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ORIGINAL ARTICLE

Effect of lower-leg trauma and knee arthroscopy on procoagulant phospholipid-dependent activity

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Abstract

Background: Lower-leg injury and knee arthroscopy are both associated with venous thromboembolism (VTE). The mechanism of VTE in both situations is unknown, including the role of procoagulant microparticles. This may provide useful information for individualizing thromboprophylactic treatment in both patient groups.

Objective: We aimed to study the effect of (1) lower-leg trauma and (2) knee arthroscopy on procoagulant phospholipid-dependent (PPL) activity plasma levels.

Methods: POT-(K)CAST trial participants who did not develop VTE were randomly selected for the current study. Plasma was collected shortly after lower-leg trauma or before and after knee arthroscopy. For aim 1, samples of 67 patients with lower-leg injury were compared with control samples (preoperative samples of 74 patients undergoing arthroscopy). Linear regression was used to obtain mean ratios (natural logarithm retransformed data), adjusted for age, sex, body mass index, infections, and comorbidities. For aim 2, pre- and postoperative samples of 49 patients undergoing arthroscopy were compared using paired *t* tests. PPL activity was measured using modified activated factor X–dependent PPL clotting assay.

Results: For aim 1, PPL activity levels were almost threefold higher in patients with lower-leg injury compared with controls, that is, mean ratio, 2.82 (95% confidence interval [CI], 1.98-4.03). For aim 2, postoperative PPL activity levels did not change significantly, that is, mean change, −0.72 mU/mL (95% CI, −2.03 to 0.59).

Conclusion: Lower-leg trauma was associated with increased plasma levels of PPL activity, in contrast to knee arthroscopy. Lower-leg trauma triggers the release of procoagulant microparticles.

KEYWORDS
arthroscopy, blood coagulation, cell-derived microparticles, knee injuries, leg injuries, venous thromboembolism

Essentials

- The effect of lower-leg injury and knee arthroscopy on the coagulation system is not well known.
- Procoagulant phospholipid-dependent (PPL) activity reflects procoagulant microparticles.
- Plasma levels of PPL activity were measured after lower-leg trauma and knee arthroscopy.
- PPL activity levels are increased threefold after lower-leg trauma and not after knee arthroscopy.

1 | INTRODUCTION

Venous thromboembolism (VTE) is associated with serious mortality and chronic morbidity.[1-3] Patients with lower-leg injury or who undergo knee arthroscopic surgery have an increased risk of VTE in the following months.[4-6] There is controversy on the optimal form of thromboprophylaxis in these patient groups: a "one-size-fits-all" approach of low-molecular-weight-heparin (LMWH) treatment does not sufficiently prevent VTE, as was concluded in two randomized controlled trials, the POT-(K)CAST trials. These trials demonstrated that the occurrence of VTE in the first 3 months following lower-leg cast application or knee arthroscopy was similar for patients treated with LMWH compared with patients who did not receive any thromboprophylaxis. For lower-leg cast immobilization (POT-CAST; (Prevention of Thrombosis Following Cast Immobilization)), 1.4% of patients treated with LMWH and 1.8% of patients in the control group developed VTE. For knee arthroscopy (POT-KAST; Prevention of Thrombosis Following Knee Arthroscopy), these risks were 0.7% and 0.4%, respectively.[7]

A possible approach to improve thrombosis prevention may be to identify new prevention targets.[5] For this, knowledge of the mechanism of how lower-leg trauma and knee arthroscopy trigger thrombus formation could be helpful. However, the exact mechanism is as yet unclear, including the role of extracellular vesicles (EVs). Over the past years, EVs in plasma, also known as "microparticles," have been shown to play an important role in clot formation in general. Moreover, their presence in plasma is found to be associated with VTE.[8,9] EVs are bilayer phospholipid membrane vesicles in plasma, derived from cell activation,[10,11] which can be triggered by inflammation and apoptosis.[12,13] The surface of EVs is enriched with negatively charged phospholipids, phosphatidylserine (PS) in particular, due to translocation from the inner layer of the membrane during the formation process.[8,11] EVs are procoagulant, as PS facilitates the assembly of positively charged coagulation factors (including prothrombin and factors VII, IX, and X) and enhances extrinsic tenase complex activity.[14-17] This phenomenon is referred to as procoagulant phospholipid-dependent (PPL) activity.[16]

To elucidate whether lower-leg trauma and knee arthroscopy trigger the release of procoagulant EVs in plasma, we aimed to establish the effects of (1) lower-leg trauma and (2) knee arthroscopy on PPL activity in plasma. For this purpose, we used data from the POT-(K)CAST trials.

