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Extracellular vesicle–associated procoagulant activity is highest the first 3 hours after trauma and thereafter declines substantially: A prospective observational pilot study

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BACKGROUND: Trauma patients have high concentrations of circulating extracellular vesicles (EVs) following injury, but the functional role of EVs in this setting is only partly deciphered. We aimed to describe in detail EV-associated procoagulant activity in individual trauma patients during the first 12 hours after injury to explore their putative function and relate findings to relevant trauma characteristics and outcome.

METHODS: In a prospective observational study of 33 convenience recruited trauma patients, citrated plasma samples were obtained at trauma center admission and 2, 4, 6, and 8 hours thereafter. We measured thrombin generation from isolated EVs and the procoagulant activity of phosphatidylserine (PS)-exposing EVs. Correlation and multivariable linear regression analyses were used to explore associations between EV-associated procoagulant activity and trauma characteristics as well as outcome measures.

RESULTS: EV–associated procoagulant activity was highest in the first 3 hours after injury. EV–associated thrombin generation normalized within 7 to 12 hours of injury, whereas the procoagulant activity of PS-exposing EVs declined to a level right above that of healthy volunteers. Increased EV-associated procoagulant activity at admission was associated with higher New Injury Severity Score, lower admission base excess, higher admission international normalized ratio, prolonged admission activated partial thromboplastin time, higher Sequential Organ Failure Assessment score at day 0, and fewer ventilator-free days.

CONCLUSION: Our data suggest that EVs have a transient hypercoagulable function and may play a role in the early phase of hemostasis after injury. The role of EVs in trauma-induced coagulopathy and posttraumatic thrombosis should be studied bearing in mind this novel temporal pattern. (*J Trauma Acute Care Surg.* 2021;91: 681–691.

LEVEL OF EVIDENCE: Prognostic/epidemiologic, level V.

KEY WORDS: Wounds and injuries; thrombin; trauma-induced coagulopathy; tissue factor; phosphatidylserine.

Trauma is a main cause of premature death.[1] Trauma-induced coagulopathy (TIC) is a severe clinical scenario present at hospital admission in about a quarter of severely injured patients and associated with higher transfusion requirements, systemic inflammation, multiple organ failure, and death.[2] Trauma-induced coagulopathy has an array of phenotypes fluctuating between a hypocoagulable and a hypercoagulable state, potentially causing both uncontrolled bleeding and thrombotic events in critically injured patients.[3] To date, there is no standard laboratory method to identify or prognosticate TIC.[3] Conventional coagulation tests such as prothrombin time with its derived measure, international normalized ratio (INR), and activated partial thromboplastin time (APTT), as well as thromboelastography, are used to stratify and guide treatment in patients at risk of TIC.[3–5] The pathophysiology of TIC is multifactorial, and the key elements are linked to platelet activation and dysfunction, dysregulated thrombin generation, activation of the protein C pathway, altered fibrinolysis, systemic inflammation, and endotheliopathy.[2,3] The main drivers of TIC are two synergistic events: tissue damage and hypovolemic shock.[2,3]

Tissue trauma and vessel injury lead to increased expression of tissue factor (TF), the main initiator of blood coagulation,[6] both by exposure of constitutively expressed TF on subendothelial cells and by induced TF expression on activated circulating monocytes.[6,7] Tissue factor can combine with coagulation factor VII/factor VIIa to generate factor IXa and factor Xa, two constituents of the tenase and prothrombinase complexes that are essential for thrombin generation. Thrombin may act in a procoagulant or anticoagulant manner dependent on localization and is important in maintaining the delicate balance between hemostasis and thrombosis.[8]

Extracellular vesicles (EVs) are submicrometer particles that are released from cells. They are delimited by a lipid membrane

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bilayer and lack a functional nucleus.[9] Some EVs bud off from the plasma membrane of a variety of activated and apoptotic cells, including blood leukocytes, platelets, erythrocytes, and endothelial cells, and they display membrane markers from their cell of origin.[10] EV-associated may carry TF and negatively charged phospholipids such as phosphatidylserine (PS).[11] The PS can activate and support coagulation by providing a surface where coagulation factors can assemble, and EVs with both TF and PS are the most procoagulant ones.[11,12] Circulating EVs are procoagulant entities that may initiate and propagate thrombin generation systemically and contribute to hemostasis after injury[13] and possibly also posttraumatic thrombosis. Increased EV-associated thrombin generation may possibly also lead to consumption of coagulation factors and thereby augment a bleeding phenotype of TIC.

Trauma patients have higher concentrations of circulating EVs early after injury, compared with healthy volunteers.[14–20] Also, EV-associated procoagulant activity is increased after injury, both at the day of admission[16,17,19,21] and 3 days thereafter.[16,19] A discernible pattern in EV-associated procoagulant activity kinetics was not found in the first 3 days after injury, but the most extreme hypercoagulable values were seen within 12 hours of injury.[17]

In this study, we aimed to describe in detail the time-course of EV-associated procoagulant activity in individual trauma patients during the first 12 hours after injury by measuring the thrombin-generating capacity of isolated EVs and the phospholipid-dependent activity of PS-exposing EVs. We explored associations between EV-associated procoagulant activity and anatomical injury severity, physiological derangement, conventional coagulation test results, and patient outcome measures.

PATIENTS AND METHODS

Study Design, Setting, and Approval

In this prospective observational pilot study, we investigated the thrombin-generating capacity of isolated EVs and the phospholipid-dependent procoagulant activity of PS-exposing EVs in biobank samples obtained with high time resolution during the first 12 hours after injury in a population of convenience recruited trauma patients. The study is part of an overarching effort to describe temporal concentration changes of several inflammation and coagulation molecules following trauma, and study design and setting have been reported in detail previously.[22] Briefly, patients 18 years or older admitted to the level I trauma center at Oslo University Hospital (OUH) Ullevål from January 2011 to January 2014 who met the criteria for trauma team activation were eligible for inclusion, except for patients with burn injuries and pregnant women. Criteria for trauma team activation were obvious severe injury, circulatory or respiratory instability, reduced level of consciousness, high-energy trauma, or other situations with a high index of concern. Patients were enrolled on arrival and followed up until 10 days, discharge from the intensive care unit (ICU) or high-dependency unit, or death, whichever came first. All parts of the study were approved by the Regional committee for medical and health research ethics (2010/2014 REK South-East D) in accordance with the Declaration of Helsinki.[22] We adhered to Strengthening the Reporting of Observational Studies in Epidemiology statement for cohort studies.[23]

