DOCTORAL THESIS

UCAM
UNIVERSIDAD CATÓLICA DE MURCIA

INTERNATIONAL DOCTORAL SCHOOL

Doctoral Program in Health Sciences

A Comprehensive Profiling of Hemostatic Activation and Thromboinflammatory Biomarkers and Cellular Indices in Predicting Outcomes in Venous Thromboembolism

Author:

Fakiha Siddiqui

Supervisors:

Prof. Dr. Manuel Monreal

Murcia, 25, September 2024

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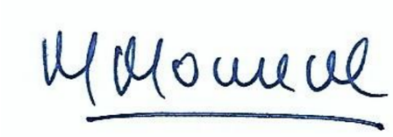
Prof. Dr. Manuel Monreal

Murcia, 25, September 2024

AUTHORIZATION OF THE DIRECTOR OF THE THESIS FOR SUBMISSION

Prof. Dr. Manuel Monreal as Director of the Doctoral Thesis "A Comprehensive Profiling of Hemostatic Activation and Thromboinflammatory Biomarkers and Cellular Indices in Predicting Adverse Outcomes in Venous Thromboembolism" by Ms. Fakiha Siddiqui in the Doctorate Program Health Science, **authorizes for submission** since it has the conditions necessary for her defense.

Sign to comply with the Royal Decrees 99/2011, in Murcia, September 25, 2024.



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ABSTRACT

Background: Despite advancements in diagnostic and therapeutic approaches, pulmonary embolism (PE) and deep vein thrombosis (DVT) in general remain challenging conditions due to their complex pathophysiological mechanisms. This thesis aims to comprehensively profile and evaluate the prognostic value of blood cellular indices and biomarkers in VTE with a specific focus on PE.

Materials and Method: This study was comprised of two independent groups of patients, namely the RIETE registry and LUMC PERT cohort. Blood count data was used from 10,000 patients from the RIETE registry and provided the basis for cellular indices calculations. Blood samples from the PERT study, comprised of 500 patients with acute VTE, were analyzed for both cellular indices and biomarker profiling. The calculation of cellular indices included NLR, PLR, and SII. Plasma samples were tested for thrombin generation potential, and a comprehensive profiling of hemostatic activation and thromboinflammatory biomarkers using ELISA and biochip array. Statistical analysis included a descriptive test (median with IQR) and a comparative analysis (Chi square and Mann Whitney U tests). Prognostic analyses were performed using ROC curves and multivariate logistic regression analysis. Data was stratified based on PE severity and 30-day mortality.

Results: Although the numbers of patients were different, both cohorts were demographically balanced. Among the VTE patients, NLR outperformed both PLR and SII for predicting mortality and major bleeding, while none of the cellular indices were predictive for VTE recurrence. Of all the cellular indices, NLR was found to be the best predictor for 30-day mortality in PE patients and demonstrated to have the highest predictive accuracy in low-risk PE patients. Furthermore, the incorporation of NLR in the clinical sPESI score enhanced the predictive power for identifying low-risk PE patients at increased risk of death. While thrombin generation potential was decreased in PE patients, thrombin generation markers such as D-dimer, F1+2, and TAT were increased. Combining the cellular indices with thrombin generation parameters amplified the predictive value for clinical outcomes in patients with PE. The relationships between thrombin generation potential, biomarkers, and cellular indices not only reinforced their relevance to the

pathology of PE but also emphasized the complexity of the disease including its severity. Additional, hemostatic activation and thromboinflammatory biomarker profiling including endothelial dysregulation, platelet activation, coagulation activation, fibrinolysis process, and inflammatory markers including tissue factor were also performed in the 500 PE patients. Most of these biomarkers were significantly elevated, consistent with PE severity, and 30-day mortality. Taken together, these data suggest that combined use of cellular indices with biomarker data may improve the risk stratification to determine outcomes in these patients. Finally, a biochip array utilizing panel profiling for cytokines, chemokines, and growth factors was employed. The elevated levels, along with cellular indices data, correlated with PE severity, and 30-day mortality outcome. These findings underscore the substantial predictive value of inflammatory markers, and cellular indices in VTE/PE.

Conclusion: This dissertation focused on the prediction of the adverse clinical outcomes in patients with VTE using cellular indices and circulating biomarkers of hemostatic activation and inflammation. The findings demonstrated their relevance to the cellular modulation and biomarker changes associated with VTE, and PE in particular. Together the RIETE registry and the PERT program at Loyola, a robust investigation was able to be undertaken to demonstrate a unique approach using molecular and cellular parameters to improve the diagnosis of VTE. Not only risk stratification and clinical outcome predictions were enhanced, but application of the new information obtained in this thesis may also have implications to improve individualized therapy and develop novel therapeutic interventions for patients with VTE. This thesis provides a novel approach to refine the understanding of the pathophysiology of VTE and to improve patient health care.

KEY WORDS

Hemostasis, thrombosis, venous thromboembolism, deep vein thrombosis, pulmonary embolism, coagulation cascade, fibrinolysis, endothelium, platelets, inflammation, adhesion molecules, inflammatory cytokines, chemokines, growth factors, mortality, bleeding.

RESUMEN

Antecedentes: A pesar de los avances recientes en el diagnóstico y tratamiento de la embolia pulmonar (EP) y la trombosis venosa profunda (TVP), siguen siendo enfermedades con complejos mecanismos fisiopatológicos. El objetivo de esta Tesis es perfilar y evaluar de manera integral el valor pronóstico de los índices celulares sanguíneos y otros biomarcadores en la trombosis con un enfoque específico en la EP.

Materiales y métodos: Este estudio está compuesto por dos grupos independientes de pacientes, uno procedente del registro RIETE y otro de la cohorte LUMC PERT. Se utilizaron datos del hemograma inicial de 10 000 pacientes del registro RIETE y proporcionaron la base para los cálculos de índices celulares. Las muestras de sangre del grupo PERT, compuesto por 500 pacientes con EP aguda, se analizaron para conocer los índices celulares y el perfil de biomarcadores. El estudio de los índices celulares incluyó el NLR, PLR y SII. Además, se estudió el potencial de generación de trombina y un perfil integral de activación hemostática y biomarcadores trombo-inflamatorios utilizando ELISA y una matriz de biochip. El análisis estadístico incluyó una prueba descriptiva (mediana con RIQ) y un análisis comparativo (pruebas de Chi cuadrado y U de Mann Whitney). Los análisis pronósticos se realizaron mediante curvas ROC y análisis de regresión logística multivariante. Los datos se estratificaron en función de la gravedad de la EP y la mortalidad a los 30 días.

Resultados: Aunque el número de pacientes era diferente, ambas cohortes estuvieron equilibradas demográficamente. Entre los pacientes con EP, el NLR superó al. PLR y al SII en su capacidad predictiva de mortalidad y sangrado grave, mientras que ninguno de los índices celulares fue predictivo de la recurrencia de trombosis. De todos los índices celulares, el NLR fue el mejor predictor de muerte a los 30 días en pacientes con EP, y además tuvo la mayor precisión predictiva en pacientes con EP de bajo riesgo. Además, la incorporación del NLR en la escala pronóstica sPESI mejoró su poder predictivo para identificar a los pacientes con EP de bajo riesgo con mayor riesgo de muerte. Si bien el potencial de generación de trombina disminuyó en los pacientes con EP, los marcadores de generación de trombina (como el dímero D, F1+2 y TAT) aumentaron. La combinación de los índices celulares con los parámetros de generación de trombina aumentó el valor

predictivo en los pacientes con EP. Las relaciones entre el potencial de generación de trombina, los biomarcadores y los índices celulares no solo reforzaron su relevancia en el pronóstico de la EP, sino que también enfatizaron la complejidad de la enfermedad, incluida su gravedad. Además, en los 500 pacientes con EP de la segunda cohorte se analizó la activación hemostática y un perfil de biomarcadores trombo-inflamatorios (desregulación endotelial, activación plaquetaria, activación de la coagulación, fibrinólisis y marcadores inflamatorios, incluido el factor tisular). La mayoría de estos biomarcadores estaban significativamente elevados, en consonancia con la gravedad de la EP y la mortalidad a los 30 días. En conjunto, estos datos sugieren que el uso combinado de índices celulares con datos de biomarcadores puede mejorar la estratificación del riesgo para determinar los resultados en estos pacientes. Finalmente, se empleó una matriz de biochips que utiliza perfiles de panel para citocinas, quimiocinas y factores de crecimiento. Los niveles elevados, junto con los datos de los índices celulares, se correlacionaron con la gravedad de la EP y el resultado de muerte a los 30 días. Estos hallazgos confirman el valor predictivo de los marcadores inflamatorios y los índices celulares en la EP. Conclusión: Esta tesis se centró en la predicción de resultados clínicos adversos en pacientes con EP utilizando índices celulares y biomarcadores de activación hemostática e inflamación. Los hallazgos demostraron su relevancia para la modulación celular y los cambios de biomarcadores asociados con la EP. Junto con el registro RIETE y el programa PERT en Loyola, se pudo realizar una investigación sólida para demostrar un enfoque único que utiliza parámetros moleculares y celulares para mejorar el pronóstico en los pacientes con EP. No solo mejoraron la estratificación del riesgo y las predicciones de resultados clínicos, sino que la aplicación de la nueva información obtenida también puede tener implicaciones para mejorar el tratamiento individualizado y desarrollar nuevas intervenciones terapéuticas para pacientes con EP. Esta Tesis proporciona un enfoque novedoso para mejorar nuestro conocimiento de la fisiopatología de la EP y mejorar la atención médica del paciente.

PALABRAS CLAVE

Hemostasia, trombosis, tromboembolismo venoso, trombosis venosa profunda, embolia pulmonar, cascada de coagulación, fibrinólisis, endotelio, plaquetas, inflamación, moléculas de adhesión, citocinas inflamatorias, quimiocinas, factores de crecimiento, mortalidad, sangrado.

DEDICATED



This thesis is dedicated to my beloved parents, Mr. Ghulam Mohammad Siddiqui (late) and Mrs. Khurshida Begum, whose unwavering support, immense strength, and boundless love have been my greatest inspiration throughout my journey, guiding me in every step I take. Their unwavering belief in me has been truly invaluable; you are my guiding light and my strength.

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‘Nothing in life is to be feared, it is only to be understood.
Now is the time to understand more, so that we may fear less’.
Marie Curie (1867-1934).

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ACRONYMS AND ABBREVIATIONS

AA, Arachidonic Acid

ADP, Adenosine Diphosphate

AF, Atrial Fibrillation

APC, Activated Protein C

ASCVD, Atherosclerotic Cardiovascular Disease

aOR, Adjusted Odds Ratio

AT, Antithrombin

ATP, Adenosine Triphosphate

AUC, Area Under the Curve

BAT, Biochip Array Technology

BNP, B-type Natriuretic Peptide

BPM, Beats per Minute

CAD, Coronary Artery Disease

cAMP, Cyclic Adenosine Monophosphate

CBC, Complete Blood Count

CCD, Charge-coupled Device

CI, Confidence Interval

cGMP, Cyclic Guanosine Monophosphate

CLRs, C-type Lectin Receptors

COAT, Collagen/Thrombin Activated

CrCl, Creatinine Clearance

CRP, C-Reactive Protein

CT, Computed Tomography

CTEPH, Chronic Thromboembolic Pulmonary Hypertension

CTPA, Computed Tomography Pulmonary Angiogram

CVD, Cardiovascular Disease

DAG, Diacylglycerol

DAMPs, Damage-associated Molecular Patterns

D-D, D-dimer

DIC, Disseminated Intravascular Coagulation

dL, Deciliter

DMSO, Dimethyl Sulfoxide

DNA, Deoxyribonucleic Acid

DNase-1, Deoxyribonuclease-1

DNase-1l3, Deoxyribonuclease-1-Like 3

DTR's, Discrete Test Regions

DVT, Deep Vein Thrombosis

ECHO, Echocardiogram

ECMO, Extracorporeal Membrane Oxygenation

ECP, Eosinophil Cationic Protein

EGD, Ethylene Glycol Dinitrate

EGF, Epidermal Growth Factor

ELAM-1, Endothelial Leukocyte Adhesion Molecule-1

ELISA, Enzyme-Linked Immunosorbent Assay

eNOS, Endothelial Nitric Oxide Synthase

EP3, E-type Prostanoid-3

EPX, Eosinophil Peroxidase

ESC, European Society of Cardiology

F, Factor

F1+2, Prothrombin Fragment 1+2

FVII, Coagulation factor VII

FIX, Coagulation factor IX

FX, Coagulation factor X

FXIII-A, Coagulation factor XIII-A

GAG, Glycosaminoglycan

GF, Growth Factor

GP, Glycoprotein

HF, Heart Failure

HMWK, High Molecular Weight Kininogen

HR, Heart Rate

HRP, Horseradish Peroxidase

IACUC, Institutional Animal Care and Use Committee

ICAM-1, Intracellular Adhesion Molecule-1

IFN-γ, Interferon-γ

IL-1α, Interleukin-1α

IL-1β, Interleukin-1β

IL-2, Interleukin-2

IL-4, Interleukin-4

IL-6, Interleukin-6

IL-8, Interleukin-8

IL-10, Interleukin-10

IL-18, Interleukin-18

IP_3, Inositol Triphosphate-3

IQR, Interquartile Range

IRB, Institutional Review Board

IU, International Units

IVC, Inferior Vena Cava

L, Liter

LMWH, Low Molecular Weight Heparin

LPS, Lipopolysaccharide

LUMC, Loyola University Medical Center

LV, Left Ventricular

LVDD, Left Ventricular Diastolic Dysfunction

MBP, Major Basic Protein

MCP-1, Monocyte Chemoattractant Protein-1

MI, Myocardial Infraction

min, Minutes

MLR, Monocyte**-**to-Lymphocyte Ratio

MRA, Magnetic Resonance Angiography

MP, Microparticle

MPA, Monocyte-Platelet Aggregates

MP-TF, Microparticle-Derived Tissue Factor

MRV, Magnetic Resonance Venography

µg, Microgram

µmol, Micromole

mg, Milligram

mL, Milliliter

mmHg, Millimeters of Mercury

mM, Millimole

NA, Not Available/Applicable

NETs, Neutrophil Extracellular Traps

NF-κB, Nuclear Factor Kappa B

ng, Nanogram

NHP, Normal Human Plasma

NO, Nitrotyrosine

NOD, Nucleotide Oligomerization Domain

NLR, Neutrophil-to-Lymphocyte Ratio

NPV, Negative Predictive Value

NS/ns, Not Significant

NT-proBNP, N-terminal Prohormone of Brain Natriuretic Peptide

O_2SA, Oxygen Saturation

OR, Odds Ratio

pg, Picogram

PAD, Peripheral Arterial Disease

PAI-1, Plasminogen Activator Inhibitor-1

PAMPs, Pathogen-associated Molecular Patterns

PAR-1, Protease Activator Receptor-1

PAR-4, Protease Activator Receptor-4

PC, Protein C

PDGF, Platelet-Derived Growth Factor

PE, Pulmonary Embolism

PERC, Pulmonary Embolism Rule-Out Criteria

PERT, Pulmonary Embolism Response Team

PESI, Pulmonary Embolism Severity Index

PF4, Platelet Factor 4

PFP, Platelet-Free Plasma

pg, Picogram

PGI_2, Prostacyclin

PGE_2, Prostaglandin E_2

PH, Potential of Hydrogen

PLR, Platelet -to-Lymphocyte Ratio

pmol, Picomole

PPP, Platelet-Poor Plasma

PPV, Positive Predictive Value

PRP, Platelet-Rich Plasma

proBNP, Pro-B-type Natriuretic Peptide

PSGL-1, P-selectin Glycoprotein Ligand-1

PTS, Post-Thrombotic Syndrome

RIETE, Registro Informatizado de Enfermedad Trombo Embólica

ROC, Receiver Operating Characteristic

RPM, Revolutions Per Minute

RV, Right Ventricular

RVDD, Right Ventricular Diastolic Dysfunction

SBP, Systolic Blood Pressure

SD, Standard Deviation

SII, Systemic-immune Inflammation Index

sGC, Soluble Guanylate Cyclase

sPESI, Simplified Pulmonary Embolism Severity Index

sTR, Seven Transmembrane Receptors

TAFI, Thrombin Activable Fibrinolysis Inhibitor

TAT, Thrombin-antithrombin Complex

TF, Tissue Factor

TFPI, Tissue Factor Pathway Inhibitor

TH, Thrombin

TIA, Transient Ischemic Attack

TLRs, Toll-like Receptors

TM, Thrombomodulin

TNF-α, Tumor Necrosis Factor-α

tPA, Tissue Plasminogen Activator

TT, Thrombin Time

TTE, Transthoracic Echocardiography

TXA_2, Thromboxane A_2

UFH, Unfractionated Heparin

USA, United States of America

VCAM-1, Vascular Cell Adhesion Molecule-1

VEGF, Vascular Endothelial Growth Factor

VTE, Venous Thromboembolism

vWF, von-Willebrand Factor

WBC, White Blood Cells

WT, Wild-type

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I – INTRODUCTION

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1.1. HEMOSTASIS

Circulating blood flow is critical to resource oxygen and nutrients to tissues and organs for sustaining life and maintaining physiologic functions, which requires veracity in the vasculature system. Hemostasis is a Greek word; *haeme* meaning blood and *stasis* meaning to stop. Hemostasis is a physiologic process which sustains blood in a free-flowing liquid state and also assists in solid clot formation to maintain the vascular integrity. In response to vascular injury, hemostasis prevents excessive blood loss through vascular constriction, plug formation and coagulation activation [1]. Hemostasis is based on certain elements; cellular elements including endothelial cells of vascular intima, platelets, and white blood cells, and a plasma component which includes coagulation and fibrinolytic proteins and their inhibitors [2].

Hemostasis involves two main phases; primary hemostasis and secondary hemostasis, each of them has distinct components and functions [1]. Primary hemostasis is achieved by the endothelial cell and platelets' response following vascular injury or desquamation of endothelial cells. It initiates with the vasoconstriction mediated through neurogenic reflexes, followed by platelet adhesion to vWF, triggering the activation and aggregation of platelets leading to the subsequent platelet plug formation. The vasoconstriction and platelet plug prevents blood loss, however primary hemostasis is a rapid and short-lived response [3]. Secondary hemostasis is mediated by coagulation activation and further platelet involvement. The release of tissue factor from endothelial cells initiates the extrinsic pathway of the coagulation cascade, activating coagulation proteins. Some of these proteins are initially inactive in the plasma, also released from the platelet granules, together, they stabilize the clot and ensure longer-term hemostasis [3].

