



The Relevance of Anti-PF4 Antibody Isotypes and Endogenous Glycosaminoglycans and their Relationship with Inflammatory Biomarkers in Pulmonary Embolism Patients

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Abstract

Introduction: Previous studies have shown that inflammation may contribute to the interplay of endogenous glycosaminoglycans (GAGs) and anti-PF4 antibodies. In this study, we quantified the levels of anti-PF4 antibody isotypes and endogenous GAGs together with inflammatory biomarkers in pulmonary embolism (PE) patients to determine whether there is a relationship in between. Identification of this relationship may provide insight to the complex pathophysiology of PE and HIT and may also be useful for development of potential prognostic, diagnostic and therapeutic interventions.

Materials and Methods: Plasma samples from PE patients (n: 210) were analyzed for anti-PF4 antibody isotypes and various thrombo-inflammatory cytokines utilizing commercially available biochip array and ELISA methods. The endogenous GAG levels in PE patients' plasma were quantified using a fluorescence quenching method. The collected data analyzed to demonstrate the relationship between various parameters.

Results: The endogenous GAG levels were increased in the PE group ($P < .05$). The levels of anti-PF4 antibody isotypes were higher in varying levels in comparison to the normal group ($P < .05$). Inflammatory cytokines have shown varying levels of increase with IL-6, IL-8 and IL-10 showing the most pronounced values. Mortality outcome was related to increased GAGs and some of the cytokines.

Conclusion: In this study, we demonstrated increased levels of anti-PF4 antibody isotypes, endogenous GAGs, and inflammatory biomarkers in a large patient cohort in PE. The levels of the endogenous GAGs and inflammatory biomarkers were associated with PE severity and mortality. More studies are needed to understand this complex pathophysiology.

Keywords

pulmonary embolism, inflammatory biomarkers, glycosaminoglycans, anti-PF4 antibodies, COVID-19

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Introduction

Venous thromboembolism (VTE), which includes deep vein thrombosis (DVT) and pulmonary embolism (PE), is the third leading cause of cardiovascular death.¹ Endothelial injury, hypercoagulability and stasis of the blood flow are described as the main factors associated with the development of VTE.² While complex pathophysiological pathways contribute to the development and progression of VTE, mounting evidence suggests that the inflammation has a major role in its development.³

Many established clinical VTE risk factors likely cause thrombosis through the inflammatory pathways.⁴ Studies have

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shown that inflammatory cascades are responsible not only for development but also resolution of thrombotic events.⁵ Furthermore, the presence of an established embolism has been shown to be a source of inflammation.⁶ The increased levels of cytokines have been shown in various epidemiologic and clinical studies of venous thrombosis. Their relevance has also been observed in cell culture studies and animal models of acute and chronic venous thrombosis. However, their causative role in acute PE remains unclear.⁷

HIT is a serious immune-mediated adverse drug reaction that is caused by the production of anti-PF4 (PF4) antibodies that target PF4 bound to heparin, leading to catastrophic venous and arterial thrombosis.^{8,9} Several studies have shown that the interplay of endothelial damage, inflammation, and the generation of anti-PF4 antibodies, may contribute to the outcome of HIT in acute PE patients.¹⁰ During the course of HIT, anti-PF4 antibody isotypes of IgG, IgM, and IgA can develop, however, only a subset of patients exposed to heparin produce IgG-specific anti-PF4 antibodies and a smaller subset of patients produce pathogenic antibodies that can cause clinical HIT syndrome.^{11,12} HIT can also occur in patients who have not been exposed to heparin which suggests the complex interplay of various factors in its development. These factors can be listed as the severity of tissue damage, exposure to heparins in different types, doses and durations, the amount and ratio of PF4 protein and polyanions that circulate in the bloodstream, activation of T-cell dependent and independent immune pathways, temporary loss of immune tolerance, activation of innate immunity and genetic predisposition.¹³ Although the relevance of anti-PF4 isotypes have been investigated in various disease states, the information in acute PE patients is scarce.

The vascular endothelium confers important functions in circulatory homeostasis. It is responsible for regulation of vascular tone, control of hemostasis and thrombosis, cellular adhesion, smooth muscle cell proliferation, and vascular inflammation.¹⁴ While the functional endothelium prevents thrombosis by producing prostacyclins, NO, and the ectonucleotidase CD39, it also has fibrinolytic properties and can produce tissue plasminogen activator (tPA) and urokinase plasminogen activator (uPA).^{15,16} In previous studies, several endothelial biomarkers have been suggested for the investigation of endothelium in thrombotic states.^{17,18} Although these biomarkers have been tested in various disease states, current knowledge on their use in acute PE is limited.

Endothelial glycocalyx is a mixture of glycosaminoglycans (GAGs) attached to proteins and regulates the access of cells and molecules in the blood to the endothelium. Studies have shown that severe inflammation can cause glycocalyx dysfunction causing enzymatic degradation (shedding) of its components.¹⁹ After an insult, matrix metalloproteases dissolve the endothelial glycocalyx, and release the endogenous GAGs into the circulation.²⁰ The shedding of endothelial glycocalyx has been shown in certain disease states such as sepsis, viral infections and cardiovascular disease as pathogenic factors and recognized to have diagnostic or prognostic implications.²¹ However, their relevance has not been determined in large scale

studies. In this regard, Heparin Red probe is a novel fluorescence assay which quantifies polyanionic sulfated GAGs with a sensitivity range of 0-6 µg/ml.²² Considering the fact that only heparins or other endogenous polyanions (eg, heparin sulfate, hyper-sulfated chondroitin sulfate, nucleic acids, polyphosphates, lipid-A) can induce the conformational changes in PF4 which is required to generate anti-PF4 antibodies, using this method may help to better understand the mechanisms of endothelial damage, inflammation and the generation of anti-PF4 antibodies that can lead to the development of HIT in acute PE.²³

The mainstay of treatment in acute PE is systemic anticoagulation.²⁴ However, the patients that present with high-risk features for decompensation, systemic anticoagulation alone may not be sufficient. In these patients' pharmacological thrombus breakdown with thrombolytic therapy or clot removal using surgical or percutaneous techniques may have great importance for the outcome.²⁵ Several institutional guidelines have been published in risk stratification of acute PE.^{26,27} While d-dimer is mostly used as a non-specific biomarker for the diagnosis of VTE, only the levels of brain natriuretic peptide (BNP) and NT-proBNP, cardiac troponin and lactate have been validated for use in risk prediction of acute PE.²⁸⁻³³ More studies are needed to find a better biomarker in diagnosis and risk prediction of acute PE.

