Hemostatic alterations in COVID-19

Coronavirus disease 2019 (COVID-19) is caused by the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2),[1,2] that, first identified in China, has spread globally. A coagulopathy is common, particularly in patients admitted to intensive care units (ICU).[3] Although controversial,[4-6] high rates of venous thromboembolism (VTE) have also been reported.[7] The International Society on Thrombosis and Haemostasis (ISTH) released a statement suggesting prophylactic low molecular weight heparin (LMWH).[8] However, the optimal strategy for prophylaxis remains controversial,[9] owing to limited knowledge on how COVID-19 affects hemostasis.

At the beginning of the pandemic, COVID-19 patients were reported to present with abnormalities mimicking the coagulopathies like disseminated intravascular coagulation (DIC) or sepsis induced coagulopathy (SIC).[10] However, a more recent study in a small group of patients severe enough to be admitted to the ICU failed to confirm DIC, because patients presented with a marked increase of D-dimer but without hypofibrinogenemia or thrombocytopenia, *i.e.*, the hallmarks of DIC with consumption coagulopathy.[3] Considering these controversial findings, we report the results obtained using an array of hemostasis measurements in COVID-19 patients, admitted first to the emergency room and then to different wards characterized by delivery of different

Table 1. Median (min-max) values of the hemostasis measurements in COVID-19 patients.

	Intensity of care			
	Low	Intermediate	High	*P*
Tests for DIC diagnosis				
PT ratio	1.02	1.12	1.06	0.0037
	(0.85-1.33)	(0.95-1.44)	(0.96.133)	
APTT ratio	0.93	0.91	0.95	0.66
	(0.79-1.22)	(0.78-1,10)	(0.78-1.15)	
Platelet count, nx10^9/L	275	362	366	0.1
	(138-480)	(120-556)	(80-584)	
Fibrinogen, mg/dL	344	471	531	0.061
	(150-861)	(285-830)	(224-1035)	
D Dimer, ng/mL	870	1347	2,217	0.009
	(203-38,847)	(525-6,910)	(564-6,410)	
Pro- and anticoagulant factors				
Factor II, U/dL	116	94	104	0.24
	(65-140)	(76-128)	(75-143)	
Factor VIII, U/dL	208	223	302	0.014
	(121-347)	(109-423)	(178-374)	
Antithrombin, U/dL	87	94	100	0.43
	(61-133)	(63-135)	(71-143)	
Protein C, U/dL	120	126	143	0.057
	(60-234)	(72-210)	(85-232)	
Protein S free antigen, U/dL	75	72	84	0.13
	(38-98)	(26-95)	(56-110)	
Endothelial-derived factors				
VWF:Ag, U/dL	262	371	466	0.00007
	(90-577)	(132-769)	(231-746)	
VWF:RCo, U/dL	210	303	383	0.00015
	(88-447)	(129-539)	(195-528)	
VWF:RCo /Ag ratio	0.85	0.86	0.81	0.34
	(0.65-1.02)	(0.62-0.98)	(0.69-1.01)	
FVIII/VWFAg ratio	0.81	0.61	0.65	0.06
	(0.40-2.05)	(0.32-1.00)	(0.40-0.97)	
Inflammation markers				
Ferritin, µg/L	380	705	788	0.017
	(32-1,587)	(124-4,081)	(212-5,064)	
C-reactive protein, mg/dL	1.00	3.32	5.05	0.0057
	(0.07-11.71)	(0.19-18.3)	(0.6-25.5)	

COVID-19: Coronavirus disease 2019; DIC: disseminated intravascular coagulation; PT: prothrombin time; APTT: partial thromboplastin time; VWF:AG: von Willebrand factor antigen; VWF:Rco: ristocetin cofactor activity; FII: factor II; FVIII: factor VIII.

levels of intensity care depending on disease severity.

After the viral diagnosis, 62 patients, depending on their severity, were consecutively admitted to three wards, characterized by low-intensity care (n=21), when hypoxia could be handled by ventilation support with high-flow nasal cannulas; intermediate sub-intensive care (n=21), when hypoxia prompted the use of continuous positive airway pressure, or high-intensity care (n=20) when hypoxia warranted intubation and mechanical ventilation in ICU. In this context, we designed the project COHERENT (COVID-19: HEmostasis, immune Response, ENdothelial perTurbation and complement), aimed to investigate the mechanism of thrombosis in COVID-19 patients. The project received approval by Comitato Etico Area2, Milano (clinicaltrials gov. Identifier: 360_2020). Patients started prophylaxis with low-dose LMWH on admission and dosages were then adjusted by attending physicians after patient transfer to the hospital wards. LMWH dosages were as follows: low-intensity, enoxaparin 70 UI/Kg once a day; intermediate-intensity, 70 UI/kg twice a day; high-intensity, 100 UI/kg once a day.