Participants

Patients were selected from the 145 patients included in the original study population[22] (Fig. 1). Severely injured patients were anticipated to have increased EV-associated procoagulant activity, and therefore, patients with critical anatomical injury severity (New Injury Severity Score [NISS],[24,25] ≥25) and pronounced physiological derangement (admission base excess [BE],[26] ≤−6) were primarily selected. In addition, some patients with low NISS and normal admission BE were selected to enable exploration of dose-response relationships between injury severity and EV-associated procoagulant activity. Finally, we have recently shown in the original study population that increasing concentration of the alarmin high-mobility group box 1 protein (HMGB1) between 3 and 6 hours after injury (HMGB1 $AUC_{3–6}$) was associated with worse outcome.[22] Therefore, some patients with a high HMGB1 $AUC_{3–6}$ were also selected for the present study. Twenty healthy volunteers with no previous history of coagulation disorders or not using any daily medication were included as a reference population.

Predictor Variables

Demographic and clinical variables were collected from the OUH Trauma Registry and ICU charts. Predefined predictor variables were age, sex, NISS,[24,25] and admission arterial BE[26] (Cobas b 221, Roche Diagnostics, Indianapolis, IN). Mechanism of injury (blunt or penetrating) was not included since only two patients (6%) had penetrating trauma (Table 1).

Conventional Coagulation Tests

Admission values of INR and APTT were available from the OUH Trauma Registry.[4,27,28] The INR was analyzed with the Owren type reagent SPA+, and APTT with the PTT-Automate reagent, both using the STA-R Evolution System (Stago, Asnieres, France). The INR was calibrated using INR calibrators certified by External Quality Assurance in Laboratory Medicine in Sweden and traceable to a World Health Organization thromboplastin.[29]

Outcome Measures

The predefined outcome measures were ventilator-free days (VFDs),[30,31] obtained from the OUH Trauma Registry and defined as days alive and off ventilator during the first 30 days after trauma,[22] and Sequential Organ Failure Assessment (SOFA) score[32] at days 0, 4, 7, and 9. Patients who were never on a ventilator received a VFD of 30. The SOFA score provides a means of objectively describing organ dysfunction in trauma patients during the ICU or high-dependency unit stay[33] and was assessed as reported previously.[34] In addition, mortality at 30 days, verified from the Norwegian Population Registry, and thrombotic and bleeding complications, diagnosed according to *International Classification of Diseases, Tenth Revision*, were obtained from the OUS Trauma Registry. The relevant *International Classification of Diseases, Tenth Revision*, codes for arterial and venous thromboembolic events were I21,

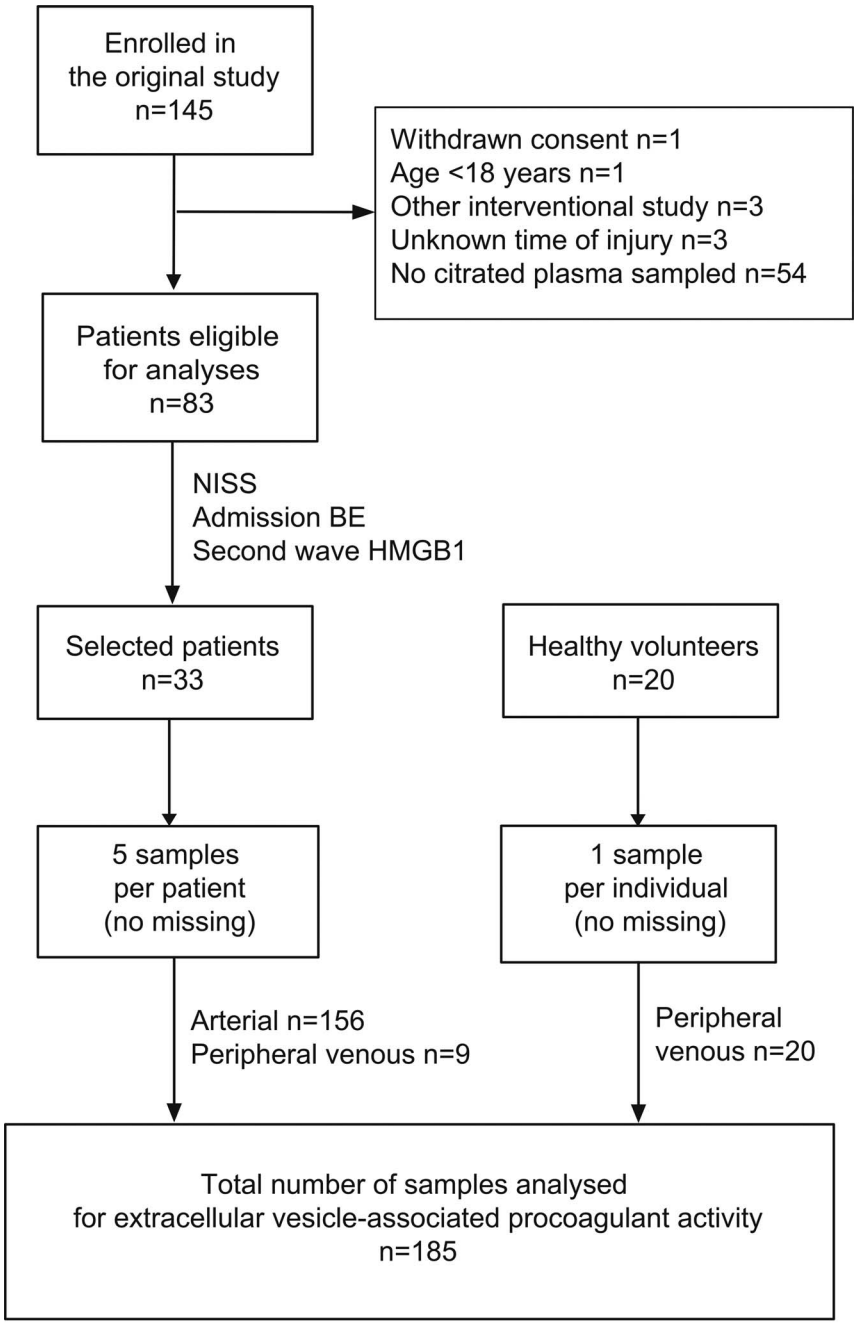

Figure 1. Flow diagram for the study population and number of samples analyzed for EV-associated procoagulant activity, according to the STROBE statement.[23] Patients were selected to constitute the study population based upon NISS, admission BE and second wave of the alarmin HMGB1 3 to 6 hours after injury.[22] STROBE, Strengthening for Reporting of Observational Studies in Epidemiology.

