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Platelet-Derived Microparticles and their Role in Sepsis

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PLATELET-DERIVED MICROPARTICLES AND THEIR ROLE IN
SEPSIS

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A thesis submitted to the faculty of The University of Mississippi in partial fulfillment of
the requirements of the Sally McDonnell Barksdale Honors College

University of Mississippi

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ABSTRACT

Causing at least 215,000 deaths in the U.S. annually- more than prostate cancer, breast cancer, and AIDS combined- and rising, sepsis is one of the most deadly medical conditions in America. Sepsis pathophysiology, however, is still incompletely understood, making it difficult for physicians to diagnosis and treat effectively. Given the time-sensitivity of treatment, researchers are now searching for new methods to identify sepsis early, developing more accurate prognostic markers, and developing new target therapeutic candidates. One biological component of interest in this research is the platelet-derived microparticle (PMP), a heterogeneous vesicle derived from the cell membrane of platelets found circulating in blood plasma. In theory, as platelets are destroyed in sepsis, PMP levels in plasma could increase and thus play an important physiological role in the cascading deregulation of the clotting mechanism characteristic of sepsis. Furthermore, because of these possible roles in sepsis, PMP concentration presents a parameter of possible prognostic value. Data collected and analyzed to date in the context of this thesis (79 of 182 sepsis patients), a correlation between PMP level and sepsis severity was observed, but in an opposite manner than expected. Rather than increasing with sepsis severity, PMP levels actually seem to decrease as the disease progresses. Additionally, PMP levels strongly parallel platelet count. These observations suggest these two markers (platelet count and PMPs) might be two different measures of the same underlying physiologic process. However, definite conclusions cannot be made until analysis of the remaining patients enrolled in this study has been completed.

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CHAPTER 1

SEPSIS

1.1 Sepsis Overview

In sepsis, an infectious insult leads to systemic inflammatory response syndrome (SIRS), an acute immune response resulting in inflammation of the entire body. Sepsis has an extremely high mortality rate of approximately 30%, depending on the definition and cohort studied, and accounts for at least 215,000 deaths per year in the United States alone. Most deaths can be attributed to the over-response and deregulation of the immune system rather than to the initial infection itself.¹³ Given the close relationship and cross-talk between the immune and coagulation systems, widespread activation of the coagulation system is common in sepsis and may serve not only as a pathophysiologic response but also a therapeutic target.

1.2 Thrombosis in Sepsis

During sepsis, clotting cascades stop functioning properly. Many pro- and anti-inflammatory cytokines are released from mononuclear and endothelial cells.¹⁴

Thrombosis occurs in later stages. Plasminogen stimulation and antithrombin-III activation take place in the fibrinolytic system, depleting fibrinolytic and fibrinogen substances.¹⁴ Coagulation abnormalities may manifest in varying degrees of severity

from minor changes in coagulation processes to wide spread microvascular thrombosis and disseminated intravascular coagulation (DIC). DIC, which simultaneously causes deleterious clotting and bleeding, results in increased platelet destruction.¹⁴ The simultaneous occurrence of aberrant coagulation in some organs and bleeding in others significantly complicates sepsis treatment, as therapeutic measures may help complications in some organs while exacerbating problems in others. If DIC becomes severe, deep vein thrombosis in various organs will result in multiple organ dysfunction syndrome (MODS), the prevailing signature of death in sepsis cases.¹⁴

1.3 Sepsis Diagnosis

One reason for the high mortality rate of sepsis is the difficulty of early diagnosis for critical care physicians. Sepsis is generally diagnosed in patients with confirmed or presumed infection and two or more of the following criteria for SIRS: (1) a body temperature greater than 38°C or less than 36°C; (2) a heart rate greater than 90 beats per minute; (3) tachypnea, manifested by a respiratory rate greater than 20 breaths per minute, or hyperventilation, as indicated by a PaCO₂ of less than 32 mm Hg; and (4) an alteration in the white blood cell count, such as a count greater than 12,000/cu mm, a count less than 4,000/cu mm, or the presence of more than 10 percent immature neutrophils (“bands”).¹⁵ Furthermore, *severe sepsis* is defined as sepsis associated with organ dysfunction, hypoperfusion abnormality, or sepsis-induced hypotension. Hypoperfusion abnormalities include lactic acidosis, oliguria, and acute alteration of mental status.¹⁵ Characteristics like hypoperfusion and high blood lactate concentrations are indicative of organ dysfunction, as both imply low oxygen delivery to organ systems.

Critical care physicians often utilize the Sequential Organ Failure Assessment (SOFA) score, which is a simple and objective score allowing for calculation of both the number and the severity of organ dysfunction in six organ systems (respiratory, coagulatory, liver, cardiovascular, renal, and neurologic).¹⁶ For the coagulatory system score, low levels of circulating platelets, also known as thrombocytopenia, corresponds to a high SOFA score, which indicates a higher likelihood of organ dysfunction. Once sepsis-induced abnormalities like hypotension can no longer be improved by infusing fluids, the patient's condition may be diagnosed as *septic shock*.¹⁷ Since most sepsis-related deaths are the result of DIC and subsequent MODS, improving current methods or finding novel ways of recognizing thrombocyte abnormalities and organ dysfunction earlier provide an important step in increasing successful sepsis treatment.

CHAPTER 2

PLATELET-DERIVED MICROPARTICLES AND THEIR POSSIBLE ROLE IN SEPSIS

2.1 Microparticle Overview

Microparticles (MPs) are a heterogeneous population of extracellular vesicles, ranging from 50 to 1000 nm, that make up a ubiquitous component of most- if not all- multicellular organisms. MPs are generally derived from the plasma membrane of eukaryotic cells, including thrombocytes, leukocytes, endothelial cells, erythrocytes, adipocytes, and various cells of the central nervous system.¹ MPs may contain many different components of the parent cell from which they originate.

2.2 Platelet-derived Microparticle Overview and Biochemistry

Platelet-derived microparticles (PMPs) are generated from the plasma membrane of thrombocytes (platelets) upon platelet activation by various physiological agonists such as collagen, thrombin, the complement membrane attack complex C5b-9, lipopolysaccharide, immune complexes, and viruses.¹⁻⁴ Apoptotic platelets also release PMP.⁵ PMP circulate freely in blood plasma, making up the most significant portion of circulating MPs. Depending on the parent cell from which they originate, PMPs may contain a wide variety of bioactive substances, membrane-anchored receptors and

adhesion molecules on their surface, allowing specific interaction and crosstalk with various target cells. Important biological molecules found in PMP include specific membrane adhesion proteins like P-selectins and integrins, tissue factor (TF) and other functional effectors that can regulate a number of processes- aggregation, adhesion, molecule expression, cell proliferation, apoptosis, endothelial migration- cytokines, and growth factors.⁶ Of most interest to the current study, however, externalization of phosphatidylserine (PS), which occurs upon platelet activation and/or platelet apoptosis, provides an efficient platform for the assembly of blood coagulation enzymes such as thrombin via the thrombin activation complex. Thrombin converts fibrinogen to fibrin, culminating in the activation of the coagulation cascade.⁶ The presence of phosphatidylserine in PMPs has been shown to induce more thrombin generation than phosphatidylserine alone, suggesting that PMPs enhance the procoagulatory response.⁷ The presence of these molecules give PMPs the potential to closely parallel platelets in immune and hemostatic function; however, their smaller size and significantly larger count in circulating blood plasma imply that they could have a much more significant effect than is currently recognized.

