UNIVERSITÀ DEGLI STUDI DI MILANO

PHD COURSE IN TRANSLATIONAL MEDICINE

PhD Thesis

Involvement of the contact pathway in COVID-19 coagulopathy

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1. Summary

Background. A novel acquired coagulopathy characterized by a severe procoagulant imbalance is common in COVID-19 patients and is associated with the clinical severity of the disease.

Aims. Our study aims at elucidating the underlying mechanisms of coagulation activation in COVID-19 patients.

Methods. The study is composed of two phases. In the first and second part, 62 and 112 symptomatic COVID-19 patients, respectively, were consecutively enrolled and stratified into 3 groups based on the intensity of care: low, requiring only high-flow oxygen by nasal cannula (21 and 23 patients); intermediate, requiring continuous positive airway pressure (20 and 47 patients); high, requiring mechanical ventilation (20 and 42 patients). During the first phase of the study blood samples were tested for markers currently used to diagnose disseminated intravascular coagulation (DIC) (PT, aPTT, fibrinogen, D-dimer, platelet count), the pro- (FII, FVIII) and anticoagulant factors (antithrombin, protein C, protein S) and those indicating endothelial perturbation (von Willebrand factor). During the second phase of the study blood samples were tested for markers of activation of the intrinsic pathway (FXI, FXII, FXIa, FXIIa) together with its physiologic inhibitor (C1-inhibitor), of the extrinsic pathway (FVII, FVIIa), global activation of the coagulation cascade (D-dimer, FDP, FM) and fibrinolysis (plasminogen, t-PA, α2-antiplasmin, PAI-1).

Results. Results of the first phase of the study demonstrated that COVID-19 coagulopathy represents a new entity, not fulfilling the criteria for DIC, characterized by endothelial activation, while the second part of the study showed a prevalent activation of the contact pathway over the extrinsic pathway of the coagulation cascade.

Conclusions. Our study showed an acquired coagulopathy associated with hyperacute inflammation, hypercoagulability, and endothelial perturbation proportional to the clinical severity

of the infection, characterized by a prevalent activation of the intrinsic pathway of the coagulation cascade, opening the possibility for targeted therapies.

2. Background

2.1. SARS-CoV-2 and COVID-19

Coronavirus disease 2019 (COVID-19) is caused by the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), ([1]; [2]) that has spread globally since the first identification in Wuhan, China, and led the World Health Organization (WHO) to declare the disease a pandemic on March 11, 2020.

SARS-CoV-2 is a single-stranded positive-sense RNA virus belonging to the genus Betacoronavirus, with surface club-shaped spikes resembling the solar corona on electron microscopy. SARS-CoV-2 has a 96% homology with a bat coronavirus, rising the hypothesis of its origin from a cross-species spill over event ([3]). SARS-CoV-2 enters the host cells by the interaction of the S1 subunit of the viral surface spike protein (S protein) and the angiotensin-converting enzyme 2 (ACE2), which is a transmembrane receptor. After the first interaction between S1 and ACE2, the serine protease Transmembrane Protease Serine 2 (TMPRSS2) cleaves the S2 binding site, facilitating its fusion with the host cell membrane and the subsequent entrance in the cell ([4]). Other than ACE2, also other molecules, such as TMPRSS2, sialic acid and extracellular matrix metalloproteinase inducer (CD147, basigin) facilitates the entrance of SARS-CoV-2 in the host cell. ACE2 is highly expressed in the lung alveolar cells and in the enterocytes of the small intestine, explaining the route of entry of the virus, but also in many other organs, such as the cardiovascular system, central nervous system, kidneys, testis, thyroid, adipose tissue and endothelial cells ([5]), indicating that SARS-CoV-2 may infect various organs other than lungs, explaining the wide variety of COVID-19 symptoms (**Figure 1**).

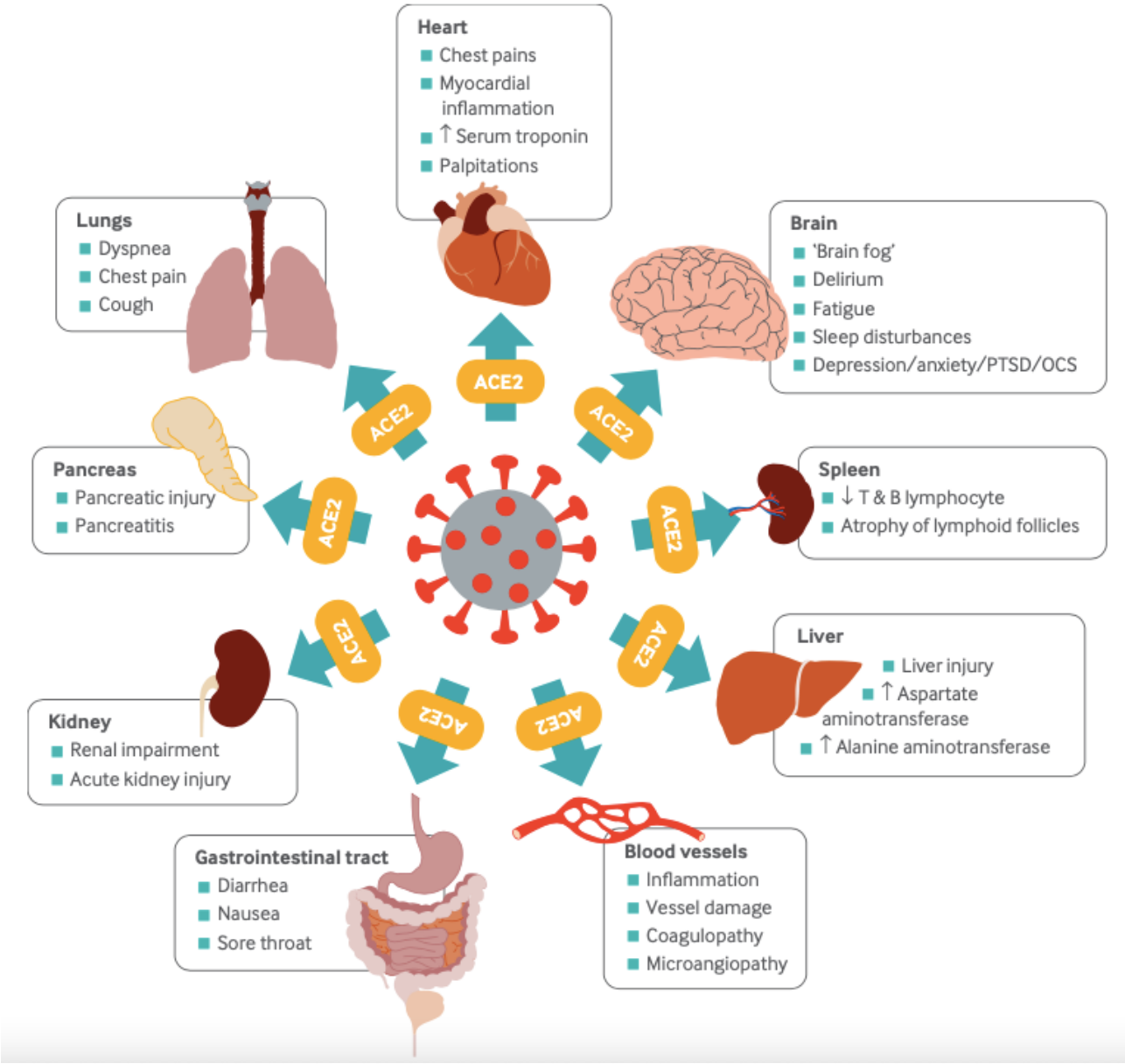

Figure 1. Distribution of ACE2 expression in different tissues and organs. Adapted from Crook et al. ([9]).

The clinical presentation is extremely heterogeneous, ranging from asymptomatic to critically ill patients with the occurrence in around 5% of patients of an acute respiratory distress syndrome (ARDS), that can progress to respiratory failure, multiorgan failure and death ([6]) (**Figure 2**). The fatality rate is 2.3% and up to 49% in critically ill patients with the most common risk factor for death (in around 10% of fatal cases) represented by a pre-existing cardiovascular disease ([7]; [8]).

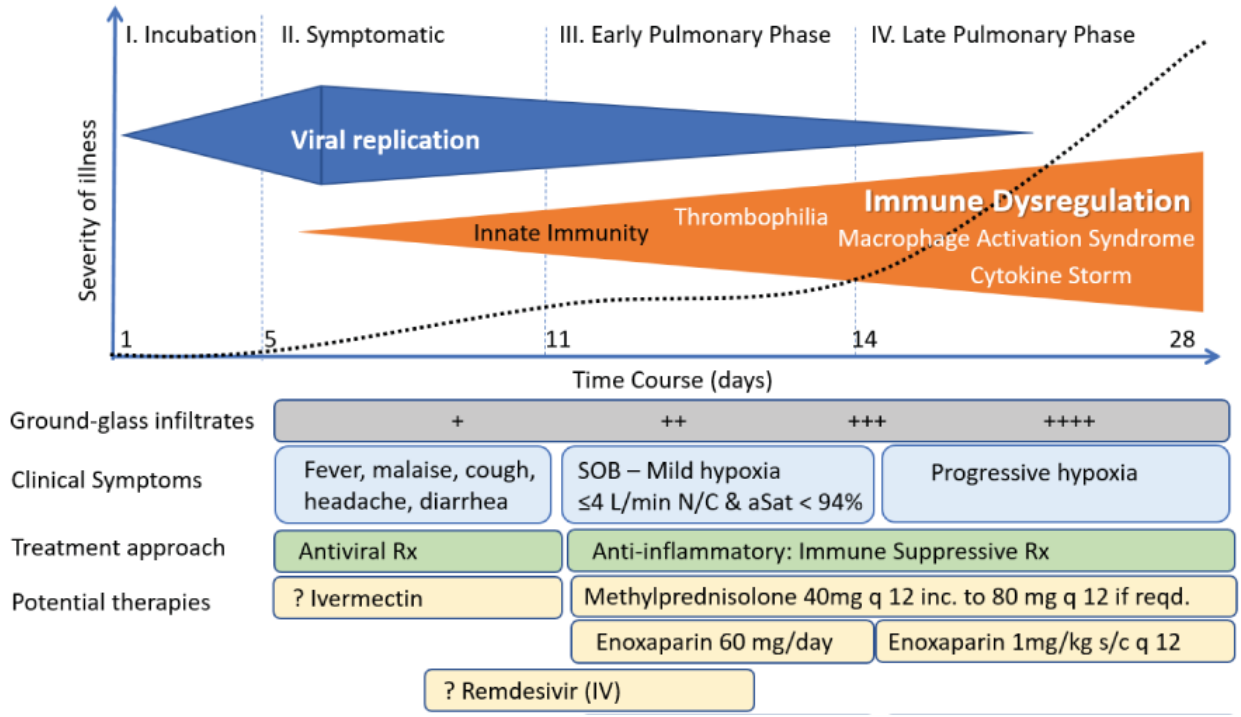

Figure 2. Typical course and stages of COVID-19 disease. Adapted from Marik et al. ([26]).

