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Presence of procoagulant peripheral blood mononuclear cells in severe COVID-19 patients relate to ventilation perfusion mismatch and precede pulmonary embolism

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ABSTRACT

Purpose: Pulmonary emboli (PE) contribute substantially to coronavirus disease 2019 (COVID-19) related mortality and morbidity. Immune cell-mediated hyperinflammation drives the procoagulant state in COVID-19 patients, resulting in immunothrombosis. To study the role of peripheral blood mononuclear cells (PBMC) in the procoagulant state of COVID-19 patients, we performed a functional bioassay and related outcomes to the occurrence of PE. Secondary aims were to relate this functional assay to plasma D-dimer levels, ventilation perfusion mismatch and TF expression on monocyte subsets.

Methods: PBMC from an ICU biobank were obtained from 20 patients with a computed tomography angiograph (CTA) proven PE and compared to 15 COVID-19 controls without a proven PE. Functional procoagulant properties of PBMC were measured using a modified fibrin generation time (MC-FGT) assay. Tissue factor (TF) expression on monocyte subsets were measured by flow cytometry. Additional clinical data were obtained from patient records including end-tidal to arterial carbon dioxide gradient.

Results: MC-FGT levels were highest in the samples taken closest to the PE detection, similar to the end-tidal to arterial carbon dioxide gradient (ETCO2 – PaCO2), a measurement to quantify ventilation-perfusion mismatch. In patients without proven PE, peak MC-FGT relates to an increase in end-tidal to arterial carbon dioxide gradient. We identified non-classical, CD16 positive monocytes as the subset with increased TF expression.

Conclusion: We show that the procoagulant state of PBMC could aid in early detection of PE in COVID-19 ICU patients. Combined with end-tidal to ETCO2 – PaCO2 gradient, these tests could improve early detection of PE on the ICU.

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1. Introduction

Coronavirus disease 2019 (COVID-19) is the result of infection with the emerged severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2). The clinical spectrum ranges from mild, flu-like symptoms to a severe viral pneumonia in a minority of cases, resulting in an acute respiratory distress syndrome (ARDS). ARDS due to COVID-19 is characterised by a high risk of venous thromboembolisms (VTE), especially pulmonary embolism (PE) in at least 25% of ICU patients [1,2]. This occurs primarily in the absence of immunity acquired through vaccination or previous infection and in the presence of a hyperinflammatory innate immune responses to SARS-CoV-2, which fail to adequately limit viral replication [3]. Once severe illness has developed, treatment is mainly supportive, complemented with well-timed immunosuppression, treatment of superinfections and thromboprophylaxis or therapeutic anticoagulants.

Thrombotic complications contribute substantially to COVID-19 related mortality and morbidity and are caused by endothelial dysfunction, coupled with a hypercoagulable state induced by a complex multifactorial mechanism collectively termed "immunothrombosis" [4]. In this process, SARS-CoV-2 virus particles and cells damaged by infection induce an innate immune response from circulating neutrophils and monocytes, which release proinflammatory, prothrombotic and anti-thrombolytic factors into the blood plasma, resulting in a hypercoagulable state [5]. Tissue factor (TF) has been identified as one of the key prothrombotic factors driving immunothrombosis in both human data and animal studies [6,7]. When activated, TF is presented on the cell membrane of circulating monocytes, neutrophils or released in microvesicles. TF subsequently binds to Factor VIIa, triggering the extrinsic coagulation pathway[8]. The procoagulant effects of TF can be functionally measured by suspending peripheral blood mononuclear cells (PBMC) in blood plasma and adding calcium ions, which will result in clot formation, a method known as a thrombin generation, or in this case fibrin generation time (FGT) assay.

Timely recognition and treatment of PE is vital to improve survival. One of the most sensitive markers in diagnostics to detect PE is the use of plasma D-dimer levels. The D-dimer, formed by degradation of fibrin by plasmin, is an indicator of both activation of coagulation and fibrinolysis. In the absence of any YEARS criteria, VTE can be safely ruled out when plasma D-dimer levels are below 1000 ng/mL, at least in non-COVID-19 patients, or below 5,00 ng/mL in the presence of one of the YEARSs criteria [9]. D-dimer levels are higher than 1000 ng/mL in most patients with severe COVID-19, which makes it of little use in COVID-19 patients. Studies in COVID-19 inpatients have investigated the benefit of empirically initiating therapeutic dose anticoagulants, based on higher D-dimer cut-offs and other clinical risk scores, although this approach has shown no benefit for intensive care patients [10]. In critically ill patients for whom pulmonary angiography is not possible, a presumptive diagnosis of PE can be made, based on clinical features such as sudden onset hypoxia, chest pain and hypotension [11]. However, hypoxia and hypotension often occur in intensive care medicine and, with the use of sedation and analgesia, chest pain can easily remain unnoticed. Furthermore, D-dimer has been proven to be a sensitive marker of disease severity in COVID-19 patients also without a proven PE, and its use as a diagnostic marker for PE in critically ill patients thereby is limited[12].

