


Dysregulation of Biomarkers of Hemostatic Activation and Inflammatory Processes are Associated with Adverse Outcomes in Pulmonary Embolism

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Abstract

Introduction: The pathophysiology of pulmonary embolism (PE) represents complex, multifactorial processes involving blood cells, vascular endothelium, and the activation of inflammatory pathways. Platelet (P), endothelial (E), and leukocyte (L)-selectin molecules may play an important role in PE pathophysiology. We aimed to profile the biomarkers of inflammation, including selectins in PE patients, and compare them to healthy individuals.

Materials and methods: 100 acute PE patients and 50 controls were included in this case control study. ELISA methods were used to quantify levels of selectins, inflammatory, and hemostatic biomarkers.

Results: In PE patients, levels of selectin molecules as compared to controls convey increased P-selectin levels (95 ng/mL vs 40 ng/mL, $p < .0001$) and decreased L-selectin levels (1468 ng/mL vs 1934 ng/mL, $p < .0001$). Significant correlations were found between selectins and Plasminogen Activating Inhibitor-1 (PAI-1), Tumor Necrosis Factor- α (TNF α), and D-dimer. Fold change between selectins and controls is compared to other biomarkers, illustrating degrees of change comparable to TNF α , alpha-2-antiplasmin, and microparticles. L-selectin levels are inversely associated with all-cause-mortality in PE patients, ($p = .040$).

Conclusion: These studies suggest that various thrombo-inflammatory biomarkers are elevated in PE patients. Furthermore, L-selectin levels are inversely associated with mortality outcomes.

Keywords

selectins, pulmonary embolism, inflammation, hemostasis, mortality

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Introduction

Pulmonary embolism (PE) is a common disease complication in the hospital setting and one of the most frequent causes of death.^{1,2} Among the many risk factors for PE development are history of cancer, surgery, and inflammation.²⁻⁴ Females who are pregnant or are taking oral contraceptives also have a higher chance of developing PE.⁴ PE has been found to be more prevalent in elderly populations, overweight individuals, as well as individuals who smoke tobacco.⁵ Clinical presentation of PE often includes hemoptysis, severe chest pain, dyspnea, and syncope.⁵ Many diagnostic tools have been extensively studied and adopted, including the Wells Criteria, D-dimer, V/Q scan, and CT angiography.^{2,5,6} These laboratory

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and confirmatory tests have made the diagnosis of PE more approachable in the clinical setting.

While diagnosis of PE has become routine clinical practice, risk stratification of the condition has been more challenging. Prediction of clinical outcomes of PE is a growing area of research, which is now exploring the role of biomarkers. Right ventricular dysfunction as well as troponin levels were studied by Becattini et.al. and used to create a predictive model for mortality outcomes in PE patients.⁷ Jovanovic et.al. examined the biomarker Brain Natriuretic peptide (BNP) and was able to illustrate a statistically significant correlation between BNP and PE mortality 30 days from onset of the diagnosis.⁸ Other studies, such as those by Surov et.al., sought out to create a predictive model for PE mortality using parameters such as troponin, pH, blood pressure, age and gender.⁹ Inflammatory and hemostatic activation biomarkers such as Alpha-2-antiplasmin, C-reactive Protein, D-dimer, MMP-9, microparticles, and TNF α have been extensively studied in venous thromboembolic conditions.^{1,3} Despite these studies about the predictive value and correlations of various parameters to mortality outcomes, adhesion molecules, such as P, E, and L-selectins, have not been examined for their clinical use in prediction of outcomes.

P-selectin is a cell adhesion molecule which is located on the outer membrane of endothelial cells and platelets.¹⁰ P-selectin interacts with other cellular components through attachment to its ligand, PSGL-1, which is located on leukocytes. This interaction permits for the cross linkages of white cell molecules, which is of substantial importance to thrombus development. P-selectin has been found to increase tissue factor levels in other white cells, further contributing to the creation of a new thrombus.^{11,12} Increased levels of P-selectin have been reported in many thrombotic conditions, including ischemic stroke and myocardial infarction.¹³ Research studies have conveyed that a substantial increase in P-selectin levels in plasma is evident within just hours of the initial development of the thrombus.¹⁴ Studies by Mosevell et.al. have demonstrated significantly elevated levels of P-selectin in PE patients and have even suggested its use as a screening biomarker.¹⁴ The specificity of P-selectin for diagnosis of PE was greater than 97% in one study.¹⁴ Metanalysis by Antonopoulos et.al. reinforced that there was an elevation of soluble P-selectin levels in patients with a prior deep venous thrombosis.¹³ Xie et.al. examined P-selectin at the mRNA level in PE patients, also demonstrating an elevated quantity of the biomarker within the experimental cohort.⁴ Additional studies of the relationship of P-selectin to other biomarkers of inflammation and hemostasis is necessary in order to more completely understand its role.

