Enoxaparin dose impacts blood cell phenotypes during mild SARS-CoV-2 infection: the observational single-center study

Liudmila Buryachkovskaya[1], Nikita Lomakin[2], Arthur Melkumyants[1,3], Julia Docenko[1], Vladimir Ermishkin[1], Victor Serebruany[4,*]

[1] *National Medical Research Center of Cardiology, 121552 Moscow, Russia*

[2] *Cardiology Division, Central Clinical Hospital of Presidential Administration, 121359 Moscow, Russia*

[3] *Institute of Physics and Technology, 141701 Moscow, Russia*

[4] *Neurology, Johns Hopkins University, Baltimore, Maryland, MD 21794, USA*

*Correspondence: vserebr1@jhmi.edu; heartdrug@aol.com (Victor Serebruany)

Coronavirus disease 2019 (COVID-19) is associated with various hemostatic abnormalities requiring constant search for better delicate antithrombotic management in these high-risk patients. The choice and the optimal dose of anticoagulant is important, but unclear, especially for mild COVID-19. Enoxaparin has been tested in several COVID trials with mixed results regarding hard clinical outcomes including mortality. We analyzed clinical, laboratory data and changes in platelets, erythrocytes and leukocytes by scanning electron microscopy on admission and at hospital discharge in patients with confirmed COVID-19 treated with enoxaparin (n = 31) and matched healthy controls (n = 32) in a retrospective observational study. The data were triaged by enoxaparin dose comparing 40 mg/daily prophylactic enoxaparin dose (PED) with 80 mg/daily therapeutic (TED) regimens. All patients experienced mild disease, none required pulmonary support, and all survived. The impact of enoxaparin dose was prominent for platelets and erythrocytes, but less evident for leukocytes. PED was associated with significant platelet activation, diminished numbers of silent nonactive discoid cells, and increased number and size of platelet microaggregates with leukocyte involvement. In contrast, TED did not cause extra platelet activation, while circulating platelet microaggregates were smaller and lacking leukocytes in their construction. PED caused significant increase of erythrocyte--platelet aggregates formation, and numerically higher proportion of circulating echinocytes. TED was associated with significant decrease of rouleaux sludge formation compared to only some trend after PED. Changes in leukocytes were less dependent on enoxaparin dose. However, PED has been associated with enhanced aggregate formation in 7 out of 10 patients, while trap net formation has been decreased in 17 out of 21 TED patients. We conclude that over hospital stay TED was superior to PED in patients with mild COVID-19. The inability of PED to adequately protect major circulating blood cells is probably due to enhanced clearance or/and diminished bioavailability of enoxaparin during COVID. These retrospective observational small sample size data may be relevant to better understanding of the mixed results in controlled outcome-driven trials exploring optimal COVID-19 anticoagulant strategies.

Keywords

COVID-19; Enoxaparin; Anticoagulation; Blood cells; Electron microscopy

1. Introduction

Numerous prospective multinational and global studies tried to clarify similarities and differences in the presentation and outcomes of COVID-19. Moreover, current viral pandemic is definitely associated with the double-digit risks for various hemostatic abnormalities including venous, or pulmonary thromboembolism, acute thrombotic and bleeding events urgently requiring to identify strategies for better delicate management in these high-risk patients [1, 2]. The life-threatening COVID-19 frequently requires antithrombotic management to prevent severe coagulopathy and disseminated intravascular coagulation [3]. Obviously the choice for the dose of anticoagulant seems important, but currently unclear, especially for mild COVID-19 [4].