2.2. COVID-19-associated coagulopathy

High rates of venous thrombotic complications ranging from 26% to 75% were reported in hospitalized patients the first months of the pandemic and a high frequency of pulmonary embolism (PE) was found on the first autopsy studies ([10]). These rates were much higher than observed in a cohort of patients affected by influenza in 2019 (PE incidence rate of 7.5%) ([11]). Thrombotic complications have been reported to be the second cause of mortality after respiratory failure in COVID-19 patients, hereby the International Society on Thrombosis and Haemostasis (ISTH) released a statement suggesting the use of prophylactic low molecular weight heparin (LMWH) in all hospitalized COVID-19 patients ([12]). However, even despite adequate antithrombotic prophylaxis, around one third of intensive care unit (ICU) patients develops a thrombotic complication ([13]). In the subsequent months it became more and more evident that COVID-19 is associated with venous thrombotic complications as well as arterial thrombosis (such as stroke and

myocardial infarction) and microvascular thrombotic complications ([14]; [15]). The incidence of these thrombotic complications is increased in patients presenting with a more severe disease and is higher in COVID-19 patients than in non-COVID-19 patients admitted in ICU or affected by other respiratory viruses such as influenza virus and Middle East Respiratory Syndrome coronavirus (MERS) ([16]). Pulmonary embolism accounted for up to 80% of the thrombotic complications in a series of ICU patients, being disproportionately high compared to the number of deep venous thrombosis, therefore suggesting primary in-situ thrombosis as the underlying mechanism, rather than embolism ([13]). All current guidelines recommend antithrombotic prophylaxis in hospitalized COVID-19 patients in the absence of contraindication. However, there is no consensus on duration and dose of anticoagulation nor indication on the continuation of prophylaxis after hospital discharge. The American College of Chest Physician (ACCP) guidelines suggest standard dose prophylaxis only in hospitalized patients ([17]), while the ISTH guidelines suggest considering intermediate dose for severe cases ([18]). Both the ISTH and the American College of Cardiology (ACC) guidelines suggest continuing the antithrombotic prophylaxis for 2 weeks after hospital discharge ([18]; [19]). The use of therapeutic doses has been suggested, but the usefulness of this regimen has been demonstrated only for moderate and not for severe COVID-19 patients ([20]). This statement is in line with the results of a study conducted by our group during the first wave of the pandemic, which showed a 60% reduction of mortality and clinical deterioration and a 50% reduction of VTE in the cohort of patients treated with high heparin dosages compared to standard dose prophylaxis, with an increased rate of major bleeding as high as 3% in the most critically ill patients ([21]). A pattern of coagulopathy was commonly observed in patients hospitalized with COVID-19, particularly in those with a more severe disease ([22]). 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) ([23]). However, a subsequent

study in a small group of severe patients admitted to the ICU failed to confirm DIC, because patients presented with a marked increase of D-dimer but without hypofibrinogenemia or thrombocytopenia, the hallmarks of DIC with consumption coagulopathy ([22]). This discrepancy could have influenced the therapeutic choices and related outcomes, so that the first part of our study was aimed to clarify the hemostasis alterations in COVID-19 patients.

COVID-19-associated coagulopathy have some characteristics of other coagulopathies (SIC/DIC) but has unique features that need to be defined for understanding the underlying pathophysiology. The role of D-dimer levels as a prognostic marker has been extensively investigated. Many studies have demonstrated the association between elevated D-dimer levels and an increased risk of mortality ([2]). Moreover, D-dimer at admission has been shown to be a useful and dose-dependent predictor of venous thromboembolism (VTE) occurrence in COVID-19 patients during admission or within 14 days after hospital discharge. D-dimer levels between 1000 and 2000 ng/mL were associated with a 2.3-fold increased risk of developing VTE compared to the reference group of patients with a D-dimer less than 1000 ng/mL. These figures increased up to 2.9-fold and to 10.7-fold for D-dimer levels between 2000-5000 ng/mL and greater than 5000 ng/mL ([24]).

2.3. Contact pathway as a link between inflammation and coagulation

COVID-19 is characterized by a systemic inflammation with elevated levels of inflammatory markers such as CRP and ferritin, and proinflammatory cytokines such as IL-1beta, IL-2R, IL-6, IL-8, IL-10 and TNF-alpha ([27]). In particular, IL-6 is directly induced by SARS-CoV-2 and IL-6 levels correlate with disease severity ([28]). Both pathogen-associated molecular patterns (PAMPs) and host derived damage-associated molecular patterns (DAMPs) are responsible for the activation of innate immune cells, mainly monocytes and macrophages, that leads to the release of proinflammatory cytokines, but also endothelial cells, all contributing to the inflammatory response ([29]). Another mechanism by

which SARS-CoV-2 induces an inflammatory response is through its target entry receptors in the host cell. Indeed, the virus enters the host cells using ACE2, thus reducing its expression on the cell surface. ACE2 has local ant-inflammatory functions, by inactivating the kallikrein-bradykinin pathway ([30]). Therefore, loss of ACE2 may lead to hyperactivation of this pathway and consequent inflammation. This inflammatory pattern associated with COVID-19 has been described as systemic inflammatory response syndrome (SIRS), like in patients with sepsis due to other causes ([31]). At the same time, a reduction in lymphocyte count, mainly CD4+ and CD8+ T cells, and a consequent reduction in CD4+ T cells-produced IFN-γ levels, have been described in COVID-19 patients, accounting for the reduced cellular immune response ([32]).

A higher white blood cell count and a higher neutrophil-to-lymphocyte ratio, together with elevated D-dimer levels are independently associated to the development of VTE, highlighting the strong relationship between coagulation and inflammation ([33]). Other observations go in the same direction, such as the described association between IL-6 and fibrinogen levels ([34]). Many different mechanisms can be responsible for the activation of the hemostatic process induced by the inflammation status. Among others, the increased expression of the tissue factor (TF) on innate immune cells and endothelial cells induced by proinflammatory cytokines directly activate the extrinsic pathway of the coagulation cascade.

Another important mechanism is represented by the endothelial damage, secondary to both the inflammation reaction and the direct infection of the endothelial cells by SARS-CoV-2, as demonstrated by the presence of viral particles within the endothelial cells ([35]). This damage results in endothelial cells activation with expression of procoagulant molecules. In particular, high levels of von Willebrand factor (vWF) together with low levels of ADAMTS13 have been reported, resulting in increased levels of high molecular weight vWF multimers that bind, through A1 domain, the gp1b glycoprotein on the platelets surface, leading to the formation of platelet-rich microthrombi in the

arterioles and capillaries ([36]). However, vWF acts not only as a bridge between the activated endothelium and the platelets, but also as a mediator of inflammation, recruiting leukocytes at sites of vascular inflammation, playing a critical role in thromboinflammation ([37]). Moreover, COVID-19 patients' endothelial cells have a lower anticoagulant potential, due to the reduced expression of thrombomodulin on their surface, because of its shedding from the endothelial surface and consequent increased plasma concentration of its soluble form ([38]).

COVID-19 associated inflammation has also been associated to hemostatic abnormalities in the coagulation and fibrinolytic system. More in detail, increased levels of plasminogen activator inhibitor-1 (PAI-1) and reduced levels of urokinase-type plasminogen activator (uPA) result in a reduced activity of the fibrinolytic system, while increased levels of the procoagulant fibrinogen and tissue factor lead to the activation of the coagulation cascade ([39]; [40]). The activation of the coagulation cascade results in thrombin generation that, in turn, activates the innate immune cells through protease-activated receptors (PARs), leading to the release of more proinflammatory cytokines ([39]).

The complement system interplay with the coagulation cascade is well known ([41]) and SARS-CoV-2 activation of the complement system through the interaction between S-protein and mannose binding lectin (MBL) of the complement lectin pathway have been recently demonstrated ([42]).

Inflammatory cytokines are also responsible for platelet activation. In COVID-19, platelets undergo a strong activation in the lung vessels, then recirculating as exhausted platelets and in 5-40% of patients a mild thrombocytopenia is observed ([43]). This feature, together with the evidence of increased circulating platelet-derived extracellular vesicles and apoptotic platelets, confirms the hypothesis of a strong platelet activation in the lung vessels ([44]). Platelet activation, in turn, sustains the inflammation response, through the release of the α-granules content, represented by cytokines, chemokines, inorganic polyphosphates and adhesion molecules, contributing to the

recruitment and activation of immune cells ([45]). Platelets interaction with neutrophils is also responsible for the induction of neutrophil extracellular traps (NETs) formation, which is a form of neutrophil deaths called NETosis ([46]). NETs are networks of extracellular fibers composed by decondensed chromatin, histone proteins and antimicrobial proteins derived from the neutrophil intracellular granules, that represent a scaffold for coagulation initiation. NETs formation is not only due to neutrophils-platelets interaction, but also by interaction of SARS-CoV-2 itself ([47]) and the neutrophils and by subsequent complement system activation ([48]). Moreover, NETs exert also a direct cytotoxic effect on the endothelium, further contributing to the endothelial damage ([49]).

All the above-mentioned mechanisms have a bidirectional interaction between the inflammation and the hemostatic pathways, pointing towards the existence of a pivotal crosstalk between inflammation and hemostasis (**Figure 3**).

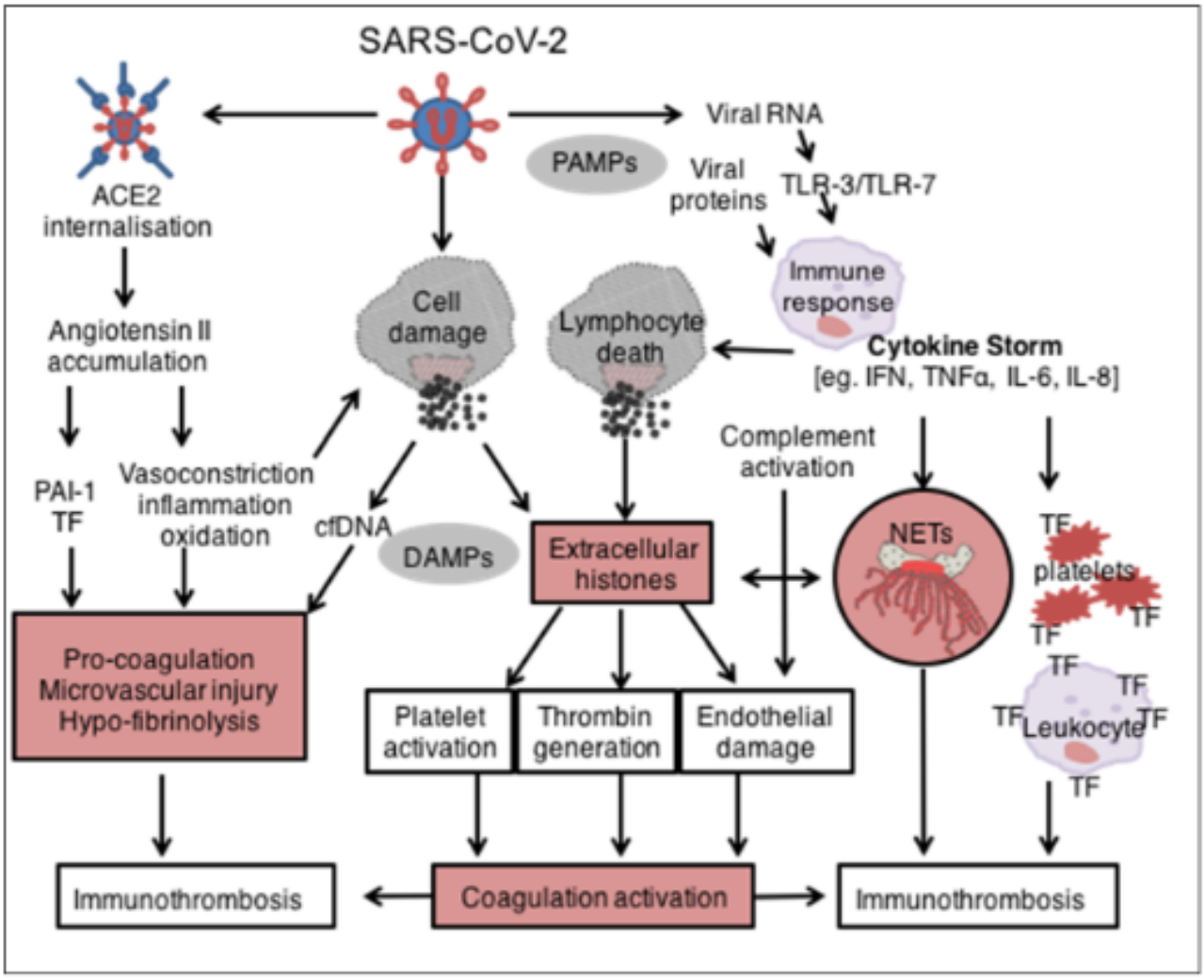

Figure 3. Mechanisms underlying immunothrombosis in COVID-19. See text for details. TF, tissue factor; TLR, Toll-like receptors; cfDNA, cell-free DNA; DAMPs, damage-associated molecular patterns; IFN, interferon; NETs, neutrophil extracellular traps; PAI-1, plasminogen activator inhibitor-1; PAMPs, pathogen-associated molecular patterns; TNFα, tumour necrosis factor alpha. Adapted from Shaw et al. ([25]).

