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Phosphatidylserine expressing platelet microparticle levels at hospital presentation are decreased in sepsis non-survivors and correlate with thrombocytopenia

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Abstract

Background: Sepsis induced platelet activation releases platelet microparticles (PMPs). PMPs express phosphatidylserine (PS) and can serve as a scaffold for the prothrombinase complex, thereby promoting coagulation. Studies of PMPs in intensive care unit sepsis patients demonstrate mixed results, while the earliest changes and potential effects of clinical interventions remain understudied. We hypothesized PMPs would be associated with patient outcome and dysfunctional coagulation shortly after emergency department presentation with sepsis.

Methods: Cohort study of patients from a single center enrolled in a previously published randomized control trial comparing two early resuscitation strategies. Adults presenting to the emergency department (ED) with suspected infection, ≥ 2 SIRS criteria, and either systolic blood pressure < 90 mmHg or lactate > 4 mmol/L were eligible. Triple positive platelet microparticles (PMPs) expressing phosphatidylserine and integrin complexes αIIb (CD41) and $\beta 3$ (CD61) were quantitated using plasma from the time of enrollment. The primary outcome was in-hospital mortality. Secondary outcomes included platelet count, disseminated intravascular coagulation (DIC), and prothrombin time (PT).

Results: 193 Patients were enrolled and 184 had samples available. In-hospital mortality was 21%. 10 (5%) patients developed DIC. Median platelet count was 197 (IQR 135, 280) and PT was 13.2 (IQR 11.9, 16.8). Median triple positive PMP counts were 932 per μL (IQR 381, 1872). PMPs were significantly lower in non-survivors (575 vs 1128, $p = 0.02$) and non-significantly lower in DIC (387 vs 942, $p = 0.17$). PMPs demonstrated a positive linear association with platelet

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Authorship

MAP conceived and designed the study, analyzed the data, wrote the manuscript

MM and RT conducted the assays and analyzed the data

DCC and SB designed the study

AEJ designed the study and provide the patient samples

All authors contributed to critical revision of the manuscript

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Disclosures

None

count ($p < 0.001$, $R^2 = 0.21$). After adjusting for platelet count, PMPs were no longer significant predictors of mortality ($p = 0.28$). We observed no association between PMPs and PT.

Conclusion: Similar to patients enrolled later in the intensive care unit, PS-expressing PMPs are lower in emergency department sepsis non-survivors. These changes primarily reflect the degree of thrombocytopenia, and an independent prognostic role was not observed. Future studies should control for platelet count in assessment of PMP prognosis in sepsis.

1. Introduction

Sepsis represents the leading cause of death in the emergency department, and a major driver of healthcare costs, representing the single most expensive inpatient condition across all payer types.(1) In-hospital mortality from sepsis exceeds 20% by most estimates,(2) rising to over 40% when shock is present.(3) Sepsis is differentiated from simple infections by a dysfunctional host immune response to the infectious agent which is often characterized by dysfunctional inflammation that drives organ failure.(4;5) Given the close linkage between the inflammatory and coagulation cascades, dysfunction of the clotting system is common and associated with poor outcomes.(6) Indeed, one of the most common and most recently advocated scoring systems for estimating sepsis severity (the sequential organ failure assessment or SOFA score) utilizes platelet count as a surrogate for coagulation system dysfunction.(7) In its most extreme form, this manifests as disseminated intravascular coagulation (DIC) with widespread formation of fibrin deposition, microcirculatory thrombosis, and consumption of coagulation factors leading to both thrombosis and bleeding.(8) While only a minority of patients manifest fulminant DIC, more subtle manifestations of microvascular coagulation can be observed in patients without DIC using sidestream darkfield video microscopy which can predict patient outcome.(9) Laboratory based markers also support the presence of non-overt DIC that is clinically occult, compounding the complexity of the diagnosis and treatment of the condition.(10) These complexities likely contribute to the largely disappointing clinical trials of anti-thrombotic strategies in sepsis to date.(11) These data indicate the importance of a better understanding of the entirety of the immune-coagulation spectrum across the continuum of sepsis cases, in order to better identify patients most likely to benefit from targeted therapeutics.