2.3 Physiological Function

The primary physiological role of platelets is to stop bleeding by coagulating at blood vessel breaks in a process called hemostasis. The clotting mechanism occurs in three steps: adhesion to molecules outside the opening, activation of chemical messengers and receptors and a cellular conformational change, and aggregation of cells through receptor bridges.⁸ In some cases, deleterious clotting can be induced in healthy blood

vessels, which may lead to obstructed blood flow. This type of aberrant coagulation is called thrombosis, and is responsible for clinical conditions such as myocardial infarction (heart attack) and cerebrovascular accidents (strokes). If blood flow is obstructed enough or is completely blocked, tissue damage may occur. Severe tissue damage can result in the failure of entire organ systems and even death. The broad importance of better understanding of thrombotic processes broadened the implications of this line of research. The analogous biochemical composition of PMPs suggests that PMP may also play a role in the coagulation cascade- both normal and aberrant- however, the degree of their effect is not well understood.

2.4 PMP Protocols and Flow Cytometry

In order to study PMPs, they must first be isolated. Due to their analogous biochemical composition, PMPs must specifically be separated from platelets for any accurate results to be possible. To achieve this separation, researchers may exploit the difference in size between PMPs and platelets through a series of centrifugations.⁹ Once isolated the research on PMPs could go in a number of directions; however, for this study, quantification of PMPs is the next step. Methods of MP quantification have been established using flow cytometry, and PMPs may be detected specifically by binding with fluorescent antibodies to platelet-specific cell markers.^{7,9-11} Antibodies to specific cell markers are conjugated to phycobiliproteins like phycoerythrin (PE) and allophycocyanin (APC) to achieve fluorescence. Furthermore, PMPs specifically expressing procoagulant activity may be quantified by also labeling them with fluorescently labeled Annexin V to detect phosphatidylserine exposure.⁷

Phosphatidylserine exposure is helps in the identification of procoagulant PMPs because it only is expressed on the outer surface of the plasma membrane in activated or apoptotic platelets.⁶⁻⁷ In order to avoid false positive detection by the flow cytometer, an appropriate negative control is utilized.¹¹ Following appropriate fluorescent labeling protocol, the flow cytometer can accurately detect PMP concentration in a sample. Flow cytometry works by streaming cells individually through a laser and recording the different wavelengths of scattered light emitted by the cell (**Figure 1.1**).¹² The fluorescent molecules of labeled PMPs are excited to a higher energy state by the laser, giving off distinct wavelengths of light as they return to their resting energy state.¹² Thus, by gating the flow cytometric data to wavelengths specific to the fluorescent platelet cell markers and Annexin V, the number of PMPs per unit of volume can be determined, yielding a PMP concentration for the sample.

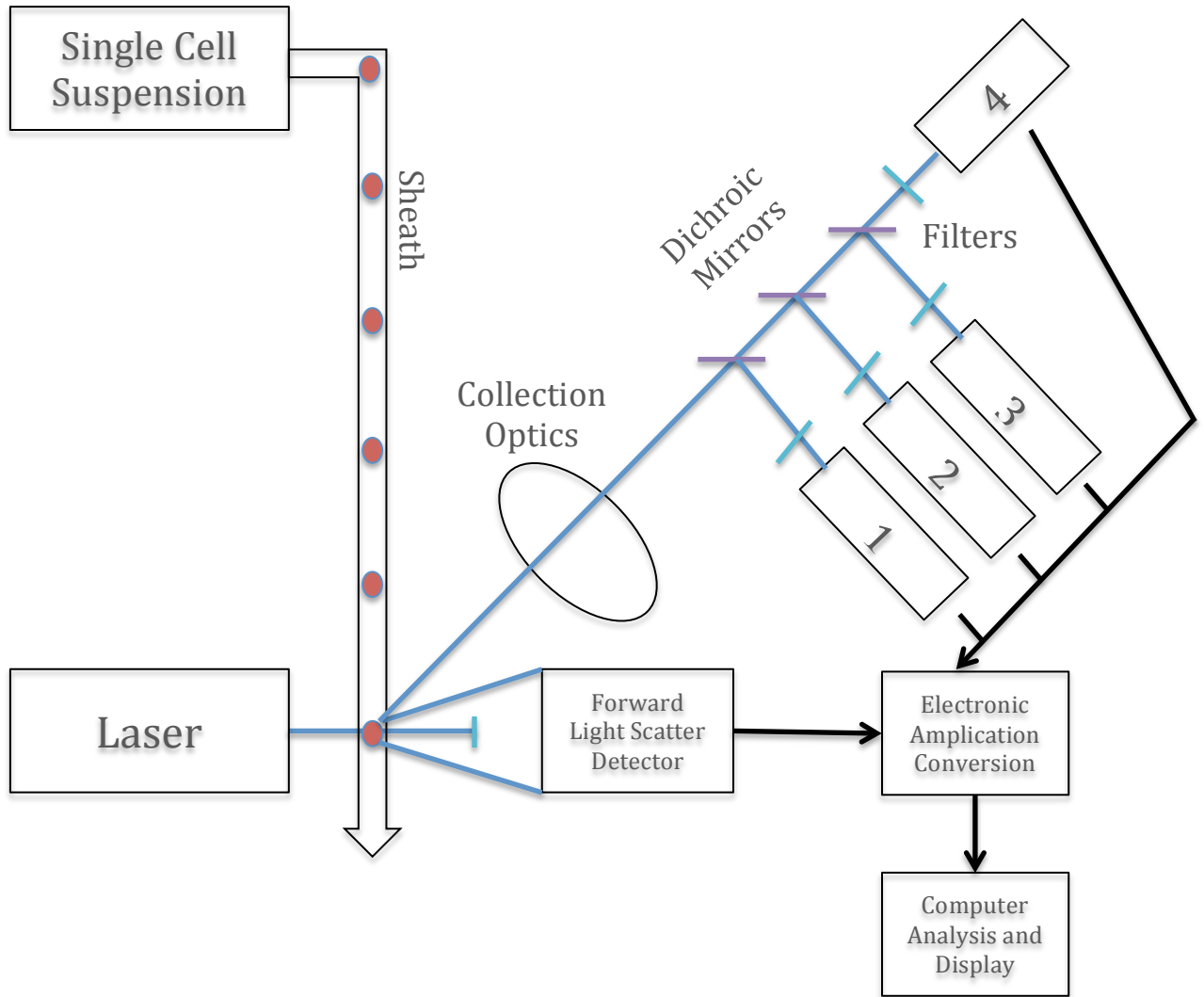


Figure 1.1 Schematic of a Flow Cytometer

2.5 Pathophysiological Role of Platelets in Sepsis

Sepsis decreases circulating platelets' hemostatic function, maintains adhesion molecule expression and secretion capability, and modulates growth factor production, suggesting that sepsis alters the hemostatic function of the platelets and increases vascular endothelial growth factor (VEGF) release in a thrombin-independent manner.¹⁸ As platelets' normal hemostatic function decreases, the number of circulating platelets also decreases as their breakdown and consumption rapidly increases. In severe sepsis cases this may manifest as DIC, leading to MODS. With platelets being increasingly broken down and consumed, it seems intuitive that severe sepsis could cause an increase in PMPs, which may exacerbate the deregulation of the coagulatory system, leading to a vicious cycle.