In this study, we sought to quantify the levels of anti-PF4 antibody isotypes and endogenous GAGs together with inflammatory biomarkers in the same patient cohort to determine whether there is a relationship between them in. Identification of this relationship may provide insight to the complex pathophysiology of PE and HIT and may be useful for development of potential prognostic, diagnostic and therapeutic interventions. These findings may also be valuable in the understanding of the pathogenesis of thrombocytopenia observed in PE patients and in COVID-19 associated reduction of platelets.

Materials and Methods

Patient Selection and Data Collection

Patients 18 years or older were recruited to participate in this study through enrollment conducted in conjunction with an ongoing IRB approved project by the Pulmonary Embolism Response Team (PERT) registry. Diagnosis of acute PE was confirmed by Computed Tomographic (CT) angiography or ventilation/perfusion imaging. Patients were classified into sub-categories of low risk, sub-massive, and massive PE according to the American College of Cardiology (ACC)/American Heart Association (AHA) guidelines. Table 1 depicts the distribution of co-morbidities in PE patients, including demographic information, collected through the review of patient electronic medical records (EMR).

Blood Samples

Whole blood samples were drawn from patients within 24 hours of confirmed diagnosis of acute PE and collected under an

Table 1. Characteristics of Patients Included in the Analysis.

	PE Patients Profiled for Anti-PF4 Ig A and Ig M (n: 119)	PE Patients Profiled for Anti-PF4 Ig G (n: 196)	PE Patients Profiled for Endogenous GAGs (n: 143)	PE Patients Profiled for Inflammatory Biomarkers (n: 98)
Age (Median ± standard deviation)	63 ± 13.5	62 ± 14.2	60 ± 14.1	62.5 ± 13.5
Gender n (%)	Female: 60 (50.4%) Male: 59 (49.6%)	Female: 100 (51.0%) Male: 96 (49.0%)	Female: 74 (51.7%) Male: 69 (48.3%)	Female: 48 (49.0%) Male: 50 (51.0%)
Body Mass Index (kg/m ² , median + IQR)	30.75 (24.84-37.37)	31.32 (25.73-37.20)	30.60 (25.83-37.09)	30.21 (25.07-37.26)
Hypertension n (%)	68 (57.1%)	106 (54.1%)	72 (50.3%)	53 (54.1%)
Diabetes Mellitus n (%)	31 (26.1%)	47 (24.0%)	29 (20.2%)	20 (20.4%)
Chronic Kidney Disease n (%)	14 (11.8%)	23 (11.7%)	18 (12.6%)	14 (14.3%)
Cancer n (%)	43 (36.1%)	62 (31.6%)	45 (31.5%)	38 (38.8%)
Acute DVT n (%)	34 (28.6%)	55 (28.1%)	39 (27.3%)	32 (32.7%)
PE Severity n (%) (ACC/AHA)	Low Risk 24 (20.2%) Sub massive 84 (70.6%) Massive 11 (9.2%)	Low Risk 52 (26.5%) Sub massive 129 (65.8%) Massive 15 (7.7%)	Low Risk 39 (27.3%) Sub massive 92 (64.3%) Massive 12 (8.4%)	Low Risk 19 (19.4%) Sub massive 70 (71.4%) Massive 9 (9.2%)
PESI Score n (%)	Class I-II (Low Risk): 33 (27.7%) Class III (Intermediate Risk): 18 (15.9%) Class IV-V (High risk): 68 (57.1%)	Class I-II (Low Risk): 63 (32.1%) Class III (Intermediate Risk): 29 (14.8%) Class IV-V (High risk): 104 (53.1%)	Class I-II (Low Risk): 43 (30.1%) Class III (Intermediate Risk): 26 (18.2%) Class IV-V (High risk): 74 (51.7%)	Class I-II (Low Risk): 24 (24.5%) Class III (Intermediate Risk): 19 (19.4%) Class IV-V (High risk): 55 (56.1%)
sPESI Score n (%)	Low Risk: 20 (16.8%) High Risk: 99 (83.2%)	Low Risk: 42 (21.4%) High Risk: 154 (78.6%)	Low Risk: 30 (21.0%) High Risk: 113 (79.0%)	Low Risk: 15 (15.3%) High Risk: 83 (84.7%)

Institutional Review Board approved protocol. Samples were collected in 3.8% (0.109 mol/L) sodium citrate tubes at the time of PE diagnosis, processed for platelet-poor plasma, and stored at -7°C prior to analysis. Control plasma samples from healthy, non-smoking, adults, aged 19 to 53, were purchased from a commercially available source (George King Biomedical, Overland Park, Kansas).

Measurement of Anti-PF4 Antibodies

The ELISA Zymutest HIA IgG/A/M® was commercially obtained (Aniara Diagnostica, West Chester, OH, USA) and performed according to the manufacturer's instructions. Fifty microliters of platelet lysate providing PF4 were placed in each well of a microtiter plate coated with biologically available UFH. Two hundred microliters of diluted control (negative or positive) or patient plasma (1:100) were added to the wells and incubated for 1 hour (h) at room temperature (RT). Unbound antibodies were then washed away and 200 μL of IgG/A/M-HRP immunoconjugate were added to the wells and incubated for 1 hour at RT. Unbound immunoconjugate was then washed away and tetramethylbenzidine substrate (TMB) was introduced into the wells (200 μL) immediately after washing. The color development was stopped by introducing 50 μL of 0.45 M sulfuric acid after exactly 5 minutes at RT. Absorbance was read at 450 nm. Each plasma sample was tested with specific assays for IgG (Zymutest HIA IgG®), IgA (Zymutest HIA IgA®) and IgM (Zymutest HIA IgM®) performed with the same procedure but using specific immunoconjugates.

