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Hepatocyte-derived Microparticles as Novel Biomarkers for the Diagnosis of Deep Venous Thrombosis in Trauma Patients

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Abstract

Venous thromboembolism is a common complication following trauma. We investigated the dynamics of plasma microparticles (MPs) levels and explored their potential as biomarkers of deep vein thromboembolism (DVT) after trauma. A total of 775 patients with traumatic fractures were recruited in this nested study. About 106 trauma patients (53 DVT subjects and 53 age-, sex-, and fracture site-matched non-DVT subjects) and 53 healthy volunteers met the enrollment criteria. MPs were characterized by transmission electron microscope, nanoparticle tracking analysis, and western blotting. Circulating levels of MPs were measured using a flow cytometer. Meanwhile, routine laboratory parameters were examined in all patients. Compared to non-DVT patients, DVT patients had higher circulating phosphatidylserine (PS) + MPs, hepatocyte-derived MPs (HMPs), PS + HMPs, and platelet-derived MPs (PMPs). Notably, PS + HMPs had the best predictive value for DVT diagnosis in trauma patients (area under the curve [AUC] 0.8939, 95% CI 0.8326 to 0.9552), which was superior to d-dimer (AUC 0.5881). The Hepatic Procoagulant Index combined plasma levels of PS + HMPs and albumin, increasing the AUC to 0.8978 (95% CI 0.8396 to 0.9561). This is the first study that addressed circulating PS + HMPs are promising biomarkers with high performance in diagnosing DVT. The Hepatic Procoagulant Index is a potential predictor of DVT in trauma patients.

Keywords

deep venous thrombosis, trauma, microparticles, biomarker, diagnosis, flow cytometric analysis

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Introduction

The total number of traumatic injuries has been increasing in the world over the past 20 years [1–3] and traumatic injury is a clear risk factor for the development of venous thromboembolism .[4,5] According to earlier studies, pulmonary embolism may occur in up to 4% of trauma victims, with a death rate ranging from 20% to 50%. Meanwhile, deep vein thrombosis (DVT) affects 2.5% to 18.91% of the traumatic population .[6–9] International guidelines point out that early detection and appropriate treatment measures can prevent DVT and reduce mortality in trauma patients.[10–13] The commonly used clinical methods for DVT diagnosis include angiography, venous ultrasonography, and plasma d-dimer assay. Angiography remains the gold standard diagnostic test for DVT. However, this option appears to be relatively high-risk for patients with coagulation disorders. Venous ultrasonography still has some drawbacks in terms of overall vascular visualization and display of collateral circulation. It requires repeated

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monitoring and highly skilled operators, although venous ultrasonography is recommended for DVT screening and monitoring. Plasma d-dimer assay is a relatively sensitive marker for DVT but lacks specificity, so d-dimer was used as an index to rule out DVT.[14] Therefore, there is an urgent need to develop new, reliable, and non-invasive biomarkers for the prediction and diagnosis of DVT.

Activated, apoptotic, and healthy cells produce extracellular vesicles. Extracellular vesicles are classified into exosomes, microparticles (MPs), and apoptotic bodies based on their size and biogenesis pathway.[15] Among them, shedding MPs are a bilayer lipid structure with a diameter of 0.1 to 1 μm .[15] MPs act as messengers in intercellular communication and contribute to the initiation and progression of various conditions by carrying signature cargoes such as proteins, nucleic acids, and lipids. MPs have important roles in regulating cellular communication, immune modulation angiogenesis, and thrombosis.[16] Since MPs retain the same surface and cytosolic proteins as their parental cells, they are regarded as promising circulating biomarkers in neoplasms.[16,17] Additionally, various non-neoplastic conditions (ie, autoimmune and cardiovascular conditions) have also clearly benefited from the research and development efforts on liquid biopsies.[17–19] Phosphatidylserine (PS)-exposing extracellular vesicles provide the surface and facilitate the generation of thrombin and tenase complex.[20,21] Therefore, PS+ MPs were considered as an important role in thrombosis. Almost all coagulation factors are synthesized by the liver, so hepatocyte-derived MPs (HMPs) may provide insights into the state of the hemostatic system. However, previous studies have not fully elucidated the link between HMPs and venous thromboembolic after trauma. Therefore, we hypothesize that HMPs provide insights into the hypercoagulable state in trauma patients.

In this study, we have evaluated circulating HMPs and platelet-derived MPs (PMPs), and other laboratory parameters. We aimed to identify a new biomarker to effectively discriminate between healthy volunteers and trauma patients with and without DVT.

Patients and Methods

Study Subjects

This is a nested case-control study. A total of 775 patients with traumatic fractures were admitted to the Beijing Jishuitan hospital's emergency department from July 2021 to July 2022. About 275 health examination patients from the same period were recruited. Patients with fractures of the pelvis, stem (femur and tibiofibular), joint (hips and patella), and foot were enrolled in the study. Individuals with any of the following were excluded: anticoagulation or antiplatelet therapy, uncontrolled infections, history of coagulation, malignant tumors, liver and kidney insufficiency, autoimmune diseases, and no bilateral lower extremity angiography. In this study, bilateral lower extremity angiography was used as the gold standard for lower extremity DVT diagnosis. All patients requiring surgery underwent preoperative angiography to clarify whether they had lower extremity DVT. Fifty-three patients were proven with lower extremity DVT within a week of admission and formed the DVT group, and the non-DVT group consisted of 53 age-, sex-, and fracture site-matched patients with negative angiographic results. The healthy control group was composed of 53 age- and sex-matched healthy volunteers (27 males and 26 females). They had normal physical indicators and did not have a history of any medical or surgical conditions including cancer, hepatitis, infections, etc. This study was approved by the Institutional Ethical Committee of the Beijing Jishuitan hospital. Our study obtained informed consent from all participants before participation and was carried out strictly following the Helsinki declaration.

Blood Sample Collection and Preparation

Peripheral venous blood samples of trauma patients were collected immediately after admission to the emergency department. The blood was collected into tubes containing ethylene diamine tetra-acetic acid, inert separating gel, coagulant, and 3.2% sodium citrate (BD Biosciences, USA). Samples were processed within 2 h. Tubes were centrifuged twice in 2 h at room temperature (RT) (25 °C), each at 2500 $\times g$ for 15 min at RT (Figure 1A) the lowest deceleration to collect platelet-free plasma (PFP). Discarding the last 0.5 cm of plasma above the buffy coat, PFP was homogenized before aliquoting and stored at −80 °C for further detection by flow cytometry (BD Cano II, Becton Dickinson, USA). Additionally, PFP was diluted with PBS and centrifuged at 17 800g for 60 min at 4 °C. Pellets were carefully washed by PBS and centrifuged again in the same way. Finally, the enriched MPs were stored at −80 °C for electron microscopy, nanoparticle tracking analysis, and western blot. Additionally, PFP randomly selected from the healthy subjects (5 males and 5 females) was pooled and aliquoted into normal pooled plasma and then stored at −80 °C.