I26, I74, I80, I63, and I64, and those for major bleeding events were I60, I61, I62, K92.0, K92.1, and K92.3.

Sample Collection and Processing

Arterial blood samples were collected from each patient at admission and 2, 4, 6, and 8 hours thereafter. If the patient did not have an arterial cannula, venous samples were obtained.[22] After a discard tube, blood was drawn into citrated tubes (Vacuette, 3.5 mL; sodium citrate, 0.109 mol/L; Greiner Bio-One, Kremsmünster, Austria) and centrifuged (2,500*g* for 15 minutes in room temperature [RT]) within 15 minutes. The supernatant was immediately transferred to sterile polypropylene tubes (NUNC CryoTubes; Thermo Fisher Scientific, Waltham, MA) and stored at −80°C. Venous blood from 20 healthy volunteers was obtained in 2014 and processed according to the same protocol.

TABLE 1. Patient Characteristics

	Trauma Patients (n = 33)	Healthy Volunteers (n = 20)
Demographic		
Sex, male:female	29 (88%): 4 (12%)	11 (55%): 9 (45%)
Age, y	39 (27–53; 18–71)	39 (32–49; 22–58)
ASA PS score, I:II:III	21 (64%): 8 (24%): 4 (12%)	
Injuries		
Mechanism of injury, blunt: penetrating	31 (94%): 2 (6%)	
ISS	26 (11–40; 1–75)	
NISS	34 (22–54; 1–75)	
Major head injury,* yes:no	18 (55%): 15 (45%)	
Admission BE [−3 to 3 mmol/L]	−4.2 (−6.1 to −1.8; −13.7 to 0.7), n = 32	
Admission BE ≤−6 mmol/L (n)	8 (24%)	
Admission INR [0.8–1.2]	1.1 (1–1.2; 0.8–1.4)	
Admission INR ≥1.3 (n)	3 (9%)	
Admission APTT [28–40 s]	33 (29–40; 26–57)	
Admission APTT ≥40 s (n)	8 (24%)	
Admission fibrinogen [1.9–4.0 g/L]	2.7 (1.9–3.2; 1.2–4.5)	
Samples		
Time from ED admission to first sample, h:min	0:10 (0:06–0:15; 0:04–1:27)	
Time from injury to first sample, h:min	1:17 (0:46–1:48; 0:29–3:38)	
Time from injury to ED admission, h:min	1:00 (0:38–1:27; 0:20–3:18)	
Hospital treatment		
Transfusions before ICU (PRBC units)	6 (3–11; 1–19), n = 9	
Hospital length of stay, d	7 (3–19; 1–35)	
ICU or HDU length of stay, d	6 (2–11; 1–35), n = 32	
Time on ventilator, d	8 (2–16; 1–35), n = 19	
Outcome measures		
VFDs	27 (0–30, 0–30)	
SOFA score [0–24]		
Day 0	9 (4–11; 1–16), n = 33	
Day 4	9 (5–12; 1–15), n = 13	
Day 7	6 (5–11; 1–14), n = 9	
Day 9	7 (2–9; 0–10), n = 7	
Dead at 30 d, yes:no	5 (15%): 28 (85%)	
Time to death, d	1 (0–5; 0–9), n = 5	
Thrombotic complications,** yes:no	2 (6%): 31 (94%)	
Bleeding complications,† yes:no	1 (3%): 32 (97%)	

Values are given as median (quartiles; range) or number (percentage), unless otherwise specified. The number of patients (n) is given when lower than the total population (N = 33). Reference limits are given in square brackets.

*Major head injury was defined as maximum Abbreviated Injury Scale severity code of ≥3 in Injury Severity Score region head or neck.

**Thrombotic complications were defined according to *ICD-10* (codes I21, I26, I74, I80, I63, and I64).

†Bleeding complications were also defined according to *ICD-10* (codes I60, I61, I62, K92.0, K92.1, and K92.3).

ASA PS, American Society of Anesthesiologists Physical Status Classification System; ED, emergency department; *ICD-10*, *International Classification of Diseases, Tenth Revision*; HDU, high-dependency unit; ISS, Injury Severity Score; PRBC, packed red blood cell.

Isolation of EVs

In this study, we have defined EVs as particles pelleted at 17,000*g* for 30 minutes. Citrated plasma from patients and healthy volunteers was thawed (15 minutes, 37°C) and lightly whirled before 400 µL plasma was transferred to a new Eppendorf tube (Biosphere SafeSeal Tube, 1.5 mL; Sarstedt, Nümbrecht, Germany). The EVs were pelleted by centrifugation (17,000*g*, 30 minutes, RT), followed by removal of 350 µL plasma, addition of 350 µL Tris-buffered saline with 0.5% *v*/*v* bovine serum albumin (TBSA), and another centrifugation (17,000*g*, 30 minutes, RT). The supernatant was removed, and the pellet was dissolved in 100 µL TBSA and vortexed for 5 seconds.

Preparation of Pooled Normal Plasma

Blood was collected in Monovette tubes (S-Monovette, 5 mL; sodium citrate, 0.106 mol/L; Sarstedt) from 10 healthy individuals (8 female; 2 male) after informed, written consent in 2017. The venepuncture was performed with a Safety-Multifly-Needle 21G (Sarstedt), and the first tube was discarded. The tubes were centrifuged (2,000*g*, 15 minutes, RT) within 1 hour, followed by transfer of the top plasma fraction to new tubes, leaving 0.5 cm plasma above the buffy coat. The plasmas were subjected to a second centrifugation (2,000*g*, 15 minutes, RT) and pooled together to constitute pooled normal plasma (PNP), which was aliquoted and stored at −80°C.