Enoxaparin is a low-molecular-weight heparin that differs substantially from unfractionated heparin in its pharmacodynamic and pharmacokinetic properties including higher ratio of anti-Xa to anti-IIa activity, more consistent release of tissue factor pathway inhibitor, weaker interactions with platelets, more consistent bioavailability, a longer plasma half-life and less bounding to plasma proteins [5, 6]. In short, enoxaparin provides a more reliable anticoagulation without the need for laboratory monitoring also offering the convenience of once-daily administration. Clinical studies have confirmed that these pharmacological advantages may translate into improved outcomes after acute thrombotic occlusions, or/and preventing venous thromboembolism, however, the enoxaparin outcome data after COVID-19 remain inconclusive [4, 7]. The enoxaparin is used at 40 mg/daily as prophylactic (PED) or therapeutic (80 mg/daily, TED) dose. Since COVID-19 is currently considered as not only a pulmonary disease, but also as the general coagulopathy, vas-

culitis targeting the crosstalk between various blood cells, the impact of enoxaparin on their constitution seems critical to understand [1, 4]. However, the validity of such associations is evidenced exclusively by random (not randomized) clinical observations, conventional blood tests and sporadic autopsy data. We utilized scanning electron microscopy to assess circulating blood cells (platelets, leukocytes, and erythrocytes) exploring their phenotypes and interactions in COVID-19 patients at hospital admission and discharge dependent on enoxaparin daily dose.

2. Methods

2.1 Patients

The detailed description is outlined elsewhere [8]. Briefly, all study participants provided the informed consent. Thirty-one patients with Polymerase-chain reaction (PCR)-confirmed COVID-19 diagnosis were admitted to the hospital from May 25 to July 22, 2020 and included in a single-center retrospective observational study. All research protocols were approved by Central Clinical Hospital of Presidential Administration Ethical Committee, and received daily enoxaparin (Clexane®, Sanofi, Paris, France) in PED (40 mg/day) or TED (80 mg/day) regimens. Thirty-two COVID-19 negative and enoxaparin free samples from matched by demographics hospital personnel constituted the control group. During the hospital stay all patients survived, and no patient was referred to Intensive Care Unit (ICU) or required pulmonary ventilation. All patients were discharged from the hospital on average of about 2 weeks frame (range 8–27 days). Two specimens (admission and discharge) of venous blood were collected for subsequent electron microscopy testing. Importantly, the sampling from COVID-19 patients and controls were collected at the same time, and no historical chart data were used in the index study protocol.

2.2 Scanning electron microscopy

In brief, blood was drawn from the cubital vein into the VACUETTE (Greiner bio-one, Vienna, Austria) tubes containing sodium citrate (3.2%). Immediately after sampling the whole blood was prefixed with glutaraldehyde (0.1%; 4.5 mL and after 30 min 20 mL from the top layer was finally fixed in 2.5% glutaraldehyde. The cells were examined with scanning electron microscope (Inspect F50; FEI Company, Eindhoven, The Netherlands).

2.3 Statistics

Continuous variables were described as mean ($\pm$ standard error) and categorical variables as percentages. Categorical variables were compared by Chi-square test. All variables before and after the end of therapy were compared applying nonparametric Mann-Whitney test. All tests were two-tailed and $p < 0.05$ was considered statistically significant. Statistical analyses were performed using IBM SPSS Statistics for Windows, Version 24.0. (Chicago, IL, USA).

3. Results

The admission variables of demographics and comorbidities in the COVID-19 patients dependent on enoxaparin dose and matched controls are summarized in Table 1.

Ten patients received PED and twice more (n = 21) were treated with TED. No patients received aspirin, thienopyridines, or other anticoagulants during their hospital stay. At presentation the arms match fairly well with regard to age, gender, smoking status, length of hospitalization, hypertension and cancer. However, the TED patients were older, much heavier, but lacking diabetics. The differences in admission and discharge laboratory indices compared to controls are summarized in Table 2.

Notably, most laboratory parameters indicate COVID-19 clinical recovery rather than differences in enoxaparin dosing. Several biomarkers although exhibit statistical difference (fibrinogen, D-dimer, erythrocyte sedimentation and respiratory rates) probably due to overall small sample size and doubled numbers in TED group when compared to the PED arm.

Changes of blood cell phenotypes dependent on enoxaparin dose are outlined in Table 3 and exhibited in Fig. 1.