Venous blood was collected, not earlier than 72 hours after the administration of LMWH prophylaxis and before the administration of the daily dose in vacuum-tubes containing 1/10 volumes of trisodium citrate 0.109 M. Specimens were centrifuged for 20 minutes at 3,000g.

Prothrombin and activated partial thromboplastin time (PT, APTT) were performed using Recombiplastin-2G and Synthasil APTT (Werfen, Orangeburg, NY, USA) with results expressed as clotting time ratios (patient-to-normal). Factor VIII (FVIII) and FII were measured by the one-stage assay based on APTT and FVIII-deficient plasma and PT-based assay and FII-deficient plasma, respectively (Werfen). von Willebrand factor antigen (VWF:Ag) and ristocetin cofactor activity (VWF:RCo) were measured by commercial kits (Werfen). Fibrinogen was measured according to Clauss. D-dimer and free protein S (PS) antigen were measured by latex-based assays (Werfen). Antithrombin and protein C (PC) activity were measured by chromogenic assays (Werfen). Platelet counts and markers of inflammation and acute-phase reactions (C-reactive-protein and ferritin) were obtained from the patients' records.

The DIC score was calculated using ISTH criteria.[11] In patients with sepsis, SIC score is more sensitive than the DIC score to detect an associated coagulopathy, thus we also calculated this score that is based on platelet count, PT-international normalized ratio (PT-INR) and the Sequential Organ Failure Assessment (SOFA) score that includes data on respiratory, cardiovascular, hepatic and renal dysfunction, but also on the presence of hemostasis alterations such as thrombocytopenia and PT-INR.

Patients characteristics did not differ in the three groups. No differences for well-known risk factors and comorbidities (age, body mass index, hypertension, diabetes) between the groups according to the intensity of care were observed. In the entire cohort we recorded three deaths and 25 thrombotic events (40%) in 25 patients, *i.e.*, 16 deep-vein thrombosis, eight pulmonary embolism and one visceral venous thrombosis.

Median (min-max) values of the hemostasis measurements in COVID-19 patients are listed in Table 1. The PT-ratio was slightly increased in patients at high- and intermediate- care intensity compared with those at low-intensity care. The APTT-ratio was slightly decreased in all patients irrespective of care intensity. Median platelet counts for patients at intermediate or high-care intensity were higher than those at low-intensity; the lowest observed platelet count (80x10^9/L) being higher the