Thrombin Generation

Hemker et al.[35] originally described thrombin generation by the calibrated automated thrombogram assay. We have used a modified protocol where isolated EVs from patient samples were added to PNP.[36] The PNP was prepared as described in the preceding section. To increase thrombin generation and to make the assay more sensitive to TF, anti–human tissue factor pathway inhibitor (anti-TFPI) antibodies (100 µg/mL final concentration, CLB/TFPI C-terminus [clone]; Sanquin Reagents, Amsterdam, The Netherlands) were preincubated in PNP (15 minutes, 37°C).[36,37] Briefly, 80 µL of PNP, preincubated with anti-TFPI antibodies, was added to wells (Thermo Immulon 2HB plate; Thermo Fisher Scientific, Waltham, MA) containing either 20 µL of EV suspension or 20 µL of calibrator. The plate was incubated for 10 minutes at 37°C before thrombin generation was initiated by automated addition of 20 µL of FluCa buffer, containing both calcium and a fluorogenic thrombin substrate. All reagents for the thrombin generation were from Thrombinoscope BV (Maastricht, The Netherlands). Fluorescence was read for 90 minutes by a Fluoroscan Ascent microplate reader (Thermo Scientific), and the thrombin generation parameters lag time, peak, endogenous thrombin potential (ETP), and velocity index were calculated by the Thrombinoscope software (Thrombinoscope BV).

Samples where thrombin generation curves did not reach baseline within 90 minutes were excluded from statistical analyses. Their measurements are not corrected for the contribution of α-2 macroglobulin-bound thrombin, and corresponding thrombin generation parameters may therefore be incorrect.[38]

To explore how EV-associated TF affected thrombin generation, isolated EVs were incubated with either 2 µL mouse monoclonal anti-TF antibodies (0.40 mg/mL, TF8-5G9 [IgG1])

or 2 µL TBSA for 15 minutes (RT). The PNP was thereafter added, and thrombin generation was initiated and measured as described previously. The hybridoma cells TF8-5G9 were a kind gift from Professor James H. Morrissey (University of Illinois), and the TF8-5G9 (IgG1) antibodies were produced at the core facility for monoclonal antibody production and assay design at OUH.

Procoagulant Activity of PS-Exposing EVs

The Zymuphen microparticle activity kit (Hyphen BioMed, Neuville-sur-Oise, France) was used to analyze the procoagulant activity of PS-exposing EVs. Citrated plasma was thawed (10 minutes, 37°C) and lightly mixed before the analysis was performed as recommended by the manufacturer. Briefly, the principle of the assay is that PS-exposing EVs in the plasma will bind to biotinylated Annexin V, which is bound to the streptavidin-coated wells. After incubation and a washing procedure, FXa, FVa, prothrombin, and Ca^{2+} are introduced. When PS-exposing EVs are present, prothrombinase complexes can form on their surfaces and convert prothrombin into thrombin. The amount of generated thrombin will depend on the amount of EVs, and thrombin activity is measured through its effect on a chromogenic substrate. The substrate releases paranitroaniline upon splitting by thrombin, and color development is measured at 405 nm. The results of the patient sample are compared with a standard curve, made from washed and lysed platelet concentrates, and expressed as PS equivalents (nM).

Statistical Analysis

Sampling times were expressed as time after admission (Fig. 2) or converted to elapsed time from injury (Supplemental Digital Content, Supplementary Fig. 2, http://links.lww.com/TA/C45, and Fig. 4). Group comparisons were performed by Fisher's exact test for categorical data and Wilcoxon rank sum test (Mann-Whitney) for continuous data. Correlation between continuous variables was assessed by Spearman correlation coefficient (ρ). A two-tailed p value of ≤0.05 was chosen to represent statistical significance.

Multivariable linear regression models with backward elimination were used to explore relationships between predictor variables and response variables at admission. The optimal model was selected using the Bayesian Information Criterion.[39] Additional mixed-model analyses were used to explore the effect of time after injury on thrombin generation parameters and procoagulant activity of PS-exposing EVs. Here, the significance level for elimination from the model was set to 0.10. Assessment of importance of the individual predictor variables was performed as variance-based sensitivity analysis.[40] Importance indices were constructed from observed combinations of measured values, since predictor variables were generally correlated. Data analyses were undertaken using JMP 13.2.0 (SAS Institute, Cary, NC).

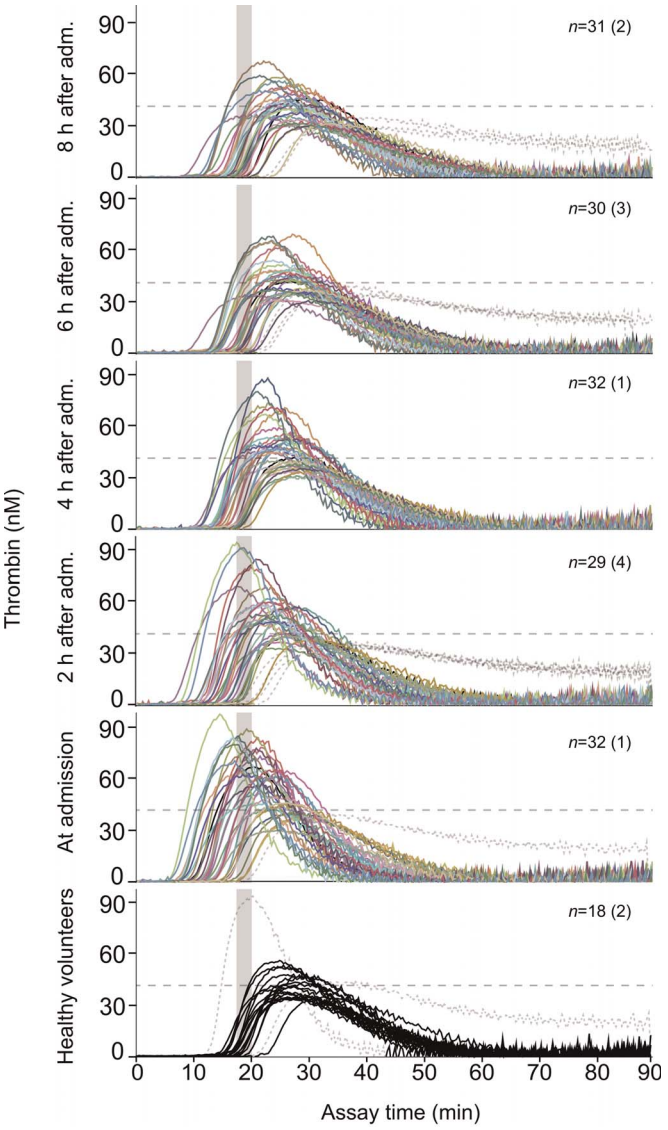

Figure 2. Thrombin generation curves for trauma patients and healthy volunteers. Extracellular vesicle–associated thrombin generation curves for trauma patients (colored, one color follows each patient) at admission and 2, 4, 6, and 8 hours thereafter and for healthy volunteers (black). Gray vertical panels represent interquartile range (quartiles, 17.6–20.0 minutes) for lag time for healthy volunteers. Dashed horizontal line represents median peak (40 nM thrombin) for healthy volunteers. Dotted gray thrombin generation curves were omitted from statistical analyses because these did not meet baseline within 90 minutes. Number of samples (n) is given for each sample time point together with the number of omitted samples in parentheses.

