Covid-19-associated coagulopathy (CoAC): thrombin burst and insufficient fibrinolysis leading to bad outcome.

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Research

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Abstract

Background: COVID-19 associated coagulopathy is characterized by a pro-thrombotic state. However, the nature of this pattern has not been comprehensively studied. We investigated the coagulation pattern of patients with COVID-19 acute respiratory distress syndrome (ARDS) comparing survivors to not survivors.

Methods: Prospective cohort study conducted in the Intensive Care Unit (ICU) of a University Hospital . Twenty COVID-19 ARDS patients received measurements of markers of thrombin generation (prothrombin fragment 1+2, PF 1+2); fibrinolysis activation (tissue plasminogen activator, tPA) and inhibition (plasminogen activator inhibitor-2, PAI-2); fibrin synthesis (fibrinopeptide A) and fibrinolysis magnitude (plasmin-antiplasmin complex, PAP, and D-dimers). Measurements were done at the ICU admission and after 10-14 days.

Results: The general pattern showed an increased thrombin generation, modest or null release of t-PA, and increased levels of PAI-2, Fibrinopeptide A, PAP and D-dimers. At baseline, non survivors had a significantly (P=0.014) higher PAI-2/PAP ratio than survivors (109, interquartile range [IQR] 18.1-216, vs. 8.7, IQR 2.9-12.6). At follow-up, thrombin generation was significantly (P=0.025) reduced in survivors (PF 1+2 from 396 pg/mL, IQR 185-585 to 237 pg/mL, IQR 120-393), whereas it increased in non-survivors. Fibrinolysis inhibition at follow-up remained stable in survivors, and increased in non-survivors, leading to a significant (P=0.026) difference in PAI-2 levels (161 pg/mL, IQR 50-334, vs. 1,088 pg/mL, IQR 177-1,565).

Conclusions: Severe patterns of COVID-19 infection (ARDS) are characterized by a thrombin burst, triggered by the release of IL-6 and other cytokines, and the consequent release of Tissue Factor. Mechanisms of fibrinolysis regulation appear unbalanced toward fibrinolysis inhibition. In survivors, this pattern ameliorates, whereas in non-survivors it worsens, leading to the environment for clinically relevant thrombi generation, that was found in 58% of non-surviving patients. Trial registration: clinicaltrials.gov (NCT04441502).

Background

COVID-19-associated coagulopathy (CoAC) is a recognized entity which determines morbidity and mortality, especially in patients with acute respiratory distress syndrome (ARDS). CoAC is characterized by elevated levels of fibrinogen and D-dimers [1-5] Different reports have shown either thrombocytosis[4,6] or mild thrombocytopenia[7,8] with variable changes in activated partial thromboplastin time (aPTT) and prothrombin time (PT).[4,9] Clinical series[10,11], autopsy reports[12] and computerized tomography angiography[13] have highlighted the clinical consequences of CoAC, represented by a number of thromboembolic complications, especially at the level of the pulmonary circulation. Conversely, hemorrhagic complications are rare, even if patterns of disseminated intravascular coagulation (DIC) have been reported in patients died due to COVID-19 infection.[3] Even if the CoAC pattern includes some early phase, thrombotic-type DIC finding, levels of endogenous anticoagulant proteins may be normal, as

well as platelet count. The clinical impact of CoAC is relevant, with a high incidence of thromboembolic complications, found in up to 50% of the patients who have been admitted to the ICU for over 2 weeks.[16] To this respect, CoAC appears as a peculiar entity, posing an important challenge and therapeutic dilemmas to the clinicians.

At present, a comprehensive analysis of the complex mechanisms underlying CoAC is lacking, with a gap in knowledge with respect to the balance between the different factors regulating thrombin generation, clot formation and fibrinolysis.

The purpose of the present study is to elucidate the mechanism(s) underlying CoAC in patients with COVID-19 ARDS through the measure of coagulation and fibrinolysis markers, and to investigate the relationship between CoAC and the outcome of COVID-19 ARDS patients mechanically ventilated in the Intensive Care Unit (ICU).

