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ORIGINAL ARTICLE

Tissue factor activity of small and large extracellular vesicles in different diseases

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Abstract

Background: Circulating procoagulant extracellular vesicles (EVs) are increased in diseases, such as cancer, sepsis, and COVID-19. EV tissue factor (TF) activity is associated with disseminated intravascular coagulation in sepsis and venous thrombosis in patients with pancreatic cancer and COVID-19. EVs are commonly isolated by centrifugation at ~20,000 g.

Objectives: In this study, we analyzed the TF activity of 2 EV populations enriched for large and small EVs in patients with either sepsis, pancreatic cancer, or COVID-19.

Methods: EVs were isolated from plasma by sequential centrifugation at 20,000 g (large EVs, LEVs) and then 100,000 g (small EVs, SEVs). We analyzed EVs from plasma prepared from whole blood samples from healthy individuals with or without lipopolysaccharide (LPS) stimulation as well as EVs from plasma samples from patients with either sepsis, pancreatic cancer, or COVID-19. TF-dependent (EV-TF activity) and TF-independent factor Xa (FXa) generation of the EVs was measured.

Results: LPS increased EV-TF activity in LEVs but not SEVs. Similarly, in 2 patients with sepsis who had EV-TF activity above the background of the assay we observed EV-TF activity in LEVs but not SEVs. Patients with pancreatic cancer or COVID-19 had circulating EV-TF activity in both LEVs and SEVs.

Conclusion: We recommend that EVs are isolated from plasma from patients by centrifugation at 100,000 g rather than 20,000 g to obtain a more accurate measure of levels of circulating EV-TF activity.

KEYWORDS
cancer, COVID-19, extracellular vesicles, procoagulant activity, sepsis, tissue factor

Essentials

- Circulating extracellular vesicles (EV)-tissue factor (TF) activity is associated with thrombosis in different diseases.
- We measured TF activity of small (20K) and large (100K) EVs in different diseases.
- TF activity was present in small and large EVs in patients with pancreatic cancer and COVID-19.
- We recommend measuring TF activity of EVs isolated from plasma by centrifugation at 100,000 g.

1 | INTRODUCTION

Levels of procoagulant extracellular vesicles (EVs) are increased in diseases, such as cancer, sepsis, and COVID-19 [1–7]. The procoagulant activity of blood-derived EVs is mainly due to the presence of the transmembrane protein tissue factor (TF) and negatively-charged phospholipids, such as phosphatidylserine (PS) [8–10]. TF-positive EVs trigger the activation of coagulation whereas PS-positive EVs amplify the coagulation cascade by facilitating assembly of coagulation protease: cofactor complexes [11–14]. Activated cells, such as monocytes, and cancer cells release TF-positive EVs into the circulation [15,16]. Indeed, levels of circulating EV-TF activity are increased in patients with cancer, bacterial and viral infections, and sepsis [17,18]. Importantly, we and others have shown that EV-TF activity is increased in patients with sepsis [19] and is associated with venous thrombosis in patients with pancreatic cancer and COVID-19 [5,20].

EVs are commonly isolated from plasma by centrifugation. For instance, 100-1000 nm EVs are isolated using centrifugation at 10,000-20,000 g [18,21–26]. Smaller EVs in the range of 40 to 100 nm can be isolated by centrifugation of plasma at high speeds (100,000-200,000 g) [26]. A recent study characterized EVs isolated from plasma using transmission electronic microscopy and found that the size of the EVs isolated using centrifugation at 20,000 g was 100 to 400 nm, whereas the size of the EVs isolated using centrifugation at 100,000 g was 30 to 100 nm [27]. Nanosight tracking analysis (NTA) also showed enrichment of large and small EVs in the 20,000 g and 100,000 g populations, respectively but there was overlap in the 2 populations using this technique [27].

In this study, we measured TF-dependent, and TF-independent factor Xa (FXa) generation of EVs. EVs were isolated from plasma samples by sequential centrifugation at 20,000 g and then 100,000 g as described previously [27]. We used plasma prepared from whole blood of healthy controls with or without lipopolysaccharide (LPS) stimulation *ex vivo* as well as plasma from patients with either sepsis, pancreatic cancer, or COVID-19. For simplicity, we will call the EVs isolated by centrifuging at 20,000 g 20K or large EVs (LEVs) and those isolated by centrifuging at 100,000 g 100K or small EVs (SEVs).

2 | METHODS

2.1 | Plasma preparation

Whole blood was collected from 6 healthy individuals (3 males, 3 females, age range: 22-50 years) who gave written consent according to a protocol (14-2108) approved by the Institutional Review Board of the University of North Carolina at Chapel Hill. Whole blood was collected from the antecubital vein into sodium citrate tubes (BD, Cat. no. 366560) with a 21G Safety-Lok Blood Collection Set (BD, Cat. no. 367281). The first 3 mL was discarded to prevent contamination with TF from the vein wall. Platelet-free plasma (PFP) samples without EV-TF activity were prepared from whole blood by centrifugation twice at 2500 g for 15 minutes at room temperature immediately after collection [28]. PFP samples with EV-TF activity were prepared from whole blood collected from healthy volunteers stimulated with LPS (10 μg/mL, Sigma–Aldrich, Cat. no. L2630) for 5 hours at 37 °C. PFP was prepared from LPS-treated whole blood as described above.

Whole blood was collected from 17 patients with sepsis. These patients were as a subset of subjects enrolled in a previous study [19]. Enrollment criteria included age >18, SEPSIS -3 criteria, and intensive care unit admission. Subjects were recruited under protocols approved by the Institutional Review Board of the University of Utah (IRB number 00102638). Written informed consent for study enrollment in accordance with the Declaration of Helsinki was given by all participants or their legal authorized representative. Blood was centrifuged (150 g, 20 minutes) to generate platelet-rich plasma. This plasma was then centrifuged (1500 g, 20 minutes) to produce platelet-poor plasma (PPP) which was frozen and stored at −80 °C.