RESULTS

Study Population

The study population consisted of 33 patients selected from the 145 originally enrolled patients[22] (Fig. 1). Four patients used acetylsalicylic acid before admission. Three others received a prophylactic dose of low-molecular-weight heparin during the blood sample collection period, encompassing seven samples in total. None of the 20 healthy volunteers used any anticoagulants. All 33 patients were followed throughout the sampling period, and no samples were missing. Patient flow through the ICU at OUH Ullevål is illustrated in Supplemental Digital Content (Supplementary Fig. 1, http://links.lww.com/TA/C44), and patient characteristics are given in Table 1. The first blood sample was

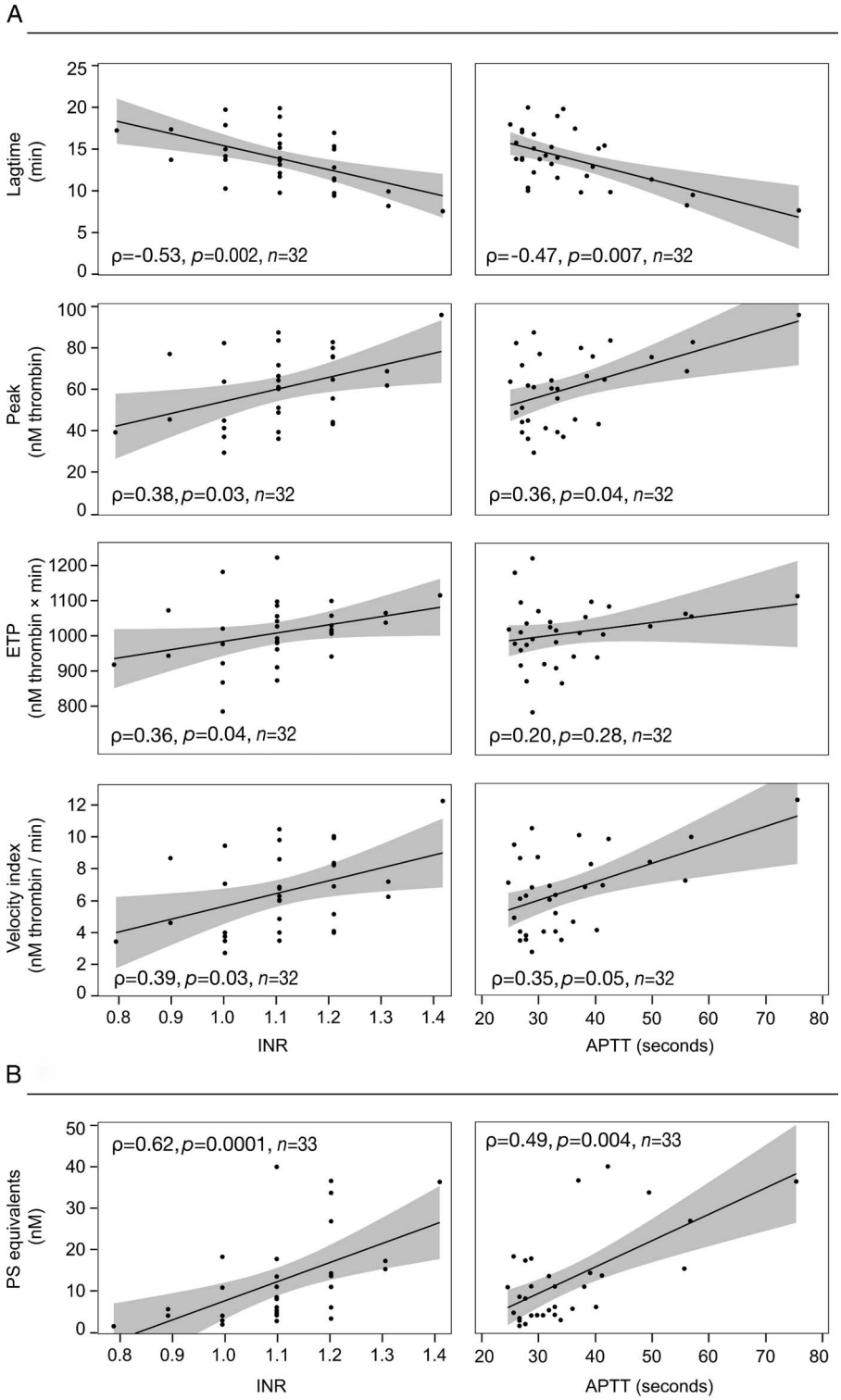

Figure 3. Associations between EV-associated procoagulant activity and conventional coagulation test results at admission. Associations between admission INR and APTT; and (*A*) admission values of EV-associated thrombin generation parameters (lag time, peak, ETP, and velocity index); and (*B*) admission levels of procoagulant activity of PS-exposing EVs (measured as PS equivalents). Regression lines with 95% confidence limits for estimated slope (shaded) are shown; nonparametric correlations are presented as Spearman's *ρ* with *p* value for two-tailed probability. n = number of samples included in statistical analyses.