Moreover, one mechanism specific of SARS-CoV-2 infection, namely the above-discussed activation of the kallikrein-bradykinin system, can be involved in the activation of the coagulation cascade as well, considering its close link with the contact pathway of the coagulation system. The contact pathway is composed by 4 plasma proteases represented by FXII, prekallikrein, high molecular weight kininogen (HMWK) and FXI. FXII is the initiator of this cascade and is strongly activated by activated platelets, platelet-derived extracellular vesicles and negatively charged polymers, such as

inorganic polyphosphates (which are stored in the platelets α-granules in complex with calcium), nucleic acids (DNA and RNA) or proteoglycans ([50]). Therefore, FXII undergoes an autocatalytic activation process with the formation of activated FXII (FXIIa) which continues this autoactivation process, but also activates prekallikrein to kallikrein, which in turn activates FXII to FXIIa, amplifying the process. This process ends with FXIIa-mediated activation of zymogen FXI to FXIa, the serine protease that initiates the coagulation cascade, resulting in thrombin generation. HMWK is not enzymatically active and acts as a cofactor for the activation of prekallikrein and factor XII and for the activation of FXI by FXIIa. The contact pathway is deeply interconnected with the coagulation system, but also with the fibrinolytic system, the complement system and the kallikrein-bradykinin pathway. Indeed, FXIIa-produced kallikrein leads to the degradation of HMWK with the formation of bradykinin, which is a potent inflammation inducer; it also results in increased levels of plasmin, counteracting the coagulation cascade activation; and activates the complement C1 complex. The main physiological inhibitor of the contact system is represented by the serine protease C1 inhibitor (C1-INH) which directly inhibits FXIIa, activated kallikrein and FXIa. It is the main regulator of the three activation pathways of the complement system, being the only inhibitor of the classical pathway through the inhibition of C1r and C1s, inhibiting the lectin pathway through inactivation of MBL-associated serine protease-1 and -2 (MASP1 and MASP2), and inhibiting the alternative pathway by binding C3b. It also inhibits to a lesser extent plasmin, tissue-type plasminogen activator (tPA) and thrombin on the endothelial cell surface. Moreover, C1-INH is the most heavily glycosylated plasma protein, and this characteristic is responsible for its binding to endothelial cell-surface adhesion molecules, competing with leukocytes binding, exerting in this way, an anti-inflammatory function. C1-INH has been predicted to interact with several SARS-CoV-2 proteins, suggesting that this key inhibitor function could be suppressed during the infection, resulting in unbalanced activation of the contact pathway of the coagulation cascade, kallikrein-bradykinin

pathway and complement pathway, resulting in a proinflammatory and procoagulant status ([51]) (**Figure 4**).

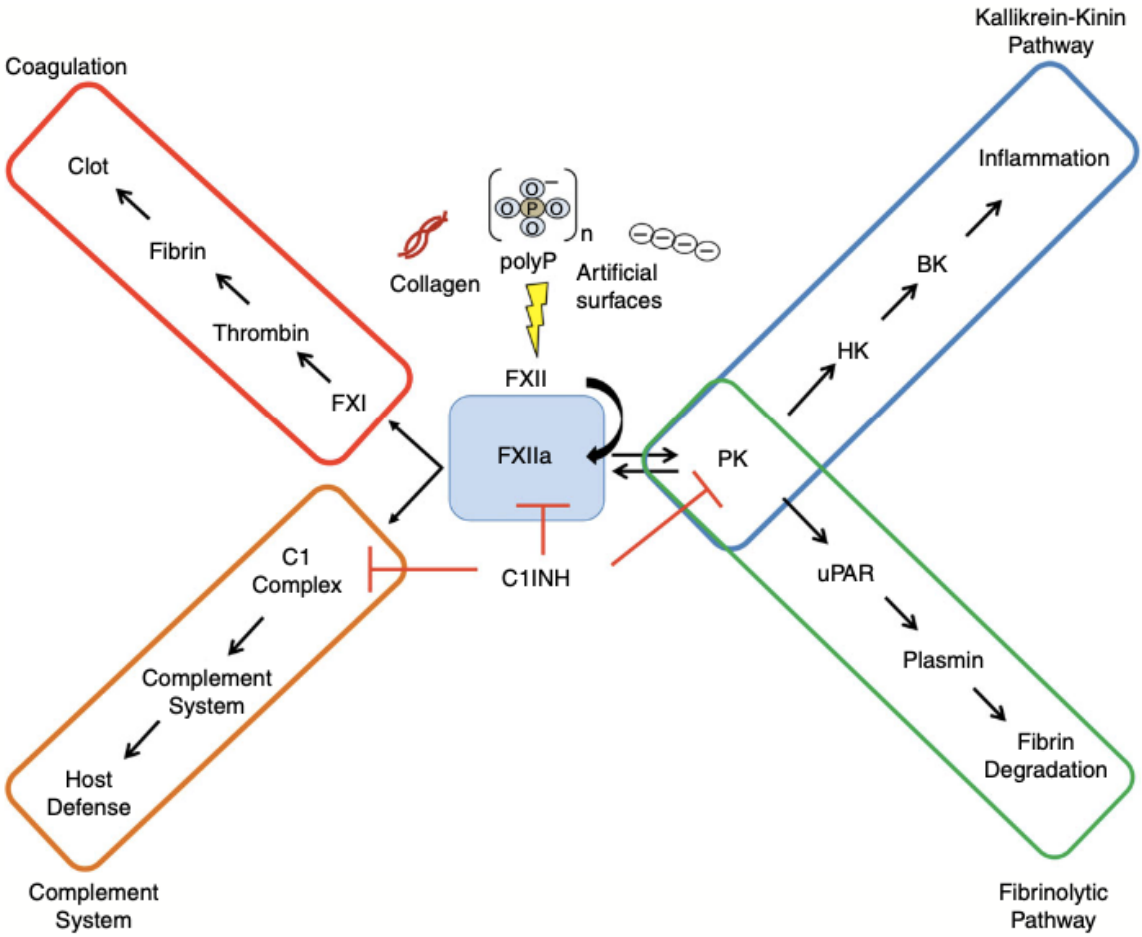

Figure 4. Factor XII and contact system–triggered pathways. The interplay between contact system and coagulation, complement system, kallikrein-kinin pathway and fibrinolytic pathway is represented. See text for details. Adapted from Long et al. ([52]).

3. Aim of the study

Considering the controversial findings on the COVID-19-associated coagulopathy reported at the beginning of the pandemic, we tried to better understand the characteristics of the SARS-CoV-2-induced coagulopathy and the mechanisms leading to the associated thrombotic complications. To do that, we planned to perform extended hemostasis measurements in COVID-19 patients admitted to our hub hospital. Subsequently, once the main features of the COVID-19 coagulopathy had been

defined by others and our work, we planned to better elucidate the pathophysiology of the coagulation activation, trying to dissect the coagulation cascade, studying the differential activation of the extrinsic and the contact pathway, with the ultimate goal to identify targets for a more precise therapeutic approach, maximizing thrombosis prevention and reducing bleeding complications.

4. Methods

4.1. Study population

All consecutive COVID-19 patients admitted to our hospital between February and April 2020 were included in the study. Depending on the severity of the disease, they were admitted to three different wards, characterized by low-intensity care, when hypoxia could be handled by ventilation support with high-flow nasal cannulas; intermediate sub-intensive care, when hypoxia prompted the use of continuous positive airway pressure; or high-intensity care when hypoxia warranted intubation and mechanical ventilation in ICU.

All patients started antithrombotic prophylaxis with low-dose LMWH at admission and dosages were then adjusted by attending physicians after patient transfer to the hospital wards. In low-intensity care wards patients were treated with enoxaparin 70 UI/Kg once daily; in intermediate-intensity with 70 UI/kg twice daily; in high-intensity with 100 UI/kg once daily.

One hundred healthy controls (50 males and 40 females; both groups homogeneous for age) were selected between healthy volunteers arrived at our Center in the pre-COVID-19 era (before 2018), characterized by no personal history of thrombosis or thrombophilia abnormalities.

4.2. Blood samples collection and processing

Venous blood was collected by venipuncture, at least 72 hours after the start 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 3.000 g within one hour and supernatant separated to obtain platelet poor plasma. All samples were quickly frozen in liquid nitrogen, stored at -80°C and thawed at 37°C for 3-4 minutes before being analyzed.

4.3. Laboratory parameters

In the first part of the study, to elucidate the characteristics of the COVID-19 coagulopathy, we tested plasma samples for markers currently used to diagnose DIC, the pro- and anticoagulant factors and those indicating endothelial perturbation, together with inflammation markers (**Figure 5**).

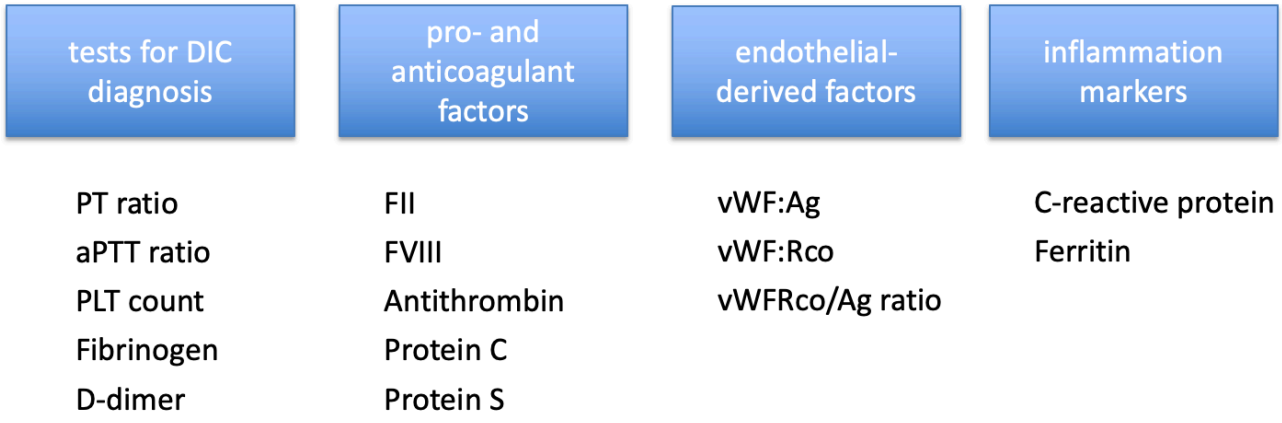

Figure 5. Laboratory tests performed during the first phase of the study.