The currently used methods for diagnosing PE rely on detecting the end net result of the immunothrombosis pathway, i.e. the presence of macrothrombi and resultant organ dysfunction. The aim of this study was to determine whether an assay measuring the procoagulant properties of PBMC, which drive immunothrombosis in COVID-19, could show potential for early detection of PE in critically ill COVID-19 patients, especially, compared to data from computed angiography (CTA) and end-tidal to arterial carbon dioxide gradient. The latter is known as a sensitive measure of ventilation to perfusion mismatch [13].

2. Methods

2.1. Study design and population

We performed longitudinal measurements of functional prothrombotic properties of PBMC's in an ICU cohort of COVID-19 patients with and without proven PE. Clinical data and samples used in this study originate from a longitudinal biorepository study (CIUM) of patients with acute respiratory distress syndrome (ARDS) and sepsis in the ICU of Erasmus Medical Centre, The Netherlands. After written informed consent, PBMC from critically ill COVID-19 patients from the first COVID-19 wave in the Netherlands were used, collected from March through to May 2020. For patients with a documented PE, monocyte fibrin generation test (MC-FGT) result before and after the diagnosis of PE were compared, from the available time points. Fig. 1 summarizes the design of this study. In addition, a case-control analysis of peak MC-FGT values of COVID-19 patients who suffered PE, compared to patients with no documented PE was performed. During the first COVID-19 wave in the Netherlands, EDTA anticoagulated whole blood was obtained for the isolation of plasma and PBMC on a weekly basis for up to 4 weeks, or until the patient was discharged from the ICU. For patients who were not conscious at the time of inclusion, deferred proxy consent was obtained, followed up with a retrospective opt-out procedure after recovery. The CIUM study protocol was approved by the institutional review board of Erasmus MC (MEC-2017-417 and MEC-2020-0222) based on World Medical Association Declaration of Helsinki: ethical principles for medical research involving human subjects including human samples and data. Healthy control PBMC were isolated from buffy coats, ordered from the Dutch blood transfusion service (Sanquin).

2.2. Fibrin generation time assay on PBMC (MC-FGT)

For this assay, we used an adapted version of a previously described protocol [14]. Blood was drawn into tubes containing EDTA for preparation of plasma and PBMC. PBMC were isolated using histopaque gradient medium, frozen in freezing medium (90% FBS, 10% DMSO) at −80 °C overnight and subsequently stored in Liquid Nitrogen until use. After thawing, PBMC were resuspended in ice cold RPMI medium, centrifuged at 400*g* for 10 min, resuspended in prewarmed R10 medium and counted. Subsequently, cells were washed twice in assay buffer: 25 nM HEPES, 137 mM NaCl, 0.1% BSA in deionized water at pH 7.4. Pooled citrated plasma from healthy donors was prewarmed to 37 °C and plated in 96 well flat-bottomed cell culture plates (80 μL/well). Subsequently, 75 μL of PBMC suspended in assay buffer (2×10^6 cells/mL) were added in duplicate to sample wells. Recombinant Tissue factor (rTF; Innovin, Siemens Healtineers, Marburg, Germany) was used as positive control and to generate a standard curve by 2-fold serial dilution in assay buffer (range 244–15.25 pg/mL). PBMC from healthy donors were used as negative controls, and stimulated using LPS, which is known to increase TF expression, for use as positive controls. Subsequently, 75 μl of assay buffer with 38 mM calcium chloride was added to the wells containing plasma with TF or PBMC, initiating the extrinsic coagulation cascade. A plate reader (Infinite F200, Tecan) was used to measure increasing absorption at 450 nm in each well in 30 s intervals, while keeping the plate at a stable temperature of 37 °C. OD values were plotted over time in minutes using a nonlinear regression curve to calculate the time until ½ of maximum OD was reached ($T_{1/2}$). Subsequently, the values obtained from the sample wells containing PBMCs were interpolated on the standard curve, to yield results in rTF equivalent concentrations.

2.3. Flow cytometry

Monocytes were isolated from PBMC using negative selection by magnetic activated cell sorting (MACS, Human Pan Monocyte Isolation Kit, Miltenyi Biotech) according to the manufacturer's instructions.