1.1.1. Coagulation

Coagulation activation is the process that transforms blood from liquid into a solid clot. Plasma contains 16 procoagulants, including enzymes, cofactors, substrates, and minerals [4]. Among these, 8 of the enzymes include serine protease (FII, FVII, FIX, FX, FXI, FXII, and prekallikrein), and transamidase (FXIII) [5]. Serine proteases are synthesized as zymogens, circulating in an inactive form. Most of these factors are synthesized by the liver through monocytes and endothelial cells. Megakaryocytes also contribute to their production. Coagulation activation and clot formation requires the activation of FX to FXa and transformation of prothrombin into thrombin (TH). Coagulation cascades consist of three distinctive pathways, including intrinsic, extrinsic, and common pathways (figure 1) [6]. Both the intrinsic and extrinsic pathways lead to the activation of Factor X to Xa, which in turns leads to the activation of FII to thrombin [7, 8]. Each pathway in the cascade results in the formation of a unique coagulation complex [6].

In response to injury, tissue factor (TF) generates during the activation of cells. Endothelial damage plays an important role and activates coagulation FVII, complexing with activated FVIIa (TF:FVIIa) which forms an extrinsic tenase or TF complex formed by TF, activated FVII, along with Ca^{+2} and PL, which becomes a potent activator of FX to FXa [9-11]. On the other hand, in intrinsic pathway, contact factors such as FXII result in the activation of intrinsic pathway of the cascade, besides FXII, prekallikrein, and high molecular weight kininogen (HMWK) also contribute to its activation. During the activation of FX, certain factors such as FXI and FIX were also activated, which results in the reduction in thrombin generation parameters. In the intrinsic pathway, intrinsic complex formed by activated FXIa, FVIIIa, along with Ca^{2+} and PL is mandatory for the activation of FX [12]. Since tissue factor and the activation of the intrinsic system both result in activation of FX, and subsequent thrombin formation occurs in the common pathway and prothrombinase complex forms (FXa:FVa:PL:Ca^{2+}) [13, 14]. Thrombin plays an especially significant role in the stabilization of clots through hydrolyzing the fibrinogen into fibrin, which was further stabilized by activated FXIII [15]. During the conversion of prothrombin into thrombin, a peptide fragment called prothrombin fragment 1+2 (F1+2) is released [16], while some of the thrombin binds with the antithrombin (AT), forming a thrombin-antithrombin complex (TAT). This complex plays a crucial role in regulating coagulation by inhibiting thrombin.

Moreover, thrombin also activates platelets, monocytes, and macrophages which results in the release of products from these cells [17]. Some of these produce localized effects such as vasoconstriction and impedance in blood flow.

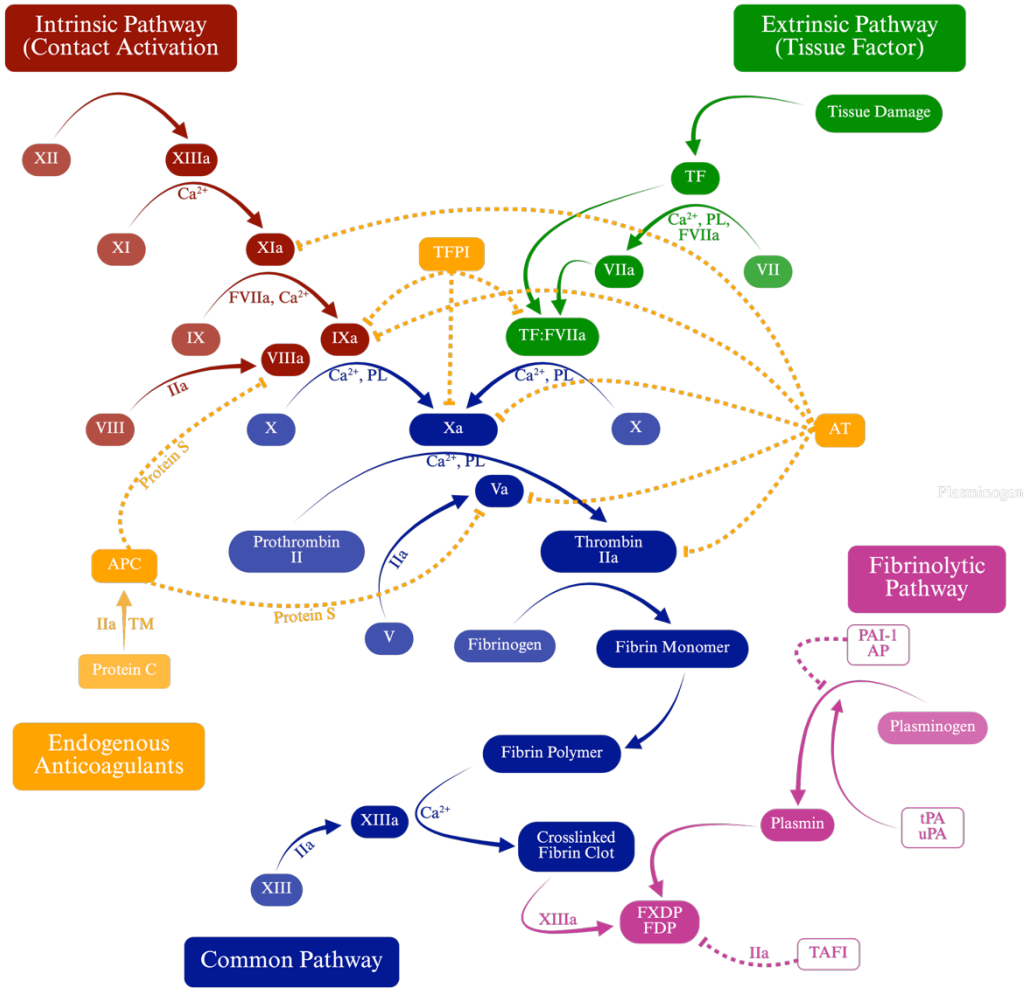


Figure 1. The Coagulation Cascade.

Coagulation cascade consists of three pathways: 1), intrinsic or contact pathway, initiated by contact with negatively charged surfaces (shown in red); 2) extrinsic or tissue factor pathway, initiated by tissue factor (shown in green); and 3) common pathway, where both the intrinsic and extrinsic pathways converge at the activation of FX that leads to the formation of crosslinked fibrin clot (shown in blue). Endogenous inhibitors including protein C, activated protein C (APC), thrombomodulin (TM), antithrombin (AT) and tissue factor pathway inhibitor (TFPI) inhibit excessive or inappropriate coagulation by inhibiting activation of coagulation at targeted points. The fibrinolysis pathway (shown in pink) is responsible for the degradation of a fibrin clot. Created in Biorender {Siddiqui, 2024 #1415}.

The activation of coagulation is a controlled process and is tightly regulated by certain cofactors and natural anticoagulants. Cofactors are plasma or procoagulant proteins which bind to their specific proteins enhancing their activity and increasing their affinity for substrates. Clotting factors including FV, FVII, FVIII and tissue factor serve as cofactors and are crucial for the formation of extrinsic tenase, intrinsic tenase, and prothrombinase complex. These complexes are required for the activation of FX and thrombin generation leading to the clot formation [18, 19]. Other coagulation proteins such as protein S, protein Z, and thrombomodulin (TM) also serve as a cofactor

The natural regulation of these zymogens is facilitated by inhibitors such as AT, tissue factor pathway inhibitor (TFPI), and protein C (PC) [20]. AT is a serine protease inhibitor which neutralizes them, however, with lower affinity. Inhibitory effect of AT is amplified to 2000-fold by heparin or heparan sulfate [21]. These glycosaminoglycans are found on the glycocalyx and released upon damage to endothelium [20], AT binds with these glycosaminoglycans which subsequently leads to the formation of TAT complex. TFPI is also a serine protease inhibitor, which regulates the extrinsic pathway of the cascade. TFPI inhibits the activation of FX and FIX via two distinctive mechanisms [22]. First TFPI binds with FXa, TFPI:FXa complex fosters the binding with TF:FVIIa complex inhibiting its activity and reduces the activation of FIX and FX. Secondly, TFPI directly binds with TF:FVIIa:Xa complex, inhibiting both FVIIa and FXa [23, 24]. Protein S also serves as a cofactor for TFPI, augmenting the affinity of FXa inhibition by 10-fold [25]. Generated thrombin binds with the thrombomodulin expressed on the endothelial cells, triggering the protein C mechanism from endothelial protein C receptor (EPCR) [25-28]. Activated protein C (APC) in the presence of protein S inhibits coagulation by hydrolyzing the FVa and FVIIIa and impedes the generation of thrombin [29].

1.1.2. Fibrinolysis

Plasminogen-Plasmin system is a crucial physiological mechanism and a fundamental player for the hemostatic equilibrium [30]. An imbalance in the fibrinolysis can lead to the persistent progression and maturation of a clot. The regulation/balance in the fibrinolysis is stringently controlled by definite activators

and inhibitors (figure 1-2) [31]. Central to this process is the conversion of fibrin-bound plasminogen by tissue-plasminogen activator (tPA) and urokinase plasminogen activator (uPA) into plasmin. This transformation is essential for the assimilation of fibrin by hydrolyzing arginine-lysine-related peptide bonds, which is critical for clot lysis.

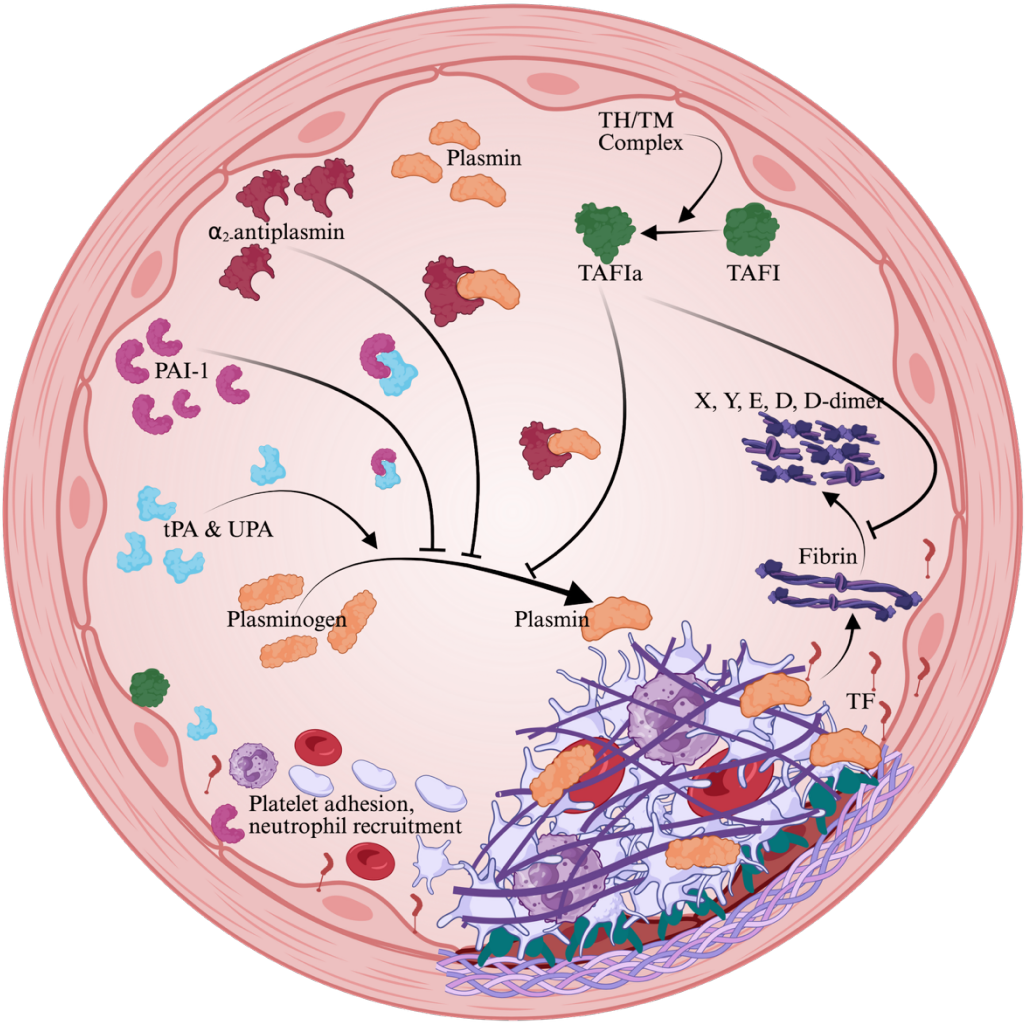


Figure 2. Fibrinolysis pathway, its activators, and inhibitors.

The main components of the fibrinolysis pathway are plasminogen and its activator tissue-plasminogen activator (tPA), both of which become bound to a fibrin clot. Conversion of plasminogen to plasmin is critical for the lysis of a blood clot. tPA converts bound plasminogen into plasmin. Plasmin digests fibrin into fibrin degradation products (FDPs) X, Y, D, E, and D-D (D-dimer). D-dimer is the product of cross-linked fibrin. Urokinase plasminogen activator (uPA) also converts plasminogen to plasmin. Plasminogen activator inhibitor-1 (PAI-1) is an important inhibitor of tPA, α2-Antiplasmin is an important inhibitor of free plasmin. TAFI

inhibits fibrinolysis indirectly by preventing the binding of plasminogen, plasmin and tPA to fibrin via cleaving the lysine residues of fibrin. Created in Biorender {Siddiqui, 2024 #1415}.

The activation for plasminogen is regulated by serine protease tPA and uPA. tPA is secreted by endothelial cells and triggers fibrinolysis by hydrolyzing fibrin-bound plasminogen into plasmin. During the depolymerization, tPA forms a covalent lysine bond with fibrin [32, 33]. uPA secreted from endothelial cells along with monocytes and macrophages, binds to the urokinase plasminogen activator receptor (uPAR), enables uPA to convert plasminogen into active plasmin, which hydrolyzes fibrin and leads to clot lysis [34]. The binding of plasminogen with tPA has a higher affinity compared to the uPA, which is due to the structural differences among them as tPA has two glycosylated kringle region while uPA has only one, which makes tPA a more efficient plasminogen activator, enhancing plasmin formation and promoting effective clot dissolution.

The key inhibitors of fibrinolysis are plasminogen activator inhibitor-1 (PAI-1), α2-antiplasmin (AP), and thrombin activatable fibrinolysis inhibitor (TAFI). PAI-1 is a serine protease inhibitor and produced from endothelial cells, megakaryocytes, monocytes, hepatocytes, and other cells, also stored in platelets [35]. PAI-1 complexes with tPA and uPA, preventing plasminogen activation and conversion into active fibrinolytic plasmin [36]. Moreover, binding of tPA with the fibrin shields the formation of tPA:PAI-1 complex. AP is a serine protease inhibitor which synthesizes in the liver with N- and C- terminal extension [37]. N-terminal linked with fibrin by FXIIIa while C-terminal has lysine binding site which binds with free plasmin to form a complex; plasmin-antiplasmin (PAP) makes it inactive [38-40]. While AP is a plasmin inhibitor, fibrin-bound plasmin remains partially protected from its effects [41, 42]. TAFI is an inactive enzyme precursor and a polycarboxypeptidase which is synthesized in the liver. TAFI activated by the thrombin-thrombomodulin complex; inhibits fibrinolysis and stabilizes the clot by impeding the formation of plasmin via slicing the carboxy-lysine residues of fibrin and counteracting the binding of tPA and plasminogen with fibrin [43]. Generation of thrombin triggers the activation of TAFI, and resultant inhibition of fibrinolysis can contribute towards a thrombotic state.

The formation of plasmin leads to the degradation of fibrin into fibrin fragments; X, Y, D, E, and D-D (D-dimer). Fragment X, Y, D, and E are the end-

product of fibrin or fibrinogen, while D-dimer is the product of cross-linked fibrin and generated via thrombin, FXIIIa, and plasmin activity [44-46].

1.1.3. Endothelium

The vasculature system includes arteries, veins and capillaries structurally organized into three distinct layers: tunica intima, tunica media, and tunica adventitia [47]. A single-layered simple squamous epithelial cells line the inner layer of vascular system; referred as endothelium, and subendothelial connective tissues are the components of vascular intima [48, 49]. The subendothelial connective tissue encompasses collagen, and fibrinogen in veins, whereas in arteries it also contains smooth muscle cells. Endothelium is tailored to the unique function in maintaining the vascular health by regulating vascular tone and inflammatory responses. Tunica media or middle layer is embraced by smooth muscles and elastic fibers, the thickness on these layers especially smooth muscles varies depending on the type of vessel, with an immense number in arteries and occasionally in capillaries which provide the regulation of diameter, and elasticity to accommodate pressure changes. Tunica adventitia or externa are comprising of connective tissues including collagen and elastic fibers, providing structural integrity, stability, and flexibility to the vessel, anchoring the vessel wall and upholds its position, and nourishment to the larger vessels itself via small blood vessels called vasa vasorum.

Integrity of endothelium plays a vital role in hemostasis and coagulation processes and provides a thromboresistant state through anti- and pro-thrombotic effects (figure 3). Intact endothelium provides anti-thrombotic environments in the system by counteracting platelet activation [50]; prevents platelet aggregation and vasoconstriction [51-53]; maintains fibrinolysis [50]; reduces coagulation activation, and generation of thrombin [51-57]. Uninterrupted and smooth surface of the endothelium ensures harmonious blood flow by counteracting platelet activation, and also prevents the platelet adhesion as intact endothelium serves as a barrier between the platelets and subendothelial collagen along with vWF [50]. It also separates the endothelial tissue factor with the circulating procoagulant proteins controlling the activation of coagulation cascade.