Prothrombin (PT) and activated partial thromboplastin time (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 II (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 the Clauss method. 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 according to the ISTH criteria ([53]). In patients with sepsis, SIC score is more sensitive than 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.

In the second part of the study, to better understand the pathways involved in the activation of the coagulation in COVID-19 patients, we tested plasma samples for indirect markers of the global coagulation activation such as fibrin degradation products together with markers of the fibrinolytic pathway and markers of activation of the extrinsic and the contact pathway (**Figure 6**).

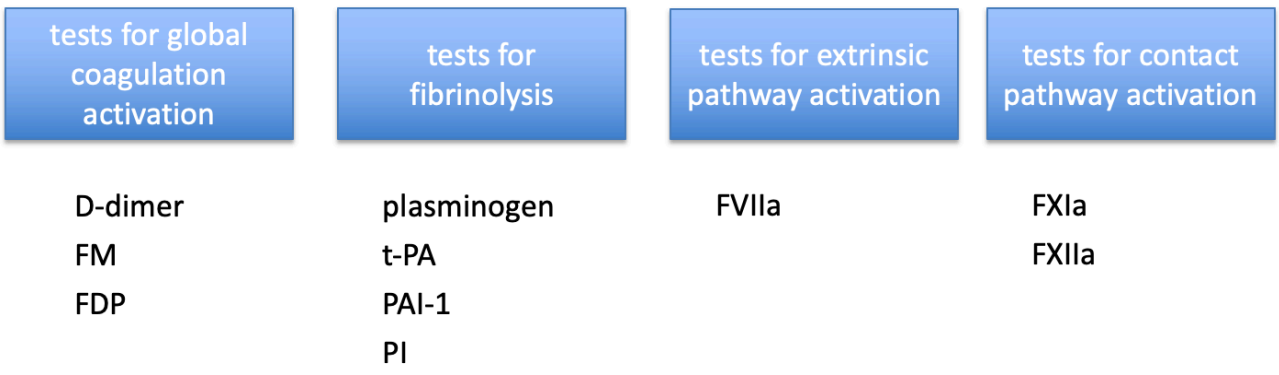

Figure 6. Laboratory tests performed during the second phase of the study.

D-Dimer (DD), Fibrin Monomer complex (FM) and Fibrin Degradation Products (FDP) levels were measured by the relevant commercial kits: Lias Auto D-Dimer, LIA FM and Lias Auto P-FDP (Sysmex Corporation, Kobe, Japan) on CS-2400 instrument (Sysmex Corporation) ([54]). Plasma levels of FVII, FXI and FXII were measured by one-stage assays using Synthasil on ACL Top instrument (Werfen, Bedford, MA, USA). Activated FVII (FVIIa) levels were measured using the HemoclotTM Factor VIIa kit (Hyphen Biomed, France), a clotting method for the quantitative determination of FVIIa activity; the method is insensitive to FVII because the reagent used is the recombinant truncated human tissue factor (rTTF) unable to promote FVII activation. The limit of detection of HemoclotTM Factor VIIa is <1 mIU/mL and the total imprecision CV <7.3%. Activated FXI (FXIa) was determined by the Biophen FXIa chromogenic assay (Hyphen Biomed, France); the commercial kit contains human FVIII, FIX, thrombin, FX, calcium and synthetic phospholipids. All the reagents are present in excess, so that the generated FXa is directly related to FXIa present in the tested sample and measured at 405 nm by its specific activity on a Factor Xa chromogenic substrate (SXa-11). The detection limit of the Biophen FXIa assay is <2.5 mIU/mL ([55]). Both FVIIa and FXIa assays were performed on CS-2400 instrument. Activated FXII (FXIIa) concentrations were quantified by the CoaChrom FXII Chromogenic (CoaChrom Diagnostica GmbH, Austria) manual method: FXII is converted to FXIIa by an activator (containing Prekallikrein and HMWK and incubated for 10 minutes at 37°C); at a fix time a kallikrein inhibitor is added to stop the activation and the produced active protease FXIIa cleaves a chromogenic substrate and releases p-nitroaniline (pNA), which can be measured photometrically at 405 nm. The results are calculated based on a curve obtained with a standard at known potency. The standard curve is linear up to 1.5 U/mL. Intra-assay CV: 5.5% at 1.00 U/mL. Detection limit: 0.05 U/mL ([56]). C1-esterase inhibitor (C1-INH) activity was quantified by a commercial chromogenic kit (Technochrom C1-INH, Technoclone GmbH, Vienna, Austria). Tissue-type plasminogen activator (t-PA) antigen was measured using a commercially available ELISA kit (Zymutest t-PA antigen, Hyphen

BioMed, Neuville sur Oise, France). The intra- and inter-assay CVs were <10%, and the lower detection limit was 0.5 ng/mL. Plasminogen activator inhibitor-1 (PAI-1) antigen was measured using a commercially available ELISA kit (Zymutest PAI-1 antigen, Hyphen BioMed) whose intra- and inter-assay CVs were 8% and 13%, respectively; the lower detection limit was 0.5 ng/mL. Plasminogen and alpha2-antiplasmin plasma levels were measured on ACL Top instrument by specific commercial chromogenic kits: HemosIL plasminogen (detection limit 2%; total imprecision CV <2.7%) and HemosIL Plasmin Inhibitor (detection limit 7%; total imprecision CV <5.9%), respectively.

One hundred plasma samples of healthy donors stocked in the pre-COVID-19 era have been used as external controls to define the normal range for FVIIa, FXIa, FXIIa, DD, FM, FDP, tPA and PAI-1.

FXIIa and FXIa plasma levels had never been analyzed in our laboratory before. Therefore, a relevant aspect of the second phase of this project was the fine-tuning of the method. After checking the scientific literature for the most suitable commercial methods available, we firstly used the Activity Colorimetric Assay Kit (Biovision, CA, USA) for FXIIa measurement, a manual micromethod. Numerous attempts to identify the correct plasma dilutions have been made, with low reproducible results. So that, we switched to the CoaChrom Factor XIIa Kit (CoaChrom Diagnostica GmbH, Austria), a manual chromogenic assay. We started a process of fine-tuning of the method, achieving a highly reproducible performance. According to the kit instruction manual, in plasma samples containing heparin, heparin should be neutralized by protamine sulphate or protamine chloride before freezing. All patients enrolled in the study were on low molecular weight heparin, but heparin was not neutralized before plasma stockage. So that, we tested plasma samples from patients not treated with heparin and known to have elevated concentrations of FXIIa, and we added increasing concentrations of heparin to obtain a range of anti-Xa activity levels from 0 to 1.5

U/mL (0, 0.1, 0.25, 0.5, 10 and 1.5) and we found no significant interference for anti-Xa activity levels as high as 0.5 U/mL.

The method for FXIa plasma level determination is also affected by the presence of heparin in the sample, due to the method itself, which is a chromogenic assay evaluating the residual anti-Xa activity. Therefore, we performed the same test as for FXIIa with plasma samples from patients not treated with heparin and known to have elevated concentrations of FXIa, and we added increasing concentrations of heparin to obtain a range of anti-Xa activity level from 0 to 1.5 U/mL (0, 0.1, 0.2, 0.3, 0.4, 0.5, 0.75, 1.0 and 1.5) and we found a significant interference for anti-Xa activity levels higher than 0.2 U/mL.

All determinations were done in triplicates.

4.4. Statistical analysis

Mean and standard deviation or median and interquartile range were used for continuous variables, and count and percentages were used to describe demographic and categorical clinical variables.

Comparisons of the coagulation parameters among patients and controls and within low, intermediate, and high intensity of care were performed using the Kruskal-Wallis non-parametric test. Far outliers, defined as values more than 3 times the interquartile range from the quartiles, according to Tukey's rule, were not considered in the analysis. To evaluate the linear correlation between two sets of data the Spearman's correlation coefficient (rho) was calculated.

To consider the possible confounding role of intensity of care, age and occurrence of thrombotic events during hospital stay on the coagulation parameters, stratification analysis by intensity of care and by occurrence of VTE, and sensitivity analysis excluding patients over 65 years were also performed.

The level of statistical significance was set to 0.05 for all the performed tests.

5. Results and Discussion

Results are reported separately for the two different phases of the study.

5.1. Study phase 1

In the first part of the study, 62 COVID-19 patients admitted to our hospital were consecutively enrolled, 21 in low-intensity care wards, 21 in intermediate sub-intensive care wards and 20 in high-intensity care units (**Figure 7**).

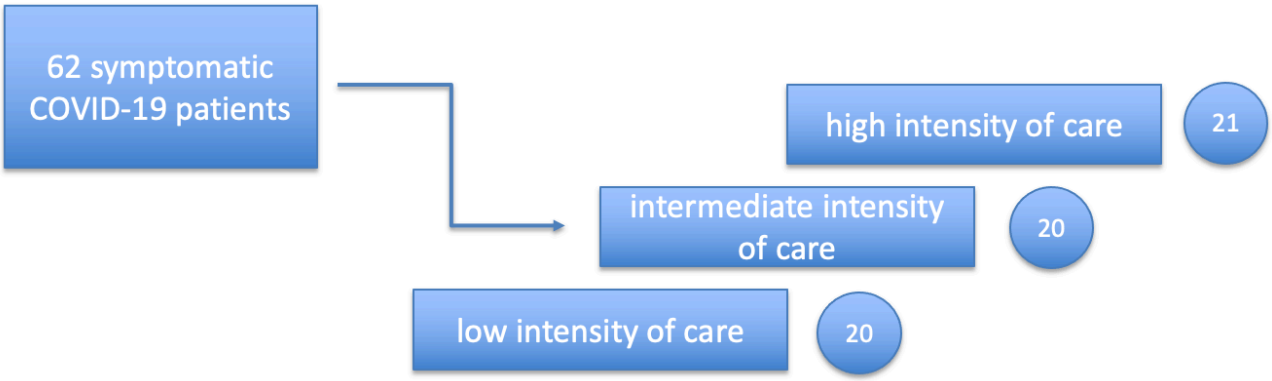

Figure 7. Distribution of the enrolled patients in the different intensity of care wards for the first phase of the study.

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 during the hospital stay three deaths and 25 thrombotic events (40%) in 25 patients, *i.e.*, 16 deep-vein thrombosis, eight pulmonary embolisms and one visceral venous thrombosis.

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 than the $50x10^9$/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, Figure 8**). 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.

	Intensity of Care			
	Low	Intermediate	High	P value
Tests for DIC Diagnosis				
PT ratio	1.02 (0.85-1.33)	1.12 (0.95-1.44)	1.06 (0.96.133)	0.0037
APTT ratio	0.93 (0.79-1.22)	0.91 (0.78-1,10)	0.95 (0.78-1.15)	0.66
Platelet count, n x 10^9/L	275 (138-480)	362 (120-556)	366 (80-584)	0.1
Fibrinogen, mg/dL	344 (150-861)	471 (285-830)	531 (224-1035)	0.061
D Dimer, ng/mL	870 (203-38,847)	1347 (525-6,910)	2,217 (564-6,410)	0.009
ISTH DIC score	**2 (0-4)**	**2 (0-3)**	**2 (0-3)**	

Table 1. Median (min-max) values of the hemostatic measurements in COVID-19 patients. Tests for DIC diagnosis and ISTH DIC score are reported.