It is increasingly recognized that widespread platelet activation can lead to the release of biologically active platelet microparticles (PMPs) comprised of components of the surface membrane. This surface membrane is particularly relevant in the discussion of procoagulant phenotypes due to the significant presence of negatively charged phosphatidylserine residues that are normally maintained on the inside of living cells due to constituent activation of flippases that maintain membrane asymmetry.(12) Following cell death (or activation in the case of platelets(13)), these negatively charged phosphatidylserine residues are exposed to the surface, and can provide the scaffold for formation of the prothrombinase complex through the interaction with factors Va and X, and leading to drastically increased rates of thrombin activation. While many cell types can lead to the formation of microparticles which may have different biologic properties *in vivo*, platelet microparticles are of particular interest in sepsis.(14;15) The vast number of platelets in circulation and the massive shifts in platelet consumption that can develop during sepsis provide ample surface area for

prothrombinases complexes, with significant potential to drive fibrin production, increase coagulation, and worsen clinical outcomes. better understanding of these processes could be useful in driving novel sepsis therapeutics targeting the coagulation system.

While data generally agree that sepsis increases PMPs, the prognostic value and whether PMPs represent a pathologic or protective role in the intensive care unit are mixed.(16–20) Furthermore, samples from these studies were generally obtained later in the patient's course after hemodynamic stabilization, administration of antibiotics, and other clinical interventions that have the potential to affect thromboinflammation. In this study, we wished to examine the prognostic role of PMPs at the earliest stages of hospital presentation in order to shed light on changes during the most proximal time periods following presentation. We hypothesized that PMPs would be altered in patients with sepsis at emergency department presentation, in patients who developed disseminated intravascular coagulation, and would be associated with laboratory evidence of dysfunctional coagulation including platelet count and prothrombin time.

2. Materials and Methods

2.1 Study population

Patients from a single emergency department participating in a previously published multicenter clinical trial conducted between 2007-2009 comparing two early resuscitation strategies for severe sepsis were enrolled. The study was IRB approved and all patients or their surrogates gave informed consent. Inclusion criteria included age > 17 years, 2 or more systemic inflammatory response criteria, and *either* hypotension following a fluid challenge *or* lactate > 4 mmol/L. Exclusion criteria included an absolute contraindication to central venous line placement, requirement for emergency surgical intervention, or inability of the patient or a surrogate to provide informed consent. Median time from hospital presentation to study entry was approximately 2 hours,(21) indicating early enrollment. All patients underwent standardized quantitative resuscitation based on iterative steps of intravenous crystalloid, vasopressors, inotropes, and/or packed red blood cells to achieve central venous pressure, mean arterial pressure, and either Scvo2 70% or lactate clearance goals based on treatment allocation. The protocol was continued until all end points were achieved or a maximum of 6 hours. The study showed a 6% (95% CI, -3% to 14%) in-hospital mortality difference favoring the lactate clearance group, confirming non-inferiority of the resuscitation strategy.(22)

2.2 Clinical and laboratory measurements and outcomes

Data were prospectively collected starting at patient enrollment, including patient demographics and clinical characteristics, hemodynamic parameters, clinical interventions, and in-hospital mortality. Clinical laboratory results performed in the emergency department (including platelet count and prothrombin time) were recorded, and the value at the closest time point to enrollment was used for the purposes of this analysis. The coagulation component of the sequential organ failure assessment (SOFA) score was calculated for each patient and assigned a number based on the scoring system (0 for platelet count >150 cells/mm³, 1 for 100-149 cells/mm³, 2 for 50-99 cells/mm³, 3 for 20-49 cells/mm³, and 4 for

<20 cells/mm³). Common sepsis-related interventions (including volume of intravenous fluid and antibiotic administration) and timing relative to enrollment and blood draw were recorded. Per the study protocol, patients were considered to meet the diagnosis of disseminated intravascular coagulation if the clinical team documented the diagnosis at any time during the patient's hospitalization.

2.3 Blood collection and processing

Plasma was gently drawn into EDTA tubes via a preexisting central venous line after a waste and placed immediately on ice and immediately taken for sample processing, typically <30 minutes. Plasma was centrifuged at 3,000g at 4 °C for 10 minutes. Aliquots of the relatively cell-free supernatant were stored at -80 °C without any additional freeze-thaw cycles until the time of measurement. On the day of measurement, samples were thawed to room temperature and centrifuged at 13,000g for 2 minutes. The resultant supernatant was centrifuged at 20,000g for 20 minutes at 4 °C to pellet the microparticles (MPs). MPs were washed with PBS buffer and then resuspended in PBS for analysis.

