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REGULAR ARTICLE

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An increase in CD62L^{dim} neutrophils precedes the development of pulmonary embolisms in COVID-19 patients

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Abstract

Objectives: A high incidence of pulmonary embolism (PE) is reported in patients with critical coronavirus disease 2019 (COVID-19). Neutrophils may contribute to this through a process referred to as immunothrombosis. The aim of this study was to investigate the occurrence of neutrophil subpopulations in blood preceding the development of COVID-19 associated PE.

Methods: We studied COVID-19 patients admitted to the ICU of our tertiary hospital between 19-03-2020 and 17-05-2020. Point-of-care fully automated flow cytometry was performed prior to ICU admission, measuring the neutrophil activation/maturation markers CD10, CD11b, CD16 and CD62L. Neutrophil receptor expression was compared between patients who did or did not develop PE (as diagnosed on CT angiography) during or after their ICU stay.

Results: Among 25 eligible ICU patients, 22 subjects were included for analysis, of whom nine developed PE. The median (IQR) time between neutrophil phenotyping and PE occurrence was 9 (7-12) days. A significant increase in the immune-suppressive neutrophil phenotype CD16^{bright}/CD62L^{dim} was observed on the day of ICU admission ($P = 0.014$) in patients developing PE compared to patients who did not.

Conclusion: The increase in this neutrophil phenotype indicates that the increased number of CD16^{bright}/CD62L^{dim} neutrophils might be used as prognostic marker to predict those patients that will develop PE in critical COVID-19 patients.

Abbreviations: BMI, Body mass index; CD, Cluster of differentiation; COVID-19, Coronavirus disease 2019; fNLF, formyl-norleucyl-leucyl-phenylalanine; ICU, Intensive care unit; LMWH, low molecular weight heparin; PE, Pulmonary embolism; SARS-CoV-2, Severe acute respiratory syndrome coronavirus 2.

*The COVPACH study group are listed in Appendix A.

1 | INTRODUCTION

Various studies reported a remarkably high incidence of pulmonary embolisms (PE) and deep venous thrombosis in patients with coronavirus disease 2019 (COVID-19) admitted to the intensive care unit (ICU) (25%-31%) despite adequate thrombosis prophylaxis.[1,2] Neutrophils can contribute to pathologic venous and arterial thrombosis by a process called 'immunothrombosis'.[3,4] Immunothrombosis is a process where a thrombus is formed by fibrin, erythrocytes and immune cells (mainly neutrophils).[5] Markers associated with elevated neutrophil extracellular traps (NET) formation have been found in severe COVID-19 patients that correlated with disease severity and thrombosis. These data indicate neutrophil involvement,[6,7] although a dominant role of neutrophil dysfunction in early severe COVID-19 has recently been refuted. As shown by previous research, a specific neutrophil subset of $CD62L^{low}$ neutrophils is more prone to form NET-like structures.[8,9] To study the role of the innate immune system in coagulopathy, we investigated the presence of neutrophil phenotypes and the expression of neutrophil activation markers/adhesion receptors in COVID-19 patients. The purpose of this study was to explore whether changes in the neutrophil compartment measured by fully automated flow cytometry are associated with the development of PE in COVID-19 patients.

2 | METHODS

A cohort study was performed, which included ICU patients aged 18-years or older with proven COVID-19 who were presented in the University Medical Center Utrecht from 19-03-2020 till 17-05-2020. Patients were excluded if they (1) were receiving therapeutic heparin because of thrombosis in other places than the lung and (2) had a medical condition (eg leukaemia, stem cell transplantation) or were using medication affecting neutrophils (eg systemic chemotherapy, azathioprine). Study groups were formed based on whether or not patients developed PE during ICU admission. The last blood sample available for flow cytometry analysis before ICU admission was analysed. For this study, a waiver for an informed consent procedure was provided by the institutional medical ethics committee under protocol number 20-284/C. In addition, in line with the academic hospital policy, an opt-out procedure was in place for the use of patient data for research purposes. All procedures performed in this study were in accordance with the 1964 Helsinki declaration and its later amendments.

