**Microvesicle phenotypes are associated with transfusion requirements and mortality in subjects with severe injuries**

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**Background**: Severe injury often results in substantial bleeding and mortality. Injury provokes cellular activation and release of extracellular vesicles. Circulating microvesicles (MVs) are predominantly platelet-derived and highly procoagulant. They support hemostasis and vascular function. The roles of MVs in survival after severe injury are largely unknown. We hypothesized that altered MV phenotypes would be associated with transfusion requirements and poor outcomes.

**Methods**: This single-centre study was approved by the Institutional Review Board. The study cohort consisted of patients with major trauma requiring blood product transfusion and 26 healthy controls. Plasma samples for MVs were collected upon admission to the emergency department (n=169) and post-resuscitation (n=42), and analysed by flow cytometry for MV counts and cellular origin: platelet (PMV), erythrocyte (RMV), leukocyte (LMV), endothelial (EMV), tissue factor (TFMV), and annexin V (AVMV). Twenty-four hour mortality is the outcome measurement used to classify survivors versus non-survivors. Data were compared over time and analysed with demographic and clinical data.

**Results**: The median age was 34 (IQR 23, 51), 72% were male, Injury Severity Score was 29 (IQR 19, 36), and 24 h mortality was 13%. MV levels and phenotypes differed between patients and controls. Elevated admission EMVs were found both in survivors (409/µL) and non-survivors (393/µL, *p* < 0.001) and persisted over time. Admission levels of PMV, AVMV, RMV, and TFMV were significantly lower in patients who died compared to survivors, but were not independently associated with the 24 h mortality rate. Patients with low MV levels at admission received the most blood products within the first 24 h. AVMV and PMV levels either increased over time or stabilized in survivors but decreased in non-survivors, resulting in significantly lower levels at intensive care unit admission in non-survivors (1,048 vs. 1,880 AVMV/µL, *p* < 0.00004 and 1,245 PMV/µL vs. 1,866 PMV/µL, *p* = 0.003).

**Conclusion**: Severe injury results in endothelial activation and altered MV phenotypes. Significant differences in specific MV phenotypes or changes over time were associated with blood product requirements and the 24 h mortality rate.

**Keywords**: extracellular vesicles; microvesicles; microparticles; transfusions; injury; trauma; coagulopathy

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Microvesicles (MVs) are a heterogeneous population of extracellular vesicles released from a variety of cells with a size ranges from 100 to 1,000 nm (1–4). They circulate in healthy subjects providing physiological maintenance functions, but are also reported in a variety of pathologic conditions (5,6). They have complex biological and molecular properties, biodistribution, and functions (1). Generally, the phenotypes of MVs reflect their parent cells by retaining cell surface proteins from the cell of origin, along with cytosolic proteins, enzymes, RNA, and miRNAs. They play an important role in cellular communication, and their analysis in peripheral blood and other body fluids could serve as an important diagnostic and prognostic tool (7–9).

Traumatic injury remains the leading cause of death for younger populations (≤44 years) worldwide and is the...
third leading cause of death in the United States (10). About one-quarter of all severe trauma patients experience acute, excessive, and uncontrolled haemorrhage that accounts for up to 30–50% of all trauma-related deaths/mortality (11–13). Haemorrhage from severe injury involves impaired coagulation, resulting in reduced clot formation and strength, and/or hyperfibrinolysis, and requires the transfusion of blood products to arrest bleeding. Injury provokes cellular activation and release of cell-derived MVs (14–23). However, despite recent advances in trauma research, the pathophysiology and mechanisms of haemostatic and cellular alterations of MVs in the early response to trauma and their roles in outcomes are not well characterized or understood.