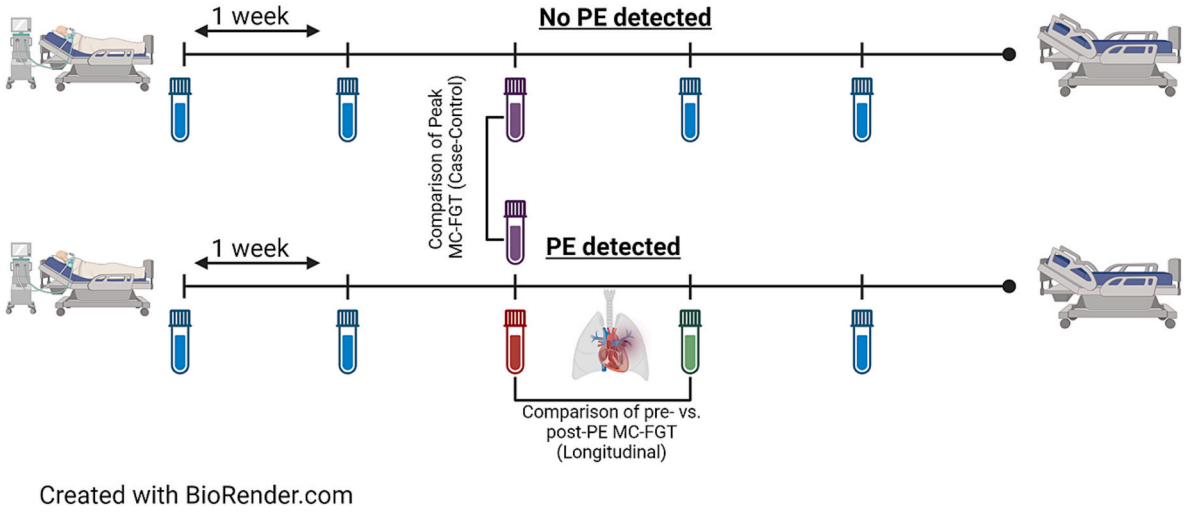

Fig. 1. Study design.

Monocyte were stained anti-CD14^{BV605} (Clone M5E2, BD, 1:100), anti-CD16^{AF700} (Clone 3G8, Biolegend, 1:100) and anti-TF^{AF647} (Clone #323519, R&D systems, 1:150) for 15 min, washed twice and resuspended in FACS buffer. Samples were measured on a BD FACSLyric. It was previously shown that TF expressed by monocytes is elevated in PBMC in COVID-19 patients.[8] These were specifically monocytes with high CD 16 expression (non-classical and intermediate subtypes). To determine whether TF measured immunocytochemically correlates with functional coagulant properties measured in MC-FGT, cell surface staining was performed on monocytes from COVID-19 ICU patients for CD 14, CD 16 and TF.

2.4. Statistics

Statistical comparisons between the PE and non-PE groups were performed using independent samples *t*-tests (after confirmation of a normal distribution, or after log transformation) for continuous variables and Chi-Squared tests for binominal variable, or Fisher's exact test when appropriate. For longitudinal analyses of MC-FGT, D-dimer and ETCO2 – PaCO2 gradients, paired *t*-tests were used, after log-transformation in the case of the MC-FGT. For case-control analyses, Mann-Whitey *U* tests were used. We made use of GraphPad Prism version 9.0.0 for Windows, GraphPad Software, San Diego, California USA, www.graphpad.com".

Table 1
Patient characteristics.

Characteristic	No CTA or CTA negative (n = 15)			CTA proven PE (N = 20)			P	All (n = 35)		
Age (Median, $IQR_{min\text{-}max}$)	62	54	70	66	63	73	0.165	65	59	72
Female (N, %)	4	27		4	20		0.700	8	8	23
BMI (Median, $IQR_{min\text{-}max}$)	30	24	33	27	25	30	0.260	28	25	31
Days in ICU (Median, $IQR_{min\text{-}max}$)	17	13	29	23	16	31	0.095	21	15	29
Days on ventilator (Median, $IQR_{min\text{-}max}$)	17	6	28	20	12	30	0.479	18	11	28
Comorbidities N, %										
Cardiovascular	1	7		4	27		–	5	5	14
Hypertension	1	7		4	27		–	5	5	14
Vascular disease	2	13		1	7		–	3	3	9
Diabetes	2	13		5	33		–	7	7	20
Neurological disease	2	13		0	0		–	2	2	6
Malignancy	3	20		1	7		–	4	4	11
Pulmonary disease	4	27		3	20		–	7	7	20
CCI (Median, $IQR_{min\text{-}max}$)	3	1	4	2	2	3	0.585	2	2	3
SOFA (Median, $IQR_{min\text{-}max}$)	6	5	8	6	5	8	0.525	6	5	8