Whole blood was collected from 10 patients with pancreatic cancer who provided written informed consent in accordance with the institutional review board of their hospital. Ambulatory patients with pancreatic cancer were eligible if they were to be scheduled for chemotherapy within 7 days or had started chemotherapy in the previous 3 months. The study was registered in ClinicalTrials.gov (identification number: NCT02095925) [29,30]. Blood was collected from the antecubital vein or a peripheral catheter into sodium citrate tubes. PPP was obtained by centrifuging blood at 1,560 g for 20 minutes at 20 °C.

Whole blood was collected from 12 patients with COVID-19 who provided informed consent with a protocol approved by the Swedish Ethical Review Board (COMMUNITY study [COVID-19 Biomarker and Immunity] dnr 2020-01653) [6]. PPP was obtained by centrifuging blood at 2000 g for 20 minutes at room temperature.

2.2 | Isolation of EVs

EVs were isolated from plasma using sequential centrifugation as described [27]. Plasma (100 μL) was added to 1 mL of HEPES buffer saline with bovine serum albumin (HBSA, 137 mmol/L NaCl, 5.38 mmol/L KCl,

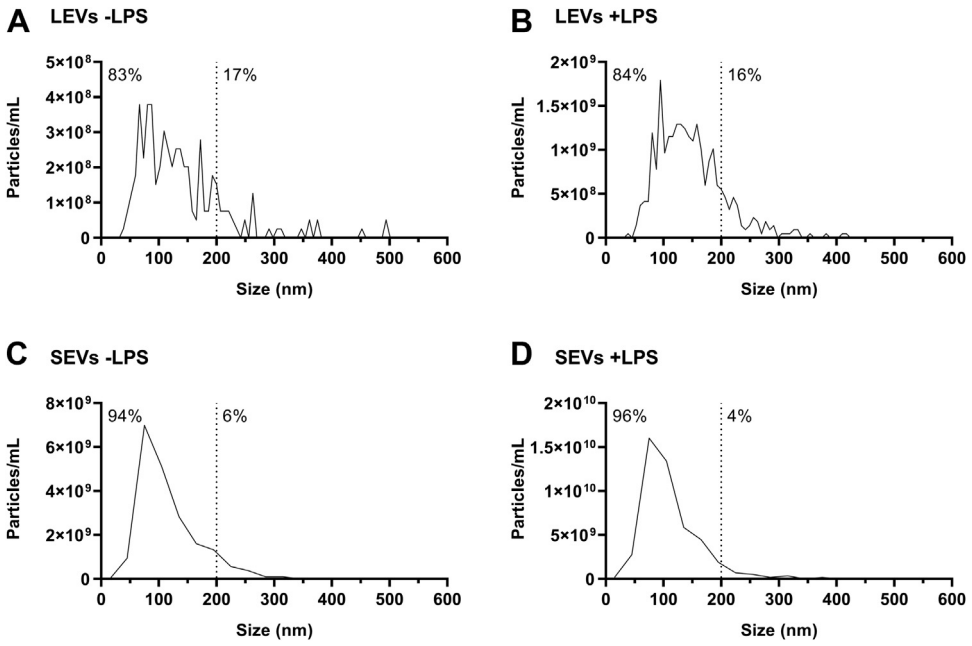

FIGURE 1 Analysis of extracellular vesicles (EVs) from plasma. Plasma was prepared from the whole blood of 6 healthy donors with or without lipopolysaccharide (LPS) stimulation. EVs were isolated from plasma by centrifugation at either 20,000 g (large EVs [LEVs]) or 100,000 g (small EVs [SEVs]). The size and number of EVs were determined using nanosight tracking analysis. The size distribution of LEVs and SEVs isolated from unstimulated (A, C) and LPS-stimulated (B, D) plasma is shown for a representative sample (donor #2). The concentration of EVs in the different samples is as follows: LEVs -LPS, 0.5 x 10^{10} particles/mL; LEVs +LPS, 2.4 x 10^{10} particles/mL; SEVs -LPS, 2.0 x 10^{11} particles/mL; SEVs +LPS, 4.6 x 10^{11} particles/mL.

5.55 mmol/L Glucose, 10 mmol/L HEPES, and 0.1% BSA, pH 7.4) and centrifuged at 20,000 g for 15 minutes at 4 °C. The supernatant and the pellet were separated. The pellet was washed with 1 mL of HBSA and centrifuged again at 20,000 g for 15 minutes at 4 °C to collect the LEVs. The supernatant was centrifuged at 100,000 g for 70 minutes at 4°C to pellet EVs that were resuspended in 1 mL of HBSA and centrifuged again to pellet the SEV. The LEV and SEV pellets were resuspended in 100 μL of HBSA.

2.3 | Nanoparticle tracking analysis

Nanoparticle tracking analysis was performed to measure the number and size of the EVs. All samples were diluted to optimal conditions for analysis (2.1 x 10^{7}-1.3 x 10^{8} particles/mL) in 20 nm filtered Dulbecco's phosphate-buffered saline (DPBS). Video recordings were made for a period of 60 seconds each, with a measurement of 11 positions using ZetaView software (version 8.05.12 SP2). The particle concentrations were corrected for the dilution necessary for NTA analysis.

2.4 | EV-TF activity

EV procoagulant activity (EV-TF activity) was measured using our in-house FXa generation assay with the use of a TF inhibitory antibody (HTF-1, BD Biosciences, Cat no. 550252) or control antibody (IgG, Sigma, Cat no. I5381) to differentiate TF-dependent from TF-independent FXa activity as previously described [21,31].

2.5 | D-dimer

D-dimer levels were measured in samples from patients with pancreatic cancer using INNOVANCE (Siemens Healthineers) and in samples from patients with COVID-19 using an automated coagulation analyzer (STACompact 3, Stago).

2.6 | Statistical analysis

Data are shown as EV FXa generation (pg/mL) for individual values. The normal distribution and homoscedasticity of the results were analyzed. The Mann–Whitney U-test was used. Correlation coefficients were analyzed using Spearman's coefficient. GraphPad Prism (version 9.0) was employed for the analyses. Data were considered statistically significant when $P < .05$, and results were expressed as mean ± SD.