Clinical Laboratory Parameters Detection

Routine blood tests were performed using ethylene diamine tetra-acetic acid-anticoagulated whole blood on a Sysmex XT4000i analyzer (Sysmex, Kobe, Japan); blood coagulation tests were performed in the PFP samples by an optical method on a Sysmex CS5100i analyzer (Sysmex, Kobe, Japan) and liver function tests were performed using plasma on the HITACHI 7600 series automatic analyzer (Hitachi, Tokyo, Japan).

Characterization of Isolated MPs

Transmission Electron Microscope of MPs

The isolation effect of MPs was verified by electron microscopy. The enriched suspensions (10 μL) of MPs were immediately dropped onto the carbon film copper grid and incubated for 1 min at RT. The liquid was dried with filter

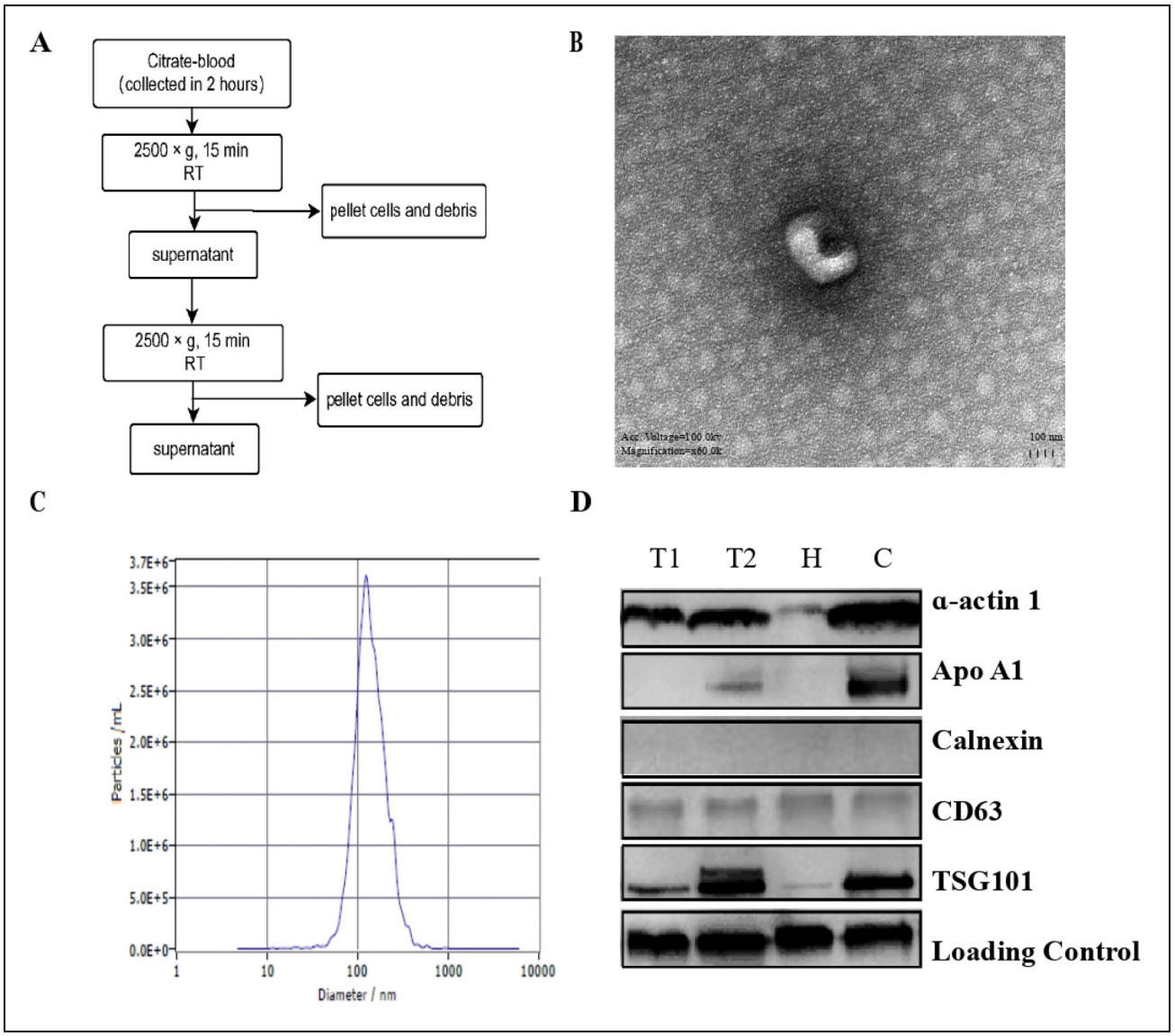

Figure 1. Characterization of Circulating MPs. (A) The workflow of the isolation of MPs. (B) Transmission electron microscopy shows the typical round morphology of MPs from plasma. (C) The size distribution of MPs by nanoparticle tracking analysis. (D) Presence of α-actin 1, CD63, and TSG101, and non-MP marker of Apo A1 and Calnexin, analyzed by western blotting. An equal protein amount of 20 μg was loaded for all samples.

Abbreviations: MP, microparticle; RT, room temperature; T1, circulating microparticles of trauma patient 1; T2, isolated microparticles of trauma patient 2; H, isolated microparticles of healthy volunteer; C, control of untreated plasma.

paper. The grids were negatively stained with uranyl acetate for 1 min and air-dried at RT. Finally, the grids were examined with an HT-7700 transmission electron microscope (Hitachi, Tokyo, Japan) at an acceleration voltage of 100 kV.

Nanoparticle Tracking Analysis of MPs

The particle size and density distribution of MPs were analyzed with a nanoparticle tracking analysis instrument (ZetaView, Particle Metrix, Meerbusch, Germany). The sample was thawed at 25.0 °C and diluted with 1 × PBS to appropriate concentrations before detection. The data were analyzed with ZetaView Software 8.05.14 SP7.