Measurement of Endogenous Glycosaminoglycans

Heparin Red method based on fluorescence quenching method was used. The reagents were obtained from Red Probes (Munster, Germany). For determination of endogenous GAG concentrations in plasma samples, the protocol of the provider for a 96-well microplate assay was followed. A mixture of 10 000 μL Heparin Red solution and 90 μL Enhancer solution was freshly prepared. Twenty microliters of the patient or healthy control sample were pipetted into a microplate well, followed by 80 μL of the Heparin Red-Enhancer mixture. Immediately after mixing, the micro-plate was introduced in the fluorescence reader (Biotek Cytation-5 microplate reader) and fluorescence recorded within 1 min.

Measurement of Inflammatory Biomarkers

A Randox Investigator Cytokine and Growth Factors High-Sensitivity Array was utilized to measure IL-2, IL-4, IL-6, IL-8, IL-10, VEGF, IFN- γ , TNF- α , IL-1 α , IL-1 β , MCP-1 and EGF (Randox Laboratories, London, United Kingdom) per manufacturer guidelines. Quantification of all factors were tested simultaneously by utilizing a sandwich chemiluminescent immunoassay using a single patient sample. D-dimer

was measured using an ELISA based immunoassay (Hyphen Biomedical).

Statistical Analysis

Circulating levels of each biomarker in PE patient plasma were compared to control plasma. Descriptive statistics were calculated utilizing SPSS (v.24.0) (SPSS Inc., Chicago, IL, USA) and GraphPad Prism version 9.2.0 (GraphPad Software, La Jolla, California). Statistical difference between PE groups and normal controls were evaluated utilizing nonparametric Mann-Whitney U, Student t-tests and Kruskal-Wallis ANOVA. Correlations utilized Spearman correlation coefficients. $P < .05$ was considered statistically significant.

Results

Patient Demographics

The results of the 210 patients profiled for anti-PF4 Ig A and Ig M (n: 119), anti-PF4 Ig G (n: 196), endogenous GAGs (n: 143) and inflammatory biomarkers (n: 98) included in the final analysis. The distribution and general characteristics of the patients were similar and are shown in Table 1.

Anti-PF4 Antibodies and Endogenous GAGs

As shown in Figure 1, the levels of anti-PF4 Ig A, Ig G and endogenous GAGs were significantly elevated in acute PE patients compared to normal healthy individuals ($P < .05$). The increase in anti-PF4 Ig M was not statistically significant ($P: .78$).

Composite data, including the average, median, and range for each anti-PF4 antibody isotype and endogenous GAGs are shown in Table 2.

As shown in Figure 2, there was no significant difference in the levels of anti-PF4 antibody isotypes according to severity of PE in the patients ($P > .05$). The levels of endogenous GAGs were significantly higher (2.3-fold increase) in massive acute PE patients ($P < .001$). There was no significant difference in the levels of anti-PF4 antibody isotypes and endogenous GAGs according to PESI and sPESI scores ($P > .05$). Composite data, including the average and standard deviation for each anti-PF4 antibody isotype and endogenous GAGs according to acute PE severity are shown in Table 3.

As shown in Figure 3, there was no significant difference in the levels of anti-PF4 antibody isotypes according to 30-day mortality of acute PE patients ($P > .05$). The levels of endogenous GAGs were significantly higher (2.0-fold increase) among patients deceased within 30 day of PE diagnosis ($P < .001$). Composite data, including the average and standard deviation for each anti-PF4 antibody isotypes and endogenous GAGs according to 30-day mortality are shown in Table 4.

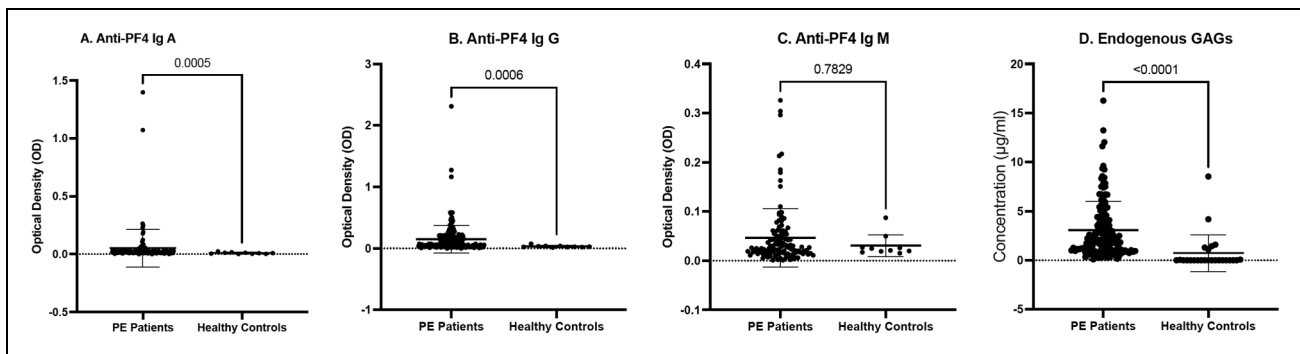


Figure 1. The Levels of Anti-PF4 Antibody Isotypes and Endogenous GAGs in acute PE patients and healthy controls. The levels of anti-PF4 Ig A, Ig G and endogenous GAGs were significantly elevated in acute PE patients compared to normal healthy individuals (Panel A, B and D). The increase in anti-PF4 Ig M was not statistically significant (Panel C).

Table 2. Levels of Anti-PF4 Antibody Isotypes and Endogenous GAGs in PE Patients and Healthy Controls.