PED regiment is associated with significant platelet activation, diminished numbers of silent nonactive discoid cells, and increased number and size of platelet microaggregates with the substantial leukocyte involvement. In contrast, doubling enoxaparin dosing did not cause extra platelet activation over hospital stay, while circulating platelet microaggregates were smaller and lacking leukocytes in their construction. Changes in leukocytes were less dependent on enoxaparin dose. Both key biomarkers (leukocyte-platelet aggregates and neutrophil extracellular traps) did not change significantly over enoxaparin dose. However, PED has been associated with enhanced aggregate formation in 7 out of 10 patients, while net formation has been decreased in 17 out of 21 TED patients. With regard to erythrocytes the differences dependent on enoxaparin dose were more profound. In fact, PED caused significant increase of erythrocyte-platelet aggregates formation, and numerically higher proportion of circulating echinocytes. Higher enoxaparin dose was associated with significant decrease of rouleaux sludge formation compared to the only slide trend with PED.

4. Discussion

The main finding of the current study is that circulated blood cells biomarkers support more aggressive anticoagulation with higher enoxaparin dose over the hospital stay in patients with mild COVID-19. Indeed, multiple indices indicative of improved rheology and blood flow in microcirculation suggest that full therapeutic dose of enoxaparin (80 mg/daily) is better than half-dose prophylactic (40 mg/daily) regimen. This is especially true with regard to diminishing platelet and erythrocyte activity, while the impact of enoxaparin dosing on leukocytes was less prominent over COVID recovery. Obviously, this project was way too small and not randomized

Table 1. Demographics and clinical characteristics in COVID-19 patients and controls.

Variable	Controls (n = 32)	COVID-19 patients	
		Enoxaparin 40 mg (n = 10)	Enoxaparin 80 mg (n = 21)
Gender (M/F; n, %)	17 (53%)/15 (47%)	5 (50%)/5 (50%)	12 (54.6%)/9 (45.4%)
Age (years, range)	54.2 ± 4.1 (25–74)	47.8 ± 4.4 (29–71)	55.1 ± 3.4 (27–74)
BMI* (kg/m^2, range)	25.1 ± 3.0 (21.8–31.4)	24.9 ± 2.9 (22.4–32.9)	29.7 ± 3.0 (23.4–34)
Hospitalization (days)	–	13.0 ± 1.2 (10–22)	13.4 ± 4.6 (8–27)
Obesity (n, %)	5 (15.9%)	1 (10.0%)	10 (47.6%)
Smoking (n, %)	4 (12.5%)	0	1 (4.8%)
Hypertension (n, %)	4 (12.5%)	5 (50%)	9 (42.9%)
Diabetes (n, %)	–	2 (20%)	0
Coronary artery disease (n, %)	–	2 (20%)	2 (9.5%)
Heart failure (n, %)	–	1 (10%)	1 (4.7%)
Cancer (n, %)	–	2 (20%)	5 (23.8%)
Renal disease (n, %)	–	1 (10%)	0
COPD**	–	0	2 (9.5%)

* BMI, body mass index; ** COPD, chronic obstructive pulmonary disease.

to claim or even suspect any outcome benefit, especially since all patients survived, and exhibited mild COVID with no need for ICU or mechanical ventilation. However, these data are important justifying future trials specifically targeting optimal anticoagulation strategies. Aside from the main message, there are few other critical issues raised by the index study, but still lacking any fundamental support, or reasonable explanation(s).

The postulate that COVID-19 is heavily associated with the prothrombotic state is currently under intensive investigation. Enoxaparin may offer advantage over conventional heparin combating few critical COVID problems including cytokine storm [9], interleukin burst [10] or lung fibrin deposition [11].