Western Blotting Analysis of MPs

The pellets of MPs were lysed with PIPA lysis and extraction buffer (Thermo Scientific, Rockford, IL, USA) for 40 min and were centrifuged for 15 min at 11 000 × *g* at 4 °C. Supernatants were transferred to a new tube and the protein concentrations were quantified with a Pierce™ BCA Protein Assay Kit (Thermo-Scientific) according to the manufacturer's protocol. We mixed the 20 μg protein solution with loading buffer (5×) and then it was heated in boiling water for 5 min. After that, samples and markers were loaded into gels for separation by 15% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (Bio-Rad, Hercules, CA, USA) after which the proteins were

transferred onto a polyvinylidene fluoride membrane (Millipore, Bedford, MA, USA). After blocking with 5% skimmed milk powder, primary antibodies were added and the membrane was incubated at 4 °C overnight on a horizontal shaker. Primary antibodies used were CD63 (ab134045, Abcam, Cambridge, UK, 1/1000), TSG101 (ab125011, Abcam, Cambridge, UK, 1/1000), α-Actinin (6487, Cell Signaling, Boston, USA, 1/500), Apo A1 (ab52945, Abcam, Cambridge, UK, 1/1000), and Calnexin (ab133615, Abcam, Cambridge, UK, 1/1000). After washing 3 times with PBS, the membrane was further incubated with the secondary antibody for 2 h at RT on a horizontal shaker. Proteins were visualized using an ultrasensitive ECL chemiluminescence staining assay kit (Beyotime) and the density of each protein band was quantified.

Flow Cytometric Analysis of MPs

PFP was immunolabeled immediately after thawing at 37 °C for roughly 3 min and was quantified after a single freeze-thaw cycle. 8 µl PFP was stained with mixed fluorescent antibodies (Allophycocyanin [APC]-labeled Annexin V [AV] [BD Pharmingen, Milan, Italy], phycoerythrin [PE]-labeled Anti-asialoglycoprotein receptor-1 [Anti-ASGPR 1] [BD Pharmingen] and phycoerythrin-cyanine7 [PE-Cy7]-labeled CD41a 1 µl each) in a Trucount absolute counting tube (BD Trucount™). 2 × AV binding buffer (Abcam, Cambridge, MA, USA) was added to a total volume of 50 µl. MPs were gently vortexed and incubated for 20 min at RT in the dark. Samples were then diluted to 400 µl with 2 × AV binding buffer and continued incubating in the dark for 10 min at RT before being analyzed by a FACSCanto II flow cytometer (Becton Dickinson, USA). The optimal amount of antibody to use was determined by titration experiments. FSC and SSC, and fluorescence data were obtained on the logarithmic scale. FSC/SSC and fluorescence properties of MPs reflected their size and cell origin. Gate limits were established as manufacturer descriptions using the Megamix-Plus SSC (BioCytex) and Megamix 7401 (BioCytex) in sizes from 0.16 to 3 µm (Figure 2). To exclude the electronic background noise, the gate of MPs was defined by 0.16 and 0.9 µm bead cloud. Negative controls and isotype controls were used to determine background fluorescence and to identify positive stained events. Before all analysis, we performed the fluorescence minus one experiment for compensation of spectral overlap. Autofluorescence of AV binding level was corrected using fluorescence signals obtained with MPs in a calcium-free PBS. Data were analyzed using the FACSDivaTM software (version 8.0.2, Becton Dickinson). The circulating MP concentration was determined according to the formula: events/µl = Positive events × Counting bead concentration / Number of Counting Beads. Additionally, normal pooled plasma has been independently proceeded 3 times a day for 3 consecutive days to ensure that the coefficient of variation (CV) was within the allowable range.

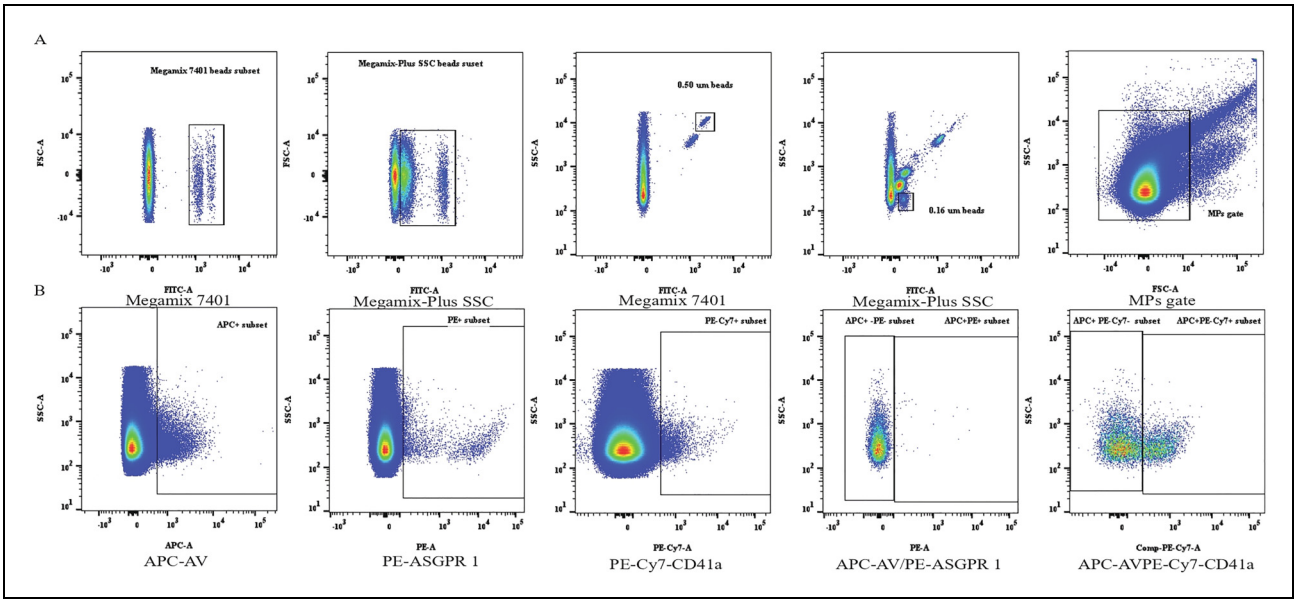

Figure 2. Gating strategy and representative images for the detection of MPs in the FACS analysis. (A) MPs gate limits in the FSC/SSC plot for MP quantification (defined as > 0.1 µm to < 1 µm) were established before analysis using the Megamix-Plus SSC and Megamix 7401 beads. MPs gate limits were set according to beads signal and according to MPs size and granularity. (B) MPs of APC + subset binding APC-labeled Annexin V, MPs of PE + subset binding PE-labeled anti-ASGPR 1 antibodies, and MPs of PE-Cy7 + subset binding PE-Cy7-labeled anti-CD41a + antibodies were selected from MPs gate and quantified. APC and PE double-positive and APC and PE-Cy7 double-positive MPs were quantified from the APC + subset region.

