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Dynamic Cytokine and Coagulation Profiling in Patients With Severe COVID-19 Evaluated for Pulmonary Embolism: A Prospective Cohort Study

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Background: COVID-19 is associated with immune dysregulation and an increased risk of thromboembolic complications, including pulmonary embolism (PE). Identification of dynamic immunological predictors of adverse outcomes remains clinically relevant. This study evaluated associations between coagulation, inflammatory, immunological markers, and in-hospital mortality in patients with severe COVID-19.

Material/Methods: In this prospective observational study, 47 hospitalized adults with severe COVID-19 and suspected PE were enrolled. Serum cytokines, coagulation parameters, and cardiac injury biomarkers were assessed at enrollment and on hospital day 5. PE was confirmed using computed tomography pulmonary angiography. Univariable and multivariable binary logistic regression was used to identify predictors of in-hospital all-cause mortality. Model discrimination was evaluated using the area under the receiver operating characteristic curve (AUC), with internal validation by bootstrap resampling.

Results: PE was diagnosed in 19 patients (40.4%), and 13 patients (27.7%) died during hospitalization. Higher standardized day-5 interleukin (IL)-9 and IL-15 levels were associated with mortality in univariable analyses, but neither remained significant after Benjamini-Hochberg false discovery rate correction across the panel of 15 cytokines measured on day 5 (IL-9, q = 0.180; IL-15, q = 0.210). In an age-adjusted model, day-5 IL-9 remained associated with mortality (adjusted odds ratio per 1-SD increase, 3.331; 95% CI, 1.289-8.608; $P = 0.013$). Apparent discrimination was moderate (AUC 0.774; 95% CI, 0.563-0.984; $P = 0.011$), but bootstrap validation showed marked coefficient instability.

Conclusions: Day-5 IL-9 levels may reflect an evolving risk in severe COVID-19, but the association was sensitive to multiple-testing correction and model instability. This study's findings are exploratory and require confirmation in larger, externally validated cohorts.

Keywords: COVID-19 • Cytokines • Interleukin-15 • Interleukin-9 • Pulmonary Embolism

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Introduction

COVID-19, which is caused by SARS-CoV-2, presents with a wide range of clinical manifestations, from asymptomatic infection to life-threatening acute respiratory distress syndrome, multiorgan dysfunction, and death. It is associated with a significant risk of thromboembolic complications, particularly pulmonary embolism (PE). Reported PE incidence among hospitalized patients ranges from approximately 1% to 40%. Real-world cohorts typically observe rates between 3% and 9%, with even higher rates in intensive care settings, contributing to increased mortality [1-3].

COVID-19–associated coagulopathy is a distinct clinical entity characterized by excessive thrombin generation, endothelial dysfunction, platelet activation, and a pro-inflammatory state leading to a hypercoagulable condition [4,5]. It is characterized by elevated D-dimer, activated partial thromboplastin time (aPTT), and fibrinogen levels, as well as decreased prothrombin activity and mild thrombocytopenia, which are frequently associated with an increased risk of thrombotic events and mortality [1,6].

Furthermore, the interplay between innate immune responses and coagulation, described as immunothrombosis, plays a pivotal role in the pathogenesis of thrombotic complications in COVID-19. Endothelial injury, neutrophil extracellular traps, and inflammatory cytokines and markers, such as interleukin (IL)-6 and C-reactive protein (CRP), promote thrombus formation in micro- and macrovascular beds [7,8].

Clinically, PE may be difficult to diagnose in patients with COVID-19 due to overlapping features with viral pneumonia and acute respiratory distress syndrome, including dyspnea, hypoxia, and chest discomfort. However, postmortem studies have revealed a high incidence of undiagnosed thromboembolic complications, underscoring the need for early identification of at-risk patients through appropriate biomarkers and imaging modalities [9,10].

IL-9 has been mechanistically linked to increased SARS-CoV-2 severity and lung inflammation and has been reported to be elevated in active COVID-19 in translational work [11]. In a cohort of critically ill patients with COVID-19 and acute respiratory distress syndrome, IL-15 levels were reported as higher over early intensive care unit (ICU) time points among nonsurvivors vs survivors, supporting its plausibility as a severity-associated cytokine [12]. In a cohort of 286 hospitalized patients with severe COVID-19, non-survivors had significantly higher serum IL-15 levels than survivors in samples collected during the first 5 hospital days, and IL-15 showed good discrimination for mortality (area under the receiver operating characteristic curve [AUC], 0.765), supporting elevated IL-15 as a prognostic marker of worse outcome [13].

Severe illness often begins about 1 week after symptom onset, and time-dependent immune dysregulation is well described; this supports serial sampling rather than baseline-only inference [14,15]. The identification of dynamic immunological predictors of adverse outcomes remains clinically relevant. However, it remains unclear whether dynamic cytokine measurements obtained on hospital day 5 provide prognostic information beyond baseline testing in patients with severe COVID-19 evaluated for suspected PE. Therefore, this study aims to evaluate the association between PE and dynamic changes in coagulation parameters (including D-dimer, prothrombin activity, aPTT, fibrinogen, and platelet count [PLT]), immunological, and inflammatory markers (serum cytokine concentrations, CRP, and white blood cell count) in patients with COVID-19. Identification of early laboratory predictors could enhance diagnostic precision, enable more effective risk stratification, and support timely therapeutic interventions.

Material and Methods

Ethics Statement

All patients provided written informed consent for participation in this study. The study was approved by the Kaunas Regional Biomedical Research Ethics Committee (No. BE-2-50, April 20, 2022).