Numerous anticoagulants in general and enoxaparin in particular is currently in late-stage clinical trials with mixed and somewhat confusing results. While in HESACOVID trial therapeutic enoxaparin improves gas exchange and decreases the need for mechanical ventilation in severe COVID-19 [12]. However, another elegant randomized unnamed trial failed to find the difference between standard prophylactic dose and intermediate dose enoxaparin in preventing death or thrombosis at 30 days in hospitalized adults with severe COVID-19 [13]. Moreover, larger INSPIRATION trial revealed that among patients admitted to the ICU with COVID-19, intermediate-dose prophylactic anticoagulation, compared with standard-dose prophylactic anticoagulation, did not result in a significant difference in the primary outcome of a composite of adjudicated venous or arterial thrombosis, treatment with extracorporeal membrane oxygenation, or mortality within 30 days [14], or longer 90 days same trial follow-up [15]. Finally, large (n = 3331) multicenter Brazilian ACTION trial revealed that in-hospital therapeutic anticoagulation with rivaroxaban or enoxaparin followed by rivaroxaban to day 30 did not improve clinical outcomes

but increased bleeding compared with prophylactic anticoagulation. Therefore, use of therapeutic-dose rivaroxaban, and other direct oral anticoagulants, should be avoided in these patients in the absence of an evidence-based indication for oral anticoagulation [16]. The very latest combined evidence of ATTACC, ACTIV-4a, and REMAP-CAP trials picked up a signal that in not critically ill patients with COVID-19, an initial strategy of therapeutic-dose anticoagulation increased the probability of survival to hospital discharge with reduced use of cardiovascular or respiratory organ support as compared with usual-care thromboprophylaxis [17]. This analysis is also in full agreement with our data suggesting that the most benefit from enoxaparin is yielded in non-critically sick COVID patients.

There are few advances worth mentioning. First, the definite proof of impaired blood cells in COVID-19 patients and dose-dependent impact of enoxaparin has been documented not post mortem or by routine clinical examination, but directly by electron microscopy in survived mild-to-moderately sick hospitalized patients. Indeed, most COVID electron microscopy studies were conducted in autopsy samples, e.g., [1, 2] rather than directly examining life cells. We recently provided evidence that endothelial cells from COVID-19 patients are damaged by SARS-CoV-2 virus resulting in platelet activation, endothelial dysfunction, and coagulopathy [8]. This finding requires delicate antithrombotic management for prevention of further risks. Second, the alarming signal that even mild COVID-19 still requires full enoxaparin dose suggests probably more severe thrombotic risk in critically sick patients requiring even more aggressive antithrombotic treatment. Third, such substantial cell disturbances existed even in mild COVID-19, and were not fully prevented by enoxaparin prophylactics what is truly unexpected and alarming. Importantly, our data are in full agreement with the high-quality pharmacokinetic study in-

Table 2. Laboratory indices at admission and discharge dependent on enoxaparin dose.

Variable	Controls (n = 32)	COVID-19 patients					
		Enoxaparin 40 mg (n = 10)			Enoxaparin 80 mg (n = 21)		
		Admission	Discharge	p-value	Admission	Discharge	p-value
Heart rate (min^{-1})	74.3 ± 2.1	90.0 ± 2.6	78.1 ± 2.5	0.01	86.5 ± 3.5	73.2 ± 1.5	0.0006
Respiratory rate (min^{-1})	16.5 ± 0.8	17.6 ± 0.5	17.0 ± 0.5	0.34	18.2 ± 0.3	16.8 ± 0.2	0.01
SpO$_2$ (%)	97 ± 1.0	97.1 ± 0.4	97.3 ± 0.5	0.73	97.4 ± 0.4	97.7 ± 0.3	0.41
Hemoglobin (g/L)	127.8 ± 4.5	133.8 ± 11.0	132.6 ± 9.9	0.85	136.7 ± 3.3	134.5 ± 3.3	0.32
Erythrocytes (10^{12}/L)	4.7 ± 0.5	4.3 ± 0.3	4.3 ± 0.3	0.83	4.5 ± 0.1	4.5 ± 0.1	0.70
Leukocytes (10^9/L)	4.7 ± 0.6	6.5 ± 1.0	5.9 ± 0.7	0.35	5.4 ± 0.4	5.6 ± 0.3	0.64
Platelets (10^9/L)	192.0 ± 7.5	168.3 ± 15.3	214.7 ± 11.9	0.02	199.4 ± 11.8	261.0 ± 18.7	0.004
Lymphocytes (%)	28.4 ± 2.9	18.5 ± 2.8	31.3 ± 5.6	0.03	28.0 ± 1.9	35.0 ± 2.4	0.02
Neutrophils (%)	54.4 ± 1.9	69.9 ± 3.4	58.4 ± 6.4	0.06	62.1 ± 2.2	52.7 ± 2.2	0.001
ESR (mm/h)	12 ± 3.1	25.6 ± 8.6	26.2 ± 10.4	0.91	32.1 ± 6.0	19.2 ± 2.6	0.03
CRP (mg/L)	4.2 ± 0.6	10.4 ± 2.6	3.6 ± 2.3	0.02	14.9 ± 5.8	1.2 ± 0.2	0.02
Creatinine (mg/dL)	82.5 ± 4.3	104.0 ± 3.8	97.8 ± 5.6	0.71	88.7 ± 3.3	84.8 ± 3.2	0.09
D-dimer (ng/L)	328 ± 15.2	225.8 ± 75.4	157.2 ± 38.3	0.16	178.9 ± 25.1	116.7 ± 15.4	0.001
Fibrinogen (g/L)	2.9 ± 0.3	4.0 ± 1.2	3.7 ± 1.1	0.15	5.0 ± 0.5	3.9 ± 0.2	0.04
Ferritin (µg/L)	85.4 ± 8.9	250.6 ± 27.3	393.2 ± 28.1	0.02	267.3.3 ± 16.7	354.2 ± 17.3	0.05
Prothrombin time (s)	12.8 ± 1.2	12.5 ± 0.31	12.1 ± 0.5	0.27	14.8 ± 2.0	11.8 ± 0.2	0.15