Abbreviations: FACS, fluorescence activated cell sorting; FSC, forward scatter; SSC, side scatter; APC, allophycocyanin; ASGPR 1, asialoglycoprotein receptor-1; PE, phycoerythrin; PE-Cy7, phycoerythrin -cyanine7. Used markers were CD41a for platelets, ASGPR 1 for hepatocytes.

Statistical Analysis

All statistical analyses were performed using IBM SPSS 26.0 and GraphPad Prism 9.0. The Shapiro–Wilk test was used to test the normality of the continuous data. Normal distribution variables were reported as mean ± standard deviation and compared using Student's T-test for 2 groups or one-way-ANOVA for 3 groups. Corresponding; non-normal distribution variables were reported as median (25th, 75th percentiles) and compared using the Mann–Whitney *U*-test or Kruskal–Wallis test. Dunn's multiple comparisons tests were performed if *P*-value was significant. Comparison of qualitative variable frequencies was calculated using Chi-square analysis. Additionally, multivariate logistic regression analysis in a forward stepwise model was used to generate adjusted odds ratios (OR) with 95% confidence intervals (CI). Spearman's correlation was run to determine the relationship between the variables of MPs subpopulations, the Hepatic Procoagulant Index, and hemostatic laboratory markers. The predictive values of the respective parameters were estimated by receiver operating characteristic (ROC) curve analysis and area under the curve (AUC) analysis with a 95% confidence interval (CI). $P < .0500$ was used as the threshold for statistical significance.

Results

Subjects' Characteristics

A total of 775 trauma patients met the inclusion and exclusion criteria and were enrolled in this study from June 2021 to June 2022 (Hospital Jishuitan, Beijing, China). Fifty-three trauma patients were diagnosed with DVT within a week of admission and matched with 53 trauma patients without DVT by age and gender. Additionally, 27 male and 26 female healthy volunteers were included in this study with ages ranging from 22 to 71 years (46.79 ± 12.25). The demographic and clinical characteristics of all the study subjects are shown in Table 1. Compared to healthy controls, trauma patients were more prone to thrombotic events with a 12.9% preoperative incidence of DVT. Compared with DVT patients, non-DVT patients had no significant difference in gender, age, previous DVT and surgery, chronic basic diseases, fracture position, time from trauma to admission, and angiography (Table 1).

Comparison of Laboratory Characteristics in Healthy Controls and Trauma Patients with or Without DVT

The laboratory characteristics of all subjects including blood coagulation tests, liver function tests, and routine blood tests are summarized in Table 2. Decreased levels of PA, APTT, and TT were found in trauma patients with or without DVT compared to healthy individuals while the d-dimer levels were increased ($P < .0001$). There were no significant differences in the levels of PA, INR, and fibrinogen between the 2 groups (Table 2). Furthermore, trauma patients without or with DVT had slightly lower levels of GGT, total protein, albumin, and prealbumin compared to healthy participants (Table 2). Furthermore, all the above indicators except albumin had no significance between the DVT and non-DVT groups. Albumin levels of the DVT group were mildly lower than the non-DVT group (42.60 g / L vs 41.30 g / L, $P = .0066$) (Supplemental Figure S1A). The levels of ALT, AST, and DBIL were not significantly different between the 3 groups (Table 2). Finally, the number of neutrophils in trauma patients was almost double the figure of healthy individuals ($P < .0010$). In contrast, the levels of RBCs were lower in trauma patients ($P < .0001$) and were even lower in the DVT group ($P = .0098$) (Supplemental Figure S1B).

Characterization of Circulating MPs

The MPs were isolated from 30 mL of plasma by differential ultracentrifugation. During centrifugations, the pellets and cells of MPs were carefully removed and washed. The morphological investigation of MPs was performed by transmission electron microscope.

Table 1. Demographic and Clinical Characteristics of Healthy Controls and Trauma Patients With or Without DVT.

Variables	Healthy Controls n = 53	Non-DVT group n = 53	DVT group n = 53	*P*-value
Male/female, n/n	27/26	21/32	22/31	.843
Age, year	46.79±12.25	53.87±14.58	57.36±14.26	.327
Risk factors before trauma, n				
Previous DVT	0	0	0	1.000
Previous surgery at past 6 months	0	0	0	1.000
Hypertension	1	5	8	.374
Diabetes, n	1	2	4	.401
Cancer history	0	0	0	1.000
Fracture position				
Pelvis/hips/femur/tibiofibular/patella/others (ankle and calcaneus), n/n/n/n/n/n		3/6/19/17/6/2	6/4/16/20/6/1	.821
Time from trauma to admission, hours <24/24~72/>72		23/19/11	24/16/13	.977
Time from trauma to angiography, hours <24/24~72/>72			12/14/27	.776

Table 2. Baseline Laboratory Characteristics in Healthy Controls and Trauma Patients With or Without DVT.

Characteristics	Healthy controls (n = 53)	Non-DVT group (n = 53)	DVT group (n = 53)	*P*-value
PT, s	11.40(11.20, 11.75)	11.54±0.62	11.50(11.15, 12.00)	.6470
PA, %	107.20±8.38	106.82±13.10[a]	107.86±16.13[a]	<.0001
INR	0.96±0.04	0.97±0.06	0.95(0.92, 1.01)	.8501
APTT, s	27.30(25.55, 28.60)	25.90(24.65, 27.30)	25.40(23.85, 26.88)[a]	.0006
TT, s	17.22±0.66	16.47±0.90[a]	16.40(15.62, 17.30)[a]	<.0001
D-dimer, mg / l FEU	0.25(0.14, 0.40)	4.11(1.68,15.91)[a]	6.89(3.68, 17.23)[a]	<.0001
Fibrinogen, mg / dl	281.90±53.84	331.96±109.87	289.20(229.45, 399.65)	.1073
ALT, IU / L	22.00(16.00 27.00)	17.00(14.00, 28.00)	20.00(16.00, 36.00)	.1208
AST, IU / L	20.00(17.75, 26.00)	21.00(17.00, 26.00)	23.00(18.50, 31.50)	.0822
GGT, IU / L	27.30(25.55, 28.60)	18.0(13.00, 26.50)[a]	22.00(14.00, 33.50)[a]	<.0001
DBIL, umol / L	3.40(2.80, 4.75)	4.00(2.65, 6.10)	4.40(3.30, 6.55)	.0941
Total protein, g / L	74.10±3.81	72.40(67.35, 76.00)	68.64±8.22a	.0004
Albumin, g / L	45.15(43.50, 46.00)	42.60(40.30, 45.40)[a, b]	41.30(38.15, 43.40)[a]	<.0001
Prealbumin, mg / L	262.14±48.14	224.48±57.53[a]	207.21±61.80[a]	<.0001
Glucose, mmol / L	29.39±3.09	28.93±4.04	28.34±4.89	.3300
RBC, $\times 10^9$ / L	4.77±0.62	4.35±0.59[a, b]	4.11(3.53, 4.38)[a]	<.0001
Platelet, $\times 10^9$ / L	256.29±59.24	213.50(188.25, 269.50)[a]	246.90±82.62	.0468
Neutrophil, $\times 10^9$ / L	4.06(3.38, 5.03)	7.17(5.10, 9.46)[a]	8.72(5.75, 11.96)[a]	<.0010
Monocyte, $\times 10^9$ / L	0.62(0.37, 0.56)	0.63±0.26[a]	0.62(0.43, 0.75)[a]	<.0001
Lymphocyte, $\times 10^9$ / L	1.88±0.58	1.22(0.98, 1.52)[a]	1.26±0.54[a]	<.0010
Hemoglobin, g / L	143.13±16.74	133.72±18.91[a, b]	122.25±19.40[a, b]	<.0001