Methods

The present study is part of a wide project (COVID-OMICS) prospectively undertaken at the IRCCS Policlinico San Donato at the beginning of the COVID-19 pandemic. The study was approved by the Local Ethics Committee of San Raffaele Hospital (Code: 75/INT/2020) and registered at clinicaltrials.gov (NCT04441502). All the survived patients gave a written informed consent. The coagulation arm was planned on 20 COVID-19 ARDS patients admitted to the ICU and mechanically ventilated.

Patient population and treatments

Twenty patients were randomly selected within our population of COVID-19 ARDS patients admitted in the ICU and mechanically ventilated. The first patient was admitted on March 27th, 2020, and the last on April 21st, 2020. During the course of their stay in the ICU, they received variable treatments according to the changing scenario of international recommendations. These included hydroxychloroquine, tocilizumab, and steroids (2 mg/kg methylprednisolone for 5 days followed by 0.5 mg/kg in the next days).

Anticoagulation was established according to a local protocol already published[6], with an aggressive regimen of low-molecular weight heparin (LMWH): 6000 b.i.d. (8000 IU b.i.d. if body mass index > 35). Additionally, antithrombin concentrate was applied to correct antithrombin activity values < 70%; antiplatelet agents were used (clopidogrel loading dose 300 mg + 75 mg/day) if platelet count > 400,000 cells/μL.

All the patients were sedated with propofol or midazolam, and mechanically ventilated under full muscle relaxant dose at baseline; survivors could still be under full mechanical ventilation support or under weaning from mechanical ventilation at the time of follow-up.

Measurements

We measured the following coagulation and fibrinolysis markers: prothrombin fragment 1.2 (PF 1+2, ELISA LS-F23736 LifeSpan BioSciences, Seattle), a marker of thrombin generation; plasmin-antiplasmin complex (PAP, ELISA LS-F21825 LifeSpan BioSciences, Seattle), a marker of plasmin generation; tissue plasminogen activator (tPA, Elisa DuoSet DY7449-05 R&D Systems, Biotechne, Minneapolis), a marker of fibrinolysis activation; plasminogen activator inhibitor 2 (PAI-2, Elisa DuoSet DY8550-05 R&D Systems, Biotechne, Minneapolis), a marker of fibrinolysis inhibition; and fibrinopeptide A (ELISA LS-F20727 LifeSpan BioSciences, Seattle), a marker of fibrin generation.

Besides these markers, we measured the ratio between PAI-2 (pg/mL) and PAP (ng/mL) as a marker of the balance between fibrinolysis inhibition and fibrinolysis amount.

Additionally, the standard hemostasis and coagulation characterization included the measure of the aPTT, INR, platelet count, fibrinogen, D-dimers, and antithrombin (AT) activity. INR and aPTT were assessed using the STA-NeoPTimal 10 and the STA-Cephascreen 10 (Diagnostica Stago), respectively; fibrinogen was measured using the Clauss-based STA-LiquidFib (Diagnostica Stago).

The blood samples were collected at different points in time: at the admission in the ICU and at variable intervals (5-7 days) from the admission in the ICU. For the purposes of the present analysis, we considered two points in time: baseline (admission in the ICU) and follow-up. This corresponded to the last measure before dead in non-survivors, and to a similar point in time in survivors.

Outcome assessment

The primary outcome was defined as survival at 45-days from ICU admission or mortality within 45 days from ICU admission. Thromboembolic events at the level of pulmonary circulation were defined on the basis of computerized tomography (CT) when available. No routine assessment of venous thrombosis at other sites was performed.

Statistics

Data are presented as number (%) or median (interquartile range, IQR). Differences between groups (survivors vs. non-survivors) were tested with non-parametric tests (Mann-Whitney U-test) and differences between baseline and follow-up within groups with a Wilcoxon Signed Rank Test. Predictive ability of the different parameters were tested with a Receiver Operating Characteristics (ROC) analysis producing c-statistics, and sensitivity, specificity, positive and negative predictive values (PPV and NPV, respectively) for the identified cut-off values. For all the tests, a two-tailed P value < 0.05 was considered significant. The statistical analyses were conducted using computerized packages (SPPS 13.0, IBM, Chicago, IL, and MedCalc, Ostend, Belgium).