Marker	PE patients			Healthy controls (n:10)			P value
	Mean \pm SEM	Median	Range	Mean \pm SEM	Median	Range	
Anti-PF4 Ig A	0.0519 \pm 0.0149	0.0190	0–1.398	0.0090 \pm 0.0021	0.0081	0.0012–0.0231	<.001
Anti-PF4 Ig G	0.1502 \pm 0.0162	0.0800	0–2.312	0.0316 \pm 0.0051	0.0265	0.0151–0.0731	<.001
Anti-PF4 Ig M	0.0465 \pm 0.0054	0.0250	0.0010–0.3260	0.0306 \pm 0.0070	0.0228	0.0153–0.0873	.78
Endogenous GAGs	PE patients 3.0760 \pm 0.2434	2.0350	0.0937–16.27	Healthy controls (n:25) 0.7328 \pm 0.3757	0.00	0–8.5425	<.001

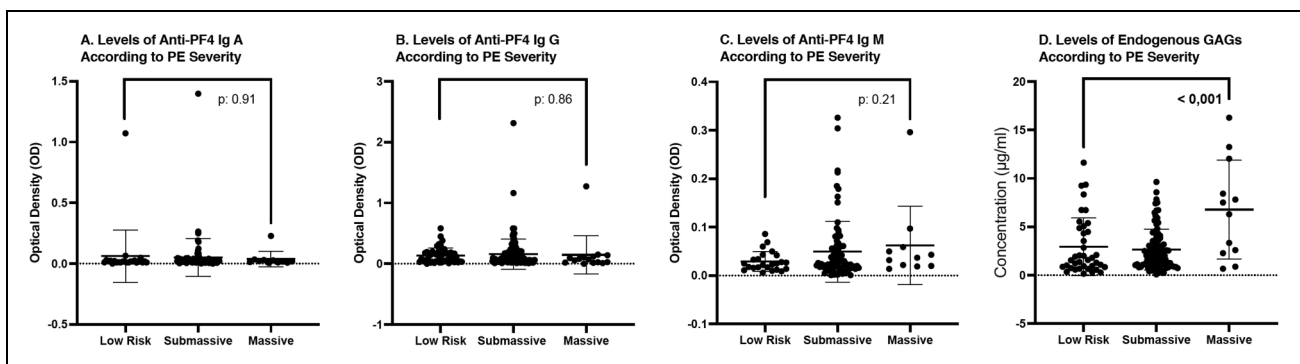


Figure 2. The Levels of Anti-PF4 Antibody Isotypes and Endogenous GAGs According to PE Severity. There was no significant difference in the levels of anti-PF4 antibody isotypes according to severity of acute PE (Panel A, B and C). The levels of endogenous GAGs were significantly higher in massive acute PE patients (Panel D).

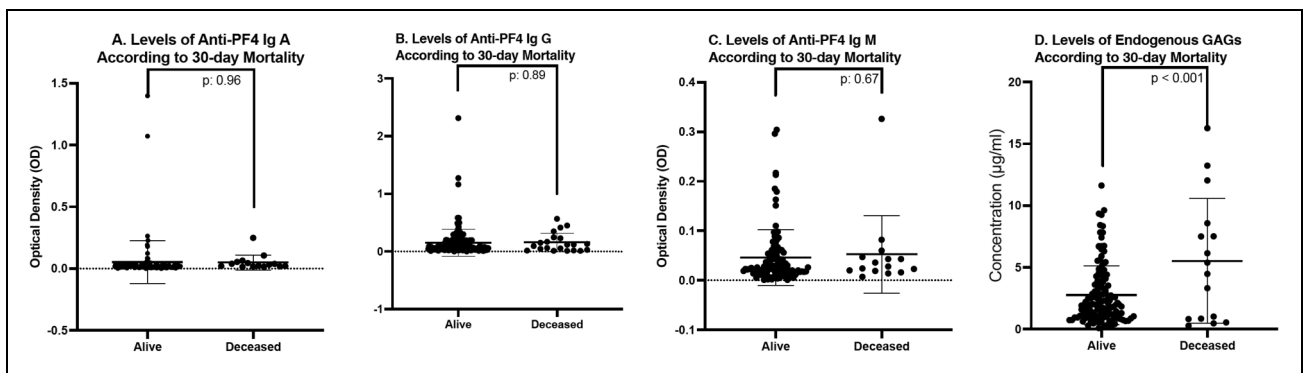
Inflammatory Biomarkers

The levels of IL-4 (1.4-fold increase), IL-6 (12.1-fold increase), IL-10 (24.5-fold increase), IL-1a (1.6-fold increase), MCP-1 (2.6-fold increase) and d-dimer (4.9-fold increase) were significantly higher according to the severity of acute PE ($P < .05$). The data, including the average and standard deviations for each biomarker according to severity of acute PE patients are shown in Table 5.

The levels of IL-2 (3.6-fold increase), IL-4 (1.3-fold increase), IL-6 (4.8-fold increase), IL-8 (5.2-fold increase), IL-10 (4.8-fold increase), VEGF (3.0-fold increase) and MCP-1 (2.3-fold increase) were significantly higher in patients who are deceased within 30 day of acute PE diagnosis. The data, including the average and standard deviations for the levels of each biomarker according to 30-day mortality are shown in Table 6.

Table 3. Levels of Anti-PF4 Antibody Isotypes and Endogenous GAGs According to PE Severity.