2.6 Possible Prognostic Value of PMPs

As sepsis deregulates the coagulatory system, resulting in the increased break down of platelets by apoptosis and macrophage consumption, we believe that PMP concentration in blood plasma may increase as they are released from platelets. This change in platelet to PMP ratio could have a significant effect on the clinical manifestation of the disease and therefore presents possible prognostic value for critical care physicians. In order to test our hypothesis that sepsis causes an increase in PMP count and the idea of PMPs being a novel, useful parameter of diagnostic and therapeutic value in severe sepsis, we first quantified PMP concentrations in severe sepsis patients and compared them to the degree of disease severity and a number of other sepsis-related clinical factors, including adverse clinical outcomes and factors, including past medical

history and demographic characteristics that may predispose a patient to developing significant complications. A significant association to any of these adverse clinical outcomes or patient death could suggest a novel target for future therapeutics, and associations to the other factors could reveal which patients are most at risk of developing complications related to sepsis-induced changes in PMP concentration.

CHAPTER 3

METHODS

3.1 Study Design and Setting

This is a secondary analysis of blood samples collected as part of a prospective, randomized clinical trial compared two resuscitation strategies for the early emergency department treatment of severe sepsis. The results of this study have presented published previously, and demonstrated non-inferiority of a lactate guided resuscitation strategy compared to central venous oxygen guided resuscitation.¹⁶ Patients admitted to the Emergency Department of Carolinas Medical Center with severe sepsis or septic shock were assessed for inclusion, which required that patients be older than 17 years with confirmed or presumed infection. The criteria for exclusion from the study were pregnancy, any primary diagnosis other than sepsis, suspected requirement for immediate surgery within 6 hours of diagnosis, an absolute contraindication to chest or neck central venous catheterization, cardiopulmonary resuscitation, transfer from another institution with a sepsis-specific resuscitative therapy underway, and advanced directive orders that would restrict the study procedure. Additionally, patients were excluded from this secondary analysis if no samples were available for analysis. Using a 24-hour day, 7-day-week method that was previously established for the routine clinical care of sepsis patients at each of the participating institutions, an alert was sent to inform clinical care

resources when patients were identified as candidates. Each enrolled patient or the patient's legally authorized next of kin provided written informed consent prior to collection of data.¹⁹ All consented to give blood for study and further use. Patient plasma samples were processed via immediate centrifugation, aliquoted, and frozen at -80 °C, transferred to the Emergency Department lab at the University of Mississippi Medical Center on dry ice, and kept frozen until the time of analysis. 79 out of the 182 patients enrolled in the study have been analyzed so far.

3.2 Definitions

For this study, patients were assessed for inclusion if they were older than 17 years of age and fit the study's definition for severe sepsis or septic shock. This included confirmed or presumed infection, having 2 or more systemic inflammatory response criteria,²⁰ and having hypoperfusion evidenced by either a systolic blood pressure lower than 90 mm Hg after a minimum of 20 mL/kg rapid volume challenge or a blood lactate concentration of at least 36 mg/dL (4 mmol/L).¹⁹

3.3 Demographic and Clinical Factors

Demographic characteristics, such as age and gender, and clinical factors, such as source of infection (pneumonia, UTI, surgical wound), past medical history (diabetes, liver disease, hypertension, CHF, malignancy, transplant), and clinical presentation (presence or absence of hypotension, lactate elevation), were obtained from patient files.

3.4 Methods of Isolation

Previously frozen patient plasma was thawed at room temperature and centrifuged at 13,000 g for 2 min. Microparticles (MPs) were isolated by centrifuging the resulting supernatant at 20,000 g for 20 min at 4°C. The resulting MP pellet was washed twice with PBS before being resuspended in 550 µL of PBS.

3.5 Preparation For Quantification By Flow Cytometry

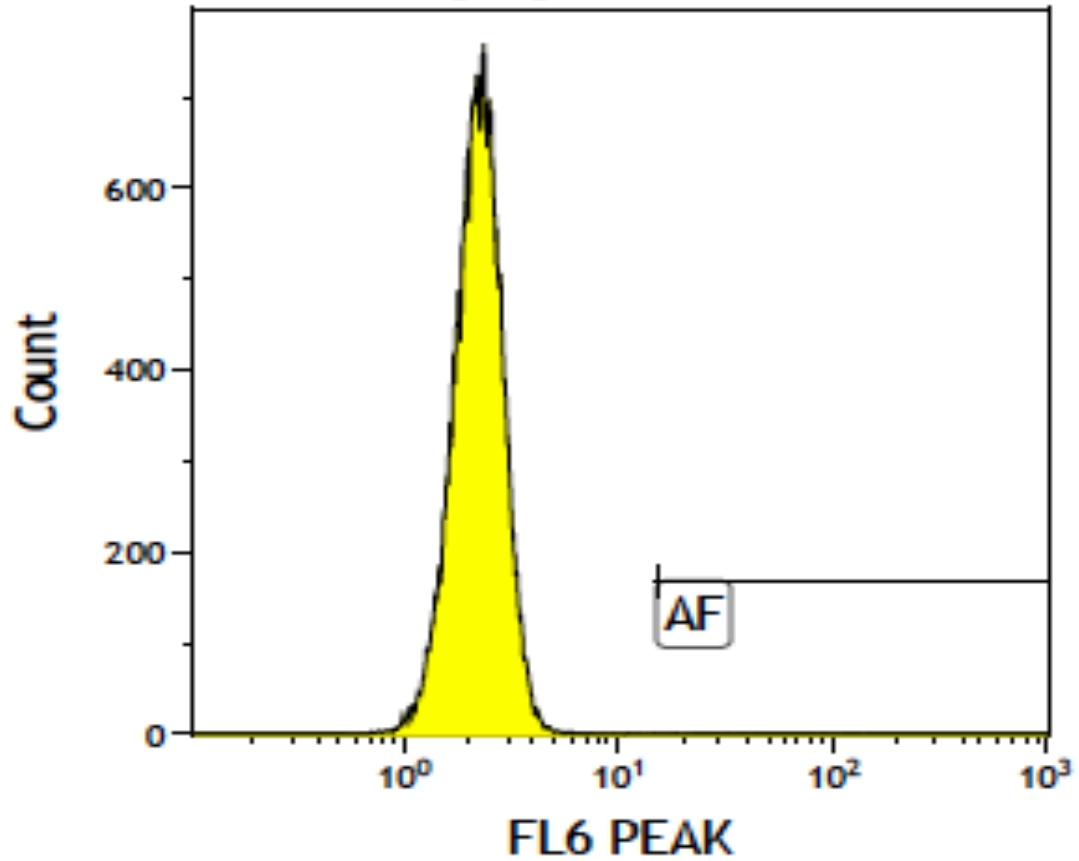
The isolated MPs were fluorescently stained with specific antibodies in order to quantify the PMP level of each patient's plasma. 50 µL of suspended MPs were added to three test tubes that would have no antibodies, control antibodies, and a triple-positive mix of antibodies added, respectively, in order of flow cytometer acquisition. Each patient was analyzed in triplicate to reduce error, yielding a total of nine test tubes per patient. sFITC-Annexin V (Beckman Coulter, Brea, CA) was used to detect phosphatidylserine (PS), PE-anti-CD61 and APC-anti-CD41 (Beckman Coulter) were used as surface markers for platelet MPs. Platelet microparticles were identified as triple-positive MPs (Annexin-V⁺/CD61⁺/CD41⁺). MPs were incubated with 50 µL antibody solution in Annexin V Binding Buffer (FITC-Annexin V, 1 µL; PE-anti-CD61, 20 µL; APC-anti-CD41, 10 µL) for 30 minutes at room temperature, protected from light. 400 µL of Annexin V-FITC binding buffer was then added and then incubated for 10 min at room temperature, protected from light. 100 µL of AccuCheck Beads (Life Technologies, Frederick, MD) were added and manually mixed thoroughly just before acquisition on the flow cytometer. Identical samples were incubated with isotype control IgG antibodies in the same manner to test for false positives. Single staining controls and