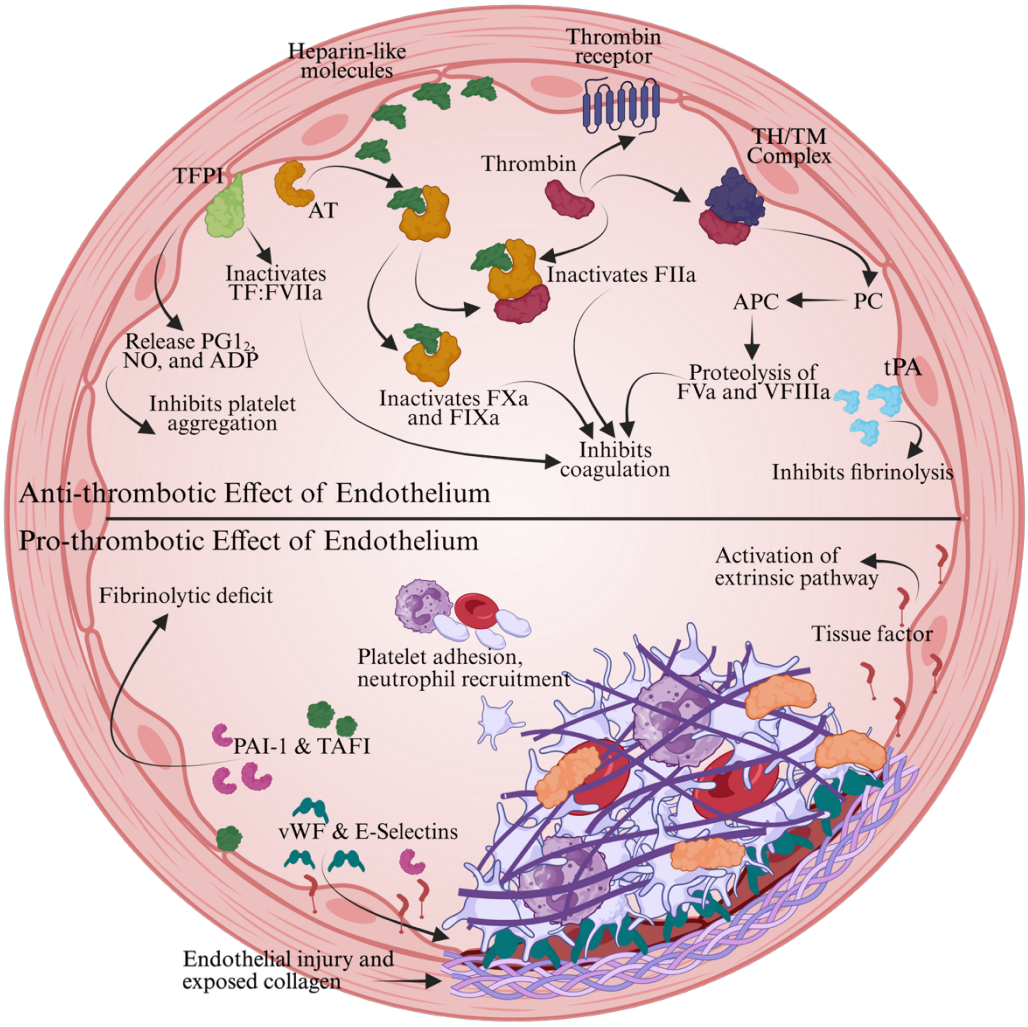


Figure 3. Anti-thrombotic, and pro-thrombotic effects of endothelium.

Antithrombotic effect of endothelium involves the release of prostacyclin which inhibits platelet activation, nitric oxide mediating vasodilation, TFPI mediating neutralization of TF and antithrombin producing inhibition of FXa, and FIIa. The pro-thrombotic effect of endothelium involves the generation of TF mediating coagulation, formation of thromboxane activating platelets, mediators of vasoconstriction such as endothelin and PAI-1 mediating compromised fibrinolysis. Created in Biorender {Siddiqui, 2024 #1415}.

Endothelial cells synthesize and secrete numerous substances including prostacyclin (PGI_2), nitric oxide (NO), TFPI, and tPA. PGI_2 is a vasodilator and a potent inhibitor of platelet aggregation [52, 53]. PGI_2 is synthesized from endothelial cells via the eicosanoid pathways. Released PGI_2 binds with the specialized prostacyclin receptors (IP receptors) on surface of platelets, binding

subsequently increases the intracellular cyclic adenosine monophosphate (cAMP) levels within the platelets; inhibits platelet activation and aggregation acting as a competitor of thromboxane A_2 (TXA_2) [52]. Besides that, PGI_2 inhibits myosin light chain kinase through protein kinase A, promoting vasodilation in the vessel [53]. NO is synthesized in endothelial cells by enzyme endothelial nitric oxide synthase (eNOS), which converts the amino acid L-arginine to NO and citrulline. Synthesized NO binds to soluble guanylate cyclase (sGC), which in turn increases the levels of cyclic guanosine monophosphate (cGMP) [51]. Elevated cGMP leads to the relaxation of smooth muscles resulting in vasodilation while increasing levels in platelets leading to the inhibition of platelet aggregation [58, 59]. TFPI, as name represents, is a protease inhibitor and primarily regulate extrinsic or TF pathway of coagulation cascade by inhibiting the activity of FX and FIX either directly or indirectly [23, 24]. In direct inhibition, it requires TFPI binding with the FXa which binds with TF complex inhibiting its activity and reducing the activation of FIX and FX. While TFPI directly inhibits both FVIIa and FXa when bound to the TF:FVIIa complex, thereby limiting coagulation activation and thrombin generation. tPA is an activator of fibrinolysis which converts the plasminogen into plasmin initiating the activation of the fibrinolytic system by cleaving the fibrinogen and fibrin into fibrin degradation products and assist the fibrinolysis [50].

Endothelial cells maintain their anti-thrombotic properties by synthesizing and expressing the main inhibitors of coagulation, including TM and heparin sulfate on their surface. TM is a glycoprotein bound on the endothelial cells that regulate the coagulation activation by binding the generated thrombin [60], TM:TH complex activates protein C, activated protein C inhibits FVa and FVIIa; key amplifiers of coagulation cascade which regulate coagulation; TM:TH complex also inhibits thrombin activity to convert fibrinogen into fibrin and activate platelets [28, 61]. Heparan sulfate is a glycosaminoglycans, and a component of endothelial glycocalyx. Expression of heparan sulfate promotes binding with antithrombin, inhibiting thrombin and FXa, regulating the coagulation activation, and generation of thrombin supporting the anti-thrombotic properties of the endothelium [20].

Damaged endothelium provides pro-thrombotic environment via platelet activation/adhesion, activation of the coagulation pathway, platelet/leukocyte recruitment, and inhibits fibrinolysis. Damaged endothelium exposed subendothelial collagen initiating platelet activation/adhesion. TF also expresses on

the impaired endothelium, that binds with the FVII activates extrinsic coagulation pathway leading to the activation of FXa and generation of thrombin [62]. vWF is a glycoprotein and stored in Weibel-Palade bodies, vWF forms a complex with platelets via binding with GPIb/IX/V glycoprotein at the site of injury [63, 64]; circulating vWF through collagen initiates platelet-endothelial cell interactions [54]; promotes thrombus formation. E-selectin (CD62, endothelial-leukocyte adhesion molecule-1; ELAM-1) involved in leukocyte recruitment facilitates thrombosis [54, 65-67]. Endothelial cells regulate the process of fibrinolysis by secreting the key inhibitors of fibrinolysis; PAI-1 and TAFI. PAI-1, a glycoprotein, inhibits fibrinolysis by complexing with tPA and uPA (PAI-1:tPA, and PAI-1:uPA) rendering them inactive, reducing plasminogen activation, leading to decrease clot lysis, endorsing the pro-coagulant environment [36, 50]. TAFI is a zymogen which is activated by thrombin-thrombomodulin complex which removes the C-terminal lysine residues from fibrin, which are essential for the binding of plasminogen and tPA to fibrin, this modification impedes the conversion of plasminogen into plasmin, further inhibiting fibrinolysis [43].

1.1.4. Platelets

The first line of defense for blood loss is mediated by platelets: anucleate fragments of cytoplasm from the bone marrow megakaryocytes and smallest circulatory cells [68]. Functionality of platelets are based on 3A's: adhesion, activation and aggregation. Structurally, platelets contain 1) surface canalicular and dense tubular system including microfibrils and microtubules; 2) cytoplasmic granules including α-granules and δ-granules; 3) receptors on the plasma membrane [69, 70]. The structure and function of platelets are intricately linked with each aspect, complementing and enhancing the other.

The crucial role of platelets towards the maintenance of hemostasis depends on three main steps as adhesion, activation, and aggregation [71]. At the site of vascular injury, platelets first adhere to the exposed subendothelial collagen, facilitated by platelet surface receptors (figure 4). These receptors bind with collagen, and vWF, anchoring the platelets to the damaged vessel wall. During activation, platelets undergo a shape change along with the release of granular content such as ADP, serotonin, and TXA_2 especially pro-coagulant factors which

amplifies the coagulation activation. In the phase of aggregation, platelets bind with one another via forming a bridge with fibrinogen and vWF which results in the formation of a platelet plug [69, 72].

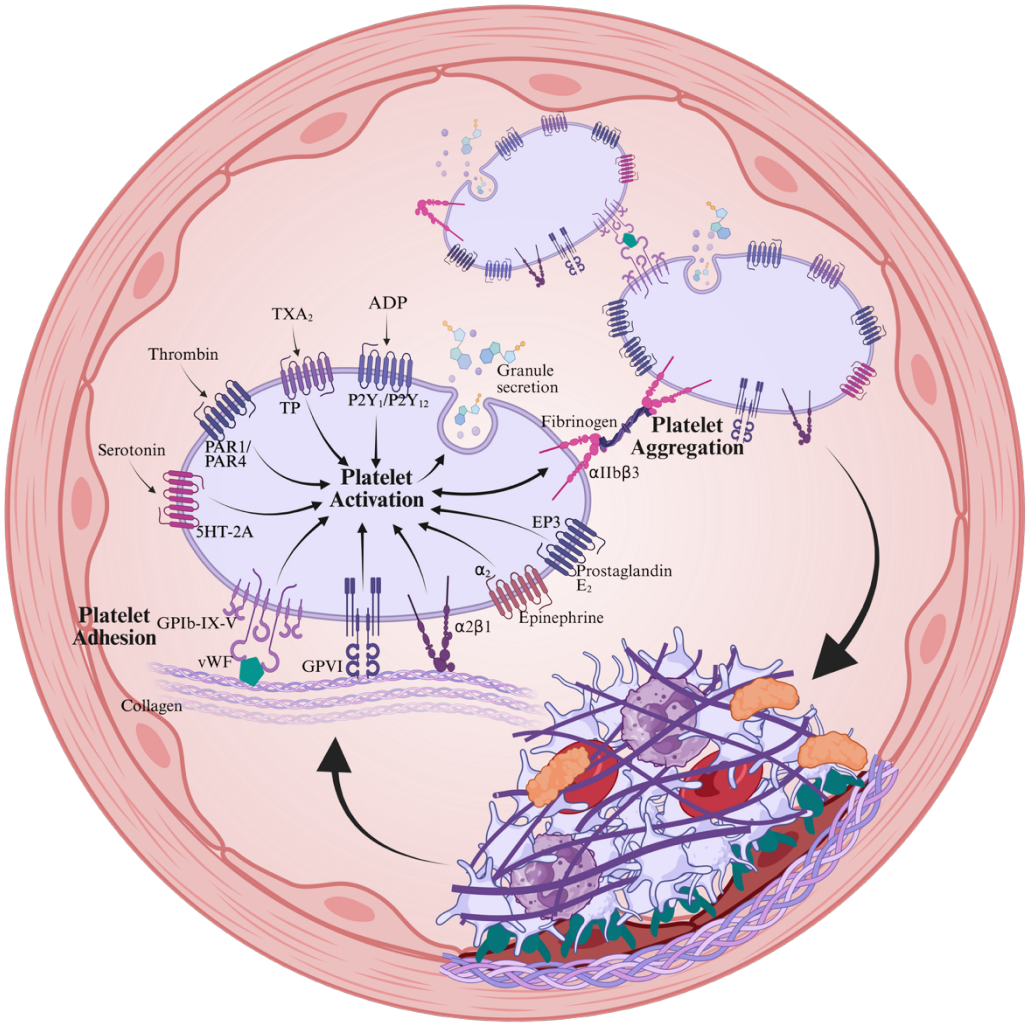


Figure 4. Platelet membrane receptors and associated activation pathways.

Endothelial damage leads to the activation of glycoprotein receptors such as GPVI for collagen and GPIb/IX/V for vWF. Binding of vWF and collagen with their receptor initiates platelet adhesion. The seven transmembrane receptors for thrombin, thromboxanae-A_2 (TXA_2), adenosine diphosphate (ADP), prostaglandin E_2, and epinephrine activates when agonist binds to their perspective receptors leads to the activation of platelets and secretion of granular content. Subsequently TXA_2, and ADP activates integrin $\alpha_{IIb}\beta_3$ for fibrinogen and vWF leads to platelet-platelet aggregation resulting in formation of platelet plug and further activation via combination of collagen, and thrombin leads to collagen/thrombin activated platelets (COAT), a procoagulant platelet stabilizes fibrin clot. Created in Biorender {Siddiqui, 2024 #1415}.

Surface canalicular and dense tubular system facilitates release of calcium, enzymes, and hemostatic proteins; and microfibrils and microtubules mediate the conformational shape change and pseudopod formation crucial for platelet adhesion and aggregation [69]. The cytoplasmic α-granules or light granules embraces fibrinogen, vWF, platelet factor 4 (PF4), FVIII, P-selectin, and growth factors which assist in functionality and recruitment of platelets at the site of injury, while δ-granules [73, 74], or dense granular bodies provides energy and signaling molecules such as ADP, ATP, calcium, and serotonin, which is also essential for the platelet activation and aggregation [75, 76]. The plasma membrane of the platelets contains receptors including Glycoproteins (GP) such as GPIa/IIa, GPIb/IX/V, GPIIb/IIIa, and GPVI which aids in the adhesion of platelets to the sub-endothelial matrix and aggregation with other platelets.; and seven transmembrane receptors (sTR) such as PAR-1, PAR-4, P2Y1, P2Y12, α_2-adrenergic, 5HT-2A, and thromboxane prostaglandin drives platelets activation, and granular content secretion in response to thrombin, and ADP [69, 77].

Endothelial damage initiates platelets adhesion to the non-platelets subendothelial collagen; directly by GPVI in high shear stress vessels or indirectly by GPIb/IX/V-vWF, GPIc/IIa (integrin $\alpha_6\beta_1$)-fibronectin, GPIc'/IIa (integrin $\alpha_5\beta_1$)-laminin, and VnR (integrin $\alpha_v\beta_1$)-vitronectin. GPVI-collagen internally activates the platelet, initiating outside-in and inside-out reaction via the activation of eicosanoid synthesis or inositol triphosphate-diacylglycerol (IP_3-DAG) activation pathways increases the affinity of GPIa/IIb for collagen; GPVI, GPIb/IX/V, and GPIa/IIb firmly attached the platelets and undergo to the conformational change in shape to ameboid formation [78]. Following, subendothelial binding, TF from endothelium generates the COAT (collagen and thrombin activated) platelets, while TXA_2, and ADP activates neighbor platelets triggering outside-in activation of GPIIb/IIIa (integrin $\alpha_{IIb}\beta_3$) and leads to interplatelet fibrinogen/vWF bridging with GPIIb/IIIa (integrin $\alpha_{IIb}\beta_3$) receptor in the presence of ionized calcium (Ca^{+2}) which initiates aggregation which forms a platelet plug [69, 72]. P-selectin from -granules induces platelet-leukocytes binding. Moreover, release of endothelial TF initiates extrinsic-tenase complex (TF:VII:Ca_2), while contents from α-granules and polar phospholipids activates intrinsic-tenase (IXa:VIIIa:phospholipids:Ca^{2+}) and prothrombinase complex (Xa:Va:phospholipids+Ca^{2+}) generates the burst of thrombin and coagulation activation.

1.1.5. Inflammation

Interplay between coagulation and inflammation also plays a role in the pathogenesis of thrombosis, a phenomenon often referred to as Immunothrombosis [79]. Systemic infection leads to the systemic coagulation activations and multi-organ failure by microvascular obstructions resulting in the dysregulation of Immunothrombosis (figure 5). Innate immune cells, particularly neutrophils and monocytes, and platelets play a pivotal role in Immunothrombosis [80]. Specialized pattern recognition receptors such as Toll-like receptors (TLRs), nucleotide oligomerization domain (NOD) like receptors, and C-type lectin receptors (CLRs) on neutrophils and platelets detect pathogens [81, 82]. Via TLR receptor binding with the bacterial product like lipopolysaccharide, and pathogen-associated molecular patterns (PAMPs) activates platelets. Platelets as a mechano-scavenger gathers bacteria to present them to neutrophils [83]. Actomyosin dependent platelet migration is mediated by αIIbβ3 integrin with surrounding fibrinogen [79]. Migrating platelets (loaded with bacteria) and neutrophils contacts via P-selectin on the platelets and P-selectin glycoprotein ligand-1 (PSGL-1) further enhance the migratory properties of neutrophils triggering activation of neutrophils resulting in formation of neutrophil extracellular traps (NETs) [83, 84]. Neutrophils undergoes to the programmed called death called as NETosis, forming NETs by releasing release of damage-associated molecular patterns (DAMPs), deoxyribonucleic acid (DNA), histones, and antimicrobial proteins (myeloperoxidase, and neutrophil elastase) [85-87]; provides platform for the platelets and red blood cells, thus promoting clot formation [88-90]. The formation of NET's was further endorsed by the platelets as platelets bound bacteria phagocytosed by neutrophils lead to the NETosis [83, 91].

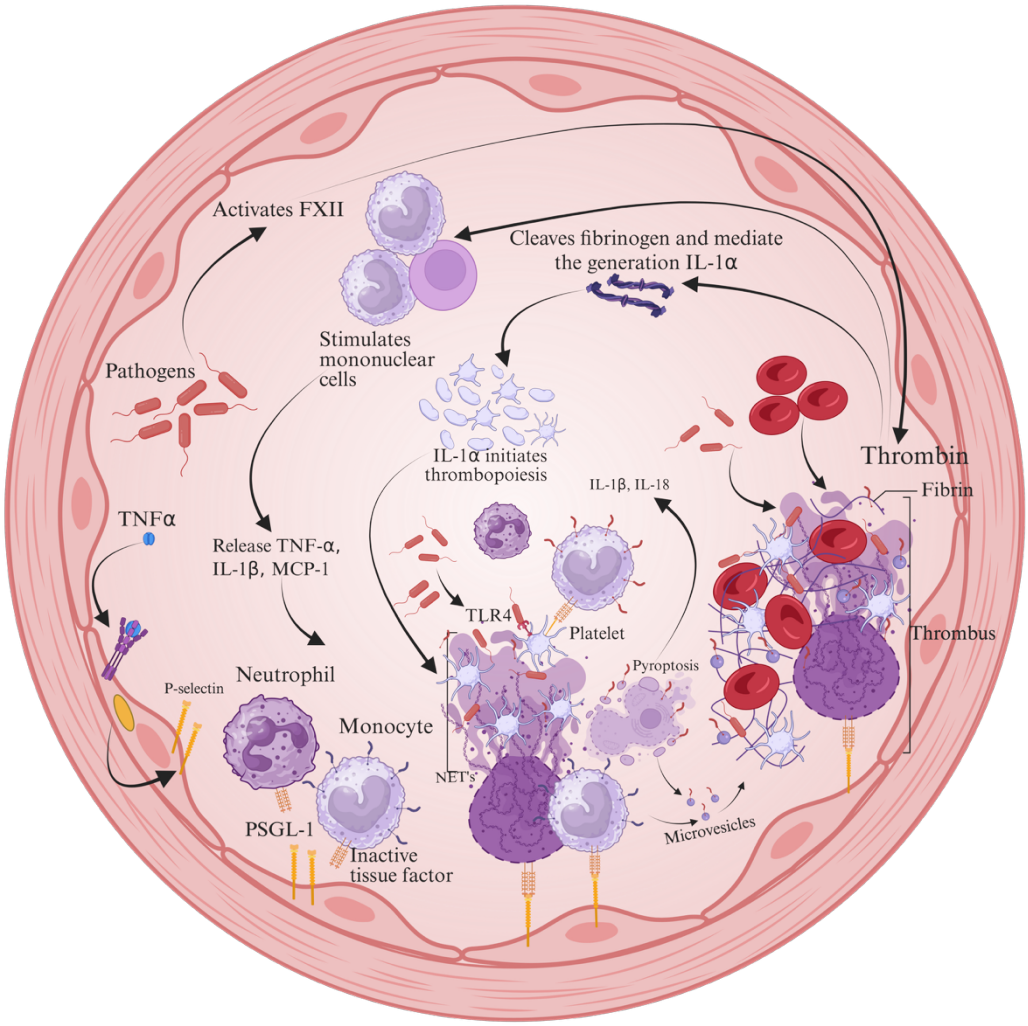


Figure 5. Interplay between inflammation and thrombosis.

