Haematological factors of SARS-CoV-2 infection aggravation: a COVID'HEMOS study

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Abstract

Since December 2019, a pandemic caused by a new coronavirus has spread to more than 170 countries around the world. Worsening infected patients requiring intensive care unit (ICU) admission, associated with 30% of mortality. A part of worsening is mediated by haemostasis deregulation. The primary aim of this study was to determine if haematological biomarkers, in addition to clinical risk factors on hospital admission, predict worsening (defined by ICU admission and/or death) in Covid-19 infected patients, and secondary, if they could predict the occurrence of thrombotic events. Thirty-five of the 99 patients got clinically worse and 8 developed pulmonary embolism (PE). Final model of the logistic regression analysis revealed that oxygenodependence (RR=7.27[1.50-19.31]), fibrinogen levels (RR=1.45[1.17-1.82]), thrombin peak (RR=1.28[1.03-1.59]), monocytes counts below 0.2 G/L (RR=2.88[1.67-3.19]) and prothrombin fragment 1+2 (F1+2) higher than 290 pM (RR=2.39[1.20-3.30]) were associated with clinical worsening. Fibrinogen level threshold of 5.5 g/L, thrombin peak measurement threshold of 99 pM and oxygenodepence allowed prediction of clinical outcome in near than 80% of our cohort. Moreover, using ROC curves, thrombin peak and F1+2 on admission with a threshold of 204 nM and 393 pM were sensitive and specific to predict PE (AUC: 85.7% Se: 70.8% Sp: 100% and AUC: 81.5% Se: 75.0% Sp: 86.8% respectively). In conclusion, we described a rapid decision tree to predict outcome of SARS-CoV-2 infected patients based on fibrinogen, TGA peak and oxygen dependence. Furthermore, thrombin peak and F1+2 seem to be specific haematological marker to predict PE, even in patients with thromboprophylaxis.

Introduction

Since December 2019, a pandemic caused by a new coronavirus has spread to more than 170 countries around the world. It started in China (1) and then spread to Europe and the United States of America. This virus called SARS-CoV-2 (1) is responsible for an infectious disease itself called Covid-19. Most patients are asymptomatic or mildly symptomatic. In symptomatic patients, the clinical manifestations are dominated by respiratory symptoms (2, 3). Nevertheless, some patients have digestive infections (4). On the other hand, there are serious lung complications that can lead to intensive care units admission for acute respiratory distress syndrome (5, 6). Some patients also had severe cardiovascular injuries (7). Many retrospective studies investigated the risk factors associated with severe forms of COVID-19, but they focused on medical background and identified age>65 years old, obesity, or cardiovascular diseases as associated with a poor prognosis. But fewer studies focused on

Biological factors to assess the risk of serious complications. Among them, an increase in D- dimer levels have been associated with severe forms of the pathology (8) and with other markers of disseminated intravascular coagulation (DIC). Clinical manifestations of these DIC were predominantly thrombotic with high venous thromboembolism rates (6).

In the study of Zhang et al. (9), patients hospitalized in intensive care unit (ICU) exhibited D- dimer levels 5 fold higher than the others. Moreover in the retrospective case series from Tongji hospital (8), mortality rate was about 11.5% with significantly higher D-dimer levels. Since the beginning of the pandemic, many

studies confirmed an increase in D-dimer level (10, 11) and a very recent paper (12) determined a cutoff value of D-dimer at 2000 µg/L in patients who were clinically worsening.

The alteration of the endothelium could originate the deregulation of haemostasis (13). In addition, sepsis promotes platelet overactivation, leading to acute respiratory distress syndrome and acute renal failure (14, 15). Recent recommendations from the International Society of Thrombosis and Haemostasis (ISTH) and a retrospective study suggest that preventive anticoagulation in patients would be associated with a better prognosis (16, 17).

Furthermore, D-dimer level is a very sensitive but not very specific marker of hypercoagulable state. Then, it is possible to assess coagulation globally, by measuring thrombin generation (18). This technique studies the initiation, propagation and inhibition of coagulation allowing the observation of hypo- or hyper-coagulable risk profiles.