Circulating platelets play a fundamental role in the haemostatic response to trauma by providing the surface for key reactions involved in clotting, and by generating highly procoagulant platelet microvesicles (PMVs). Although circulating MVs are predominantly of platelet origin and support hemostasis and vascular function, MVs can be released by various cell types including endothelial cells and circulating blood cells. Generally, MVs may express phosphatidylserine (PS), adhesion receptors, and tissue factor (TF), rendering them highly procoagulant. We and others have previously demonstrated that elevated procoagulant PMVs contribute to enhanced thrombin generation in healthy donors (24–26), and low PMVs are associated with trauma-induced coagulopathy (14). The role of MVs in the requirements for blood product transfusions and subsequent survival after severe injury is largely unknown. We hypothesized that altered MV phenotypes would be associated with transfusion requirements and poor outcomes. Thus, the aim of this study was to determine the potential association of MV counts and cellular origin (phenotypes) after trauma with blood product transfusions and the 24 h mortality rate.

Materials and methods
The study was conducted at the single Level 1 trauma centre in the US (Houston, Texas). The Institutional Review Board approved the protocols. The study included trauma patients meeting the criteria for the highest level trauma activation who were administered blood products. A total of 169 patients were selected. The study excluded prisoners; individuals under 16 years old; and patients who had been transferred from other hospitals. Finally, the study excluded any patient who had more than 5 min of cardiopulmonary resuscitation prior to or during the first 30 min of hospitalization. Twenty-six healthy controls of both genders (69% male, aged 18–59 years) were used as a comparison group.

Research blood samples for MVs were collected at admission to the emergency department (ED) (n = 169), of whom 42 had a post-resuscitation sample drawn upon admission to the intensive care unit (ICU). Blood was collected into sodium citrate (3.2%, 0.109 M) vacutainer tubes (Becton Dickson, Franklin Lakes, NJ) and processed immediately by double centrifugation, 15 min at 2,500 g, followed by 2 min at 13,000 g, at room temperature; and plasma was frozen at −80 °C until use. Prior to analysis, samples were quickly thawed in a 37 °C water bath. Cellular MV counts and phenotypes were analysed with Gallios flow cytometer (3 laser, Beckman Coulter, Inc., Miami, FL) and Kaluza analysis software v1.2. The MV analysis gate (< 1 μm) was set up with a blend of fluorescent beads (0.5, 0.9, and 3 μm), among which the 2-submicron beads covered the expected size range for MVs (0.5 to 1 μm) (Megamix, BioCytex, Marseille, France), according to the ISTH SSC First Collaborative Workshop (in which our laboratory participated) (27). MVs acquired within the pre-set MV scatter gate in the FS versus SS dot plot were further analysed in a specific fluorescence cyograms gate on this MV scatter gate (Fig. 1). MV phenotypes included platelet (PMV), red blood cell (RMV), leukocyte (LMV), endothelial MV (EMV), phosphatidylserine positive (AVMV), and tissue-factor-bearing MVs (TFMV), which were identified using the following monoclonal antibodies: CD41-APC (BioLegend, San Diego, CA, 6.25 μg/mL, 5 μL, IgG1k, clone HIP8), CD62P-PC5 (BD Pharmingen, San Jose, CA, 6 μg/mL, 5 μL, IgG1k, clone AK-4), CD235a-AF750 (Beckman Coulter, 50 μg/mL, 2.5 μL, IgG1, clone 11E4B-7-6 (KC16)), CD45-Pacific Blue (Beckman Coulter, 100 μg/mL, 2.5 μL, IgG1k, clone J33), CD144-FITC (BD Pharmingen, San Jose, CA, 12.5 μg/mL, 5 μL, IgG1k, clone 55-7H1), annexin V-V500 and 10 × binding buffer (BD Horizon, 50 μg/mL, 2 μL) and CD142-PE (BD Pharmingen, 12.5 μg/mL, 5 μL, IgG1k, clone HTF-1). To remove antibody aggregates, for each panel, antibodies were combined and filtered using centrifugal filter units (Ultrafree-MC, 0.22 μm, Millipore, Billerica/Bedford, MA) prior to staining. The non-specific binding (background fluorescence) was set up by using plasma only (no antibodies), antibodies only in IsolFlow (no plasma), and/or using fluorescence minus one staining. The procedure details: 25 μL of plasma was stained with appropriate premixed antibodies for 30 min at room temperature in the dark, diluted with 500 μL of IsolFlow sheath fluid or with 1 × binding buffer for annexin V staining, and immediately analysed. Events were acquired at medium flow rates. Counting beads (25 μL, Flow-Count, Beckman Coulter) with known concentration were used for absolute MV counts. Acquired data were compared over time (ER admission versus ICU admission), and contrasted with clinical data.