Marker	Low risk (Mean \pm SD; n:24)	Sub massive (Mean \pm SD; n:84)	Massive (Mean \pm SD; n:11)	P value
Anti-PF4 Ig A	0.0620 \pm 0.2155	0.0510 \pm 0.1560	0.0368 \pm 0.0637	0.91
Anti-PF4 Ig M	0.0290 \pm 0.0203	0.0494 \pm 0.0628	0.0626 \pm 0.0809	0.21
Anti-PF4 Ig G	Low risk (Mean \pm SD; n:52) 0.1359 \pm 0.1206	Sub massive (Mean \pm SD; n:129) 0.1564 \pm 0.2489	Massive (Mean \pm SD; n:15) 0.1462 \pm 0.3155	0.86
Endogenous GAGs	Low risk (Mean \pm SD; n:39) 2.9489 \pm 2.9689	Sub massive (Mean \pm SD; n:92) 2.6461 \pm 2.0975	Massive (Mean \pm SD; n:12) 6.7821 \pm 5.0924	<0.001

**Figure 3.** The Levels of Anti-PF4 Antibody Isotypes and Endogenous GAGs According to 30-day Mortality. There was no significant difference in the levels of anti-PF4 antibody isotypes according to 30-day mortality of acute PE patients (Panel A, B and D). The levels of endogenous GAGs were significantly higher among patients deceased within 30 day of PE diagnosis (Panel D).**Table 4.** Levels of Anti-PF4 Antibody Isotypes and Endogenous GAGs According to 30-day Mortality in Acute PE Patients.

Marker	Alive (Mean \pm SD; n: 104)	Deceased (Mean \pm SD; n: 15)	P value
Anti-PF4 Ig A	0.0522 \pm 0.1730	0.0503 \pm 0.0602	.96
Anti-PF4 Ig M	0.0457 \pm 0.0563	0.0525 \pm 0.0781	.67
Anti-PF4 Ig G	Alive (Mean \pm SD; n:175) 0.1494 \pm 0.2343	Deceased (Mean \pm SD; n:21) 0.1563 \pm 0.1623	.89
Endogenous GAGs	Alive (Mean \pm SD; n:127) 2.7669 \pm 2.3769	Deceased (Mean \pm SD; n:16) 5.5274 \pm 5.0521	<0.001

Correlations between Inflammatory Biomarkers, Anti-PF4 Antibody Isotypes and Endogenous GAGs

Anti PF4 Ig A were correlated with IL-2 (Spearman r : 0.294; P : <.01), IL-6 (Spearman r : 0.251; P : .01) and TNFA (Spearman r : 0.3168; P : <.01). Anti PF4 Ig G were correlated with IL-6 (Spearman r : 0.291; P : <.01) and d-dimer (Spearman r : 0.239; P : <.01). Anti PF4 Ig M were correlated with IL-2 (Spearman r : 0.347; P : .001) and MCP-1 (Spearman r : -0.245; P : .02). The levels of endogenous GAGs were correlated with TNF- α (Spearman r : 0.413; P : <.001), IL-10 (Spearman r : 0.357; P : <.01), IL-8 (Spearman r : 0.324; P : <.01), IL-1 β (Spearman r :

0.253; P : .03), IL-1 α (Spearman r : 0.241; P : .04) and VEGF (Spearman r : -0.257; P : .03). Correlations between cytokine concentrations, Anti PF4 antibody isotypes and endogenous GAGs in acute PE patients are shown in Table 7, with bolded values indicating the significant statistical correlation (P <.05).

As shown in Figure 4, we observed significant correlations between the levels of anti-PF4 antibody isotypes. Anti-PF4 Ig G was correlated with anti-PF4 Ig A (r =0.438, P <.001) and anti-PF4 Ig M (r =0.242, P <.01) and anti-PF4 Ig A was correlated with anti-PF4 Ig M (r =0.424, P <.001). However, we did not observe any significant correlation between the levels of anti-PF4 antibody isotypes and endogenous GAGs.

Table 5. Levels of Inflammatory Biomarkers According to PE Severity (n: 98).

Marker	Low Risk (n:19) Mean \pm SD	Submassive (n:70) Mean \pm SD	Massive (n:9) Mean \pm SD	P value
IL-2	0.5974 \pm 0.5932	1.4424 \pm 2.7177	1.5167 \pm 0.9867	.36
IL-4	1.5705 \pm 0.4951	1.8001 \pm 0.5743	2.3167 \pm 0.9131	.01
IL-6	22.0700 \pm 28.2276	58.5574 \pm 106.1068	267.1400 \pm 327.4290	<.001
IL-8	20.3826 \pm 31.8997	46.0836 \pm 107.8305	116.5678 \pm 218.5944	.11
IL-10	1.2895 \pm 1.8854	1.6087 \pm 1.9855	31.6411 \pm 45.7101	<.001
VEGF	29.7147 \pm 33.5555	25.1127 \pm 35.8555	19.1633 \pm 17.5963	.74
IFN- γ	0.2742 \pm 0.3110	0.3380 \pm 0.4659	0.2856 \pm 0.3759	.82
TNF- α	1.8242 \pm 0.7162	2.8134 \pm 3.9524	3.3689 \pm 2.3085	.44
IL-1 α	0.1889 \pm 0.1236	0.1787 \pm 0.1198	0.3211 \pm 0.2977	.02
IL-1 β	0.8658 \pm 0.8139	1.4980 \pm 2.2358	2.5678 \pm 1.8841	.11
MCP-1	128.4768 \pm 70.2241	153.4181 \pm 149.0921	338.2378 \pm 211.6992	.001
EGF	36.3011 \pm 36.8114	28.6001 \pm 35.3324	19.0978 \pm 16.7696	.45
D-dimer	2799.71 \pm 2230.44	7374.94 \pm 5702.56	13845.19 \pm 10143.66	<.001

Table 6. Levels of Inflammatory Biomarkers According to 30-Day Mortality (n: 98).