Thus the aim of this study was to evaluate if haematological biomarkers, in addition to clinical risk factors on hospital admission, are able to predict clinical worsening in Covid-19 infected patients, and secondary, if they could be used as specific biomarker of the occurrence of thrombotic events.

Methods

Patients

Between March the 16th and May the 1st, 2020, 100 Covid-19 patients hospitalized in Covid- 19 dedicated medical units were prospectively recruited (clinicaltrials registration number: NCT04367662). Information was given to the patients and the citrated plasma from the initial blood test less than 24h after the admission was collected, double centrifuged according to French Group of Hemostasis and Thrombosis (GFHT) guidelines and frozen at - 80°C within 4 hours after collection. Clinical, radiological and biological relevant data were also collected.

The study was performed in accordance with the Declaration of Helsinki. The institutional review board and a national ethical committee approved the study, and a national anonymous data collection was declared (Authorization protocol number: 2020-A00914-35).

Computed tomography imaging

As defined by European Society of Radiology (19) , finding Covid-19 pneumonia in computed tomography scan were:

- A scale of disease extension (<10%, 10-25%, 25-50%, 50-75%, > 75%)
- Condensation type (nodular, linear or both)
- Radiological abnormalities localisation (unilateral, bilateral)

Assays

During initial blood test prothrombin time (PT), activated partial thrombin time (aPTT) (Diagnostica Stago – Asnières sur Seine- France) and D-dimer (Vidas® DEX2 – Biomérieux – Marcy l'étoile – France) assays were performed.

After defrost, several coagulation tests were assayed:

- Fibrinogen (STA-Liquid Fib– Diagnostica Stago – Asnières sur Seine- France), Fibrin monomers (STA-Liatest® FM – Diagnostica Stago – Asnières sur Seine- France) and chromogenic antithrombin assays (StachromATIII – Diagnostica Stago – Asnières sur Seine - France) were realized on STA®RMax (Diagnostica Stago – Asnières sur Seine- France);
- VWF:GPIb-binding activity (InnovanceVWAc – Siemens Healthcare- Marburg – Germany) was assayed on BCS XP (Siemens Healthcare- Marburg –Germany)
- Prothrombin fragments 1+2 were assayed with Enzygnost® F1+2 (Siemens

Healthcare- Marburg –German) on Diasonrin Etimax.

- Complete blood count was performed on EDTA samples on XN-1000 (Sysmex, Villepinte, France).
- Thrombin generation assay (TGA) was triggered by a low concentration of tissue factor (TF) (1pM) and a normal concentration of phospholipids (PPP low reagent, Diagnostica Stago, Asnières sur Seine, France). TGA was measured by Calibrated Automated Thrombography and Fluorocan Ascent Fluorometer (Thermoscientific Labsystems, Helsinki, Finland).

International Society of Thrombosis and Hemostasis Disseminated intravascular Coagulation score

Disseminated intravascular coagulation score (DIC) was calculated with ISTH criteria recommendation (20). Briefly, the scoring system included platelet count, prothrombin time, fibrinogen, and D-dimer or fibrin monomer.

Data and statistical analysis

The primary objective of the study was to assess baseline haemostasis predictors of clinical worsening on admission. Patients were considered to be clinically worsening' if they were transferred to the intensive care unit or died and clinically 'improving' if not. For patient characteristics, data were expressed as median [interquartile range or IQR], n (%), or n/N (%), where N is the total number of patients with available data. P values comparing clinical improving to clinical worsening are from χ^2 test, Fisher's exact test, Chi Square with *Yates' correction* for continuity, Spearman correlation or Mann-Whitney U test when appropriate.