SpO$_2$, peripheral blood oxygen saturation; ESR, erythrocyte sedimentation rate; CRP, C-reactive protein.

Table 3. Changes of cell phenotypes in mild COVID-19 dependent on enoxaparin dose.

| Variable Hospitalization	Controls (n = 32)	Enoxaparin 40 mg (n = 10)		p-value	Enoxaparin 80 mg (n = 21)		p-value
		Admission	Discharge		Admission	Discharge	
Platelets							
Resting discoidal (%)	85.3 ± 3.2	65.9 ± 4.1	50.9 ± 7.0	0.049	58.6 ± 4.8	57.2 ± 4.0	0.75
Activated spheres (%)	14.2 ± 4.4	31.0 ± 3.9	46.7 ± 6.9	0.035	38.5 ± 4.7	40.2 ± 3.9	0.69
Microaggregates (n)	0	2.4 ± 1.0	5.2 ± 1.4	0.12	7.7 ± 2.3	8.6 ± 2.1	0.44
Leukocytes							
Leukocyte-platelet aggregates (%)	0	38.6 ± 7.7	46.4 ± 7.5	0.36	43.3 ± 5.0	45.8 ± 4.5	0.63
Neutrophil extracellular traps (%)	0	6.6 ± 2.9	3.5 ± 1.4	0.86	4.0 ± 1.5	3.0 ± 1.3	0.42
Erythrocytes							
Erythrocyte-platelet aggregates (%)	0	18.4 ± 2.3	26.9 ± 4.3	0.048	20.7 ± 3.5	19.6.1 ± 2.5	0.79
Echinocytes (%)	0	20.4 ± 6.7	26.8 ± 8.9	0.78	29.3 ± 6.7	22.8 ± 5.6	0.44
Rouleaux (n)	0	3.1 ± 0.7	2.7 ± 0.8	0.15	5.5 ± 1.1	3.0 ± 0.8	0.044

dicative of impaired enoxaparin metabolism in COVID-19 patients. The finding that in ICU COVID-19 patients, exposure to enoxaparin is reduced due to an increase in the volume of distribution and clearance is of critical importance explaining why PED is suboptimal to attain thromboprophylactic anti-Xa levels [18]. Enoxaparin is already recommended to prevent venous thromboembolism allows clinicians to collect clinical data in real-world and help answering this crucial question for the optimal management of COVID-19 patients beyond trial setting. Finally, the benefit of enoxaparin may be related to non-anticoagulant drug properties [19] although claiming "pleiotropy" is usually tricky suggesting serious gaps in knowledge. Importantly, enoxaparin exhibits direct anti-inflammatory properties [20], reduce viral entry into host cells [8, 21, 22] and neutralize circulating histones [23].

