The Activin/Follistatin-axis is severely deregulated in COVID-19 and independently associated with in-hospital mortality

Evgenia Synolaki[1], Vasileios Papadopoulos[2*], Georgios Divolis[1*], Olga Tsahouridou[3], Efstratios Gavriilidis[2], Georgia Loli[3], Ariana Gavriil[1], Christina Tsigalou[4], Nikolaos R. Tziolos[5], Eleni Sertaridou[6], Bhanu Kalra[7], Ajay Kumar[7], Petros Rafailidis[8], Arja Pasternack[9], Dimitrios T. Boumpas[5], Georgios Germanidis[3], Olli Ritvos[9], Simeon Metallidis[3], Panagiotis Skendros[2,10,#] and Paschalis Sideras[1,#]

[1]Biomedical Research Foundation Academy of Athens, Center for Clinical, Experimental Surgery & Translational Research, Athens, Greece; [2]First Department of Internal Medicine, University Hospital of Alexandroupolis, Democritus University of Thrace, Alexandroupolis, Greece; [3]First Department of Internal Medicine, AHEPA University Hospital, Aristotle University of Thessaloniki, Thessaloniki, Greece; [4]Laboratory of Microbiology, University Hospital of Alexandroupolis, Democritus University of Thrace, Alexandroupolis, Greece; [5]Fourth Department of Internal Medicine, Attikon University Hospital, National and Kapodistrian University of Athens Medical School, Athens, Greece; [6]Intensive Care Unit, University Hospital of Alexandroupolis, Alexandroupolis, Greece; [7]AnshLabs, Webster, Texas, USA; [8]Second Department of Internal Medicine, University Hospital of Alexandroupolis, Democritus University of Thrace, Alexandroupolis, Greece; [9]Department of Physiology, Faculty of Medicine, University of Helsinki, Helsinki, Finland; [10]Laboratory of Molecular Hematology, Democritus University of Thrace, Alexandroupolis, Greece;

[*]These authors contributed equally to the study.

[#]These authors supervised equally the study.

Corresponding author: Dr. Paschalis Sideras; Biomedical Research Foundation Academy of Athens, Center for Clinical, Experimental Surgery & Translational Research, Athens, Greece; tel: +302106597183; e-mail: sideras@bioacademy.gr

Alternate corresponding author: Dr. Panagiotis Skendros; [2]First Department of Internal Medicine, University Hospital of Alexandroupolis, Democritus University of Thrace, Alexandroupolis, Greece; tel: +302551351090; e-mail: pskendro@med.duth.gr

Brief Summary

The Activin/Follistatin-axis is significantly deregulated in the context of COVID-19 pathophysiology. FACT-CLINYCoD, a novel scoring system based on peripheral blood Activin A, B and Follistatin assessment in conjunction with classical biomarkers permits dynamic prediction of COVID-19 outcome supporting real-time clinical decision.

Abstract

Background: Activins are members of the TGFβ-superfamily implicated in the pathogenesis of several immuno-inflammatory disorders. Based on our previous studies demonstrating that over-expression of Activin-A in murine lung causes pathology sharing key features of COVID-19, we hypothesized that Activins and their natural inhibitor Follistatin might be particularly relevant to COVID-19 pathophysiology.

Methods: Activin-A, Activin-B and Follistatin levels were retrospectively analyzed in 574 serum samples from 263 COVID-19 patients hospitalized in three independent centers, and compared with common demographic, clinical and laboratory parameters. Optimal-scaling with ridge-regression was used to screen variables and establish a prediction model.

Result: The Activin/Follistatin-axis was significantly deregulated during the course of COVID-19, correlated with severity and independently associated with mortality. FACT-CLINYCoD, a novel disease scoring system, adding one point for each of Follistatin>6235pg/ml, Activin-A>591pg/ml, Activin-B>249pg/ml, CRP>10.3mg/dL, LDH>427U/L, Intensive Care Unit (ICU) admission, Neutrophil/Lymphocyte-Ratio>5.6, Age>61, Comorbidities>1 and D-dimers>1097ng/ml, efficiently predicted fatal outcome in an initial cohort (AUC: 0.951; 95%CI: 0.919-0.983, $p<10^{-6}$). Two independent cohorts that were used for validation indicated similar AUC values.

Conclusions: This study unravels strong link between Activin/Follistatin-axis and COVID-19 mortality and introduces FACT-CLINYCoD, a novel pathophysiology-based tool that allows dynamic prediction of disease outcome, supporting clinical decision making.

Key words: COVID-19, SARS-CoV-2, Activin, Follistatin, outcome

Introduction

COVID-19 pandemic constitutes presently the most prominent public-health issue worldwide [1]. The disease follows a bimodal pattern. The initial phase of COVID-19 relates to viral pathogenic effects and is characterized by mild "flu-like" symptoms [2]. However, approximately one week after onset-of-symptoms, ~20% of patients develop moderate to severe acute respiratory distress syndrome (ARDS), thereby increasing substantially in-hospital mortality [2–5]. Various comorbidities and aging negatively affect COVID-19 outcome [3,6]. Accumulating evidence indicate that excessive activation of innate-immunity, deregulated neutrophils and thrombotic microangiopathy characterize the maladaptive host-response that drives the pathophysiology of severe COVID-19 [7–11]. These pathomechanisms lead to rapid progression of hypoxemic respiratory failure and protean clinical manifestations that involve almost every organ [12,13]. Monitoring hospitalized COVID-19 patients, in the midst of multiple continuously changing parameters is challenging [2,3,5,13]. Therefore, development of novel therapeutic strategies and bed-to-bench tools permitting day-to-day prediction of patient outcome is of utmost importance for such dynamically evolving and clinically heterogeneous disease [12,14–16].

Activin-A and Activin-B are members of the Transforming Growth Factor-β (TGF-β)-superfamily implicated in the regulation of numerous aspects of inflammation and/or tissue remodeling [17–19]. Follistatin, a physiological Activin inhibitor, binds to them, induces endocytosis and proteolytic degradation and modulates their bioavailability [20]. Activins and Follistatin, are synthesized continuously in healthy tissues [21]. However, in immuno-inflammatory conditions, epithelial, endothelial,

interstitial stroma cells and immune cells secrete higher levels that can be detected in serum as biomarkers of local or systemic stress [17–19].