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Diabetes	2	13		5	33		–	7	7	20
Neurological disease	2	13		0	0		–	2	2	6
Malignancy	3	20		1	7		–	4	4	11
Pulmonary disease	4	27		3	20		–	7	7	20
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Table 2
Clinical characteristics of COVID-19 patients without a CTA proven PE including anticoagulant treatment, MC-FGT and ETCo2-PaCo2 gradient results. The occurrence of PE could not be ruled out in some cases during high MC-TGT results. Allo-SCT = Allogeneic Stem Cell Transplantation, Y/N = Yes/No, VKA = Vitamin K antagonist, LMWH = Low Molecular Weight Heparin, CML = Chronic Myeloid Leukemia, RV/LV ratio = Right Ventricle-to-Left Ventricle diameter ratio, DOAC = Direct Oral Anticoagulant, FVIII = Factor VIII, NSCLC = Non-Small Cell Lung Carcinoma. D0 = Day of ICU admission, UFH = Unfractionated Heparin.

Id	Age	Sex	CTA	Anticoagulant	MC-FGT						$PaCO_2$	$ETCO_2$	$PaCO_2$	$ETCO_2$	Description
			Y/N Day Result		Peak	Nadir	At peak D-dimer	At nadir D-dimer	At peak $PaCO_2$/$ETCO_2$	At nadir $PaCO_2$/$ETCO_2$	D0	D0	At nadir MC-FGT	At nadir MC-FGT	
1	59	F	No	Nadroparin 5700 IU day 0–3. Therapeutic dose from day 3 onward.	51	10	2.25	4.12	2.3	1.8	5.6	3.3	6.5	4.7	Prone ventilation. Deceased
2	74	F	No	Pre-hospital VKA. therapeutic Nadroparin during ICU stay.		14		2.82		1.1			5.6	4.5	No CTA because of existing indication for therapeutic anticoagulation. Recovered.
3	53	M	No	Nadroparin 5700 IU bid	112	6	4.03	2.71	2.1	0.8	6.9	4.8	6.20	5.4	No aXa levels measured. Recovered
4	62	F	No	Nadroparin 5700 IU bid		10		1.57		1.4			5.6	4.2	cT4NxM0 IIIb NSCLC
5	61	M	Day 0 No PE	Nadroparin 5700 IU bid	77	3			1.5	0.1	7.5	6	4.8	4.7	CTA report: suboptimal contrast. tr. Pulmonalis 31 mm RV/LV ratio 1.2 no central PE. Plain CT 12 weeks later: normal a. pulmonalis
6	77	M	No	Nadroparin 5700 IU od; day 14 Dabigatran	37		0.74		1.7		6.1	4.4			Thrombophlebitis upper extremities. DOAC started after AF de novo with high CHA_2DS_2-VASc. Deceased.
7	66	M	No	Nadroparin 5700 IU od	66	4	4.62	1.42	1.1	0.5	5.1	4	4.3	3.8	2 × 72 h prone position
8	61	M	No	Nadroparin 5700 IU od		9		0.66		0.6			5.00	4.4	Pre-existing pulmonary sarcoidosis. Recovered.
9	54	M	No	Nadroparin 5700 IU od; 1–5 bid		9		2.6		0.7			6	5.3	2× prone position. Control CT complete recovery; first PBMC sample day 7
10	29	M	day 8 No PE	Nadroparin 5700 IU bid	79		2.29								No mechanical ventilation. 2017 CML treated with Allo-SCT
11	40	M	Plain thorax CT	Nadroparin 5700 IU bid	89	3	6.6	1.6	0.7	0.1	5.4	4.7	5.80	5.70	72 h proning during peak FGT. Recovered
12	75	M	Plain thorax CT	Nadroparin 5700 IU bid	117		2.5								No ET-CO_2 measurement recorded. Hemoptysis reported on day 5
13	70	F	day 0 No PE	Nadroparin 5700 IU od		4		2.95		1.7			6.40	4.7	
14	64	M	day 0 No PE	DOAC pre hospital. Therapeutic UFH during ICU stay.	59				2		7.5	5.5			anti Xa during FGT 0.25
15	66*	M	day 0 No PE	Nadroparin 5700 IU	–	0	–	5.5		2.0	5.5	3.5			Follow up: massive PE; deceased; no samples available after 02–04

3. Results

3.1. Patients

To assess the performance of the MC-FGT in COVID-19 ICU patients, PBMC were collected from 35 ICU patients, admitted during the first wave of COVID-19 in the Netherlands. Patient baseline characteristics are shown in Table 1. Detailed information individual patients in the non-PE group is presented in Table 2. Both groups were similar regarding disease severity at the time of admission (SOFA score), comorbidities (CCI), age, sex and BMI. Of the 35 COVID-19 patients, 20 had a computed tomography angiography (CTA) proven PE. CTA was performed because of a clinical suspicion of PE. The remaining 15 patients either had a negative CTA during their hospitalization ($n = 5$) or did not have a CTA performed ($n = 10$). Two patients did not undergo CTA, because they had an existing indication for therapeutic anticoagulation (Atrial Fibrillation with an elevated CHA2DS2-VASc score), which was continued. The remaining eight subjects were not suspected of having PE or VTE but were treated with prophylactic- or intermediate dose low molecular weight heparin (LMWH), at the discretion of the intensive care specialist.