Studied Sample

An observational prospective study was conducted in Lithuania, in 2022 to 2024. Seventy consecutive hospitalized adults with severe COVID-19 were screened during the study period, and those who met clinical criteria for suspected PE (according to European Society of Cardiology [ESC] guidelines for the diagnosis and management of acute PE [16], eg, dyspnea, chest pain, presyncope or syncope, or hemoptysis) underwent computed tomography pulmonary angiography (CTPA). Among patients who underwent CTPA, those with available cytokine measurements and complete baseline assessment were enrolled, yielding a final cohort of 47 patients (47 out of 70 eligible cases; 18 men, 29 women), with PE confirmed or excluded by CTPA. The main reasons for exclusion from the study were as follows: severe mental disability (4 cases excluded), unavailable cytokine assay (6 cases), unavailable day-5 cytokine samples (5 cases), and refusal to consent (8 cases). In addition to SARS-CoV-2 confirmation (with nucleic acid amplification testing), routine evaluations included chest imaging and standard laboratory testing, including D-dimer, prothrombin activity/international normalized ratio, aPTT, fibrinogen, and PLT. The inclusion criterion was laboratory-confirmed SARS-CoV-2 infection, with PE status determined by CTPA. A diagnosis of SARS-CoV-2 was confirmed according to the European

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Society of Clinical Microbiology and Infectious Diseases and the Infectious Diseases Society of America guidelines on the diagnosis of COVID-19 in force at the time [17,18]. Severe COVID-19 was defined by the presence of tachypnea (respiratory rate ≥ 30 breaths per minute), oxygen saturation of 93% or lower at rest (on ambient air), or a ratio of arterial oxygen partial pressure to fractional inspired oxygen of less than 300 mm Hg [14,19]. The exclusion criteria were as follows: age under 18 years, severe mental disability, no CTPA, contraindication to contrast/CTPA, unavailable cytokine assay, unavailable day-5 cytokine samples, and refusal to consent.

Because enrollment was restricted to patients with severe COVID-19 who met clinical criteria for suspected PE and underwent CTPA (with cytokine testing available), this was a selected convenience cohort and may not be generalizable to all hospitalized patients with severe COVID-19. Accordingly, the proportion of CTPA-confirmed PE should be interpreted as the diagnostic yield within this suspected-PE cohort rather than as an estimate of population incidence. No formal a priori power calculation was performed, because this prospective study enrolled all eligible patients during the study period; accordingly, the cohort should be viewed as a convenience sample and all regression analyses as exploratory low-events models.

The primary outcome was in-hospital all-cause mortality.

Methods of Examination

In this study, we analyzed data from serum cytokine concentrations, inflammatory markers, cardiac damage biomarkers (amino-terminal pro-B-type natriuretic peptide and high-sensitivity cardiac troponin I), coagulation parameters, and radiology studies (chest X-ray and CTPA), as well as other relevant clinical and laboratory variables. All these tests were performed on the day of enrollment. Serum cytokine concentrations, coagulation parameters, and cardiac damage biomarkers were analyzed at enrollment and 5 days later. The day-5 time point was selected based on prior evidence demonstrating time-dependent changes in cytokine profiles among patients with severe COVID-19 [14,15,20].

The serum cytokine concentrations were measured from the serum using a Luminex performance assay multiplex kit (Bio-Techne Corporation, Minneapolis, MN, USA) and analyzed with a certified Luminex analyzer (Luminex 200; Luminex Corp, Austin, TX, USA). A human cytokine fixed panel included 15 well known cytokines: interferon (IFN) α-2, IFN-γ, IL-1α, IL-1β, IL-1 receptor antagonist, IL-2, IL-3, IL-4, IL-6, IL-7, IL-9, IL-10, IL-15, IL-33, and vascular endothelial growth factor. On the day of the assay, all fresh and previously frozen serum samples were centrifuged at 16 000 × g for 4 minutes immediately prior to use.

A diagnosis of PE was confirmed according to the ESC guidelines for the diagnosis and management of acute PE [16]. We adhered to these guidelines because other researchers conclude that, in COVID-19, the diagnostic pathway of PE should not be altered [21-23].

Statistical Analysis

The IBM Statistical Package for Social Sciences Statistics for Windows (IBM Corp, Armonk, NY, USA) was used for data analysis. The Shapiro-Wilk and Kolmogorov-Smirnov tests were used to check whether the continuous variables followed a normal distribution. All the continuous variables were not distributed normally. Thus, for descriptive statistics continuous data are presented as the median and interquartile range, and non-parametric tests were used to test statistical hypotheses. For categorical variables, presented as numbers (%), the Fisher exact test was used for comparisons between the groups. Two independent variables were compared using the Mann-Whitney U test. Two dependent variables were compared using the Wilcoxon signed-rank test. The Spearman correlation coefficient (r_S) was used to assess relationships between serum cytokine concentrations, coagulation parameters, inflammatory markers, and cardiac injury biomarkers and other clinical, laboratory, and imaging data. The following thresholds were used to interpret correlation coefficients: r_S = 0-0.30, very weak; r_S = 0.31-0.50, weak; r_S = 0.51-0.70, moderate; r_S = 0.71-0.90, strong; and r_S = 0.91-1.00, very strong. A univariable and multivariable binary logistic regression model was used to identify independent predictors of in-hospital mortality during the in-hospital treatment period. Candidate variables considered for logistic regression included demographic variables (age and sex), PE status, and the prespecified candidate cytokines (day-5 IL-9 and IL-15), as well as other baseline and day-5 clinical and laboratory variables. Given the limited number of outcome events (n = 13 deaths), multivariable analyses were restricted to parsimonious age-adjusted models with no more than 1 additional predictor. Therefore, IL-9 and IL-15 were evaluated in separate age-adjusted models and were not entered simultaneously. Potential collinearity among candidate predictors, particularly between cytokines, was assessed by inspection of pairwise correlations, and predictors were not jointly modeled when collinearity or events-per-variable constraints could compromise model stability. We z-standardized key biomarkers (mean = 0, standard deviation [SD] = 1) to place them on a common scale, allowing effect estimates to be interpreted per 1-SD increase and improving comparability across predictors measured in different units. Discrimination of the multivariable model was assessed using the AUC. Given multiple comparisons within the cytokine panel, we performed a Benjamini-Hochberg false discovery rate (BH-FDR) sensitivity analysis separately for each time point (baseline and day 5) across the 15 cytokines (m = 15 tests per time point), using

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the univariable mortality regression *P* values; we report both the raw *P* values and BH-FDR–adjusted *P* values (q values). We also performed 2000 bootstrap resamples to internally validate the regression model and obtain more robust estimates of performance and uncertainty by quantifying optimism and sampling variability. Given the limited event count, all regression models were interpreted as exploratory low-events models rather than clinically deployable prediction tools. No baseline or day-5 biomarker values or key covariates were missing in the enrolled analytic cohort because no patient died or was discharged before day 5; therefore, no imputation procedures were required. Two-sided *P* values < 0.05 were considered statistically significant.