We have previously described over-expression of Activin-A and Follistatin in bronchoalveolar lavage (BAL) of ARDS patients [18,22]. Moreover, we showed that ectopic expression of Activin-A in murine lungs causes ARDS-like pathology [22], which shares cardinal features of COVID-19 pathophysiology. These include, neutrophils infiltration in the lung, alveolar epithelial and endothelial cell damage, systemic hyper-coagulant state and a deregulated cytokine response [5,7,8,18,23].

In view of these findings we hypothesized that the Activins might be particularly relevant to COVID-19. To validate this hypothesis, we analyzed sera from COVID-19 patients and found that Activin-A, Activin-B and Follistatin, herein referred to as the Activin-Follistatin-axis (A/F-axis) were significantly upregulated during the crucial period when patients tend to deteriorate. Of note, these A/F-axis components were independently associated with disease severity and in-hospital mortality. Based on that, we developed and validated a scoring-system for prediction and monitoring of COVID-19 outcome in real-life using Activins, Follistatin and common clinical/laboratory parameters.

Materials and methods

Study design

This is a retrospective study with a single endpoint, the final outcome (survival or death). Three national reference hospitals from distant regions of Greece participated in the study (Supplementary Figure 1A). An initial cohort of 117 consecutive COVID-19 patients hospitalized at University Hospital, Alexandroupolis and “AHEPA” Hospital, Thessaloniki from March 10, 2020 and had an outcome until

July 7, 2020 was endorsed and 314 randomly acquired samples were analyzed (Table 1 and Supplementary Figure 1B). Two independent validation cohorts belonging to the "second COVID-19 wave" between end of July and early October 2020 were introduced: The first included 28 consecutive COVID-19 patients derived from a distinct hospital ("Attikon" Hospital, Athens) contributing 35 samples and the second included 118 consecutive patients derived from Alexandroupolis and "AHEPA" hospitals contributing 225 samples (Supplementary Table 1). Activin-A, Activin-B, Follistatin, and standard-of-care (SOC) laboratory parameters including absolute-neutrophil-count (ANC), absolute-lymphocyte-count (ALC), neutrophil/lymphocyte-ratio (NLR), C-reactive protein (CRP), lactate dehydrogenase (LDH), ferritin and D-dimers, were analyzed. All laboratory variables were measured concomitantly in serum (or citrated plasma in case of D-dimers) samples derived from the same venipuncture. Measurements of Activins/Follistatin levels were performed with the leftover sera. The study conformed to the TRIPOD statement [24] and is aligned with the Helsinki declaration. The study protocol design was approved by the Local Scientific and Ethics Committees and Institutional Review Boards of the University Hospital of Alexandroupolis (Ref. No. 803/23-09-2019 and Ref. No. 87/08-04-2020), AHEPA University Hospital of Thessaloniki (Ref. No. 1789/2020) and ATTIKON University Hospital of Athens (Ref. No. 487/3-9-2020). Patients' records were anonymized and de-identified prior to analysis so confidentiality and anonymity were assured.

Immunoassays

Activin-A, Activin-B and Follistatin serum levels were measured using enzyme-linked immunosorbent-assay (ELISA) (Ansh-Labs, Webster, TX, USA) according to manufacturer's instructions.

Statistical analysis

To elucidate the role of A/F-axis molecules, we carried out an analysis based on samples and not on patients; for that purpose, we initially used three separate general linear models, each one incorporating outcome as binary dependent variable (survival/death), as well as Activin-A, Activin-B, Follistatin and either multiplicity of sampling, or period of sampling, or reference hospital as independent variables to discriminate whether the latter three variables were potential confounders or true predictors. Having validated the prognostic value of A/F-axis molecules as predictors of outcome, we further examined their independent prognostic value across 14 variables of interest, including potential confounders, which were transformed to binary ones through nominal optimal scaling along with discretization to two groups. Imputing of missing data was added using the CATREG automated selection function of SPSS, ridge-regression was selected for regularization and 10-fold cross-validation was added. Results were adapted to a point-system scoring, where one additive point was given for each parameter included in the unfavorable category as suggested after discretization; binary-regression was utilized to mathematically approach outcome probability. Evaluation of the scoring systems was based on Area-Under-Curve (AUC) as determined from Receiver Operating Characteristic (ROC) analysis. Optimal-Scaling procedure was utilized to detect whether the scoring

systems could predict response to certain treatment options. More details regarding the Materials and Methods is provided in Supplementary Materials.

Results

The Activin/Follistatin axis is highly deregulated in COVID-19 non-survivors

To validate the hypothesis that the A/F-axis is linked to COVID-19 pathophysiology we analyzed serum levels of Activin-A, Activin-B and Follistatin in a cohort of 117 COVID-19 patients. The day-of-symptom-onset (disease-day) was used to align the data derived from serum samples. Activin-A, Activin-B and Follistatin, were substantially increased, particularly in samples from non-survivors (Figures 1 and 2, and Supplementary Table 2). Increased levels of these proteins were observed approximately 7-28 days from onset of symptoms (Figures 1, 2). Classical parameters such as ANC, ALC, NLR, CRP, LDH and D-dimers were increased within the same time window (Figure 1). However, whereas CRP, neutrophils and NLR were elevated already from first week of disease and D-dimers were gradually increasing over time, A/F-axis proteins and LDH were upregulated during the second week, when COVID-19 leads to hospitalization (Figure 2). Interestingly, comparative analysis of A/F-axis components and classical parameters in survivors *vs* non-survivors on the basis of disease-status at serum sampling (Supplementary Figure 2) demonstrated upregulation of Activin-A, Activin-B and Follistatin in sera from both severe and critical, disease status (DS) 3 and DS4, non-survivors. Neutrophils, CRP and D-dimers were upregulated in samples from DS3 non-survivors, as well as, DS4 survivors and non-survivors; LDH levels were particularly elevated in DS4 non-survivors.