Inflammation leads to the activation of neutrophils and monocytes which undergo to NETosis and Pyroptosis and release granular content. This results in the generation of microvesicles TF and cytokines and facilitates thrombus formation. Generated thrombin leads to the cleaves fibrinogen and activates monocytes leading of generation of various cytokines facilitates inflammation. Created in Biorender {Siddiqui, 2024 #1415}.

Activated monocytes or macrophages facilitates pyroptosis; a programmed cell death which is dependent on caspase-1 and gasdermin D, resulting the releases of inflammatory mediators such as interleukin-1β (IL-1β) and interleukin-18 (IL-18), and TF-bearing microvesicles, which further facilitate clot formation by extrinsic pathway [92]. Contrary, extracellular DNA was degraded by deoxyribonuclease-1 (DNase-I) and deoxyribonuclease-1 like 3 (DNase1L3)

prevents further activation of coagulation. While inflammation cause thrombosis, thrombosis can also trigger inflammation [93]. Thrombin stimulates mononuclear cells which in turn release cytokines and chemokines such as IL-1β, tumor necrosis factor-α (TNF-α), and monocyte chemotactic protein-1 (MCP-1), further cleaves fibrinogen and activates IL-1α, provides a direct link between coagulation-inflammation [94]. Besides fibrinogen, thrombin also activates IL1-α which stimulates thrombopoiesis by inducing the fragmentation of megakaryocytes in the marrow and rapidly replace platelets [95]. While platelet consumption due to infection leads to the activation of stem-like megakaryocyte progenitors, replenished platelets [96].

1.1.6. White Blood Cells

The role of white cells has been known to contribute towards the coagulation activation. Neutrophils and monocytes adhere to the intact endothelium within hours of flow reduction [97]. Neutrophil plays an imperative role in the activation of coagulation and hemostasis. Activated neutrophils trigger NETosis; a regulated cell death and releases their nuclear material such as decondensed chromatin, proteases, DNA, histones, and secretory granular contents in form of extracellular structure called as NETs [93, 98]. NETs have both prothrombotic and proinflammatory effects. Histones have capability to bind with adhesion protein such as vWF, fibrinogen, and fibronectin, histone/vWF interaction activates the endothelial cell, stimulates platelets (aggregation and adhesion) leads to the thrombus formation [86, 87]. Histone also impairs activation of thrombomodulin-dependent protein C which leads to the thrombin generation [99]. Moreover, NETs associated elastase and protease degrade anticoagulants via TFPI [100]. Although extracellular trap production has also been observed in monocytes, eosinophils, and basophils, neutrophils are considered the primary producers of these traps in venous thromboembolism (VTE) [101].

Eosinophilia also plays a role in thrombosis [102-105]. Degranulation of eosinophils leads to the release of cytotoxin proteins including major basic protein (MBP), eosinophil cationic protein (ECP), eosinophil peroxidase (EPX), and ethylene glycol dinitrate (EGD), leads to the damage of vascular endothelial cells, besides that they increase the activity of TF, FVII, FX and FXII, promotes

hypercoagulable state [106], and platelet activation [107]. Eosinophils via directly activating TF [108], and releasing the specific leukotrienes are central toward the activation of extrinsic pathway of coagulation cascade and enhancing platelet aggregation. Endothelial damage either by direct infiltration of eosinophils in the vessel wall or by expression of CD40 Ligand (CD40L) and the CD40/CD40L system plays an important role in inflammation and leads to the coagulation and platelet activation. Moreover, MBP-enriched eosinophil extracellular DNA traps also contribute to platelet activation [109].

Monocytes, traditionally known for their phagocytic role in innate immunity, contribute to VTE through several mechanisms including initiating coagulation via tissue factor (TF) presentation and enhancing thromboinflammation through inflammasome activation [92]. Receptor ligand binding leads to the activation of monocyte-platelet aggregates (MPA) [110]. Interaction between monocytes and platelets mediated by P-selectin/PSGL-1 results in release of IL-8, IL-1β, TF, and MCP-1, superoxide anion production and activation and translocation of nuclear factor kappa B (NF-κB) which trigger coagulation and accelerate thrombosis [111, 112]. MPA also involves in different thromboembolic diseases including VTE, coronary heart disease, unstable angina pectoris, and acute myocardial infarction through several mechanisms [113, 114]. Monocytes-derived EVs have also shown trigger for the generation of thrombin [115]. In contrast, lymphocytes being a component of innate and adaptive immune system contribute to thrombosis developments with interaction of other members of cellular network.

1.1.7. Abnormal Hemostasis

Imbalance or disruption in the physiological mechanisms that regulate blood clotting, due to any pathologic condition, results in abnormal hemostasis either uncontrolled bleeding (hemorrhage) or pathological clot formation (thrombosis). Any of these conditions could be life-threatening.

Thrombosis is the formation of clot in the vessel wall and the clot referred to as thrombus. The formation of thrombus impedes the normal blood flow potentially leading to multiple complications. Besides that, clot can dislodge from its original site or break into smaller pieces released into the blood circulation and travels through the bloodstream to the distant vessels, the process referred to as

embolization and the dislodged thrombus referred to as embolus. Lodging of embolus into other vessels can cause blockages and damage to the associated organ or system. Depending on the vessels, thrombosis can be venous thrombosis, arterial thrombosis, and microvascular thrombosis. The most common destination of the emboli includes lungs, brain, or limbs, potentially resulting in severe and life-threatening conditions like pulmonary embolism (PE), stroke, or peripheral artery disease (PAD).

Bleeding is another sequala of abnormal hemostasis which could be minor to major. A hemorrhage is a major bleeding which is potentially fatal. Bleeding complications could be acquired, or congenital. Bleeding disorders could be associated with platelets referred as primary, coagulation factors related bleeding or secondary, and unregulated fibrinolysis. Bleeding associated with the platelets depends on the quantity or quality of platelets. Thrombocytopenia is the most common quantitative platelet disorder, it could be due to excessive platelet destruction or consumption or related to the production from the bone marrow. The qualitative disorder of platelets could be due to the defective functionality of platelet or secretion of granular content also dispose the patients to bleeding disorders. The most common quantitative platelet disorder includes Bernard-Soulier disease (GPIb,IX,V receptor dysfunction), Glanzmann thrombasthenia (GPIIb/IIIa receptor), or storage pool disease (granule deficiency) [116-120]. The deficiency in the clotting factors is typically defined as having less than 30% of the normal levels of those factors. The most common factor deficiencies include vWF diseases, Hemophilia A, and Hemophilia B (Christmas disease) [121, 122] while some other rare single factor deficiencies include Dysfibrinogenemia, Rosenthal syndrome, Stuart-Prowar factor deficiency (FX deficiency), Prothrombin deficiency, and fibrin-stabilizing factor deficiency (FXIII deficiency) [123-126]. Some of the acquired coagulopathies include: trauma-induced coagulopathy which is usually a fatal hemorrhage; liver disease coagulopathy is associated with the deficiency of prothrombin, FV, FVII, FVIII, FIX, FX, and fibrinogen as they are synthesized by the liver; vitamin K deficiency affects the levels of FII, FVII, FIX, and FX as they are dependent on the availability of vitamin K during their synthesis; and renal failure also leads to thrombocytopenia, along with factor deficiencies. Fibrinolysis is the physiological process of clot breakdown, if unregulated it can contribute to the bleeding disorder and inhibitors control the

clot resolution [127]. Excessive tPA activity or deficiencies related to PAI-1 or AP may disrupt the balance resulting in excessive clot lysis and increasing the risk of bleeding. The steady fibrinolysis prevents uncontrolled bleeding while ensuring effective clot removal.

1.2. THROMBOEMBOLISM

Thrombus and embolus both are Greek words, "Thrombos" means a clot, while "embolus" means thrown or plug. The concept of thrombus and embolus was conceived by Virchow's (1854), per Robb Smith (1955) and the term thrombus was coined by Galen who summarized it in *Gesammelte Abhandlungen* in 1856. Thrombosis is defined as a pathological transition of blood from a fluid state to a solid or semi-solid state within a blood vessel. In thrombosis a platelet plug or fibrin clot is formed in the vessel wall which obstructs the circulation, called thrombus. Thromboembolism is a condition where formed thrombus subsequently breakdown or isolate from the vessel wall, travel from origin of location through the circulation to another location and cause an obstacle in a blood vessel. The dislodge or fragment of the clot is denoted as emboli or embolus. Thrombosis is a multifaceted condition resulting from stasis in the circulation and abnormalities in the coagulation system, coagulation control mechanism, platelet function, endothelium or inflammatory cytokines. Depending upon the type of vasculature, thrombosis can be arterial thrombosis, venous thrombosis and microvascular thrombosis. Mortality related with thromboembolic conditions account for 1 in 4 deaths globally in 1990 [128].

In 1856, a German Pathologist Rudolf Virchow's delineate the process of intravascular thrombus formation and its potential consequences including embolization and pulmonary obstruction. Virchow's triad is an integrated concept of thrombosis (figure 4), proposing three major factors which contribute towards the pathogenesis of thromboembolic disease, includes endothelial damage, hypercoagulability, and vascular stasis.

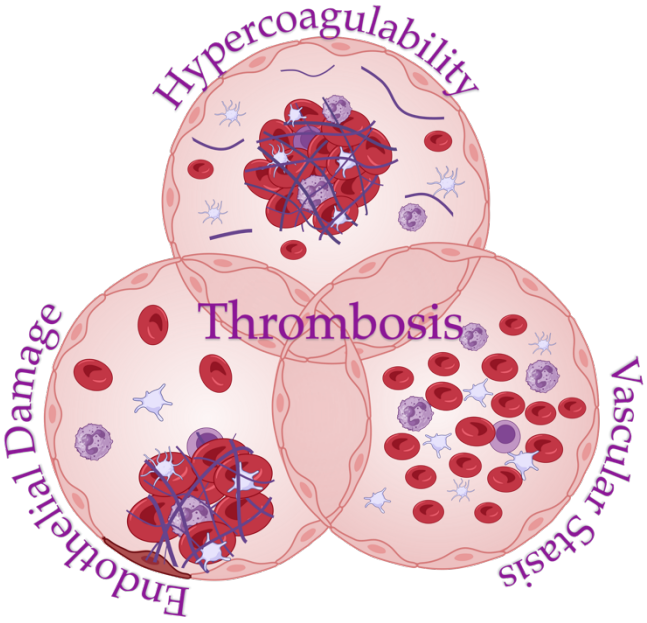


Figure 6. Virchow's Triad.

Virchow's Triad describes three conditions as endothelial damage, hypercoagulability and vascular stasis that must be present for thrombosis to occur. Endothelial damage leads to the formation of TF and procoagulant material leading to a hypercoagulable state with thrombin generation, and stasis causes blood flow impedance. Created in Biorender {Siddiqui, 2024 #1415}.

Endothelial damage provides procoagulant environments by expression of the TF, exposure of subendothelial collagen, platelet and leukocyte recruitments, loss of anticoagulant properties, and inhibition of fibrinolysis. Hypercoagulability or thrombophilia refers to a condition in which blood has an increased tendency towards the clot formation which could be due to imbalance in the coagulation system. Both the congenital and acquired conditions may lead to hypercoagulable state. Congenital factors including deficiencies or mutations in the proteins or factors involved in coagulation, such as AT deficiency, protein C or S deficiency, prothrombin G20210A, factor V leiden mutation, or hyperfibrinogenemia [129]. Acquired conditions includes antiphospholipid syndrome, inflammation, certain cancers, leukemia, sepsis, use of oral contraceptives, obesity, smoking, or trauma contribute to the risk of developing VTE. Vascular stasis or turbulent blood flow results in slowing or halting of blood flow, especially in veins. This reduced blood flow facilitates the accumulation of blood cells and coagulation factors leading to clot formation. Activation of coagulation is favored in the locations with static flow

in the vasculature, stasis can be primary associated with the medical conditions such as atrial fibrillation (AF), varicose veins, and heart failure; or secondary stasis resulting from immobility such as long-haul flights or prolong bed rest.

1.1.1. Arterial Thrombosis

Arterial thrombosis refers to the formation of a blood clot within an artery. Arterial thrombosis is mostly associated and linked with atherosclerosis which contributes towards the endothelial injury. Atherosclerosis is a chronic inflammatory condition characterized by the plaque buildup or fatty deposits (atheroma) in the arteries [130]. Atherosclerosis leads to the hardening of the arteries, restricting blood flow by narrowing down the lumen, potentially leading to the atherosclerotic cardiovascular disease (ASCVD). The most common arteries associated with arterial thrombosis are coronary arteries, cerebral arteries, and peripheral arteries.

Clinical manifestation of arterial thrombosis varies depending on the arteries affected. In coronary artery disease (CAD), thrombosis can lead to myocardial infraction (MI) or angina pectoris. When cerebrovascular arteries or the carotid end of arteries are blocked by thrombus, it may result in ischemic stroke or a transient ischemic attack (TIA). Thrombosis in peripheral arteries can lead to peripheral arterial disease (PAD), characterized by obstructive symptoms in the limbs. Cardiovascular disorders are the leading cause of mortality and morbidity globally [131]. Arterial thrombosis-related deaths are among the leading of cardiovascular mortality globally. The global death rate related with CVD has been increase significantly from 12.1 million in 1990 to 18.6 million deaths in 2019 which increases to 20.5 million deaths in 2021 [132]. In US, cardiovascular conditions result in a death in every 33 seconds, and responsible for 1 in every 5 deaths [133]. Coronary artery disease accounts for the death of approximately 371,506 individuals in 2022 [133, 134]. About 805,000 individuals encounter MI, of those 200,000 experienced recurrent MI, 1 in every 40 seconds suffered with MI, 20% MIs are usually silent [135]. Stroke is the second leading cause of CVD globally. In US, >795,000 individuals in every 40 seconds suffers with stroke each year, with 610,000 individuals experiencing the first episode of stroke [135]. Stroke-related death in US accounts for 17.5% of cardiovascular disease resulting in one death in every 3

minutes and 11 seconds in 2022 [133]. Approximately, 6.5 million people with the age group of 40 and older in US have PAD [136], individuals with PAD are at increased risk of emerging MI or stroke [137]. Approximately 85% of MI and 80% of stroke are associated with PAD [138].

Risk factors contributing to arterial thrombosis can be categorized as environmental, natural or medical illnesses [139]. Environmental risk factors most importantly include smoking, abdominal obesity, sedentary lifestyle, mechanical devices (such as mechanical valve or drug-eluting stent) [140]. Natural factors encompass patient-related characteristics such as age, gender, pregnancy, the use of oral contraceptives, and congenital thrombophilia such as hyperfibrinogenemia, hyperhomocysteinemia, PAI-1 polymorphism [141-144]. Medical illnesses, especially atrial fibrillation, hypercholesterolemia, hypertension, diabetes, cancer, or chronic inflammatory diseases are associated with arterial thrombosis [145-147].

The pathophysiology of arterial thrombosis follows Virchow's triad with emphasis on endothelial injury and hypercoagulable state [148]. Unstable atherosclerotic plaque plays a central role in the initiation of clot formation. Atherosclerotic plaque damages the endothelial lining of the vessel wall, exposing the subendothelial matrix [149, 150]. This exposure of subendothelial lining, exposed the collagen and vWF which promotes platelet aggregation; exposure of prothrombotic substances such as TF activates the coagulation cascade [149, 151-153]. Activated platelets, along with vWF forms an arterial platelet plug. Hypercoagulable state whether due to congenital or acquired disorders also contributes to the arterial thrombosis. Some of the common congenital thrombophilia associated with arterial thrombosis includes hyperhomocysteinemia. Acquired conditions such as antiphospholipid syndrome, chronic inflammatory conditions such as rheumatoid arthritis or systemic lupus erythematosus, procoagulant producing cancers, use of oral contraceptives, obesity, smoking, or trauma contributes towards the pathogenesis of arterial thrombosis. Arterial thrombosis is a pale thrombus, mainly composed of platelets and fibrin may exhibit lines of zahn.

1.2.1. Venous Thrombosis

Venous thrombosis refers to the formation of a blood clot within the vein. Venous thrombosis is a chronic illness and possesses a major global health burden in terms of mortality and morbidity. VTE includes deep vein thrombosis (DVT) and pulmonary embolism (PE), but it can also occur in other locations such as splenic, splanchnic cerebral and arm veins. DVT refers to the formation of clot in the deep veins, especially in lower extremities.

VTE and arterial thrombosis share many common risk factors (figure 7) which can be broadly categorized into environmental, natural and medical illness [139]. The environmental risk factors of VTE includes smoking, prolonged periods of immobility such as during long flights or surgeries, use of mechanical devices such as shunts plays a role [154]. Natural factors encompass patient-related characteristics such as age, gender-specific factors like pregnancy, the use of oral contraceptives or hormone replacement therapy; and congenital thrombophilia factor V Leiden mutation, prothrombin gene mutation, deficiency of protein C or S or antiphospholipid antibody syndrome [155, 156]. Medical illnesses especially cancer, trauma, surgeries of note orthopedic, or gynaecologic surgeries, history of DVT or PE, congestive heart failure, varicose veins are some of the risk factors for VTE [157-159].