Marker	Alive (n: 85) Mean \pm SD	Deceased (n: 13) Mean \pm SD	P value
IL-2	0.9553 \pm 1.2005	3.4438 \pm 5.3547	<.001
IL-4	1.7289 \pm 0.4840	2.2877 \pm 1.0819	.002
IL-6	46.9501 \pm 95.5216	225.5269 \pm 277.6988	<.001
IL-8	30.5240 \pm 82.8662	159.0531 \pm 203.4784	<.001
IL-10	2.8560 \pm 14.1820	13.7785 \pm 22.7636	.02
VEGF	20.0978 \pm 22.2937	60.5100 \pm 65.8268	<.001
IFN- γ	0.3129 \pm 0.4200	0.3723 \pm 0.5054	.64
TNF- α	2.5236 \pm 3.6071	3.6469 \pm 1.8706	.27
IL-1 α	0.1885 \pm 0.1385	0.2285 \pm 0.2058	.36
IL-1 β	1.3406 \pm 2.0668	2.3438 \pm 1.6560	.09
MCP-1	141.2945 \pm 112.7693	324.1877 \pm 264.6509	<.001
EGF	29.7336 \pm 34.2046	25.8654 \pm 36.7835	.70
D-dimer	6885.66 \pm 6453.72	8366.60 \pm 5768.08	.43

Discussion

One of the important features of our study is the novel observation of the increased levels of heparin red sensitive GAGs in the PE patients. While the level of endogenous GAGs widely varied in the PE population, these GAGs may have formed complexes with the PF4 which is released from the platelet upon activation with thrombin or other activators. This complex is similar to the complex formed with the heparin and may have triggered the allosteric changes in the PF4 resulting in the generation of HIT-like antibodies. This process is illustrated in Figure 5. The generated antibodies subsequently play the role in the up-regulation of the thrombo-inflammatory biomarkers. In the profiling of the PE patients for these biomarkers, marked elevation were noted with significant relationships with the HIT antibodies. This is suggestive of regulatory role of these antibodies in triggering the high levels of inflammatory biomarkers, most likely there is an interplay between these antibodies and the inflammatory mediators. Interestingly, the GAG levels did not correlate with the

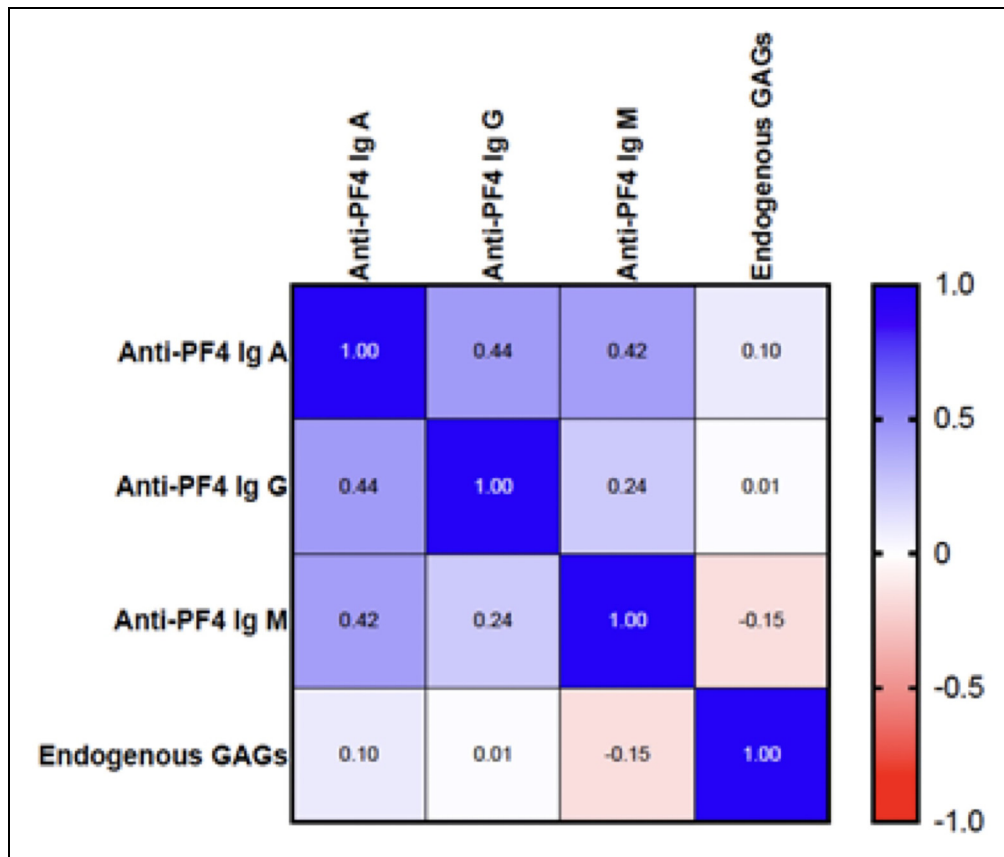
elevation of anti-heparin-PF4 antibodies. However, there was an apparent relationship with some of the inflammatory biomarkers which may be suggestive the direct role of endothelial-released GAGs and inflammatory process. These observations are suggestive of important pathophysiologic role of endogenous GAGs in mediating the observed vascular pathogenesis in PE and related syndromes.

In this study, our results have shown that the levels of anti-PF4 Ig A, Ig G and endogenous GAGs were significantly elevated in acute PE patients compared to normal healthy individuals. Interestingly, we did not observe a significant difference in anti-PF4 Ig M levels. In the classical T cell-dependent pathway of HIT immune response, Ig M antibodies are produced following primary antigen exposure. Following secondary antigen exposure, memory B cells rapidly differentiate into plasma cells that produce high-affinity IgG antibodies.³⁴ However, T cell-independent antibody response of HIT is predominated by IgM with no secondary IgG antibody responses.³⁵ Additionally, antibody class switching from IgM to IgG and Ig A is characteristic of a T cell-dependent response.³⁶ The reason that we did not observe an increase in anti-PF4 Ig M in acute PE patients may be related with the predominance of the T-cell dependent antibody production in our patient group. This finding could be explained if the inflammatory process in these patients started before the time of the diagnosis. Additionally, this observation was also evident in the correlation analysis.