Statistical analysis
Descriptive statistics are expressed as the median and interquartile range (IQR: Q1–Q3 in which Q1 and Q3 are the first and third quartiles) for continuous variables and proportions (%) for categorical variables. Parametric data were compared using a Student’s t-test (unpaired or paired,
2 tails), and non-parametric using the Kruskal–Wallis test. Correlation analysis was performed with Pearson’s correlation test. Twenty-four hour mortality is the outcome measurement that was used to classify survivors versus non-survivors. An exploratory logistic regression modelling was performed to identify candidate associations between admission MV phenotypes and outcomes worthy of further research. Multiple-regression analyses were performed using Stata Software Version 12.1 (College Station, TX). Mortality rate at 24 h and total blood products transfused were selected as the outcomes of interest (dependent variables), with cell-derived MVs acting as independent variables, while controlling for age and platelet count. P-values of 0.05 or less were considered a crude indicator of potential significance.

**Results**

**Patient characteristics**
Patient demographics and injury characteristics are presented in Table I. These patients represent a severely injured and primarily blunt cohort. During the first 24 h of admission, all 169 patients were transfused. The 24 h mortality rate was 13% (22/169).

**Table I.** Trauma patients characteristics (n = 169)

|                      | Median (IQR) | N (%) |
|----------------------|--------------|-------|
| Age (years)          | 34 (23, 51)  | 122 (72) |
| Male                 | 29 (19, 36)  | 47 (28)  |
| ISS                  | 231 (185, 276) | 169 (100) |
| Penetrating          | 1.3 (1.1, 1.5) | 169 (100) |
| Platelet count, 10^9/L | 11 (4, 21)   | 169 (100) |
| INR                  | 24 h transfused, total blood products, U | |
| 24 h RBC, U          | 5 (2, 10)    | 169 (100) |
| 24 h FFP, U          | 6 (3, 11)    | 154 (91)  |
| 24 h platelets, U    | 12 (6, 18)   | 46 (27)   |
| 24 h mortality       | 22 (13)      | 169 (100) |

ISS, Injury Severity Score; INR, International Normalized Ratio.
Microvesicle phenotypes
In comparison to healthy controls, both the MV levels and their relative phenotypic distributions were different in trauma patients and controls. Injured patients at admission had significantly higher levels of EMVs, RMVs, LMVs, and TFMVs, while PMV and AVMV levels were lower but failed to reach statistical significance, most likely as a result of the high variance in the patient cohort (Table II), confirming our previous results (14). Phenotypic distributions of MVs were different in trauma patients and healthy controls, for which 87% of all MVs were of platelet origin in controls, but only 63% in patients, while EMVs were less than 1% in controls but 12% in patients. Figure 1a–c shows MV size gate settings with fluorescent beads as well as a representative example of cytograms for PMVs, AVMVs, EMVs, TFMVs, LMVs, and RMVs in a healthy donor and an injured subject, respectively. There were no gender-specific differences in any of the MV phenotypes studied, nor between blunt and penetrating injuries. The Injury Severity Score (ISS) correlated weakly with PMVs and AVMVs (r = −0.226, p < 0.05 and r = −0.181, p = 0.02), and platelet counts with PMVs (r = 0.219, p < 0.05).