2 | METHODS

2.1 | Study population

Data of individuals with lower-leg injury and individuals scheduled for knee arthroscopy who participated in the POT-CAST and POT-KAST trials, respectively, were used.[7] As described previously, the aim of these trials was to study the effectiveness of LMWH as prophylactic treatment in these populations by assessing the occurrence of symptomatic VTE in the first 3 months. In both trials, patients were randomly assigned to either LMWH in prophylactic dose or no prophylaxis.[7] Both trials took place between March 2012 and January 2016. POT-CAST participants were enrolled upon presentation at the emergency department with a lower-leg injury, while POT-KAST participants were included when they were scheduled for elective knee arthroscopy. Patients who were aged ≥18 years and who did not meet following exclusion criteria were eligible for inclusion: history of VTE, current use of anticoagulant therapy (except antiplatelet medication), contraindications for LMWH, pregnancy, mental or physical disability to fulfill study requirements, or insufficient knowledge of the Dutch language. All participants completed a questionnaire on putative thrombotic risk factors. Specific details about lower-leg injuries and knee arthroscopic procedures were derived from medical records. A blood sample was provided by POT-CAST (leg injury) participants shortly after trauma, at all times before any surgical procedure took place. POT-KAST participants provided two blood samples: one preoperative sample (drawn within 4 hours before surgery) and one postoperative sample (drawn within 4 hours after surgery). All individuals who were randomized to prophylactic LMWH received the first dose of LMWH after blood sampling. In this way, measurements of PPL activity in plasma were not affected by LMWH. The Medical Ethics Committee of Leiden University Medical Center approved both trials, and all patients who participated in the trials provided informed consent.

2.2 | Current study

For this study, individuals were randomly selected from both POT-(K)CAST populations who (1) did not develop VTE and (2) had blood sample(s) available. Details of these selections are depicted in the flowchart (Figure 1). Predisposition to VTE distorting the association