Furthermore, Anti-PF4 Ig M and Ig A have shown the strongest correlations with IL-2, and anti-PF4 Ig G have shown the strongest correlations with IL-6. It is important to note that IL-2 is a well-known cytokine that promotes the activation, proliferation, and differentiation of helper and regulatory T cells.³⁷ On the other hand, IL-6 which is produced in response to tissue damage, is a strong proinflammatory cytokine. It can further stimulate T-cell functions and is a potent B cell activator.³⁸⁻⁴⁰ Additionally, the levels of endogenous GAGs were significantly correlated with TNF- α , IL-10, IL-8, IL-1 β , IL-1 α and VEGF, which are mostly nonspecific pro-inflammatory

Table 7. Correlations between Inflammatory Biomarkers, Anti-PF4 Antibody Isotypes and Endogenous GAGs.

Markers	Anti-PF4 Ig A		Anti-PF4 Ig G		Anti-PF4 Ig M		Endogenous GAGs	
	Spearman R	P value	Spearman R	P value	Spearman R	P value	Spearman R	P value
IL-2	0.294	<.01	0.181	.09	0.347	.001	0.019	.87
IL-4	-0.074	.49	0.011	.91	0.102	.34	0.060	.626
IL-6	0.251	.01	0.291	<.01	0.016	.88	0.203	.09
IL-8	0.042	.69	-0.029	.78	-0.141	.19	0.324	<.01
IL-10	-0.016	.88	-0.042	.70	-0.089	.41	0.357	<.01
VEGF	0.171	.122	0.102	.34	0.090	.40	-0.257	.03
IFN- γ	-0.040	.71	0.020	.85	0.079	.46	0.053	.66
TNF- α	0.212	.04	0.074	.49	-0.039	.72	0.4130	<.001
IL-1 α	-0.069	.52	0.049	.65	0.098	.36	0.241	.04
IL-1 β	0.145	.18	0.012	.91	0.139	.19	0.253	.03
MCP-1	-0.007	.94	-0.177	.10	-0.245	.02	0.173	.15
EGF	-0.195	.07	-0.009	.93	-0.136	.20	0.130	.28
D-dimer	0.133	.14	0.239	<.01	0.033	.72	0.136	.26

**Figure 4.** Heatmap that shows the correlations between the levels of anti-PF4 isotypes and endogenous GAGs.

biomarkers except IL-10 and VEGF. These findings indicate that, in contrary to production of anti-PF4 antibodies, the release of endogenous GAGs is less dependent of T-cell specific pathways.⁴¹ In parallel to this, although correlations have been observed in between anti-PF4 antibody isotypes, there were no correlations between the levels of endogenous GAGs and anti-PF4 antibodies. This reminds that although inflammation

have a role in their formation, different inflammatory pathways are responsible for their development.

In our current study we were able evaluate all the tested biomarkers according to severity and 30-day mortality. Among these biomarkers, while IL-6 (12.1-fold increase), IL-10 (24.5-fold increase) and MCP-1 (2.6-fold increase) have shown the strongest fold increase for acute PE severity, IL-2

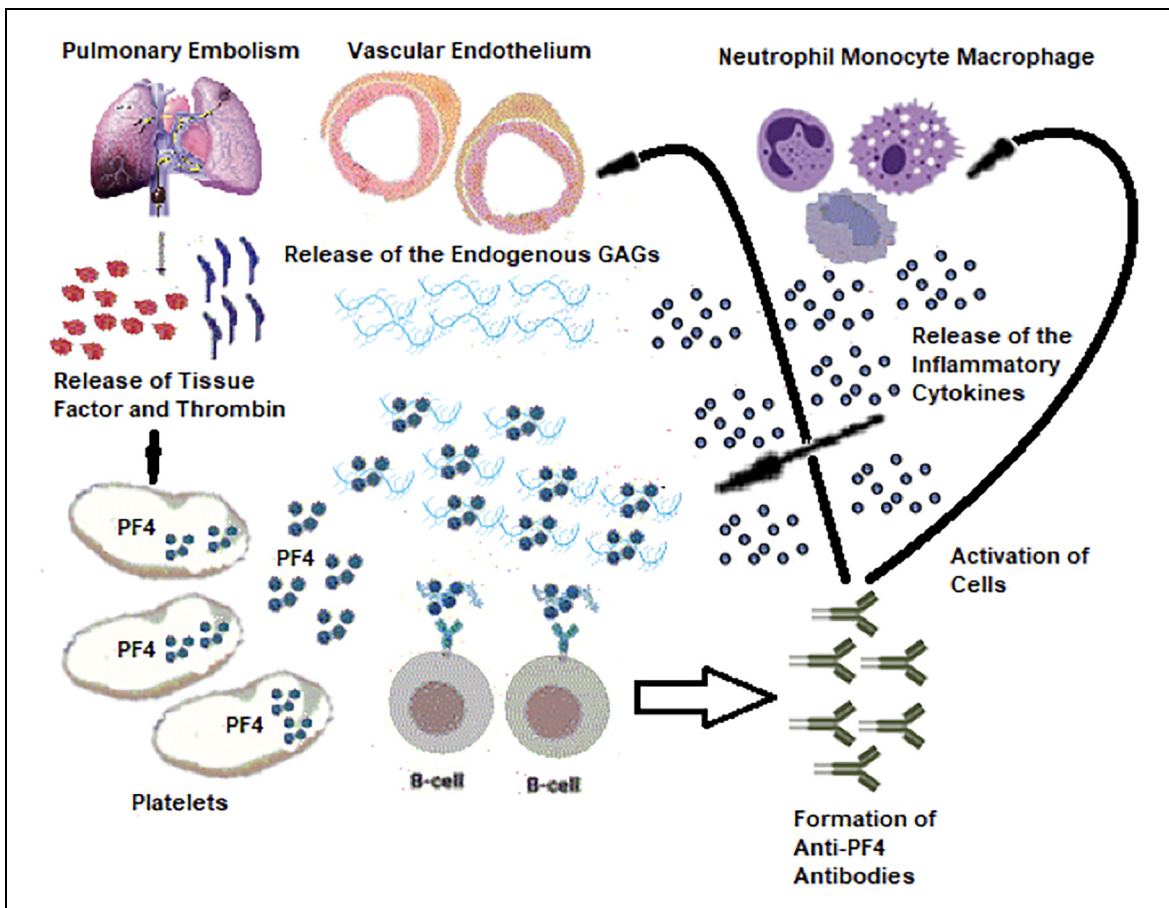


Figure 5. Pulmonary embolism results in the generation of thrombin and tissue factor which are capable of activating platelets and cells. Damaged endothelium results in the generation of GAGs which complex with the PF4 released from platelets. This complex results in the generation of anti-heparin-PF4 antibodies which in turn further amplify activation of cells and platelets resulting in the formation of inflammatory mediators.