3 | RESULTS

3.1 | Analysis of EVs

We analyzed the EVs isolated from PFP from whole blood from 6 healthy donors with or without LPS stimulation using NTA. The number of SEVs was higher (2.5–4.2 fold) than the number of LEVs in both unstimulated (LEVs 1.5 ± 1.1 x 10^{10} particles/mL, $n = 6$, mean ± SD vs SEVs 6.4 ± 5.5 x 10^{11} particles/mL, $n = 6$, mean ± SD) and LPS-

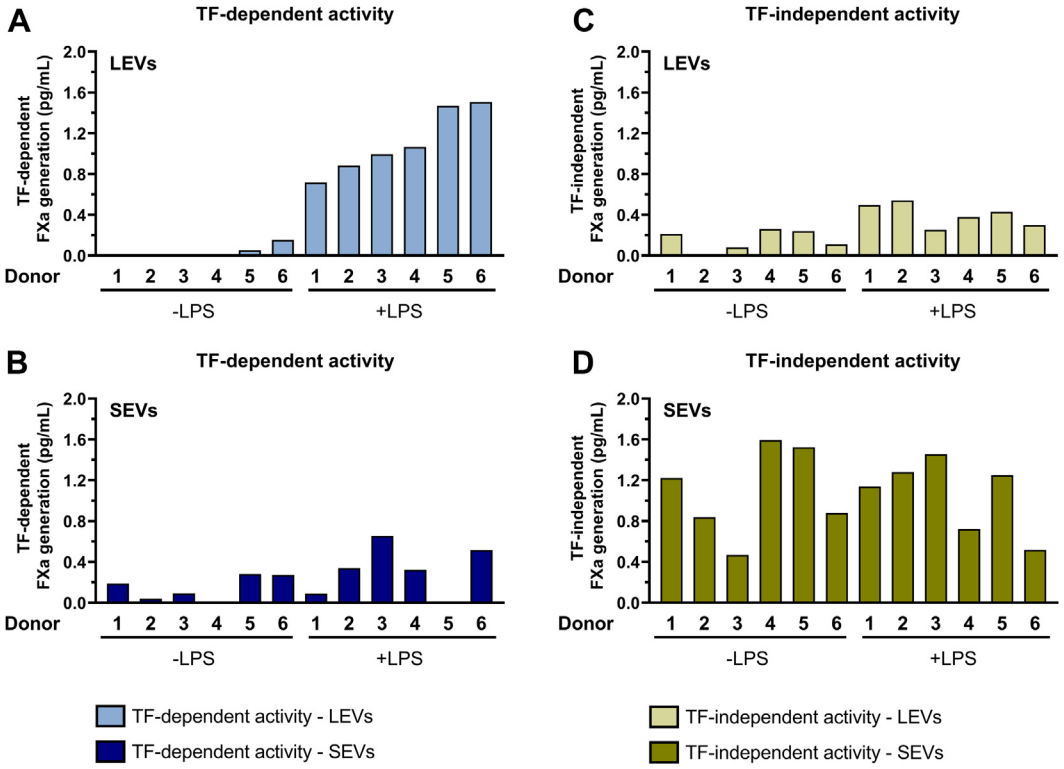

FIGURE 2 Procoagulant activity of extracellular vesicles (EVs) from plasma prepared from untreated and lipopolysaccharide (LPS)-treated whole blood from healthy donors. Plasma was prepared from the whole blood of 6 healthy donors with or without LPS stimulation. EVs were isolated from plasma by centrifugation at 20,000 g (large EVs [LEVs]) or 100,000 g (small EVs [SEVs]). Tissue factor (TF)-dependent (A, B) and TF-independent (C, D) factor (F) Xa was measured in LEVs and SEVs.

stimulated samples (LEVs 1.9 ± 0.6 x 10^{10} particles/mL, n = 6, mean ± SD vs SEVs 4.9 ± 3.1 x 10^{11} particles/mL, n = 6, mean ± SD). LPS stimulation did not significantly change the number of LEVs or SEVs (see above).

The LEV population had a broader size distribution compared with the SEV population (Figure 1). We used a cut of 200 nm to compare the 2 populations. The LEV population had a higher number of EVs that were larger than 200 nm compared with the SEV population for both the unstimulated (LEVs 16 ± 11%, n = 6, mean ± SD vs SEVs 4 ± 2%, n = 6, mean ± SD, P <.002) and LPS-stimulated (LEVs 21 ± 6%, n = 6, mean ± SD vs SEVs 5 ± 1%, n = 6, mean ± SD, P <.002) samples. However, there was a significant overlap in the size of the EVs in the LEV and SEV populations when analyzed by NTA, which is consistent with a recent study [27]. Interestingly, there was no association between the number of particles in a sample and the procoagulant activity of the EVs (data not shown).

3.1 | Procoagulant activity of EVs isolated from plasma of healthy donors with or without LPS stimulation

We first analyzed the procoagulant activity of LEVs and SEVs from PFP prepared from whole blood from 6 healthy donors with or without LPS stimulation. We and others have shown that LPS increases levels of EV-TF activity in LEVs [10,23,32–34]. In this study, LPS increased EV-TF activity in all 6 donors in the LEVs but not in the SEVs (Figure 2A, B). The range of EV-TF activity in LEVs in different donors was 0.7-1.5 pg/mL (Figure 2A). This is consistent with the heterogeneity in LPS induction of TF expression in monocytes between different donors [10,33,35]. We observed higher levels of TF-independent FXa generation in SEVs compared to LEVs (Figure 2C, D). LPS slightly increased TF-independent FXa activity of LEVs but not TF-independent FXa activity of SEVs (Figure 2C, D).

3.2 | Procoagulant activity of EVs from patients with sepsis

We analyzed the procoagulant activity of EVs isolated from PPP from 17 patients with sepsis. Clinical characteristics of the patients are shown in Table 1. Only patients #16 and #17 had a level of EV-TF activity in LEVs that was above the background of the assay (0.50 pg/mL) (Figure 3A) [10]. This is consistent with another study in which we observed 4 of 35 patients with sepsis having detectable EV-TF activity [19]. Interestingly, neither of these 2 patients had increased levels of EV-TF activity in the SEVs (Figure 3B). Two other patients (#11 and #13) had EV-TF activity in SEVs that was above the

TABLE 1 Demographic, clinical, routine laboratory, and plasma biomarkers of patients with sepsis.