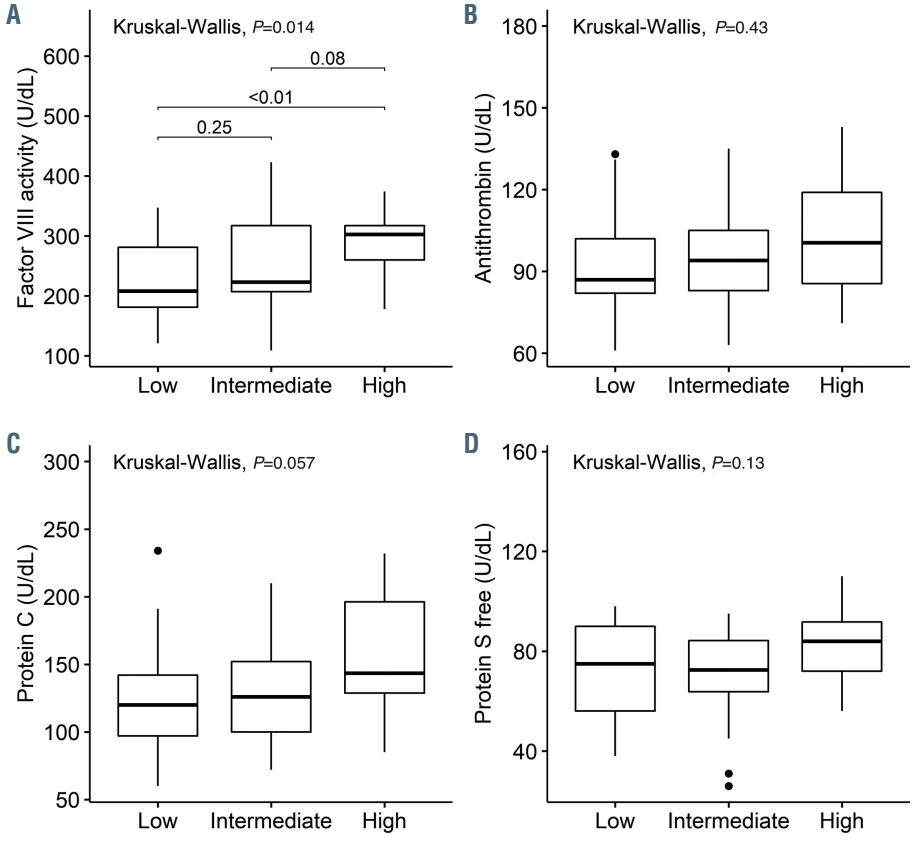

Figure 1. Box plots of results for (A) factor VIII, (B) antithrombin, (C) protein C and (D) protein S for patients at low, intermediate and high intensity of care.

50x10^9/L threshold value for DIC. Fibrinogen for patients admitted to the three care-intensity wards were higher than the upper limit of the normal range, with a gradient of increase across the care intensities and with values in patients at high-intensity care as high as 1,035 mg/dL. The lowest fibrinogen level (150 mg/dL) measure was higher than the 100 mg/dL DIC score threshold value incorporated to assign points. A similar trend of positive association with the level of care intensity was observed for D-dimer; as median values ranged from 870 ng/mL (low-intensity) to 1,347 ng/mL or to 2,217 ng/mL (intermediate- or high-intensity care) (Table 1). The median (min-max) DIC score for the whole patient cohort was 2 (range, 0-4), with only one patient scoring 4. SIC scores were similar in the three groups, all being below the cut-off of 4. Median FVIII, already high (208 U/dL) in low-intensity patients, was increased steadily in intermediate (223 U/dL) and high-intensity (302 U/dL) patients. Median antithrombin varied from 87 U/dL (low-intensity) to 100 U/dL (high-intensity). PC was increased in low-intensity patients (120 U/dL) and was further increased in intermediate (126 U/dL) or high-intensity (143 U/dL) care patients. PS free antigen was lower than 100 U/dL, with small variations according to the intensity of care (Table 1; Figure 1). Median VWF:Ag was high in patients at low-intensity (262 U/dL) and was further increased in intermediate (371 U/dL) and high-intensity (466 U/dL) care patients. VWF:RCo values paralleled those of VWF:Ag, albeit at a lower level, and the VWF:RCo/VWF:Ag ratio ranged between 0.85 (low), 0.86 (intermediate) and 0.81 (high) care intensity (Table 1; Figure 2). The median FVIII/VWF:Ag ratio ranged between 0.81 (low), 0.61 (intermediate) and 0.65 (high) care intensity. Median ferritin was extremely high, *i.e.*, 380 µg/L (low), 705 ng/mL (intermediate) and 788 ng/mL (high) care intensity. C-reactive protein was 1.00 mg/dL (low), 3.32 mg/dL (intermediate) and 5.05 mg/dL (high-intensity) care patients (Table 1).

Several studies reported that COVID-19 patients have an acquired coagulopathy with an increased risk of VTE in critically ill patients.[4-7] However, the frequency varies greatly and there is still an unsettled strategy for prophylaxis.[12] Therefore, besides the need of well-designed randomized clinical trials, we deemed crucial to better mechanistically understand thrombosis, with the ultimate goal to implement more targeted approaches to management. We, therefore, investigated coagulation in infected patients hospitalized on the basis of their clinical severity in three different intensity-care wards by employing an array of measurements centralized in the same laboratory, with special emphasis on those used to diagnose DIC and SIC, the pro- and anticoagulant factors and those indicating endothelial perturbation. Our results did not confirm DIC, as high DD was the only compatible result, while other parameters indicating consumption coagulopathy, as low fibrinogen and platelet counts, were normal or often increased. Furthermore, none of the patients had a DIC score of 5 or more (the threshold indicating a