Transfusions and MVs
When analysed by transfusion requirements, patients with the lowest admission levels of TFMVs (p = 0.001) were transfused with the highest volumes of total blood products (RBC + FFP + PLT) within the first 24 h (Fig. 2a); for the AVMVs, the only significant difference in the number of blood products received was between patients with the very lowest (Q1) and the very highest (Q4) MV counts (p = 0.026), most likely as a result of the high variance in AVMVs among patients (Fig. 2b). Similar trends were observed for PMVs (p = 0.061) and RMVs (p = 0.057). At the same time, there was a significant correlation (p = 0.011) between LMVs and higher volumes of transfused blood products, that is, the highest levels of LMVs were associated with a significantly higher volume of transfused blood products (Fig. 2c). In order to determine the contribution of MV phenotypes to patient transfusion requirements, multiple-regression analyses were performed while controlling for age and platelet count. Analysis showed that only low TFMV was a significant, independent predictor of total blood product transfusions (Correlation coefficient −0.056, p < 0.05, 95% Confidence Interval (−0.09, −0.03)).

24 h mortality rate and MVs
Twenty-two patients (13%) died within 24 h of admission. Admission levels of AVMVs, PMVs, RMVs, and TFMVs were significantly lower in patients who died within 24 h of admission compared to survivors (all p < 0.005) (Fig. 3). There was no difference in age, gender, type of injury (penetrating, blunt), or admission platelet counts between patients who died and those who survived, however deceased patients were more severely injured (ISS 36 vs. 29, p = 0.002). There was a weak correlation between platelet count and MVs in survivors (r = 0.197, p < 0.05). In order to determine the contribution of MVs to 24 h mortality rate, multiple-regression analyses were performed while controlling for age and platelet count; MV levels were not independently associated with 24 h mortality rate.

Time effect (ED admission to ICU admission) and MVs
Forty-two patients had blood samples collected at two time points: pre-resuscitation (at admission to the ED) and post-resuscitation (upon the admission to ICU). Graphics are presented in Table III. The 24 h mortality rate for this cohort was 17% (7/42). When MV levels were compared over time, AVMV and PMV levels were either increased or remained at the ED levels in patients who survived, but decreased in non-survivors, resulting in significantly lower levels at ICU admission in non-survivors (1,048 vs. 1,880 AVMV/μL, p < 0.00004; 1,245 versus 1,866 PMV/μL, p = 0.003) (Fig. 4a and b). Percentage changes in MVs from ER to ICU in survivors versus non-survivors were calculated. In patients who survived, PMV and AVMV counts were not significantly changed; in non-survivors, PMV and AVMV levels decreased by 39% (p < 0.05) and 41% (p < 0.01), respectively.

Table II. MVs in controls and injured patients

|                  | Controls (n = 26) | Injured patients (n = 169) | p    |
|------------------|------------------|---------------------------|------|
|                  | Median (IQR)     | % of total MVs            | Median (IQR) | % of total MVs | p    |
| Phosphatidylserine, annexin V (AVMV)/μL | 3,282 (2,424–5,364) | 8.67 (4.89) | 2,529 (1,504–4,152) | 63.18 | NS |
| Platelet (PMV)/μL | 2,624 (1,765–4,332) | 86.87 | 2,371 (1,372–3,465) | 302 (110–399) | 9.52 | <0.001 |
| Erythrocyte (RMV)/μL | 191 (148–254) | 4.27 | 304 (162–456) | 10.46 | <0.05 |
| Leukocyte (LMV)/μL | 185 (151–328) | 6.40 | 399 (286–525) | 11.95 | <0.00001 |
| Endothelial (EMV)/μL | 23 (19–39) | 0.64 | 227 (163–285) | 4.89 | <0.00001 |
| Tissue factor (TFMV)/μL | 67 (55–87) | 1.82 | 87 | 0.05 | NS |
Discussion