2.4 Antibody labeling of microparticles

The resuspended microparticles were separated into 3 different preparations, each performed in triplicate: no added antibodies, IgG isotype control antibodies, and a combination of FITC-Annexin V [to detect phosphatidylserine (PS)] and PE-anti-CD61 (integrin complex beta3) and APC-anti-CD41 (integrin complex alphaIIb) to detect MPs of platelet origin. Antibodies were obtained from Beckman Coulter (Brea, CA). Platelet MPs were defined as triple-positive MPs (Annexin-V⁺/CD61⁺/CD41⁺). A representative histogram and gating strategy is provided in Figure 1. Immediately following incubation in a light-free environment, 100 µL of AccuCheck Counting Beads (Life Technologies, Frederick, MD) were manually mixed thoroughly and added just before acquisition on the flow cytometer.

2.5 Flow cytometry and platelet microparticle quantitation

Single staining controls and control IgG antibodies were used to establish fluorescence compensation settings and to set up positive regions using a Gallios 6-color flow cytometer (Beckman Coulter; Indianapolis, IN). Forward and side scatter parameters were plotted on logarithmic scales to best cover a wide size range. The MP-gate was determined by bead diameters with MPs being defined as particles < 1.0 µm in size. Following manufacturer instructions, comparisons of 2 types of fluorescent beads (low and high intensity for FL2) were performed and confirmed to be within the allowed variance of the assay. Total bead quantitation and quality control are illustrated in Figure 2. A minimum of 10,000 microspheres were read and PMP concentrations were calculated in accordance with the numbers of counting beads using the equation provided by the manufacturer [Absolute Count (PMPs/µL) = (number of events counted / total number of beads counted) × number of AccuCheck Counting Beads per µL]. The average of the three calculated PMP counts performed in triplicate were used for subsequent statistical analyses.

2.6 Statistical analysis

Clinical data were described and compared between groups using simple descriptive statistics, including chi-square, Fisher exact, Student's t-test, and Mann-Whitney-U, as appropriate. Patients were divided into groups based on clinical variables of interest and compared. Given a highly non-normal distribution, PMP data were log transformed for further statistical analysis. PMP data in the manuscript and tables are presented as concentrations for the purposes of data interpretation, but statistical comparisons (and reported p-values) were made using log-transformed data and t-tests.

In order to determine the relationship between platelet count and PS-expressing PMPs, a linear regression model using platelet count as the dependent variable and PMPs as the independent variable was constructed. Association between intravenous fluid volume prior to enrollment and PMPs were similarly assessed. Finally, to determine the independent prognostic value of PMPs compared to platelet count in the prediction of in-hospital survival, both variables were entered into a logistic regression model, with and without an interaction term. The same analysis strategy was performed to determine the association of PMPs to laboratory prothrombin time. Given our unexpected observations, we conducted a post-hoc analysis of any platelet microparticles (CD61⁺/CD41⁺) and any phosphatidylserine expressing microparticles (Annexin-V⁺) using the same analysis strategy.

Flow cytometry data were analyzed using manufacturer provided software (Kaluza 1.33; Beckman Coulter, Indianapolis IN). Data were analyzed using STATA 15.0 (StataCorp; College Station, TX). All tests were two-sided and p-values of <0.05 were considered significant.

3. Results

3.1 Clinical and laboratory characteristics of the cohort

Of the 300 patients enrolled in the parent trial, 193 were enrolled at the site and 184 had samples available for analysis. Consistent with the parent trial, patients with available samples were older (59 ± 18 years), severely ill with a median SOFA score of 7 (IQR 4, 9), and in-hospital mortality of 21%, consistent with ED based studies of early sepsis resuscitation. Patient demographics and clinical characteristics of the cohort are provided in Table 1. Median platelet count was 197 (135, 280) while the percentage of patients exhibiting coagulation SOFA scores of 0-4 at enrollment were 70.1%, 16.9%, 7.6%, 3.8%, and 1.6%, respectively. Emergency department prothrombin times were measured in 113 patients (61%) and demonstrated median values of 13.2 (IQR 11.9, 16.8). Of the cohort, 10 patients (5%) received a clinical diagnosis of disseminated intravascular coagulation.