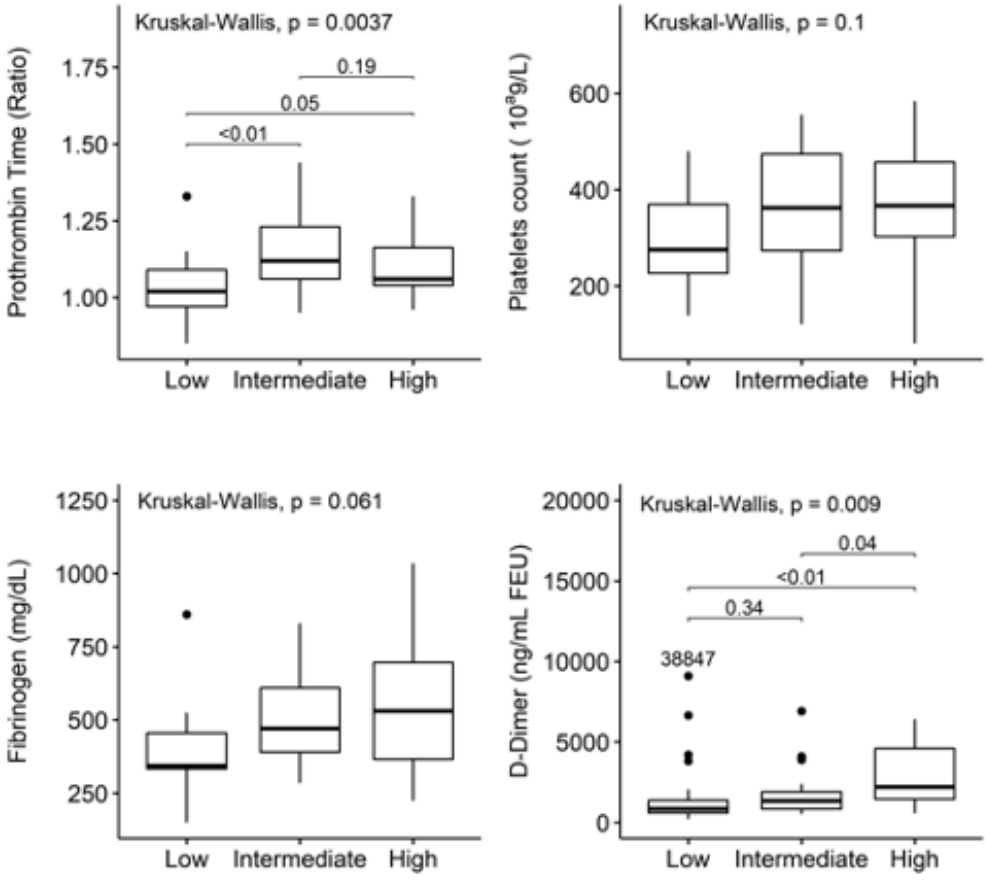

Figure 8. Box plots of results for tests for DIC diagnosis for COVID-19 patients at low, intermediate, and high intensity of care.

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 2; Figure 9**).

	Intensity of Care			
	Low	Intermediate	High	P value
Pro- and Anticoagulant Factors				
Factor II, U/dL	116 (65-140)	94 (76-128)	104 (75-143)	0.24
Factor VIII, U/dL	208 (121-347)	223 (109-423)	302 (178-374)	0.014
Antithrombin, U/dL	87 (61-133)	94 (63-135)	100 (71-143)	0.43
Protein C, U/dL	120 (60-234)	126 (72-210)	143 (85-232)	0.057
Protein S free antigen, U/dL	75 (38-98)	72 (26-95)	84 (56-110)	0.13

Table 2. Median (min-max) values of the hemostatic measurements in COVID-19 patients. Tests for pro- and anticoagulant factors are reported.

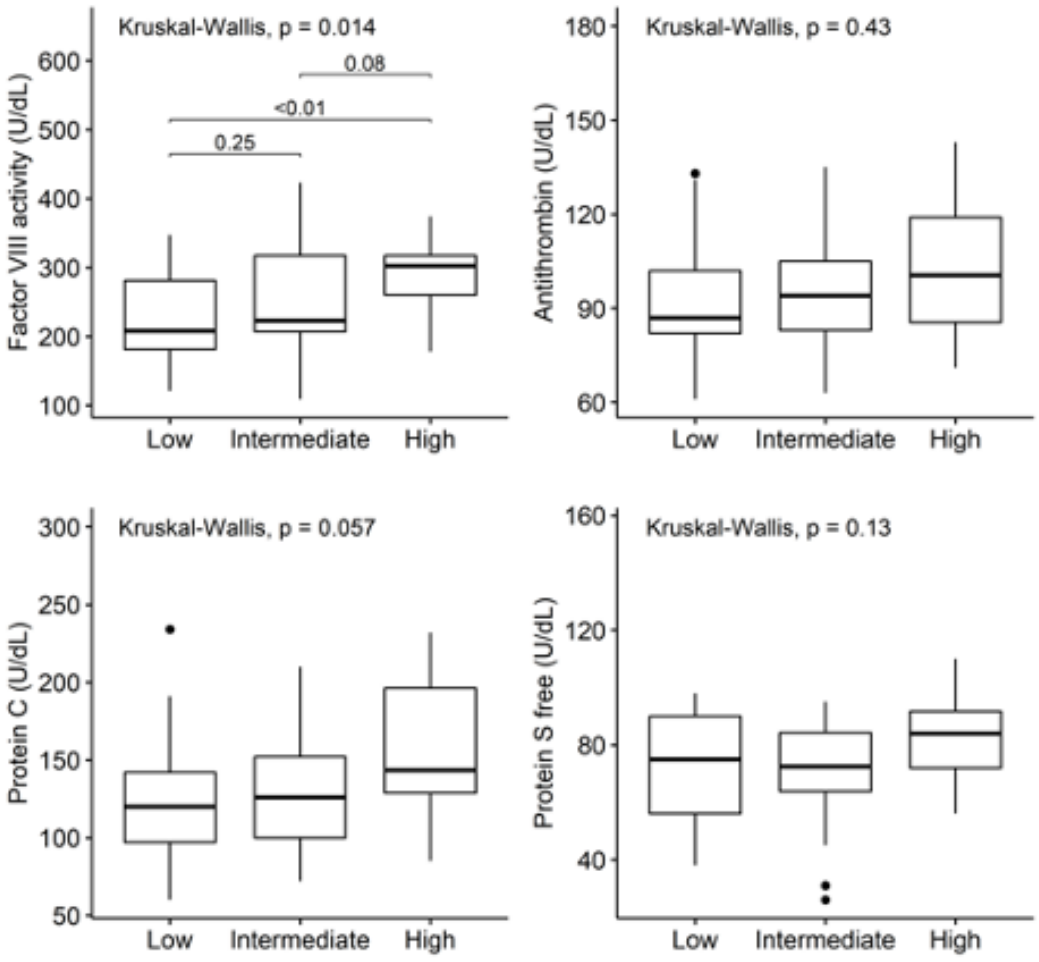

Figure 9. Box plots of results for tests for pro- anti anticoagulant factors for COVID-19 patients at low, intermediate, and high intensity of care.

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 3; Figure 10**). The median FVIII/VWF:Ag ratio ranged between 0.81 (low), 0.61 (intermediate) and 0.65 (high) care intensity.

	Intensity of Care			
	Low	Intermediate	High	P value
Endothelial-derived Factors				
VWF:Ag, U/dL	262 (90-577)	371 (132-769)	466 (231-746)	0.00007
VWF:RCo, U/dL	210 (88-447)	303 (129-539)	383 (195-528)	0.00015
VWF:RCo/Ag ratio	0.85 (0.65-1.02)	0.86 (0.62-0.98)	0.81 (0.69-1.01)	0.34
FVIII/Ag ratio	0.81 (0.40-2.05)	0.61 (0.32-1.00)	0.65 (0.40-0.97)	0.06

Table 3. Median (min-max) values of the hemostatic measurements in COVID-19 patients. Tests for markers of endothelial activation are reported.

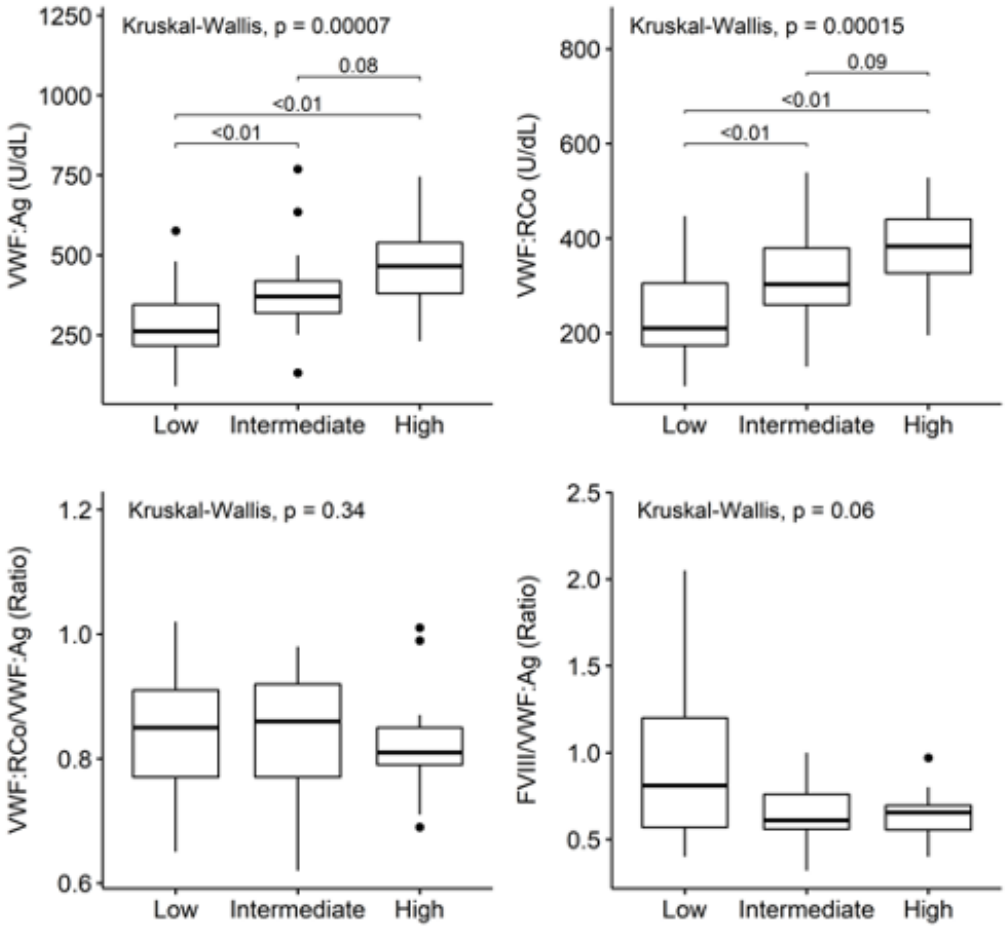

Figure 10. Box plots of results for von Willebrand factor antigen (vWF:Ag), vWF ristocetin-cofactor (vWF:RCo), VWF:RCo/Ag ratio and factor VIII (FVIII)/VWF:Ag ratio for COVID-19 patients at low, intermediate, and high intensity of care.