To assess whether the basis of analysis (per sample/per patient), the day of sampling (disease-day at sampling) as well as the origin of patients (hospital of admission)

might constitute confounders that blur the results, three discrete general linear models including Activin-A, Activin-B and Follistatin as dependent variables and outcome along with either multiplicity of samples per patient or sampling period (1-7/8-14/15+ day of sampling) or reference hospital as independent ones were introduced. These models demonstrated that Activins and Follistatin did not correlate with any of these potential confounders (Supplementary Table 3). Therefore, our efforts to construct a predictive model were based on the analysis of all the samples of the initial cohort rather than the analysis of independent patients. Thus for subjects where several samples were drawn at different disease stages, each sample was treated as an independent entry and its score was computed separately.

The Activin/Follistatin-axis in the dynamically shifting phenotypic heterogeneity of COVID-19

COVID-19 is characterized by clinical heterogeneity and a dynamically changing phenotype [2,3,5,12]. We hypothesized that this behavior must be mirrored by analogous fluctuations in the expression profiles of key biomarkers. We therefore investigated the correlations between different biomarkers and final outcome in our sample collection. The correlation of Activin-B levels to LDH, and Activin-A levels to CRP and NLR are shown as representative comparisons (Figure 3 and Supplementary Figure 3). In Activin-B vs LDH and Activin-A vs CRP, some samples exhibited good correlation but others did not and in Activin-A vs CRP or NLR comparisons, the parameters did not correlate at all. To understand better this finding, we highlighted in similar plots the groups of samples derived from non-survivors AL022, AL034,

AL035, AL062, AL063 and AX112 for which multiple serial samplings were available (Figure 3 and Supplementary Figure 3). This depiction highlighted the marked and dynamic phenotypic heterogeneity among patients and samples of the same patient collected at different disease-days. For example, for patients AL022 and AL035, disease progression and eventual death were associated with concordant changes in Activin-B and LDH levels. However, patients AL063 and AX112, were characterized by substantial changes in Activin-A, Activin-B and CRP levels and for patients AL035 and AL062 disease progression was associated with eventual CRP and NLR reduction. Interestingly, patient AL022, who received a dose of Tocilizumab one day after admission, exhibited for ~10 days transient reduction of LDH, CRP, NLR, Activin-A and Activin-B, before reverting and dying on disease-day 28. Therefore, in addition to patient intrinsic phenotypic variability, treatments could also influence biomarker profile during hospitalization. From all the above we concluded that a broader spectrum of biomarkers, including both early and later modulated ones, had to be integrated to develop a meaningful disease scoring/monitoring-system.

The FACT-CLINYCoD score for dynamic monitoring of COVID-19 outcome and treatment

Considering the heterogeneous phenotypic changes characterizing COVID-19 progression, we aimed to build a simple and clinically meaningful scoring system to discriminate survivors from non-survivors. To this end, we first demonstrated that Follistatin, Activin-A, Activin-B, CRP, LDH, ICU-admittance, NLR, age, comorbidities and D-dimers performed well in predicting disease outcome, as judged by ROC analysis (Supplementary Figure 4). Notably, among all these parameters, Follistatin

exhibited the highest AUC (0.857). We then converted all these continuous variables, which could independently predict outcome, to binary through selection of optimal cutoffs using optimal-Scaling procedure along with ridge-regression (Supplementary Figure 5 and Supplementary Table 4). Based on these data, we constructed a ten-point scoring-system where one additive point was arbitrarily given for each parameter included in the unfavorable category as suggested after discretization, namely if Follistatin>6235 pg/ml, Activin-A>591 pg/ml, Activin-B>249 pg/ml, CRP>10.3 mg/dL, LDH>427 U/L, ICU admission, NLR>5.6, Years of Age >61, Comorbidities>1 and D-dimers>1097 ng/ml (Table 2, Supplementary Table 4). We evaluated this score with ROC analysis, yielding to an AUC value of 0.951 (95% CI: 0.919-0.983) ($p<10^{-6}$), that indicates outstanding discrimination in foretelling survivors from non-survivors (Figure 4A). This score was titled FACT-CLINYCoD, being an acronym of *F*(ollistatin), *ACT*(ivins), *C*(RP), *L*(DH), *I*(CU admission), *N*(LR), *Y*(ears of age), *Co*(morbidities) and *D*(-dimers). Every additive point increases the odds ratio (OR) for dying 3.781 times (Supplementary Table 5). A score ≥4 has 90.8% sensitivity and 87.5% specificity to predict fatal outcome, whereas a score ≥5 has 81.5% sensitivity and 95.7% specificity. The FACT-CLINYCoD score distinguishes survivors from non-survivors at admission and outcome and monitors accurately disease progression independently of disease-day or status (Figure 4B,C,D). The probabilities of death at different disease-days in non-surviving patients AL022, AL034, AL035, AL062, AL063 and AX112 are shown in Figures 3 and Supplementary Figure 3, next to each sample analyzed. Interestingly, some of these patients had very high scores already at first sampling. However, others, such as AX112 and AL035 had low scores

at admission and either gradually or rapidly deteriorated and eventually died. The latter type of patients highlights the value of continuous monitoring for COVID-19.

Considering that various treatment modalities might affect the value of FACT-CLINYCoD score to predict final outcome, we performed ROC analysis separately for samples corresponding to each treatment (Supplementary Figure 6). Interestingly, all relevant AUCs were similar, thus indicating that the predictive value of FACT-CLINYCoD score was not affected by current therapies. Therefore, this scoring system may be used for monitoring response to treatment.

Validation of the FACT-CLINYCoD score during the second wave of COVID-19

The FACT-CLINYCoD score was at first validated using an independent cohort of 28 consecutive patients contributing 35 random samples. Although this small cohort differed significantly from the initial regarding mortality rate, age, disease-status, treatments, sampling approach, and day-from-disease-onset (Supplementary Table 1), a similar AUC was observed (0.958, 95%CI: 0.872-1.000, P=0.032, Figure 4A). A second independent validation cohort of 118 consecutive patients contributing 225 samples, differing significantly regarding sex-ratio, number of comorbidities, disease-status, treatments, and sampling approach (Supplementary Table 1) was again found to exhibit an AUC of 0.924 (95%CI: 0.890 - 0.958, $P<10^{-6}$, Figure 4A). Both validation cohorts shared similar AUCs with the initial cohort (P=0.880 and 0.256, respectively).