Results

Overall, we reported 8 (40%) survivors and 12 (60%) non-survivors. Table 1 reports the general characteristics and standard coagulation tests in the patient population. Factors being significantly

associated with mortality were age and the CT evidence of pulmonary thromboembolism, found in 7 (35%) patients all belonging to the non-survivors group, with a significant (P=0.015) between-groups difference.

Table 2 reports the coagulation parameters related to thrombin generation and fibrinolytic profile of the patient population, at baseline and follow-up. The median time between baseline and follow-up was 17 days (IQR 14-24 days) in survivors, and 13 days (IQR 6-22 days) in non-survivors (P=0.134).

At baseline assessment, the only significant (P=0.014) difference between survivors and non-survivors was a ten-time higher value of the PAI-2/PAP ratio in non-survivors. In both groups the values of fibrinogen, D-dimers, PAI-2, PF 1+2, PAP, and Fibrinopeptide A were above the normal range reported in the literature. Conversely, the value of tPA was within the normal range. At follow-up, non-survivors had a significantly higher value of D-dimers and PAI-2 (P=0.003 and P=0.026, respectively).

There were within-groups differences between values at baseline and follow-up. Survivors showed a significant (P=0.025) decrease of PF 1+2, and a non-significant decrease of fibrinogen, tPA, PAI-2, PAP and PAI-2/PAP ratio. Non-survivors showed a non-significant increase in D-dimes, PAI-2, PF 1+2, while PAI-2/PAP ratio remained stable.

In the overall patient population, PAP concentrations were strongly and directly correlated with Fibrinopeptide A concentrations at baseline (R^2: 0.98, P=0.001) and moderately correlated at follow-up (R^2:0.51, P=0.001). Conversely, D-Dimers were not dependent on Fibrinopeptide A levels (figure 1).

The ability of predicting mortality of the different markers measured at baseline was investigated with an ROC analysis. The only parameters with a c-statistics $\geq$ 0.80 were D-dimers and the PAI-2/PAP ratio, with values of 0.813, and 0.875, respectively (figure 2). The best cut-off values (best fit between specificity and sensitivity) were found at a level of

1.13 µg/mL for D-Dimers and 12.9 for the PAI-2/PAP ratio. These values correspond to a

sensitivity of 83.3% for D-Dimers and of 83.3% for PAI-2/PAP ratio, and a specificity of 62.5 % for D-Dimers and 87.5% for PAI-2/PAP ratio. Considering a prevalence of mortality of 55% (the one recorded in the whole patient population admitted to the ICU in our Institution), the PPV and NPV for D-dimers were 73.1% and 75.4%, respectively, while for PAI-2/PAP ratio they were 89.1% and 81.1%, respectively.

Discussion

Our results provide an interpretation of the already known pro-coagulant pattern of patients with ARDS due to COVID-19 infection, and, to our knowledge, this is the first investigation of the coagulation profile based on markers of thrombin generation and fibrinolysis. Previous studies with viscoelastic tests had already stressed that the main finding in these patients is an abnormally increased clot firmness, with no signs of hyperfibrinolysis or even fibrinolysis shutdown.[6, 15-17] However, analyses based on standard or

viscoelastic tests remain inconclusive with respect to the nature of this pattern. From this respect, our study suggests an interpretative view of the major factors determining the CoAC, and on their differences in survivors and non-survivors.

Thrombin generation

Thrombin generation cannot be assessed with standard or viscoelastic tests. In the first case, variable values of aPTT and PT have been reported[1-6], but their changes obviously reflect even the effects of the antithrombotic therapies. In the second, the reaction times (measured with heparinase) did not show a decreased value suggestive for an increased thrombin generation.[15,17] We addressed thrombin generation by measuring PF 1+2, a marker of prothrombin cleavage to thrombin. We found values ranging from 20 to over 2,300 pg/mL at baseline (median 442 pg/mL), and from 20 to over 3,300 pg/mL at follow-up (median 371 pg/mL). The normal values of PF 1+2 in healthy subjects is between 11 and 22 pg/mL, and hence a strong thrombin generation is present in COVID-19 ARDS patients. In other models of severe sepsis, median values of 100-200 pg/mL were reported[18]; in our series, thrombin generation is almost double these values. Of notice, thrombin generation behaved differently in survivors and non-survivors. At baseline, there were no significant differences between groups; however, in survivors, thrombin generation significantly decreased at follow-up, whereas it remained stable or increased in non-survivors.