Clinical characteristics	Patients with sepsis (n = 17)
Sex (male/female)	(9/8)
Age (y)	57 ± 34.5
Survival (%)	86
Diabetes (%)	10
Hypertension (%)	42
Mechanical ventilation (%)	10
SOFA	9.0 ± 3.8
Duration of ICU stay (d)	3.7 ± 8.5
Duration of hospital stay (d)	6.5 ± 6.0
WBC count (x10^9/L)	14.5 ± 7.83
Platelet Count (x10^9/L)	188.5± 126.3

Results are presented as median ± IQR for continuous variables and number for categorical variables.

BMI, body mass index; ICU, intensive care unit; SOFA, sequential organ failure assessment; WBC, white blood cell.

background of the assay (Figure 3B). A few patients with sepsis had elevated levels of TF-independent FXa activity in both the LEVs and the SEVs (Figure 3C, D).

TABLE 2 Demographic, clinical, routine laboratory, and plasma biomarkers of patients with pancreatic cancer.

Clinical characteristics	Patients with pancreatic cancer (n = 10)
Sex (male/female)	(6/4)
Age (y)	62 ± 15
Stage	
III	1
IV	9
D-dimer (ng/mL)	1100 ± 5400

Results are presented as median ± IQR for continuous variables and number for categorical variables.

3.3 | Procoagulant activity of EVs from patients with pancreatic cancer

We analyzed the procoagulant activity of EVs isolated from PPP from 10 patients with pancreatic cancer. We selected patients with a range of EV-TF activity in LEVs based on results observed in a previous study [29]. The clinical characteristics of the patients are shown in Table 2. Patients #5-10 had levels of EV-TF activity in the LEVs that were above the background of the assay (Figure 4A). Interestingly, patients #5 to #10 also had high levels of EV-TF activity in the SEVs (Figure 4B). The level of EV-TF activity in the SEVs in the 6 positive patients was higher than the level of EV-TF activity in the LEVs (Figure 4A, B). In general, patients #5 to #10 had higher levels of TF-independent FXa generation in LEVs and SEVs compared with patients

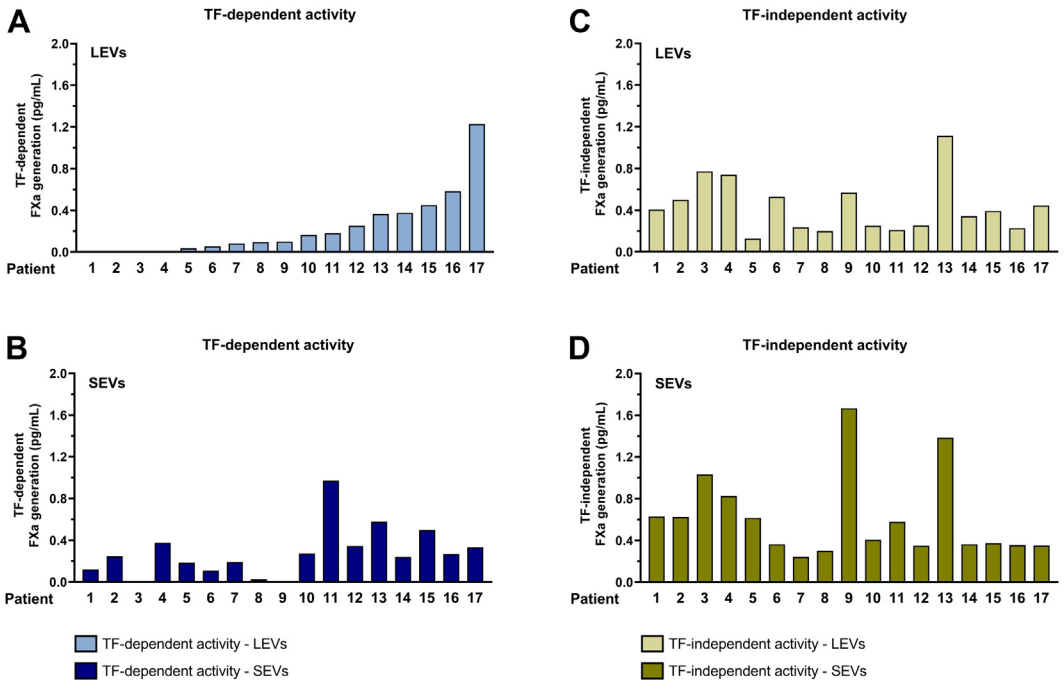

FIGURE 3 Procoagulant activity of extracellular vesicles (EVs) from plasma from patients with sepsis. We analyzed plasma from 17 patients with sepsis. EVs were isolated from plasma by centrifugation at 20,000 g (large EVs [LEVs]) or 100,000 g (small EVs [SEVs]). Tissue factor (TF)-dependent (A, B) and TF-independent (C, D) factor (F) Xa was measured in LEVs and SEVs.