Contribution of the A/F-axis in the predictive power of FACT-CLINYCoD

To further substantiate the significance of Activins and Follistatin for the predictive power of the FACT-CLINYCoD scoring system we performed an ad-hoc analysis in all 574 samples derived from both initial and validation cohorts using area under

precision-recall curves (AUPRC) (Supplementary data). As a result, we found that FACT-CLINYCoD score is superior to the "F/A axis-depleted (CLINYCoD) score; the difference between the relevant AUPRCs is 4.8% (95% CI: 2.6%-7.5%), P<0.05 (Supplementary Figure 7A). Moreover, FACT-CLINYCoD score is even superior to "Activins-depleted" (F-CLINYCoD) score; the difference between the relevant AUPRCs is 1.5% (95% CI: 0.3%-3.2%), P<0.05 (Supplementary Figure 7B). These results demonstrate the contribution of all three A/F-axis proteins in the predictive power of the FACT-CLINYCoD scoring system.

The value of FACT-CLINYCoD score to guide timely treatment

Optimal-Scaling procedure was performed along with ridge-regression to investigate whether FACT-CLINYCoD score could guide timely administration of current SOC treatments [25] such as low-molecular-weight heparin (LMWH), remdesivir and dexamethasone (or equivalent doses of alternative glucocorticoids) on data available from 574 samples after pooling the initial and validation cohorts. Both LMWH (P<0.001) and dexamethasone (P=0.026), when administered in FACT-CLINYCoD score ≤2 (P<0.001), were independently correlated with favorable outcome. This was not the case for remdesivir (P=0.071), most possibly due to the limited number of available samples (Supplementary Table 6). Although the utility of FACT-CLINYCoD in guiding timely treatment administration and predicting therapeutic responses looks promising, the relatively small number of patients dictates caution in interpreting these findings.

Discussion

This study provides evidence suggesting that inflammation and tissue stress-related proteins Activin-A, Activin-B, and Follistatin (A/F-axis) are tightly associated with severity and outcome of COVID-19. Their upregulation was prominent in non-survivors and was independently related to in-hospital mortality. Of note, combination of A/F-axis components with common clinical and laboratory parameters permits prediction of COVID-19 outcome throughout the course of the disease.

Identification of high-risk patients and death-prediction are of particular importance especially in strained health-care systems. Hence, several studies attempted to develop scoring systems to predict timely disease severity and mortality [4,6,14,15,26–28]. Most of these models were based on demographic parameters and instantaneous evaluation of subjective symptoms (i.e. dyspnea) combined with single measurements of common laboratory markers, such as neutrophil or lymphocyte counts, LDH, CRP and several proinflammatory cytokines [4,6,14,15,26,28,29]. These models are undoubtedly useful; however, they emphasize prediction of final-outcome at admission and thus may fail to predict some non-survivors most likely due to the complex and often erratic development of COVID-19 and the downregulation of key biomarkers at late disease-stages (Figure 3 and Supplementary Figure 3).

Considering the above, we exploited the findings presented herein to develop a more dynamic COVID-19 monitoring-system. Specifically, we utilized the measurement of Activin and Follistatin levels, based on their deregulation described herein and their previous implication in sepsis/ARDS, neutrophil-mediated

inflammation, coagulopathy, endothelial cell stress, angiogenesis and post-inflammatory pulmonary fibrosis/remodeling, all of which are key characteristics of COVID-19 pathophysiology [7,8,18,22,30]. Moreover, we reasoned that upregulation of A/F-axis could indicate tissue-damage in the lung, vasculature or other vital peripheral tissues, thus complementing information derived from other biomarkers reporting tissue-damage (i.e. LDH) or coagulation/vascular injury (i.e. D-dimers) [2,15,27,28]. Finally, taking into consideration the differences in kinetics of A/F-axis components and other clinical biomarkers like NLR, CRP, LDH and D-dimers (Figure 2) we combined them all to develop a more generalizable monitoring-system that will not only make early predictions but will rather allow readjustment of predictions during disease progress [31].

In the pre-COVID-19 era, the A/F-axis was evaluated as predictor of outcome in critical-care patients [32]. Increased levels of Activin-A and Activin-B in sera from ICU patients with acute respiratory failure could predict 90-days and 12-months survival with reasonable accuracy (~80%). Follistatin did not provide any extra predictive value [32]. A/F-axis components were measured also in critically-ill, influenza-A (H1N1), patients, but no significant association with disease severity was established [33]. However, while preparing the current manuscript, consistent with our conclusions, a preprint study reported Follistatin among the circulating markers of endothelial damage associated with in-hospital mortality in a small number of COVID-19 patients [34].

Although the exact mechanism linking Activins to COVID-19 pathogenesis remains unknown, the importance of A/F-axis in the disease is reflected on the predictive value of FACT-CLINYCoD score, the superiority of which was demonstrated by the ad-

hoc analysis vs the “F/A axis-depleted” CLINYCoD and “Activin-depleted” F-CLINYCoD scores (Supplementary Figure 7A,B). The FACT-CLINYCoD model, which depends on A/F-axis by 3/10, demonstrates an almost perfect AUC (0.951±0.032) as well as a very satisfactory sensitivity (81.5%) and specificity (95.7%) of scores ≥5 to predict fatal outcome. In agreement with this, the key role of A/F-axis components, in particular Follistatin, in the prediction of fatal outcome at any time of disease course (Figure 4B,C) is underlined by the relevant ROC analysis (Supplementary Figure 4) and ridge-regression (Supplementary Figure 5). The FACT-CLINYCoD score constitutes a balanced, robust and flexible tool efficiently intertwining pathophysiology of A/F-axis, clinical profile (by means of NLR, LDH, D-dimers and CRP) and key parameters that are well established to affect mortality (ICU admission, age, and comorbidities), capable of providing dynamic outcome prediction.