Fibrinogen and fibrin generation

Elevated fibrinogen levels are confirmed in our patient population, as already highlighted in other studies.[6,15,16] Fibrinogen levels are decrease by 35% at follow-up in survivors, and by 16% only in non-survivors. Fibrin generation was addressed by measuring Fibrinopeptide A, a marker of fibrinogen cleavage to fibrin. Normal levels of Fibrinopeptide A range between 0.1 and 2 ng/mL, with a mean at 0.50 ng/mL.[19] In our series, Fibrinopeptide A largely exceeded the upper limit of the normal range, both in survivors and non-survivors, at baseline and follow-up, with a trend toward higher values in survivors. Hence, as a logical consequence of the increased thrombin generation, fibrin generation is increased as well, and continues unabated from baseline to follow-up. Fibrinopeptide A increases in patients with bacterial and virus sepsis, as a consequence of the cross-link between inflammation and coagulation. The values found in our series are in the range of what previously found in patterns of bacterial sepsis, severe sepsis, and septic shock.[20]

Fibrinolysis activation

Tissue plasminogen activator is a fibrinolytic agent released mainly by endothelial cells as a response to fibrin generation. Its normal values are around 10,000 pg/mL[21,22], but in conditions of systemic inflammatory reaction syndrome or severe sepsis its values are usually higher (> 10,000 pg/mL)[23], with reported values up to 50,000-70,000 pg/mL in non-survivors.[24]

Quite surprisingly, in our series, the median value of tPA was at the lower limits of normal range both at baseline and follow-up, and both in survivors and non-survivors, with only one case reaching 20,000 pg/mL. Therefore, it apparently seems that fibrinolysis was not activated in these patients, despite an increased thrombin (and fibrin) generation.

Fibrinolysis inhibition

Plasminogen activator inhibitor-2 is a powerful inhibitor of fibrinolysis, released by monocytes. It is usually undetectable in plasma from normal subjects, and it is considered an inhibitor of urokinase-plasminogen activator acting mainly at an extravascular level.[24]

Due to its ability to act at the level of interstitial tissues (including lung interstitium) and to its non-detectability in normal subjects (except in pregnant women), we have measured PAI-2 as a marker of fibrinolysis inhibition. Previous studies highlighted that in septic patients PAI-2 becomes detectable, with values of 500-1,000 pg/mL in survivors and up to 30,000 pg/mL in non-survivors.[24]

In our series, elevated values of PAI-2 were observed especially in non-survivors, and at follow-up the level of PAI-2 was 6-folds that of survivors, with a significant between-groups difference. Therefore, fibrinolysis was inhibited, and the extent of inhibition at follow-up was associated with mortality.

The net effect on fibrinolysis

The markers of fibrinolysis in our series were the PAP complexes and the D-Dimers. PAP is a marker of plasmin formation and of plasmin ability to counteract fibrin generation. Not by chance, we could observe a strong relationship between PAP and the marker of fibrin generation Fibropeptide A. Levels of PAP are usually greatly increased under conditions of inflammation and sepsis, with levels exceeding 1,000 ng/mL.[25] We could only observe a modest increase of PAP with respect to the reported normal range of 19-27 ng/mL[25], more pronounced in survivors than in non-survivors. Therefore, fibrinolysis appears in a shutdown condition, as the result of the balance between increased antifibrinolytic agents (PAI-2) and stable fibrinolytic agents (tPA). This shutdown appears more pronounced in non-survivors, where the PAI-2/PAP ratio is significantly higher than in survivors.