3.2. MC-FGT, D-dimer and ETCO2 – PaCO2 gradient in COVID-19 patients with proven PE

From patients diagnosed with PE, sample collection time relative to diagnosis was categorized as: 1. Timepoint available closest to the positive CTA conforming PE (sample drawn within 5 days before positive CTA), 2. Timepoint available before the positive CTA confirming PE (>5 days) and 3. ≥ 48 h after the CTA confirming PE. As controls, we analysed MC-FGT in PBMC from 4 healthy individuals. Clinical data from timepoints matching the PBMC collection dates was extracted from patient records, including D-dimer levels and ETCO2 – PaCO2 gradients. D-dimer levels (Fig. 2a), were highly elevated (>1 μg/mL) in 18/19 (95%) of patients who suffered PE at all available timepoints (1 patient missing D-dimer data $n = 20$). D-dimer levels within 48 h before detection of PE (mean 7,2 SD 5.3) did not significantly differ from D-dimer levels >5 days before the detection of PE or 48 h after ($p = 0.07$). When comparing ETCO2 – PaCO2 gradient (Fig. 2b), a significant difference is observed ($p = 0.0003$) with a mean gradient of 2 kPa (+/− 1.2) closest to PE versus 0.9 (+/− 0.7) >5 days before PE. This gradient also significantly decreased to 1.4kpa (+/− 1.2) 48 h after treatment with anticoagulants ($p = 0.04$). When performing the same analysis on the MC-FGT assay results, a similar effect was observed, with the highest values measured at the closest timepoint before PE (Fig. 2c), significantly higher compared to >48 h after PE ($p = 0.02$) and compared to healthy controls ($p = 0.003$). For the earliest timepoint (>5 days before PE) only 4 paired samples were available, all of which were either higher compared to the >48 h after PE timepoint, or lower than the closest before PE timepoint, but the difference was not statistically significant.

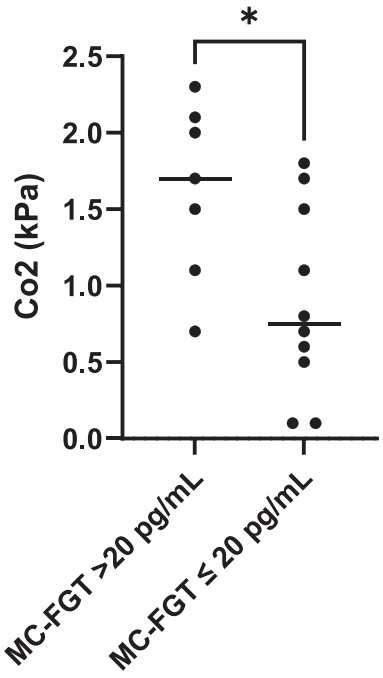

Fig. 3. Peak MC-FGT results in patients without a proven PE compared to those without a proven PE. * = $P < 0.05$. PE = pulmonary embolism MC-FGT = modified fibrin generation time.