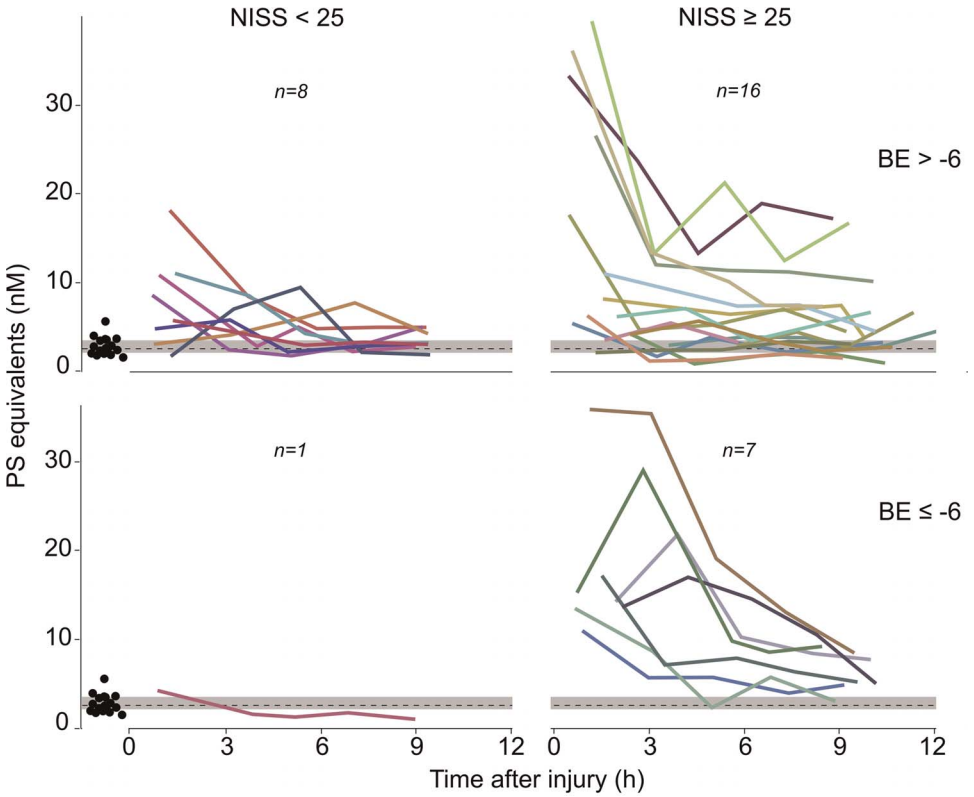

Figure 4. Activity of PS-positive EVs. The procoagulant activity of PS-exposing EVs (measured as PS equivalents) as a function of time after injury for trauma patients (colored, one color follows each patient), stratified by NISS and admission BE. Results from healthy volunteers (n = 20) are shown as black symbols with dashed horizontal lines representing median (2.6 nM) and gray horizontal bars representing their interquartile range (quartiles, 2.1–3.5 nM). The BE was missing for one patient. n = number of samples.

collected at a median of 1 hour and 17 minutes (quartiles, 46 minutes to 1 hour and 48 minutes) after injury.

EV-associated Thrombin Generation Outliers

Ten samples from seven patients and one sample from one healthy volunteer displayed thrombin generation curves that did not reach baseline within 90 minutes, one sample displayed a flat curve at 4 hours after admission, and one healthy volunteer had extremely short lag time and high peak. These samples (marked as gray dotted curves in Fig. 2) were omitted from the statistical analyses.

When anti-TF antibodies were added to the assay, thrombin generation curves did not reach baseline within 90 minutes for two patient admission samples and five samples among healthy volunteers. In addition, the same volunteer mentioned previously was an extreme outlier. These samples were omitted from statistical analyses and figures.

EV-Associated Thrombin Generation in Trauma Patients Versus Healthy Volunteers

Compared with healthy volunteers, EV-associated thrombin generation curves obtained from trauma patients showed a hypercoagulable pattern with shorter lag time and higher peak, ETP and velocity index at admission and 2 hours after admission (all p values ≤0.01) (Fig. 2 and Table 2). At 4 hours after admission, only lag time and ETP were different between patients and healthy volunteers (both p values <0.008), and at 6 and 8 hours after admission, none of the thrombin generation parameters were different.

Time Course of EV-Associated Thrombin Generation During the First 12 hours After Injury

To be able to describe EV-associated thrombin generation as function of time after injury, blood sample time points were converted to elapsed time from injury. Supplemental Digital Content (Supplementary Fig. 2, http://links.lww.com/TA/C45) shows thrombin generation parameters as a function of time after injury, stratified by NISS and BE. Lag time was shortest among patients with NISS of ≥25 during the first 3 hours after injury and gradually normalized within 9 to 12 hours after injury. Peak, ETP, and velocity index were high within the first 3 hours among patients with NISS of ≥25, but kinetics curves were highly variable and hard to distinguish from less injured patients. In mixed-model analyses, time after injury was the most important predictor of all four thrombin generation parameters, together with NISS and age for lag time, and together with admission BE for peak and velocity index, Supplemental Digital Content (Supplementary Table 1, http://links.lww.com/TA/C47).

In summary, EV-associated thrombin generation curves in trauma patients showed a hypercoagulable pattern in the first

TABLE 2. Thrombin Generation Parameters and PS Equivalents

	Lag Time (min)	Peak (nM thrombin)	ETP (nM thrombin × min)	Velocity Index (nM thrombin/min)	PS Equivalents (nM)
	Median (quartiles; range) n *p* value	Median (quartiles; range) n *p* value	Median (quartiles; range) n *p* value	Median (quartiles; range) n *p* value	Median (quartiles; range) n *p* value
Healthy volunteers	18.5 (17.6–20.0; 16.0–24.5) 18	40 (34–46; 33–55) 18	894 (844–969; 809–1,048) 18	4.4 (3.8–5.1; 3.4–6.9) 18	2.6 (2.1–3.5; 1.5–5.6) 20
At admission	13.6 (11.2–16.3; 7.5–19.7) 32 < 0.0001	61 (44–75; 29–95) 32 0.0001	1,043 (978–1,091; 818–1,247) 32 < 0.0001	6.9 (4.2–9.0; 2.8–12.9) 32 0.002	8.6 (4.2–16.2; 1.7–39.4) 33 < 0.0001
2 h after admission	15.5 (13.2–17.4; 8.2–21.6) 29 0.0001	51 (40–60; 32–91) 29 0.001	1,028 (975–1,088; 842–1,275) 29 < 0.0001	5.2 (4.5–6.6; 3.1–10.9) 29 0.01	5.7 (2.8–10.3; 0.9–35.4) 33 0.0006
4 h after admission	17.3 (15.0–18.8; 11.7–20.9) 32 0.008	46 (36–54; 30–85) 32 0.10	998 (888–1,072; 792–1,153) 32 0.005	4.6 (3.9–6.1; 2.9–12.2) 32 0.40	4.8 (3.1–9.6; 1.2–21.2) 33 0.001
6 h after admission	17.7 (15.3–19.3; 10.4–22.1) 30 0.10	42 (35–49; 30–67) 30 0.52	970 (865–1,012; 805–1,107) 30 0.11	4.3 (3.7–5.4; 3.1–7.6) 30 0.96	3.6 (2.5–7.6; 1.7–18.9) 33 0.01
8 h after admission	18.7 (16.3–19.7; 10.2–25.5) 31 0.43	42 (35–49; 30–66) 31 0.85	936 (846–1,006; 777–1,071) 31 0.49	4.3 (3.5–5.4; 2.9–7.3) 31 0.72	4.5 (3.0–6.6; 0.9–17.2) 33 0.002