Univariate logistic regression analysis of clinical outcome (improving or worsening) were performed using the following variables as predictors: age, sex, oxygen dependency, tobacco consumption,

radiological scale of disease extension (dichotomized to lower or higher than 25 %), body mass index (BMI), hypertension, diabetes, respiratory disease (including COPD and/or asthma and/or other causes of respiratory disease), aPTT ratio (higher than 1.15), blood lymphocyte count (lower than 1G/L), blood monocyte count (lower than 0.2 G/L), neutrophil to monocyte ratio, neutrophil to leucocyte ratio, D-dimer (higher than 1000 µg/L), fibrinogen, TGA parameters (ETP, peak, velocity), fibrin monomers (higher than 6 µg/ml), VWF:GPIb-binding activity (higher than 250%) and F1+2 (higher than 290 pM). Significant predictors under unadjusted analysis were further analyzed by multiple logistic regression analysis (full model). Then, based on the Akaike Information Criterion (AIC), irrelevant variables were eliminated from the full model by backward variable selection to obtain the final model. Results from the logistic regressions were expressed as relative risk (RR) [95% confidence interval]. Finally, a decision tree based on the predictors retained in the logistic regression final model was built using recursive partitioning method with the following parameters: minimum number of observations that must exist in a node in order for a split to be attempted = 30; minimum number of observations in any terminal node = 6; k-fold cross-validation = 9.

The secondary objective aimed to assess if some haematological parameters could be used to predict the risk of thrombosis occurrence during hospitalization. We evaluated Prothrombin fragment1+2, D-dimer, VWF:GPIb-binding activity and thrombin peak in TGA using receiver operator characteristic (ROC) curve analysis. For each parameter, the optimal threshold value was determined using the Youden index corresponding to the point for which (sensitivity + specificity-1) is maximum and 95% confidence interval for sensitivity and specificity of this threshold was computed with 2000 stratified bootstrap replicates.

Data and statistical analysis and captions were performed using *R v4.0.0* software (21) and the following software packages: *pROC* (22), *MASS* (23), *caret* (24) , *sjstats* (25) , *rpart* (26) and *rpart.plot* (27).

Results

One hundred patients were recruited and followed up to hospital discharge or death. One patient opposed participation after analysis. With World Health Organization classification of Covid-19 severity in admission, 23 patients had pneumonia, 51 patients had severe pneumonia and 26 patients had acute respiratory distress syndrome. During hospitalization, patients were considered to be clinically worsening (n = 35) if they were transferred to the intensive care unit (n = 28) or died (n = 12) and clinically improving if not. Five patients had anticoagulant treatment before admission for atrial fibrillation. During hospitalization, 46 patients had prophylactic anticoagulation. Nine patients developed venous thrombosis: 5 and 3 pulmonary embolisms in clinical worsening and improving group respectively, and 1 superficial venous thrombosis in clinical improving group. Only one patient who had developed thrombosis did not have thromboprophylaxis. None patient developed arterial thrombosis. Demographic and clinical data were reported in Table 1. Age and oxygen dependency at the time of admission were significantly different between groups. As expected, anticoagulation instauration and hospitalization duration were reported significantly different between groups as well as the radiological scale of disease extension.

In biological markers, we observed a non-significant difference in lymphocytes blood count<1G/L and significantly difference between clinical worsening and improving for monocyte blood count<0.2G/L. Moreover, neutrophil/lymphocyte ratio was not significant different, and we demonstrated that neutrophil/monocyte ratio was increased in worsening group. Fibrinogen levels and D-dimer were also increased in worsening group.. Fibrin monomers and antithrombin levels were not significantly different. International Society Thrombosis and Haemostasis DIC score was calculated at the time of admission either with D-dimer or with Fibrin monomer. We observed a significant difference between worsening and improving patients with ISTH DIC score with D-dimer and no difference with fibrin monomer scores were significantly different (2 [2-3] vs 2 [2-2], and 0 [0-0.25] vs 0 [0-1] respectively with D-dimer and Fibrin monomer).

VWF:GPIb-binding activity were also different ($p<0.01$ and $p<0.05$ respectively). Coagulation activation was studied thanks to thrombin generation assay and prothrombin fragments 1+2 measurement with a significant difference amongst the two groups.

Predictive factors for clinical worsening

As described in *Data and statistical analysis* section, clinical, radiological and biological parameters were used as predictors for logistic regression analysis in order to determine predictors of clinical worsening outcome (Table 2). Each variable was independent with Spearman correlation and was not confounding (Table 1 supplemental data). Final model of the logistic regression analysis revealed that oxygenodependance (RR=7.27 [1.50-19.31]; p=0.045), monocytes below 0.2G/L (RR=2.88 [1.67-3.19]; p=0.015), fibrinogen levels (RR=1.45 [1.17-1.82] per g/L increase; p=0.005), prothrombin fragments 1+2 higher than 290 pM (RR=2.39 [1.20-3.30]; p=0.023) and peak of the TGA assay (RR=1.28 [1.03-1.59] per 50 nM increase; p=0.043), were associated with an increased risk of clinical worsening (Table 2).