2.1 | Study procedure

In addition to the standard-of-care laboratory tests, an 4 mL or 9 mL Vacutainer® sodium heparin blood tube (Greiner Bio-One, Kremsmünster, Austria) was drawn.[10] The blood tube was placed in the automated AQUIOS CL® 'Load & Go' flow cytometer (Beckman Coulter, Miami, FL, USA), which combines automated sample preparation with automated single-cell analysis using flow cytometry.[11]

The blood tube was placed in the automated AQUIOS CL® 'Load & Go' flow cytometer (Beckman Coulter, Miami, FL, USA), which combines automated samples preparation with automated single-cell analysis using flow cytometry. A cassette filled with blood tubes is placed in the machine, and the device reads the barcodes on the blood tubes and automatically mixes and pipettes the blood and proceeds with antibody staining. After 15 minutes of incubation, 335 µl of lysing reagent A is added to lyse the blood cells, followed by 100 µl of lysing reagent B which slows the reaction caused by reagent A and preserves the white blood cells. Finally, the prepared sample is aspirated for analysis. Absolute leucocyte count is based on an electronic-volume measurement.

For this research purpose, a customized antibody mix was made and tested in the absence and presence of the bacterial/mitochondrial-derived stimulus N-formyl-norleucyl-leucyl-phenylalanine (fNLF) (BioCat GmbH, Heidelberg, Germany) with an end concentration of 10^{-5}M. The antibody panels consisted of CD16-FITC (clone 3G8, Beckman Coulter, Miami, FL, USA), CD11b-PE (clone Bear1, Beckman Coulter), CD62L-ECD (clone DREG56, Beckman Coulter) and CD10-PC5 (clone ALB1, Beckman Coulter).

2.2 | Analysis of flow cytometry data

For in-depth analysis, data files were exported from the AQUIOS CL® and imported into FlowJo® analysis software (Tree Star Inc, Ashland, OG, USA). Based on forward scatter and side scatter, polymorphonuclear leucocytes were gated. Eosinophils were identified and excluded from the polymorphonuclear leucocytes gate, based on CD16/CD62L expression. Hereafter, neutrophil markers were analysed in both the absence and presence of fNLF (10 µM), to analyse resting and activated neutrophils, respectively. Phenotypes of neutrophils were identified by the expression of CD16 and CD62L as described in detail before.[12]

2.3 | Clinical data

Baseline characteristics including age, sex, body mass index (BMI), immunocompromised (according to the International

Classification of Disease 9th revision), history of thrombotic disease (including DVT, PE, factor V Leiden, thrombosis due atrial fibrillation) and cardiovascular diseases (including hypertension, diabetes mellitus, cerebrovascular accident, myocardial infarction, peripheral artery and venous disease, aneurysms), and prehospital maintenance anticoagulation therapy and thromboprophylaxis during hospital stay (direct oral anticoagulant (DOAC), antiplatelet drug, vitamin K antagonist, low molecular weight heparin (LMWH)). At day of sample drawn, clinical values were collected at the time the patient needed the most oxygen support therapy. The following values of vital functions and laboratory were retrieved: fraction of inspired oxygen (FiO2) (%), respiratory rate (/min), pulse rate (/min), systolic blood pressure (mm Hg), diastolic blood pressure (mm Hg), temperature (°C), laboratory values CRP (g/L), leucocytes ($x10^9$/L) and arterial saturation (%). Baseline characteristics, clinical values, cell counts and neutrophil markers were compared between the PE group and the non-PE group.

Development of PE was diagnosed based on computed tomography angiography (CTA) assessed by a radiologist, showing partial or complete intraluminal filling defects in lobar, segmental and/or subsegmental pulmonary arteries. PE was considered absent in all other cases (ie either negative findings on CTA or no imaging performed). Medical conditions affecting neutrophils were defined according to the International Classification of Disease 10th revision. The development of acute kidney injury (AKI) was defined as having at least stage 2 of kidney injury, based on the AKIN classification.[13]

2.4 | Statistical analysis

Baseline characteristics and neutrophil receptor expression were compared between patients who developed PE and patients who did not. The worst measured daily clinical parameters were used for analysis. Continuous data were shown as median with interquartile range (IQR). Fisher's exact/chi-squared test and Mann-Whitney *U* test were used for dichotomous and continuous data, respectively. Statistical significance was defined as a $P < 0.05$. All data were analysed with Stata® version 13.0 (StataCorp LP, College Station, TX, USA), and GraphPad Prism version 7 (GraphPad software inc., San Diego, CA, USA) was used for data visualization.