An obvious emerging question is whether the A/F-axis is a suitable target for COVID-19 therapeutics. Indeed, several studies have proposed Follistatin or soluble Activin type-II receptors as therapeutics for sepsis, ARDS and fibrotic disorders [18,35]. Although increase of Activin-A and -B could be interpreted in favor of utilizing such therapeutics, the dramatic increase of Follistatin, often at stoichiometry that surpasses substantially the sum of Activins-A and -B in serum, could argue against it.

Neutrophils can release preformed Activin-A upon arrival in inflamed tissues [18,36], whereas, other inflammatory cells such as monocytes, $CD4^+$ T cells and tissue resident cells can secrete Activin-A later on [18,21,37]. Neutrophils do not secrete Activin-B and therefore this factor must be produced by other cells, probably acting as a biomarker of vascular stress and hypoxia [38,39]. Follistatin can be produced locally or released systemically from distant organs such as liver [18]. The tissue

origin of the increased A/F-axis components in sera of COVID-19 patients and the actual Activin/Follistatin stoichiometry in the affected tissues are unknown. Therefore, the suitability of the A/F-axis as therapeutic target for COVID-19 warrants further careful investigation.

Another promising aspect of this study is the potential utility of FACT-CLINYCoD to monitor response to various treatments targeting SARS-CoV-2 (antivirals) or hyper-inflammatory host reactions (heparin, corticosteroids) since the value of this scoring system to predict mortality was not disturbed by various treatments. Our preliminary results showed favorable outcome when LMWH and dexamethasone was commenced at FACT-CLINYCoD score ≤2. This is consistent with emerging clinical data linking reduced mortality with early administration of LMWH in all hospitalized COVID-19 patients [40] and supports the detrimental role of immunothrombosis in COVID-19 [7]. Moreover, current clinical data derived from randomized clinical studies recommend administration of dexamethasone as early as the patient needs oxygen supply [25].

Finally, the postulated implication of the A/F-axis in other infectious diseases associated with in-hospital mortality [17–19,22], leaves open the possibility that the FACT-CLINYCoD score may be applicable to other diseases in addition to COVID-19.

Overall, potential limitations of this study could be considered. First, the possible inherent weaknesses due to the retrospective nature of our findings, although they were well-validated. Second, the applicability of the FACT-CLINYCoD score requires the implementation of Activins/Follistatin measurements in daily laboratory routine.

In conclusion, A/F-axis dysregulation is tightly associated with poor outcome of COVID-19. FACT-CLINYCoD, a novel pathophysiology-driven monitoring-system, enables dynamic prediction of disease outcome and may support real-time medical decision. Prospectively, large-scale, multinational validation of this calculator, as well as investigation of the mechanisms linking A/F-axis to COVID-19 pathogenesis is definitely warranted.

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FIGURES LEGENDS

Figure 1. **Activin/Follistatin-axis components are upregulated in the serum of COVID-19 patients during the critical period when patients deteriorate.** Serum levels of A/F-axis components and other key inflammatory parameters in the blood of COVID-19 patients plotted over time according to the days of symptom onset. Vertical dotted lines indicate time-windows of first, second and more than two-week intervals used for statistical analysis. Horizontal dotted lines indicate the thresholds determined by optimal scaling (Supplementary figure 5 and Supplementary table 4).

Figure 2. **Levels of A/F-axis components are upregulated selectively in non-survivor severe or critically-ill COVID-19 patients during the critical second week from disease onset.** Concentrations of Activin/Follistatin-axis components in serum and levels of other key inflammatory parameters in the blood of COVID-19 patients plotted over time according to the days of symptom onset and grouped per week. Data are expressed as mean±SEM analyzed using one-way analysis of variance with Tuckey's post-hoc test. Asterisks represent comparison of the indicated group vs the survivors of week 1 (0-7 days from onset of disease). Asterisks above a horizontal line represent comparison between the groups under the line segment (*P<0.05, **P< 0.01, ***P < 0.001 and ^{ns}non-significant)

Figure 3. The dynamic phenotypic heterogeneity of COVID-19 patients is evident when serum relative levels of A/F-axis components are plotted against classical biomarkers. A) Correlation between Activin-B and LDH (upper panel) or Activin-A and NL-ratio levels (lower panel) in all acquired serum samples from our cohort. B) Serial samples collected from the indicated non-survivor patients ALO63 and ALO22. C) Serial

samples collected from non-survivor patients ALO35, ALO62 and AX112. Vertical and horizontal dotted lines indicate the thresholds determined by optimal scaling (supplementary figure 5 and Supplementary table 4).

Arrows indicate the direction of disease progression towards death. Numbers within the circular points indicate disease-day. Percentages indicate the probabilities of death estimated by the FACT-CLINYCoD scoring system developed herein. TOCI: Tocilizumab

Figure 4. The FACT-CLINYCoD scoring system may enable prediction of day-to-day prognosis of disease outcome and real-time clinical decision making. ROC analysis for FACT-CLINYCoD score of the initial and two validation cohorts (A). The FACT/CLINYCoD scoring system distinguishes survivors from non-survivors both at admission and at the final outcome (B), irrespectively of disease-day (C) and disease status (D). Data are expressed as mean±SEM analyzed using one-way analysis of variance with Tuckey's post-hoc test. Asterisks above a horizontal line represent comparison between the groups under the line segment. Asterisks without a line underneath in C and D represent comparison of the indicated group with the survivors in the first group of the corresponding graph (*P<0.05, **P< 0.01, ***P < 0.001 and ^{ns} non-significant).