Obviously, there is an immediate need to design and implement a "prudent" separate trial on whether high-dose enoxaparin is indeed beneficial in patients with COVID-19, and which particular patients will benefit the most [24]. The proposal for such trial with real hard clinical endpoints is quite urgent [24] especially since the entire issue of optimal thromboprophylaxis is constantly challenged [25], and we need some hard and clear evidence as soon as possible.

5. Limitations

The main shortcoming of this study is a retrospective cohort design, and small sample size, which may limit the power to detect differences between enoxaparin doses. Obviously, these data are preliminary, and somewhat lack the definite clinical message. Also, the COVID-19 group did not match ideally with controls. Most importantly, there were substantial differences in cell damage within COVID-19 groups,
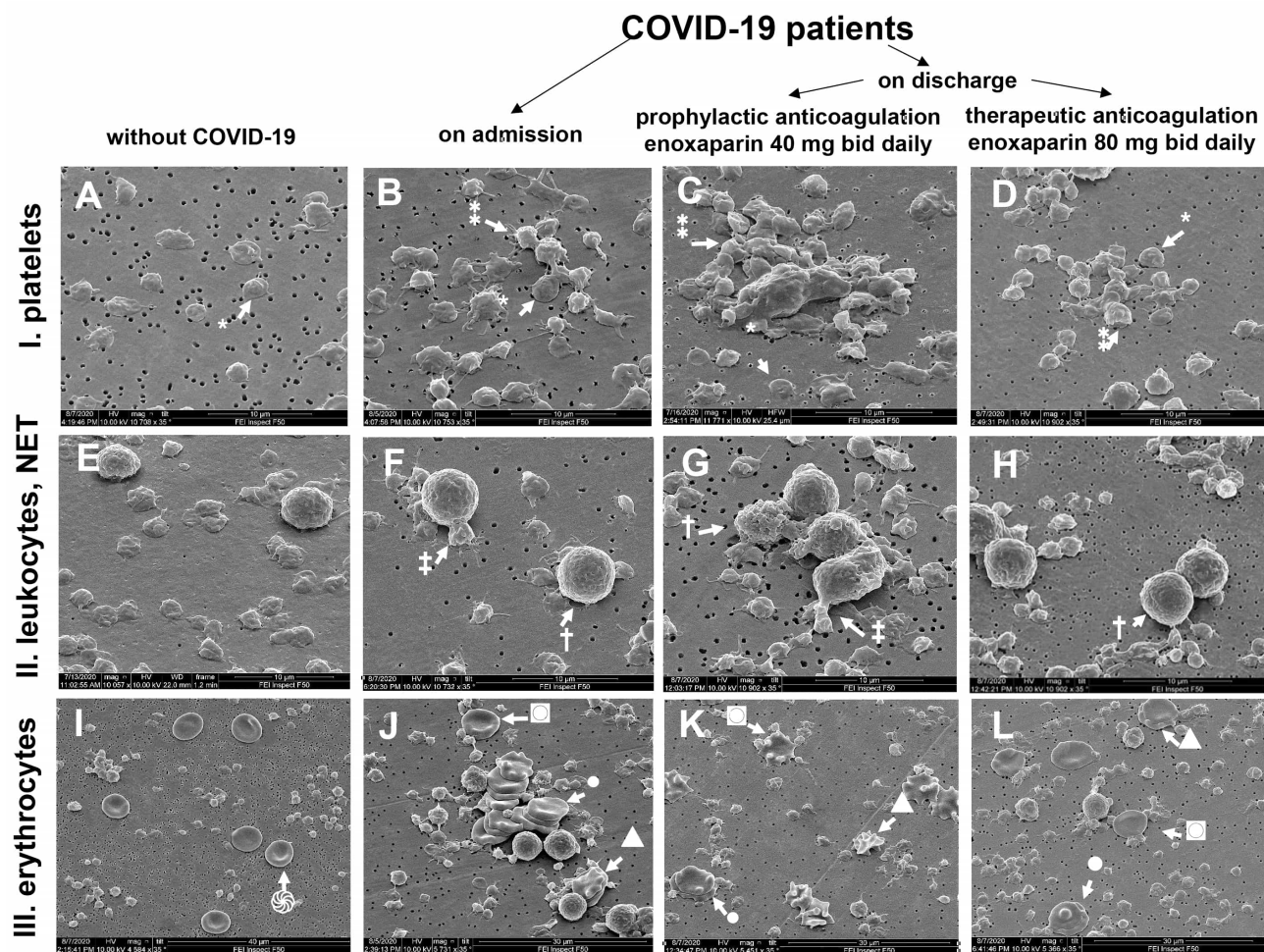
Fig. 1. Effect of prophylactic and therapeutic enoxaparin on blood cell phenotypes in mild COVID-19 patients. I. Platelets. Mostly discs in controls (A). Less quantity, activation, sphere transformation, at COVID-19 admission (B). Platelet characteristics at discharge are dependent on enoxaparin dose. PED is associated with decreased activity and microaggregate formation (C), in contrast to TED anticoagulation (D). Arrows indicate normal discoid platelets (∗) and activated spherical platelets (⁂). Magnification × 10 000 for all cell images. II. Leukocytes. No clusters or neutrophil extracellular traps in controls (E). Leukocyte-platelet aggregates (LPA) and neutrophil extracellular traps at COVID admission exhibited by N-arrows (F). Increased LPA (appr 30%) after PED, but tendency towards less neutrophil extracellular traps (G). No difference after TED (H). Arrows indicate LPA (†) and neutrophil extracellular traps (‡). Magnification × 10 000 for all cell images. III. Erythrocytes. Biconcave shape with no rouleaux formation in controls (I). Transformation of erythrocytes into echinocytes, formation of erythrocyte-platelet aggregates and erythrocyte rouleaux (J). Increased number of echinocytes and erythrocyte–platelet aggregates (EPA) and no difference in rouleaux after PED (K) with less changes after TED (L). Arrows indicate normal biconcave erythrocytes (🥏), echinocytes (▲), erythrocyte-platelet aggregates (◙) and erythrocyte rouleaux (●). Magnification × 5 000 for all cell images.