3 | RESULTS AND DISCUSSION

A total of 26 patients (PCR positive for SARS-CoV-2) were initially admitted to the COVID-19 ward at the UMC Utrecht and later transferred to the ICU. After exclusion, 22 unique patients were included for further analyses (Figure 1). All patients were receiving LMWH thromboprophylaxis (dalteparin sodium) according to the hospital guidelines for thromboprophylaxis from the first day of hospital admission based on the Padua Prediction Score.[14] Nine (41%) patients developed a PE, of whom two patients developed larger embolisms (one saddle embolism and one central embolism) and seven patients developed smaller embolisms (two subsegmental, one segmental and four both segmental and subsegmental). Two patients developed an additional thrombotic complication during therapeutic coagulation therapy with heparin: one patient developed an ischaemic stroke 11 days after PE and one patient a deep venous thrombosis four days after PE. The percentage of PE development in our cohort (41%) is relatively high compared to other studies investigating PE development at the ICU, where prevalence of 20%-30% were shown.[15-17]

Baseline characteristics, clinical parameters at the day of blood sample analysis and clinical outcomes are shown in Table 1. Patients who developed a PE had significantly lower BMI compared to patients who did not develop a PE

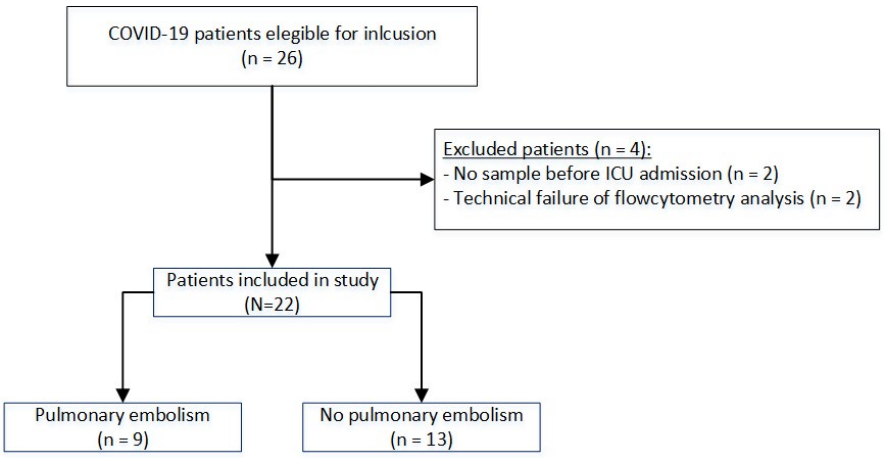

FIGURE 1 Flowchart of patient inclusion

TABLE 1 Differences between characteristics in COVID-19 patients who did and did not develop a pulmonary embolism

	No pulmonary embolism n = 13		Pulmonary embolism n = 9		*P*-value
Baseline characteristics					
Age, y	69.4	(65.8, 73.7)	59.2	(51.4, 60.7)	0.066
Gender, (m/f)	8/5	(62%/38%)	4/5	(57%/43%)	1.00
BMI, kg/m^2	31.9	(30.7, 37.5)	23.8	(20.4, 28.0)	<0.001
Hypertension	6	(46%)	2	(22%)	0.69
Diabetes mellitus	3	(23%)	1	(11%)	1.00
History of thrombotic disease	0	(0%)	1	(11%)	1.00
History of cardiovascular disease	11	(85%)	5	(56%)	0.18
Maintenance anticoagulation therapy prehosptial	8	(62%)	2	(22%)	
Vitamin K antagonist	4	(31%)	2	(22%)	
Antiplatelet drug	3	(23%)	0	(0%)	
Direct oral anticoagulant	1	(8%)	0	(0%)	
No therapy	5	(38%)	7	(78%)	
Thromboprophylaxis during admission	13	(100%)	9	(100%)	
LMWH	7	(54%)	8	(89%)	
LWMH + antiplatelet drug	4	(31%)	1	(11%)	
Vitamin K antagonist	2	(15%)	0	(0%)	
Time in hospital before ICU admission, d	2	(0, 3)	2	(1, 5)	0.38
Clinical parameters at the day of blood sample analysis					
FiO2, %	90	(60, 90)	90	(90, 90)	0.21
Respiratory rate, /min	31	(26, 35)	30	(26, 35)	0.91
Systolic blood pressure, mm Hg	135	(121, 152)	130	(120, 138)	0.57
Diastolic blood pressure, mm Hg	79	(67, 99)	80	(77, 90)	0.86
Heart rate, beats per minute	98	(82, 110)	85	(81, 97)	0.25
Temperature, °C	38.4	(37.7, 39.1)	38.0	(37.4, 39.0)	0.44
Arterial saturation, %	95	(90, 97)	94	(92, 98)	0.75
Clinical outcomes					
Mortality	2	(15%)	1	(11%)	
Acute kidney failure during ICU admission	0	(0%)	6	(67%)	0.001