E-selectin is an adhesion molecule that is unique in that it is only found on the endothelium. Under normal conditions, E-selectin expression is limited to microvessels, but this can be amplified in pathologic, inflammatory, states.¹⁵ Many studies have conveyed that one of the primary functions of E-selectin is to permit rolling or migration of leukocytes along the endothelium in pathologic states.¹⁶ In fact, recent studies have conveyed that this function is not limited to

E-selectin, but rather all three selectins can partake in this role. In-vivo studies have illustrated that L-selectins have the fastest rolling speed, followed by P then E-selectin.¹⁷ Past studies have conveyed that E-selectin levels become more upregulated in inflammatory states, specifically citing molecules such as TNF α . It has also been suggested that a co-expression of P and E-selectin molecules takes place in inflammatory states, illustrating a co-dependent relationship between the two.¹⁷ Further support for this codependency among P and E-selectin are reported in mice models which showed much smaller thrombi in the absence of both P and E-selectin versus in the absence of only P-selectin or E-selectin.¹⁵ However, despite this initial upregulation, studies have conveyed a substantial decline in levels of E-selectin, likely due to its breakdown in lysosomes shortly after translation.¹⁷ Further supporting this concept, Xie et.al. reported a lower level of E-selectin mRNA in PE patients, specifically.⁴

L-selectin is an adhesion molecule that is located on the outside surface of leukocyte molecules.¹⁸ More specifically, L-selectin has been found on T-cells, B-cells, neutrophils, monocytes, and natural killer cells.¹⁷ This position allows for leukocytes to translocate across and through the endothelium to reach locations of injury or inflammation.^{18,19} This concept has been proven through knock-out studies of L-selectin, which have subsequently led to impaired rolling of leukocytes in in-vivo studies.¹⁷ It has been reported that quantifiable levels of L-selectin can be found in normal healthy individuals.¹⁷ Upregulation of L-selectin levels is associated with inflammatory states; this concept has been illustrated by knock-out L-selectin mice demonstrating lower levels of inflammation than those who possess intact L-selectin.¹⁵ Xie et.al. studied selectin molecules at the mRNA level, reporting higher levels of L-selectin in PE patients. This, the researchers noted, suggested the abundant recruitment of white cells during the development of a PE.⁴

The current study aims to quantify levels of selectins in PE patients and controls, correlate selectin levels to other biomarkers of hemostatic activation and inflammation, compare degree of magnification of selectins in PE patients to degree of magnification of other hemostatic and inflammatory biomarkers, and examine potential correlations of selectins to mortality outcomes. These studies will provide further elucidation of the mechanistic role of selectin molecules in PE pathogenesis, while allowing for investigation of another biomarker that can be indicative of negative patient outcomes.

Materials and Methods

Patient Population

Patients 18 years old and over presenting to Loyola University Medical Center (LUMC) with acute PE between March and June 2019 were included in a prospective database for the PE Response Team (PERT) Trial, and consenting patients underwent phlebotomy for research purposes following hospital admission. The age ranged from 18 to 101 (Mean = 63 \pm 16

years). The gender of the group was equally balanced. Diagnosis was made using CT angiography or ventilation perfusion (VQ) scan confirmed by PERT team. A random selection of patients with varying PE severity (as defined by the European Society of Cardiology classification system) were selected for inclusion in this study using a random number generator.²⁰ The study was approved by the Institutional Review Board.

Quantification of Biomarker Levels

Blood was drawn from all patients within 72 hours after PE diagnosis. All samples were de-identified. Plasma was isolated via centrifugation of the sample, which was subsequently aliquoted and frozen until use. Control citrated plasma (N = 50) of normal human volunteers, apparently free of any on board medication, was purchased from the vendor George King Biomedical, Inc., Overland Park, KS. This control cohort has been used for the comparison of the biomarker levels in all studies carried out in conjunction with the PERT program.