3.2 No difference in platelet microparticles between clinical subgroups

Median platelet microparticle counts were 932 PMPs / μ L (IQR 381, 1871). PMPs did not differ significantly by demographics (age, gender, race, or ethnicity), past medical history, or source of infection. PMPs observed within these clinical groups are presented in Table 2.

3.3 Pre-enrollment interventions were not associated with differences in PMPs

Of the 184 patients, 80 received antibiotics prior to blood draw. PMPs did not significantly differ in patients receiving antibiotics prior to the blood draw. Of the 120 (62%) patients in this cohort receiving emergency department vasopressors, 27 (23%) were treated prior to blood draw, and PMPs similarly did not differ significantly between groups (Table 2). Finally, median intravenous fluid volume administered prior to enrollment and blood draw was 2 L (IQR 1.5, 2.9). We observed no relationship between pre-enrollment fluid administration and PMPs either visually or by linear regression ($p = 0.99$).

3.4 Relationship between PMPs, organ failure, and survival

We observed no association between PMP and the total emergency department SOFA score ($p = 0.71$), nor any individual organ failure score (respiratory, cardiovascular, renal, liver, or central nervous system) with the exception of the coagulation score which was inversely related to PMPs. Patients with the least amount of coagulation system dysfunction as assessed by platelet count had the highest levels of PMPs at 1215 (IQR 632, 2361), as compared to 32 (IQR 25, 132) in those with the highest coagulation system dysfunction.

PMPs were significantly lower among patients suffering in-hospital mortality as illustrated in Figure 3 [575 (IQR 205, 1154) vs 1128 (IQR 464, 2265), $p = 0.02$]. PMPs were non-significantly lower in patients with DIC [387 (IQR 190, 1841) vs 942 (IQR 418, 1884), $p = 0.17$], likely reflecting a relatively low incidence of this clinical outcome ($n = 10$, 5%).

3.5 Relationship between PMPs, platelet count, prothrombin time, and liver disease

We observed no significant linear relationship between PMP and prothrombin time at enrollment ($p = 0.74$), and visual inspection did not suggest an alternative (U-shaped, logarithmic, or other) relationship between the variables. PMPs demonstrated a significant positive linear relationship with platelet count (Figure 4, $p < 0.001$, $R^2 = 0.21$). Platelet counts were significantly lower in patients who died [166 (IQR 63, 229) vs. 208 (IQR 149, 295); $p = 0.01$]. While both platelet count and PMPs were significant predictors of mortality ($p = 0.01$, $p = 0.03$) in univariate models, these lost significance in multiple logistic regression modeling ($p = 0.06$ and 0.28 , respectively). As expected, platelet counts were lower in the small number of patients with liver failure [$n = 12$; 142 (IQR 120, 192) vs 210 (IQR 139, 294); $p = 0.01$]. However, addition of this risk factor did not significantly alter the multivariate model.

3.6 Microparticle subpopulations

We subsequently analyzed whether different subpopulations of microparticles demonstrated different results. We repeated the analysis of in-hospital mortality using levels of either platelet microparticles (regardless of PS expression) or phosphatidylserine expressing microparticles (regardless of integrin expression). Both groups were significant predictors of mortality under a univariate analysis ($p < 0.01$ and $p = 0.03$), though failed to demonstrate independent predictive significance when platelet count or total SOFA score were added to the models, consistent with our primary analysis (data not shown).

4. Discussion

In this study, we enrolled and measured PS expressing platelet microparticles in 184 emergency department patients with sepsis very shortly after hospital presentation and gathered detailed clinical data regarding their course and survival. We observed no significant differences based on underlying patient demographics or comorbidities, making these unlikely confounders in our study. We observed PMPs at hospital presentation were significant predictors of mortality but upon further analysis, we observed that PMPs were highly associated with platelet count. After multivariate modeling, PMPs were no longer a significant independent predictor of mortality. These data suggest that early after hospital presentation, low platelet microparticles primarily represent a surrogate marker of thrombocytopenia rather than an independent predictor of mortality. In order to determine the independent role of PMPs in predicting and potentially modulating sepsis outcomes, future studies should consider normalizing values to platelet count.