Abbreviations: PT, prothrombin time; PA, prothrombin time activity; INR, international normalized ration; APTT, activated partial thromboplastin time; TT, thrombin time; ALP, alanine aminotransferase; AST, aspartate aminotransferase; GGT, γ-glutamyl transpeptidase; DBIL, direct bilirubin; RBC, red blood cell.
[a]$P < .05$, and the difference is statistically significant when compared with healthy controls.
[b]$P < .05$, and the difference is statistically significant when compared with patients with DVT.

Using a transmission electron microscope intact and round MPs with typical bilayer lipid structures were observed (Figure 1B). Nanoparticle tracking analysis also indicated that the MPs had a relatively narrow size distribution and the peak diameter for the MPs was 148.9 nm (Figure 1C). Compared to untreated plasma, the common MP markers CD63, TSG101, and α-actin 1 were detected in both the trauma group and healthy group by western blotting and negative markers of Calnexin and Apo A1 were absent (Figure 1D).

Circulating MPs Concentration of Healthy Participants

Circulating MPs concentrations were measured in 53 healthy individuals (27 males and 26 females). The MPs values of 5 parameters (PS + MPs, HMPs, PMPs, PS + HMPs, and PS + PMPs) were also not significantly different at different genders and ages (20-34 years [n = 13], 35-45 [n = 11], 45-55 [n = 16] and ≥ 55 [n = 13]) (Table 3). Intra-assay CVs for PMPs and HMPs were 3.93% and 6.99%, respectively; inter-assay CVs were 4.79% and 7.32%, respectively.

Comparison of the Gross Profile of the Circulating MPs Concentration in Healthy Controls and Trauma Patients With or Without DVT

Table 4 presents the concentrations of circulating MP subgroups measured in trauma patients with or without DVT and healthy subjects. The circulating levels of PS + MPs were lower in the non-DVT group but were similar between healthy subjects and trauma patients with DVT. It was observed that HMPs and PS + HMPs were significantly elevated in trauma patients with DVT but lower in trauma patients without DVT (both $P < .0001$). Therefore, trauma patients with DVT had markedly increased levels than those without DVT (Figure 3 A-D). PMPs and PS + PMPs were reduced in trauma patients (both $P < .0001$). Of note, the DVT group had higher levels of PS + PMPs than the non-DVT group ($P = .011$). But such a difference was not found in the levels of PMPs ($P = .077$) (data not shown).

Independent Risk Factors for DVT

Multivariable models for the prediction of a DVT were performed with a logistic regression model with MPs levels and those variables that were significantly different between patients with and without DVT, including albumin, and hemoglobin. Moreover, according to previous research, other factors with important impacts on DVT, such as fibrinogen, d-dimer, count of leukocyte, and platelet, were also included in the multivariate logistic regression analysis.[22] PS + HMPs and albumin were identified as significant independent risk factors for preoperative DVT after trauma (Table 5). High levels of PS + HMPs ($P = .0001$) and low levels of albumin ($P = .021$) were associated with an increased risk of DVT. This new indicator, the Hepatic Procoagulant Index, was computed using the following formula: Hepatic Procoagulant Index = (0.442) × PS + HMP (counts / μl) + (−0.183) × albumin (g / L) + 4.644. As

Table 3. Circulating MPs of Healthy Controls.

Concentration (count / μl)	Gender			Age, y				
	Males (n = 27)	Females (n = 26)	*P*-value	21-34 (n = 13)	35-44 (n = 11)	45-54 (n = 16)	55-90 (n = 13)	*P*-value
PS + MPs	2443.89±1036.00	2738.49±1126.43	.3261	2581.84±1265.23	2356.41±1063.17	2754.15±959.68	2587.29±1133.45	.8380
HMPs	555.69±158.94	477.72(394.18, 593.73)	.3006	500.56±172.47	476.71±145.29	615.03±195.80	541.83±132.11	.1479
PMPs	1210.18±421.14	1176.90(947.48, 2011.51)	.5805	1461.51±893.99	1138.12±540.52	1456.24±500.26	1207.59±373.45	.4085
PS + HMPs	3.88(3.14, 5.34)	4.30±2.40	.7076	4.33±2.37	3.95(2.97, 6.75)	3.87(3.07, 4.86)	4.52±2.20	.9543
PS + PMPs	1091.18±595.15	1280.08±649.55	.2745	1216.81±771.12	1114.72±673.78	1239.94±567.12	1140.35±546.84	.9508

Abbreviations: MP, microparticle; PS, phosphatidylserine; HMP, hepatocyte-derived MPs; PMP, platelet-derived MPs.

Table 4. The Concentration of Circulating MPs in Healthy Controls and Trauma Patients With or Without DVT.

Concentration (count / μl)	Healthy controls (n = 53)	Non-DVT group (n = 53)	DVT group (n = 53)	*P*-value
PS + MPs	2432.94(1849.14, 3593.56)	1496.93(943.11, 2461.58)[a, b]	2312.31(1825.09, 3254.40)	<.0010
HMPs	542.85(397.27, 634.78)	466.66±163.01[b]	576.61(436.06, 832.64)	<.0010
PMPs	1177.02(1026.52, 1627.84)	867.31±531.17[a]	902.18(697.12, 1425.87)[a]	<.0001
PS + HMPs	3.86(3.04, 5.46)	3.34(2.66, 4.96)[b]	9.47(6.38, 13.24)[a, b]	<.0010
PS + PMPs	1183.85±623.73	638.32(253.12, 877.77)[a, b]	812.40(473.60, 1214.64)[b]	<.0001

[a]$P < .05$, and the difference is statistically significant when compared with healthy controls.
[b]$P < .05$, and the difference is statistically significant when compared with patients with DVT.

shown in Figure 3 (E), compared to trauma patients without DVT, the Hepatic Procoagulant Index was higher in those who had DVT (0.8268 vs 0.1764, $P < .0001$).