Commercially available Enzyme Linked Immunosorbent Assays (ELISA) for Alpha-2-antiplasmin, C-reactive Protein, D-dimer, MMP-9, microparticles, TNFa, and P, E, and L-selectin are performed according to enclosed package procedures. Normal samples comprised of 50 plasma samples collected from healthy male and female individuals. An additional mixed pool of plasma obtained from 50 healthy male and 50 healthy female volunteers, referenced as Normal Human Plasma (NHP), was also used for comparison purposes.

Table 1. Biomarkers of Hemostatic Dysregulation, Inflammatory Process, and Selectins in Patients with PE and Their Comparison with Normal Controls

Parameter	Median	Mean ± SD	Fold Change (PE/	
		(SEM)	NHP	NHP)
D-Dimer (ng/mL)	5032	6986 ± 5879 (446)	202	34.6
MMP-9	216	815 ± 4472 (397)	51	16
CRP (ug/mL)	13	36 ± 51 (4)	4	9
PAI-1 (ng/mL)	60	69 ± 46 (3)	22	3.1
Microparticles (nM)	59	80 ± 64 (5)	29	2.8
TNFa (pg/mL)	64	98 ± 107 (12)	49	2
Alpha-2 Anti-Plasmin (%)	83	90 ± 41 (3)	100	0.9
P-Selectin (ng/mL)	77	95 ± 65 (7)	46	2.1
E-Selectin (ng/mL)	60	65 ± 33 (3)	74	0.9
L-Selectin (ng/mL)	1353	1468 ± 588 (59)	1806	0.8

All values represent mean ± 1 SD (SEM) of PE patients and their comparison with normal controls. In addition, the median values are also provided for comparison purposes. Fold changes in the biomarker levels is calculated in reference to NHP.

Data Analysis

Mean, median, standard deviation, and standard error of the mean were calculated for Alpha-2-antiplasmin, C-reactive Protein, D-dimer, MMP-9, microparticles, TNFa, and P, E, and L-selectin. Fold change of PE patients as compared to the mixed pool of healthy male and female volunteers, referenced in the paper as normal human plasma or NHP, was calculated by dividing the mean of the biomarker in PE patients by the biomarker level in NHP. Biomarkers were then ranked by fold change from greatest fold change to smallest fold change. Two-tailed Wilcoxon Mann Whitney tests were used to compare selectin levels in PE patients and 50 human controls. Statistical tests with p = .05 were executed using Graphpad Prism Software. Spearman correlational studies relating P, E, and L-selectin, PAI-1, TNFa, and D-dimer were performed using Graphpad Prism Software. Selectin levels were then correlated to patient outcomes in terms of mortality using Wilcoxon Mann Whitney tests. Significance was assigned at alpha = .05.

Results

Comparison of Inflammatory and Hemostatic Biomarker Levels

Table 1 shows the mean values of each of the individual biomarkers in comparison to normal. The mean value of D-dimer in PE patients is 6986 ng/mL, MMP-9 is 815 ng/mL, CRP is 36 ug/mL, PAI-1 is 69 ng/mL, microparticles is 80 nM, P-selectin is 95 ng/mL, TNFa is 98 pg/mL, alpha-2-antiplasmin is 90%, E-selectin is 65 ng/mL, and L-selectin is 1468 ng/mL. When compared to the internal control, NHP, the biomarker that was most magnified in the PE patient cohort was D-dimer, with a magnification of 34.6. After D-dimer the biomarkers that were amplified in PE patients were MMP-9 (fold change 16.1), CRP (fold change of 9.0), PAI-1 (fold change of 3.1), Microparticles (fold change of 2.8 nM), P-selectin (fold change of 2.1), and TNFa (fold change of 2.0). Alpha-2-antiplasmin, E-selectin, and L-selectin had lower levels in PE patients when compared to NHP. Fold change of alpha-2-antiplasmin was 0.9, fold change of E-selectin was 0.9, and fold change of L-selectin was 0.8. Figure 1 shows a composite of the individual distribution profiles of each of these biomarkers, illustrating the inter-quartile ranges.