control IgG antibodies were used to check fluorescence compensation settings and to set up positive regions on the Gallios flow cytometer (Beckman Coulter).

3.6 PMP Quantification By Flow Cytometry

MP analysis was performed using monodisperse fluorescent beads (Megamix, BioCytex, Marseille, France) of three diameters (0.5, 0.9, and 3 μ m). Forward and side scatter parameters were plotted on logarithmic scales to best cover a wide size range. The MP-gate was determined by the Megamix bead diameters with MPs being defined as particles < 1.0 μ m in size. 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 (cells/ μ L)= (number of cells counted/total number of beads counted) x number of AccuCheck Counting Beads per μ L). The number of cells counted was determined by subtracting the number of false positives like in **Figure 3.1** from the number of triple-positives in like **Figure 3.2**. The total number of beads counted was taken from graphs produced by the flow cytometer like in **Figure 3.3**. Since flow cytometry analysis of platelet microparticles was performed in triplicate, the average of the three calculated PMP concentrations was used for each patient.

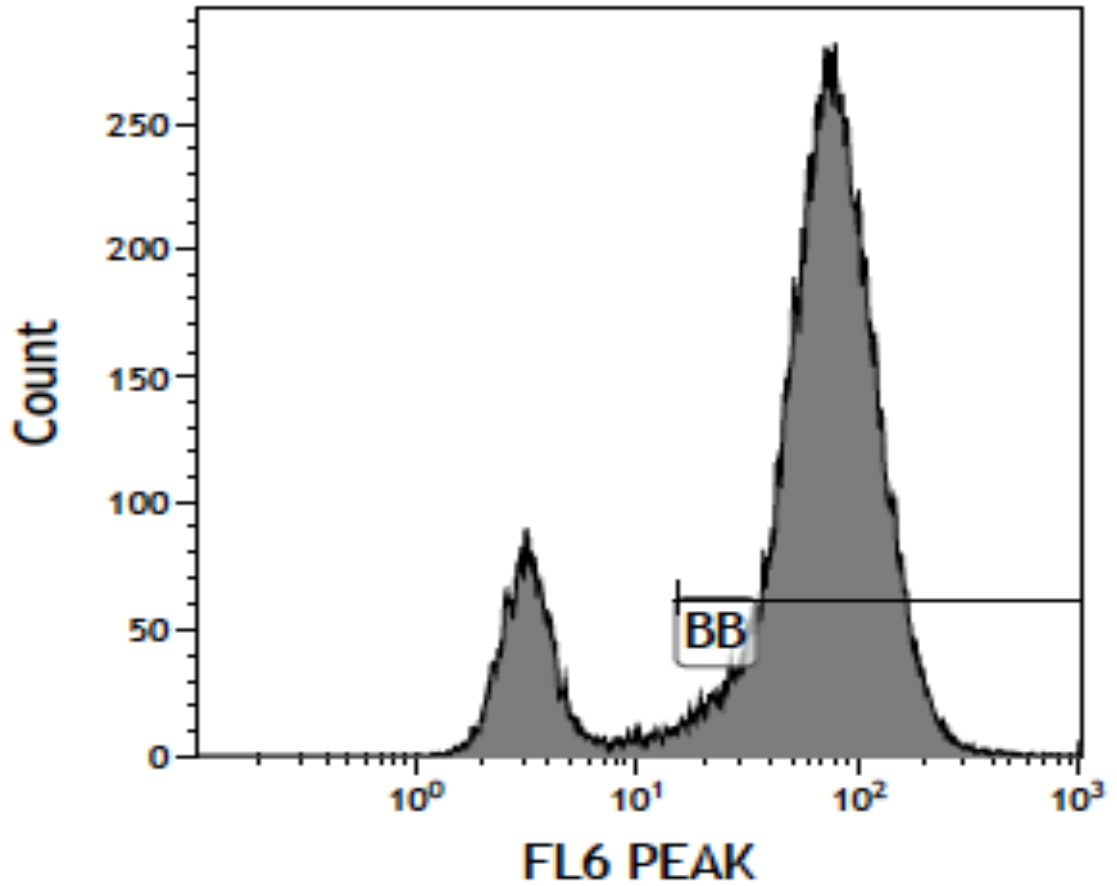
Data Set 5: 1111-1113 PMP 121416
wwd 014 421
[AB] FL6 PEAK