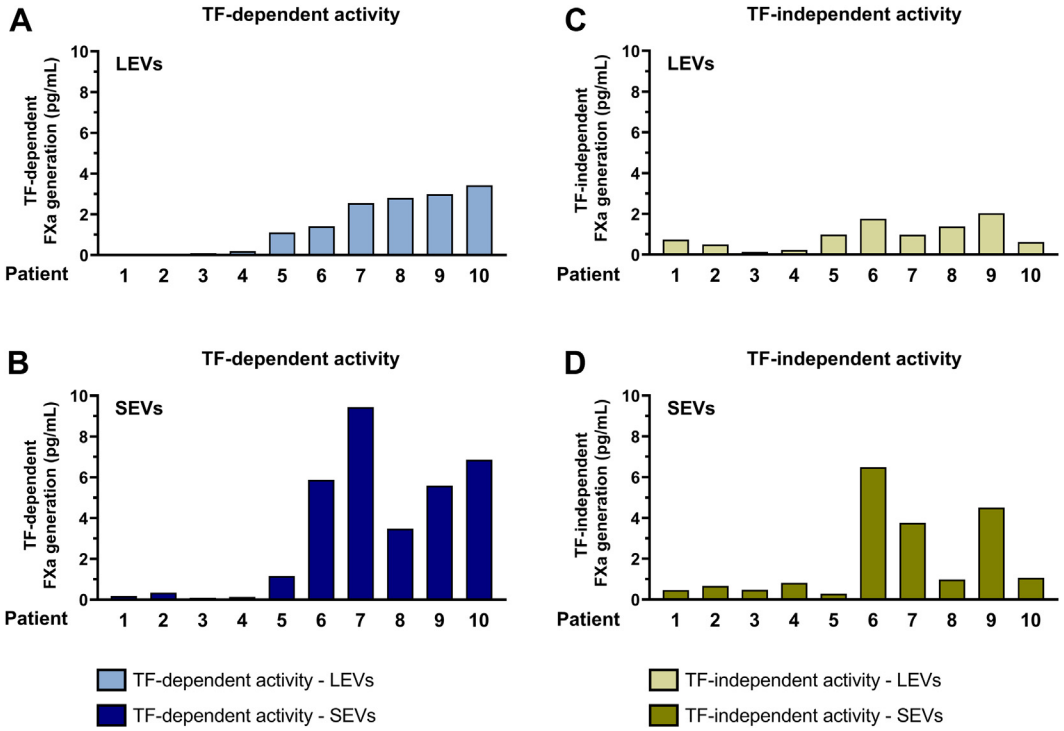

FIGURE 4 Procoagulant activity of extracellular vesicles (EVs) from plasma from patients with pancreatic cancer. We analyzed plasma from 10 patients with pancreatic cancer. EVs were isolated from plasma by centrifugation at 20,000 g (large EVs [LEVs]) or 100,000 g (small EVs [SEVs]). Tissue factor (TF)-dependent (A, B) and TF-independent (C, D) factor (F) Xa was measured in LEVs and SEVs.

#1 to #4, who had a lower level of EV-TF activity (Figure 4C, D). Some of the patients with pancreatic cancer had higher levels of TF-independent FXa generation in the SEVs compared to the LEVs (Figure 4C, D). There was a moderate correlation between the levels of total EV-TF activity (20K + 100K) with levels of D-dimer (r = 0.709, P =.03) in patients with pancreatic cancer.

3.4 | Procoagulant activity of EVs from patients with COVID-19

We analyzed samples from 12 patients with COVID-19. We selected patients with a range of EV-TF activity in LEVs based on results from a previous study [6]. The clinical characteristics of the patients are shown in Table 3. Patients (#7-12) had levels of EV-TF activity in the LEVs that was above the background of the assay (Figure 5A). Five of the 6 of these patients had elevated levels of EV-TF activity in the SEVs (Figure 5B). Four of the 6 patients also had higher levels of TF-independent FXa generation in LEVs (Figure 5C). All 6 of the patients (#7-12) have high levels of TF-independent FXa generation in SEVs compared with the patients (#1-6) that had low levels of EV-TF activity in the LEVs (Figure 5D). There was a moderate correlation between the levels of total EV-TF activity (20K + 100K) with levels of D-dimer (r = 0.683, P =.02) in patients with COVID-19.

4 | DISCUSSION

Procoagulant EVs are released into the circulation in a variety of diseases and likely contribute to the pathologic activation of coagulation. Most studies to date have focused on measuring the

TABLE 3 Demographic, clinical, routine laboratory, and plasma biomarkers of patients with COVID-19.

Clinical characteristics	Patients with COVID-19 (n = 12)
Sex (male/female)	(10/2)
Age (y)	64 ± 32
Oxygen requirement (L/min)	1.0 ± 8.0
Ventilation	
No support	3
Cannula	7
Nasal high flow	1
Intubation	1
Duration of hospitalization (d)	4 ± 5
WBC count (x10^9/L)	6.0 ± 2.9
D-dimer (ng/mL)	1604 ± 2296

Results are presented as median ± IQR for continuous variables and number for categorical variables.

WBC, white blood cell.