Classification tree of clinical outcome

Based on the predictors of the final model of the logistic regression, a classification tree was built in order to establish a hierarchical ranking of predictors to classify patients between clinical worsening and improving. Fibrinogen levels below 5.5 g/L was associated with clinical improving (N=35/40, 87.5%). For patients with higher value than 5.5 g/L, a TGA peak below 99 nM is also predictive of favourable outcome (N=11/12, 90.9 %). Then, for patients with fibrinogen higher than 5.5 g/L and TGA peak higher than 99 nM, patients had a better clinical outcome prognosis if they did not depend of oxygen when compared with patients who need it (N=5/6, 83.3% and N=28/42,66.7% respectively). This classification tree provided an accuracy of 79%, a sensitivity of 88%, a specificity of 67%, a positive predicted value of 78% and a negative predicted value of 80% (Figure 1).

Predictive factors for pulmonary embolism

Based on the 8 pulmonary embolisms of our cohort, we evaluated haemostasis test to predict thromboembolism event occurrence. Using ROC curves, thrombin peak and prothrombin fragments 1+2

on admission with a threshold of 204,0 nM and 393 pM were sensitive and specific to predict PE (Figure 2A: AUC: 85.7% Se: 70.8% Sp: 100% and Figure 2B AUC: 81.5% Se: 75.0% Sp: 86.8% respectively). The ROC curve of D-dimer (with a threshold of 2558 µg/L) and of VWF:GPIb-binding activity (with a threshold of 305%) had also a good sensibility and specificity to predict thrombosis development(Figure 2C: AUC: 77.5% Se: 75.0% Sp: 89.0% and Figure 2D AUC: 76.0% Se: 75.0% Sp: 75.8% respectively).

Discussion

Our study aimed to demonstrate with clinical, radiological and haemostasis markers the possibility to predict clinical worsening in Covid-19 patients and to determine the best thrombotic event predictor.

Since December 2019, several clinical and biological markers were associated with poor prognosis in Covid-19 patients. Elderly, increase body mass index, hypertension (28), diabetes and male gender (29) were regularly associated with mortality. Our study only confirmed elderly as a potential predictor under unadjusted hypothesis and a trend for gender, HTA and diabetes that could be due to our limited sample size (99 patients of which 35 worsening). As expected, oxygen therapy in preadmission was a predictive factor to develop worsening SARS-CoV-19. Chest computed tomography examination is recommended for the detection of lesions, early diagnosis, and assessment of the disease extension (19). Interestingly in CT scan, pulmonary disease extension >25 % was associated with a rapid progression of Covid-19 pneumonia and worsening in our patients.

Lymphopenia is a prominent part of severe Covid-19. A meta-analysis described lymphopenia<1.5 G/L predicting the severity clinical outcomes (30), however lymphopenia is defined as under 1 G/L in our laboratory and more than 78% of our cohort exhibited lymphocytes level below 1.5 G/L (56% below 1G/L). Interestingly, our results suggest a non- significant relationship between lymphopenia and SARS-CoV-2 severity. To our knowledge, we first describe that monocytopenia below 0.2 G/L could be related to Covid-19 severity. This is in accordance with the fact that a decrease monocyte count is associated with poor prognosis in sepsis (31). Recruitment of monocytes is essential for effective control and clearance of viral, bacterial, fungal and protozoal infections (32) . The inflammatory recruitment failure is also possible explanation to aggravation.

Yan *et al.* reported a correlation between neutrophil to lymphocyte ratio at hospital admission and all-cause in-hospital mortality (33). In our cohort, neutrophil to lymphocyte ratio was not associated with intensive care unit hospitalization and death. Interestingly, the fact that neutrophil to monocyte ratio was increased in worsening patients could be explained by a reduced monocyte count.