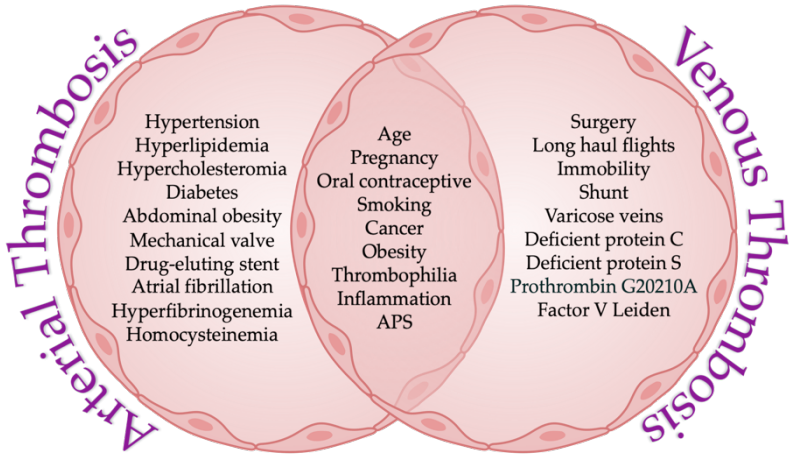


Figure 7. Comparisons of risk factors of arterial and venous thrombosis.

The Venn diagram presents the risk factors of arterial thrombosis (left side) and venous thrombosis (right side). Both arterial and venous thrombosis has some common risk factors. Arterial thrombosis includes the traditional

risk factors of atherosclerosis while venous thrombosis may result from an imbalance between coagulation factors and their inhibitors. Created in Biorender {Siddiqui, 2024 #1415}.

The pathophysiology of VTE follows all three foci of Virchow's triad. Stagnant blood flow in venous sinus and deep veins triggers the activations of coagulation mechanisms leading to the formation of small fibrin deposits. Vascular injury and hypoxia inhibit the natural anticoagulants and promote the release of TF further activating the coagulation cascade providing the hypercoagulable state. Injury to the vessel wall also leads to the expression of vWF and adhesion molecules promotes the platelets and leukocyte recruitments also promotes the release of procoagulant tissue factor microparticles leads to the activation of extrinsic coagulation pathway. Thrombophilia plays a role towards the hypercoagulable state and further promotes clot formation. Dislodged proximal portion of the clots travels upwards from heart to the pulmonary artery leads to the PE. Contrary to arterial thrombi, venous thrombi are predominantly composed of red cells mixed with some platelets and fibrin meshwork.

1.3. VENOUS THROMBOEMBOLISM

Swelling in the ankle extended to leg and thigh experienced by a 20-year-old Raoul or Guillot (born in 1247) represents one of the earliest documented case of DVT, as described by Dexter and Folch-Pi (1974) [160]. Raoul was very fortunate as he was cured of a disease within the 9 days after confessing his sins before the bones of Saint Louis (Louis IX). Although, emboli were suggested by Bonetus in 17th century, van Swieten in 18th century, and Allibert and François in 19th century. The idea of the pulmonary embolism had evolved by Virchow's (1856), following his autopsy of a young man who was admitted to the Charity Hospital, Berlin in 1844, and died suddenly. Despite having rheumatic fever, patient experienced pain, rapid pulsation and elevated temperature in his right thigh. Virchow's found a clot firmly adhered to the vessel wall of right crural vein, and also a coiled clot in pulmonary artery. Later Virchow performed 76 autopsies and out of those he identified 18 cases with venous thrombi and in 11 he found emboli in the pulmonary arterial system. He found venous thrombosis in all the cases however only one on them was an embolic cases. Virchow described the pathogenesis of pulmonary embolism as a process in which:

New masses of coagulum deposit themselves from the blood upon the end of the thrombus layer after layer. The thrombus is prolonged beyond the mouth of the branch into the trunk in the direction of the current of the blood, shoots out in the form of a thick cylinder farther and farther, and becomes continually larger and larger. It is these prolonged plugs that constitute the source of real danger; it is in them that ensues the crumbling away, which leads to secondary occlusions in remote vessels. Thus, we see that as a rule all the thrombi from the periphery of the body produce secondary obstructions and metastatic deposits [emboli] in the lungs [161].

The sudden death associated with the clot in the lower extremities and lungs was explained by Lee in 1835, which was further reinforced by Baron in 1838, who observed pulmonary clots in three patients. However, the concept of embolization was still not fully developed, until Cruveilhier (1852) contemplated the likelihood of thrombi detaching from their site of origin.

Venous thromboembolism is defined as a clot formed in the deep veins considered as DVT. DVT is the most predominant form of VTE, typically originating in the iliac, popliteal, and femoral vein of the calves and upper legs [162]. When the proximal end of the venous thrombus fragments, the free-floating clot swiftly move from the right chambers of the heart to the lungs, lodges to the pulmonary artery, obstructs the blood flow, leading to ischemia and necrosis of the lungs tissue referred as PE [163]. Approximately, 95% of the PE originates from the clot that forms in the deep veins specifically from leg and calf veins.

1.3.1. Epidemiology of DVT and PE

VTE, that consists of pulmonary embolism (PE), and deep vein thrombosis (DVT) is one of the leading causes of vascular disease and cardiovascular death globally [164]. The incidence of VTE is estimated to be ~1–2 per 1,000 person-years which accounts for ~8% lifetime risk of VTE in Europe and the USA [165-167]. Annually, the incidence of VTE in US, among people of European descent, is approximately between 104 to 183 cases per 100,000 individuals [168]. An American Heart Association report from 2021 estimated that approximately 370.000 cases of PE, 857,000 cases of DVT, and 1,220,000 total cases of VTE occur in

the USA annually [136]. A modelling study estimated that the annual VTE incidence in six countries in Europe was 296,000 cases of PE and ~466,000 cases of DVT [169]. According to Center for Disease Control (CDC), around 900,000 people in the United States will be diagnosed with venous thromboembolism (including DVT or PE) and approximately 60,000 to 100,000 people will die of it per year [170]. Around 25% of the cases with PE encountered sudden death. The recurrence rate is also high and is about 33% of cases for DVT/PE within 10 years. The data is less or unavailable in other parts of the world.

It is widely accepted that PE is the more severe form and responsible for most of the mortality cases of VTE [171]. From 250,000 PE cases, ~10% to 15% of the individuals die within the 3-months of PE diagnosis. Approximately, 70% to 80% of the PE originates from DVT of the lower extremities of pelvis, ~6% from the DVT of upper extremities [172, 173]. 1-year risk of mortality is reported to be ~20% after a PE diagnosis, sometimes from VTE but often from other conditions that provoked the event [174]. With the advancement and increased use of pulmonary angiography, the diagnosis of PE has been subsequently increasing from 62 per 100,000 in 1998, to 112 per 100,000 and 120 per 100,000 in 2016 [175-177]. In western countries, the annual incidence of PE is about 60 to 120 cases per 100,000 in general population, of those 14% and 20% of the cases encountered an in-hospital mortality and 90-day mortality respectively [175, 178, 179]. A study from Denmark (between 1999-2018) on the incidence and 1-year mortality related with PE reported the incidence of PE with the age, with 350 cases per 100,000 among people >75 years older compared to 50 per 100,000 among people young and age <50 years [180].

Patients presenting with PE often have significant underlying comorbidities that predispose them to PE and impact their prognosis. Among these comorbidities, cancer associated thrombosis (CAT) is a well-known consequence of cancer diagnosis which is associated with worsened survival, morbidity, need for hospitalization and potential delay or interruption of cancer specific therapy [181]. It has been first described in 1823 by Jean-Baptist Bouillaud, and later explained by Trousseau in 1865 and referred it as “Trousseau Syndrome” [182-184] Cancers, including solid cancers such as gastric, pancreatic, lung, gynecologic, and testicular cancers; and hematologic cancer including lymphoma and acute leukemia are mostly associated with VTE. National Cancer Institute (NCI), the estimated 20 million new cases of cancer with 6 million deaths in 2024 [185]. There

is an increase incidence (4-20%) of clinically overt deep vein thrombosis (DVT) in patients with cancer, who are at 4 to 6.5-fold higher risk of developing thrombotic events. The survival rate for cancer patients with VTE also reduced to 2.2 to 3.7% [186-188]. Although the developments in medicine have led to many investigations which have provided a better understanding of CAT mechanisms, the exact pathophysiology remains incompletely elucidated.

1.3.2. Pathophysiology of DVT and PE

The three pillars of VTE, first articulated by Rudolph Virchow's in 1856, identify three core factors: vascular stasis, endothelial damage, and hypercoagulability as Virchow's Triad [148]. Vascular stasis and endothelial damages are the two arms riving the formation of venous thromboembolism (VTE) [189].

Vascular stasis plays a central role in the pathogenesis of VTE [190]. Inert blood flow in the veins, especially in the venous sinuses and in the deep veins of calf and thighs (femoral, popliteal or iliac) provides a procoagulant environment by the activation of coagulations factors. Normal luminal blood flow flushes out the activated coagulation factors and platelets with the endothelial surface, along with natural anticoagulants, neutralizes the procoagulant environments, and inhibits clot formation. Venous stasis results in the disruption of natural balance leading to a permissive environment, for the accumulation of coagulation factors and platelets, and initiates thrombus formation. Epidemiological factors such as postoperative state, obesity elderly people, long flight, cancer mass, or limb immobilization which reduces muscles contractions, and lowering the pumping action of the veins, resulting stagnant blood flow and increases the risk of VTE [139, 154, 155, 191-194].

Thromboresistance plays a key role in maintaining the integrity of endothelium preventing thrombus formation. This resistant constrain by platelets adhesion to the subendothelial surface and interactions with vWF or adhesion molecules; inhibits platelet aggregation and adhesion by prostacyclin and NO mechanism; prevents activation of circulating clotting factors or cofactors. Endothelium losses its thromboresistant state due to the damage by triggering the releases of TF results in the activation of extrinsic coagulation pathway; expression

of vWF and adhesion molecules such as P-selectin or E-selectin promotes platelets adhesion and aggregation along with leukocytes recruitment, thus initiating clot formation. Damage in the endothelial barrier further disrupts the release of natural anticoagulants, facilitates the activation of extrinsic or intrinsic tenase complex (required phospholipid surface) and is also favorable towards thrombus formation. Epidemiological factors such as surgery, trauma, cancer, or inflammatory conditions can irritate or damage the vessel, thereby contributing to an increased risk of clot formation [157, 195].

Circulating coagulation factors also known as serine proteases are initially present as inactive zymogens. The activation of these factors is crucial for initiating coagulation cascade, when dysregulated thus providing hypercoagulable state and increased risk of thrombosis. Activation of both the intrinsic and extrinsic coagulation pathways can contribute to clot formation by activating the common pathway [13, 14]. Extrinsic pathway is triggered by the release of TF from endothelial surfaces, or TF-bound microvesicles from monocytes, often in response to endotoxin or cytokines. TF binds with FVII forming a FVIIA-phospholipid bound TF which activates Factor X (FX) to FXa and subsequently conversion of prothrombin into thrombin facilities clot formation [9-11]. Foreign surfaces or negatively charged surfaces are essential for the activation of FXII [196]. Pathogen-associated molecular patterns (PAMPs) or damage endothelium provides negatively charge surface and the contacts of FXII with them activates, begins activation of intrinsic pathways. Epidemiological factors such as thrombophilia by designing a pro-coagulant environment or hypercoagulable state favors excessive clot formation. Congenital thrombophilia including deficiency of protein C or S, prothrombin G20210A mutation, factor V leiden mutation or dysfibrinogenemia [197-199]; or acquired thrombophilia result from factors such as pregnancy, use of oral contraceptives, TF-releasing cancer, or antiphospholipid antibody, significantly increase the risk of developing DVT [200-204].

Fragments or dislodged proximal portion of the clots travels from the venous system, right side of the heart, obstruct pulmonary artery leads to the PE. Most of the PE originates from DVT. Symptoms of PE ranges from asymptomatic to hemodynamic changes resulting death. The obstruction of the pulmonary vasculature with vasoconstriction resulting in the release of various vasoconstrictors including serotonin, and TXA2 from platelets; thrombin from

plasma; and histamine, and endothelin from tissues; obstruction along with the vasoconstrictors increases pulmonary vascular resistance, and afterload on right ventricle [205]. Under normal condition, right ventricular pressure ranges from 30 to 40 mm Hg, however a significant increase in the pressure can cause drastic change results in right ventricular dilation, tricuspid regurgitation, and hypokinetic, leads to failure of right side of the heart [206]. Leftward shift of the interventricular septum, reducing left ventricular distensibility, impaired the filling of left ventricle, decrease left ventricle output leads to systemic hypotension [206-208]. The increased right ventricular pressure also compressed the right coronary artery, leading to reduced subendocardial perfusion and constraint oxygen supply to myocardium, further compromising cardiac function [207]. The levels of cardiac biomarkers such as troponin, pro-B-type natriuretic peptide (pro-BNP) and B-type natriuretic peptide (BNP) increased due to due right ventricular microinfarction and right ventricular overload [209-213].

Obstruction affects the blood flow in the pulmonary vascular bed, leading to arterial hypoxemia [214-216]. Imbalance in the ventricular perfusion resulting in inadequate gas exchange from low ventilation to perfusion areas; shunt due to the opening of patent foramen ovale because of elevated right atrial pressure leads to arterial hypoxemia. Gas exchange is significantly impaired in patients with acute PE, leading to decreases arterial hypoxemia along with increase in alveolar-arterial oxygen tension gradient, disruption in gas exchange increases the dead space of the lungs; contributes to hypercapnia (elevated carbon dioxide levels) and respiratory alkalosis [217].

1.3.3. Diagnosis of DVT and PE

Vague signs and symptoms of DVT and PE is a diagnostic challenge and only <20% of patients presented with the symptoms diagnosed with suspected VTE. Approximately 10% of the patients reported chest pain and dyspnea are related to PE. Several studies have reported <10% cases in Europe and <5% cases in US further diagnostic evaluation for pulmonary embolism [218, 219]. The diagnosis of VTE is perplexing, comprises of sequential workup from clinical presentations, and probability, along with biomarker levels and imaging helps in the risk stratification and the management of DVT or PE.

1.3.3.1. Clinical Presentation

Throbbing pain (80% to 90%), swelling (80%), redness (25%) warmth, tenderness on palpation (75% to 85%) are the classical presentation of DVT [220, 221]. Besides that's presence of Homan's sign and leg heaviness are not definitive but indication of DVT. While chest pain (60% to 70%), breathlessness (80%), hemoptysis (5% to 13%), tachycardia (65% to 70%), hypoxemia (70%) are the typical symptoms of PE [222]. Syncope as an initial finding of symptomatic PE presents in association with the hemodynamic changes that leads to shock, hypotension and sudden death. Around 5% to 10% of patients presented with Proximal DVT accounts for ~40% of symptomatic PE cases, which is lower in distal DVT with 25% cases [223, 224].

1.3.3.2. Clinical Probability Tests

Clinical pretest probability assessment test by gestalt is mandatory for the assessment and further diagnostic imaging for DVT or PE. Imaging is the standard of care for the confirmed diagnosis of VTE [225], however the cost-effectiveness, time consumption and radiation is not suitable for all the suspected VTE cases. Thus, avoiding image in all suspected VTE, extensive work-up is required within 24 hours of clinical presentation of VTE for the initiation of the management option [225].

The probability assessment test for DVT by Well's score has been derived by Well, P S et al. (1995, 1997, 2003) with 529, 593, and 1096 patients respectively; proposed the likelihood of DVT [226-228]. The scores were generated by incorporating the risk factor, signs, and symptoms of the patients (table 1), the score ranges from 0-9 and classifies patients into 2 categories: ≤0 points, low/unlikely (prevalence 5%); 1-2 points, moderate (prevalence 17%); ≥3 points high/likely (prevalence 17-53%). Scarvelis D et al. in 2006 performs the validation studies by evaluating multiple studies used to predict DVT by Well's score; including 14 studies with 8239 patients supported the scores for predicting risk of DVT [229].

Table 1. Well's score for the diagnosis of DVT.

Predictors	*Points*
Active cancer	*+1*
Paralysis, paresis, or recent plaster cast on lower extremities	*+1*

Recent immobilization >3 days or major surgery within the past 4 days	*+1*
Localized tenderness of deep venous system	*+1*
Swelling of entire leg	*+1*
Calf swelling >3 cm compared with asymptomatic side	*+1*
Unilateral pitting edema	*+1*
Collateral superficial veins	*+1*
Previously documented DVT	*+1*
Alternate diagnosis at least as likely as DVT	*-2*

A total point score for a given patient is obtained by summing the point for each predictor when present. The score corresponds with the following risk classes: ≤0,low unlikely; 1-2, moderate; ≥3 high/likely.

Clinical assessment for identifying PE include, the Well's score, the Pulmonary Embolism Rule-Out Criteria (PERC), and revised Geneva score and YEARS score. The well's score, revised Geneva score, and YEARS criteria for PE used to gestalt the likely hood of PE by classifying patients into low, intermediate and high risk of PE or either likely or unlikely for PE. The scores are based on the clinical characteristics, and predisposing factor to PE, along with likelihood of PE diagnosis in revised Geneva score.

Well's score for assessing the likelihood of PE diagnosis was established by Wells, P S et al. (2001) and considered as the most widely used criteria for the diagnosis of PE [230]. The scoring system was derived from a cohort of 930 patients with suspected PE, and includes 7 core predictors-comprising risk factors, sign, and symptoms, as well as 1 subjective variable related to the clinician's likelihood of PE diagnosis (table 2). Wolf S J et al. (2004) performed the validation of the score by utilizing 120 patients [231]. The original score ranges from 0-12.5 points and classifies patients into different risk categories using either three-tier or two-tier models. Three-tier models stratify patients as follows: 0-1 points, low (prevalence 1.3%); 2-6 points, intermediate (prevalence 16.2%); >6 points, high (prevalence 37.5%), or ≤4 points, unlikely (prevalence 8%); ≥5 points, likely (prevalence 41%). Alternatively two-tier models classify patients into two groups: ≤4, unlikely (prevalence 8%); ≥5 points, likely (prevalence 41%). Gibson N S et al. in 2008 simplified the Well's score using a cohort of 3306 consecutive patients with suspected PE. his simplified version reduced the score range to 0-7 points and categorized patients two 2 main classes: ≤1 point, unlikely (prevalence 11%); >1 point, likely (prevalence 36%).

Table 2. Well's score for the diagnosis of PE.

Predictors	Wells' Score	
	Original[a]	Simplified[b]
Clinical signs and symptoms of DVT	+3	+1
PE is #1 diagnosis or equally likely	+3	+1
Heart rate >100 beats per minute	+1.5	+1
Immobilization at least 3 days or surgery in the previous 4 weeks	+1.5	+1
Previous, objectively diagnosed PE or DVT	+1.5	+1
Hemoptysis	+1	+1
Active cancer with treatment within 6 months or palliative	+1	+1

[a] A total point score for a given patient is obtained by summing the point for each predictor when present. The score corresponds with two different risk classes: 0-1, low; 2-6, intermediate; >6, high or ≤4, unlikely; ≥5, likely.