Gate Number	%Total	%Gated	
All	49,854	99.71	100.00
AF	19	0.04	0.04

Figure 3.1 Screenshot of False Positive Detection by Flow Cytometer

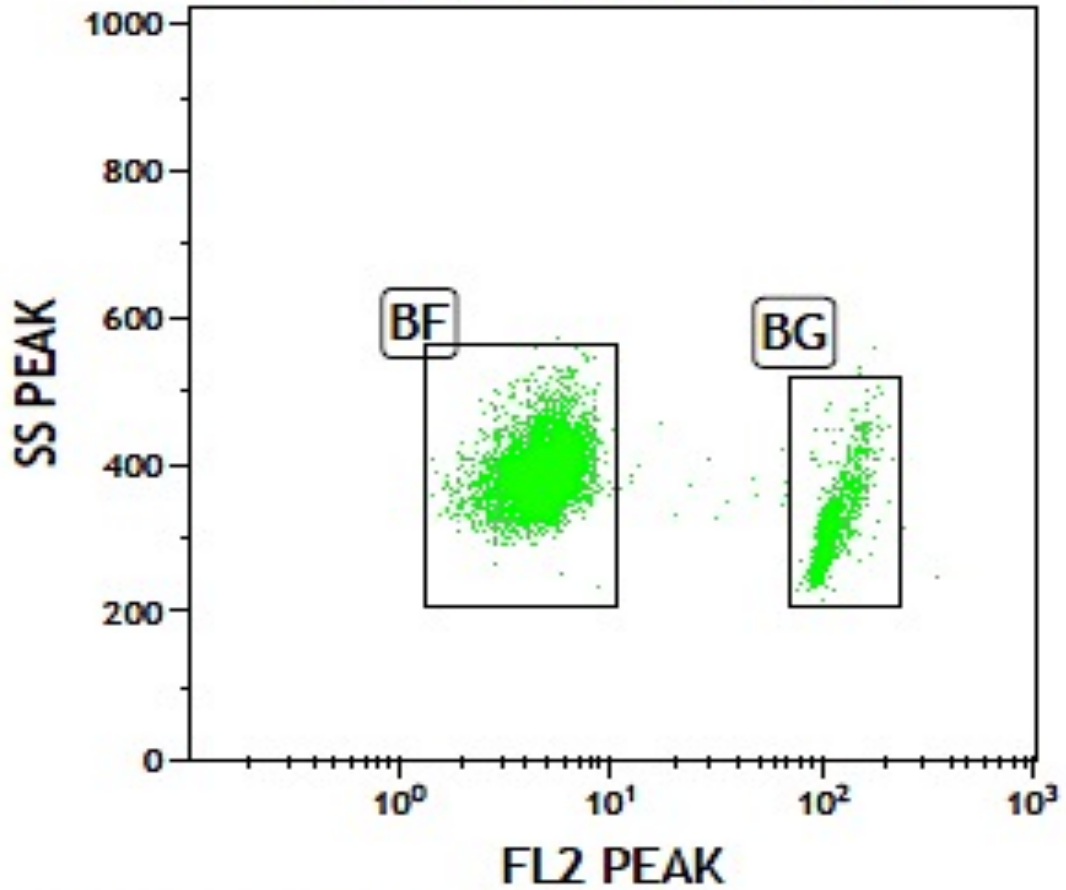
Data Set 6: 1111-1113 PMP 121416
 wwd 015 422
 [AX] FL6 PEAK



Gate Number	%Total	%Gated
All	39,843	79.69
BB	33,475	66.95

Figure 3.2 Screenshot of Triple-Positive MP Detection by Flow Cytometer

Data Set 6: 1111-1113 PMP 121416
 wwd 015 422
 [BE] FL2 PEAK / SS PEAK



Gate Number	%Total	%Gated
All	9,191	18.38
BF	4,402	47.89
BG	4,766	51.86

Figure 3.3 Screenshot of Accucheck Bead Detection by Flow Cytometer

3.7 Statistical Methods

Statistical tests were performed with the PMP concentrations of the 79 patients that were analyzed. Despite log transformation, PMP distributions remained non-parametric, and therefore two-sample Wilcoxon rank-sum (Mann-Whitney) tests were performed with PMP levels to test for statistically significant differences between patients meeting the primary of in-hospital death versus survival. Differences in PMP levels among a number of clinical factors including the source of infection (pneumonia, UTI, surgical wound), past medical history (diabetes, liver disease, chronic renal insufficiency, congestive heart failure, hypertension, malignancy, transplant), clinical presentation (lactate elevation, spontaneous bacterial peritonitis), and sex were similarly tested. Linear regression was used to assess for statistically significant associations between PMP level and both age and platelet count. A Kruskal-Wallis equality-of-populations rank test was performed on the patients' SOFA coagulation scores, a determination of how sick someone is to assess the association between PMPs and severity of illness as assessed by organ failure. Mann-Whitney tests were then done with PMP based on the SOFA coagulation score of the patient to test further for statistically significant correlations to in-hospital death or survival. Logistic regression was used to see if both PMP and platelet counts were independent predictors of death. All tests were two-sided, and p-values of < 0.05 were considered significant.

CHAPTER 4

RESULTS & DISCUSSION

4.1 PMP Concentrations

So far, the average PMP concentrations for 79 of the 182 enrolled severe sepsis patients have been determined. Seeing that the concentrations do not follow a normal distribution curve (**Figure 4.1**), the moments of the actual distribution were determined. Of note, log transformation did not lead to a normal distribution, so data were analyzed based on raw values without transformation and using non-parametric statistics. Data analysis revealed median, mean, standard deviation, skewness, and kurtosis values of 761.0, 1250.1, 1516.1, 2.6, and 11.9, respectively. 63% of our PMP concentrations fall below the mean, and consequently 27% rest above it. Outliers of 8059 and 8308 PMPs/ μ L account for much of this skew. The statistical density of PMP levels decreases appreciably with increasing concentration (**Figure 4.1**). Comparing the determined PMP concentrations to each of the factors below will clarify the significance of this distribution.