Median ferritin was extremely high, *i.e.*, 380 mg/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 care intensity) (**Table 4, Figure 11**).

	Intensity of Care			
	Low	Intermediate	High	P value
Inflammation markers				
Ferritin, µg/L	380 (32-1,587)	705 (124-4,081)	788 (212-5,064)	0.017
C-reactive protein, mg/dL	1.00 (0.07-11.71)	3.32 (0.19-18.3)	5.05 (0.6-25.5)	0.0057

Table 4. Median (min-max) values of the inflammation markers in COVID-19 patients.

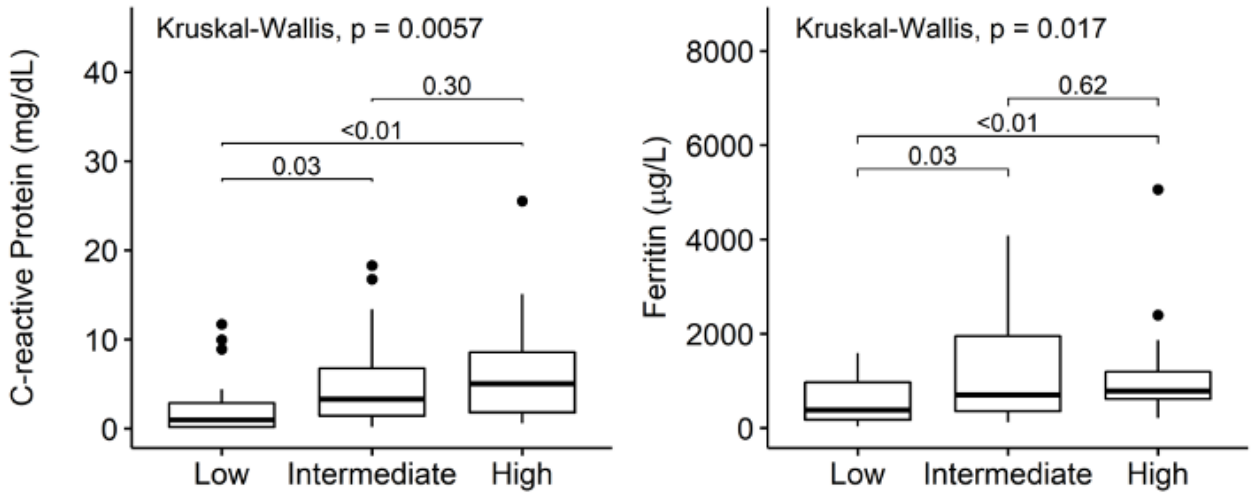

Figure 11. Box plots of results for the inflammation markers for COVID-19 patients at low, intermediate, and high intensity of care.

To better mechanistically understand thrombosis in COVID-19 patients we 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 high likelihood of DIC according to the ISTH criteria) ([53]). 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). Alike, 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. The clinical picture of hospitalized COVID-19 patients in Milan differed not only from DIC ([22]) but also from other disorders characterized by hypercoagulability and endothelial perturbation, triggered by systemic inflammation, such as the hemophagocytic lymphohistiocytosis/macrophage activation syndrome ([57]) and bacterial sepsis ([58]). 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.

5.2. Study phase 2

In the second part of the study, the initial cohort of patients was expanded and a total of 111 COVID-19 patients were consecutively included, 26 in low-intensity care wards, 42 in intermediate sub-intensive care wards and 43 in high-intensity care units (**Figure 12**).

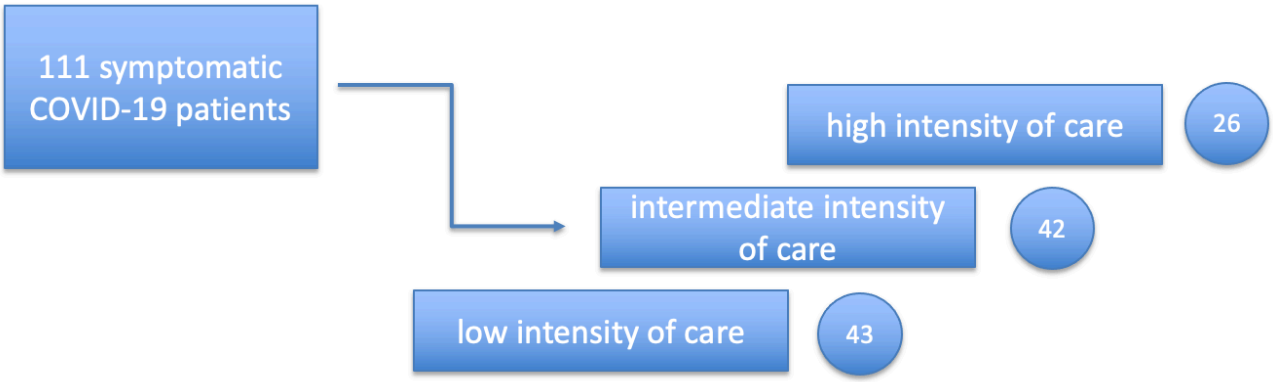

Figure 12. Distribution of the enrolled patients in the different intensity of care wards for the second phase of the study.

The median age at enrollment was 59 (±12) with 34 patients older than 65 years. Thirty-two patients developed a venous thromboembolic event with one of these patients also having an ischemic stroke (**Table 5**). Twelve patients (11%) died while admitted in the hospital.

Variables	Controls (N= 100)	COVID-19 patients (N= 111)
Male, n (%)	50 (50%)	70 (63%)
Age, mean (sd)	43 (10)	59 (12)
Age<65	47	77
Age≥65	3	34
Intensity of care		
Low		26
Intermediate		42
High		43
Thrombosis (arterial or venous)		32*

Table 5. Demographic and clinical characteristics of the patients enrolled in the second part of the study.

* All patients had a venous thrombosis, and one patient had an ischemic stroke together with a venous thrombosis.

Fibrin degradation products (DD, FDP, FM) plasma levels were significantly higher in COVID-19 patients compared to normal controls, with a gradient of increase across the three care intensities, confirming the results of the first part of the study (**Table 6, Figure 13**).

Parameters	Normal Range	Controls (n=100)	Patients tot (n=111)	Group Low (n=26)	Group Interm. (n=42)	Group High (n=43)
Ddimer (µg/mL)	0-2.0	0.1[0-0.3]	2.8[1.2-5.6]	0.9[0.7-1.4]	2.65[1.2-5]	4.8[2.65-8.4]
FDP (µg/mL)	<2.5	2.4[2.4-2.4]	4.5[2.7-7.9]	2.4[2.4-3.95]	3.9[2.7-6.7]	7.0[4.08-10.8]
FM (µg/mL)	0.5-4.2	1.9[1.6-2.3]	2.3[1.9-3.3]	2.0[1.5-2.95]	2.2[1.9-3.0]	2.6[2.1-3.88]

Table 6. Median values and their interquartile range of the fibrin degradation products in controls and COVID-19 patients.

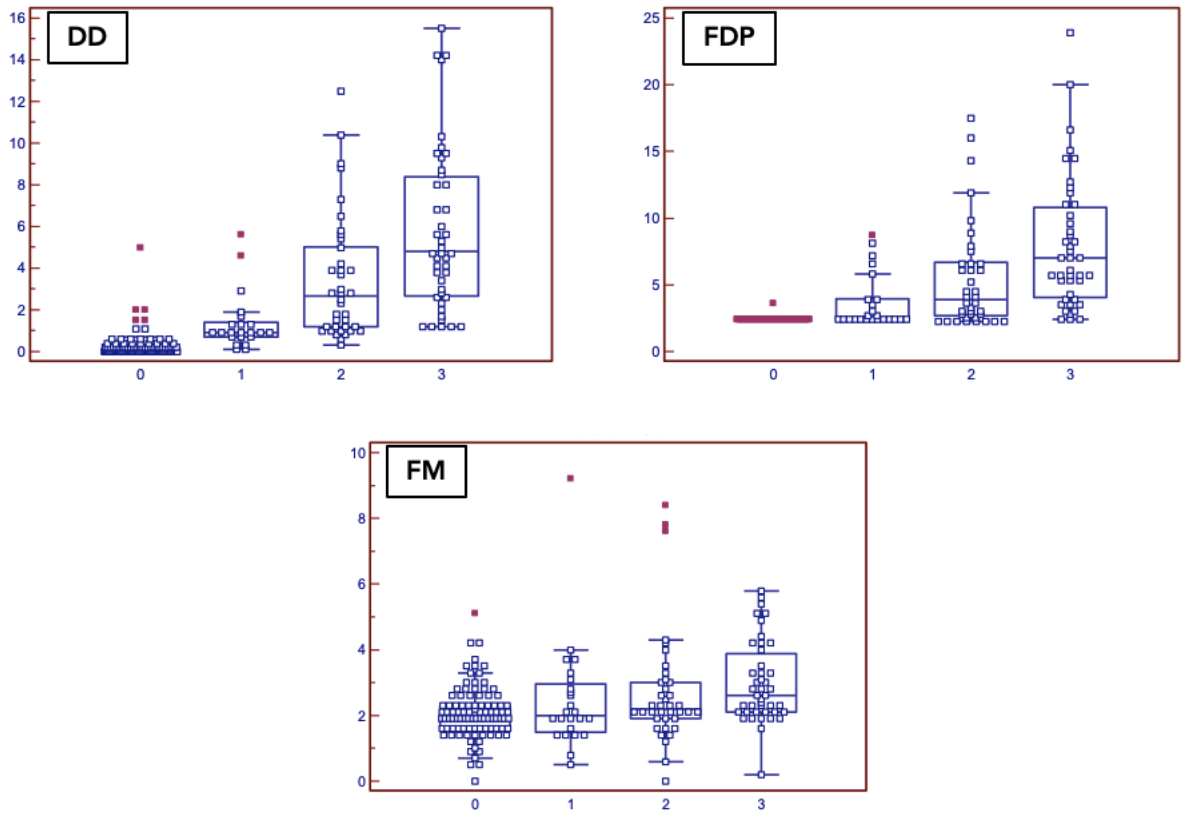

Figure 13. Box plots of the fibrin degradation products values in controls and the COVID-19 patients in the three intensity of care levels. On the x axis are reported the patients' categories (0, healthy controls; 1, low-intensity of care COVID-19 patients; 2, intermediate-intensity of care COVID-19 patients; 3, high-intensity of care COVID-19 patients), while on the y axis plasma levels of the tested parameters are reported (all in ug/mL).

Plasma levels of non-activated factor VII, factor XI and factor XII were within the normal range, excluding congenital or acquired deficiencies of these clotting factors, that should have been taken into account if present, while examining their active forms.

Plasma levels of activated FVII were significantly lower in COVID-19 patients compared to controls with no differences between the three care intensities. This observation could be a consequence of a reduced production due to a relatively lower activation of the extrinsic pathway or to a consumption of FVIIa by bond with tissue factor or other molecules on platelet or leucocytes plasma membrane. We speculate that in an initial phase of the disease, activation of the extrinsic pathway as a consequence of the endothelial damage directly induced by the virus itself and indirectly by the inflammation response represents the main activation pathway of the coagulation cascade. In a second phase, in condition of sustained inflammation response, the contact pathway is activated as well and becomes the main mechanism responsible for the coagulation activation ([52]). In our study patients with overt SARS-CoV-2 infection have been included, and blood samples were obtained many days after symptoms onset, supporting the hypothesis of a predominant role of the contact pathway in advanced phases of the COVID-19 coagulopathy.