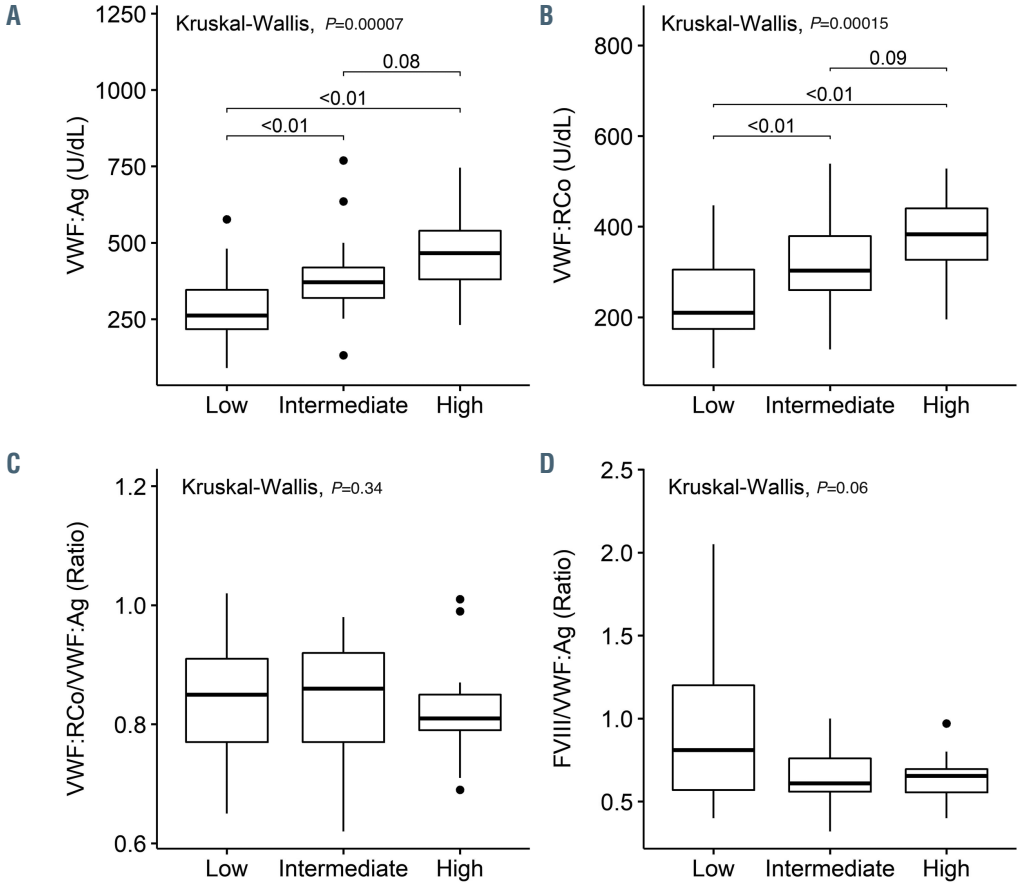

Figure 2. Box plots of results for (A) von Willebrand factor (VWF) antigen (VWF:Ag), (B) VWF ristocetin-cofactor (VWF:RCo), VWF:RCo/Ag ratio and factor VIII (FVIII)/VWF:Ag ratio for low-, intermediate- and high-intensity care patients.

high likelihood of DIC according to the ISTH criteria).[11] The vast majority of patients had a score of 2 or less and only one had a score of 4, driven by remarkably high levels of D-dimer (38,847 ng/mL). Similarly, SIC scores were similar in the three groups and were all below the cut-off value of 4 and these patients, thus, differed from those with sepsis. FVIII, one of the most potent procoagulants, was strikingly increased with a gradient from low- to high-intensity care, suggesting a state of hypercoagulability roughly proportional to disease severity. VWF:Ag was even higher than FVIII, causing a proportional reduction of the FVIII/VWF:Ag ratio to the degree of disease severity and, thus, suggesting that endothelial cell perturbation concurs with hypercoagulability to explain mechanistically the clinical manifestations of VTE associated with COVID-19. These views are supported by the findings of Goshua *et al*.[13] who recently showed that VWF and D-dimer were significantly higher in ICU *versus* non-ICU patients.

Overall, the above findings are consistent with a complex crosstalk between inflammation, hemostasis and endothelial cells that, once activated during inflammation, acquire a prothrombotic phenotype which in turn contributes to the procoagulant imbalance. These findings are mechanistically plausible with the increased VTE risk in COVID-19 patients, with a possible added contribution from fibrinolysis derangement not explored in this study.

The clinical picture of hospitalized COVID-19 patients in Milan differed not only from DIC[3] but also from other disorders characterized by hypercoagulability and endothelial perturbation, triggered by systemic inflammation, such as the hemophagocytic lymphohistiocytosis/macrophage activation syndrome[14] and bacterial sepsis.[15] The reasons for such differences may be caused by the evaluation of patients at different disease stages and/or the early start of LMWH prophylaxis, even though striking hypercoagulability was present notwithstanding the implementation of prophylaxis.

In conclusion, this study in COVID-19 patients characterizes an acquired coagulopathy associated with hyperacute inflammation, hypercoagulability and endothelial perturbation broadly proportional to the clinical severity of the infection and to the levels of intensity of care needed by the patients.

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