Comparison of Selectin Levels in PE Patients and Controls

Figure 2 shows the distribution profile of selectin levels in both PE patients and controls. In PE patients, P-selectin levels were increased to 95 ng/mL as compared to 40 ng/mL in the control group. E-selectin levels in PE patients were slightly increased at 65 ng/mL, as compared to control levels of 61 ng/mL. L-selectin levels in PE patients were reduced at 1468 ng/mL,

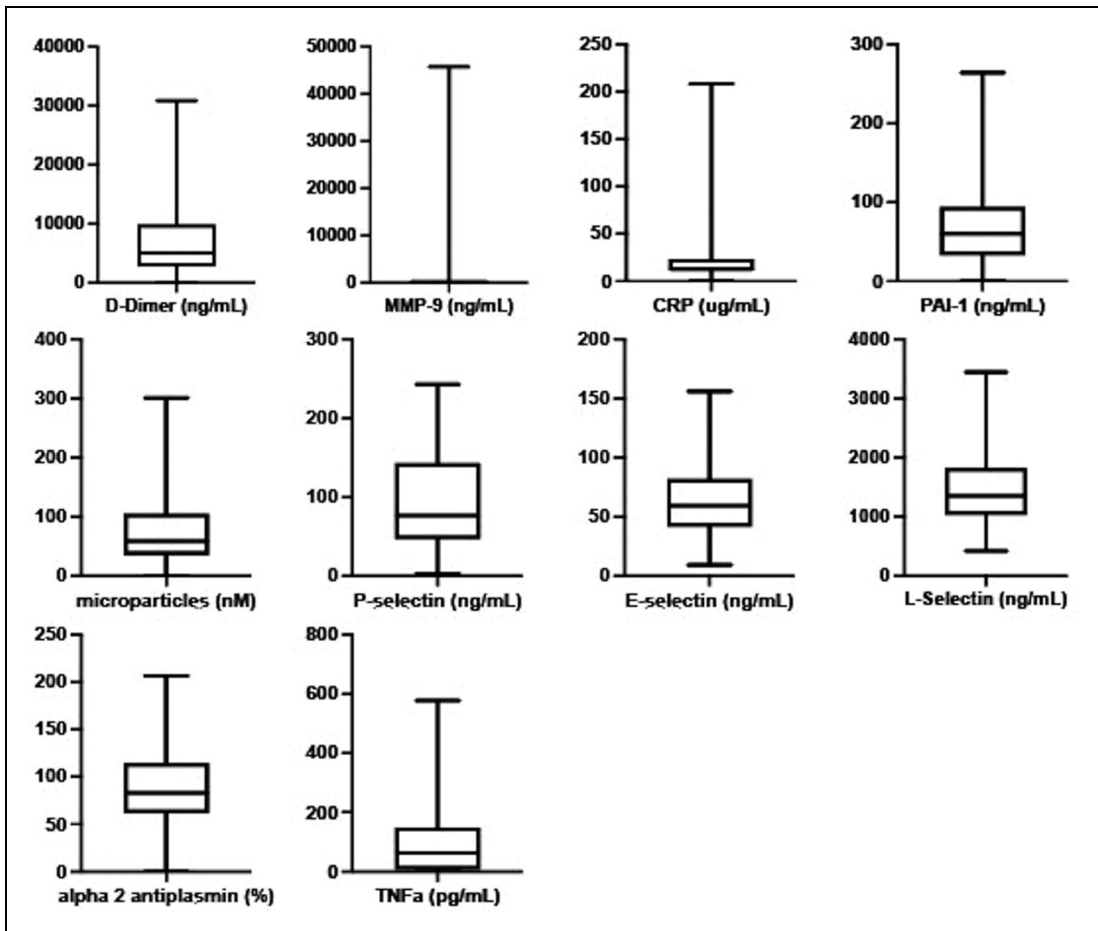


Figure 1. A Composite Representation of the Distribution Profile of Various Biomarkers in PE Patients.