especially at hospital discharge. Short duration of follow-up might limit our understanding of the delayed impact of enoxaparin on vascular-endothelial interplay which is currently entirely unclear. Importantly, we also did not analyze the potential direct impact of enoxaparin on endothelium exclusively limiting our experiments to circulated cells. It will also be critical to prove whether the observed findings are indeed directly related to enoxaparin. Further experiments including in vitro assessment with endothelial cells together with X-a plasma serial measurements to reproduce similar damage will be mandatory to prove direct influence. Obviously, the index data are preliminary and descriptive with low incidence of parametric statistics due to small sample size and

substantial differences in clinical characteristics and electron microscopy indices. It will be also interesting to include the COVID-19 negative controls receiving enoxaparin for the better elucidation and triaging of the virus role in this pathology. Finally, the control used in the current stud may affect the interpretation of findings. It will be important to match hospitalized patients with similar comorbidities not requiring enoxaparin.

We conclude that over hospital stay TED was superior to PED in patients with mild COVID-19. The inability of PED to adequately protect mayor circulating blood cells is probably due to enhanced clearance or/and diminished bioavailability of enoxaparin during COVID. These observational data may

be relevant to the mixed results of controlled outcome-driven trials exploring optimal anticoagulant strategies for COVID-19.

Conflict of interest

The authors declare no conflict of interest. Victor Serebruany is serving as one of the Editorial Board members of this journal. We declare that Victor Serebruany had no involvement in the peer review of this article and has no access to information regarding its peer review. Full responsibility for the editorial process for this article was delegated to Alpo Vuorio.

Data availability statement

Data are the property of the authors and can become available by contacting the corresponding author.

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