Measurements from patients in each time category are compared with healthy volunteers. *p* Values represent two-tailed probability for Wilcoxon rank sum test (Mann-Whitney U) with correction for ties. EV-associated thrombin generation parameters and Procoagulant activity of PS-exposing EVs (PS equivalents). Where the thrombin generation curve did not reach baseline within 90 minutes, all corresponding parameters were omitted because they may be affected. One sample had a flat thrombin generation curve at 4 hours after admission and was omitted.

hours 3 hours after injury and thereafter gradually normalized within 7 to 12 hours.

EV-Associated Thrombin Generation, Trauma Characteristics, and Conventional Coagulation Tests

Increasing NISS and decreasing BE were both associated with shorter lag time at admission (ρ= −0.51, p = 0.003; ρ =0.45, p = 0.01) (Supplemental Digital Content, Supplementary Table 2 and Supplementary Fig. 3, upper panel), but NISS was the only significant predictor in the optimal multivariable linear regression model (R^2 = 0.25, p = 0.004) (Supplemental Digital Content, Supplementary Table 3, http://links.lww.com/TA/C49). When anti-TF antibodies were added to the assay, the significant effects of both NISS and admission BE disappeared (ρ = −0.03, p = 0.88; ρ = 0.27, p = 0.15) (Supplemental Digital Content, Supplementary Fig. 3, http://links.lww.com/TA/C46, lower panel); however, lag time in trauma patients (median, 17.2 minutes [quartiles, 15.5–18.8 minutes; range, 13.4–22.5 minutes]) remained significantly shorter than in healthy volunteers (median, 21.1 minutes [quartiles, 19.1–23.0 minutes; range, 17.9–25.5 minutes]) (p < 0.0001).

Multivariable linear regression rendered admission BE the only significant predictor for peak, ETP, and velocity index at admission (all p values ≤0.05) (Supplemental Digital Content, Supplementary Table 3, http://links.lww.com/TA/C49). Increasing admission INR was associated with shorter lag time and higher peak, ETP and velocity index (all p values ≤0.04), and increasing admission APTT with shorter lag time and higher peak and velocity index (all p values ≤0.05) (Fig. 3A and Supplemental Digital Content, Supplementary Table 2, http://links.lww.com/TA/C48).

In summary, a more hypercoagulable EV-associated thrombin generation profile at admission was associated with increasing NISS, lower admission BE, increasing INR, and prolonged APTT.

At 2 hours after admission, the only significant associations were between increasing admission APTT and shorter lag time as well as higher peak (both p values ≤0.03), whereas no significant associations were found at 4 hours after admission (Supplemental Digital Content, Supplementary Table 2, http://links.lww.com/TA/C48). Remarkably, significant associations between EV-associated thrombin generation parameters and age, NISS, admission BE, and APTT again appeared at 6 hours after admission, approximately 7 hours after injury.

EV-Associated Thrombin Generation and Outcome

There were significant associations between shorter lag time at admission and the outcome measures SOFA score at day 0 and VFD (both p values ≤0.02) (Supplemental Digital Content, Supplementary Table 2, http://links.lww.com/TA/C48). The SOFA score at days 0 and 7, and VFD were also associated with

hypercoagulable values of EV-associated thrombin generation parameters at 6 hours after admission, approximately 7 hours after injury (Supplemental Digital Content, Supplementary Table 2, http://links.lww.com/TA/C48).

Procoagulant Activity of PS-Exposing EVs During the First 12 hours After Injury

The procoagulant activity of PS-exposing EVs, measured as PS equivalents, was elevated after trauma (Fig. 4 and Table 2). The activity gradually declined during the first 12 hours after injury but remained significantly higher than in healthy volunteers at all sampling time points (all p values ≤0.01). In mixed model analyses, time after injury was the most important predictor of procoagulant activity, with contributions from admission BE and age (Supplemental Digital Content, Supplementary Table 1, http://links.lww.com/TA/C47).

Decreasing admission BE was associated with increasing procoagulant activity of PS-exposing EVs at admission and 2 and 6 hours thereafter, whereas increasing NISS was associated with increasing procoagulant activity only at 8 hours after admission (all p values ≤0.05) (Supplemental Digital Content, Supplementary Table 2, http://links.lww.com/TA/C48). The BE and age were the only significant predictors for procoagulant activity at admission in the optimal multivariable linear regression model (R^2 = 0.28, p = 0.01) (Supplemental Digital Content, Supplementary Table 3, http://links.lww.com/TA/C49). The INR and APTT were associated with procoagulant activity throughout all sampling time points (all p values ≤0.004) (Fig. 3B and Supplemental Digital Content, Supplementary Table 2, http://links.lww.com/TA/C48). The SOFA score at day 0 was associated with procoagulant activity throughout all sampling time points (all p values ≤0.04), whereas VFD was associated with procoagulant activity only at 4 and 8 hours after admission (both p values = 0.04).

In summary, the procoagulant activity of PS-exposing EVs was increased in the first 3 hours after injury and thereafter declined to levels just above healthy volunteers within 7 to 12 hours. Increased procoagulant activity early was associated with admission BE, INR, APTT, and SOFA score at day 0.

DISCUSSION

In this study we demonstrated with two different assays that EV-associated procoagulant activity was highest in the first 3 hours after injury and thereafter gradually declined. The thrombin-generating capacity of isolated EVs normalized within 7 to 12 hours after injury, whereas the procoagulant activity of PS-exposing EVs declined to a level just above that of healthy volunteers. This implies that EVs have a transient hypercoagulable function after injury and presumably play a role in the early phase of hemostasis.

New to this study is the repeated-measures design with five samples obtained with 2-hour intervals after admission. These high time resolution measurements enabled us to discern a pattern not previously described. In line with others, we found that trauma patients had higher EV-associated procoagulant activity at admission compared with healthy volunteers.[16,17,19,21] However, where others have only reported the most extreme hypercoagulable values of EV-associated thrombin generation within 12 hours of injury without finding any clear pattern among trauma patients,[17] we found a consistent temporal pattern with the most hypercoagulable values in the first 3 hours followed by a gradual decline and normalization within 7 to 12 hours of injury.