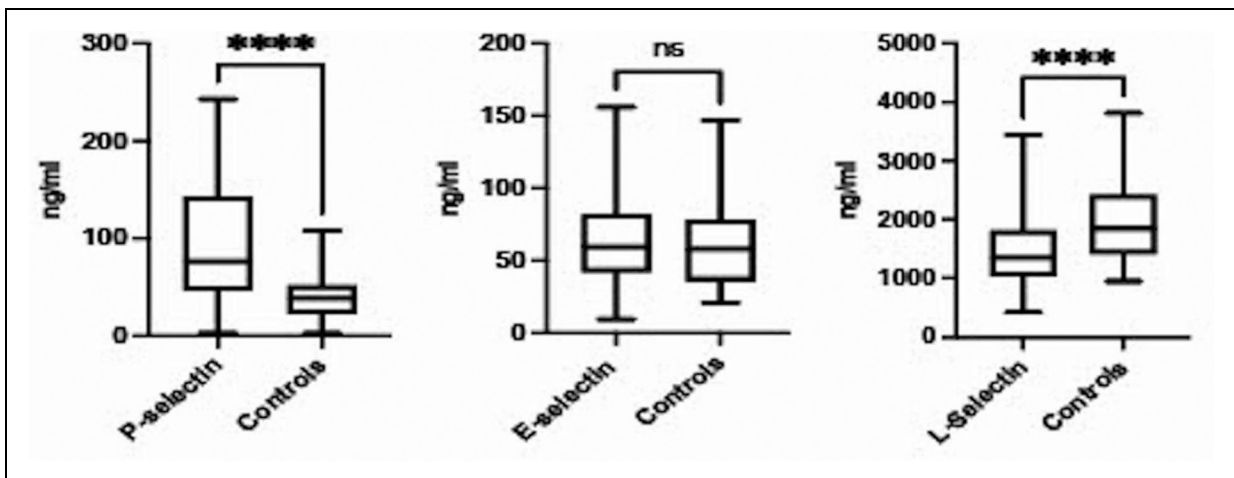


Figure 2. A Comparison on P, E, and L-selectin Levels in PE Patients as Compared to Normal Controls.

as compared to control levels of 1934 ng/mL. Non-parametric Wilcoxon Mann Whitney results on the data indicated that P-selectin levels were significantly magnified, with $p < .0001$. L-selectin levels were significantly decreased, with $p < .0001$. E-selectin levels indicated no significant change as compared to normal controls, with $p = 0.51$.

Correlational Studies Among Hemostatic and Inflammatory Biomarkers

Correlational relationships among P, E, and L-selectin, TNFa, PAI-1, and D-dimer are performed. Figure 3 shows composite correlation coefficients among various biomarkers investigated. P-selectin and E-selectin had a correlation coefficient of 0.43. P-selectin and PAI-1 demonstrated a correlation coefficient of 0.39. P-selectin and D-dimer had a correlation coefficient of 0.28. Finally, TNFa and L-selectin had a correlation coefficient of 0.24.

Among each biomarker pair, four pairs of biomarkers were found to be statistically significant. Figure 4 shows the correlation curves for these four pairs of biomarkers. The most strongly correlated were P and E selectin, with $r = .43$ ($p = .00001$, 95% CI = 0.247 to 0.580, $n = 100$). P-selectin and PAI-1 exhibited the second strongest correlation with $r = .39$ ($p = .00006$, 95% CI = 0.206 to 0.554, $n = 98$). P-selectin and D-dimer had the third strongest correlation with $r = .28$ ($p = .01$, CI = .064 to .472, $n = 84$). The final biomarker pair that was statistically significant was L-selectin and TNFa, with $r = .24$ ($p = .033$, 95% CI = .013 to .451, $n = 76$).

Association of Selectins and All-Cause Mortality

As shown in Table 2, patient outcomes in terms of mortality were tabulated. Of the 100 patients in whom selectin levels

were studied, 88 were living at the time of analysis and 12 were deceased at that time. Mean levels of P, E, and L-selectin in living PE patients were compared to those who were deceased at the time of analysis. Interestingly, the mean P-selectin levels in the deceased patients was 80 ng/mL, which was marginally increased compared to the level in living patients, 75 ng/mL. E-selectin levels in deceased patients were also slightly elevated at 69 ng/mL as compared to the mean level in living patients 58 ng/mL. L-selectin levels in deceased patients had an average of 1046 ng/mL as compared to the level in survivors, 1391 ng/mL, and this difference was statistically significant, with $p = .040$. P-selectin and E-selectin did not exhibit statistical significance when related to all-cause mortality, with p -values of 0.252 for P-selectin and 0.504 for E-selectin respectively.