Correlation Between MPs Variables, Hepatic Procoagulant Index, and Hemostatic Laboratory Markers in DVT Group and non-DVT Group

We found positive correlation between PS + MPs and d-dimer, and between HMPs and PA (Table 6). Moreover, HMPs levels were inversely correlated with PT and INR (Table 6). But we did not find relationships between other MPs subpopulations, Hepatic Procoagulant Index, and hemostatic laboratory variables (Table 6).

Diagnostic Performance of Parameters for Prediction of DVT After Trauma

To determine the diagnostic power of circulating MPs and to set a diagnostic threshold for DVT diagnosis in trauma patients, ROC curve analyses were performed for circulating MPs, and Hepatic Procoagulant Index and compared with plasma d-dimer levels (Figure 4). The optimal cut-off value for PS + HMPs was 5.00 counts/μl using the Youden index. PS + HMPs (AUC 0.8939, $P < .0001$) had better diagnostic power than HMPs, PS + MPs, PS + PMPs, and d-dimer (Figure 4). Detailed information is shown in Table 7. The Hepatic Procoagulant Index included levels of PS + HMPs and albumin, further enhancing the ability of PS + HMPs to diagnose DVT in trauma patients. The AUC of the Hepatic Procoagulant Index reached 0.9150 ($P < .0001$, 95% CI 0.8700 to 0.9590), as depicted in Figure 4. Hepatic Procoagulant Index discriminated between non-DVT and DVT trauma patients with a 77.40% sensitivity, 93.20% specificity, 89.13% PPV, and 80.00% NPV (Table 7).

Discussion

As a strong transient provoking factor, trauma contributes to 20% of all venous thromboembolic cases.[3] The post-thrombotic syndrome is a serious long-term complication that lowers the quality of life. However, DVT can develop with few symptoms or even asymptomatically.[11] Therefore, there is a significant unmet need to develop biomarkers for DVT. TT, PT, and APTT are conventional global coagulation predictors which are widely used in the setting of coagulation system conditions. While isolated prolongation of TT, PT, and APTT reflect coagulation factor deficiencies in the extrinsic and intrinsic pathways, respectively .[23] As a product of fibrin proteolysis by plasmin, elevated levels of d-dimer indicate the ongoing fibrinolysis of complex fibrin.[24] In this study, we found trauma patients had higher d-dimer levels and shortened PA, APTT, and TT than healthy control. Overall, trauma facilitates the activity of coagulation and fibrinolytic systems. Previous studies suggested that hypoalbuminemia was a significant independent risk factor for serious complications even death after hip fracture.[25] Furthermore, mounting data suggested that

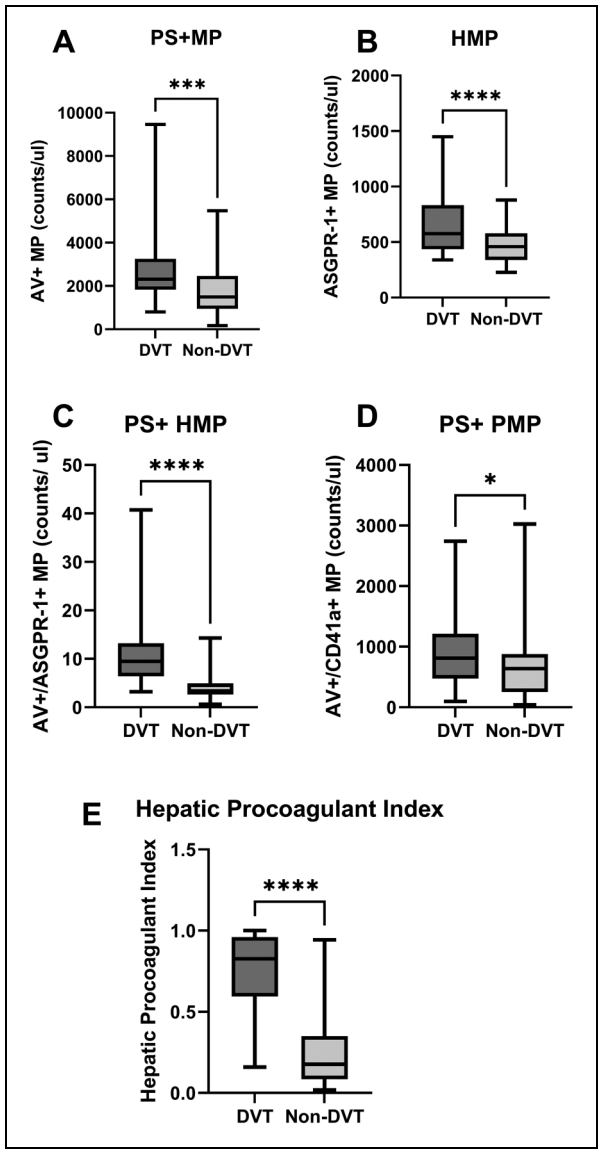

Figure 3. Comparison of circulating MPs indicators between trauma patients with or without DVT, including (A) PS + MPs ($P = .0003$), (B) HMPs ($P < .0001$), (C) PS + HMPs ($P < .0001$), (D) PS + PMPs ($P = .0395$) and (E) Hepatic Procoagulant Index ($P < .0001$). *$P < .05$; **$P < .01$; ***$P < .001$; ****$P < .0001$. $P < .05$ is statistically significant. The horizontal bars represent medians and the vertical bars the 2.5th and 97.5th percentiles for all box plots shown. Non-DVT and DVT stand for patients without and with DVT after traumatic lower extremity fractures, respectively.

Abbreviations: AV, Annexin V; PS, phosphatidylserine; HMP, hepatocyte-derived MPs; PMP, platelet-derived MPs; DVT, deep venous thrombosis; non-DVT, non-deep venous thrombosis.

Table 5. Independent Risk Factors for DVT.