Note: Data are presented as median (IQR) or number (%).

Abbreviations: FiO2, fraction of inspired oxygen; IQR, interquartile range; LWMH, light molecular weight heparin; y, year.

Percentage of missing values: FiO2—5%; Respiratory rate—5%; Systolic blood pressure—5%; Diastolic blood pressure—5%; Heart rate—9%; Arterial saturation—18%; all others: no missing values.

(23.8 (20.4-28.0) vs 31.9 (30.7-37.5) kg/m^2; respectively $P < 0.001$).[15] Furthermore, patients who developed a PE during ICU admission developed AKI more frequently than patients who did not develop PE (6/9 (67%) vs 0/13 (0%); $P = 0.001$). Several studies showed a high incidence of AKI in patients with COVID-19,[18-20] and only limited evidence is available on the correlation with pulmonary embolisms. Only one Chinese postmortem study found fibrin deposits in glomerular loops, supporting the concept that AKI might be caused by coagulation dysregulation and microcirculatory dysfunction.[21]

Results of the analysis of blood samples prior to admittance to the ICU are shown in Table 2. Both study groups were characterized by a median stay of 2 days at the COVID-19 ward before ICU admission. The median number of days from collection of the blood sample until

TABLE 2 Differences between blood samples prior to admittance to the ICU in COVID-19 patients

	No pulmonary embolism n = 13 (%)		Pulmonary embolism n = 9 (%)		*P*-value
Samples	13	(100%)	9	(100%)	
Time from blood sample until ICU admission, d	0	(0, 0)	0	(0, 1)	0.47
Time from blood sample until diagnosis of PE, d			9	(7, 12)	
tWBC, $x10^6$/mL	6.7	(5.6, 8.2)	8.3	(7.2, 10.6)	0.07
Granulocyte count, $x10^6$/mL	5.8	(5.4, 7.8)	7.4	(6.1, 9.7)	0.09
Neutrophil count, $x10^6$/mL	5.8	(5.2, 7.8)	7.4	(6.1, 9.6)	0.09
Eosinophil count, $x10^4$/mL	4.3	(2.1, 7.1)	2.5	(1.6, 5.3)	0.19
Lymphocyte count, $x10^6$/mL	0.56	(0.48, 0.67)	0.59	(0.45, 0.79)	0.51
Monocyte count, $x10^6$/mL	0.21	0.19, 0.33)	0.23	(0.17, 0.35)	0.83
CRP, g/L	122	(100, 170)	255	(160, 283)	0.003

Note: Data are presented as median (IQR) or number (%).

Abbreviations: IQR = interquartile range, CRP = C-reactive protein, tWBC = total white blood cell count.

Percentage of missing values: CRP—9%; Total leucocyte count—9%; all others: no missing values.