Results

Because the primary outcome was in-hospital all-cause mortality, the mortality analyses are reported below in a dedicated subsection. PE-related comparisons are presented first as descriptive secondary analyses.

Descriptive Statistics of the Studied Sample

Baseline characteristics were comparable between the PE and non-PE groups (**Tables 1, 2**). The median age of the study participants was 78 years (range, 64-86). PE was diagnosed in 19 of 47 (40.4%) patients, of which 15 patients (78.9%) had small branch PE and 4 patients (21.1%) had large branch PE; on CTPA scans, 8 patients (42.1%) presented with signs of acute right ventricular pressure overload.

Given the CTPA-based inclusion of symptomatic patients with suspected PE, this proportion should be interpreted as the diagnostic yield of CTPA within a selected high-risk cohort rather than the incidence of PE among unselected severe COVID-19 admissions.

Serum Cytokine Concentrations, Coagulation Parameters, and Cardiac Injury Biomarkers

In this study, we quantified serum cytokines, coagulation parameters, and cardiac injury biomarkers at baseline and on day 5. Differences between COVID-19 patients with confirmed PE and those without confirmed PE were assessed using the Mann-Whitney U test. The results are presented in **Tables 3 and 4**. Across the 15-cytokine panel, most cytokines showed no statistically significant differences between PE and non-PE groups at either baseline or day 5 (**Table 4**). Overall, these findings suggest that, within this CTPA-evaluated suspected-PE cohort, the measured biomarker profiles provided limited discrimination between PE and non-PE status. These PE-group comparisons were descriptive and exploratory and should not be overinterpreted on the basis of isolated nominal *P* values.

We additionally assessed within-group changes in these markers from baseline to day 5 separately for patients with confirmed PE and for those without confirmed PE, using the Wilcoxon signed-rank test. The results are presented in **Tables 3 and 4**.

Associations Between Serum Cytokine Concentrations, Coagulation Parameters, Inflammatory, Cardiac Injury Biomarkers, and Clinical Data

Associations between serum cytokine concentrations, coagulation parameters, inflammatory, cardiac injury biomarkers, and clinical data were evaluated, and baseline parameters were

Table 1. Main characteristics of the studied sample (demographics and vaccination).

Variable	PE group, n (%)/median (Q1-Q3)	Non-PE group, n (%)/median (Q1-Q3)	P value
Sex (male/female)	8 (42.1) / 11 (57.9)	10 (35.7) / 18 (64.3)	0.891
Age (years)	76 (64.00-86.00)	79 (64.00-84.75)	0.679
Body mass index (kg/m^2)	28.63 (23.42-31.22)	27.68 (22.33-33.17)	0.893
Smoker (Y/N)	2 (10.5) / 17 (89.5)	8 (28.6) / 20 (71.4)	0.168
Vaccination status (Y/N)	13 (68.4) / 6 (31.6)	14 (50.0) / 14 (50.0)	0.341
Prior COVID-19 (Y/N)	2 (10.5) / 17 (89.5)	2 (7.1) / 26 (92.9)	1.000
Prior venous thromboembolism (Y/N)	1 (5.3) / 18 (94.7)	1 (3.6) / 27 (96.4)	1.000
History of cancer (Y/N)	3 (15.8) / 16 (84.2)	4 (14.3) / 24 (85.7)	1.000
Hospitalization in the past year (Y/N)	5 (26.3) / 14 (73.7)	7 (25.0) / 21 (75.0)	1.000

Abbreviations: PE, pulmonary embolism; Q1, first quartile; Q3, third quartile; Y, yes; N, no.

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Table 2. Main characteristics of the studied sample (comorbidities, clinical management, and laboratory tests).

Variable	PE group, n (%)/median (Q1-Q3)	Non-PE group, n (%)/median (Q1-Q3)	P value
Deep vein thrombosis (Y/N)	1 (5.3) / 18 (94.7)	1 (3.6) / 27 (96.4)	1.000
Pneumonia (Y/N)	14 (73.7) / 5 (26.3)	23 (82.1) / 5 (17.9)	0.496
Arterial hypertension (Y/N)	16 (84.2) / 3 (15.8)	21 (75.0) / 7 (25.0)	0.718
Ischemic heart disease (Y/N)	8 (42.1) / 11 (57.9)	10 (35.7) / 18 (64.3)	0.891
Chronic heart failure (Y/N)	7 (36.8) / 12 (63.2)	9 (32.1) / 19 (67.9)	0.984
Diabetes (Y/N)	4 (21.1) / 15 (78.9)	7 (25.0) / 21 (75.0)	1.000
Atrial fibrillation (Y/N)	3 (15.8) / 16 (84.2)	6 (21.4) / 22 (78.6)	0.720
Cerebrovascular disease (Y/N)	10 (52.6) / 9 (47.4)	12 (42.9) / 16 (57.1)	0.718
COPD (Y/N)	1 (5.3) / 18 (94.7)	4 (14.3) / 24 (85.7)	0.635
Asthma (Y/N)	0 (0.0) / 19 (100.0)	3 (10.7) / 25 (89.3)	0.262
Gout (Y/N)	4 (21.1) / 15 (78.9)	2 (7.1) / 26 (92.9)	0.204
Dyslipidemia (Y/N)	2 (10.5) / 17 (89.5)	3 (10.7) / 25 (89.3)	1.000
Hypothyroidism (Y/N)	1 (5.3) / 18 (94.7)	3 (10.7) / 25 (89.3)	0.638
Anticoagulation therapy (Y/N)	19 (100.0) / 0 (0.0)	28 (100.0) / 0 (0.0)	0.404
Glucocorticosteroid therapy (Y/N)	5 (26.3) / 14 (73.7)	6 (21.4) / 22 (78.6)	0.737
Antiviral therapy (Y/N)	8 (42.1) / 11 (57.9)	9 (32.1) / 19 (67.9)	0.698
Oxygen therapy (days)	5 (2-11)	6 (3.25-9.75)	0.867
In-hospital stay (days)	14 (9-18)	8.5 (6.00-15.75)	0.096
Admission to ICU (Y/N)	2 (10.5) / 17 (89.5)	3 (10.7) / 25 (89.3)	1.000
In-hospital death (Y/N)	4 (21.1) / 15 (78.9)	9 (32.1) / 19 (67.9)	0.616
White blood cell count ($\times 10^9$/L)	9.87 (5.33-11.48)	7.79 (5.65-10.85)	0.461
Red blood cell count ($\times 10^{12}$/L)	4.37 (4.14-4.86)	4.34 (3.82-4.58)	0.537
Hemoglobin (g/L)	133.2 (118-144)	127.1 (114.23-139.23)	0.319
C-reactive protein (mg/L)	60.79 (41.10-85.10)	47.40 (27.35-60.38)	0.079
Potassium (mmol/L)	4.20 (3.71-4.50)	4.00 (3.63-4.45)	0.571
Sodium (mmol/L)	137.4 (134.9-139.9)	140.0 (135.6-143.0)	0.442
Creatinine (µmol/L)	80.70 (56.50-99.99)	74.5 (54.1-95.4)	0.468
Blood glucose (mmol/L)	8.2 (6-8.6)	6.50 (5.44-8.80)	0.412