Discussion

The pathophysiology of PE is a complex process where the dysregulation of the coagulation process and inflammatory activation plays an important role.^{1,2} The profiling of biomarkers of hemostatic activation and measurement of inflammatory mediators provides additional insight into the pathogenesis of PE not previously evaluated in clinical settings. The biomarkers analyzed were also compared with plasma samples collected from normal healthy volunteers to examine the changes in the circulating levels of these analytes. The major findings in this study include that P-selectin levels are significantly elevated in PE patients, L-selectin levels are significantly reduced, and that statistically significant correlational relationships do exist between the selectin molecules and other biomarkers of hemostasis and thrombosis. These correlations demonstrate that adhesion molecules are important markers for the inflammatory state and hematologic dysregulation found in PE.

Our study found a marked increase in D-dimer level, suggesting endogenous formation of thrombin associated with thromboembolic complication.² Additionally, microparticles from the endogenous consumption of thrombogenic membranes of cells were also elevated in the PE cohort as compared to the reference group.^{21,22} Such markers of inflammation including MMP-9, a matrix degrading protease, and CRP were also increased in our PE cohort. The PAI-1 levels which indicate the increased inflammatory process and fibrinolytic deficit were also elevated in the PE cohort.^{23,24} TNFa, which represents a hallmark of tissue factor activation of macrophages in various inflammatory conditions, was also elevated in the PE cohort.²⁵ Overall, our study demonstrates an upregulation in hemostatic biomarkers and inflammatory cytokines. The PE cohort showed wide variations which may be related to the difference in the pathophysiologic state and individual predisposition of recruited PE patients. In this study, the patients were randomly recruited and were not selected on the basis of established criteria. However, upon the recruitment of additional patients, the analysis of a larger cohort may provide enough sample size to carry out subgroup analysis. The purpose of the study was to identify measurable biomarkers to predict

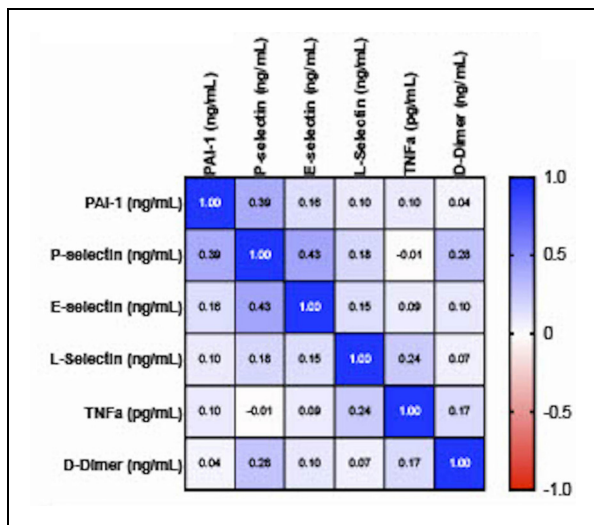


Figure 3. A composite of the Correlation of Various Biomarkers of Hemostatic Activation, Inflammation, and Selectins in PE Patients Compared to Normal Controls.