Within this scenario, a particular aspect is represented by D-Dimers behavior. D-Dimers are a fibrin degradation product, and therefore their increase, found both in survivors and (to a larger extent) in non-survivors should be interpreted as marker of increased fibrinolysis. However, contrary to PAP, D-Dimers have no correlation with fibrin generation (as represented by Fibrinopeptide A). Therefore, their raise cannot be ascribed solely to the increased levels of fibrin. Actually, the source of D-Dimers increase in COVID-19 patients is still a matter of debate, and the role of extravascular fibrin degradation (namely in the interstitial and alveolar lung space) has been hypothesized.[26] A possible interpretation is that the

large concentration of substrate (fibrinogen) generates large amounts of fibrin, and that even in presence of a limited fibrinolysis, the gross amount of fibrin generates high levels of D-Dimers.

The overall picture that can be drawn from our results, is summarized in figure 3.

In both survivors (Panel A) and non-survivors (Panel B) there is an initial phase characterized by the release of proinflammatory cytokines and consequent burst of thrombin generation (reasonably mediated by monocyte and endothelial release of tissue factor). The endothelial cells show a very modest release of tPA. At this stage, both thrombin generation and tPA release do not differ between survivors and non-survivors. Conversely, in non-survivors the release of PAI-2 is higher than in non-survivors, switching the balance between fibrinolysis stimulation and inhibition toward the latter. In both groups there are similar and very high levels of substrate (fibrinogen), and an important increase of fibrin generation. However, fibrinolysis appear more efficient (higher PAP values) in survivors than in non-survivors. In both cases it is likely that an initial thrombi formation may intervene (elevated D-Dimers).

The two pathways clearly diverge at follow-up. In survivors, thrombin generation, PAI-2 and tPA release decrease, as well as fibrinogen levels. Fibrinolysis appears maintained and D-Dimers are stable. Conversely, in non-survivors, thrombin generation and PAI-2 increase, and tPA decreases, with a further shutdown of fibrinolysis and an important increase in D-Dimers. This is likely to represent a condition were thrombi formation may become uncontrolled and clinically relevant.

Thromboembolism, mortality and its predictors, and therapeutic implications

The prothrombotic pattern of CoAC has a relevant clinical impact. Other studies already highlighted the high risk of thromboembolic events in these patients.[10, 12-14] It is not the purpose of the present study to address the link between thromboembolic events and mortality. However, it deserves to be quoted that we could observe 7 events of pulmonary thromboembolism (either of minor or major degree) in our patient population, and all of them were diagnosed in patients who lately died. This confirms the uncontrolled thrombi formation in non-survivors, as depicted in figure 3.

The definition of the pathway of CoAC from the onset to the final outcome is of course important providing that (i) an early recognition of patients at high risk of mortality is feasible, and (ii) adequate diagnostic measures and therapies may be established.

With respect to the first issue, our data suggest a high predictive ability of the ratio PAI-2/PAP (cut-off at 12.9) early after the patient is tracheally intubated and placed under mechanical ventilation, and a moderate predictive ability of D-Dimers (cut-off at 1.13 μg/mL). Patients with values above these thresholds deserve a pulmonary CT scan angiography for early detection of micro/macro thrombi.

Conclusions

The pathophysiological process leading to negative outcome may be divided into 3 phases. Therapeutic choices are not the purpose of our study, but some possible interventions may be hypothesized, based on our time-related data.

The first is the containment of the inflammatory reaction with blunting of the pro-inflammatory cytokines. The drugs of choice are steroids: a very recent report demonstrated that, in patients under mechanical ventilation, dexamethasone therapy reduces mortality by 35%.[28] The second is the containment of thrombin generation: heparin is the most commonly used drug, but even direct thrombin inhibitors (argatroban and bivalirudin) may be considered. In our series, we used an aggressive dose of LMWH; this led to a successful reduction of thrombin generation in survivors, but not in non-survivors. It can be hypothesized that in this case, switching to intravenous unfractionated heparin could induce a greater containment of thrombin generation.

Finally, the third phase is tackling the fibrinolysis shutdown. In presence of elevated levels of fibrinolysis inhibitors, and with documented CT scan pulmonary thromboembolism, it seems reasonable to consider the administration of rtPA to prevent further thromboembolic events and to trigger thrombi dissolution.