Abbreviations: PE, pulmonary embolism; Q1, first quartile; Q3, third quartile; Y, yes; N, no; COPD, chronic obstructive pulmonary disease; ICU, intensive care unit.

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Table 3. Coagulation parameters and cardiac damage biomarkers at baseline and on day 5.

Variable	PE group, baseline, median (Q1-Q3)	Non-PE group, baseline, median (Q1-Q3)	PE group, day 5, median (Q1-Q3)	Non-PE group, day 5, median (Q1-Q3)	*P* value[1]	*P* value[2]
PLT (× 10^9/L)	223.7 (172.9-269.0)	248.4 (154.4-319.0)	278.6 (202.4-346.8)	323.0 (246.7-399.5)	0.584/ 0.302	**0.006/ 0.003**
D-dimer (mg/L)	3.33 (1.39-12.63)	2.320 (1.250-6.163)	1.52 (0.73-2.26)	1.63 (0.78-3.07)	0.270/ 0.682	**< 0.001/ 0.030**
Fibrinogen (g/L)	4.39 (3.90-5.83)	4.98 (4.01-5.36)	4.82 (3.76-5.05)	4.15 (3.58-5.26)	0.944/ 0.711	0.112/ **0.015**
PA (%)	81 (64-92)	88.5 (73.0-107.3)	74.0 (66.0-81.0)	82.0 (64.0-99.5)	**0.041/** 0.412	0.320/ 0.064
INR	1.09 (1.03-1.21)	1.05 (0.97-1.15)	1.16 (1.12-1.20)	1.13 (1.00-1.26)	0.108/ 0.382	0.209/ 0.063
aPTT (s)	42.3 (36.3-49.3)	36.4 (36.3-49.3)	36.4 (33.3-40.9)	36.1 (30.9-42.5)	0.096/ 0.643	**0.044/** 0.181
NT-proBNP (pmol/L)	222.0 (88.6-686.0)	164.5 (54.7-472.0)	89.1 (43.5-536.0)	116.5 (33.4-236.5)	0.215/ 0.543	**0.022/** 0.063
Hs-cTnI (µg/L)	0.05 (0.02-0.20)	0.01 (0.01-0.06)	0.04 (0.01-0.05)	0.01 (0.01-0.02)	**0.012/ 0.032**	**0.016/ 0.010**

PE, pulmonary embolism; Q1, first quartile; Q3, third quartile; PLT, platelet count; PA, prothrombin activity; INR, international normalized ratio; aPTT, activated partial thromboplastin time; NT-proBNP, amino-terminal pro-B-type natriuretic peptide; Hs-cTnI, high-sensitivity cardiac troponin I. *P* value[1]: PE group vs non-PE group at baseline/PE group vs non-PE group day 5; statistically significant differences are in bold (Mann-Whitney U test). *P* value[2]: PE group at baseline vs day 5/non-PE group at baseline vs day 5; statistically significant differences are in bold (Wilcoxon signed-rank test).

analyzed. The analysis revealed weak to moderate correlations. All statistically significant results are presented in **Table 5**.

We also evaluated the potential associations between PE status and selected demographic and clinical characteristics, including sex, age, vaccination status, and comorbidities, in the overall cohort and in stratified analyses of the PE and non-PE subgroups. Categorical comparisons were performed using the Fisher exact test. No statistically significant associations were identified in either the overall or subgroup analyses.

Outcome Analysis

Overall, in-hospital mortality was 13 of 47 (27.7%) and did not differ significantly by PE status (**Table 2**). To identify independent predictors of fatal outcomes, we fitted a multivariable binary logistic regression model with in-hospital death as the dependent variable. At baseline, no cytokine was associated with in-hospital mortality, and none remained significant after BH-FDR correction across the 15 baseline cytokine tests (lowest raw *P* values: IL-4, $P = 0.108$, $q = 0.867$; IL-15, $P = 0.122$, $q = 0.867$). Given the limited number of fatal events (n = 13), multivariable analyses were prespecified as parsimonious age-adjusted logistic regression models; day-5 IL-9 and IL-15 levels were examined in separate models (not entered simultaneously) to limit overfitting and potential collinearity. Inspection of pairwise correlations among the prespecified candidate cytokines did not reveal marked collinearity requiring variable exclusion; IL-9 and IL-15 were nevertheless modeled separately because of events-per-variable constraints and conceptual overlap.