Plasma levels of activated FXII and FXI were higher in COVID-19 patients compared to the controls with a gradient of increase across the three care intensities. However, FXIIa and FXIa plasma levels are not reliable with the used tests for plasma levels of anti-Xa activity higher than 0.5 and 0.2 U/mL, respectively, as stated above in the Methods paragraph. This interference is due to the physiological in vivo mechanism of action of heparin, that inhibits, other than FXa and FIIa, also FIXa and FXIa, even though at a minor extent. Due to the physiologic negative feedback of FXIa on FXIIa, the inhibition of FXIa by heparin results also in an indirect inhibition of FXIIa. Moreover, the method for FXIa plasma level determination is affected by the presence of heparin in the sample due to the method itself, which is a chromogenic assay evaluating the residual anti-Xa activity. Anti-Xa activity

due to heparin in patients' plasma was between 0 and 1.38 U/mL, with only 10 patients showing an anti-Xa activity >0.5 and only 6 patients >1.0 U/mL. Therefore, overall, the impact of heparin for FXIIa determination is negligible. On the other hand, a significant proportion (30 patients) of plasma samples had an anti-Xa activity >0.2 U/mL, making the test for FXIa unreliable on the actual values of this parameter in the tested samples. More in detail, the presence of heparin reduces the detected concentration of FXIa, but in a non-linear way, avoiding the possibility to identify a correction factor to determine the actual levels of FXIa. So that, the method underestimates the plasma levels of FXIa in presence of heparin. Therefore, the finding of statistically significant more elevated plasma levels of FXIa in COVID-19 patients compared to the controls is reliable and represents an underestimation of the real situation. However, a comparison of FXIa levels between the three care intensity groups is not feasible, since all patients are on treatment with heparin and, as stated above, no linear correlation between heparin levels in terms of anti-Xa activity and FXIa levels does exist. Other than heparin, there are other FXIa inhibitors in plasma, including antithrombin, glycosaminoglycans, C1-INH and protein Z-dependent protease inhibitor/protein Z complex (ZPI-PZ). No data on the impact of these inhibitors during the assay performance, and during the sample handling are available.

C1-INH plasma levels were increased in COVID-19 patients compared to the normal range and higher in the intermediate and high compared to the low intensity care group (**Table 7, Figure 14**).

Parameters	Normal Range	Controls (n=100)	Patients tot (n=111)	Group Low (n=26)	Group Interm. (n=42)	Group High (n=43)
FVIIa (mU/mL)	6.6-83.8	40.05[24.9-52.3]	27.5[16.45-38.87]	29.9[22.9-40.65]	26.7[12.6-39.6]	26.7[17.5-33.5]
FXIa (mU/mL)	2-31.3	5.5[2.0-14.4]	11.3[4-22.3]	19[4.9-22.1]	8.7[2.7-20.3]	11.2[4.7-22.4]
FXIIa (mU/mL)	3.0-13.7	7.2[5.7-9.0]	11.15[9.4-13.25]	10 [9.05-12..45]	10.9[8.25-12.87]	11.9[10.2-14.2]
C1-INH (%)	70-130		180.52±47.4	164.1±34.2	197.0 ±49.1	198.38±43.0

Table 7. Median values and their interquartile range for markers of extrinsic (FXIIa) and contact pathway (FXIa, FXIIa, C1-INH) of the coagulation cascade.

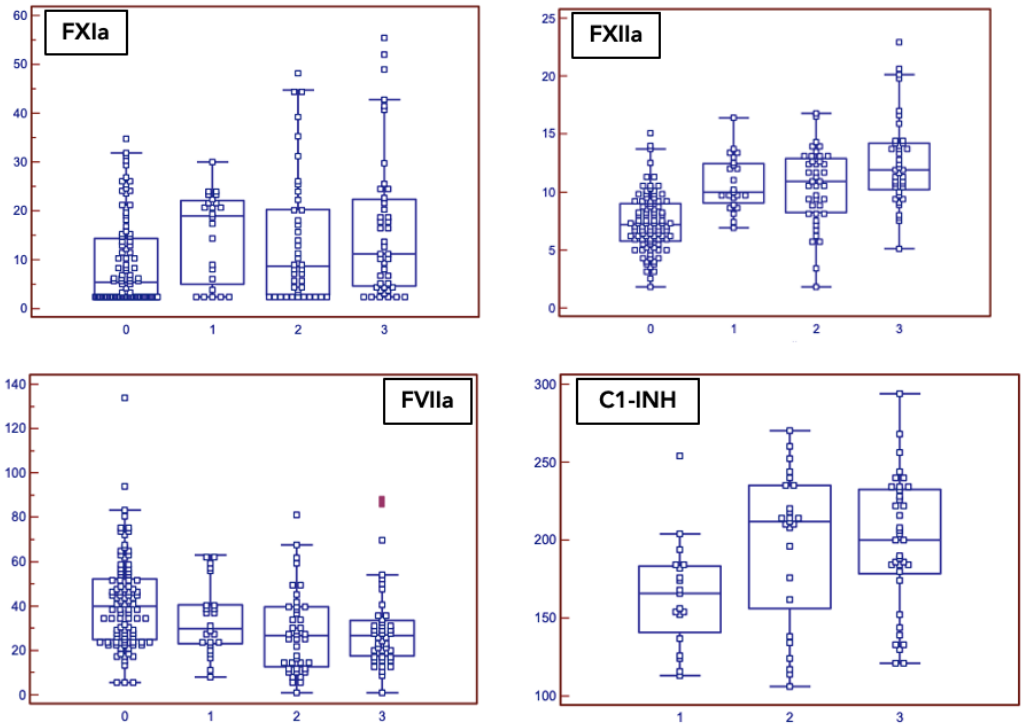

Figure 14. Box plots of the markers of extrinsic (FXIIa) and contact pathway (FXIa, FXIIa, C1-INH) of the coagulation cascade in controls and the COVID-19 patients in the three intensity of care levels. On the x axis are reported the patients' categories (0, healthy controls; 1, low-intensity of care COVID-19 patients; 2, intermediate-intensity of care COVID-19 patients; 3, high-intensity of care COVID-19 patients), while on the y axis plasma levels of the tested parameters are reported (mU/mL for FXIa, FXIIa, FVIIa, and % for C1-INH).

All the fibrinolytic pathway parameters were in the normal range, with no significant differences between the three intensity groups, except t-PA for which we found increased levels in the low and intermediate intensity group compared to the high intensity group. PAI-1 plasma levels were significantly higher in every patients group compared to controls (**Table 8, Figure 15**).

Parameters	Normal Range	Controls (n_20)	Patients tot (n=111)	Group Low (n=26)	Group Interm. (n=42)	Group High (n=43)
PAI-1 Ag (ng/mL)	1.5-35.3	4.2 [2.04-7.34]	30.6±10.2	29.7±11.1	31.6±10.0	30,3±10.0
tPA Ag (ng/mL)	6.1-26.0	13.4 [9.4-17.3]	16.1[3.3-30.6]	17.4[8.7-28]	30[5.8-43.1]	5.8[1.5-24.3]
Alfa2AP (%)	98-122		117.43±13.2	115.56±14.4	116.59±11.2	119.30±14.3
PLG (%)	80-132		109.63±20.0	109.63±24.4	109.86±16.5	114.35±20.6

Table 8. Median values and their interquartile range for parameters of the fibrinolytic system.

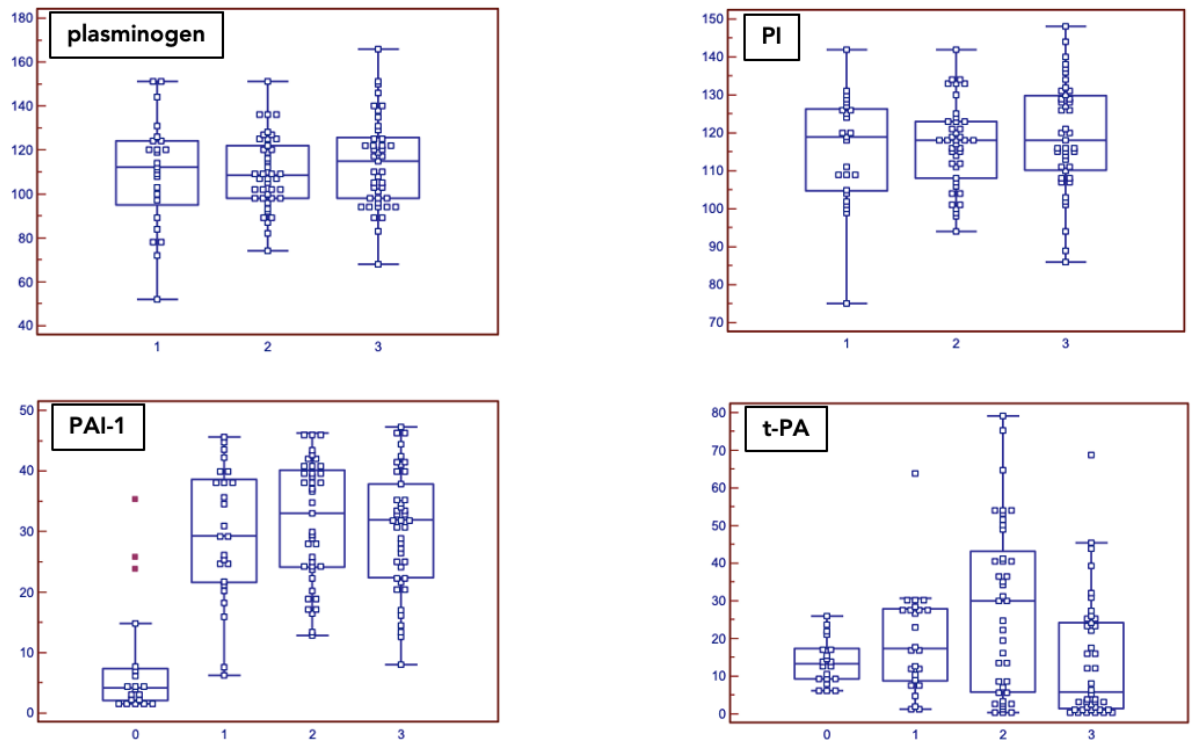

Figure 15. Box plots of the parameters of the fibrinolytic system. On the x axis are reported the patients' categories (0, healthy controls; 1, low-intensity of care COVID-19 patients; 2, intermediate-intensity of care COVID-19 patients; 3, high-intensity of care COVID-19 patients), while on the y axis plasma levels of the tested parameters are reported (% for plasminogen and PI, and ng/mL for PAI-1 and t-PA).

The influence of acute thrombosis on coagulation parameters is well known and is the rational for guidelines to advising against thrombophilia testing in this setting. In our cohort, 32 patients out of 111 (29%) had a thrombotic event, potentially influencing the investigated biological parameters. Moreover, among patients who developed thrombosis, a proportion was sampled before the thrombotic event, while the others after, representing an additional confounding factor. Among patients that didn't develop thrombosis, 20 were in low, 24 in intermediate and 24 in high care intensity. Among patients who developed a thrombotic event, those who was sampled before were 1, 5 and 6 in the low, intermediate, and high care intensity group, and those who was sampled after were 4, 8 and 2 in the three care intensity groups. For 6 patients (2 in the intermediate and 4 in the high intensity of care level) the information about the temporal relationship between blood sampling and thrombosis onset was missing. Therefore, we performed a sensitivity analysis for the presence of thrombosis for each considered parameter, showing no influence of thrombosis. No stratification for intensity of care level have been performed, due to the low number of patients in each group (**Figure 16**).