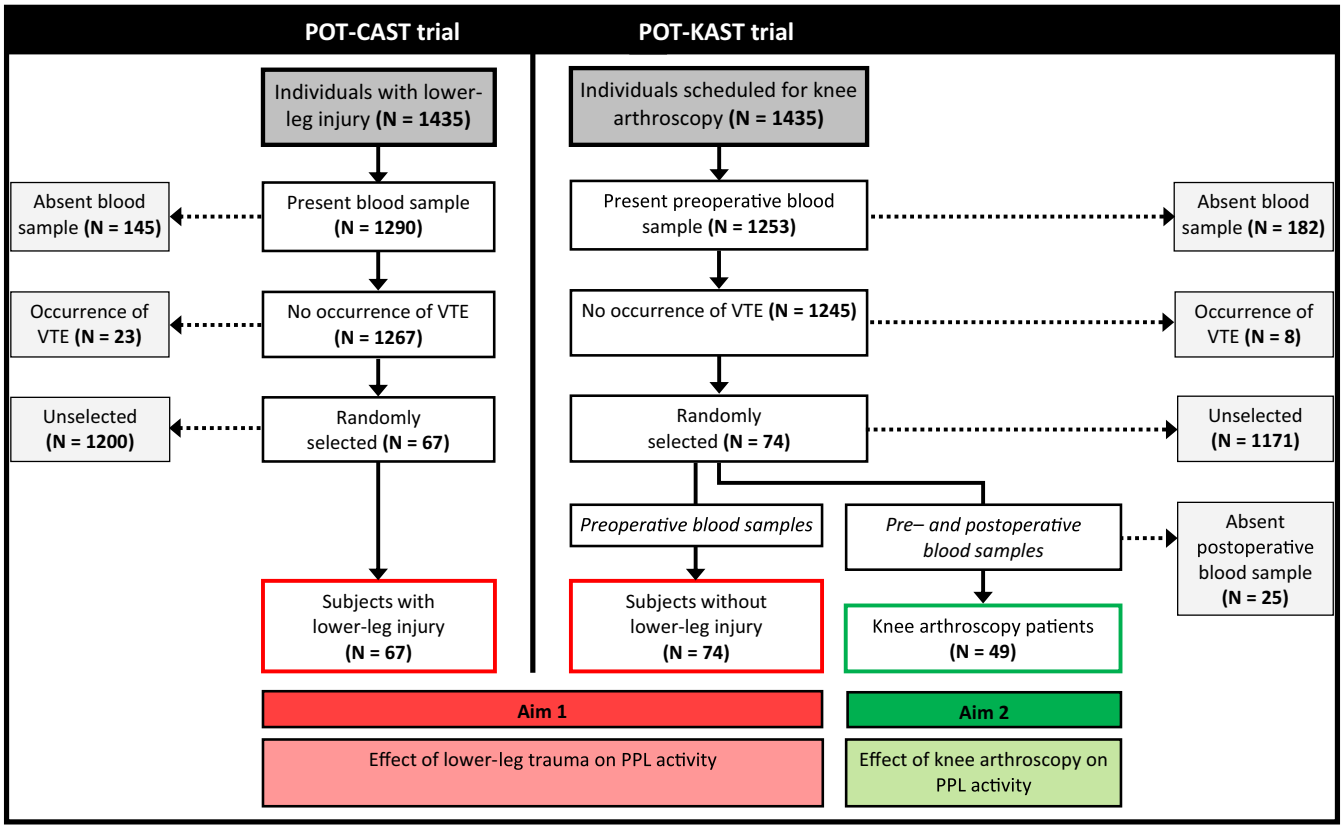

FIGURE 1 Flowchart patient selection from the POT-CAST and POT-KAST trials. PPL, procoagulant phospholipid-dependent; VTE, venous thromboembolism

between lower-leg injury or knee arthroscopy and PPL activity levels was avoided by including only those individuals who did not develop VTE during trial follow-up.

For the first research question, that is, whether lower-leg trauma is associated with increased levels of PPL activity, samples of 67 randomly chosen individuals with lower-leg trauma (POT-CAST trial) were used for measurements, further referred to as "subjects with lower-leg injury." As control samples, we used the preoperative blood samples (ie, at baseline) of 74 randomly selected patients undergoing knee arthroscopy, further referred to as "subjects without lower-leg injury." We chose to use these samples as control samples because they were processed, stored, and analyzed in the same way. Furthermore, the most prevalent indications for arthroscopy for these subjects were either unexplained complaints (diagnostic indication) or persisting residual complaints after minor knee trauma in the distant past (for which physical therapy appeared inadequate). Hence, at the time of blood draw, no acute trauma was present that could have affected PPL activity. For the second research question, that is, whether knee arthroscopy was associated with increased PPL activity levels, pre- and postoperative blood samples were compared for 49 of the 74 randomly selected patients undergoing knee arthroscopy, further referred to as "knee arthroscopy patients." Of the remaining 25 patients, no postoperative blood sample was available; therefore, they were excluded for these analyses.

2.3 | Blood collection and laboratory measurements

Blood was collected from all participants through vena puncture in the cubital vein and drawn into vacuum citrate tubes (0.105 M, 3.2%). These tubes were centrifuged during 10 minutes at 2500 *g* at 18°C. The resulting plasma was aliquoted and stored at −80°C within 4 hours after vena puncture.

For current PPL measurements, the citrated platelet-poor plasma (PPP) samples were thawed for the first time. Modified activated factor X (factor Xa)-dependent PPL clotting assay was used, as described previously.[18] In short, PPP samples were centrifuged at a speed of 13 500 *g* for 2 minutes to generate platelet-free plasma (PFP). Test plasma was mixed in equal amounts of 25 µL with 25 µL PPL-depleted plasma (phospholipid-depleted plasma [PPLDP]), incubated for 2 minutes before the reaction was initiated by the addition of 100 µL prewarmed factor Xa reagent (0.1 U/mL bovine factor Xa in 15 mM calcium chloride, 100 mM sodium chloride and 20 mM HEPES buffer, pH 7.0). A commercially available standardized reagent containing 0.1% of rabbit brain cephalin in a buffered solution was used as calibrator (UPTT; Bio/Data Corporation, Horsham, PA, USA). Clotting tests were carried out in duplicate on a StarT4 instrument (Diagnostica Stago, Parsippany, NJ, USA). PPL levels were measured in seconds of clotting time and converted to mU/mL, by the use of the UPTT calibrator. PPLDP was prepared

from pooled citrated PFP (n = 18) centrifuged at 100 000 *g* for 60 minutes at 16°C (Beckman Optima LE-80K Ultracentrifuge, rotor SW40TI; Beckman Coulter, Indianapolis, IN, USA), stored in aliquots of 0.5 mL and frozen until further use. The PPL assay displayed a low coefficient of variation of ≤4% and variation between runs was adjusted for by an internal standard (pooled PFP).