This study demonstrates that severe injury induces alterations in circulating MV phenotypes and that low-admission MV levels are associated with 24 h transfusion requirements and MV changes over time with death at 24h. Phenotypic distributions of MVs were different in trauma patients when compared to healthy controls. Injured patients had significantly higher EMV, RMV, TFMV, and LMV levels, and a trend toward lower admission levels of PMVs and AVMVs, although not of statistical significance, confirming our previous results (14). There were no age- or gender-specific differences in the MV counts and phenotypes of injured subjects in our study, although they were reported to be different in healthy people (28). The finding that PMVs and AVMVs were not significantly different between the controls and the patients is most likely due to the high variance in both populations, particularly in the levels of the predominant PMVs. The levels of different circulating MV types in injured patients were reported to be both decreased (17), and elevated (15,16,18,20). The majority of circulating MVs in trauma patients were generated by platelets and expressed phosphatidylserine (annexin V-positive staining), indicating their procoagulant phenotype. We and others have reported that MVs circulate in healthy subjects and contribute to thrombin generation (24/C1/26), and it has been reported that PMV counts analysed by flow correlate with their functional activity (29). A trend toward lower admission levels of PMV and AVMV may indicate that, after trauma, either the procoagulant response to injury is impaired or procoagulant MVs are already engaged in clotting processes and interactions with other cells or MVs, and therefore cannot be detected as single vesicles in circulation. The continued release of procoagulant MVs (from platelets) would be beneficial for the maintenance of procoagulant activity.

Our results showed a weak correlation between PMVs and platelet count at admission. It is recognized that circulating platelet counts often decrease after severe injury, which may be accompanied by the loss of platelet function. Activated platelets become exhausted and refractory and are not capable of producing MVs, or are cleared from circulation. The continuing release of MVs of injured subjects in our study, although they were reported to be different in healthy people (28). The finding that PMVs and AVMVs were not significantly different between the controls and the patients is most likely due to the high variance in both populations, particularly in the levels of the predominant PMVs. The levels of different circulating MV types in injured patients were reported to be both decreased (17), and elevated (15,16,18,20). The majority of circulating MVs in trauma patients were generated by platelets and expressed phosphatidylserine (annexin V-positive staining), indicating their procoagulant phenotype. We and others have reported that MVs circulate in healthy subjects and contribute to thrombin generation (24/C1/26), and it has been reported that PMV counts analysed by flow correlate with their functional activity (29). A trend toward lower admission levels of PMV and AVMV may indicate that, after trauma, either the procoagulant response to injury is impaired or procoagulant MVs are already engaged in clotting processes and interactions with other cells or MVs, and therefore cannot be detected as single vesicles in circulation. The continued release of procoagulant MVs (from platelets) would be beneficial for the maintenance of procoagulant activity.

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Fig. 2. Admission TFMV, AVMV, and LMV levels and total blood products transfused within the first 24 h. Differences in blood product transfusion by (a) TFMV, (b) AVMV, and (c) LMV level quartiles were determined by the Kruskal–Wallis test. (a) *Denotes p < 0.0001 compared to Q1; (b) *Denotes p < 0.005 compared to Q1; (c) *Denotes p < 0.05 compared to Q1; **Denotes p < 0.001 compared to Q1.

Fig. 3. Admission MV levels in trauma patients, survivors versus non-survivors. Box plots depict differences in MV levels for each cell of origin between patients who were alive versus non-survivors 24 h after admission. *Denotes p < 0.005 compared to alive.
from platelets after injury would be beneficial for the maintenance of procoagulant capacity. In line with this, the presence of procoagulant MVs was inversely correlated with transfusion requirements, further reflecting an impaired or suppressed procoagulant response to major trauma. Our previous report demonstrated lower MV levels in severely injured patients with coagulopathy and substantial bleeding (14). Similar findings of a correlation of MVs with transfusions were reported in two other trauma studies (15,16). However, unlike Windelov and colleagues, who did not detect any tissue-factor-positive MVs in their trauma patients, we report here that patients with the lowest admission TFMVs were transfused with the highest volumes of blood products, emphasizing important role of procoagulant MV phenotype in the course of resuscitation. Interestingly, Curry et al. reported a correlation between the number of TFMVs at admission and the number of RBCs transfused within the first 24 h, and significantly higher TFMVs in the RBC recipients compared to trauma patients who did not receive RBCs (15). It is important to note that although elevated circulating levels of TFMVs have been reported in a variety of disorders (30,31), it has also been recognized that the immunodetection of TFMVs is highly impacted by the TF-antibody clones used for detection as well as the presence of non-specific fluorescent particles in antibody solutions (5,32/33). In line with this, our TFMV results should be interpreted with caution when compared with studies that apply different TF clones.