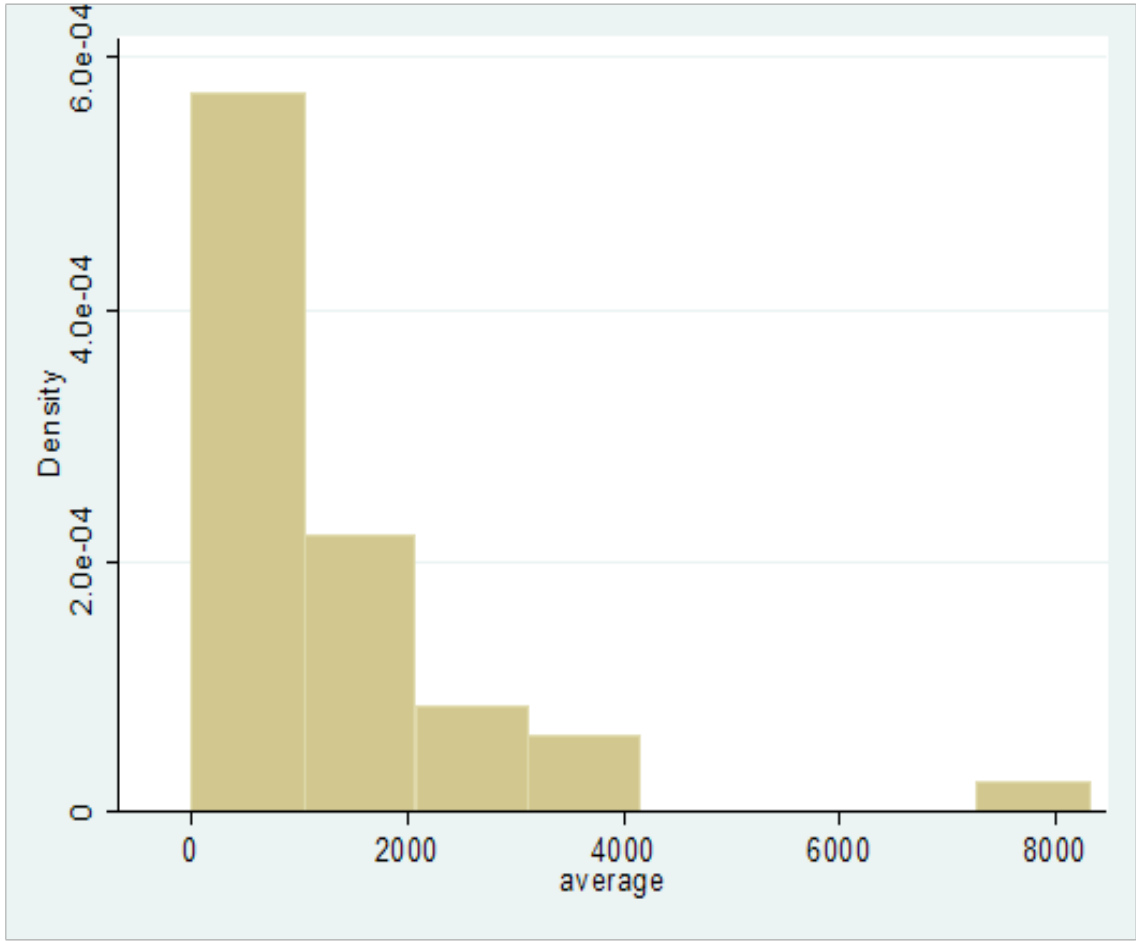


Figure 4.1 Distribution of Observed PMP Concentrations

4.2 PMP Concentrations Compared to In-Hospital Death or Survival

One of the main goals of this research was to investigate if PMP count is associated with adverse clinical outcomes and patient death, as a significant association might suggest a novel target for future therapeutics. In order to do so, a number of clinical factors were recorded for each sepsis patient and compared to the determined PMP concentrations. Statistical tests were performed for each factor to establish if a significant correlation exists. First, the patients' PMP count was compared to the incidence of in-hospital death or survival at the time of plasma extraction. Of the 79 patients studied so far, 13 (16%) died, while 66 (84%) survived. **Figure 4.2** shows this data as a box and whisker graph, noting median, quartiles, and outlier PMP counts by death or survival. A Mann-Whitney rank sum test gave a statistically significant p-value of 0.04 (**Table 4.1**). Thus, higher levels of PMP in plasma were associated with patient survival, establishing a positive correlation between PMP level and survival.

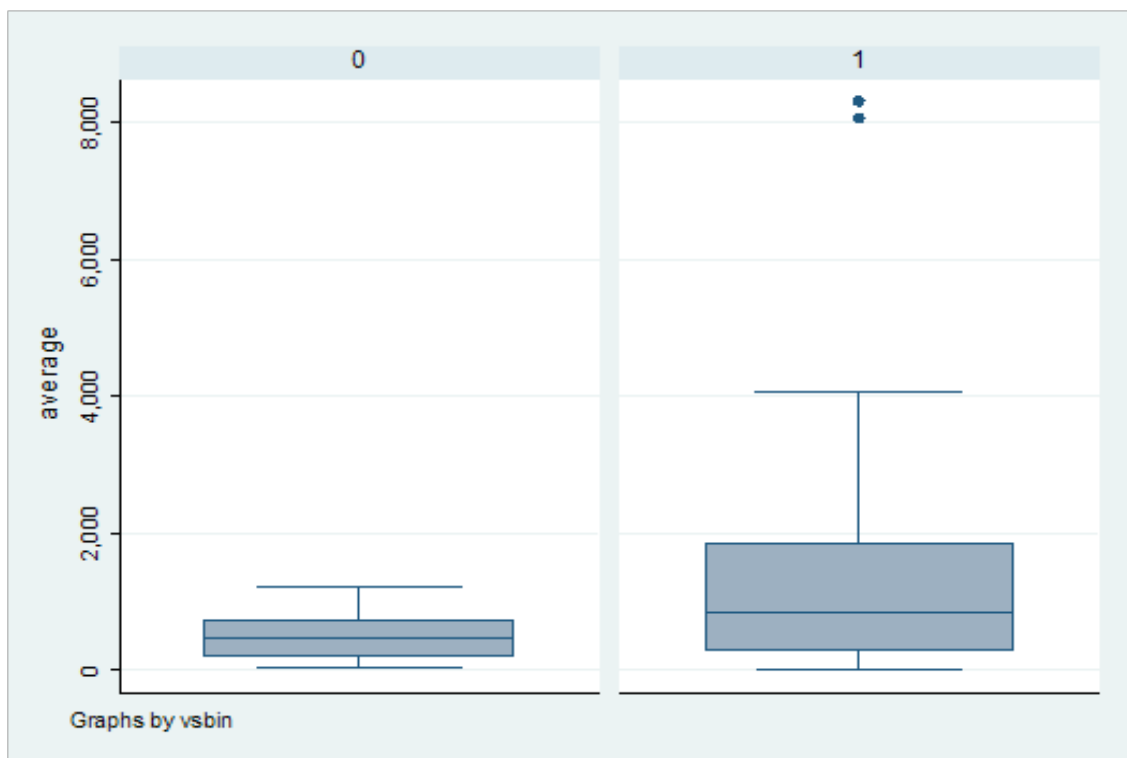


Figure 4.2 PMP Concentrations Separated by In-Hospital Death or Survival
 *Death = 0; Survival = 1

Two-sample Wilcoxon Rank-sum (Mann-Whitney) Test			
0	13	365	520
1	66	2795	2640
combined	79	2795	3160
		P > z	0.0404

Table 4.1 Statistical Test for Correlation Between In-Hospital Death or Survival and PMP Concentration

4.3 The Association of PMPs with SOFA Coagulation Score and Platelet Count

Once a correlation had been established between PMP count and mortality, we wanted to expound on this idea further to see if PMP concentration could indicate not only the death or survival of the patient but also the specific degree of disease severity. In sepsis, critical care physicians utilize the SOFA scoring system to determine sepsis severity by quantifying dysfunction of organ systems. For the coagulatory system, the degree of dysfunction is established by assessing platelet count (**Table 4.2**). A Kruskal-Wallis equality-of-populations test was performed to determine if PMP concentration varies with circulating platelet level (**Table 4.3**). With a $P < .05$ (**Table 4.3**), the results indicate that a relationship does exist between SOFA coagulatory score and PMPs and, thus, between platelet and PMP count. However, contrary to expectations, PMP count, like platelet count, trended downward on average with increasing SOFA coagulation score (**Table 4.3, Figure 4.3, Figure 4.4**). A logistic regression was performed with PMP and platelet counts to determine if these two measures represent independent predictors of death or whether they are co-linear, suggesting they may represent alternate measurements of the same underlying phenomenon (**Table 4.4**). Analyzing the 79 patients so far, these two factors do not appear to be independent predictors (**Table 4.4**), meaning they may be two different measures of the same underlying physiologic process. However, the p-value approaches statistical significance for independence, so further analysis of the remaining patients will be important to further investigate our hypotheses.