2.4 | Statistical analysis

2.4.1 | Aim 1: Effect of lower-leg injury on PPL activity

Subjects with and without lower-leg injury were compared in terms of plasma levels of PPL activity expressed in milliunits per milliliter, using descriptive statistics and linear regression models. Data had to be transformed using natural logarithms (ln), since PPL was not normally distributed. To obtain geometric means, the ln of all PPL values was computed, after which the mean of ln-PPL was calculated and retransformed by calculating *e^(mean ln-PPL)*, resulting in the geometric mean. In linear regression models, ln-PPL was included as the dependent variable. Since the outcome estimate of linear regression is mean difference, for ln-transformed variables, this involves *ln(b) − ln(a)*, which equals *ln(b/a)*. Therefore, by retransforming the outcome of linear regression, that is, *e^(ln(b/a))*, a mean ratio (ie, *b/a*) is obtained. The geometric means and mean ratios were obtained with their 95% confidence intervals (95% CIs). The mean ratios were adjusted for age, sex, body mass index (BMI), comorbidities and infections (in the past 2 months). BMI was included as a continuous variable in the model. Comorbidities included chronic obstructive pulmonary disease (COPD), liver disease, kidney disease, rheumatoid arthritis, multiple sclerosis, heart failure, hemorrhagic stroke, and arterial thrombosis.

To observe whether timing of blood sampling affected our results, we plotted PPL activity levels against the time between lower-leg trauma and blood sampling in a scatterplot, in which the slope of the regression line was calculated. Furthermore, we performed a sensitivity analysis, calculating the crude and adjusted mean ratio restricted to subjects in whom blood was sampled on day 0 or day 1 after the trauma occurred. In addition, PPL activity levels were stratified for types of injury ordered by magnitude.

2.4.2 | Aim 2: Effect of knee arthroscopy on PPL activity

The comparison of pre- and postoperative PPL activity levels in patients undergoing arthroscopy led to paired differences. Since these differences were normally distributed, paired mean changes were calculated along with their 95% CIs.

All analyses were performed using Stata 16.0 (StataCorp, College Station, TX, USA; http://www.stata.com). Figures were composed using Prism version 9.0.1 (GraphPad Software, San Diego, CA, USA; http://www.graphpad.com).

2.5 | Sample size considerations

Before this study, we performed sample size calculations in which we aimed to achieve a power of 90% and a level of significance of 0.05 (two-sided). Based on the results of a pilot study that we performed before the current study, we anticipated a mean difference or change of 3 mU/mL with a standard deviation of 4 mU/mL, resulting in a minimal sample size of 76 individuals in total (ie, 38 subjects with and 38 subjects without lower-leg injury) for aim 1 and 22 individuals (ie, pairs) for aim 2.

3 | RESULTS

3.1 | Aim 1: Effect of lower-leg injury on PPL activity

Subjects with and without lower-leg injury were comparable in terms of general patient characteristics (Table 1). Both patient groups involved slightly more men (56.7% and 54.1%, respectively) and had a similar median age (54.9 and 51.0 years, respectively), and the majority had BMI >25 (68.2% and 69.9%, respectively). Most lower-leg injuries concerned an ankle fracture (32.8%) or foot fracture (62.7%). Of all injuries, 13.4% were treated surgically. In a majority of the subjects, blood was sampled within 24 hours after trauma occurred (69.7%).

Plasma levels of PPL activity in both patient groups were right-skewed (Figure 2). Subjects with lower-leg injury had a geometric mean PPL of 4.63 mU/mL (95% CI, 3.81-5.62), while this was 1.62 mU/mL (95% CI, 1.23–2.14) in subjects without lower-leg injury, resulting in an almost three times higher mean plasma level of PPL activity (mean ratio, 2.86; 95% CI, 2.02-4.03). Adjusting for age, sex, BMI, comorbidities, and infections in the past 2 months hardly affected this result (mean ratio, 2.82; 95% CI, 1.98-4.03; Table 3). Figure S1 shows that mean levels of PPL activity remained the same over time, although there were more extreme values (both increased and decreased) observed on day 0 or 1 after lower-leg trauma occurrence as compared with a later blood draw. In line with this observation, the sensitivity analysis,that is, restricted to subjects with blood sampling on day 0 or 1 after trauma (57 subjects in total, which involves 85% of all subjects with lower-leg injury), showed that mean ratios were only slightly higher: a crude ratio of 2.94 (95% CI, 2.04-4.24) and an adjusted ratio of 3.05 (95% CI, 2.10-.45). Stratified analyses showed that PPL activity was increased in all different subtypes of lower-leg injury compared to subjects without, although the magnitude of lower-leg injury did not seem to correlate with PPL activity (Figure 3).