Risk factors	β value	OR value	OR of 95% CI	*P*-value
PS + HMPs, count / L	0.442	1.556	1.299, 1.862	<.0001
Albumin, g / L	−0.183	0.833	0.713, 0.973	<.0210

hypoalbuminemia caused hyperfibrinogenemia and platelet aggregability.[26] The albumin levels of the DVT group were slightly lower than those without DVT, which demonstrates the association between plasma albumin levels and the risk of DVT. There are dramatic changes in the number of circulating immune cells following the trauma.[27,28] Elevated leukocyte and platelet counts as well as a reduction in hemoglobin are blood count analysis parameters that have proven helpful in risk assessment.[22] Consistent with this, we observed that neutrophil numbers almost doubled after trauma while lymphocytes declined which underscored a functional link between activation of these immune cells in trauma patients. Moreover, we found that erythrocytes and hemoglobin were reduced after trauma, which was more pronounced in patients with DVT. This is consistent with previous studies had found that anemia (OR 1 to 2) significantly affects the risk of thrombosis.[22]

Previous publications had described MPs' function as contributors to the physiological balance of vascular homeostasis and also the immune system.[29,30] Since MPs can be sampled via easily accessible peripheral blood, they are promising non-invasive biomarkers for disease detection and progression monitoring.[17,18] In this study, we showed that the circulating HMPs and PMPs were significantly increased in trauma patients, especially those with DVT. These findings not only matched the pathophysiology of the disease but also revealed novel insights into its pathogenesis. Typically, PS distribute exclusively in the cytoplasmic leaflet .[31] Driven by Ca^{2+}-dependent enzymes, PS are rearranged and exposed on the surface of stressed cells.[16,20] About half of MPs detected expose anionic PS on their surface and thus are positive for annexin V.[32] PS-exposing extracellular vesicles provide the surface and facilitate the generation of thrombin and tenase complex .[20,21]We found elevated PS + MPs and PMPs concentrations in trauma patients with DVT compared to the non-DVT group, and these changes likely reflected a more extensive activation of the coagulation system in the DVT group. In addition, we also found positive relationships between circulating PS + MPs level and plasma d-dimer level. This result suggested that elevated levels of PS + MPs were associated with a hypercoagulable state. Interestingly, in vitro studies indicated that MPs of healthy individuals were found no procoagulant ability.[33] PMPs also failed to facilitate thrombin generation without procoagulant stimulations.[34] Consistent with this, we found that higher levels of circulating PS + MPs and PMPs in the healthy group did not function as procoagulants. The trauma resulted in the exposure of TF on monocytes or vessel walls, which amplified the coagulation cascade by interacting with MPs. Furthermore, a previous study found that PMPs were completely cleared from circulation within 50 min .[35] Therefore, trauma subjects especially those with DVT depleted a large amount of circulating clotting substances, and we could speculate that they were in a state of continuous production of more MPs.[36,37] One PMP was more procoagulant and almost equal to one activated platelet,[38] so high levels of PMPs and PS + MPs may suggest a hypercoagulable state. Additionally, PMPs could deposit on monocytes via P-selectin and indirectly amplify the coagulation

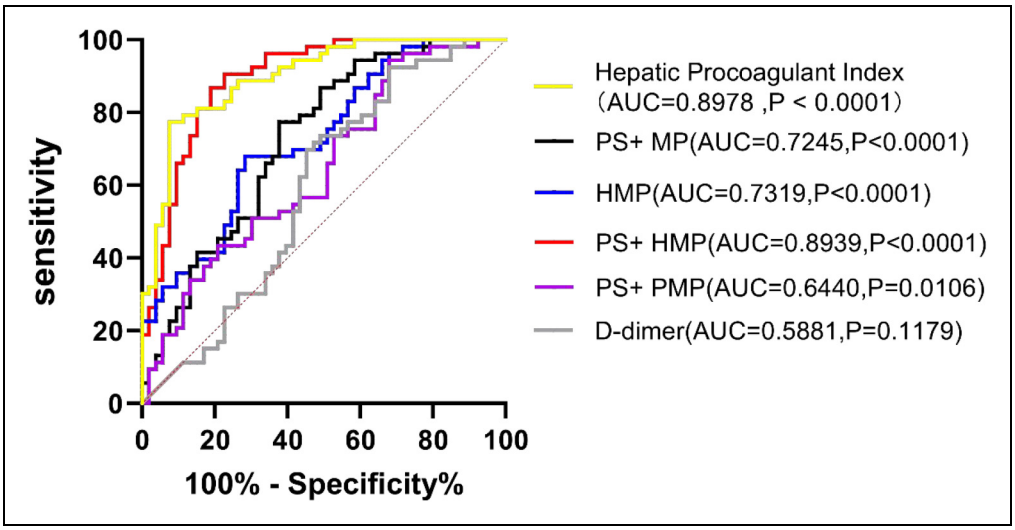

Figure 4. Receiver operating characteristic curve analysis of circulating MPs indicators for DVT in trauma patients. PS + MPs are presented by a black line. HMPs are shown by a blue line. PS + HMPs are exhibited by a red line. PS + PMPs are manifested by a purple line. D-dimer is displayed by a grey line. Hepatic Procoagulant Index is presented by a yellow line.
Abbreviation: AUC, area under the curve.

Table 6. Correlation Between MPs Variables, Hepatic Procoagulant Index, and Hemostatic Laboratory Markers in DVT Group and Non-DVT Group.

Variables		PS + MPs	HMPs	PMPs	PS + HMPs	PS + PMPs	Hepatic Procoagulant Index
PT, s	*R*	0.010	−0.305	−0.109	−0.003	0.060	0.073
	P-value	.915	.001	.266	.974	.539	.458
PA, %	*r*	−0.004	0.290	0.058	0.005	−0.048	−0.040
	P-value	.970	.003	.557	.962	.624	.684
INR	*r*	0.014	−0.293	−0.055	−0.009	0.062	0.038
	P-value	.887	.002	.578	.927	.531	.698
APTT, s	*r*	−0.074	0.000	0.082	−0.183	−0.007	−0.171
	P-value	.456	.998	.405	.061	.947	.080
TT, s	*r*	0.007	0.091	−0.013	0.059	−0.142	−0.032
	P-value	.945	.354	.893	.549	.148	.747
D-dimer, mg / l FEU	*r*	0.194	−0.145	0.079	0.062	0.189	0.068
	P-value	.047	.137	.419	.529	.052	.487
Fibrinogen, mg / dl	*r*	−0.043	−0.06	0.098	−0.051	0.117	−0.013
	P-value	.662	.544	.316	.605	.231	.899

