% CI: 1.729-7.124; *P*=0.001), and C-reactive protein in mg/L (OR 1.005; 95% CI: 1.001-1.009; *P*=0.010). OR: odds ratio; CI: confidence interval; AUR: area under the curve; FVIIa: activated FVII; AT: antithrombin: Pka: plasma kallikrein; C1INH: C1 esterase inhibitor; FXIa: activated factor XI; α1AT: α1-antitrypsin; FIXa: activated factor IX; T:AT: thrombin in complex with antithrombin; vWF:Ag: von Willebrand factor antigen; ICU: intensive-care unit.

percoagulability and thrombosis. Previous data suggest ongoing activation of the intrinsic pathway, thrombin formation, and vascular damage for up to 3 months after onset of COVID-19.[7] Future studies are needed to study the role of coagulation and vascular damage in long COVID-19.
Our study has several limitations. First, this cohort was collected at the beginning of the pandemic when thrombotic events were not routinely screened for and thrombosis could have been missed. Second, the limited number of follow-up samples may affect interpretation of data. Our data, however, benefit from the prospective design and inclusion of a large and well-defined cohort of patients with COVID-19. Moreover, our data reflect the natural course of disease because most patients were not treated with glucocorticosteroids and/or immunosuppressive agents.
In conclusion, we showed that thrombin formation, particularly via the intrinsic pathway, is critical for COVID-19's hypercoagulability to occur. Thrombin formation and vascular damage are important markers of disease severity, thrombosis, and mortality. The intrinsic pathway may therefore be a potential target for the treatment of this devastating disease. Future studies should address whether our findings can be extrapolated to other (viral) respiratory conditions or not.

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HC and HMHS received funding for research from Bayer and Pfizer; they are stakeholder in Coagulation Profile. HC is a consultant for Alveron and has served at advisory boards for Bayer, Pfizer, Daiichi, Leo and Gilead. CPR is co-inventor of a patent describing use of low anticoagulant heparins in sepsis and owned by Maastricht University. CPR is a scientific consultant for Matisse Pharmaceuticals and Annexin Pharmaceuticals. All other authors have no conflicts of interest to disclose.

Contributions

MHB and SAMEGT collected and managed data and enrolled patients; MHB, SMJK and MN designed and performed statistical analyses, and interpreted data; DPCD performed statistical analyses; MHB wrote the first draft of the manuscript; JP, MP, IH and PP enrolled patients; JPA, RY, and JGMCD collected and stored samples and managed data; MN, HMHS and HC performed experiments and interpreted data; MN, HMHS, HC, CPR and PP supervised experiments, interpreted data, and wrote portions of the manuscript. All authors critically reviewed the manuscript and approved the final version.

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Data-sharing statement

The original data of this study can be obtained upon reasonable request from the corresponding author.

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