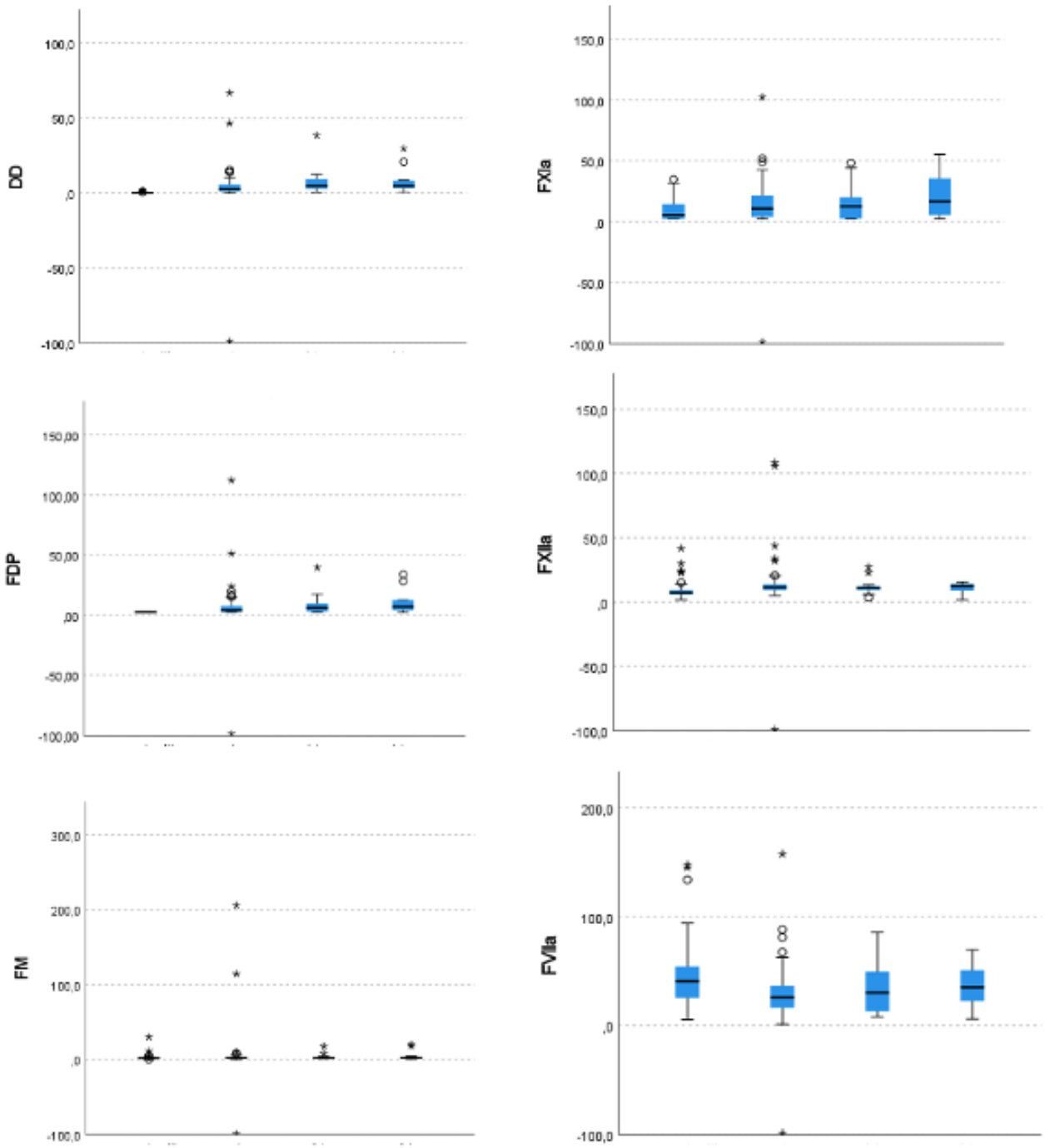

Figure 16. Box plots showing no influence of thrombosis on fibrin degradation products, FXIa, FXIIa and FVIIa. On the x axis are reported (from left to right) the healthy controls, COVID-19 patients without thrombosis, COVID-19 patients who developed thrombosis with the plasma sample collected before thrombosis onset and COVID-19 patients who developed thrombosis with the plasma sample collected after thrombosis onset, while on the y axis plasma levels of the tested parameters are reported (ug/mL for DD, FDP and FM, and mU/mL for FXIa, FXIIa and FVIIa).

Together with thrombosis, many other medical conditions can influence the coagulation parameters, such as cardiovascular or immunologic diseases. Data on comorbidities were not systematically collected in our study, so that adjustment for these data has not been possible. However, considering age as the main risk factor for thrombosis and for the presence of comorbidities, we performed a sensitivity analysis for older age, considering 65 years as a cut-off. Thirty-nine patients were older than 65 years, and the statistical analysis repeated excluding these patients showed the same results of the global analysis. Due to the limited number of patients a subanalysis for the three care-intensity group was not performed.

As expected, the three fibrin degradation products (DD, FDP, FM) showed a positive linear correlation (rho >0.6, p<0.0001). No linear correlation was found between FXIIa or FXIa and fibrin degradation products. The lack of a linear correlation doesn't mean a lack of association of the extrinsic and contact pathway on a hand and the global coagulation activation on the other hand, but simply that this relationship is not well described by a linear correlation. Indeed, there is no direct association between these two mechanisms, but many intermediate passages do exist. Moreover, these are not simultaneous mechanisms, but one is the result of the activation of the first two, while they have all been tested on the same plasma sample reflecting only one timepoint. FXIIa plasma levels were found to correlate positively with plasminogen (rho 0.236, p 0.016), PAI-1 (rho 0.232, p 0.02) and PI (rho 0.197, p 0.04), showing a direct relationship between contact pathway activation and the fibrinolytic cascade, both the activator (plasminogen) and inhibitory pattern (PAI-1 and PI). Indeed, the close relationship between contact pathway and the fibrinolytic pathway has been described. The main link is represented by kallikrein that directly converts plasminogen to plasmin, the effector protein of the fibrinolytic pathway, but also FXIa and FXIIa, even though at a minor extent, are able to activate plasminogen to plasmin. Moreover, thrombin generation resulting from the activation of the contact pathway is also responsible for the

dampening of the fibrinolytic system via activation of the thrombin activatable fibrinolysis inhibitor (TAFI). Moreover, there is also a negative feedback of the fibrinolytic system on the contact pathway carried out by PAI-1 on FXIIa ([52]) (**Figure 17**).

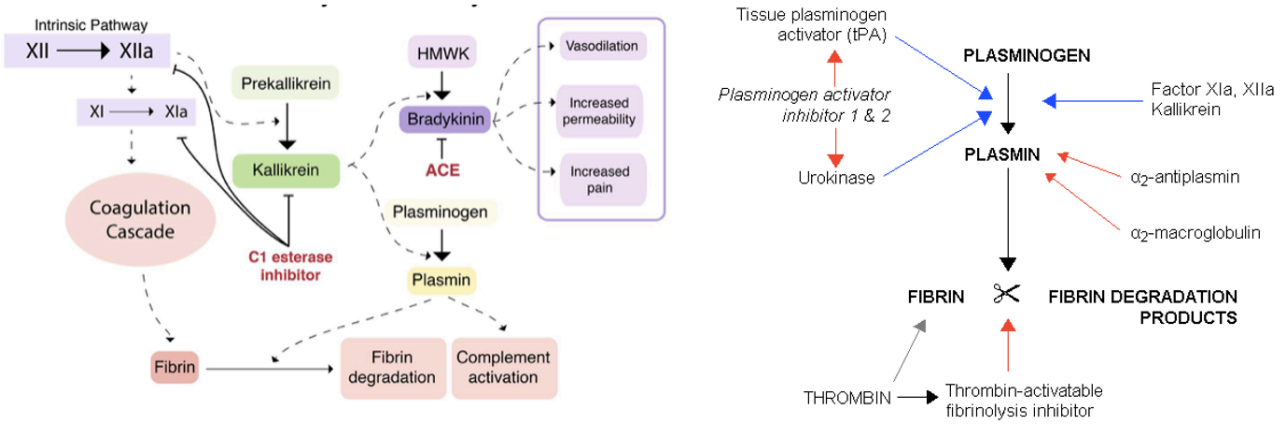

Figure 17. Connection between contact pathway and fibrinolytic system. See text for details.

As to fibrin degradation products and fibrinolysis parameters, no correlation was found, corroborating the hypothesis that the increased levels of fibrin degradation products is a consequence of the coagulation cascade activation and not secondary to hyperfibrinolysis. tPA is the only fibrinolytic parameter that showed a negative correlation with DD (rho -0.342, p 0.0007), FDP (rho -0.397, p 0.0001) and FM (rho -0.233, p 0.019), supporting the above reported hypothesis. C1-INH positively correlated with DD (rho 0.470, p<0.0001) and FDP (rho 0.394, p=0.0001) in line with the central role of C1-INH in negatively regulating the activated contact pathway.

6. Conclusions

Several studies reported that COVID-19 patients have an acquired coagulopathy with an increased risk of VTE in critically ill patients ([59]; [60]; [13]; [61]). However, the frequency varies greatly and there is still an unsettled strategy for prophylaxis ([62]). Therefore, besides the need of well-designed randomized clinical trials, we deemed crucial to better mechanistically understand this coagulopathy, with the ultimate goal to implement more targeted approaches for management of thrombotic complications.

Our study showed an acquired coagulopathy associated with hyperacute inflammation, hypercoagulability, and endothelial perturbation proportional to the clinical severity of the infection and to the levels of intensity of care needed by the patients.

Overall, our 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. The contact pathway is also activated and becomes predominant over the extrinsic pathway contributing to the hyperactivation of the coagulation cascade and leading to thrombus formation.

COVID-19 patients have also an increased risk of bleeding, with a rate of bleeding event of 4.8% (3.1% in non-critically ill patients and 7.6% in ICU patients), with a 2.3% of major bleeding rate, that rises to 5.6% in critically ill patients. Among the episodes of major bleedings, the most frequent were deep muscle bleedings (23%), gastrointestinal (14%), central venous catheter-associated (14%) or otorhinolaryngological (14%) ([60]; [63]). These high rates of bleeding events, together with the reported elevated rates of thrombotic events, point out the urgent need of establishing the best anticoagulant treatment for COVID-19 patients, to maximize the prevention of thrombotic events without increasing bleeding complications. In this scenario, the evidence of a prominent role of the activation of the contact pathway in COVID-19 associated coagulopathy is strikingly important,

considering the emergence of new drugs targeting this pathway, that are efficacious in resolution of thrombosis without an increase of bleeding events. Indeed, congenital or acquired deficiencies of the contact system factors, i.e., FXII, prekallikrein, HMWK and C1-INH, are not associated with impaired hemostasis. Together with this observation, data obtained from murine models, shows that FXII and the contact pathway are crucial for thrombosis development, but not in hemostasis, making FXII a promising therapeutic target to limit thrombosis without increasing bleeding risk ([64]). In conclusion, the evidence of a crucial role of the contact pathway in a disease characterized by an elevated risk of thrombosis together with a high risk of bleeding while on anticoagulant treatment, is of remarkable importance considering that drugs targeting this pathway are currently under advanced phases of investigation.

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