In univariable binary logistic regression, several variables were associated with in-hospital all-cause mortality. Higher IL-9 and IL-15 levels at day 5 were associated with increased odds of death (odds ratio [OR] per 1-SD increase, 3.295; 95% CI, 1.293-8.394; $P = 0.012$; and OR per 1-SD increase, 2.582; 95% CI, 1.110-6.006; $P = 0.028$, respectively). After BH-FDR adjustment across the 15 day-5 cytokine tests (m = 15), the associations with mortality were no longer statistically significant (IL-9: raw $P = 0.012$, $q = 0.180$; IL-15: raw $P = 0.028$, $q = 0.210$), and should therefore be interpreted as exploratory. ICU admission and longer duration of oxygen therapy were also associated with increased odds of in-hospital all-cause mortality (OR, 14.667; 95% CI, 1.453-148.045; $P = 0.023$ for ICU admission; OR per day of oxygen therapy, 1.127; 95% CI, 1.003-1.268; $P = 0.045$). Other candidate variables did not show evidence of association with mortality at the univariable level (all $P > 0.05$) (**Tables 6, 7**).

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Table 4. Serum cytokine concentrations at baseline and on day 5.

Variable	PE group, baseline, median (Q1-Q3)	Non-PE group, baseline, median (Q1-Q3)	PE group, day 5, median (Q1-Q3)	Non-PE group, day 5, median (Q1-Q3)	*P* value[1]	*P* value[2]
IFN-α2 (pg/mL)	1.229 (0.410-2.068)	1.060 (0.621-1.862)	0.083 (0.410-2.452)	1.172 (0.730-1.75)	0.995/ 0.908	0.861/ 0.936
IFN-γ (pg/mL)	0.560 (0.415-2.375)	0.784 (0.399-1.998)	0.476 (0.232-1.779)	1.033 (0.323-4.392)	0.595/ 0.314	0.650/ 0.888
IL-1α (pg/mL)	4.904 (3.845-5.705)	5.364 (4.44-6.045)	4.904 (4.438-8.033)	6.270 (4.671-7.268)	0.242/ 0.199	0.069/ **0.031**
IL-1β (pg/mL)	0.9160 (0.581-1.569)	0.916 (0.581-1.407)	0.916 (0.581-1.569)	1.245 (1.081-1.730)	0.692/ 0.202	**0.042/ 0.020**
IL-1ra (pg/mL)	1236.79 (742.34-1532.87)	645.43 (414.55-1146.89)	1050.84 (596.70-1721.00)	623.97 (484.19-1129.072)	**0.034/** 0.131	0.570/ 1.000
IL-2 (pg/mL)	1.283 (0.895-1.522)	1.100 (0.997-1.415)	1.100 (0.997-1.630	1.309 (1.100-1.496)	0.569/ 0.780	0.600/ 0.237
IL-3 (pg/mL)	5.601 (4.432-6.987)	5.402 (3.857-6.987)	6.190 (3.857-9.422)	6.190 (4.624-7.791)	0.691/ 0.458	0.209/ **0.017**
IL-4 (pg/mL)	0.437 (0.308-0.577)	0.420 (0.350-0.542)	0.420 (0.282-0.629)	0.455 (0.350-0.665)	0.757/ 0.458	0.556/ 0.469
IL-6 (pg/mL)	10.528 (5.863-22.971)	9.064 (6.037-18.328)	7.981 (3.821-60.518)	7.981 (4.491-19.064)	0.554/ 0.961	0.925/ 0.627
IL-7 (pg/mL)	6.918 (4.712-10.246)	10.443 (7.494-13.792)	8.270 (4.299-14.101)	11.149 (7.254-14.619)	**0.009/** 0.268	0.177/ 0.968
IL-9 (pg/mL)	42.335 (34.574-50.096)	50.096 (26.655-65.413)	34.574 (18.735-50.096)	57.754 (34.574-76.794)	0.334/ **0.043**	0.928/ 0.409
IL-10 (pg/mL)	49.514 (30.443-62.339)	46.306 (32.810-85.042)	36.764 (27.297-55.909)	46.306 (35.182-57.515)	0.342/ 0.564	0.510/ 0.184
IL-15 (pg/mL)	1.709 (1.012-2.171)	1.9390(1.204-2.466)	1.370 (1.039-1.823)	1.997 (1.482-2.882)	0.505/ **0.030**	0.572/ 0.126
IL-33 (pg/mL)	4.329 (2.243-7.642)	2.243 (1.173-4.329)	4.329 (2.243-5.357)	3.293 (1.173-5.357)	**0.033/** 0.419	0.754/ 0.131
VEGF (pg/mL)	245.652 (166.755-337.885)	177.173 (95.301-385.930)	204.958 (164.788-415.046)	218.848 (111.434-311.600)	0.389/ 0.805	0.233/ 0.263

Abbreviations: PE, pulmonary embolism; Q1, first quartile; Q3, third quartile; IFN-α2, interferon alpha-2; IL, interleukin; IL-1ra, IL-1 receptor antagonist; VEGF, vascular endothelial growth factor. *P* value[1]: PE group vs non-PE group at baseline/PE group vs non-PE group day 5; statistically significant differences are in bold (Mann-Whitney U test). *P* value[2]: PE group at baseline vs day 5/non-PE group at baseline vs day 5; statistically significant differences are in bold (Wilcoxon signed-rank test).

These univariable findings were subsequently evaluated in multivariable models adjusted for age. Specifically, we fitted 2 separate age-adjusted models. Model 1 included day-5 IL-9 level and age, and model 2 included day-5 IL-15 level and age (**Table 8**). Discrimination of the multivariable model between fatal and nonfatal outcomes was evaluated using the AUC. In model 1, higher standardized IL-9 was associated with increased odds of in-hospital mortality (adjusted OR per 1-SD increase, 3.331; 95% CI, 1.289-8.608; $P = 0.013$). Model 1 showed moderate apparent discrimination (AUC = 0.774; 95% CI, 0.563-0.984; $P = 0.011$). In model 2, higher standardized IL-15 was also associated with increased odds of in-hospital mortality (adjusted OR per 1-SD increase, 2.745; 95% CI, 1.129-6.676; $P = 0.026$). In contrast, model 2 showed only modest apparent discrimination between lethal and nonlethal outcomes, which did not reach statistical significance (AUC = 0.714; 95% CI, 0.484-0.943; $P = 0.068$).

However, model 1 bootstrap resampling (2000 iterations) produced a very wide bias-corrected and accelerated (BCa) 95% bootstrap CI for the IL-9 coefficient, indicating limited coefficient stability and potential overfitting in this small cohort.