In conclusion, our results stress the leading role of thrombin generation and most of all of fibrinolysis shutdown in determining the environment for pulmonary micro/macro vascular thrombosis and negative outcomes. Therapeutic implications require adequate randomized or case-control trials to achieve the required evidence of success.

List Of Abbreviations

aPTT: activated partial thromboplastin time

ARDS: acute respiratory distresssyndrome

CoAC: COVID-19 associated coagulopathy

CT: computerized tomography

DIC: disseminated intravascular coagulopathy

ICU: intensive care unit

INR: international normalized ratio

IQR: interquartile range

LMWH: low molecular weight heparin

NPV: negative predictive value

PAI: plasminogen activator inhibitor

PAP: plasmin-antiplasmin

PF 1+2: prothrombin fragment 1+2

PPV: positive predictive value

PT: prothrombin time

ROC: receiver operating characteristics

tPA: tissue plasminogen inhibitor

Declarations

Ethics approval and consent to participate: The study was approved by the Local Ethics Committee of San Raffaele Hospital (Code: 75/INT/2020) and registered at clinicaltrials.gov (NCT04441502). All the survived patients gave a written informed consent.

Consent for publication: not applicable

Availability of data and materials: the dataset used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Competing interests: the authors declare that they have no competing interests.

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Author contributions

Marco Ranucci designed the study, analyzed the data and wrote the manuscript. Clementina Sitzia performed the Elisa tests; Ekaterina Baryshnikova and Rosanna Cardani collected the blood samples and participated in the study design and data analysis; Umberto Di Dedda helped in collecting blood samples, data analysis, and critically revised the manuscript; Fabio Martelli was responsible for the whole COVID-OMICS study design and participated in the coagulation arm study design; Massimiliano Corsi Romanelli participated in data analysis and critically revised the manuscript.

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Tables

Due to technical limitations, tables 1 and 2 are only available as a download in the supplemental files section

Figures

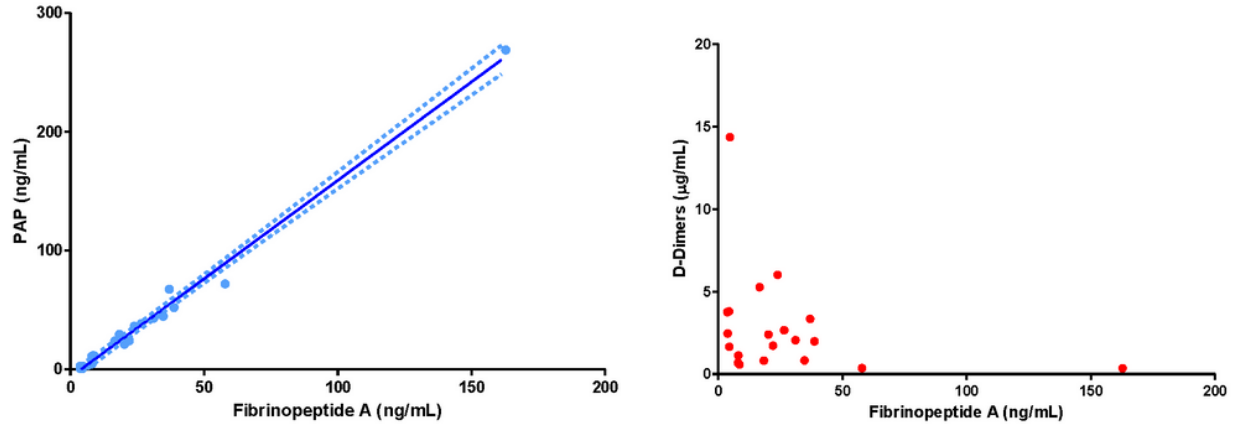

Figure 1

Figure 1

Association between plasma levels of Fibrinopeptide A and Plasmin-AntiPlasmin (PAP) complexes (panel A) and D-Dimers (Panel B). Data in the text.