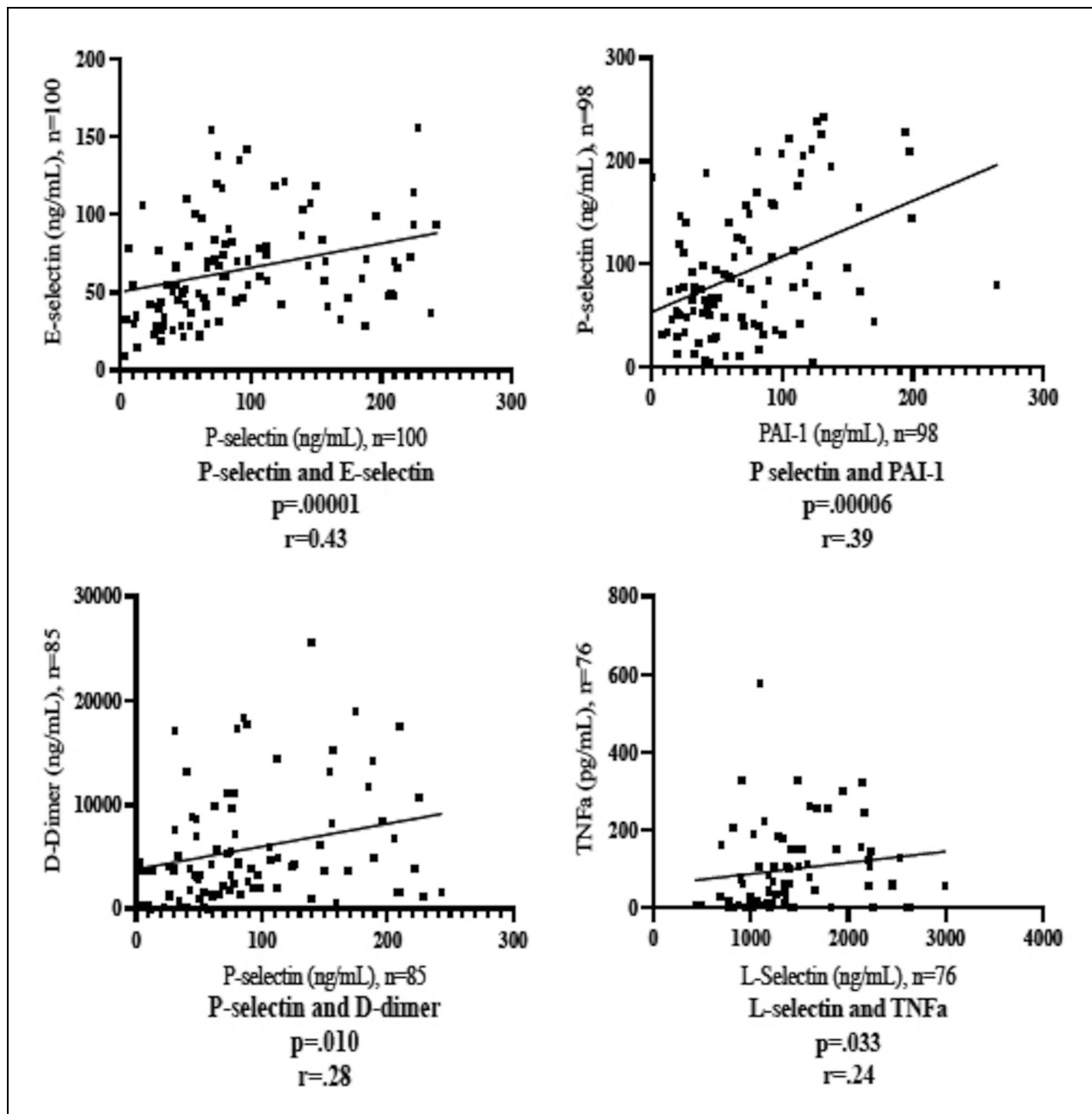


Figure 4. Correlation Plots of Various Biomarkers Showing Significant Relationships in the PE Patient Cohort.

outcomes and the nature of the pathogenesis of the patient population in the PERT program.

Table 2. Selectin Levels and Their Relationship with Mortality Outcome in PE cohort.

Selectin	Survivor Selectin Levels (ng/mL)	Non-Survivor Selectin Levels (ng/mL)	P-value
P-selectin	75 (44-126)	80 (59-197)	0.252
E-selectin	58 (41-84)	69 (52-74)	0.504
L-selectin	1391 (1093-1826)	1046 (868-1302)	0.040

The mortality outcome in terms of survivor and non-survivors was calculated utilizing Wilcoxon-Mann-Whitney U Tests for the PE cohort.