In regard to blood product transfusions, it is important to note that all patients in our study were transfused. One of the criteria for inclusion in the study was that patients would receive blood products, resulting in an all-transfused patient cohort. Therefore, our patient population

Table III. Demographics of injured patients with blood sample collected at ED and ICU (n = 42)

|                          | Survivors (n = 35) | Non-survivors (n = 7) | p     |
|--------------------------|-------------------|-----------------------|-------|
| Age (years)              | 34 (23, 51)       | 24 (21, 41)           | NS    |
| Male                     | 24 (69)           | 5 (71)                | NS    |
| ISS                      | 29 (25, 36)       | 41 (35, 47)           | 0.047 |
| Penetrating              | 10 (29)           | 2 (28)                | NS    |
| Platelet count, 10^9/L   | 246 (182, 291)    | 195 (178, 260)        | NS    |
| Transfused, TBP, U       | 26 (11, 46)       | 21 (9, 83)            | NS    |
| 24 h RBC, U              | 11 (7, 15)        | 12 (4, 31)            | NS    |
| 24 h FFP, U              | 11 (5, 19)        | 9 (5, 27)             | NS    |
| 24 h platelets, U        | 6 (6, 18)         | 30 (24, 33)           | NS    |

ISS, Injury Severity Score; INR, International Normalized Ratio; TBP, total blood products.

Fig. 4. PMV and AVMV levels in trauma patients at the ER and ICU admission, survivors versus non-survivors. Blood was collected on a subset of enrolled patients upon admission to the ICU. Box plots depict differences in (a) AVMV and (b) PMV levels upon admission to the ER versus ICU between patients who were alive or did not survive 24 h after initial admission. (a) *Denotes p = 0.00004 comparing AVMV levels in the ICU between survivors and non-survivors; (b) *Denotes p = 0.003 comparing PMV levels in the ICU between survivors and non-survivors.
is potentially different from those of other reported trauma studies. It would be useful to have a group of severely injured patients who did not receive blood transfusions and compare the MV response of a patient who does and does not require a post-trauma blood transfusion. Injury and transfusion might play distinct roles in the resulting MV profiles and/or functions, and it is likely that severely injured patients who require blood transfusions exhibit different MV profiles compared to injured patients who do not bleed and hence do not need blood products, as reported (17). A study by Windelov et al. reported lower levels of AVMVs at admission in trauma patients transfused with more than 10 units of RBCs, when compared with both patients who did not receive any transfusion, and those who received 1–10 units of RBCs (16), while Curry et al. reported higher admission levels of TF+ PMVs in patients who received RBCs compared to non-transfused patients (15). The further difference between our study and other trauma studies is that we analysed MVs association with total volume of blood products (RBC, plasma, and platelets) compared to the transfused RBCs reported in other studies. Furthermore, we found a correlation between admission LMVs and the volume of total blood product transfused. It might be assumed that the inflammatory response to injury, reflected by increased LMVs, causes increased endothelial leak and dysfunction, requiring higher transfusion volumes. MVs are recognized for their participation in inflammatory responses (5,17), and might contribute to post-traumatic complications, which deserves further study.

In the current study, lower admission MV levels were found in patients who died within 24 h of admission, compared to survivors. However, after multivariate analysis, the lower levels were not independently associated with death at 24h, but MV changes over time (ER versus ICU) were associated with death at 24h. There are controversial reports of an association between MVs and mortality rates. Windelov et al. found no difference in the PMV levels of survivors and patients who died within 24 h or 28 days (16). Jacoby et al. reported a 24 h mortality rate in trauma patients that was associated with higher levels of admission PMVs (35), while Curry et al. reported significantly lower PMVs in patients who died (15).