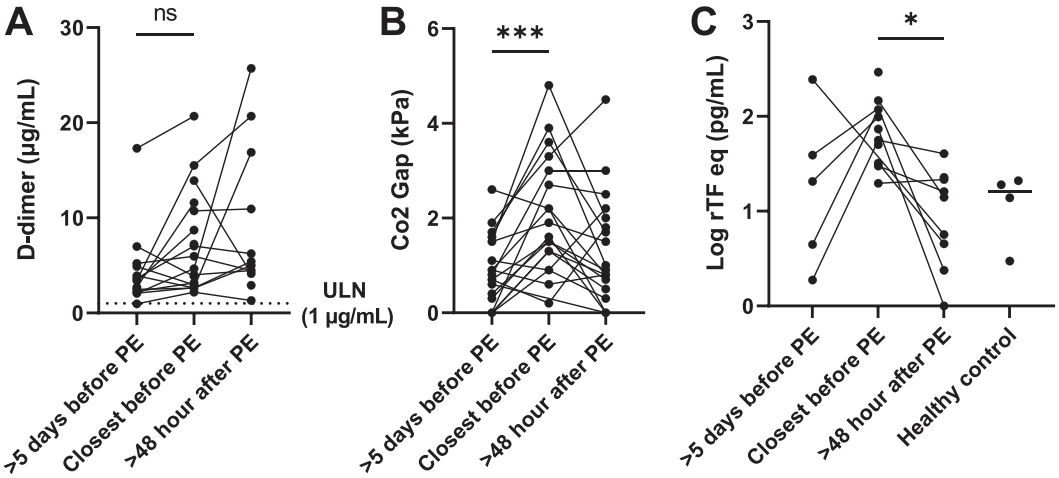

Fig. 2. D-dimer, ET CO2-PaCO2 gradient and MC-FGT in COVID-19 patients with CTA proven PE. A. 19 patients with computed tomography angiography proven pulmonary embolism had D-dimer levels above 1 μg/mL. There is no statistically significant difference in d-dimer level closest to the detection of PE compared to a sample taken before PE, with D-dimer remaining elevated after the start of treatment. B. Closest before detection of PE, a significant increase of the ETCO2 – PaCO2 gradient is observed, with a subsequent decrease after initiation of anticoagulant treatment. C. MC-FGT levels significantly higher closely before PE compared to >48 h after and compared to healthy controls (line indicates the median). Black dots are individual values, with connecting lines between values originating from the same patients. ULN = Upper Limit of Normal, NS = nonsignificant. * = $P < 0.05$, *** = $P < 0.0001$.

3.3. MC-FGT, D-dimer and ETCO2 – PaCO2 gradient in COVID-19 patients with or without a CTA proven PE

Subsequently, a case-control analysis was performed between patients with or without a CTA proven PE. Because of the observed increase in MC-FGT in the longitudinal analysis of patients with proven PE, peak observed MC-FGT values in both groups were used for comparison. Median peak MC-FGT in patients with proven PE was 109 pg/mL (range 17–292), versus 51 pg/mL patients without a proven PE (range 0–117, $p = 0.02$), whereas the highest MC-FGT in healthy controls was 21 pg/mL (range 3–21). Despite this observed statistically significant difference, several patients without a proven PE showed MC-FGT levels higher than healthy controls (Fig. 3). This raised the possibility that these patients were in a similar procoagulant state to the proven PE group. Raising the possibility of undiagnosed PE, or microvascular thrombosis in the lungs eluding detection by CTA. To test this hypothesis, an additional subgroup analysis was performed, comparing the ventilation perfusion mismatch by end tidal CO2-PaCO2 gradient measured in patients without a CTA proven PE at timepoints where MC-FGT was above that of healthy controls (>20 pg/mL) versus below (≤20 pg/mL). This revealed a significantly higher ET Co2-PaCo2 gradient in timepoints with elevated MC-FGT (Fig. 4, $p = 0.04$), suggesting a ventilation-perfusion mismatch, which can have multiple causes, including PE. Individual MC-FGT values and their corresponding ET Co2-PaCo2 gradient used for this analysis are listed in Table 2. Patient details offer explanation for increased MC-FGT results. For instance, patient 6 suffered from thrombophlebitis. In specific cases [1,3,5,12] CTA was not performed or suboptimal. Of specific interest is patient 15. For this patient only a sample drawn at inclusion was available for MC-FGT testing, which yielded a low result. On that day also a negative CT scan was available. Follow-up sampling was not performed. However, this patient deceased due to a CTA proven massive PE in the following days. Of specific interest is the high end tidal to PaCo2 gap already present early after ICU admission (Table 2).

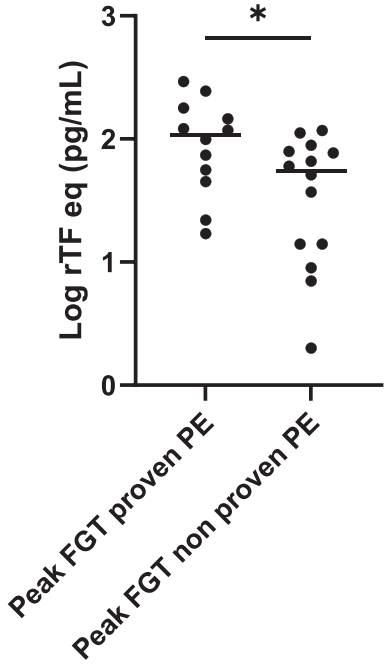

Fig. 4. End tidal CO2 PaCO2 gradient taken concurrently with elevated versus non-elevated MC-FGT measurements from patients without a CTA-proven PE. * = P < 0.05.