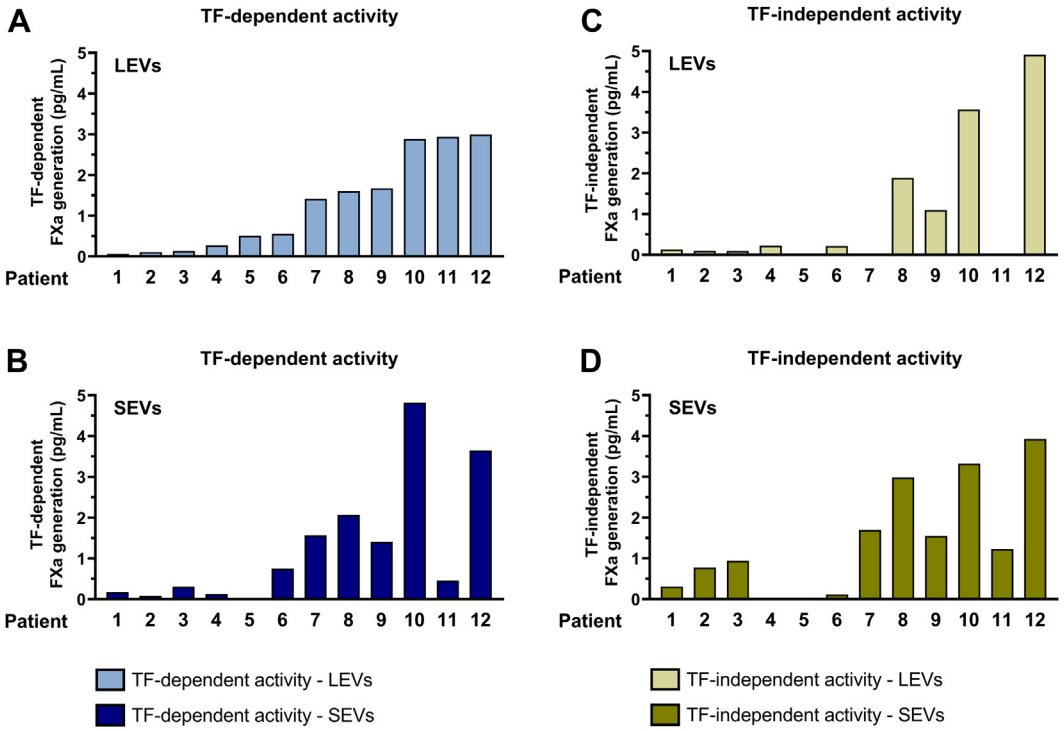

FIGURE 5 Procoagulant activity of extracellular vesicles (EVs) from plasma from patients with COVID-19. We analyzed plasma from 12 COVID-19 patients. EVs were isolated from plasma by centrifugation at 20,000 g (large EVs [LEVs]) or 100,000 g (small EVs [SEVs]). Tissue factor (TF)-dependent (A, B) and TF-independent (C, D) factor (F) Xa was measured in LEVs and SEVs.

procoagulant activity of EVs isolated by centrifugation of plasma at 20,000 g [10,23,32–34]. In this study, we used NTA to analyze EVs isolated from plasma using centrifugation at either 20,000 g (LEVs) or 100,000 g (SEVs). As expected, the SEV population was smaller than the LEV population. In addition, there were higher numbers of SEVs compared to LEVs. Interestingly, the number of particles was not associated with the procoagulant activity of the EVs, which suggested that a mixture of PS+ and PS- EVs was present in the LEV and SEV populations.

LPS stimulation of whole blood from healthy donors increased levels of EV-TF activity in LEVs but not in SEVs. This is consistent with our previous study, in which we showed that the level of TF activity in EVs isolated from plasma from LPS-stimulated whole blood by centrifugation at 20,000 g was similar to that observed in EVs isolated by centrifugation at 100,000 g [10]. Similarly, 2 patients with sepsis had increased levels of EV-TF activity in LEVs but not in the SEVs.

We have shown that elevated levels of EV-TF activity in LEVs are associated with venous thromboembolism in patients with pancreatic cancer [21,36,37]. In this study, we found that patients with pancreatic cancer with high levels of EV-TF activity in LEVs also had high levels of EV-TF activity in SEVs. Interestingly, levels of EV-TF activity in the SEVs were higher than the levels of EV-TF activity in the LEVs. There was a moderate correlation between levels of total EV-TF activity (20K + 100K) and levels of D-dimer in the patients with pancreatic cancer. Some patients with pancreatic cancer who have high levels of EV-TF activity also had higher levels of TF-independent FXa generation in LEVs and SEVs.

We and others have shown that levels of EV-TF activity in LEVs are increased in COVID-19 patients and are associated with thrombosis [5,19,38]. Here, we found that patients with high levels of EV-TF activity in LEVs also had high levels of EV-TF activity in SEVs. Activated monocytes have been shown to express TF in COVID-19 patients and are the likely source of TF+ EVs [39,40]. There was a moderate correlation between levels of total EV-TF activity (20K + 100K) and levels of D-dimer in the patients with COVID-19. Some patients with COVID-19 with high levels of EV-TF activity also had high levels of TF-independent FXa generation in LEVs and SEVs.

The data from patients with either pancreatic cancer or COVID-19 indicate that isolation of EVs by centrifugation at 100,000 g would give a more accurate measure of the total amount of EV-TF activity in each patient rather than isolation of EVs by centrifugation at 20,000 g.

An important consideration when measuring TF activity of EVs is that FVIIa can cleave FX to FXa on a membrane surface independently of TF [41]. Indeed, it has been shown that the concentration of FVIIa is linearly correlated to TF-independent FXa generation [42]. The commercial Human Tissue Factor Chromogenic Assay Sense Activity Kit (AssayPro) claims to measure TF activity in EVs. This assay is like our in-house FXa generation assay but does not include an anti-TF antibody. Therefore, this assay cannot distinguish between TF-dependent

and TF-independent FXa generation of EVs and can only measure total FXa generation. A recent study sponsored by the Vascular Biology Scientific and Standardization Committee of the International Society on Haemostasis and Thrombosis assessed the ability of 14 functional FXa generation assays to measure TF activity in EVs isolated from plasma (Bonifay et al., presented at the Scientific and Standardization Committee Meeting of International Society on Thrombosis and Haemostasis 2022 Congress). Of these 14 assays, 6 used an anti-TF antibody to inhibit TF procoagulant activity, 3 used an anti-TF antibody to capture the EVs and 5 did not use an antibody. Assays that used an anti-TF antibody to inhibit TF-dependent procoagulant activity were superior to those that did not.

Recently, Krishnamachary et al. [27] used the Human Tissue Factor Chromogenic Assay Sense Activity Kit to measure FXa generation of LEVs and SEVs from plasma obtained from patients with moderate and severe COVID-19. As described above, this assay measures total FXa generation rather than TF activity. Krishnamachary et al. [27] showed that FXa generation of LEVs and SEVs was elevated in patients with severe COVID-19 compared with that of EVs from healthy donors. In addition, both types of EVs had similar levels of FXa generation.

The main sources of EVs in plasma are platelets and megakaryocytes [8,13,43]. In this study, we did not determine the cellular origin of PS and EVs. However, we speculate that the majority of these EVs are derived from platelets and megakaryocytes. Interestingly, we found that TF-independent FXa activity in our in-house assay strongly correlates with PS equivalents measured using the Zymuphen MP-activity kit (Hyphen BioMed) [44]. This suggested that this TF-independent FXa generation is mainly due to PS.

The study has a few limitations. The number of patients with either sepsis, pancreatic cancer, or COVID-19 is small. PPP from the 3 groups of patients was prepared using slightly different centrifugation speeds and therefore we cannot compare the absolute numbers of EV-TF activity and TF-independent FXa generation between the groups. We did not collect race/ethnicity data of the healthy controls and patients used in this study. We do not believe that this impacts the conclusions of the study.