process by transferring their cargo and surface receptors,[39] thus activated PMPs may also be associated with inflammation leading to DVT. Compared to healthy controls, we did not observe the increased levels of PMPs in the trauma group, as opposed to a previous study.[40] This could be explained by different detection methods and injury patterns. Since almost all coagulation substances are synthesized by the liver, HMPs may be deeply involved in coagulation activation. However, most clinical studies of HMPs solely focused on liver conditions, whereas data linking HMPs and the coagulation system are limited. A previous study reported that TF expression-deficient mice had reduced levels of TF in the liver and MPs and attenuated activation of the coagulation cascade, while it was not reduced in TF expression-deficient myeloid cells.[41] Another study found that the high-affinity PS-binding protein greatly blocked factor Xa production by MPs from Fas death receptor agonist treated hepatic progenitor cell (HPCs),[42] suggesting increased TF procoagulant activity through a mechanism involving increases of PS externalization on the surface of MPs. Therefore, HMPs may play a major role in the activation of the coagulation cascade. In concordance with this study, our study observed that dramatically higher levels of HMPs were found in trauma patients with DVT. Thus, we could speculate that the increase in HMPs (with or without PS expression) may predict DVT in patients with trauma. Interestingly, we found a positive correlation between HMPs and PA, which

Table 7. Diagnostic Values of the Identified Indicators.

Diagnostic values	Cutoff value[a]	Sensitivity%	Specificity%	PPV%	NPV%	AUC	AUC of 95% CI	*P*-value
PS + MPs, (count / μl)	1804.00	60.34	75.81	67.21	73.33	0.7245	0.6281 to 0.8208	<.0001
HMPs, (count / μl)	521.00	66.04	73.58	71.43	68.42	0.7319	0.6383 to 0.8256	<.0001
PS + HMPs, (count / μl)	5.00	90.57	77.36	80.00	89.13	0.8939	0.8326 to 0.9552	<.0001
PS + PMPs, (count / μl)	324.8	94.34	32.08	58.14	85.00	0.6440	0.5394 to 0.7486	.0106
D-dimer, mg / l FEU	1.835	92.45	32.08	57.65	80.95	0.5881	0.4775 to 0.6987	.1179
Hepatic Procoagulant Index	0.594	77.36	92.45	92.45	77.36	0.8978	0.8396 to 0.9561	<.0001

[a]All cutoff values were determined by the Youden index.

indicated that HMPs levels were linked to the coagulation function of the liver. Moreover, HMPs levels were inversely correlated with PT and INR, thus supporting the hypothesis that increased levels of HMPs were associated with the hypercoagulation state. To the best of our knowledge, this is the first study on the clinical application of HMPs in the diagnosis of posttraumatic DVT. Of note, we found the area under the ROC curve for PS + HMPs was 0.8939, yielding a discriminative performance superior to d-dimer (Table 6). Based on circulating concentrations of PS + HMPs, the Hepatic Procoagulant Index incorporated plasma levels of albumin. The area under the ROC curve for the Hepatic Procoagulant Index reached 0.9150 (Table 6). The assessment of DVT risk by the circulating PS + HMPs levels could assist clinicians in recognizing and evaluating the potential dangers of DVT in trauma patients. The detection of circulating HMPs is a robust and reliable parameter to assist in the diagnosis of DVT. This detection is simple and non-invasive to obtain samples, with a short detection time and low cost.

Previous studies of our group had found higher levels of plasmin–α2-antiplasmin complex and thrombin–antithrombin complex in posttraumatic DVT subjects.[43] These results were linked to enhanced fibrinolytic system activation and higher coagulation system activation. In addition, we also observed neutrophil extracellular traps,[44] and levels of complement 3 and complement 4 (unpublished) were increased in the DVT group rather than the non-DVT group. The previous data showed that increased HMPs induced by inflammatory activity, and HMPs quantity strongly correlated with disease severity. When hepatic stress and inflammatory state were relieved, the hepatic MPs level also showed the same downward trend.[45] In our study, the increased levels of HMPs may reflect the fact that trauma led to the release of a large number of inflammatory mediators, which resulted in the activation of hepatocytes and the generation of large amounts of hepatocyte HMPs and coagulant substances. In turn, HMPs carried cargoes of the mother cell into circulation and could be undertaken by other cells. Vascular endothelial cells easily engulfed extracellular vesicles in areas of low magnitude shear stress.[46] In previous studies, our team observed significantly increased levels of thrombomodulin and tissue factor pathway inhibitor in trauma patients with DVT.[47] In addition, the content of endothelial tissue factor MPs was increased significantly (results not published), which indicated endothelial dysfunction in trauma patients with DVT. Overall, the uptake of vesicles by endothelial cells with the concomitant occurrence of endothelial dysfunction and the role they play in DVT in trauma patients deserve further exploration.

However, this study has several limitations. First, the further clinical applications of PS + HMPs need to be validated in larger sample sizes, although flow cytometry is reliable for use in the clinical setting. Second, we only investigated PS + MPs, HMPs, and PMPs and ignored other subgroups. Besides, not all inflammatory markers could be detected due to the limitations of emergency samples. The present study was focused on the change in circulating MP concentrations. The mechanisms behind the dynamic change in MPs and the coagulation system still need to be explored in future studies and they may pave the path to assessing the dynamics of the coagulation system.

Conclusion

Collectively, trauma patients suffer a high risk of DVT. The circulating HMPs are sensitive markers to identify patients at higher risk of developing DVT. Collectively, trauma patients suffer a high risk of DVT. The circulating PS + HMPs are sensitive markers to identify patients at higher risk of developing DVT. The Hepatic Procoagulant Index can assist clinicians in the identification and diagnosis of DVT in trauma patients.

Highlights

- Compared to healthy individuals, the coagulation time was shorter while the d-dimer level was higher in trauma patients. Trauma patients with DVT had significantly higher levels of PS + MPs, PMPs, and HMPs compared to healthy controls and non-DVT patients.
- PS + HMPs and albumin were independent risk factors for preoperative DVT after trauma. Patients with DVT had a higher hepatic procoagulant index compared with trauma patients without DVT.
- There was positive correlation between PS + MPs and d-dimer and between HMPs and PA. Moreover, HMPs levels were inversely correlated with PT and INR.
- The circulating PS + HMPs are sensitive markers to identify patients at higher risk of developing DVT. The

Hepatic Procoagulant Index can assist clinicians in the identification and diagnosis of DVT in trauma patients.

Data Availability Statement

All data generated or analyzed during this study are included in this published article.

Declaration of Conflicting Interests

The authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Ethics Approval and Consent to Participate

The study was conducted following the Declaration of Helsinki, and approved by the Institutional Ethical Committee of the Beijing Jishuitan hospital.

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Supplemental Material

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