3.2 | Aim 2: Effect of knee arthroscopy on PPL activity

Postoperative plasma levels of PPL activity were measured in 49 patients undergoing knee arthroscopy. These patients were mostly men (57.1%), and the median age was 51.0 years. The majority were

TABLE 1 Aim 1: General characteristics of subjects with and without lower-leg injury

	Subjects with lower-leg injury (N = 67)	Subjects without lower-leg injury (N = 74)
Sex		
Male, n (%)	38 (56.7)	40 (54.1)
Age		
Median, y (IQR)	54.9 (46.1-60.6)	51.0 (44.0-57.3)
BMI		
<20 kg m^{-2}, n (%)	0 (0.0)	1 (1.4)
20–25 kg m^{-2}, n (%)	20 (30.3)	21 (28.8)
25–30 kg m^{-2}, n (%)	30 (45.5)	33 (45.2)
>30 kg m^{-2}, n (%)	16 (24.2)	18 (24.7)
At least one comorbidity		
Yes, n (%)	12 (18.2)	6 (8.2)
Infection in past 2 months		
Yes, n (%)	8 (12.5)	7 (9.6)
Smoking		
Yes: currently, n (%)	18 (27.7)	19 (26.0)
Yes: formerly, n (%)	26 (40.0)	20 (27.4)
Current use of oral contraceptives[a]		
Yes, n (%) of women	1 (3.7)	3 (9.1)
Malignancy in past year		
Yes, n (%)	1 (1.5)	0 (0.0)
ABO blood type		
Homozygote O, n (%)	26 (41.9)	33 (45.2)
Heterozygote O, n (%)	31 (50.0)	33 (45.2)
Homozygote non-O, n (%)	5 (8.1)	7 (9.6)
Factor V Leiden		
Yes: heterozygote, n (%)	2 (3.2)	3 (4.1)
No, n (%)	60 (96.8)	71 (95.9)
Administration of prophylactic LMWH after blood sampling		
Yes, n (%)	31 (46.3)	38 (51.4)
Type of lower-leg injury		
Achilles tendon rupture, n (%)	2 (3.0)	NA
Ankle distortion, n (%)	1 (1.5)	
Ankle fracture, n (%)	22 (32.8)	
Foot fracture, n (%)	42 (62.7)	
Surgical treatment of injury		
Yes, n (%)	9 (13.4)	NA
Time between trauma and blood sampling		
Within 24 h, n (%)	46 (69.7)	NA
Within 7 days, n (%)	17 (25.8)	
After 7 days, n (%)	3 (4.5)	

BMI, body mass index; IQR, interquartile range (25th–75th percentile); LMWH, low-molecular-weight heparin; NA, not applicable.
[a]Including hormonal therapy.

meniscectomies (69.4%), and in 98% a tourniquet was used. Most patients underwent surgery under general anaesthesia (69.4%; Table 2). The preoperative geometric mean of PPL activity was 1.35 mU/mL (95% CI, 1.00-1.82), and postoperatively this was 1.05 mU/mL (95% CI, 0.78-1.40). The mean change was −0.72 mU/mL (95% CI, −2.03 to 0.59; Table 3).

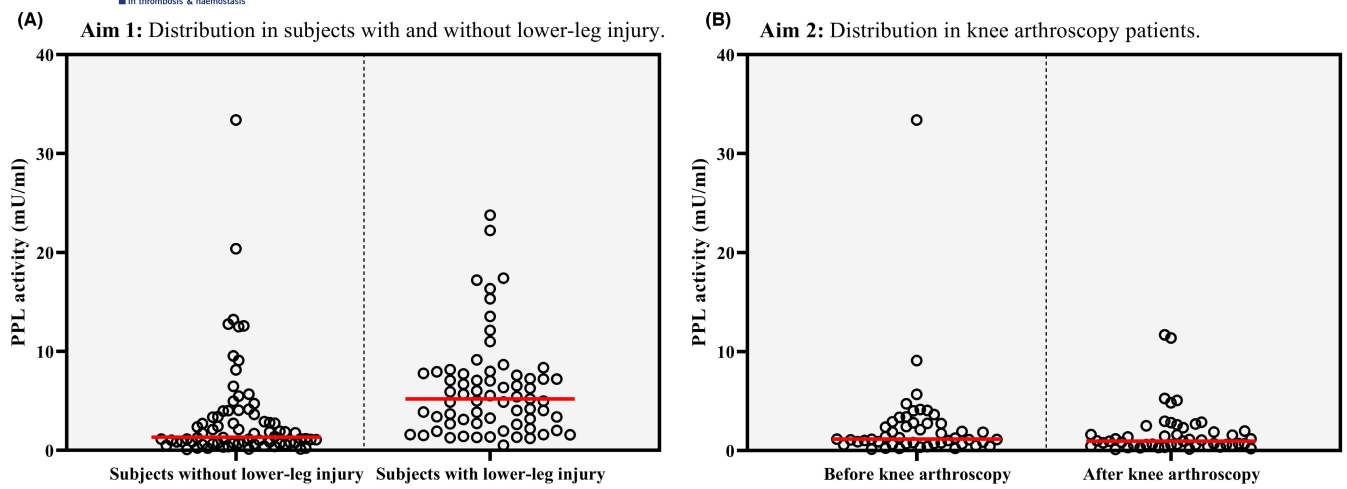

FIGURE 2 Distribution of procoagulant phospholipid-dependent activity per study population for both aims, with medians indicated as lines