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Table 5. Correlation between baseline blood markers and clinical data.

Variable	OTD (rs; *P*)	Hs-cTnI (rs; *P*)	NT-proBNP (rs; *P*)	IFN-γ (rs; *P*)	IL-1ra (rs; *P*)	IL-6 (rs; *P*)	IL-10 (rs; *P*)
D-dimer	0.313; 0.032	0.396; 0.006	0.293; 0.046	-0.463; 0.002	0.410; 0.006	0.415; 0.006	-0.328; 0.003
Variable	**OTD (rs; *P*)**	**D-dimer (rs; *P*)**	**IFN-γ (rs; *P*)**	**IL-1ra (rs; *P*)**	**IL-2 (rs; *P*)**	**IL-6 (rs; *P*)**	
Hospital days	0.603; < 0.001	0.402; 0.005	-0.339; 0.026	0.365; 0.016	0.342; 0.025	0.420; 0.005	N/S
Variable	**PA (rs; *P*)**	**IL-1α (rs; *P*)**	**IL-1ra (rs; *P*)**	**IL-7 (rs; *P*)**	**VEGF (rs; *P*)**		
PLT	0.295; 0.044	0.347; 0.023	-0.525; < 0.001	0.409; 0.006	0.351; 0.021		
Variable	**VEGF (rs; *P*)**	**Fibrinogen (rs; *P*)**					
CRP	0.455; 0.002	0.395; 0.006					
WBC	0.354; 0.020	N/S					

Abbreviations: OTD, oxygen therapy duration; rs - Spearman's correlation coefficient; Hs-cTnI, high-sensitivity cardiac troponin I; NT-proBNP, amino-terminal pro-B-type natriuretic peptide; IFN, interferon; IL, interleukin; IL-1ra, IL-1 receptor antagonist; PA, prothrombin activity; VEGF, vascular endothelial growth factor; PLT, platelet count; CRP, C-reactive protein; WBC, white blood cell count; N/S, not significant.

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Specifically, the BCa 95% bootstrap CI for the IL-9 coefficient ranged from -0.156 to 3.584. Coefficients were unstable on bootstrap resampling, and AUC estimates were imprecise. These findings should therefore be interpreted as exploratory.

Discussion

In the present study, we evaluated the interplay between coagulation parameters, inflammatory and immunological markers, and clinical outcomes, with a particular focus on serum cytokine concentrations measured dynamically during hospitalization. The principal exploratory finding was that higher serum IL-9 measured on hospital day 5 was associated with increased odds of in-hospital all-cause mortality, and that a parsimonious day-5 model incorporating IL-9 showed moderate apparent discrimination. However, internal validation using bootstrap resampling revealed substantial coefficient instability, underscoring the exploratory nature of these findings.

Cytokines, Immunothrombosis, and COVID-19 Severity

COVID-19–associated coagulopathy is increasingly recognized as a manifestation of immunothrombosis, driven by endothelial dysfunction, platelet activation, dysregulated cytokine release, and excessive thrombin generation [4,7,10]. Most prior studies have focused on classical inflammatory biomarkers, particularly CRP and D-dimer, which consistently correlate with disease severity and mortality. Although IL-6 is also associated with severity and mortality, a direct link to thrombotic risk has not been established [6,10]. In contrast, the prognostic role of T-cell–associated cytokines, such as IL-9 and IL-15, has been far less explored in clinical cohorts. These biological considerations should be interpreted only as context, because, in the present study, the day-5 IL-9 and IL-15 findings were nominally significant in univariable analyses (raw $P = 0.012$ and $P = 0.028$) but did not persist after BH-FDR correction ($q = 0.180$ and $q = 0.210$).

IL-9 is a pleiotropic cytokine primarily produced by Th9 cells and innate lymphoid cells, with documented roles in immune amplification, mast cell activation, endothelial interaction, and tissue inflammation. Emerging experimental and translational data suggest that IL-9 contributes to airway inflammation, mast cell activation, and viral propagation in severe COVID-19, representing a Forkhead box protein O1–dependent Th9 cell–mediated immunopathological pathway [11,24]. Clinical data regarding IL-9 elevation in severe COVID-19 remain limited and inconsistent. Sadhu et al demonstrated elevated IL-9 mRNA expression in peripheral blood mononuclear cells from patients with active COVID-19, with experimental evidence linking IL-9 to adverse outcomes, including increased viral load and airway inflammation [11]. The apparent discordance between our results and those of Kalantar et al [25], who found no baseline IL-9 differences between critically ill COVID-19 patients and controls, may be explained by the following: (1) measurement at different time points (our day-5 measurements capture sustained immune activation not evident at baseline); (2) different patient populations (our cohort with suspected PE may select for immunothrombotic phenotypes); or (3) findings in our study that reflect a type I error given the exploratory nature and small sample size (deaths = 13). Accordingly, our data do not establish IL-9 as a prognostic biomarker; rather, they

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Table 6. Univariable binary logistic regression model to identify patients at risk of in-hospital all-cause mortality (demographic and clinical variables).

Variable	OR (95% CI)	P value[1]
Age (years)	1.003 (0.957-1.052)	0.894
Sex (male)	1.632 (0.613-11.298)	0.193
Smoking	1.157 (0.249-5.370)	0.852
Comorbidities		
Ischemic heart disease	1.010 (0.271-3.757)	0.989
Arterial hypertension	1.692 (0.308-9.285)	0.545
Chronic heart failure	1.307 (0.346-4.933)	0.693
Atrial fibrillation	3.692 (0.414-32.942)	0.242
Diabetes	1.714 (0.406-7.247)	0.464
History of cancer	2.250 (0.428-11.824)	0.338
Chronic obstructive pulmonary disease	1.600 (0.162-15.820)	0.688
Asthma	1.333 (0.110-16.089)	0.821
Hypothyrosis	9.900 (0.924-106.029)	0.058
Gout	1.364 (0.218-8.523)	0.740
Dyslipidemia	0.625 (0.063-6.180)	0.688
Total number of comorbidities	1.146 (0.826-1.591)	0.415
Vaccination status (vaccinated)	0.792 (0.214-2.924)	0.726
Imaging studies (baseline)		
Infiltration (chest X-Ray)	4.320 (0.489-38.126)	0.188
Pulmonary embolism (CTPA)	1.776 (0.457-6.910)	0.407
Management		
Bed days (days)	1.050 (0.978-1.128)	0.175
Oxygen therapy duration (days)	1.127 (1.003-1.268)	**0.045**
Intensive care unit admission	14.667 (1.453-148.045)	**0.023**
Anti-SARS-CoV-2 antivirals	2.333 (0.542-10.046)	0.255
Inflammatory markers (baseline)[2]		
White blood cell count (SD)	0.715 (0.346-1.480)	0.366
C-reactive protein (SD)	1.456 (0.794-2.670)	0.224