It has been previously demonstrated that fibrinogen and D-dimer were whereas antithrombin was normal or mildly decreased in worsening patients compared with clinical improving (34-36). In the review, published by Violi *et al.* (37), it was resumed that D-dimer, PT and aPTT were often compared between survivors and non-survivors or severe and non- severe patients. In all cases, D-dimer level was significantly higher in severe or non survivors patients than in non-severe or survivors patients

respectively. Our results suggest that fibrinogen<5.5 g/L was associated with a better outcome in our patients, with a high discriminate power and in a more specific manner than D-dimer does.

Several studies have described DIC in some Covid-19 patients. In the study of Fogarty et al. (38), DIC was rare and appeared in the late stage disease. In two others studies (8, 39) DIC was significantly more frequent in non survivors than in survivors. In contrast in the 24 patients from Panigada's report (34) DIC was not evidenced. With International Society Thrombosis and Haemostasis score, we demonstrated DIC score increase with D-dimer, in worsening patients with more than 75% with a DIC score below of 3. With fibrin monomer, more than 75% worsening patients had a DIC score below of 1. Furthermore, the increase of platelet and fibrinogen, associated with normal prothrombin time in our patients explain normal DIC score results.

Interestingly, our results demonstrated that the association of fibrinogen level, thrombin peak measurement and oxygen dependency was an easy-to-apply model that could predict near than 80% of clinical outcome. Of note, we observed 33/35 patients with oxygenodependence in clinical worsening group among which 26 had fibrinogen level higher than 5.5 g/L and TGA peak higher than 99 nM suggesting the ability of these last two parameters to predict clinical outcome.

In the Study of Panigada *et al.* (34), von Willebrand factor antigen and ristocetin cofactor activities were very increased. In Poissy et al. study (40), factor Willebrand antigen levels seem to be associated with a greater PE risk. Our study demonstrated an increase in von Willebrand factor activity in patient whose worsening. However, VWF:GPIb-binding activity was not the most predictive factor of thrombotic development during hospitalization, the AUC sensibility and specificity being 76.0%, 75.0% and 75.8% respectively, with a threshold 305 %.

In the literature, up until now, there was no description of thrombin generation profile in Covid-19 patients. Three studies looked at procoagulant profile thanks to thromboelastography (34, 36) or viscoelastic tests (35). In these studies, hypercoagulant profile was demonstrated in patients with acute respiratory distress syndrome (ARDS) or admitted to ICU. TGA has already been used to evaluate hypercoagulability (41) and acute ischemic stroke development (42). The fact that SARS-CoV-2 virus induces severe endothelial injury associated with intracellular virus and disrupted endothelial cell membranes (43) make TGA an interesting tool to predict clinical outcome of SARS-CoV 2 infected patients since microangiopathy and occlusion of alveolar capillaries from lung patients with Covid-19 were founded to be secondary to widespread vascular thrombosis (43). Interestingly, the global thrombin formation, reflected by ETP, is not increase (990 nM.min [718-1237] vs 1132 nM.min [905-1465] for improving and worsening patient respectively) but we observed an increase in peak and velocity in clinically worsening patients. Activated coagulation is an expected response to the inflammatory through several procoagulant pathways. Usually, thrombin generation participate to host response, but when exaggerated, it is associated with thrombosis. The endothelium supports an extensive repertoire of natural anticoagulant. However during sepsis, activated endothelium increase TF expression within the vasculature is considered a pivotal step in initiating and sustaining coagulation. The concept of thrombosis associated with inflammation is

known as thromboinflammation (44). Overall, the results of TGA support the concept that the hypercoagulability is associated with endothelial dysfunction, due to the profound activation and amplification of coagulation.