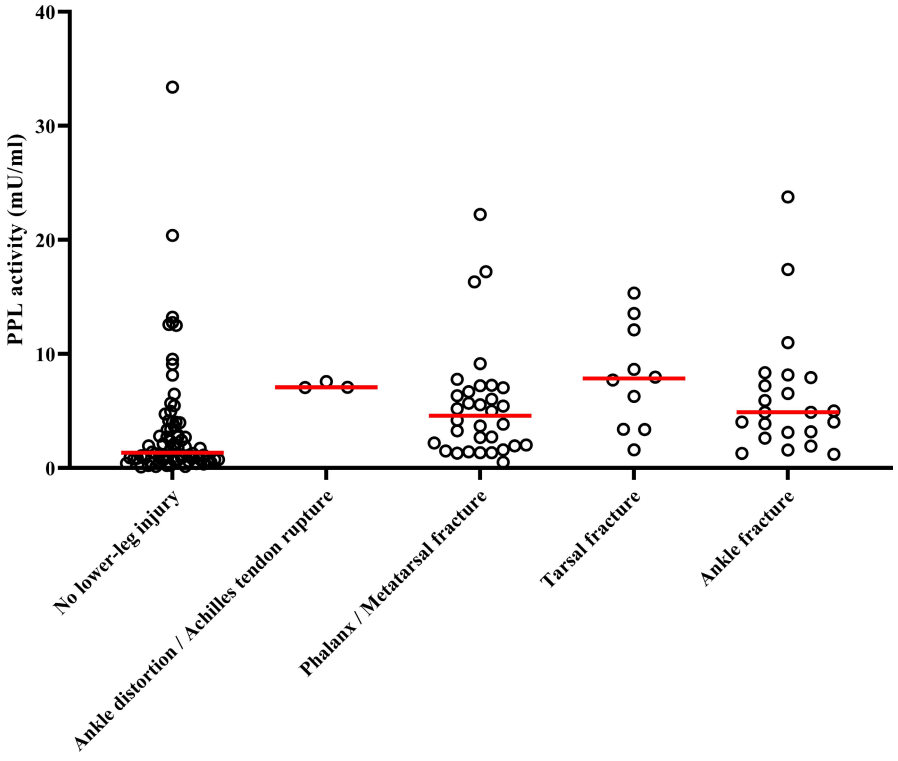

FIGURE 3 Aim 1: Distributions of procoagulant phospholipid-dependent activity per injury type ordered by magnitude with medians indicated as lines

4 | DISCUSSION

We found lower-leg injury to be associated with almost threefold increased plasma levels of PPL activity, indicating that lower-leg trauma triggers the release of PS-bearing microparticles. In contrast, the "trauma" of knee arthroscopy (generally in combination with tourniquet use) did not affect PPL activity levels.

A positive association between lower-leg injury and release of procoagulant EVs has not been studied before. In contrast, the effect of major injury on EVs has been reported earlier,[19-21] although the results of these studies cannot be compared easily with our results. Major trauma has a greater impact on the coagulation system than lower-leg trauma because of factors such as high-volume blood loss, blood transfusion, consumption of clotting factors, and multiple injuries (and its associated tissue damage).[19,20] Partly based on these studies, we can speculate on the mechanism by which lower-leg trauma triggers EV release: Vessel wall damage induced by trauma activates endothelial cells and platelets, which are both main providers of EVs (especially platelets for 70%–90%). As a result, PS is translocated to the cell's surface, which initiates the formation of EV.[11,22-24] EVs are procoagulant due to the presence of PS on their surface, expressed as PPL activity, and tissue factor (TF). PS and TF stimulate thrombus formation through thrombin generation, by promoting positively