Abbreviations: OR, odds ratio; CTPA, computed tomography pulmonary angiography; SD, standard deviation.
[1]: Statistically significant results are in bold. [2]: Key biomarkers were standardized using z-transformation (mean = 0, standard deviation = 1), ORs therefore represent the change in odds of mortality per 1-SD increase in biomarker concentration.

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Table 7. Univariable binary logistic regression model to identify patients at risk of in-hospital all-cause mortality (cytokine panel, coagulation panel, and cardiac injury biomarkers).

Variable	OR (95% CI)		P value[2]
	Baseline	Day 5	
Coagulation panel[1]			
Platelets (SD)	1.150 (0.615-2.151)	1.176 (0.578-2.392)	0.661 / 0.654
D-dimer (SD)	1.147 (0.613-2.147)	1.696 (0.830-3.467)	0.668 / 0.147
Fibrinogen (SD)	0.853 (0.357-2.037)	0.969 (0.454-2.067)	0.720 / 0.935
Prothrombin activity (SD)	1.200 (0.628-2.291)	0.690 (0.322-1.480)	0.581 / 0.340
International normalized ratio (SD)	0.310 (0.000-380.853)	0.728 (0.161-3.296)	0.747 / 0.680
aPTT (SD)	1.752 (0.906-3.388)	0.883 (0.419-1.861)	0.096 / 0.744
Cardiac damage biomarkers[1]			
Hs-cTnI (SD)	0.663 (0.256-1.716)	0.441 (0.121-1.601)	0.397 / 0.213
NT-proBNP (SD)	1.117 (0.614-2.033)	1.256 (0.658-2.400)	0.716 / 0.490
Serum cytokine concentrations[1]			
Interferon alpha-2 (SD)	0.546 (0.111-2.685)	0.871 (0.389-1.952)	0.457 / 0.737
Interferon gamma (SD)	0.756 (0.282-2.024)	0.497 (0.151-1.634)	0.578 / 0.250
Interleukin-1 alpha (SD)	1.137 (0.586-2.206)	0.913 (0.391-2.134)	0.705 / 0.834
Interleukin-1 beta (SD)	1.045 (0.537-2.032)	1.369 (0.652-2.875)	0.897 / 0.406
Interleukin-1 receptor antagonist (SD)	0.878 (0.435-1.775)	1.619 (0.773-3.390)	0.718 / 0.202
Interleukin-2 (SD)	1.511 (0.750-3.045)	1.838 (0.833-4.057)	0.248 / 0.132
Interleukin-3 (SD)	1.030 (0.526-2.016)	1.198 (0.587-2.444)	0.931 / 0.619
Interleukin-4 (SD)	0.324 (0.082-1.282)	0.750 (0.234-2.407)	0.108 / 0.629
Interleukin-6 (SD)	1.212 (0.646-2.271)	1.501 (0.737-3.055)	0.549 / 0.263
Interleukin-7 (SD)	1.248 (0.639-2.437)	1.188 (0.554-2.549)	0.517 / 0.657
Interleukin-9 (SD)	1.257 (0.666-2.373)	3.295 (1.293-8.394)	0.480 / **0.012**
Interleukin-10 (SD)	0.898 (0.432-1.869)	3.334 (0.779-14.260)	0.774 / 0.104
Interleukin-15 (SD)	1.722 (0.865-3.427)	2.582 (1.110-6.006)	0.122 / **0.028**
Interleukin-33 (SD)	0.555 (0.102-3.002)	1.406 (0.664-2.976)	0.494 / 0.373
Vascular endothelial growth factor (SD)	1.519 (0.791-2.918)	1.504 (0.724-3.127)	0.210 / 0.274

Abbreviations: OR, odds ratio; aPTT, activated partial thromboplastin time; Hs-cTnI, high-sensitivity cardiac troponin I; NT-proBNP, amino-terminal pro-B-type natriuretic peptide; SD, standard deviation. 1: Key biomarkers were standardized using z-transformation (mean = 0, standard deviation = 1), ORs therefore represent the change in odds of mortality per 1-SD increase in biomarker concentration. 2: Baseline/day 5, statistically significant results are in bold.

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Table 8. Age-adjusted exploratory multivariable logistic regression models for in-hospital all-cause mortality.

Model	Predictor	Adjusted OR (95% CI)	*P* value	Apparent AUC (95% CI)	Bootstrap summary
Model 1 (age + day-5 IL-9)	Day-5 IL-9 (per SD increase)	3.331 (1.289-8.608)	0.013	0.774 (0.563-0.984)	BCa 95% bootstrap CI: -0.156 to 3.584; Bias: 1.353; Mean: 2.363; SE: 14.981
Model 2 (age + day-5 IL-15)	Day-5 IL-15 (per SD increase)	2.745 (1.129-6.676)	0.026	0.714 (0.484-0.943)	BCa 95% bootstrap CI: 0.034-66.214; Bias: 1.601; Mean: 2.804; SE: 21.819

Abbreviations: OR, odds ratio; AUC, area under the receiver operating characteristic curve; IL, interleukin; SD, standard deviation; BCa, bias-corrected and accelerated; SE, standard error.

suggest that day-5 IL-9 may be an exploratory correlate of an evolving severe immune phenotype that warrants confirmation.

IL-15, another cytokine evaluated in our study, plays a key role in natural killer cell activation and CD8+ T-cell survival [26]. Elevated IL-15 concentrations have been reported in severe COVID-19 and have been linked to hyperinflammatory phenotypes and immune exhaustion [27,28]. In our cohort, IL-15 should therefore be regarded as an exploratory signal rather than a stable independent predictor.