SOFA Coagulation Score					
	0	1	2	3	4
Platelet Count (x10 ³ /μL)	≥150	<150	<100	<50	<20

Table 4.2 SOFA Coagulation Scoring

Kruskal-Wallis Equality-of-populations Rank Test		
SOFA Coag Score	Observed	Rank Sum
0	57	2546.00
1	11	335.50
2	6	135.50
3	4	136.00
4	1	7.00
	P	0.0398

Table 4.3 Statistical Test for Determining If PMP Concentration Varies With Platelet Count

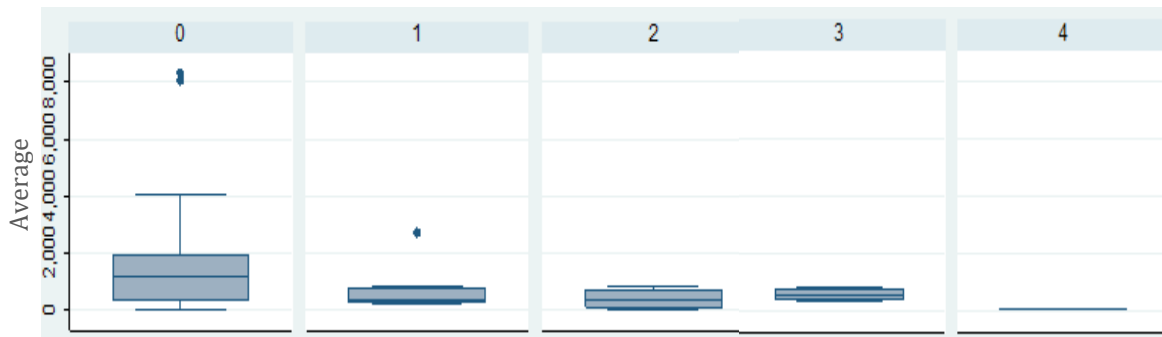


Figure 4.3 PMP Concentrations Separated By SOFA Coagulatory Score

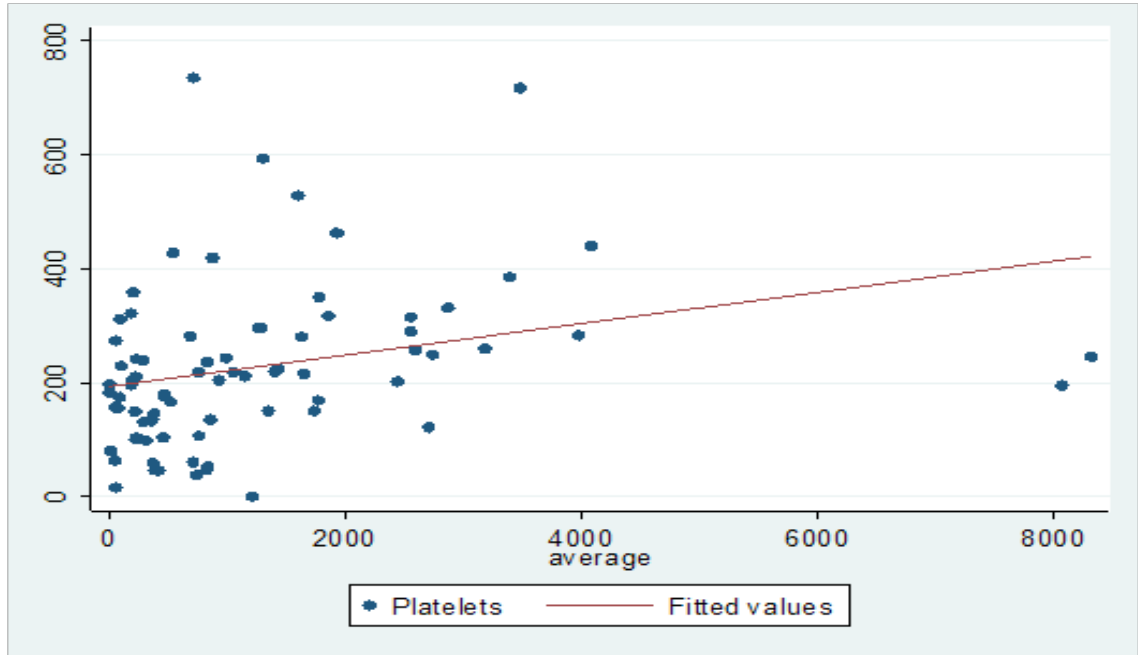


Figure 4.4 PMP Concentration vs. Platelet Count

Logistic Regression of PMP Average and Platelet Count						
	Odds Ratio	Std. Err.	z	P > z	[95% Conf. Interval]	
Average	1.0011	0.0005	2.01	0.044	1.0000	1.0022
	Odds Ratio	Std. Err.	z	P > z	[95% Conf. Interval]	
Average	1.0008	0.0006	1.35	0.179	0.99964	1.0019
Platelets	1.0072	0.0038	1.90	0.057	0.99977	1.0147
	Odds Ratio	Std. Err.	z	P > z	[95% Conf. Interval]	
Platelets	1.0036	0.0015	2.46	0.014	1.0007	1.0065

Table 4.4 Statistical Analysis of PMP Concentration and Platelet Count As Independent Predictors of Death

4.4 PMP Concentrations Compared to Other Clinical Factors

Once the above correlations to death and disease severity had been established, as well as the relationship to platelet count, PMP concentrations were compared to a number of other clinical factors. PMPs were analyzed by Mann-Whitney tests to determine if a relationship to the original source of infection that lead to each patients case of severe sepsis exists. Three categories for the source of infection were analyzed: pneumonia, urinary tract infection (UTI), and surgical wound (**Table 4.5**). The same tests were performed on seven past medical history factors: diabetes, liver disease, chronic renal insufficiency (CRI), congestive heart failure (CHF), hypertension, malignancy, and transplant (**Table 4.6**). Two clinical presentation factors were also evaluated: lactate elevation and spontaneous bacterial peritonitis (SBP) (**Table 4.7**). Lastly, the effects of sex and age on PMP level were considered (**Table 4.8**). None of these factors were significantly associated with PMP level. While we cannot definitely state there is no relationship between these factors and PMPs, given the moderate sample size we would expect any potential relationships to be relatively small with small effect sizes.