Table 1. Characteristics of patients (n=117) and samples (n=314); "numbers in parenthesis correspond to percentages (%)"

Parameter	patients (n=117)	samples (n=314)	P
Age (years)			
Mean (±SD)	61.3±15.9	61.6±15.7	0.863
Sex			
Male	63 (53.8)	161 (51.3)	0.635
Female	54 (46.2)	153 (48.7)	
Number of comorbidities			
0	28 (24.0)	69 (22.0)	0.813
1	33 (28.1)	79 (25.2)	
2	26 (22.7)	72 (22.9)	
3	21 (17.9)	60 (19.1)	
4	6 (5.1)	28 (8.9)	
5	3 (2.6)	6 (1.9)	
Disease status (DS) at sampling			
DS1	39 (33.4)	95 (30.2)	0.363
DS2	27 (23.1)	74 (23.6)	
DS3	32 (27.4)	71 (22.6)	
DS4	19 (16.2)	74 (23.6)	
Treatment at sampling			
Hydroxychloroquine	58 (49.6)	98 (31.2)	0.358
Azithromycin	69 (59.0)	125 (39.8)	
Antibiotics (other)	103 (88.0)	257 (81.8)	
Lopinavir/Ritonavir	57 (48.7)	112 (35.7)	
Remdesivir	4 (3.4)	10 (3.2)	
Tocilizumab	3 (2.6)	5 (1.6)	
Anakinra	12 (10.3)	31 (9.9)	
LMWH	91 (77.8)	254 (80.9)	
Corticosteroids	20 (17.2)	52 (16.6)	
Colchicine	8 (6.8)	14 (4.5)	
CVVDHF	1 (0.9)	4 (1.3)	
Final Outcome			
Survival	93 (79.5)	233 (74.0)	0.256
Death	24 (20.5)	81 (26.0)	
Sampling approach			
Single	40 (34.2)	40 (12.7)	<0.001
Multiple	77 (65.8)	274 (87.3)	
Day from disease onset at sampling			
Day 1-7	37 (31.6)	55 (17.5)	0.004
Day 8-14	43 (36.8)	124 (39.5)	
Day 15+	37 (31.6)	135 (43.0)	
Follow-up period			
Median (IQR)	19.0 (14.3-26.0)	19.0 (15.0-18.0)	0.070
Hospital			
Alexandroupolis	68 (58.1)	194 (61.8)	0.488
AHEPA	49 (41.9)	120 (38.2)	

CVVDHF: continuous venovenous hemodiafiltration; Corticosteroids: dexamethasone or equivalent doses of alternative glucocorticoids; LMWH: low molecular weight heparin

Table 2. FACT-CLINYCoD scoring and predictability of outcome.

	Samples from survivors	Samples from non-survivors	Probability of death (%) ǂ
FACT-CLINYCoD score			
0	44	0	0.2 (0.1-0.3)
1	54	2	0.8 (0.5-1.1)
2	42	0	2.8 (2.0-3.6)
3	43	4	9.9 (7.2-12.6)
4	17	6	29.4 (22.7-36.1)
5	7	13	61.6 (52.6-70.6)
6	1	10	85.6 (80.7-90.5)
7	1	13	95.7 (94.1-97.3)
8	0	11	98.8 (98.4-99.2)
9	0	6	99.7 (99.6-99.8)
10	0	0	99.9 (99.8-100.0)

ǂ *approximation as derived from probability equation based on corresponding binary regression model along with ±95% CI in parentheses.*