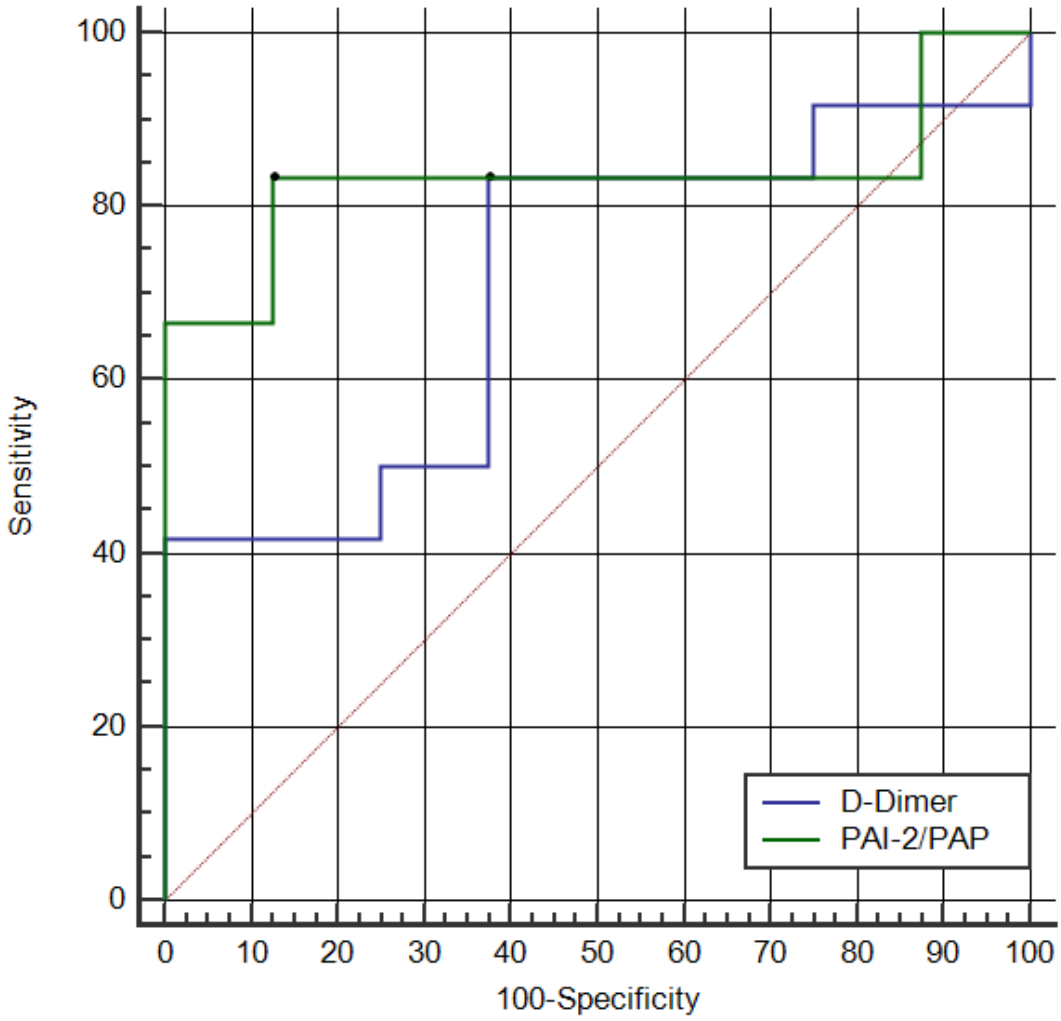

Figure 2

Receiver Operating Characteristics analysis for predictive ability of mortality. PAI2: Plasminogen Activator Inhibitor-2; PAP: plasmin-antiplasmin complexes. Data in the text.