(3.6-fold increase), IL-6 (4.8-fold increase), IL-8 (5.2-fold increase), IL-10 (4.8-fold increase), VEGF (3.0-fold increase) and MCP-1 (2.3-fold increase) have shown prominent increases for 30-day mortality. IL-6 is maybe the most studied cytokine during inflammation and has been associated as a risk factor for VTE. IL-6 promotes coagulation without affecting fibrinolysis. It increases TF production and factor VIII transcription, along with fibrinogen production. It also enhances platelet production and endothelial activation.^{42,43} In our patient group, higher levels of IL-6 were associated with severity and 30-day mortality of acute PE. IL-8 is a chemokine that activates integrin-mediated adhesion of neutrophils and a powerful trigger for adhesion of monocytes to vascular endothelium. IL-8 has been shown to induce tissue factor on monocytes, facilitates leucocyte recruitment, and contributes to the induction of a procoagulant surface by triggering the adhesion of monocytes to the endothelium.⁴⁴ In our study, higher levels of IL-8 were associated with 30-day mortality.

IL-10 is an anti-inflammatory cytokine with immunosuppressive properties. In the previous studies, the association of IL-10 with VTE have yielded mixed results. While some of these

studies reported no significant association, other studies have reported increasing or decreasing levels of IL-10 in relation with thrombosis.⁴⁵⁻⁴⁷ In our study increased levels of IL-10 have shown significant relationship with the severity and mortality of acute PE. This finding may be the result of a counter-regulatory response of IL-10 to high level of systemic inflammation in acute PE patients. MCP-1 is a potent and specific activator of monocytes and causes migration of monocytes in vitro. In venous thrombosis MCP-1 levels increase during natural resolution. MCP-1 treatment increased the organization and resolution of thrombi.⁴⁸ In our study, higher levels of MCP-1 were associated with severity and 30-day mortality of acute PE.

The role of VEGF in venous thrombosis is mostly investigated in cancer-associated thrombosis. VEGF is secreted in response to hypoxia, which stimulates angiogenesis by its downstream pathways.^{49,50} In PE it has been shown that its levels can be increased.⁵¹ In our study, higher levels of VEGF were associated with the mortality of PE. It is important to note that approximately 30-40% of patients in our study group had a cancer diagnosis. IL-2 has not been previously reported in association with VTE. However, IL-2 administration has been associated with

microvascular thrombosis and it has been shown to increase platelet activation. Additionally, IL-2 therapy is known to cause the activation of vascular endothelial cells through the induction of cytokines such as TNF and IL-1.^{52–54} In our patient group, higher IL-2 levels were associated with 30-day mortality. Additionally, the levels of endogenous GAGs were also elevated according to severity (2.3-fold increase) and 30-day mortality (2.0-fold increase) in acute PE. This finding could suggest that glycocalyx shedding has a causative and potentially prognostic role in response to inflammatory cascades in acute PE patients. However anti-PF4 isotype levels did not show any difference for these outcomes.

Finally, although COVID-19 patients were not included in our study cohort, findings from our current study may also provide some insight to the pathophysiology of COVID-19 disease and recently reported rare events after COVID-19 vaccinations.⁵⁵ Previous studies have shown an increased incidence of thrombotic events especially acute PE in COVID-19 infections.⁵⁶ Additionally, while endothelial damage is a main component of COVID-19 associated coagulopathy, there is also an increased frequency of HIT antibody production reported.⁵⁷ Additionally, complex interactions of spike protein with endogenous GAGs and inflammatory mediators have been observed in vaccine induced thrombotic thrombocytopenia (VITT) patients.⁵⁸ From this point of view, future research on interventions targeting vascular endothelium particularly endothelial glycocalyx with GAGs, the role inflammatory cytokines, regulatory pathways of immune system may be of major importance in individual management of COVID-19 patients and rare events after COVID-19 vaccinations.

Study Limitations

In this study the timing of blood sample collection varied up to 48 hours. The observed variations may partly be due to difference in blood collection times. This study is based on single sample analysis and due to logistic reasons, follow-up samples were not collected and analyzed. Future studies should consider collecting sequential samples in follow-up analysis. Additionally, we did not consider the previous anticoagulant history and the type of anticoagulant used immediately after diagnosis. For this study, it is important to know the history of heparinization in terms of type of heparin and mode of administration. Additionally, we were not able to completely consider the clinical characteristics of the patients while including in this study. The generation of anti-PF4 antibodies and its binding role with heparin is detected in several other illnesses (particularly in cancer patients) other than acute PE. Despite these limitations the study clearly points to the increased thrombo-inflammatory response associated with the release of endogenous GAGs and presence of higher titer of anti-PF4 antibody isotypes.

Conclusion

In this study, we investigated the levels of anti-PF4 antibody isotypes, endogenous GAGs and inflammatory cytokines in a

large group of acute PE patients. Our results, including the relationship between the tested parameters and their relevance for the severity and mortality for acute PE, are of major importance. From this point of view, our study represents the first definitive approach to quantify endogenous GAGs and demonstrates their relationship to the generation of HIT antibodies and upregulation of inflammation in acute PE patients. More studies are needed to understand the complex pathophysiology of HIT and acute PE.

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Authors' Note

Ethical approval for the collection of residual blood samples was obtained through the Loyola University Chicago Institutional Review Board (LU#2094572). Written informed consent was obtained from participants for the use of their anonymized information.





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