3.4. TF expression in monocyte subsets of COVID-19 patients in the ICU

Classical, non-classical and intermediate monocyte subsets were gated using CD 14 and CD 16 markers (Fig. 5, panel A and B). A longitudinal analysis of monocyte subsets, yielded a pattern of high non-classical and intermediate monocytes at the closest timepoint before PE, which normalized in the post-PE timepoints (Fig. 5, panel C). Patients without a proven PE also had more of these monocytes, both at the timepoints coinciding with peak and nadir MC-FGT results (Fig. 5, panel C). Both patients with proven PE and non-proven PE had similar average levels of CD16-expressing monocytes, but in patients with proven PE, there were more nonclassical monocytes, whereas in non-proven PE patients, intermediate monocytes were overrepresented (Fig. 5, Panel D). There was no positive correlation between immunocytochemically measured TF or CD16 expression on the monocyte cell surface and function coagulability measured in the MC-FGT assay (Pearson's R2 of 0.02 and 0.06 respectively). However, there was highly significant co-expression of TF and CD16 in monocytes (Fig. 4, panel E).

4. Discussion

In this study, we investigated whether a functional coagulation assay on PBMC can be used as a means of measuring procoagulant changes in COVID-19 patients potentially leading to immunothrombosis. D-dimer levels were highly elevated in all subjects and at all timepoints, not contributing to discriminate between PE and non-PE. Longitudinal analysis of COVID-19 ICU patients who developed PE during the course of their disease showed an increase in MC-FGT values shortly before the diagnosis was made radiographically, suggesting this assay could have future potential as an early diagnostic tool. Furthermore, when CTA is not possible a combination of MC-FGT and ET CO2 gap could help deciding to start therapeutic anticoagulation at an earlier stage. Peak MC-FGT results were higher in patients who suffered PE, compared to those without a diagnosis of PE during their ICU stay, although MC-FGT levels were elevated above healthy control levels in both groups. There may be a distinct possibility of missed PE diagnosis in this patient cohort for multiple reasons: [1] obtaining CTA scans in mechanically ventilated patients is not always feasible given the risk associated with transportation, especially in patients ventilated in prone position, [2] a negative CTA for PE does not exclude the possibility of one occurring subsequently and [3] microvascular thrombosis may not have been detected in CTA of patients classed as not having had a PE. We attempted to address this by correlating the MC-FGT with the ET Co2-PaCo2 gradient, which demonstrates a relation with ventilation/perfusion mismatch, a hallmark of PE. However, an elevated ET Co2-PaCo2 gradient may also be an indicator of atelectasis or other reasons for ventilation perfusion mismatch[15]. Therefore, the combination of ET Co2-PaCo2 gradient with an assay to determine the procoagulant state of a patient could be superior to current diagnostics.

It is not clear what the relative contribution is of different cell types to the procoagulant effect seen in the MC-FGT assay, but previous work suggests that both monocytes and neutrophilic granulocytes are the major contributors[4]. We found a dominant presence of CD16+ monocyte subsets, in previous studies identified as the population responsible for tissue factor mediated procoagulant changes[4]. We were not able to find a positive correlation between MC-FGT and tissue factor expression on these monocyte subsets. This is likely due to the fact that the TF antibody used binds both active and inactive forms of TF and that TF excreted in microvesicles is not detected by staining the cell membrane. In addition to secreting TF, neutrophils will also undergo NETosis during clot formation, which is also likely a contributing factor to pulmonary thrombosis. Previously published work has demonstrated that NETs are present in high levels in plasma of patients included in this cohort [16,17]. Very little is known about the extent to which these cell types are able to regulate immunothrombotic processes through expression of inhibitory proteins, such as Tissue Factor Pathway