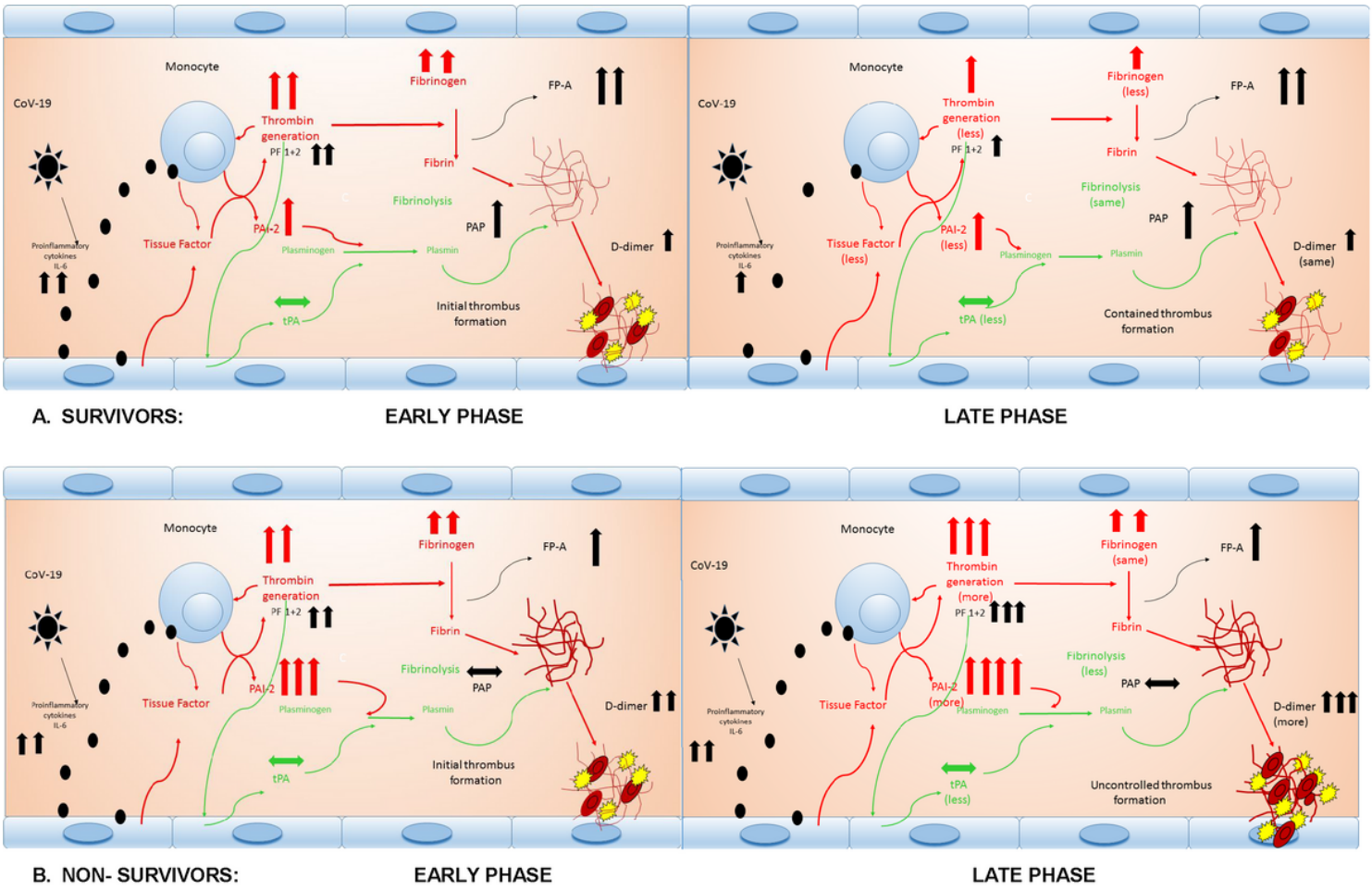

Figure 3

Figure 3

The chain of inflammatory and hemostatic reactions in survivors and non-survivors, from baseline to late phase. FP-A: fibrinopeptide A; IL-6: interleukin-6; PAI-2: plasminogen activator Inhibitor-2; PAP: plasmin-antiplasmin complex; PF 1+2: prothrombin fragment 1+2; t-PA: tissue plasminogen activator. Red lines are pro-thrombotic, green lines anti-thrombotic.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- Table1.png
- Table2.png