Figure 1

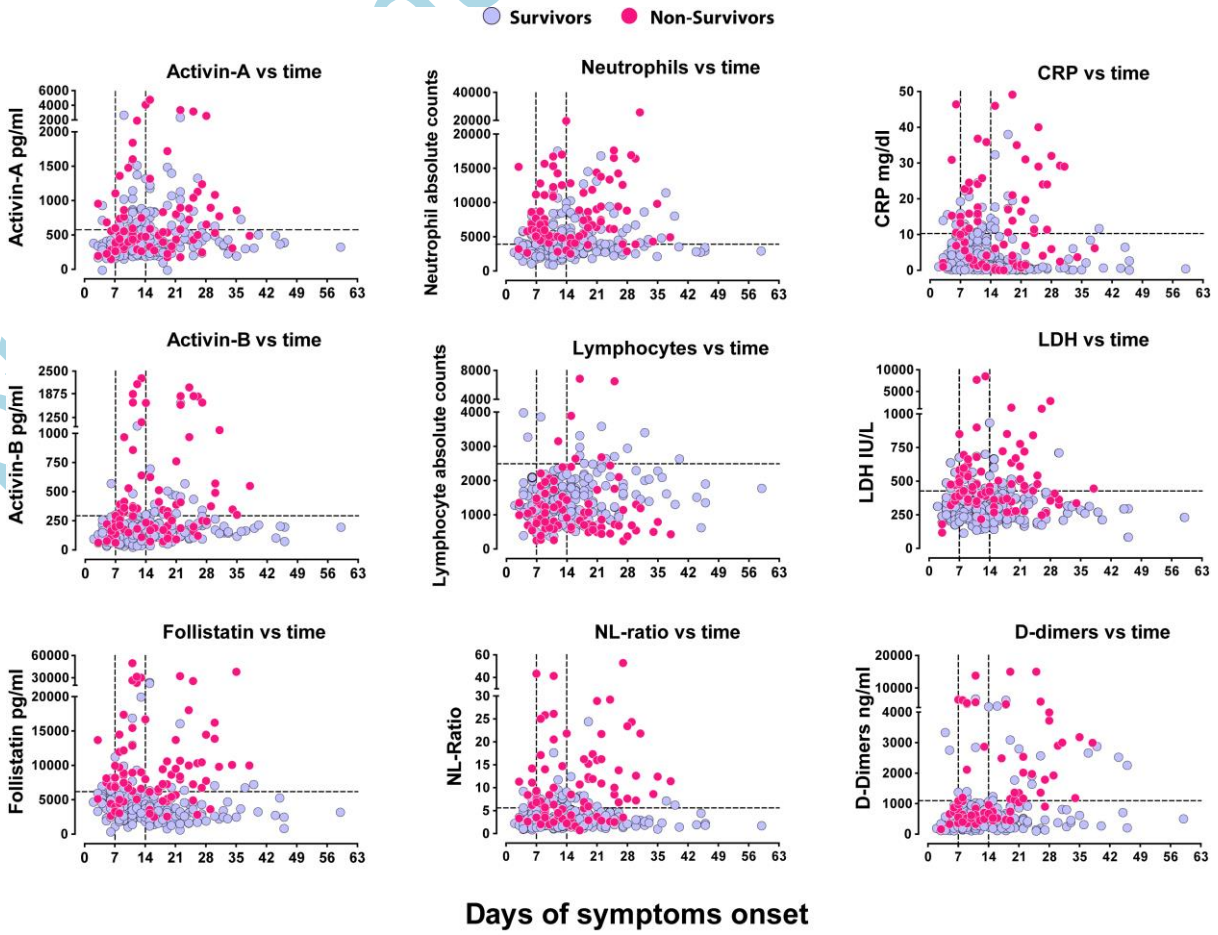


Figure 2

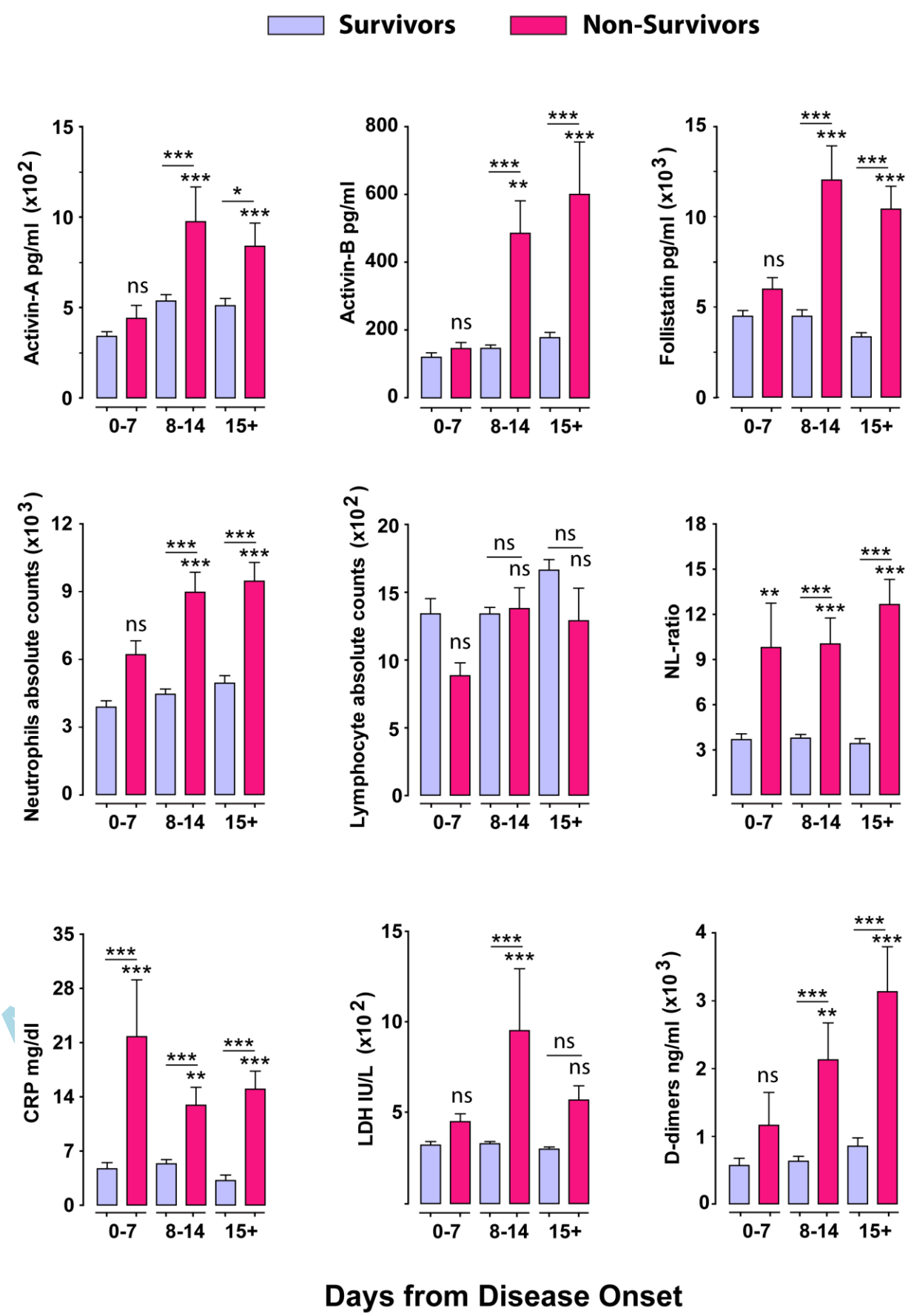

Survivors
Non-Survivors
Activin-A pg/ml (x10 2)
Activin-B pg/ml
Follistatin pg/ml (x10 3)
Neutrophils absolute counts (x10 3)
Lymphocyte absolute counts (x10 2)
NL-ratio
CRP mg/dl
LDH IU/L (x10 2)
D-dimers ng/ml (x10 3)
0-7
8-14
15+
ns
Days from Disease Onset