One of the strengths of this study was its proximal enrollment of patients, with a median time to enrollment from hospital presentation of only 2 hours, providing insight into the changes during the most proximal time period. This is important, as the most successful sepsis interventions to date have been most effective when deployed in this time frame before the clinical trajectory becomes more difficult to impact. Our analysis was also unique in that we were able to assess the potential effect of certain sepsis clinical interventions that could impact levels of circulating PMPs and add heterogeneity to the results. Early enrollment allowed minimization of these clinical interventions and procedures, such as intravenous fluid resuscitation that contributes to hemodilution or bacterial lysis through the administration of bactericidal antibiotics, which can promote lipopolysaccharide release and potentially thromboinflammation. While the number and complexity of interventions were minimized by the early enrollment window, they were not eliminated as sepsis is a time sensitive condition with multiple treatments initiated early upon patient presentation. However, in our analysis we observed no evidence to suggest these interventions were associated with significant differences in measured levels of PMPs.

Putting this work into perspective within the literature, while data seem to agree that sepsis increases levels of circulating PMPs,(23;24) their role in pathogenesis, organ failure, and survival remains unclear with conflicting reports. One study demonstrated increased PMPs associated with organ failure (specifically renal injury(25)), while another observed increased PS expressing microparticles in septic shock non-survivors(26). However, other studies have suggested a potential protective role of PMPs. One study administered PMPs from patients with sepsis into a preclinical model to demonstrate a role in the maintenance of vascular function,(27) while other clinical studies suggest higher PMPs are associated with reduced mortality and lower organ failure(28) and are diminished in patients with acute respiratory distress syndrome(29). It has been hypothesized that these changes occur early, but data supporting that hypothesis are lacking and represent a gap addressed by this report. This study is relatively large compared to others in the literature, and provides evidence to support the studies that suggest elevated PMPs in sepsis represent a favorable prognostic marker. Furthermore, it seems that these changes occur early in the disease course and do not appear to result from clinical interventions administered to the patient. However, given

the broad range of levels demonstrated in Figure 3, there exists significant overlap between groups. Moreover, the prognostic value of these PMPs was eliminated after normalizing for platelet count, leaving questions regarding whether PMPs play a unique role in sepsis pathophysiology, or are merely a complex surrogate marker of platelet homeostasis. There are several weaknesses of this study that deserve consideration. While we assessed the effect of both PS and non-PS expressing platelet microparticles, as well as PS expressing microparticles in general, there exists considerable diversity in the makeup of microparticles, (30) potentially with unique biologic effects and different prognostic value. Second, we utilized rudimentary clinical assessments of coagulation including platelet count and prothrombin time, and alternative measures such as thrombin generation or thromboelastography might have detected associations between PMPs and changes in coagulation profile that we did not observe in this relatively large, but pragmatic study. As a result, we cannot comment on the changes in non-overt or clinically occult DIC. Third, it is possible that platelet PMPs have independent prognostic potential, but that our study was underpowered to detect it. While this remains a possibility, the clinical significance of such a finding is unclear in such a heterogeneous population, and future studies might consider addressing this question in more homogenous cohorts of sepsis patients (stratified by DIC score(31)). Similarly, several subpopulations, such as those with liver disease, were small and it remains a possibility that the role of PMPs is different in certain cohorts of patients. Finally, it could be that patient cohorts enrolled in the emergency department and intensive care unit represent overlapping, but phenotypically distinct populations of patients with sepsis. Subsequent longitudinal studies of PMPs throughout the clinical course of sepsis, with attention to these various factors would be desirable to address these various possibilities.

5. Conclusion

Phosphatidylserine-expressing platelet microparticles are lower in emergency department sepsis non-survivors, though this primarily reflects thrombocytopenia and was not an independent predictor of mortality in this study. Future studies of PMPs in sepsis should consider longitudinal study design and normalization for platelet count in interpretation.