[b] A total point score for a given patient is obtained by summing the point for each predictor when present. The score corresponds with the following risk classes: ≤1, unlikely; >1, likely.

The PERC was developed by Kline J A et al. (2004), aiming to exclude PE patients at low and very-low-risk without the need for further diagnostic testing. The PERC was derived from a cohort of 3148 patients, and its validation involved two different populations of 1427, and 382 patients. The criteria were based on 9 predictors which includes risk factors, signs, and symptoms (table 3). The presence of any of these predictors does not rule out PE from the diagnosis. The PERC was also validated by Kline J A et al. in 2008 with 8138 patients with suspected PE [232].

Table 3. Pulmonary Embolism Rule-Out Criteria (PERC) to rule out PE.

Predictors	Points[a]
Age ≥50 years	+1
Heart rate ≥100 beats per minute	+1
Oxygen saturation on room air <95%	+1
Unilateral leg swelling	+1
Hemoptysis	+1
Recent surgery or trauma (≤4 weeks ago requiring treatment with general anesthesia)	+1
Hormone use (oral contraceptives, hormone replacement or estrogenic hormones use in males or female patients)	+1

[a] All of the predictors must be negative to rule out patients.

Revised Geneva score for assessing the clinical probability of PE was developed by Le Gal, Grégoire et al. (2006) [233]. The score was derived from a cohort that includes 965 patients with acute PE. For internal validation, a cross-

validation approach was employed, dividing the cohort into 10 random and equal-sized groups. External validation studies were conducted with an additional cohort of 756 patients [233]. Similar to Well's score, the Revised Geneva score also utilizes the 8 core predictors including risk factors, signs, and symptoms (table 4). The score ranges from 0-22 points and classifies patients into 3 classes, <3 points, low (prevalence 9%); 4-10 points, intermediate (prevalence 27.5%); >11 points, high (prevalence 71.7%). In 2008, Klok, F A et al. further validated the revised Geneva score and compared with wells score in 300 patients [234]. And the score was further simplified and categorize into 2 classes, ≤2 points, unlikely (prevalence 13%); >2 points, likely (prevalence 42%) [225].

Table 4. Revised Geneva score for the diagnosis of PE.

Predictors	*Revised Geneva Score*	
	Original[a]	*Simplified*[a]
Age >65	*+1*	*+1*
Previous DVT or PE	*+3*	*+1*
Surgery (under general anesthesia) or lower limb fracture in past month	*+2*	*+1*
Active cancer (solid or hematologic malignant condition, currently active or considered cured <1 year)	*+2*	*+1*
Unilateral lower limb pain	*+3*	*+1*
Hemoptysis	*+2*	*+1*
Heart rate 75-94	*+3*	*+1*
Heart rate ≥95	*+5*	*+2*
Pain on lower limb palpable and unilateral	*+1*	*+1*

[a.] *A total point score for a given patient is obtained by summing the point for each predictor when present. The score corresponds with two different risk classes: <3, low; 4-10, intermediate; >11, high.*

[b.] *A total point score for a given patient is obtained by summing the point for each predictor when present. The score corresponds with the following risk classes: ≤2, unlikely (prevalence 13%); >2, likely (prevalence 42%).*

YEARS criteria is another clinical assessment tool which stratifies the patients according to likelihood of patients with PE, developed by van der Hulle T et al. (2017) [235]. The criteria are used for hemodynamically stable patients ≥18 years old. The algorithm was derived from a cohort of 3465 patients clinically suspected PE and only 3 predictors from Well's score were incorporated (table 5). The score ranges from 0-3 which classifies patients into 2 classes, 0 point, low risk (prevalence 3%); ≥1 points, high-risk (prevalence 23%). Kabrhel C et al. and Freund Y et al. have

consecutively validated the YEARs score in a cohort of 1789, and 1414 patients suspected of PE [236].

Table 5. YEARS criteria for the diagnosis of VTE.

Predictors	*Points*[a]
Clinical signs of DVT	*+1*
Hemoptysis	*+1*
PE is most likely diagnosis	*+1*

[a] *A total point score for a given patient is obtained by summing the point for each predictor when present. The score corresponds with the following risk classes: 0 point, low-risk; ≥1, high-risk.*

1.3.3.3. ***D-dimer***

D-dimer is a fibrin degradation product, only marker for the diagnosis of VTE. Due to its high sensitivity and negative predictive value, patients with a unlikely or non-high pretest clinical probability assessment should undergo D-dimer testing. Patients with likely or high clinical probability assessment should directly undergo non-invasive imaging without testing for D-dimer by using ultrasonography for DVT or multislice or spiral chest CT scan for PE. D-dimer assays can differ in their method of measurement. Sensitivity for VTE and threshold for defining positive or negative test results. Quantitative ELISA have a high sensitivity (>95 %) but a low specificity for VTE [237]. Negative test result (usually <500 ug/L) using a highly sensitive D-dimer assay excludes VTE in patients with assessed as low-risk clinical probability for PE [238, 239]. However, the diagnostic potential of D-dimer weakens in elderly patients (>65 years), pregnant women, during infections, cancer, or chronic inflammatory conditions such as collagen vascular disease [239-241]. Newer diagnostic strategies by using a higher threshold to define either a non-high clinical pretest probability or a negative D-dimer (e.g. age-adjusted D-dimer) results have been validated in prospective studies, although requiring further investigation [242, 243]. In pregnant women D-dimer testing can be used in combination with YEARS criteria or revised Geneva score to exclude PE [241, 244].

1.3.3.4. ***Imaging***

1.3.3.4.1. Imaging for Suspected DVT

Compression ultrasonography is the first line and the most widely used imaging for the suspected DVT. In this diagnostic method, failure to fully compress the lumen of the veins with ultrasound probe is complementary of DVT. In clinical practice, two strategies are commonly used, proximal (or limited) leg ultrasonography or whole leg ultrasonography. Proximal leg ultrasonography is performed by scanning the common femoral and popliteal vein regions between the groin and the calf trifurcation. The whole leg ultrasonography is performed between the groin and ankle regions providing additional information about the deep veins of the calf. DVT can be excluded in all patients with a normal whole leg ultrasound or those assessed as having an unlikely pretest clinical probability with a normal proximal leg ultrasound. Patients assessed as having a likely pretest probability and normal leg ultrasound, should undergo a serial proximal ultrasound to exclude a proximal extension of a distal DVT and anticoagulation can be withheld between serial ultrasounds. Whole leg ultrasonography is reserved for patients assessed as having a likely pretest probability and a positive D-dimer test result.

Computed tomography (CT) can be used for establishing the diagnosis of DVT. CT venography can detect femoropopliteal venous thrombosis comparable with ultrasonographic evaluation and CT venography. However, due to use of contrast agents and radiation exposure the use of CT in this setting remains limited. There are protocols under development for imaging of both the pulmonary arteries and the subdiaphragmatic deep veins (including the legs) at the same session, with no additional contrast medium or venipunctures beyond what is required for a CT pulmonary angiogram [245-249].

Magnetic resonance venography (MRV) is as accurate as contrast venography for the diagnosis of DVT. Although the diagnostic accuracy of magnetic resonance venography is comparable to that of contrast venography, outcome data are lacking. In addition, the present high cost of magnetic resonance venography makes it unlikely that it will gain prominence as a noninvasive test for DVT. However, magnetic resonance venography is a useful approach when

contrast venography is required but precluded because of allergy to contrast material [250, 251].

Contrast venography has long been considered the reference test for the diagnosis of DVT. However, it is not recommended as an initial screening due to patient discomfort and difficulty in obtaining an adequate study [252].

1.3.3.4.2. Imaging for Suspected PE

Ventilation–perfusion (V/Q) scintigraphy and CT pulmonary angiography (CTPA) are the most extensively validated imaging modalities for diagnosing pulmonary embolism and have similar diagnostic accuracy. CTPA often remains the only option in many centers without local access to V/Q scintigraphy. Other diagnostic imaging modalities have been studied, but only ultrasonography can be considered as an alternative initial test for patients with suspected pulmonary embolism. The diagnosis of pulmonary embolism can be considered as established in patients with a confirmed proximal deep vein thrombosis and signs and symptoms of pulmonary embolism. Conversely, a normal leg ultrasound does not exclude pulmonary embolism.

PE patients may have echocardiographic abnormalities suggestive of acute PE including increased right ventricular (RV) size, decreased RV function, and tricuspid regurgitation. In cases of high risk or massive PE, these abnormalities become more prominent, and echocardiography can be useful for rapid presumptive diagnosis to justify the use of thrombolytic therapy. Additional echocardiographic findings suggestive of PE include RV thrombus and regional wall motion abnormalities that spare the right ventricular apex ("McConnell's sign"). The presence of RV dysfunction or an RV thrombus detected by echocardiography has prognostic implications [253].

The use of magnetic resonance angiography (MRA) for the diagnosis of PE is limited by respiratory and cardiac motion artifact, suboptimal resolution, complicated blood flow patterns, and magnetic susceptibility effects from the adjacent air-containing lung. However, technologic advances offer promise for an expanded role of MRA in the future [254, 255].

Pulmonary angiography is the definitive diagnostic technique or "gold standard" in the diagnosis of acute PE. It is performed by injecting contrast into a

pulmonary artery branch after percutaneous catheterization, usually via the femoral vein. A filling defect or abrupt cutoff of a small vessel is indicative of an embolus. A negative pulmonary angiogram excludes clinically relevant PE [256].

1.4. RISK STRATIFICATION OF PE

Despite advancements in diagnostic and therapeutic approaches, managing PE remains challenging, specifically due to its diverse clinical manifestations and underlying pathophysiological mechanisms. While the mortality seems to be more related to the hemodynamic impact of the thromboembolic event in the acute period, the longer-term prognosis is more influenced by the presence of underlying comorbidities [257]. The prognosis of PE patients varies widely, and the predictors of prognosis are not utterly understood. To better characterize the severity and prognosis of PE patients, several clinical prediction tools have been published [258], including Pulmonary Embolism Severity Index (PESI) and the simplified PESI (sPESI). These scores have been developed by compounding indices of cardiopulmonary impairment with age and comorbidities to stratify patients into different risk categories. The European Society of Cardiology (ESC) guidelines have further refined risk stratification by incorporating biomarkers and imaging modalities [259]. Prognostic biomarkers, such as troponin, NT-proBNP, cellular indices, and D-dimer, have emerged as valuable tools for risk stratification in acute PE. These biomarkers provide insight into the biological processes underlying adverse outcomes, such as right ventricular strain and systemic inflammation, and may improve the accuracy of existing prognostic models.

1.4.1. Pulmonary Embolism Severity Index (PESI)

PESI score is used to assess the severity and predict short-term mortality risk in patients with PE. The score was developed by a Aujesky D, et al. (2005). PESI score was derived from a cohort of 10354 patients, the score was subsequently validated in two separate cohorts; the internal validation utilized a cohort of 5177 patients, while the score was validated externally on a cohort of 21 patients [260]. PESI score has been developed by using 11 baseline clinical characteristics including demographics, comorbidities, and clinical presentation of the patients (table 6). The total score for a patient is computed by summing their age up in years

and a point assigned for each present predictor. Each of the predictors has a different weight on the scoring. The calculated score stratifies patients into the 5 following classes, 65 or less, class I; 66 to 85, class II; 86 to 105, class III; 106 to 125, class IV; and ≥125, class V. PESI score has been also prospectively validated in the cohort of 375 patients [261].

1.4.2. Simplified Pulmonary Embolism Severity Index (sPESI)

Simplified PESI also estimates the short-term 30-day mortality in patients with PE. The scoring system was developed by modifying the PESI score by Jiménez D, et al. (2010). sPESI score was derived from a cohort of 995 patients for and a cohort of 7106 patients was used for the validation purposes [262]. In the sPESI score, history of heart failure and chronic lung disease was collectively considered as a single variable of chronic cardiopulmonary disease (table 6). sPESI score stratifies patients into two classes with score 0 as low-risk PE patients; score 1 or more as high-risk PE patients. A meta-analysis of 21 global studies of 50,021 patients, including 4,991 experienced all-cause mortality, effectively compared the effectiveness of PESI and sPESI score in predicting all-cause mortality or PE-related mortality.

Table 6. Original and Simplified Pulmonary Embolism Severity Index (PESI) score.

Variables	*PESI score[a]*	*sPESI score[b]*
Age >80 years	*Age in years*	*+1*
Male sex	*+10*	*-*
History of cancer	*+30*	*+1*
History of heart failure	*+10*	*+1[c]*
History of chronic lung disease	*+10*	
Pulse >110 beats/min	*+20*	*+1*
Systolic blood pressure <100 mm Hg	*+30*	*+1*
Respiratory rate >30 breaths/min	*+20*	*-*
Temperature <36 °C	*+20*	*-*
Altered mental status	*+60*	*-*
Arterial oxyhemoglobin saturation levels <90%	*+20*	*+1*

[a] A total point score for a given patient is obtained by summing the patient's age in years and the point for each predictor when present. The score corresponds with the following risk classes: 65 or less, class I; 66 to 85, class II; 86 to 105, class III; 106 to 125, class IV; and ≥125, class V.

[b] A total point score for a given patient is obtained by summing the points. The score corresponds with the following risk classes: 0, low-risk; 1 or more, high-risk. Empty cells indicate that the variable is not included.

[c.] *The variables were combined into a single category of chronic cardiopulmonary disease.*

1.4.3. European Society of Cardiology (ESC) Guideline

In 2014, the European Society of Cardiology (ESC) issued guidelines on the diagnosis and management of PE. The guidelines offer recommendations for prognostic assessment and management of patients with acute PE, including those with special considerations such pregnancy or cancer. The guideline was revised in 2019 with some modifications. These guidelines include the risk stratification of PE by utilizing all the component predictors from PESI and sPESI scores along with other markers including elevated troponin or NT-proBNP and right ventricle (RV) dysfunction parameters (table 7). Patients are stratified into low, intermediate; intermediate-high, and intermediate-low, and high-risk patient groups. According to ESC stratification low-risk PE patients have <1% to 2% risk of in-hospital mortality, while the risk of this adverse outcome is between 5% and 10% for high-risk groups.

Table 7. Classification of pulmonary embolism severity and the risk of early (in-hospital or 30-day) death.

Early mortality risk		*Indicators of risk*			
		Hemodynamic instability[a]	*Clinical parameters of PE severity and/or comorbidity: PESI class III-V or sPESI ≥1*[a]	*RV dysfunction on TTE or CTPA*[b]	*Elevated cardiac troponin levels*[c]
High		+	*(+)*[d]	+	*(+)*
Intermediate	*high*	-	+[e]	+	+
	low	-	+[e]	*One (or none) positive*[d]	
Low		-	-	-	*- (If assessed)*

[a.] *One of the following clinical presentations: cardiac arrest, obstructive shock (systolic BP <90 mmHg or vasopressors required to achieve a BP >90 mmHg despite an adequate filling status, in combination with end-organ hypoperfusion), or persistent hypotension (systolic BP <90 mmHg or a systolic BP drop >40 mmHg for >15 min, not caused by new-onset arrhythmia, hypovolaemia, or sepsis).* [b.] *Prognostically relevant imaging (TTE or CTPA) findings in patients with acute PE, >1.0.* [c.] *Elevation of further laboratory biomarkers, such as NT-proBNP >600 ng/L, H-FABP ≥6 ng/mL, or copeptin >24 pmol/ L, may provide additional prognostic information. These markers have been validated in cohort studies, but they have not yet been used to guide treatment decisions in randomized controlled trials.* [d.] *Haemodynamic instability, combined with PE confirmation on CTPA and/or evidence of RV dysfunction on TTE, is sufficient to classify a patient into the high-risk PE category. In these cases, neither calculation of the PESI nor measurement of troponins or other cardiac biomarkers is necessary.* [e.] *Signs of RV dysfunction on TTE (or CTPA) or elevated cardiac biomarker*

levels may be present, despite a calculated PESI of I-II or an sPESI of 0. Until the implications of such discrepancies for the management of PE are fully understood, these patients should be classified into the intermediate-risk category.

1.4.4. BOVA Score

Becattini Outcomes Venous Thromboembolism Assessment (BOVA) score has been designed to predict short-term PE related outcomes in hemodynamically stable patients. BOVA score has been developed by combining the six different studies with 2874 hemodynamically stable patients with PE. BOVA et al. (2014) has been designed for normotensive PE patients who are at intermediate- and high-risk of developing PE-related complications [263]. BOVA score incorporate some of the predictors from PESI, sPESI, and ESC guideline (table 8). BOVA score stratifies normotensive patients in the following three different stages, stage I stratifies patients with the score of 0-2 as low-risk, stage II stratifies patients with the score of 3-4 as intermediate-risk, and stage III stratifies patients with the score >4 as high-risk patients. The effectiveness of BOVA score was subsequently validated in two different cohorts of 1083 and 639 normotensive patients with acute PE by Carolina Fernández C, et al. in 2015, and Bova C, et al. in 2018 [264, 265].

Table 8. Original and Modified BOVA score.

Variables	*BOVA Score*	
	Original[a]	*Extended*[a]
Systolic blood pressure (90-100 mm Hg)	*+2*	*+2*
Elevated cardiac troponin	*+2*	*+2*
RV dysfunction on echocardiography	*+2*	*+2*
Heart rate >110 bpm	*+1*	*+1*
Elevated lactate	*---*	*+3*

[a.] *A total score for a given patient is obtained by summing the points. The score corresponds with the following risk staging: 0-2 points, stage I (low-risk); 3-4, stage II (intermediate-risk), >4 stage III (high-risk).*

[b.] *A total score for a given patient is obtained by summing the points. The score corresponds with the following risk staging: 0-2 points, stage I (low-risk); 3-4, stage II (intermediate-risk), >5 stage III (high-risk).*

Although there are some risk scores for the stratification of patients and outcome analysis, these are off limited value, as current models based on clinical and imaging findings often fail to fully capture the heterogeneity of patient outcomes. The results of this thesis provide integrated cellular and biomarker

parameters to calculate a reliable prediction models. This study may provide a comprehensive and robust approach using two independent cohorts from RIETE and LUMC PERT.

1.5. BLOOD CELLULAR INDICES AS PROGNOSTIC MARKERS

The blood cellular counts and blood cellular indices have been evaluated in various studies in the literature. These studies have shown that the blood cellular indices may provide both diagnostic and prognostic information in different diseases. The prognostic value of cellular indices is tested in various pathologic states. It has been speculated that may be also relevant to thrombotic and endothelial markers. Neutrophil-to-lymphocyte ratio (NLR), platelet-to-lymphocyte ratio (PLR), and systemic immune-inflammation index (SII; NLR*platelets) are the most studied blood cellular indices and have been related to pathophysiology of VTE [266], and used to risk stratify and predict adverse outcomes.