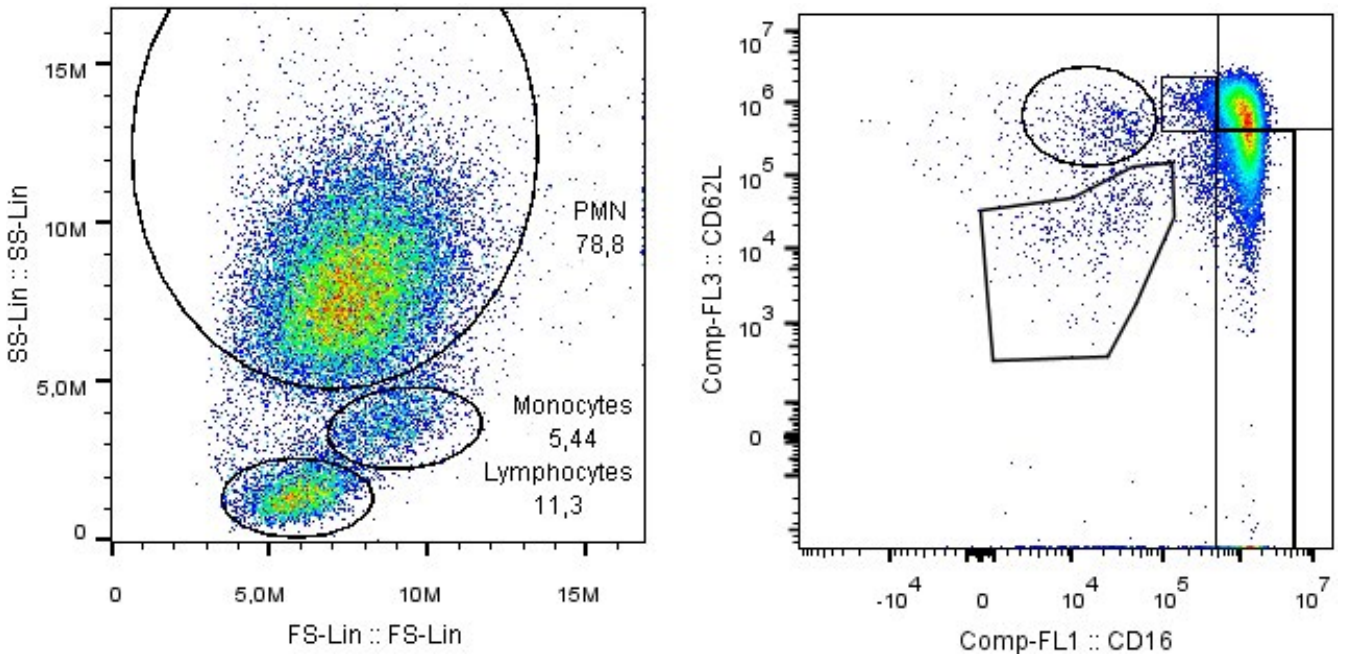

FIGURE 2 Gating strategy of the flowcytometric data. Gating strategy as performed in FlowJo® analysis software. The fNLF- and fNLF + samples were separately analysed. For both samples, a forward and side scatter plot was first made to set gates for the lymphocyte-monocyte and PMN populations (left plot). In the right plot, the CD16/CD62L expression of the PMN population is displayed. Gates were set to distinguish four populations based on expression of CD16 and CD62L as described before[12]

the development of PE was 9[7-12] days. C-reactive protein (CRP) was significantly higher in the PE group compared to the non-PE group ($P = 0.003$; Table 1). This was in line with other studies also describing a higher CRP in patients developing PE.[15-17]

Neutrophil phenotypes were gated based on CD62L and CD16 expression, as described before.[10,12] Gating strategy of CD16/CD62L neutrophil plots is shown in Figure 2.

As can be seen in Figure 3A-B, the median fluorescent intensity (MFI) of neutrophil markers CD11b and CD10 did not differ