According to many reports, Covid-19 exposes patients to a particularly high risk for venous thromboembolism (45, 46). The prevalence of pulmonary embolism in the ICU is near 20% (40). ISTH and the American Society of Hematology recommended prophylactic dose of low molecular weight heparin to prevent thrombotic event (47-49). Even with thromboprophylaxis, 7.7% of patients in academic hospital of Milan developed thrombotic event (50). The association between Padua score>4 and D-dimer>1000 µg/L had a sensitivity of 88.52% and a specificity of 61.43% for screening risk for DVT (51). In our study, the AUC sensibility and specificity being 77.5%, 75.0% and 89.0% respectively, with a threshold 2555 µg/L. No study was reported about prothrombin fragments 1+2 in Covid-19. Prothrombin fragments 1+2 are less impacted by inflammation than D-dimer (52). In our study on admission, thrombin peak was extremely specific (with 100%) and prothrombin fragment 1+2 was sensitive to predict PE. The association between different haematological markers could help to predict, and potentially, increase anticoagulation thromboprophylaxis. Our results supported a possible worsening and PE prediction with prothrombin fragments 1+2, even with a small number of cases.

Nevertheless, our study presents several limitations. First, we have a low number of included patients. However, our objectives (clinicaltrials: NCT04367662) were to determine a rapid method to help clinician for patient discharged. Despite our 99 patients, we have a robust algorithm to predict worsening. Second, we had a low number of thrombotic events. However, the rate was in accordance with other studies with patient who developed a pulmonary embolism.

Conclusion

In conclusion, we identified biological markers predictive of an evolution towards clinical complications: high fibrinogen levels at admission were predictive of secondary admission in intensive care unit or death. Furthermore, thrombin peak and prothrombin fragment 1+2 seem to be specific haematological marker to predict thrombotic event, even in patients with thromboprophylaxis. Our results should therefore be considered as exploratory and deserve confirmation.

Declarations

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Author contributions

1. Billoir designed the research, performed analysis, analyzed and interpreted the data and wrote the manuscript. V. Le Cam Duchez analyzed and interpreted the data and wrote the manuscript. T. Duflot critically revised the manuscript and checked the statistical methods and results. K. Alexandre, M. Roger, S. Miranda, O. Goria, LM. Joly, M. Demeyere, G. Feugray,
2. Etienne included patients and discussed the obtained results and critically revised the manuscript. All authors read and approved the final version of the manuscript.

Disclosure of Conflict of Interests

Authors declare no conflict of interest

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Tables

Due to technical limitations, tables 1-2 are only available as downloads in the supplemental files section.

Figures

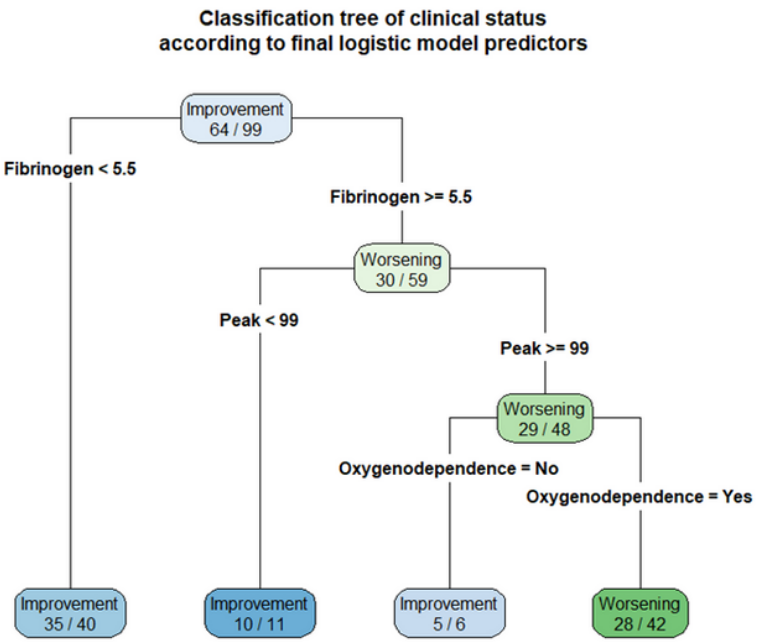

Figure 1. Classification three of clinical status according to final logistic model predictors

Figure 1

Classification three of clinical status according to final logistic model predictors