1.5.1. Neutrophil-to-Lymphocyte Ratio (NLR)

NLR has been evaluated in several studies in the setting of VTE. Ates et al. have identified NLR as an independent predictor of massive PE in 639 patients comprised of acute PE. In this study, NLR was found to have a good diagnostic accuracy with an AUC of 0.893 for this outcome. Kasapoglu et al. also reported that NLR levels were significantly higher in patients who died within 30 days in the 550 patients comprised of acute PE [267]. Interestingly, NLR was not an independent predictor of death for overall patients in this study. But subgroup analysis in patients without comorbidities showed that NLR was an independent predictor of mortality. Duman et al. have shown that an NLR was predictive for 30-day, 6-month and 1-year mortality in a cohort of 828 PE patients [268].

1.5.2. Platelet-to-Lymphocyte Ratio (PLR)

Several studies also evaluated the PLR as a diagnostic and prognostic tool for acute VTE. The study of Ates et al. identified PLR as an independent predictor of massive acute PE in 639 patients. In this study, PLR was found to have a good

diagnostic accuracy with an AUC of 0.877 for this outcome. Kasapoğlu et al. reported higher levels of PLR in 550 acute PE patients who have deceased within 30-days of acute VTE [267]. However, PLR was not an independent risk factor in the multivariable analysis in this study. Duman et al, have reported that there was no significant difference in PLR levels between deceased and surviving subgroups in 828 PE patients [268]. Additionally, PLR was also not an independent risk factor for death in this study. Kundi et al. have found that higher levels of PLR in patients with high sPESI scores was independently associated with in-hospital mortality among 646 patients with acute PE. However, Ghaffari et al. did not find a difference in PLR between patients with and without major cardiopulmonary adverse events in 492 acute PE patients [269]. But PLR was associated with in-hospital mortality with an AUC of 0.610 in this study. In the recent literature, PLR was also considered as a predictor of the VTE occurrence in cancer patients and after surgery. Grilz et al. identified a significant association between PLR and the occurrence of VTE in 1469 cancer patients. Yao et al. identified a higher preoperative PLR than postoperative PLR in 733 patients after total joint replacement. In this study, the postoperative PLR was independently associated with the occurrence of DVT with an AUC of 0.513 and 0.561 for preoperative PLR and postoperative PLR respectively. Furthermore, Kurtipek et al, have reported higher PLR values in 71 patients with acute PE compared to healthy controls, suggesting that PLR may be associated with pulmonary artery endothelial cell dysfunction.

1.5.3. Systemic Immune-Inflammation Index (SII)

The predictive value of SII was also tested in several previous studies. These studies have shown that it can also be a useful tool for both the diagnosis and prognosis of mortality in VTE. Gok et al. reported elevated SII levels in 442 patients with acute PE. SII was also higher in patients who had in-hospital mortality [270]. SII was an independent predictor of massive APE with an AUC of 0.957. Peng et al. reported an increased SII in patients with VTE compared to those without VTE. In this study, SII was an independent predictor of VTE after hip fracture of elderly patients.

1.6. BIOMARKERS IN PE

A biological marker (biomarker) is a defined characteristic that is measured as an indicator of normal biological processes, pathogenic processes, or biological responses to an exposure or intervention, including therapeutic interventions [271]. In medical practice, biomarkers are used for various purposes, serving a wide range of applications. These include diagnostic biomarkers for detecting or confirming the presence of a disease or condition, monitoring biomarkers for tracking disease progression or treatment effectiveness, response biomarkers for evaluating therapeutic responses, predictive biomarkers for identifying individuals likely to experience favorable or unfavorable outcomes, prognostic biomarkers for predicting the disease course, such as recurrence or progression, safety biomarkers for assessing treatment-related risks, and susceptibility/risk biomarkers for identifying individuals at increased risk of developing a disease.

An ideal biomarker should meet the following criteria: it should be either binary (present or absent) or quantifiable in an objective manner; measurable through an assay that is easily adaptable to routine clinical practice with a rapid turnaround time suited to the clinical setting; the assay should be both sensitive and specific; and most importantly, the biomarker should be detectable using readily accessible specimens [272]. Additionally, the availability of sufficient clinical populations for conducting and validating studies also presents a significant challenge in biomarker discovery.

In the setting of VTE, biomarkers usually complement clinical assessment, ECG and cardiac imaging in the diagnosis, risk stratification, triage and management of patients with acute PE [273]. Despite extensive basic and clinical research conducted by numerous groups worldwide, only a few biomarkers, such as D-dimer, troponins (troponin I and T), brain natriuretic peptides (B-type BNP and N-terminal-proBNP) and lactate have achieved widespread clinical acceptance and been incorporated into clinical practice guidelines.

D-dimer, known for its high negative predictive value, is used in the diagnosis and risk stratification of PE [259, 274]. Troponins, which indicate cardiomyocyte injury, are employed to predict prognosis, including right ventricular dysfunction and adverse cardiovascular outcomes. BNP levels increase due to right ventricular pressure overload from acute PE, reflecting myocardial

stretch and correlating with right ventricular dysfunction and mortality. Lactate, a marker of the imbalance between tissue oxygen supply and demand, indicates severe PE with overt or imminent hemodynamic compromise. However, like D-dimer, all these biomarkers have a low specificity and positive predictive value for adverse outcomes in normotensive patients with PE. Despite this, low levels of these biomarkers can effectively exclude unfavorable clinical outcomes, given their high sensitivity and negative predictive value. The role of alternative biomarkers is not yet well-defined. For this reason, there is a critical need for the development of novel biomarkers to enhance diagnostics, prognostics, and treatment in PE.

Several studies have evaluated various biomarkers in the setting of VTE, however most of them evaluated biomarkers of a single system, such as endothelium or coagulation or inflammation; or having small sample size. In this study, we have investigated the prognostic significance of biomarkers associated with endothelial injury, platelets and coagulation activation, fibrinolysis, and inflammation which have been associated with adverse outcomes in previous studies. Endothelial markers evaluated in this dissertation include Von-Willebrand factor (vWF), E-selectin, and Tissue Factor. Platelet activation marker include P-selectin. Marker of fibrinolysis includes Tissue-Plasminogen Activator (tPA), Plasminogen Activator Inhibitor-1 (PAI-1), Thrombin Activable Fibrinolysis Inhibitor (TAFI), D-dimer, Prothrombin Fragment 1+2 (F1+2), Thrombin-antithrombin Complex (TAT). Inflammatory biomarkers analyzed include C-Reactive Protein (CRP), the Interleukins (IL) IL-1α, IL-1β, IL-2, IL-4, IL-6, IL-8, and IL-10, Tumor Necrosis Factor-α (TNF-α), Interferon-γ (IFN-γ), Monocyte Chemoattractant Protein-1 (MCP-1). Growth factors including Epidermal Growth Factor (EGF), and Vascular Endothelial Growth Factor (VEGF). A description of each marker follows.

1.6.1. Endotelial Markers

While endothelium plays a significant role in VTE pathogenesis, endothelial markers by helping assess the endothelial functions and reflecting the level of endothelial damage can be novel biomarkers in VTE. Although several endothelial markers (e.g., vWF, E-selectin, TF, ICAM-1, and VCAM-1) have been identified for

assessing VTE [275], none of them are completely validated, and their importance remains unclear in the setting of VTE [67, 276, 277].

1.6.1.1. ***Von-Willebrand Factor (vWF)***

VWF is a glycoprotein that is primarily produced by endothelial cells and is stored in Weibel-Palade bodies in the endothelium [278]. It is also produced in platelets and stored in α-granules to a lesser extent [279, 280]. During an acute VTE event vWF can be rapidly released into the circulation and high levels of vWF reported in various VTE types such as lower extremity DVT, portal vein thrombosis, cerebral venous thrombosis, and cancer-associated thrombosis [275, 281, 282]. Additionally, elevated levels of vWF have been associated with VTE related complications such as PTS, CTEPH, and cancer-associated thrombosis [283, 284].

1.6.1.2. ***E-selectin***

E-selectin is another glycoprotein expressed from activated endothelium that facilitates thrombosis, directly modulating neutrophil and monocyte activity. E-selectin resides primarily in the endothelium and is associated closely with P-selectin to recruit leukocytes to sites of inflammation [66]. Studies on E-selectin have yielded varying results. In some cases, E-selectin levels were found to be lower in DVT patients compared to controls, or not significantly elevated [285, 286]. However, E-selectin levels have also been shown to be higher in more severe VTE cases, suggesting a potential association with disease severity in certain patient groups [287]. This variability highlights the need for further research to clarify the role of E-selectin as a biomarker in VTE.

1.6.1.3. ***Tissue Factor***

Tissue factor (TF), also known as thromboplastin, is a 47-kDa transmembrane glycoprotein located on the surface of various cells, including fibroblasts, endothelial cells, pericytes, and certain cancer cells [288, 289]. TF plays a crucial role in the activation of the extrinsic pathway of the coagulation cascade. Most circulating TF is present as microparticles (MPs) which are the small membrane fragments, also referred as microvesicles [290]; which are generated from the activated or apoptotic cells [291]. Several studies have reported the linked between

the expression of MP-TF with different diseases including VTE, sepsis, sickle crisis [277, 292-294]. An additional study reported CAT patients exhibit significantly higher levels of microparticle-tissue factor (MP-TF) compared to those without VTE [295, 296].

1.6.2. Platelet Activation Markers

Activated platelets contribute to thrombus formation by promoting coagulation and recruiting inflammatory cells, making them a key component in the progression of VTE. Several biomarkers of platelet activation, such as P-selectin, platelet factor 4 (PF4), and platelet derived microparticles provide insights into the degree of platelet involvement in VTE.

1.6.2.1. ***P-selectin***

P-selectin is primarily produced in platelets as well as endothelial cells and stored in platelet α-granules and Weibel-Palade bodies in endothelial cells [297, 298]. Upon activation of platelets, P-selectin potentiate leukocyte-endothelial, leukocyte-platelet, and leukocyte-leukocyte interactions, the release of tissue factor rich microparticles from neutrophils, the upregulation of tissue factor on monocytes, and thus the facilitation and amplification of VTE [66, 299-301]. Elevated levels of P-selectin have been associated with an increased risk of VTE and recurrence of venous thrombosis [302, 303].

1.6.3. Coagulation Factors

Elevated plasma levels of coagulation factors have been closely linked to the pathogenesis of VTE [304, 305]. In observational studies, coagulation factors such as fibrinogen, factor VIII, factor IX, and factor XI, are commonly found in higher concentrations in VTE patients [306, 307]. Marked factor V activity elevation has been found to be associated in severe COVID-19 patients with VTE.

1.6.3.1. ***Factor VII***

Factor VII is a vitamin K–dependent serine protease glycoprotein with a pivotal role in hemostasis and coagulation, synthesized in liver as a single chain

molecule of Mr ~50,000 [308]. Structurally, FVII contains a Gla domain (gamma-carboxyglutamic acid-rich), a short hydrophobic segment (often considered as part of the Gla domain), two epidermal growth factor (EGF)-like domains, and the C-terminal serine protease domain [309]. It is well known that blood coagulation is initiated by the formation of a complex between TF and activated factor VII (FVIIa) [310]. Although a large number of studies associated elevated levels of factors VII with cardiovascular disease particularly in coronary artery disease, elevated levels of FVII have not been consistently associated with VTE and the information scarce [311, 312]. Aberrant expression of factor VII has been shown in cancer associated thrombosis [313].

1.6.3.2. ***Factor IX***

Factor IX is a vitamin K–dependent glycoprotein coagulation factor, which is activated through the intrinsic pathway as well as the extrinsic pathway. FIX, like other vitamin K dependent glycoprotein, contains four domains: Gla domain, two tandem epidermal growth factor (EGF)-like domains, and a C-terminal serine protease domain [314]. Several studies have reported that factor IX levels increase with age as well as with oral contraceptive use [315, 316]. High levels of Factor IX have been associated with an elevated risk of VTE [317].

1.6.3.3. ***Factor X***

FX is a serine protease that plays an essential role towards thrombin generation, synthesized in liver as single chain polypeptidise (80 kDa.) Structurally, FX contains catalytic domain, epidermal growth factor (EGF)-like domain, and Gla (Gamma-carboxyglutamic acid) domain [318]. The activation of FX is dependent on the intrinsic or extrinsic tenase complex, which further forms a prothrombinase complex and activates thrombin [12, 13]. Although FX is known as a therapeutic target, inhibiting the activation of FX serves as an oral anticoagulant, role of FX is not in the setting of VTE has not been reported [319].

1.6.3.4. ***Factor XIII-A***

FXIII-A is a subunit of FXIII. FXIII is a clot-stabilizing factor; a 325 kDa protransglutaminase belongs to a transglutaminase-like superfamily, involves in post-translational modifications of proteins and generation of isopeptide bonds

resistant to proteolytic degradation [320]. FXIII is present in both the plasma and cellular components (such as monocytes, megakaryocytes) of blood [321]. Structurally, FXIII contains two catalytic A subunits (about 83 kDa) and two non-catalytic or inhibitory B subunits (about 80 kDa) that form heterotetramers (FXIII-A2B2). Thrombin activates FXIII by cleaving the FXIII-A subunit at Arg37-Gly38, regulated by Ca^{2+}, and activation peptide released, and Activation of platelet FXIII-A2 occurs. Besides fibrin monomer, FXIII-A catalyzes the formation of intermolecular bonds between α2-antiplasmin, fibronectin, vitronectin, thrombospondin, and collagen. Several studies have reported the decrease level of FXIII and FXIII-A in patients with DVT, however the reduced levels/activity of FXIII is not associated with the risk of recurrent VTE [322, 323].

1.6.4. Markers of Fibrinolysis

Impaired endogenous fibrinolysis may lead to thrombotic events. It has been reported that patients with intermediate-risk PE exhibit significantly longer lysis and are primarily influenced by plasma concentrations of PAI-1, and TAFI [324]. Prolonged clot lysis time affected by the plasma concentrations of TAFI, plasminogen, α2-antiplasmin, and PAI-1 has been linked to venous thrombosis. Hypo-fibrinolysis has been associated with an increased risk of DVT [325]. Measuring the levels or activity of some of the major marker of fibrinolysis includes TPA, PAI-1, TAFI, D-dimer, prothrombin fragment 1+2, and TAT [325].

1.6.4.1. ***Tissue-Plasminogen Activator (tPA)***

tPA, a major regulator of fibrinolysis, secreted by endothelial cells and released in response to vascular injury or clot. tPA triggers fibrinolysis by hydrolyzing fibrin-bound plasminogen into plasmin, during the depolymerization tPA forms a covalent lysine bond with fibrin. Increase level of tPA has been associated with postoperative thrombosis [326]. tPA has been used as a thrombolytic agent for acute PE patients with hemodynamic instability or shock.

1.6.4.2. ***Plasminogen Activator Inhibitor 1 (PAI-1)***

PAI-1 is a serine protease inhibitor and produced from endothelial cells, megakaryocytes, monocytes, hepatocytes, and other cells, also stored in platelets.

PAI-1 complexes with tPA and uPA, preventing plasminogen activation and conversion into active fibrinolytic plasmin. Moreover, binding of tPA with the fibrin shields the formation of tPA:PAI-1 complex. Studies on the mouse model have demonstrated a significant role of PAI-1 in the formation and resolution of venous thrombi. PAI-1 deficient mice have shown a high resistance towards venous thrombosis along with high capacity to lyse experimental plasma clots in a PE model [327]. The role of PAI-1 towards VTE is controversial, some studies have shown association of PAI-1 with VTE. [326, 328], while some not [329]. One study reports an increase in plasma levels of PAI-1 antigen not only associated with worse PE severity but also linked with 1-year mortality in patients with acute PE [330].

1.6.4.3. ***Thrombin Activatable Fibrinolysis Inhibitor (TAFI)***

TAFI is an inactive enzyme precursor and a polycarboxypeptidase which is synthesized in liver. TAFI activated by the thrombin-thrombomodulin complex; inhibits fibrinolysis and stabilizes the clot by impeding the formation of plasmin via slicing the carboxy-lysine residues of fibrin and counteracting the binding of tPA and plasminogen with fibrin. On off the study showed the association between increased levels of TAFI and risk of venous thrombosis [326, 331]. Another study reports reduced level of TAFI associated with mortality in patients with PE [330].

1.6.4.4. ***D-dimer***

D-dimer, a small protein fragment and the product of cross-linked fibrin, generated via thrombin, FXIIIa, and plasmin activity and suggestive of both the coagulation and fibrinolysis pathway. Increased levels of D-dimer are extensively used as a prognostic marker for the diagnosis of VTE, employed in the clinical setting to rule out VTE in patients' moderate clinical probability assessment. A study on patients with suspected VTE proposed D-dimer as a significant diagnostic marker for VTE with high sensitivity (94%) and a high negative predictive value (95%) when measured with ELISA method [332].

1.6.4.5. ***Prothrombin Fragment 1+2 (F1+2)***

F1+2 cleaved from the amino-terminal end of prothrombin during its conversion into active thrombin and considered as a marker of hypercoagulable state. During the conversion of thrombin, prothrombinase enzyme complex cleaves

prothrombin into two sites, cleavage at Arg271-Thr272 generates F1+2 while cleavage at Arg320-Ile321 (prothrombin 2) along with FXa generates thrombin. Several studies have measured the levels of F1+2 for patients with VTE in various clinical settings, few studies have shown the elevation of plasma F1+2 is associated with VTE [333-335], however it is not considered as a predictive marker for recurrent VTE [336].

1.6.4.6. ***Thrombin-antithrombin Complex (TAT)***

TAT complex is formed by binding of thrombin, with antithrombin (AT) in a form of complex; thrombin-antithrombin complex (TAT). TAT plays a crucial role in regulating coagulation by inhibiting thrombin activity. It has been shown that the TAT levels were significantly elevated in patients with VTE compared to without VTE went for lower limb or pelvis surgery [337]. Conversely one study reports the change in TAT levels are not for patients with suspected DVT [338].

1.6.5. Inflammatory Markers

Inflammation also plays a role in the pathogenesis of VTE [339]. Cytokines are diverse group of small proteins and act as a signaling protein, which is involved in regulating the growth, differentiation and the activity of immune and blood cells. They release from the innate immune cells. As a signaling molecule they facilitate the control the inflammatory response [340]. While chemokines belong to a large family of small proteins, they serve as key mediators in recruiting immune cells to sites of inflammation, infection, or injury [340]. Several studies have reported increased levels of inflammatory markers (CRP, IL-6, IL-8, MCP-1, and TNF-α) has been associated with the risk of VTE [341], contrary to that a population-based study have not shown any link of inflammatory markers (IL-1β, IL-6, IL-8, and TNF-α) with VTE [342].