Source of Infection			
	Pneumonia	UTI	Surgical Wound
P > z	0.9172	0.8927	0.9387

Table 4.5 Mann-Whitney Tests for Correlation Between Source of Infection and PMP Concentration

Past Medical History							
	Diabetes	Liver Disease	CRI	CHF	Hypertension	Malignancy	Transplant
P > z	0.5952	0.3921	0.6620	0.9240	0.9413	0.0996	0.5610

Table 4.6 Mann-Whitney Tests for Correlation Between Past Medical History and PMP Concentration

Clinical Presentation		
	Lactate Elevation	SBP
P > z	0.5832	0.2401

Table 4.7 Mann-Whitney Tests for Correlation Between Clinical Presentation and PMP Concentration

Other Clinical Factors		
	Age	Sex
P > z *	0.537	0.7120

Table 4.8 Statistical Tests for Correlation Between Age/Sex and PMP Concentration
*Sex was analyzed by a Mann-Whitney test, while regression analysis was used for age.

4.5 Discussion

With only 79 of 182 enrolled patients having been analyzed, our results thus far represent only preliminary findings; however, the initial data does suggest a few interesting corollaries between PMPs and sepsis- in large part contrary to our initial hypothesis. The statistical distribution of the data from our enrolled patients gave an initial indication that PMP concentrations tend to manifest mostly in lower levels in severe sepsis (**Figure 4.1**). One of the main focuses of this study was to determine if PMP concentration could be a useful parameter of prognostic value in severe sepsis cases and if PMP count could be used to determine the likelihood of a patient's outcome. In light of the remarkably high mortality rate of sepsis,¹³ the first issue that we examined was whether or not PMP levels in circulating blood plasma are indicative of in-hospital death or survival. The statistical analysis of PMP counts separated by in-hospital death or survival revealed a significant correlation between PMP level and patient outcome. Nearly every enrolled patient who had died from severe sepsis complications had a PMP concentration less than 1500 PMPs per μL , while this number exhibited about an average concentration for living patients. These data suggests that PMP is a significant predictor of survival and could serve as a prognostic marker.

Since PMP levels, like platelet count¹⁶, were lower rather than higher in patients with a negative outcome, this trend- although significant- was contrary to the expectations of our hypothesis. To test the parallel between PMP and platelet count further, we performed a statistical analysis on PMP counts separated by the patient's SOFA coagulation score, a quantitative measure of sepsis severity, which is scored according to the patient's platelet count. Analysis once again showed a statistically

significant direct relationship, as PMPs seem to mirror platelets, decreasing with progressive disease severity. It is possible, therefore, that having fewer platelets circulating decreases the chance of generating PMPs. In order to determine if this parallel between PMPs and platelets occurs independent of each other, PMP concentration was then compared directly to platelet count with a logistic regression test. This analysis revealed that PMP and platelet count are not independent factors but instead seem to cancel each other out. This suggests that the same underlying physiologic process can be attributed to these measures. Thus, while PMPs do represent a prognostic marker, the same data appears to be available via a common clinical, easy to perform test in platelet count. This suggests against the true clinical utility of PMPs as a prognostic marker.

Although we knew that platelets and PMPs should share many physiologic functions, it was thought that the destruction of platelets in severe sepsis would result in the propagation of PMPs. Assuming this preliminary data holds up when more patients are analyzed, the results raise some doubts toward the marginal prognostic value of PMP concentrations in severe sepsis patients for critical care physicians, as platelet counts are a much easier and cost efficient measure. Despite having an outcome opposite to what we had theorized, the results raise novel questions to be answered by future research. Most notably, if completion of the remaining samples demonstrates that PMPs are an independent predictor of mortality after controlling for initial platelet count, it could be that after platelet count is considered, PMPs do contribute to mortality all other things being equal. However, it is too early to draw any conclusions until completion of the remaining samples.

The exact physiologic mechanism affecting both PMP and platelet counts in severe sepsis can likely be attributed to the interaction of number of different processes. Previous research has attributed low platelet count in septic patients to overabundance of cytokines, endothelial damage, and the suppression of bone marrow.¹⁴ Since cytokine overabundance and endothelial damage should contribute to an increase in PMPs, bone marrow suppression may be occurring at a sufficient magnitude to outweigh those effects, yielding PMP and platelet counts that mirror each other as we have observed in our results; however, further research would be necessary to confirm this. Also, these results still do not elucidate much about the role that PMPs play in sepsis but merely show that concentrations parallel those of platelets; so further inquiry could still be done. Specific pathophysiologic effects of PMPs *in vivo* were not investigated in the current study, but would be an area for future research as well. Another important question involves PMPs and platelets' close relationship to severe sepsis and the degree to which a septic patient's natural platelet generation and composition predisposes them to descending into severe cases. Research has already shown that sepsis induces alterations in platelets, leading to their cascading dysfunction;¹⁸ however, the underlying reasons causing one individual to be more susceptible than another to these deleterious effects of sepsis are not yet well understood. Further research on PMPs will be necessary to determine if they play a role in these sepsis-specific effects on the coagulatory system.

Apart from PMPs' close relationship to platelets and patient outcome, our preliminary data demonstrated no other significant corollaries between PMP concentration and the other fourteen sepsis-related clinical factors that were considered.

Eliminating these possible confounding factors, this further indicates PMPs' specific association to platelets in sepsis.

Aside from the obvious issue of this initial data only representing roughly half of our total number of enrolled patients, a few other limitations of this study warrant discussion. For one, the small size of MPs may affect the precision of flow cytometric analysis.²¹⁻²³ We attempted to minimize these effects by appropriate gating and choosing of monodisperse beads, yet it remains possible that some particularly small PMPs were excluded. Antibody aggregates may also be of concern²³; however, our addition of false-positive detection in our protocol should make their effects on our data minimal.¹¹ Additionally, the lack of variable standardization in flow cytometric quantification limits our data in terms of comparability to other similar studies. These variables notably include the instruments and the fluorescently labeled antibodies- both positive and negative- used among other factors. Finally, alternative or larger clinical cohorts may find more significant relationships. However, given a moderately sized cohort, we believe our study was adequately powered to detect what we would interpret as clinical meaningful associations.

4.6 Conclusion

The preliminary data of our research establishes a close relationship between PMP and platelet count, revealing that the two measures are most likely affected by the same underlying physiologic processes. PMP concentration does correlate with the degree of sepsis severity and patient survival; however, platelet count provides a much more convenient parameter for critical care physicians in determining the severity of

sepsis and short-term prognosis. Although PMPs may not represent a useful new parameter for sepsis diagnosis, the outcome of this research could establish the basis for further studies examining why these two measures parallel so closely despite the destruction of platelets observed in sepsis. Analysis of the remaining patients enrolled in this study will be necessary to confirm the results established in this thesis.

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