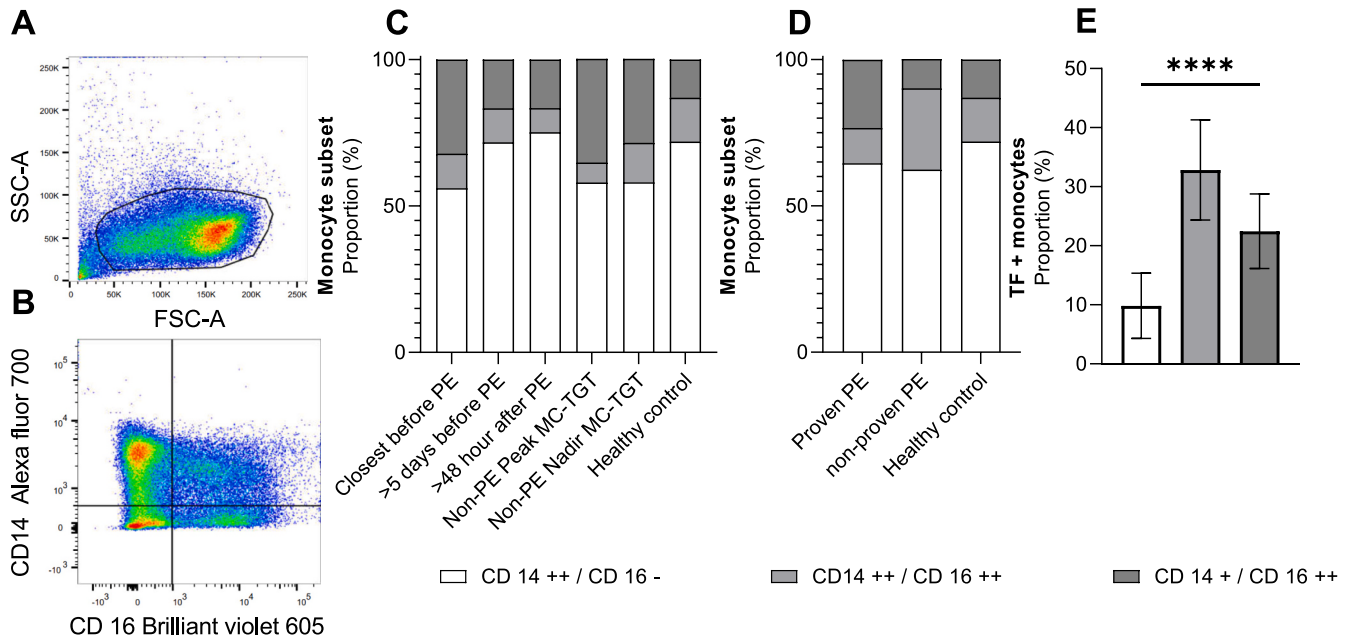

Fig. 5. Relative tissue factor expression in classical (CD14++/CD16-), intermediate (CD14 ++/CD16 ++) and nonclassical (CD14 +/ CD16 ++) monocyte subsets, determined by flow cytometry using cell-surface staining of monocytes isolated from COVID-19 ICU patients' monocytes. A. Forward-sideward scatter plot of MACS-separated monocytes of a healthy donor. B. Monocyte subset gating on the same healthy donor. C. monocyte subset distribution in longitudinal analysis groups. D. Average monocyte subset distribution across all timepoints of patients with proven PE, versus non-proven PE. E. Percentage of TF-expressing monocytes in monocyte subsets of COVID-19 ICU patients.

Inhibitor (TFPI) [18]. A more mechanistic approach is needed to study the exact role of monocytes in pulmonary immunothrombosis.

Our study has several limitations. First of all The discrepancy in comorbidities and length of stay between the control and PE patient groups raises concerns about the comparability of these two cohorts. Despite random selection from the CIUM study samples as non PE categorized patients, the potential for confounding factors influencing our findings cannot be ignored. It is worth emphasizing that our study inherently involves comparing individuals with proven pulmonary embolism to actually those with a possible pulmonary embolism, adding another layer of complexity to the comparability issue within the study design. Where also the question remains if current golden standard CTA indeed is golden standard for detecting pulmonary thrombosis.

Furthermore, due to a limited sample size, we were unable to use the MC-FGT assay at the scale required to evaluate its utility as a potential diagnostic tool. A prospective cohort study in ICU patients with severe infectious diseases who are at risk of developing immunothrombotic pathology, could serve to evaluate novel approaches to diagnosing PE, including functional cell-based coagulation assays such as MC-FGT. The use of fresh PBMCs, combined with a simplified isolation protocol, such as erythrocyte lysis buffer or simple centrifugation and resuspension in buffer, could make this assay more feasible for routine diagnostic procedures in the future. Direct translation to clinical use of the described MC-FGT is currently challenged by the labour-intensive nature of the test in its existing design. Moreover, its usability in non-immunothrombosis-related pulmonary embolism cases and specificity in the context of hyperinflammation and other causes of pro coagulant changes in peripheral blood mononuclear cells require further investigation. Nevertheless, we find promise in combining this test with a practical bedside assay, such as the ET Co2-PaCo2 gradient, which could potentially enhance its clinical applicability and relevance. Further research in this direction is warranted to fully realize the potential of our findings in clinical practice, for instance in a prospective design where MC-FGT is added to current PE diagnostics.

Author contributions

MR, TL, JM, KB, EVG and MG were responsible for the modified fibrin generation assay.

MR, TL, HE, DG, JvdA for CIUM cohort management and inclusion.

MR, MG for data analysis and writing of first manuscript.

MR, RDV, KS were responsible for FACS analysis.

MDM, MK, JDT, BV, DG, HE, JVA, RDV, DM, EvG and KS for data interpretation and shaping the manuscript.

All authors contributed to manuscript drafting.

All authors have read and approved the final manuscript.

MG is the guarantor, had final responsibility for the decision to submit for publication an attests that all listed authors meet authorship criteria.

Ethical approval

Studies involving human participants were conducted in accordance with institutional guidelines and the declaration of Helsinki. The CIUM study protocol was approved by the institutional review board of Erasmus MC (MEC-2017-417 and MEC-2020-0222).

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Declaration of Competing Interest

All the authors declare no conflicts of interest.

Data availability

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

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