In conclusion, measurement of the procoagulant activity of EVs isolated using centrifugation at 100,000 g gives a more comprehensive picture of the levels of procoagulant EVs in patients with different diseases than measurement of the procoagulant activity of EVs isolated using centrifugation at 20,000 g. Future efforts should attempt to standardize the measurement of the procoagulant activity of EVs in plasma.

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ETHICS STATEMENT

Healthy individuals and patients provided informed consent. The study's protocols were approved by: the Institutional Review Board of the University of North Carolina at Chapel Hill (protocol 14-2108); the Institutional Review Board of the University of Utah (IRB number 00102638); the institutional review board of the hospital and registered in ClinicalTrials.gov (identification number: NCT02095925); the Swedish Ethical Review Board (COMMUNITY study [COVID-19 Biomarker and Immunity] dnr 2020-01653).

AUTHOR CONTRIBUTIONS

A.T.A.S. and N.M. designed experiments, interpreted data, and edited the manuscript. A.T.A.S. and S.A. conducted experiments, analyzed data, and wrote the manuscript. R.A.C., E.A.M., and M.T.R. provided the plasma samples from patients with sepsis and edited the manuscript. N.v.E. and R.N. provided the plasma samples from patients with pancreatic cancer and edited the manuscript. A.R., S.H., and C.T. provided the plasma samples from patients with COVID-19 and edited the manuscript. Y.H. edited the manuscript.

RELATIONSHIP DISCLOSURE

There are no competing interests to disclose.

REFERENCES

[1] Marchetti M, Tartari CJ, Russo L, Panova-Noeva M, Leuzzi A, Rambaldi A, et al. Phospholipid-dependent procoagulant activity is highly expressed by circulating microparticles in patients with essential thrombocythemia. *Am J Hematol*. 2014;89:68–73.

[2] Camoin-Jau L, Sabatier F, Arnoux D, Anfosso F, Bardin N, Veit V, et al. Endothelial microparticles: a potential contribution to the thrombotic complications of the antiphospholipid syndrome. *Thromb Haemost*. 2004;91:667–73.

[3] Ardoin SP, Shanahan JC, Pisetsky DS. The role of microparticles in inflammation and thrombosis. *Scand J Immunol*. 2007;66:159–65.

[4] Tesselaar MET, Romijn FPHTM, Van Der Linden IK, Prins FA, Bertina RM, Osanto S. Microparticle-associated tissue factor activity: a link between cancer and thrombosis? *J Thromb Haemost*. 2007;5:520–7.

[5] Guervilly C, Bonifay A, Burtey S, Sabatier F, Cauchois R, Abdili E, et al. Dissemination of extreme levels of extracellular vesicles: tissue factor activity in patients with severe COVID-19. *Blood Adv*. 2021;5:628–34.

[6] Rosell A, Havervall S, Von Meijenfeldt F, Hisada Y, Aguilera K, Grover SP, et al. Patients with COVID-19 have elevated levels of circulating extracellular vesicle tissue factor activity that is associated with severity and mortality—brief report. *Arterioscler Thromb Vasc Biol*. 2021;41:878–82.

[7] Hisada Y, Sachetto ATA, Mackman N. Circulating tissue factor-positive extracellular vesicles and their association with thrombosis in different diseases. *Immunol Rev*. 2022;312:61–75.

[8] Tripisciano C, Weiss R, Eichhorn T, Spittler A, Heuser T, Fischer MB, et al. Different potential of extracellular vesicles to support thrombin generation: contributions of phosphatidylserine, ttissue factor, and cellular origin. *Sci Rep*. 2017;7:6522.

[9] Østerud B, Latysheva N, Schoergenhofer C, Jilma B, Hansen JB, Snir O. A rapid, sensitive, and specific assay to measure TF activity based on chromogenic determination of thrombin generation. *J Thromb Haemost*. 2022;20:866–76.

[10] Lee RD, Barcel DA, Williams JC, Wang JG, Boles JC, Manly DA, et al. Pre-analytical and analytical variables affecting the measurement of plasma-derived microparticle tissue factor activity. *Thromb Res*. 2012;129:80–5.

[11] Mackman N, Tilley RE, Key NS. Role of the extrinsic pathway of blood coagulation in hemostasis and thrombosis. Arterioscler. *Thromb Vasc Biol*. 2007;27:1687–93.

[12] Geddings JE, Mackman N. Tumor-derived tissue factor–positive microparticles and venous thrombosis in cancer patients. *Blood*. 2013;122:1873–80.

[13] Reddy EC, Rand ML. Procoagulant phosphatidylserine-exposing platelets. *Front Cardiovasc Med*. 2020;7:15.

[14] Aleman MM, Gardiner C, Harrison P, Wolberg AS. Differential contributions of monocyte- and platelet-derived microparticles towards thrombin generation and fibrin formation and stability. *J Thromb Haemost*. 2011;9:2251–61.

[15] Basavaraj MG, Olsen JO, Østerud B, Hansen J-B. Differential ability of tissue factor antibody clones on detection of tissue factor in blood cells and microparticles. *Thromb Res*. 2012;130:538–46.

[16] Yu JL, Rak JW. Shedding of tissue factor (TF)-containing microparticles rather than alternatively spliced TF is the main source of TF activity released from human cancer cells. *J Thromb Haemost*. 2004;2:2065–7.

[17] Key N, Mackman N. Tissue factor and its measurement in whole blood, plasma, and microparticles. *Semin Thromb Hemost*. 2010;36:865–75.

[18] Hisada Y, Alexander W, Kasthuri R, Voorhees P, Mobarrez F, Taylor A, et al. Measurement of microparticle tissue factor activity in clinical samples: A summary of two tissue factor-dependent FXa generation assays. *Thromb Res*. 2016;139:90–7.

[19] Campbell RA, Hisada Y, Denorme F, Grover SP, Bouck EG, Middleton EA, et al. Comparison of the coagulopathies associated with COVID-19 and sepsis. *Res Pract Thromb Haemost*. 2021:5.

[20] Hisada Y, Mackman N. Cancer cell-derived tissue factor-positive extracellular vesicles: biomarkers of thrombosis and survival. *Curr Opin Hematol*. 2019;26:349–56.