Conflicting reports on MV levels in various studies most likely arise from differences in the methodologies used (the selection and/or affinity and specificity of antibodies, and instrument sensitivity) or even differences in trauma populations and circulating blood cell counts over time during the course of trauma and transfusions. These discrepancies definitely open doors for new studies on the role and clinical significance of MVs in the course of trauma and later complications including, but not limited to, inflammation, sepsis, thrombosis, and multiple organ failure (17,35,36).

Importantly, by analysing plasma samples at two time points, pre- and post-resuscitation, we demonstrated that patients whose MV levels were either maintained or increased had positive outcomes compared to those whose levels declined. This is particularly important for AVMVs and TFMVs, two of the most procoagulant MV phenotypes (37). Interestingly, despite high volumes of transfused RBCs, there were no major changes in RMV levels post-resuscitation. Dhillon et al. recently reported that the transfusion of stored RBCs was not associated with increased RMVs (38). Potentially, RMVs might be smaller in size and undetectable by the standard flow cytometry, or their clearance is very fast and efficient. Although it has been reported that RMVs have procoagulant properties (39–41), circulating platelets may be the most important source of MVs involved in the body’s response to severe injury.

Of note, apart from their well-recognized roles in hemostasis, PMVs also participate in endothelial repair. PMVs may modulate endothelial function in vitro by changing their receptor expression and secretome (42), and quickening endothelial repair in vivo. We detected significantly elevated EMVs after trauma and could speculate that, in the presence of elevated EMV levels, reflecting endothelial injury, low PMV levels in non-survivors may in fact contribute to poor endothelial repair. Thus, the maintenance or increase in PMV levels during resuscitation would be beneficial for endothelial repair and recovery. Some studies reported low levels of EMVs after injury (15,17,18), potentially indicating that the endothelium was not disrupted, or resulting from differences in the antibodies used to identify EMVs. There was neither a difference in circulating EMV levels pre- and post-resuscitation in our study subjects, nor an association with the 24 h transfusion or 24h mortality rates, which suggests that, after trauma and transfusions, injured endothelium takes more time to be repaired. This is in line with that fact that vascular complications occur later in the course of trauma, beyond 24 h.

Upcoming results from our recently completed large multicentre trauma study (PROPPR), in which samples were collected at multiple time points post-injury, should provide some answers (44).

Several potential limitations are important for the interpretation of our exploratory analysis of the relationship between MV values and outcomes. First, and as are other trauma studies, ours is a single-centre experience; further work is needed to confirm and extend these findings. Second, a relatively small number of patients had a second sample collected, and a small number of study subjects died. Third, the size detection limits of standard flow cytometry are well known, and miss MVs of smaller sizes. Consequently, reported absolute MV counts may be underrepresented. Finally, the transfusion requirements results could potentially be affected by the number of patients who died soon after hospital admission.
and did not live long enough to receive more blood products (45). Overall, the purpose of the current study was not to construct a comprehensive predictive model. The data presented here provide an informative description of MV phenotypes in resuscitated trauma patients. Further studies may identify novel molecular markers among MVs and potential therapeutic targets.

Conclusions
Severe injury results in alterations in MV counts and phenotypic distribution. Elevated MV levels reflect vascular and blood cells activation or injury and procoagulant phenotypes. Lower admission levels of procoagulant MVs are associated with higher transfusion requirements; MV decrease over time is associated with the 24h mortality rate, while an increase or maintenance of MV levels during resuscitation is associated with survival. The monitoring of circulating MV levels during the course of trauma may potentially serve as a biomarker for the prediction of transfusions or survival or other complications.

Author’s contributions
Contributions: NM designed the research, performed experiments, analysed and interpreted data, and wrote the manuscript. YWW performed experiments. JBH and KZ assisted in study design and the editing of the manuscript. JCC assisted with data analysis and the editing of the manuscript. CEW designed the research and assisted in data analysis and interpretation.

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