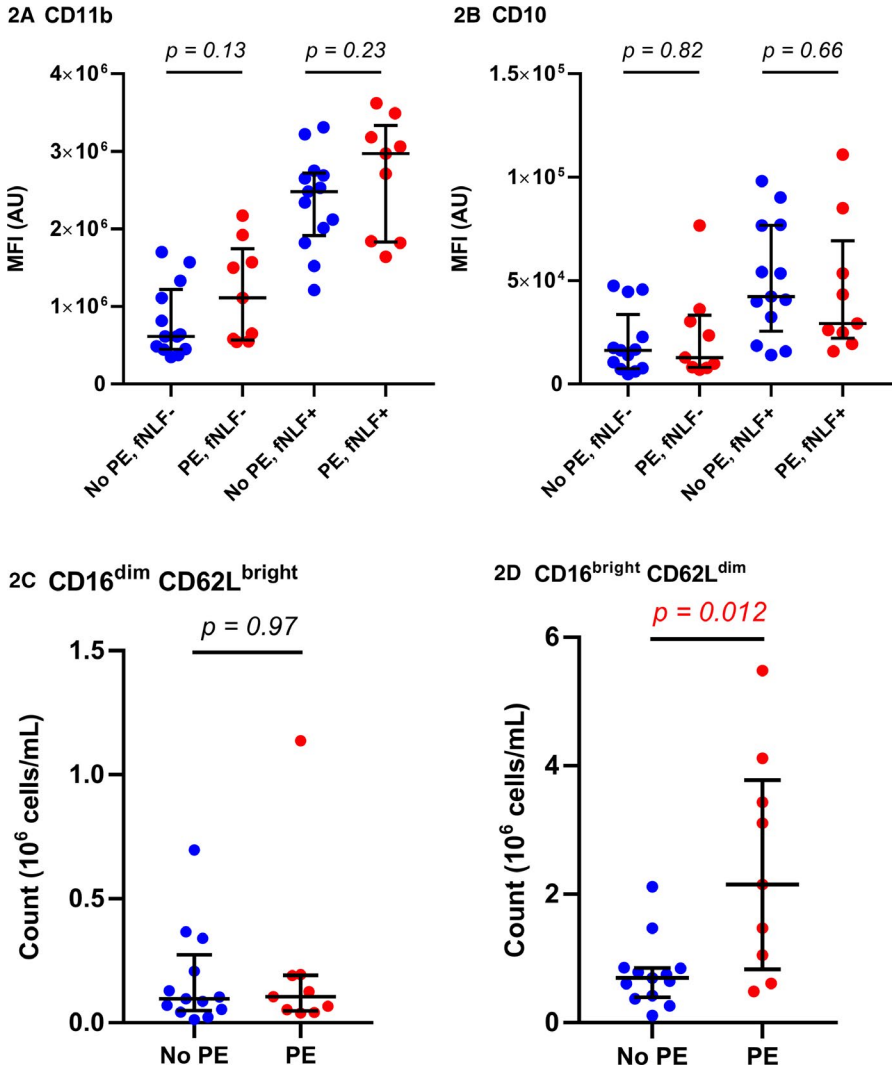

FIGURE 3 Differences in neutrophil receptor expression between COVID-19 patients who did and did not develop pulmonary embolism during hospital admission. Expression of neutrophil markers CD11b (A) and CD10 (B) with and without in vitro addition of a bacterial stimulus (10 μM fNLF). Neutrophil phenotypes based on the markers CD16/CD62L in whole blood: C) $CD16^{dim}/CD62L^{bright}$ cells; D) $CD16^{bright}/CD62L^{bright}$ cells; and E) $CD16^{bright}/CD62L^{dim}$ cells. Abbreviations: AU = arbitrary units, PE = pulmonary embolism, fNLF = N-Formyl-norleucyl-leucyl-phenylalanine

between study groups. In both groups, no differences in responsiveness to fNLF were found (Figure 3A-B). The overall younger $CD16^{dim}/CD62L^{bright}$ neutrophils with a banded shaped nucleus were not different between the groups (Figure 3C). In contrast, a statistically significant increase in $CD16^{bright}/CD62L^{dim}$ was observed in the PE group compared to the non-PE group ($P = 0.014$; Figure 3E) several days before the development of PE. Figure 4 visually shows the difference between a patient who develops a pulmonary embolism compare to a patient who did not. Example plots are shown of 2 different patients at the emergency department, −1 day before ICU admission and at the day of ICU admission. It is clear that the most $CD16^{bright}/CD62L^{dim}$ neutrophils are present at the day of ICU admission.

Causation is difficult to establish but their mere presence in the peripheral blood is a very promising predictor for development of PEs in COVID-19. On the other hand, these $CD16^{dim}/CD62L^{bright}$ neutrophils might be causally involved in the pathogenesis of COVID-19 due to their putative role in immunothrombosis.[6,7,22] Their low expression of L-selectin (CD62L) might result in a lowered capacity for cell adhesion to the endothelial wall, which might lead to an increased dwelling time in the vasculature. The increased dwell time and their increased propensity for undergoing cell death, including NETosis, can then facilitate to the development of PE in COVID-19.[6,7,22] Previous studies have indeed shown that NETosis is important for in immunothrombosis and correlates with the development of PE in COVID-19 patients.[6,7,22] However, further research with increased sample sizes is required and longitudinal data are needed to investigate the role of neutrophils in the pathogenesis of PE in COVID-19.