Dynamic Biomarkers and Exploratory Prognostic Modeling

A notable methodological aspect of the present study is the use of day-5 laboratory data to develop an exploratory prognostic model for subsequent in-hospital mortality. Importantly, no deaths or hospital discharges occurred before day 5, minimizing immortal-time bias and enabling a clinically meaningful day-5 risk re-assessment time point. Our day-5 sampling provided a clinically meaningful reassessment point without immortal-time bias; however, day-5 cytokine levels may reflect an established disease trajectory rather than early determinants of outcome. Therefore, elevations in IL-9 and IL-15 should be interpreted as exploratory prognostic signals accompanying progressive immune dysregulation and organ injury, and their value for early decision-making requires confirmation in larger cohorts with earlier longitudinal sampling and external validation. This approach aligns with growing evidence that dynamic changes in inflammatory and immune markers, rather than baseline values alone, provide superior prognostic information in COVID-19 [15,29].

The day-5 IL-9–based model demonstrated moderate discrimination (AUC = 0.774), comparable to or exceeding that reported for many single-biomarker models in COVID-19 cohorts [30,31]. However, given the small sample size and limited number of events in the present study, the reported AUC represents apparent performance and is likely optimistic. This discrimination estimate represents apparent (in-sample) performance because the model was developed and evaluated in the same dataset. The model was intentionally highly parsimonious (age plus a single cytokine predictor) given the small number of outcome events (n = 13) and therefore should not be interpreted as a comprehensive clinical prediction tool. Furthermore, bootstrap resampling yielded extremely wide bias-corrected confidence intervals for the IL-9 coefficient, indicating model instability and overfitting risk, consistent with the substantial model uncertainty and instability in this small cohort. These findings emphasize that statistical significance alone is insufficient in prognostic research, and that internal validation is essential for transparent interpretation.

PE and Mortality

PE was frequent in our cohort (40.4%), which is consistent with the results of previous studies in severe COVID-19 populations [32-34]. However, the presence of PE was not associated with in-hospital mortality in our cohort. Consistent with this finding, we also observed that most cytokines and routine coagulation markers did not differ between PE and non-PE groups at either baseline or day 5. Therefore, although the cohort was assembled around suspected PE and CTPA evaluation, the present study provides limited evidence that the evaluated biomarker panel can discriminate CTPA-confirmed PE from PE-negative severe COVID-19 in this selected population. Although CTPA-confirmed PE reflects clinically important macrovascular thromboembolism, its lack of association with mortality in our cohort does not exclude a major contribution of microvascular immunothrombosis in severe COVID-19 cases. COVID-19 lung injury is linked to endothelial dysfunction and diffuse pulmonary microthrombi that are not captured by CTPA and may occur independently of PE; therefore, outcomes

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may be driven more by microvascular thromboinflammation than by imaging-detected emboli alone. This finding is consistent with some reports, including those by Yousaf et al, Miro et al, and the meta-analysis by Gomez et al, but contrasts with larger studies that demonstrate an increased mortality risk associated with PE. For example, Gul et al reported an adjusted hazard ratio of 1.36 (95% CI, 1.20-1.55), and the meta-analysis by Fu et al found an OR of 2.21 (95% CI, 1.30-3.76; $P = 0.003$) [2,3,35-37]. These discrepancies likely reflect differences in study populations, baseline disease severity, PE diagnosis timing, and thromboprophylaxis protocols.

Although ICU admission and longer oxygen therapy duration were associated with mortality in univariable analyses, these variables were not entered into multivariable models because they reflect post-admission disease severity and clinical course. Inclusion of such downstream variables could introduce overadjustment bias and limited clinical utility for early risk stratification. Therefore, only baseline covariates and laboratory tests on day 5 were considered in adjusted analyses.

Strengths and Limitations

The strengths of this study include its prospective design, standardized cytokine profiling using multiplex assays, and dynamic assessment of biomarkers, allowing evaluation of evolving immune responses. However, several limitations must be acknowledged. The sample size was small, with only 13 deaths, limiting the complexity and stability of multivariable models. Accordingly, bootstrap resampling indicated extremely wide bias-corrected confidence intervals, coefficient instability, and imprecise AUC estimates, and the prognostic findings should be interpreted as exploratory. Moreover, in a BH-FDR sensitivity analysis across the day-5 cytokine panel, the IL-9 and IL-15 associations with mortality did not remain significant, underscoring the exploratory nature of these findings. External validation was not performed, and calibration metrics beyond discrimination could not be robustly assessed. Our restriction to a suspected-PE cohort undergoing CTPA likely enriched for immunothrombotic phenotypes and limits generalizability; thus, the observed PE prevalence should be interpreted as CTPA yield in a selected cohort, consistent with prior CTPA-based COVID-19 studies, rather than as a population estimate. Additionally, because PE status was not associated with mortality, and most cytokines did not differ between PE and non-PE groups, our data provide limited support for biomarker-based differentiation of CTPA-confirmed PE in severe COVID-19, and this question warrants larger dedicated diagnostic studies. Finally, cytokine measurements were obtained at predefined time points and may not capture rapid immune fluctuations.

Conclusions

In hospitalized patients with severe COVID-19 and suspected PE, day-5 IL-9 may be an exploratory marker of evolving risk, but this signal was sensitive to BH-FDR correction across the cytokine panel and to bootstrap-detected model instability. Dynamic immune profiling may support day-5 clinical reassessment; however, these findings require confirmation in larger multicenter cohorts with earlier longitudinal sampling and external validation before IL-9 can be considered for prognostic use.

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Institutions Where Work Was Done

This research was conducted in 3 centers of Kaunas Hospital of the Lithuanian University of Health Sciences (Baltijos str. 120, 47116, Kaunas, Lithuania; Laisvės av. 17, 44320, Kaunas, Lithuania; Josvainių str. 2, 47144, Kaunas, Lithuania) in the Departments of Infectious Diseases, Internal Diseases, Internal Medicine Diagnostics, and Intensive Care.

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