Figure 3

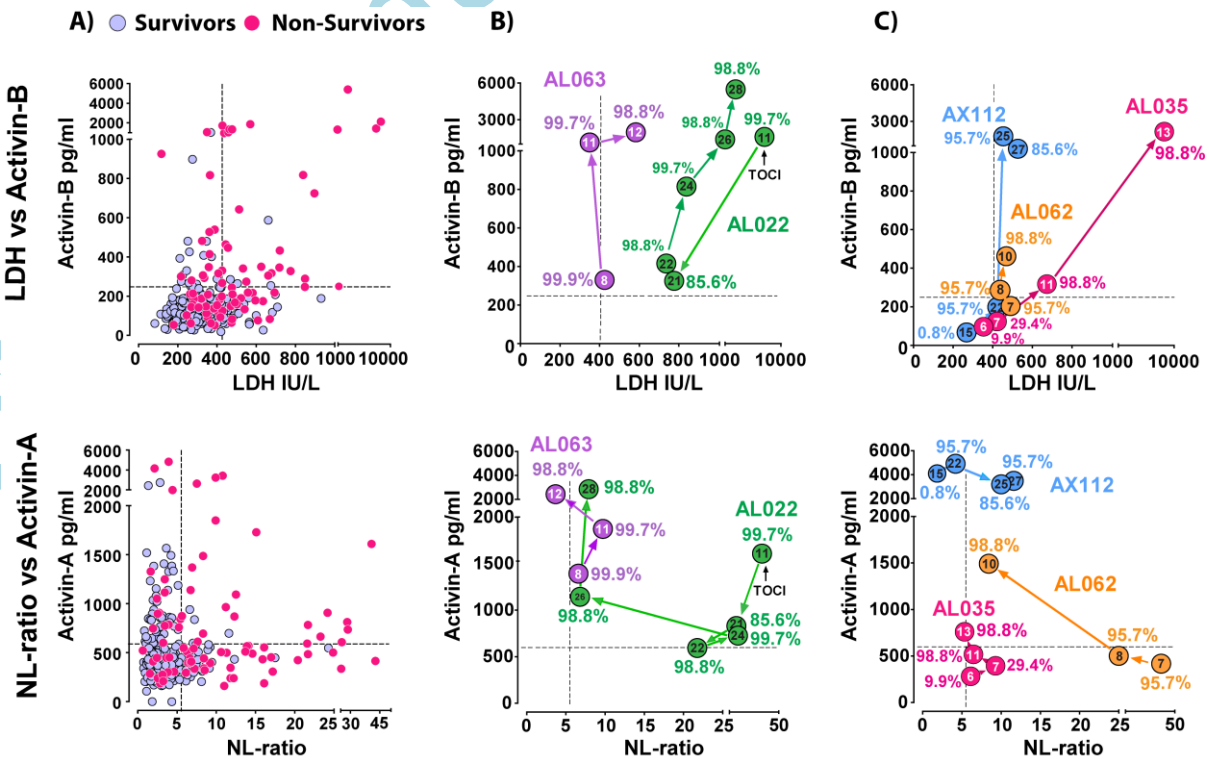
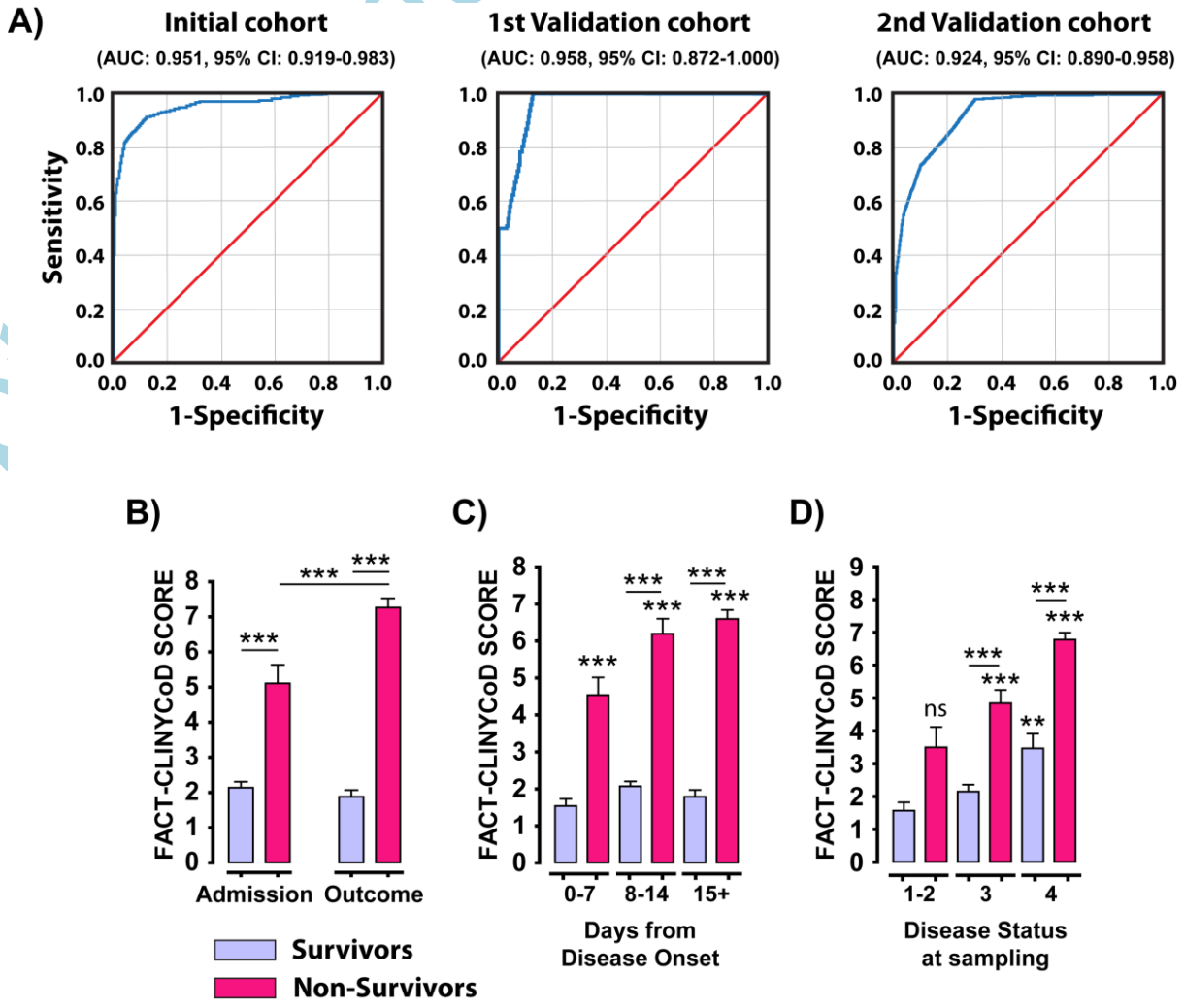


Figure 4


A)
Initial cohort
(AUC: 0.951, 95% CI: 0.919-0.983)
1st Validation cohort
(AUC: 0.958, 95% CI: 0.872-1.000)
2nd Validation cohort
(AUC: 0.924, 95% CI: 0.890-0.958)
Sensitivity
1-Specificity
B)
C)
D)
FACT-CLINYCoD SCORE
Admission
Outcome
Survivors
Non-Survivors
0-7
8-14
15+
Days from Disease Onset
1-2
3
4
Disease Status at sampling
ns