TABLE 2 Aim 2: General characteristics of patients undergoing knee arthroscopy

	Patients undergoing knee arthroscopy (N = 49)
Sex	
Male, n (%)	28 (57.1)
Age, y	
Median (IQR)	51.0 (42.5-57.0)
BMI	
<20 kg m^{-2}, n (%)	1 (2.1)
20–25 kg m^{-2}, n (%)	15 (31.3)
25–30 kg m^{-2}, n (%)	19 (39.6)
>30 kg m^{-2}, n (%)	13 (27.1)
At least one comorbidity	
Yes, n (%)	4 (8.3)
Infection in past 2 months	
Yes, n (%)	4 (8.3)
Smoking	
Yes: currently, n (%)	14 (29.2)
Yes: formerly, n (%)	16 (33.3)
Current use of oral contraceptives[a]	
Yes, n (%) of women	1 (5.0)
Malignancy in past year	
Yes, n (%)	0 (0.0)
ABO blood type	
Homozygote O, n (%)	22 (44.9)
Heterozygote O, n (%)	21 (42.9)
Homozygote non-O, n (%)	6 (12.2)
Factor V Leiden	
Yes: heterozygote, n (%)	2 (4.1)
No, n (%)	47 (95.9)
Administration of prophylactic LMWH after blood sampling	
Yes, n (%)	27 (55.1)
Type of procedure	
Meniscectomy, n (%)	34 (69.4)
Diagnostic arthroscopy, n (%)	2 (4.1)
Removal of loose bodies, n (%)	2 (4.1)
Other, n (%)	2 (4.1)
Multiple procedures, n (%)	9 (18.4)
ASA classification	
ASA I, n (%)	26 (57.8)
ASA II, n (%)	18 (40.0)
ASA III, n (%)	1 (2.2)
Type of anesthesia	
General, n (%)	34 (69.4)
Spinal, n (%)	15 (30.6)
Use of thigh tourniquet	
Yes, n (%)	48 (98.0)
Total duration of knee arthroscopy[b]	
Median, min (IQR)	20.0 (15.5-24.5)
Duration of surgery[c]	
Median, min (IQR)	12.0 (10.0-16.0)

ASA, American Society of Anesthesiologists; BMI, body mass index; IQR, interquartile range (25th–75th percentile); LMWH, low-molecular-weight heparin.

[a]Including hormonal therapy.

[b]Total duration was from the time patient received anaesthesia to the time patient left the operating room.

[c]Duration of surgery was defined from the time of incision to the time of wound closure.

charged coagulation factors and initiating (the extrinsic pathway of) the coagulation cascade, respectively.[15,25] It depends on the cell origin and the mechanism of cell activation whether PS or TF is present on EVs. For that matter, endothelial cells release negligible amounts of TF + EVs upon apoptosis.[26,27]

To obtain more insight into this process, two additional analyses regarding the association between lower-leg trauma and PPL activity levels were performed. First, since it can be hypothesized that a greater amount of tissue damage (ie, severe injuries) invokes more cell activation and thereby more release of PS+EVs, we stratified the analyses for magnitude of lower-leg injury. PPL activity, however, did not seem to correlate with the extent of the injury. Second, it is likely that PS+EV release is highest upon cell activation and decreases over time. This could have led to underestimation of our results, since not all patients had their blood drawn directly after trauma and the half-life of EVs is relatively short (≈5 hours in case of platelet-derived EVs).[28] In line with this theory, we observed that there were more extreme values of PPL activity levels on day 0 and day 1 after lower-leg trauma as compared with levels obtained by later blood draws. However, the mean levels were the same over time. The sensitivity analysis restricted to those subjects with blood sampling on day 0 or day 1 also showed that the association between lower-leg trauma and PPL activity levels was only slightly stronger within this subset of patients.

In contrast to lower-leg trauma, knee arthroscopy did not affect plasma levels of procoagulant EVs. Although knee arthroscopy is a short and minimally invasive procedure, we hypothesized that a small effect would be expected since knee arthroscopy leads to some extent of tissue damage. In addition, a thigh tourniquet blocks arterial blood circulation to the leg, in order to perform surgery in a "dry field," causing local hypoxia. Bovill et al[29] reasoned that hypoxia activates HIF-1 and Egr-1 pathways, which are involved in TF+EV release from endothelial cells. A few studies found an increase of TF+EVs following knee replacement, but for knee arthroscopy, the effect of tourniquet use has not been studied before. The neutral results we obtained might be explained by the fact that the timing at which PPL activity was measured after knee arthroscopy, that is,

TABLE 3 Effect of lower-leg injury and knee arthroscopy on plasma levels of PPL activity

		Mean ratio (95% CI)[b]	
Aim 1: Lower-leg injury	**Geometric mean (95% CI)[a]**	**Crude**	**Adjusted[c]**
PPL activity (mU/mL)			
No lower-leg injury (N = 74)	1.62 (1.23 to 2.14)	Reference	Reference
Lower-leg injury (N=67)	4.63 (3.81 to 5.62)	2.86 (2.02 to 4.03)	2.82 (1.98 to 4.03)
Blood draw on day 0 or 1 after trauma (N = 57)	4.76 (3.87 to 5.87)	2.94 (2.04 to 4.24)	3.05 (2.10 to 4.45)
Aim 2: Knee arthroscopy		**Mean change (95% CI)**	
PPL activity (mU/mL)			
Preoperative (N = 49)	1.35 (1.00 to 1.82)	−0.72 (−2.03 to 0.59)	
Postoperative (N = 49)	1.05 (0.78 to 1.40)		

Abbreviations: CI, confidence interval; PPL, procoagulant phospholipid-dependent.