[21] Khorana AA, Francis CW, Menzies KE, Wang J-G, Hyrien O, Hathcock J, et al. Plasma tissue factor may be predictive of venous thromboembolism in pancreatic cancer. *J Thromb Haemost*. 2008;6:1983–5.

[22] Mackman N, Sachetto ATA, Hisada Y. Measurement of tissue factor-positive extracellular vesicles in plasma: strengths and weaknesses of current methods. *Curr Opin Hematol*. 2022;29:266–74.

[23] Vallier L, Bouriche T, Bonifay A, Judicone C, Bez J, Franco C, et al. Increasing the sensitivity of the human microvesicle tissue factor activity assay. *Thromb Res*. 2019;182:64–74.

[24] Nielsen T, Kristensen AF, Pedersen S, Christiansen G, Kristensen SR. Investigation of procoagulant activity in extracellular vesicles isolated by differential ultracentrifugation. *J Extracell Vesicles*. 2018;7:1454777.

[25] Gheldof D, Haguet H, Dogné J-M, Bouvy C, Graux C, George F, et al. Procoagulant activity of extracellular vesicles as a potential biomarker for risk of thrombosis and DIC in patients with acute leukaemia. *J Thromb Thrombolysis*. 2017;43:224–32.

[26] Jeppesen DK, Hvam ML, Primdahl-Bengtson B, Boysen AT, Whitehead B, Dyrskjøt L, et al. Comparative analysis of discrete exosome fractions obtained by differential centrifugation. *J Extracell Vesicles*. 2014;3:25011.

[27] Krishnamachary B, Cook C, Kumar A, Spikes L, Chalise P, Dhillon NK. Extracellular vesicle-mediated endothelial apoptosis and EV-associated proteins correlate with COVID-19 disease severity. *J Extracell Vesicles*. 2021:10.

[28] Lacroix R, Judicone C, Mooberry M, Boucekine M, Key NS, Dignat-George F. Standardization of pre-analytical variables in plasma microparticle determination: results of the International Society on Thrombosis and Haemostasis SSC Collaborative workshop. *J Thromb Haemostasis*. 2013;11:1190–3.

[29] Van Es N, Hisada Y, Di Nisio M, Cesarman G, Kleinjan A, Mahé I, et al. Extracellular vesicles exposing tissue factor for the prediction of venous thromboembolism in patients with cancer: A prospective cohort study. *Thrombosis Res*. 2018;166:54–9.

[30] Van Es N, Di Nisio M, Cesarman G, Kleinjan A, Otten H-M, Mahé I, et al. Comparison of risk prediction scores for venous thromboembolism in cancer patients: a prospective cohort study. *Haematologica*. 2017;102:1494–501.

[31] Hisada Y, Mackman N. Measurement of tissue factor activity in extracellular vesicles from human plasma samples. *Res Pract Thromb Haemostasis*. 2019;3:44–8.

[32] Mackman N, Hisada Y, Archibald SJ. Tissue factor and its procoagulant activity on cancer-associated thromboembolism in pancreatic cancer: Comment by Mackman et al. *Cancer Sci*. 2022;113:1885–7.

[33] Archibald SJ, Hisada Y, Bae-Jump VL, Mackman N. Evaluation of a new bead-based assay to measure levels of human tissue factor antigen in extracellular vesicles in plasma. *Res Pract Thromb Haemostasis*. 2022;6.

[34] Franco C, Lacroix R, Vallier L, Judicone C, Bouriche T, Laroumagne S, et al. A new hybrid immunocapture bioassay with improved reproducibility to measure tissue factor-dependent procoagulant activity of microvesicles from body fluids. *Thromb Res*. 2020;196:414–24.

[35] Sovershaev MA, Egorina EM, Gruber FX, Olsen JO, Østerud B. High tissue factor-expressing human monocytes carry low surface CD36: application to intersubject variability. *J Thromb Haemostasis*. 2007;5:2453–60.

[36] Bharthuar A, Khorana AA, Hutson A, Wang JG, Key NS, Mackman N, et al. Circulating microparticle tissue factor, thromboembolism and survival in pancreaticobiliary cancers. *Thromb Res*. 2013;132:180–4.

[37] Kasthuri RS, Hisada Y, Ilich A, Key NS, Mackman N. Effect of chemotherapy and longitudinal analysis of circulating extracellular vesicle tissue factor activity in patients with pancreatic and colorectal cancer. *Res Pract Thromb Haemost*. 2020;4:636–43.

[38] Bolouri H, Speake C, Skibinski D, Long SA, Hocking AM, Campbell DJ, et al. The COVID-19 immune landscape is dynamically and reversibly correlated with disease severity. *J Clin Invest*. 2021;131:e143648.

[39] Hottz ED, Azevedo-Quintanilha IG, Palhinha L, Teixeira L, Barreto EA, Pão CRR, et al. Platelet activation and platelet-monocyte aggregate formation trigger tissue factor expression in patients with severe COVID-19. *Blood*. 2020;136:1330–41.

[40] Mackman N, Grover SP, Antoniak S. Tissue factor expression, extracellular vesicles, and thrombosis after infection with the respiratory viruses influenza A virus and coronavirus. *J Thromb Haemost*. 2021;19:2652–8.

[41] Telgt DS, Macik BG, McCord DM, Monroe DM, Roberts HR. Mechanism by which recombinant factor VIIa shortens the aPTT: activation of factor X in the absence of tissue factor. *Thromb Res*. 1989;56:603–9.

[42] Bom VJJ, Bertina RM. The contributions of Ca2+, phospholipids and tissue-factor apoprotein to the activation of human blood-coagulation factor X by activated factor VII. *Biochem J*. 1990;265:327–36.

[43] Flaumenhaft R, Mairuhu A, Italiano J. Platelet- and megakaryocyte-derived microparticles. *Semin Thromb Hemostasis*. 2010;36:881–7.

[44] Avenick D, Kidd L, Istvan S, Dong F, Richter K, Edwards N, et al. Effects of storage and leukocyte reduction on the concentration and procoagulant activity of extracellular vesicles in canine packed red cells. *J Vet Emerg Crit Care*. 2021;31:221–30.