4 | CONCLUSION

In conclusion, this study is the first to investigate a role of neutrophils in the development of PE in COVID-19 patients. The increase of $CD16^{bright}/CD62L^{dim}$ neutrophils might possibly provide the missing link between altered hemostasis and malfunction of the immune system in the pathogenesis of PE in critical COVID-19 patients.

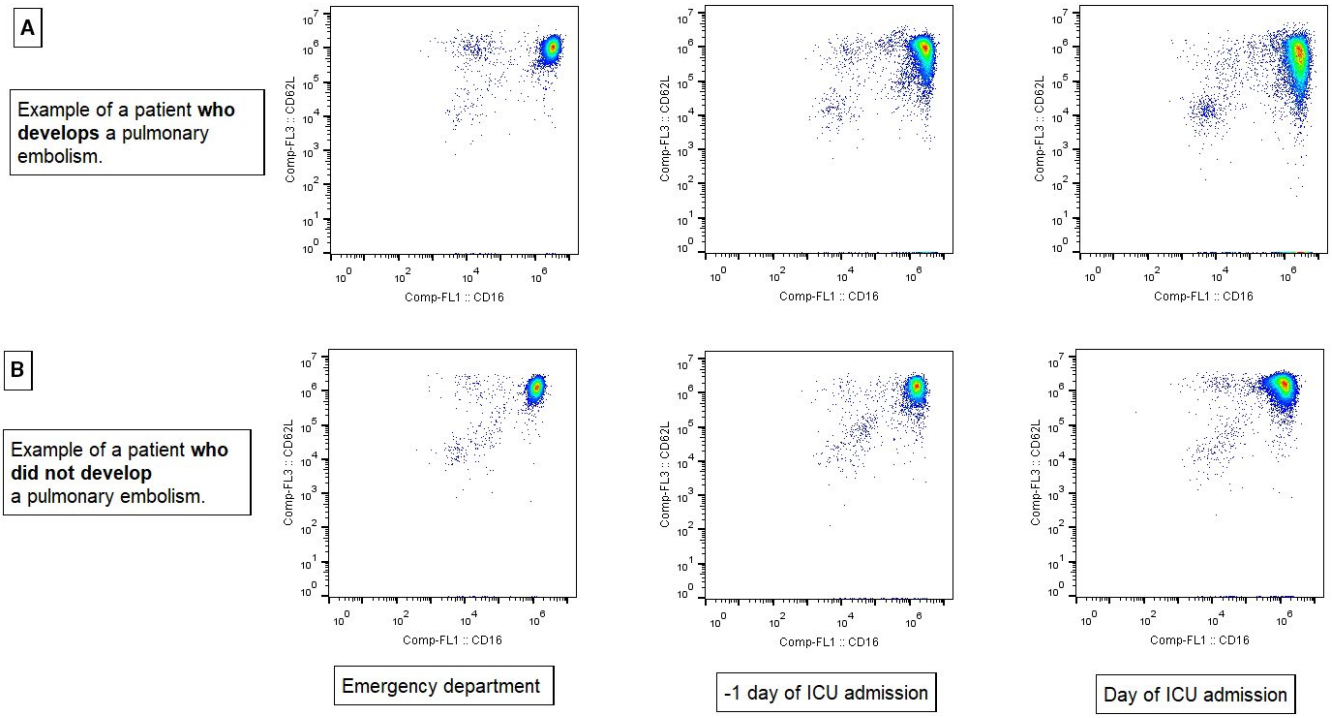

FIGURE 4 Increase of $CD16^{bright}/CD62L^{dim}$ neutrophils in the blood of a patient developing PE during hospital admission. This figure shows a representative difference in the occurrence of $CD16^{bright}/CD62L^{dim}$ neutrophils between a patient who developed a pulmonary embolism (A) compared to a patient who did not (B). The data are obtained at the emergency department, −1 day before ICU admission and at the day of ICU admission

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CONFLICT OF INTEREST

All authors declare that they have no potential conflict of interest.

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APPENDIX A

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