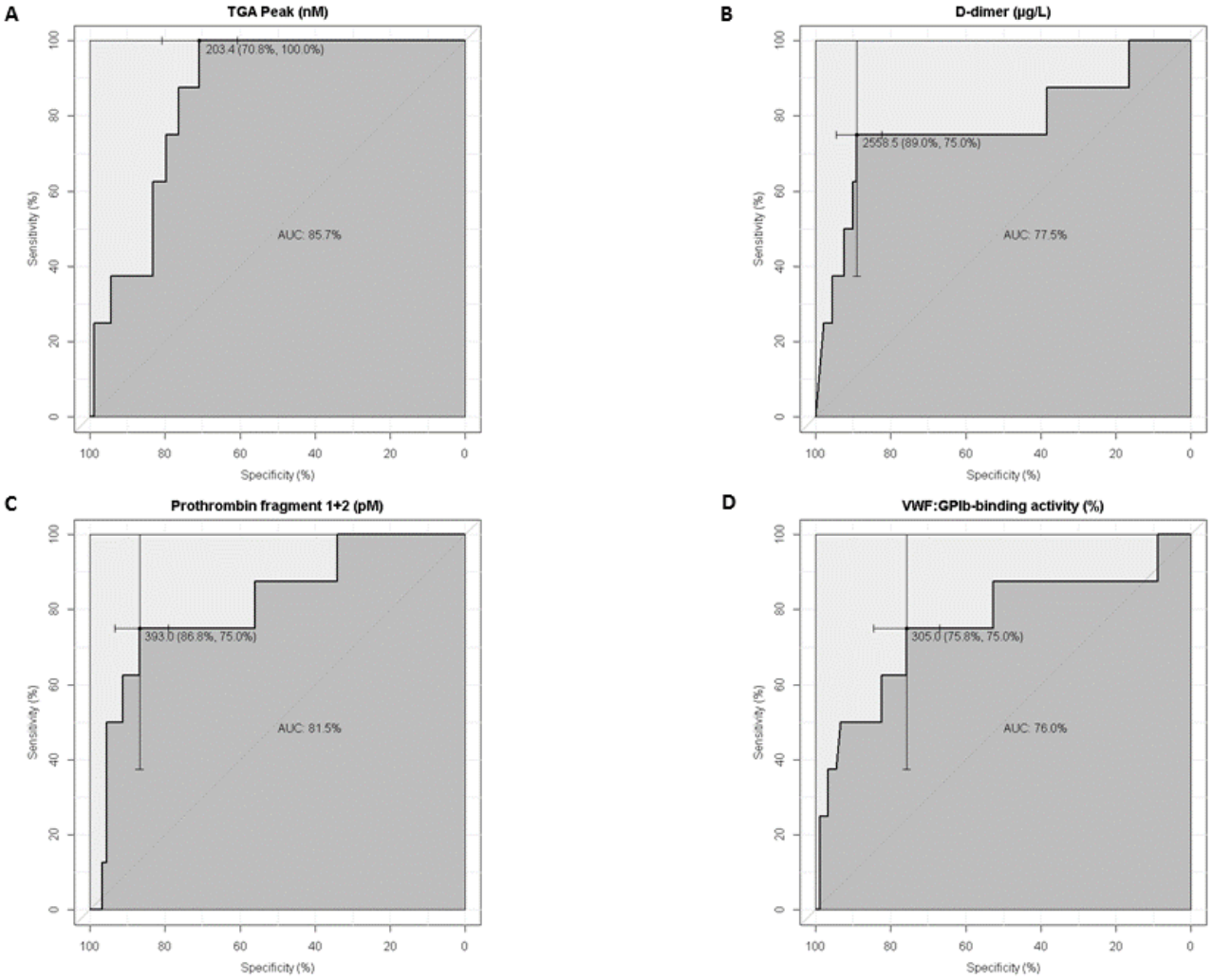

Figure 2. ROC curve model to predict thrombotic event in COVID patients. (A) Thrombin peak. **(B)** Prothrombin fragment 1+2. **(C)** D-dimer. **(D)** VWF:GPIb-binding activity.

Figure 2

ROC curve model to predict thrombotic event in COVID patients. (A) Thrombin peak. (B) Prothrombin fragment 1+2. (C) D-dimer. (D) VWF:GPIb-binding activity.

Supplementary Files

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