A reason for why we found a homogeneous temporal pattern of EV-associated thrombin generation might be that EVs were isolated from the patients' own plasma and resuspended in PNP before thrombin generation was measured. This method is not affected by consumptive coagulopathy or the presence of pharmacological anticoagulants in the patients' plasma and may therefore more explicitly evaluate the functional properties of EVs in injured patients.

In trauma patients, a dose-dependent procoagulant effect of platelet-derived EVs on thrombin generation and clot strength has been demonstrated in vitro,[13] and it is reasonable to believe that the increased EV-associated procoagulant activity after injury that we and others[14,16,17,19,21] observe is, at least in part, related to increased concentrations of circulating EVs.[14,16–20] Both a decreased release and a rapid clearance of EVs could explain the early decline in EV-associated procoagulant activity.

Data on when, where, and how EVs are cleared from the circulation are scarce. The half-life of platelet-derived EVs has been estimated to be approximately 5 hours after transfusion of platelet concentrates, containing large amount of EVs, to thrombocytopenic patients suffering from various hematological diseases,[41] whereas a few experimental animal models have indicated clearance of EVs from the circulation already within 5 to 10 minutes after injection.[42,43] Moreover, data have indicated that EVs can be endocytosed by endothelial cells in lungs and liver[44] and cleared by Kupffer cells in the liver.[43] In murine models, EVs can be embodied in arterial thrombi.[45] We speculate that, in trauma patients, EVs may be incorporated into clots and thereby cleared from the circulation.

Tissue injury and ongoing cellular hypoxia contribute to release of procoagulant EVs.[46] Supporting this, we found that patients with severe injury characteristics had the highest early EV-associated procoagulant activity after trauma. Appropriate resuscitation of trauma patients helps to restore deranged physiology and improves cellular hypoxia,[47] and presumably diminishes EV release as normal homeostasis is reestablished.

Interestingly, the associations between EV-associated procoagulant activity and degree of anatomical injury severity as well as physiological derangement were not significant at 2 and 4 hours after admission but reappeared 6 hours after admission, similar to associations with conventional coagulation tests and outcome measures. This could conceivably be an effect of transfusions, but number of units of packed red blood cells received from trauma room admission until ICU admission had no significant effect in multivariable post hoc analyses (data not shown). We have previously found a biphasic release of the innate danger signal HMGB1 following trauma with a second surge 3 to 6 hours after injury in the most severely injured and physiologically deranged patients[22] and, at the same time, an increase in terminal complement complex, the end product of the complement cascade.[34] Taken together, these data point to the period 3 to 7 hours after injury as particularly active for molecules of the innate immune and coagulation systems.

Addition of anti-TF antibodies to the EV-associated thrombin generation assay eliminated the shortening of admission lag

time with increasing NISS and decreasing admission BE, implying that TF contributes to accelerate EV-associated thrombin generation in an injury severity-dependent fashion.

Trauma-induced coagulopathy is a consequence of tissue injury and hemorrhagic shock and commonly presents clinically with hypocoagulability early after injury.[3] We found that patients with abnormal conventional coagulation tests had the highest EV-associated procoagulant activity early after trauma. The increased EV-associated procoagulant activity may contribute to hemostasis in the bleeding patient, or on the other hand, it may aggravate consumption of coagulation factors and be a reason for increased INR and prolonged APTT, with the clinical consequence of bleeding tendency.

There are several limitations to this study. The design is observational, and one can only describe associations, not causative relations. The sample size is small, limiting the generalizability of results and making it challenging to find reliable associations between EV-associated procoagulant activity and other variables where events were few, that is, in patients with thromboembolic or bleeding events. The small sample size also makes it difficult to compare subpopulations, such as patients with and without major head injury. Moreover, patients were selected and may therefore not represent the general trauma population. There was an unequal distribution of sex among patients, and between patients and healthy volunteers. Transfusion of blood products may affect measurements of EVs because they are EV rich.[48] A total of nine patients (27%) received transfusion of blood products as part of in-hospital standard care during the blood sampling period, and some patients also received packed red blood cells as part of prehospital standard care. Some patients received anticoagulants, but this is unlikely to have influenced the results because the patient's plasma was washed away before EV-associated procoagulant activity measurements. Knowing the concentration and origin of EVs could have strengthened the interpretation of results. However, because measurement of EVs by flow cytometry have limitations,[49] we chose to focus on functional EV-associated procoagulant activity. Double centrifugation is recommended for optimal measurements of EVs.[50] In this study, citrated plasma samples were centrifuged only once before freezing, which may lead to residual platelets that fragment during a freeze/thaw cycle and thus affect subsequent EV analyses. Only three patients had INR of ≥1.3, two of them were among the eight patients with APTT of >40 seconds. We used Owren INR, which is not sensitive to deficiencies of fibrinogen and factor V, as these coagulation factors are part of the reagent. Factor V is the coagulation factor that most often is reduced in trauma.[51] Probably more patients would have had an abnormal INR if we had used Quick INR, as the Quick method is also sensitive for deficiencies of fibrinogen and factor V.[4]

In conclusion, we describe a novel temporal pattern of EV-associated procoagulant activity that suggests that EVs may have a transient hypercoagulable function after injury and thus may play a role in the early phase of hemostasis. Larger prospective observational and experimental studies are needed to further clarify the functional role of EVs in trauma-induced coagulopathy and posttraumatic thrombosis. We suggest that the temporal pattern described here should be kept in mind when such studies are designed.

AUTHORSHIP

T.E. initiated the study and built the OUH trauma registry. M.H. and C.E.H. contributed with specific expertise in laboratory methods and knowledge on EVs and coagulopathy. K.G.B. contributed with expertise on biomarkers and molecular medicine. T.E., C.E.H., M.H., W.O., and I.N.R. designed the study. I.N.R., W.O., and T.E. recruited and followed up patients, and obtained and processed samples. M.H. performed measurements of EVs. I.N.R. and T.E. performed the statistical analyses. I.N.R., M.H., and C.E.H. drafted the article. All authors critically evaluated and discussed the ongoing analyses, critically revised the article, and approved the final version.

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DISCLOSURE

The authors declare no conflicts of interest.

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