[a]Natural logarithmic (re)transformed data resulting in geometric means.

[b]Mean ratios as a result of natural logarithm retransformation.

[c]Adjusted for age, sex, body mass index, comorbidities, infections in past 2 months.

within 4 hours, was too early to measure an effect. However, after knee replacement, the peak of EVs was measured at 2.5 hours after the surgery, and was still elevated after 8 hours.[30] That is, the postoperative time frame to measure PPL activity in our study seems to be appropriate. Furthermore, it is possible that administration of intravenous saline affected postoperative PPL activity levels. However, patients received at most 0.5 L of saline during surgery. Also, considering that this would be excreted by the kidneys in a short time period, it is not likely this could have resulted in a strong dilution of PPL plasma levels.

A strength of our study was that we used modified factor Xa-dependent clotting assay. This is a functional assay that measures the activity, that is, procoagulant contribution, of EVs, which is unknown when only the amount of EVs is measured. Since presence of PS on EVs induces a high procoagulant ability, and given that our measurement approach strongly correlates with the amount of PS+EVs present in plasma,[31,32] our findings give an adequate indication of the association between lower-leg trauma or knee arthroscopy and the expression of procoagulant EVs in plasma. Moreover, this clot-based assay has been studied in healthy volunteers,[18] and characterized as a convenient and fast measuring technique with a high sensitivity, suitable for use in clinical practice.[31,32] Other strengths of our study were that all samples were collected before LMWH was administered, so our results were not affected by LMWH. Finally, for knee arthroscopy, patients served as their own control group and for subjects with lower-leg injury, a similar control group was used with application of the same methods.

Some limitations of our study should also be mentioned. First, PPL was measured ≈6 years after blood collection. It is possible that sample storage in the freezer at −80°C affected plasma levels of EVs and thereby those of PPL activity. Such a storage effect would have affected all samples equally, but a small effect of such nondifferential misclassification on the comparison cannot be excluded. Second, the modified factor Xa-dependent clotting assay only measures the amount of PS+EVs and is insensitive to TF+EVs.[18] While TF contributes to the procoagulant state of EVs, the actual procoagulant state of EVs could be even higher than we have measured. Finally, no information was available on the race of POT-(K)CAST participants. Although there is limited knowledge about the effect of race on plasma levels of (procoagulant) EVs, a study comparing African and White Americans did not show any differences in circulating EVs, suggesting that any effect of race in our study was minimal.[33]

Information about the effect of lower-leg injury and knee arthroscopy on the release of procoagulant EVs is useful in order to understand the mechanism of VTE following both events, which is as yet unclear. Understanding the mechanism on its turn could be helpful to improve currently applied thromboprophylaxis strategies by the identification of new and more effective prevention targets for VTE. This is necessary, given that the VTE risk remains high under a prophylactic dose of LMWH (especially in patients with a lower-leg cast).[5,7,34-36] Additional research is necessary, where it should be established whether PPL activity levels are elevated on the long term (for which longitudinal data are necessary), and if and for how long these elevations are associated with increased VTE risk.

In conclusion, lower-leg trauma was associated with increased PPL activity levels in plasma, while after knee arthroscopy no increase was demonstrated. Therefore, it can be assumed that release of PS+EVs is part of the mechanism of hypercoagulability in patients with lower-leg injury and that the mechanism of thrombus formation differs, at least to some extent, between lower-leg trauma and knee arthroscopy.

AUTHOR CONTRIBUTIONS

SCC designed the current study and set up the original trials with RGHHN. In both trials, samples and data were collected by BN and

RAA. LW, NL, CR, and JBH set up and performed PPL activity measurements. CET performed data analyses and wrote the initial draft of the manuscript. CET, BN, WML, and SCC interpreted the results and wrote the manuscript. All authors read the manuscript and contributed to the final version.

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RELATIONSHIP DISCLOSURE

All authors declare no competing financial or other interests.

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SUPPORTING INFORMATION

Additional supporting information may be found in the online version of the article at the publisher's website.

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