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Highlights

- PS-expressing PMPs were measured in sepsis patients shortly after presentation
- Platelet microparticles were decreased in patients who died in-hospital
- PMP counts were highly associated with platelet count
- PMPs were not independent predictors of death after adjusting for platelet count

Figure 1: Gating strategy employed for the detection of phosphatidylserine expressing platelet microparticles using a representative patient sample. CD61+/CD41+/Annexin V+ microparticles were quantitated, and an average of assays performed in triplicate was used for subsequent data analyses. CB – counting beads (see Figure 2).

Figure 2:

Illustration of the method of platelet microparticle quantitation using a representative patient sample. Absolute number of CD61+/CD41+/Annexin V+ microparticles (based on the gating strategy in Figure 1) were quantitated using the following formula: [number of triple positive events counted (25,373) / total number of type A + type B beads counted (4,704 + 5,570)] × (991) = 2,247 PMPs/μL. Quality control of the percentage of Type A (low intensity FL2) versus type B (high intensity FL2) beads was verified.

Figure 3: Boxplots demonstrating PS+/CD41+/CD61+ platelet microparticles in non-survivors as compared to survivors (*: $p = 0.02$).

Figure 4:
Relationship between enrollment platelet count and platelet microparticle count ($R=0.21$, $p<0.001$).

Table 1 -

Baseline demographics and clinical characteristics of the cohort.

	<i>Cohort n=184</i>
Age; years (SD)	59 (18)
Sex; n, (%)	
Male	96 (52)
Female	88 (48)
Race n, (%)	
White	103 (56)
African American	78 (42)
Asian	2 (1)
Other	1 (1)
Ethnicity; n, (%)	
Hispanic	12 (7)
Non-Hispanic	172 (93)
Past medical history; n, (%)	
Hypertension	103 (56)
Diabetes mellitus	58 (32)
Congestive heart failure	25 (14)
Chronic renal insufficiency	31 (17)
Liver disease	12 (7)
Source; n (%)	
Pneumonia	56 (30)
Urinary	53 (29)
Intraabdominal	30 (16)
Skin / Soft tissue	15 (8)
Surgical wound	6 (3)
Labs (enrollment)	
White blood count; cells/mm ³ (IQR)	12.4 (7.8, 17.5)
Platelets; cells/mm ³ (IQR)	197 (135, 280)
Creatinine; mg/dL (IQR)	1.8 (1.2, 2.9)
Total Bilirubin; mg/dL (IQR)	1.1 (0.7, 1.8)
Prothrombin time (PT), seconds	13.2 (11.9, 16.8)
Severity of illness	
SOFA score; n (IQR)	7 (4, 9)
Lactate; mmol/L (IQR)	3.6 (1.8, 6.3)
Outcomes; n (%)	
Disseminated intravascular coagulation	10 (5)

	<i>Cohort n=184</i>
In-hospital mortality; n (%)	36 (21)

* IQR-Interquartile range

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Table 2 -

Comparison of PS+ platelet microparticles between various clinical subgroups.

	<i>PS+ platelet microparticles / μL (IQR)</i>		<i>p-value</i>
	Present	Absent	
Past medical History			
Hypertension	907 (378, 1745)	1128 (382, 2052)	0.76
Diabetes mellitus	1025 (481, 1899)	912 (371, 1859)	0.69
Congestive heart failure	725 (308, 1154)	1025 (382, 2052)	0.38
Chronic renal insufficiency	614 (378, 1725)	949 (387, 1884)	0.86
Liver disease	520 (348, 1272)	981 (385, 1892)	0.65
Source			
Pneumonia	1032 (416, 2353)	906 (379, 1849)	0.26
Urinary tract infection	905 (374, 1745)	949 (418, 2146)	0.94
Skin / soft tissue infection	1595 (583, 2765)	921 (378, 1838)	0.47
Intraabdominal	976 (418, 2408)	933 (379, 1841)	0.49
Surgical wound	1741 (707, 3070)	926 (379, 1841)	0.30
Interventions prior to blood draw			
Antibiotics	879 (366, 2159)	987 (446, 1715)	0.18
Vasopressors	919 (583, 1737)	906 (341, 2283)	0.76
Outcomes			
Disseminated intravascular coagulation	387 (190, 1841)	942 (418, 1884)	0.17
In-hospital survival	1128 (464, 2265)	575 (205, 1154)	0.02

* IQR-Interquartile range