Since the pathogenesis of PE is multifactorial, the data collected on various biomarkers was subjected to correlation analysis. In this analysis, P-selectin strongly correlated with PAI-1 ($r = 0.39$), suggesting the role of platelets in regulating PAI-1. It has been reported previously that platelets secrete anti-fibrinolytic mediators, such as alpha-2-antiplasmin, however, in our study the relationship of PAI-1 and P-selectin is suggestive of the role of P-selectin in regulating PAI-1.^{24,26,27} P-selectin was also the only adhesion molecule which was significantly increased in the PE cohort. Interestingly, P-selectin levels also correlated significantly with D-dimer, suggesting the role of this adhesion molecule in enhancing the level of thrombin generation and subsequent formation of fibrin

degradation product, as measured by D-dimer levels.²⁸ L-selectin levels demonstrated a significant correlation with TNF α levels, which is consistent with the activation of white cells and their subsequent role in upregulating TNF α .¹⁵ Overall, profiling multiple biomarkers provides a comprehensive evaluation of the complex pathways and systemic molecular and cellular dysregulation that occurs in PE.

The adhesion molecules, namely P-selectin, E-selectin, and L-selectin, showed variable responses in PE patients. P-selectin levels were increased in PE patients, L-selectin levels were lowered, and the E-selectin levels were not significantly different from the normal control cohort. The increase in P-selectin suggests that the activation of platelets plays a prominent role in the thrombogenesis in PE. It is thought that P-selectin levels are augmented in PE patients due to the platelet aggregation necessary for initial thrombus formation.^{10,29} Levels of free L-selectin are predicted to have been diminished due to consumption of leukocytes within the thrombus itself, reducing the level that is free to circulate in the patient plasma. While E-selectin levels have been demonstrated to be correlated to P-selectin levels, perhaps due to their nature of sharing a common ligand, PSGL-1, the level of E-selectin between PE patients and controls did not differ significantly.⁴ Due to known mechanistic roles of D-dimer and PAI-1 in thrombus formation, the statistically significant correlation of P-selectin to these markers conveys that P-selectin may too share a responsibility in thrombogenesis.^{28,30}

L-selectin has previously been reported to be upregulated in states of inflammation, and since TNF α is a well documented marker of inflammation, its correlation to this biomarker further supports this concept.^{12,26} The correlation of the lowering of circulating L-selectin levels to all-cause mortality is of particular importance. Since L-selectin is an adhesion molecule, it is likely that circulating L-selectin is decreased because of its adhesion to endothelial surfaces where the honing of leukocytes takes place during thrombo-inflammatory processes. It is thought that because L-selectin is so strongly connected to the inflammatory state, this relationship to mortality derives from greater levels of inflammation in non-survivors as compared to survivors.¹² This paves the path for future management of PE in the form of greater focus on anti-inflammatory therapy to improve prognostic outcome.

Study Limitations

This study was limited by the inclusion of a small sample size of 100 PE patients, which were recruited in conjunction with the PERT program. This study, therefore, represents a pilot study to determine the prognostic significance of some of the markers in PE patients. A larger number of patient inclusion may provide further validation of the observations noted in this study. The study was also limited by inclusion of a heterogeneous group of patients, including low, intermediate and high risk severity PE. Additionally, within the patient population there was also the presence of other comorbidities, which were more prevalent than in our controls which could

potentially skew the data analyzed. Further, this study only utilized select biomarkers which represent different pathogenic processes. This study can be further augmented by the inclusion of other biomarkers of inflammation and hemostatic activation. In this study only one baseline sample was collected at the time of confirmed diagnosis of PE, which represented a 72-hour span after the onset of PE. Differences in the timing of specimen collection may also have contributed to the observed variations noted as medical therapies given may impact biomarkers in addition to the evolving inflammatory changes of PE over time. Despite these limitations, this study has provided valuable information in the understanding of the pathogenesis of PE and the interrelationship between the hemostatic and inflammatory processes in mediating the complex pathophysiology of PE. The information generated may be useful in the risk stratification and overall management of PE patients.

Conclusion

In this pilot study, P, E and L-selectins are significantly correlated with some of the known biomarkers of hemostatic activation and inflammation in the setting of PE. Levels of L-selectin were also found to be inversely associated with mortality outcomes in PE patients. The observed elevation of other biomarkers is suggestive of the interrelationship of selectins and these mediators in the overall pathophysiology of PE. Profiling of these biomarkers in an integrative manner and inclusion of other adhesion molecules may provide additional insight into the molecular pathogenesis of PE-associated vascular dysfunctions and hemodynamic changes. This pilot study warrants further investigation in a larger cohort of PE patients to validate the prognostic value of these biomarkers for the optimal management of PE.

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Declaration of Conflicting Interests








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