1.6.5.1. ***C-Reactive Protein (CRP)***

CRP is an acute phase reactant protein which belongs to pentraxin superfamily or and used clinically as a marker of infection or inflammation. Tissue injury or infection radically increases its synthesis almost 10,000-fold [343]. Circulatory CRP is in inert pentameric form (pCRP), undergoes confirmation

change (pCRP*) involves binding with phosphocholine-enriched membranes which subsequently dissociates into monomeric CRP (mCRP) [343]. Both pCRP* and mCRP destabilized isoforms of CRP demonstrates pro-inflammatory and pro-thrombotic properties associated with Immunothrombosis [344, 345]. Elevated levels of high sensitivity CRP has been found not only in patients with VTE [346], but CRP levels are also linked with unprovoked VTE compared to provoked [347]. Elevated CRP also showed an association with the first episode of VTE [344, 345, 348].

1.6.5.2. Tumor Necrosis Factor-α (TNF-α)

TNF-α, is the master regulator of inflammation and a critical player in the cytokine network. It is produced by a variety of cell types including macrophages, T cells, and keratinocytes [349]. In vitro studies have shown that TNF-α promotes a hypercoagulable state by stimulating the production of tissue factor by endothelial cells and mononuclear phagocytes, inducing complement activation and releasing endothelial protein C receptor and inhibiting thrombomodulin production [350]. Activated coagulation factors further sustain this proinflammatory and procoagulant state by upregulating cytokine production, including TNF-α, through interactions with protease-activated receptors on inflammatory cells [351]. This procoagulant loop explains the role of TNF-α in thrombosis development. Additionally, its role in fibrinolysis and angiogenesis has also been demonstrated [352].

1.6.5.3. Interleukin-1α (IL-1α) and Interleukin-1β (IL-1β)

IL-1α, and IL-1β are key regulators of diverse inflammatory processes including thrombo-inflammation. They are mainly secreted from stimulated monocytes and macrophages and, to a lesser degree, neutrophils, keratinocytes, epithelial and endothelial cells, lymphocytes, smooth muscle cells, and fibroblasts [353, 354]. Both IL-1β and IL-1α signal through the IL-1 receptor, activating the NF-κB signaling pathway resulting in transcription of multiple proinflammatory genes [355, 356]. IL-1β is particularly involved in NLRP3–IL-1β axis resulting in inflammasome activation. Inhibition of IL-1β has shown promising results in treatment of patients with thrombosis including COVID-19 [357-359].

1.6.5.4. ***Interleukin-2 (IL-2)***

IL-2 is a well-known cytokine that promotes the activation, proliferation, and differentiation of helper and regulatory T cells [360]. However, there is limited knowledge regarding its association with VTE [360]. IL-2 administration has been associated with microvascular thrombosis increased 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 [361-363].

1.6.5.5. ***Interleukin-4 (IL-4)***

IL-4 is secreted by type II T helper lymphocytes (TH2 lymphocytes), can inhibit the synthesis and expression of proinflammatory markers such as IL-6, IL-8, and TNF by inhibiting type I T helper lymphocytes (TH2 lymphocytes) and stimulate B and Th2 lymphocytes, thus reducing the inflammatory response [364]. IL-4 has cytotoxic and antitumor effects, inhibits the induction of nitric oxide synthase, inhibits the release of superoxide by macrophages, and exerts numerous anti-inflammatory actions. IL-4 promotes fibrinolysis by stimulating monocytes to produce t-PA [365]. The allelic frequency of IL4 −589 T allele was significantly increased in female VTE patients in contrast to men where no differences were observed [366]. Despite limited information on IL-4 in VTE, our findings suggest elevated levels of IL-4 in patients with PE. This is consistent with our previous studies [367-369].

1.6.5.6. ***Interleukin-6 (IL-6)***

IL-6 is a multifunctional cytokine crucial to innate immunity, with diverse roles in host defense, immune cell regulation, proliferation, and differentiation [370]. It is secreted by various cell types, including monocytes and macrophages following Toll-like receptor activation, fibroblasts and endothelial cells in response to IL-1, and B and T cell subsets during viral infections [371]. IL-6 is central to the immune response, acute-phase reaction, and homeostasis, exhibiting both pro- and anti-inflammatory effects based on the physiological context [372]. Under normal conditions, IL-6 serum levels are low (1–5 pg/mL), but they can increase significantly during inflammatory diseases, such as sepsis, ARDS, and COVID-19, as well as autoimmune diseases and cancer, making IL-6 a good target for therapeutic intervention [373]. Elevated levels of IL-6 are well-documented in VTE,

with several studies highlighting its potential role in predicting adverse outcomes, including mortality, VTE recurrence, post-thrombotic syndrome (PTS), and chronic thromboembolic pulmonary hypertension (CTEPH) [367-369, 374-381]. Supporting the previous literature, our findings demonstrated that IL-6 levels were significantly elevated in PE patients compared to healthy controls.

1.6.5.7. ***Interleukin-8 (IL-8)***

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 [382]. Additionally, IL-8 has a role in thrombus organization, enhancing neovascularization and contributing to thrombus resolution [383, 384]. In a systematic review and network analysis, elevated plasma levels of IL-8, along with other inflammatory markers such as CRP, IL-6, MCP-1, and TNF-α, have been associated with a two- to six-fold increased risk of DVT [339].

1.6.5.8. ***Interleukin-10 (IL-10)***

IL-10 is an anti-inflammatory cytokine with immunosuppressive properties. In previous studies, the association of IL-10 with VTE has 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 [367-369, 374-381, 385, 386]. Additionally, IL-10 has been associated with mortality, development of PTS and CTEPH in previous studies [387-391].

1.6.5.9. ***Monocyte Chemoattractant Protein-1 (MCP-1)***

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 [392, 393]. Another study has shown that MCP-1 upregulation participates in macrophage chemotaxis and thrombolysis after DVT formation and blockade of MCP- 1 weakens its thrombolysis effects [394]. The levels of MCP-1 have been associated with CTEPH in several studies [391, 395, 396].

1.6.5.10. Interferon-γ (IFN-γ)

IFN-γ is a proinflammatory cytokine with diverse properties. Several studies evaluated the role of IFN-γ in venous thrombosis. In an animal model study of DVT, IFN-γ–deficient mice developed smaller thrombi with lower collagen content, increased matrix-metalloproteinase-9 (MMP-9) activity, and enhanced thrombus recanalization compared to wild-type (WT) mice [397]. Additionally, blocking IFN-γ accelerated thrombus resolution in WT mice in this study. In another study, thrombus formation and NET production were reduced in Tbet-deficient (transcription factor T- box expressed on T cells-deficient) and IFN-γ–deficient mice, highlighting the importance of Tbet/IFN-γ–expressing cells in thrombosis [398]. In this study, NK cell-dependent IFN-γ production was shown to be essential for thrombus development by promoting NET formation by neutrophils. Collectively these studies have shown IFN-γ is involved in thrombus formation and resolution. Several other studies have also report elevated levels of IFN-γ in the context of PE patients [367-369].

1.6.6. Growth Factors

Growth factors are crucial in the development and progression of VTE. Several studies have highlighted their role in either activating endothelial cells or promoting inflammation within the vascular system, both of which contribute to a pro-thrombotic environment. Elevated levels of some of the growth factors such as EGF, VEGF, PDGF, and TGF-β have been reported in conjunction with VTE [367, 399].

1.6.6.1. Vascular Endothelial Growth Factor (VEGF)

It has been shown that VEGF expression may enhance thrombus recanalization, organization and resolution in animal models [400, 401]. VEGF is secreted in response to hypoxia, which stimulates angiogenesis by its downstream pathways [402]. The role of VEGF in venous thrombosis is mostly investigated in cancer-associated thrombosis (CAT). In these studies, the VEGF levels were higher in VTE patients compared to controls and associated with a higher risk of VTE in CAT [403, 404]. One study that investigated the risk of VTE and serum levels of VEGF did not find any significant association in glioma patients [405]. In another

study, the levels of VEGF was higher in CAT patients and this was associated with mortality and poorer outcomes in CAT patients [406]. A few studies evaluated VEGF in patients without cancer. One study has found that VEGF levels were higher in high-risk PE patients compared with high-intermediate-risk PE patients suggesting that VEGF can be used as a biomarker in clinical risk scoring and estimation of thrombus burden in patients with acute PE [407]. Another study has shown elevated levels of VEGF in military trauma patients with VTE [408]. Increased levels of VEGF were also observed in CTEPH patients pointing out its role in this complication [409, 410].

*1.6.6.2. **Epidermal Growth Factor***

EGF is a high-affinity ligand of the epidermal growth factor family that activates epidermal growth factor receptors (EGFR) [411]. EGFR is a receptor tyrosine kinase that initiates complex intracellular signaling cascades, leading to cell cycle progression, proliferation, and mitosis. The EGF/EGFR interaction exerts a potent mitogenic effect, particularly on epithelial tissues, fibroblasts, and endothelial cells [412, 413]. Furthermore, EGF and EGFR are frequently overexpressed in certain malignancies, which is associated with a heightened risk of thrombosis in cancer patients [414, 415]. Notably, treatment with anti-EGFR therapies has also been linked to an increased risk of thrombosis in these patients [416, 417]. Despite current data, knowledge regarding EGF levels in VTE patients remains limited. One study reported decreased EGF levels in VTE, though this study had a small sample size of 50 VTE patients and 25 healthy controls [418].

1.7. RIETE AND RELATED REGISTRIES

Currently, most of the published information on VTE comes from clinical trials or observational studies [419]. Clinical trials, with their strict inclusion and exclusion criteria, fixed doses of anticoagulant drugs or specific interventions, and precisely defined follow-up, primarily focus on safety and efficacy. While these trials provide high-level evidence, they often involve well-defined populations that exclude more complex patients commonly observed in routine clinical practice. In contrast, traditional observational studies are often limited to small patient populations and single center institutions. Because nearly a quarter of patients with

VTE have at least one exclusion criterion keeping them out of clinical trials, real-life data is crucial for understanding their needs and gaining a comprehensive view of the problem [420]. To address these issues, registry studies have proven to be the most effective approach.

While many national and international health registries exist, several of them which are dedicated specifically to VTE have also been developed. Among them, the most well-known VTE registries are the Registro Informatizado de Enfermedad Trombo Embólica (RIETE) registry, the Global Anticoagulant Registry in the FIELD - Venous Thromboembolism (GARFIELD-VTE) registry, the Contemporary Management and Outcomes in Patients with VTE (COMMAND VTE) registry, the Italian Pulmonary Embolism Registry (IPER), and the Swiss Venous Thromboembolism Registry (SWIVTER), Prevention of Thromboembolic Events—European Registry in Venous Thromboembolism (PREFER), and Dresden NOAC registry.

1.7.1. RIETE Registry

The RIETE Registry is an ongoing international multicenter prospective registry of consecutive patients with acute VTE [421]. Initiated in Spain in 2001, it expanded to other countries following the translation of the database into English in 2007. As the oldest and largest ongoing VTE registry, it includes participants from 24 countries across Europe, North America, and South America. While many other registries have paused patient collection, RIETE continues to enroll patients and has currently collected data from over 100,000 VTE patients all over the world. RIETE aims to evaluate real-world outcomes such as recurrent VTE, major bleeding, mortality, and associated risk factors. It offers insights into VTE treatment in everyday clinical practice, providing a contrast to the controlled conditions of randomized clinical trials. Although not designed to assess the relative efficacy and safety of different therapies, RIETE generates hypotheses and provides valuable real-world feedback to guide the design of future randomized studies.

1.7.2. GARFIELD-VTE Registry

GARFIELD-VTE Registry is designed to examine real-life clinical treatment practices and outcomes of VTE worldwide [422]. It is a prospective, multicenter,

observational study that enrolled 10,685 patients and followed for a minimum of 3 years between May 2014 and January 2017 across 28 countries in Europe, the Americas, Asia, Africa, and Australia.

1.7.3. COMMAND VTE Registry-1 and -2

The COMMAND VTE Registry-1 and -2 are multicenter, physician-initiated retrospective cohort studies focusing on acute symptomatic VTE patients in Japan [423, 424]. COMMAND VTE Registry-1 enrolled 3,027 consecutive patients with acute symptomatic VTE from January 2010 to August 2014 before the introduction of direct oral anticoagulants. COMMAND VTE Registry-2 was the follow-up study, that incorporated DOACs, enrolled 5,197 consecutive patients with acute symptomatic VTE across 31 centers in Japan from January 2015 to August 2020.

1.7.4. PREFER in VTE Registry

The PREFER in VTE Registry was a prospective observational study conducted from January 2013 to July 2014. It involved 3,545 patients across seven European countries: Austria, France, Germany, Italy, Spain, Switzerland, and the UK [425]. The study focused on characteristics of patients with acute VTE, anticoagulation treatment and quality of life assessments. The follow-up period was 12 months.

1.7.5. Italian Pulmonary Embolism Registry (IPER)

The Italian Pulmonary Embolism Registry (IPER) enrolled patients with confirmed acute pulmonary embolism (PE) across 47 hospitals in Italy [426]. The registry aimed to capture data on the demographics, clinical characteristics, management, and outcomes of patients diagnosed with PE in routine clinical practice. By 2012, it had recruited 1,716 patients and the study is now completed.

1.7.6. SWIVTER Registry

The SWIVTER Registry, is the national registry of Switzerland dedicated to VTE, conducts retrospective data analysis using prospectively collected clinical

data [427]. It enrolls patients from various hospital settings, including university and non-university facilities, across different time periods. This registry collected data on patient demographics, clinical features, and treatment outcomes, providing national insights, informing guidelines, and serving as a research foundation.

1.7.7. Dresden NOAC Registry

The Dresden NOAC Registry is a large national prospective observational study based in the administrative district of Dresden (Saxony), Germany [428]. Since 2011, an active network of over 230 registered physicians and hospitals has been enrolling patients using non-vitamin K antagonist oral anticoagulants (NOACs). The registry focuses on patients with VTE or non-valvular atrial fibrillation (AF) and includes data from private practices and community hospitals in Saxony.

1.7.8. LUMC PERT Project

PE is a leading cause of preventable hospital deaths, contributing to upto 10% mortality in hospitalized patients. Given its complex presentation and high mortality, timely diagnosis and treatment are critical. At Loyola University Medical Center, the Pulmonary Embolism Response Team (PERT) plays an essential role in expediting the evaluation and management of patients with suspected acute PE. PERT is a multidisciplinary team consisting of cardiologists, radiologists, clinical pharmacists, and potentially cardiothoracic or vascular surgeons, who work in close collaboration with the primary medical team. Upon activation by the primary physician, the PERT rapidly assesses the patient, facilitates imaging, and determines the need for advanced therapies, such as catheter-directed thrombolysis (CDT) or surgical thrombectomy. Throughout the hospital stay, PERT provides ongoing patient management, ensuring optimal therapeutic interventions are administered in a timely manner. This coordinated approach offers an ideal framework to investigate the relationship between thromboinflammatory biomarkers, cellular indices, and their potential impact on patient outcomes in PE. Understanding these biomarkers in the context of the PERT model may lead to more personalized and effective management strategies for

II – JUSTIFICATION

II - JUSTIFICATION

The RIETE registry and LUMC PERT cohort provide a large database to validate the cellular indices in two independent patient populations. Results from these large databases also establish the integration of biomarkers with cellular indices. This data can be used for prediction of clinical outcomes and to further understand the pathophysiology of cellular activation processes, and the biomarkers generated. The role of thrombo-inflammation is taken into account with reference to biomarkers generated, particularly the role of endothelium, and tissue factor. The large database allows for a comprehensive account of biological crosstalk. Moreover, this comprehensive biomarker analysis, in conjunction with pulmonary embolism studies carried out at Loyola, represents the first extensive study to provide new, previously unknown information on the diagnosis of PE and the role of different pathways involved in the pathogenesis of VTE.

III – OBJECTIVES

III - OBJECTIVES

3.1 STATEMENT OF PURPOSE

The purpose of this dissertation is multi-fold. Identification of new biomarkers and validation of their importance in the pathophysiology of PE will be undertaken. The obtained data will be used to improve the understanding of the complex pathophysiology of PE, with a particular focus on the role of thrombo-inflammatory biomarkers generated from cells and organ specific mediators. Further, the blood cellular indices and their role in the regulation of cellular function will be dissected, with a focus on the central role of endothelium as a regulator of cellular and protease networks involved in the activation process leading to VTE. Integration of the data will be used to determine the interdependence and regulatory role of both cells and mediators. These studies will serve to improve the diagnosis of VTE by introducing new biomarkers, to understand the molecular and cellular biological processes in development of VTE, and eventually to develop the new diagnostic tools and new therapeutic and interventional modalities. The study results can be used to propose new mathematical models that will correlate the cellular mediators and other biomarkers. Thrombosis and other hemostatic aberrations leading to inflammatory conditions represent a complex array of pathophysiologic complications which need to be further explored. It is the objective of this study to understand the role of cellular indices and biomarkers in clarifying the pathophysiology of PE.

3.2 HYPOTHESIS

The primary hypothesis of this study is that cellular indices such as NLR, PLR, and SII as a determinants of the thrombo-inflammatory response are predicative of clinical outcomes in VTE patients.

Additionally, the role of cellular markers of hemostatic activation and thrombo-inflammation is determined.

3.3. SPECIFIC AIMS

3.3.1. Specific Aim 1: The prognostic value of blood cellular indices as NLR, PLR, and SII in patients with VTE

Aim1: To determine the prognostic value of blood cellular indices as NLR, PLR, and SII in patients with VTE and their relevance with the 90-day outcomes (study from RIETE Registry)

Sub-Aim 1a: To assess the prognostic value of NLR, PLR, and SII in patients with VTE for all-cause mortality within the first 90-day of diagnosis

Sub-Aim 1b: To assess the prognostic value of NLR, PLR, and SII for major bleeding within the first 90-day of diagnosis in the patients with VTE

Sub-Aim 1c: To assess the prognostic value of NLR, PLR, and SII for VTE recurrence within the first 90-day of diagnosis in the patients with VTE

3.3.2. Specific Aim 2: Comparative prognostic value of blood cellular indices as NLR, PLR and SII in patients with acute PE

Aim 2: To compare the prognostic value of NLR, PLR, and SII in identifying PE patients at increased risk for death within the first 30-day (study from the RIETE registry and LUMC PERT program)

Sub-Aim 2a: To assess the prognostic significance of NLR, PLR, and SII in patients with acute PE for all-cause mortality within the first 30-day of diagnosis

Sub-Aim 2b: To assess the prognostic significance of NLR, PLR, and SII in patients with acute PE, stratified by low-risk, intermediate-risk, and high-risk PE, as determined by the European Society of Cardiology classification for all-cause mortality within 30-day of diagnosis

3.3.3. Specific Aim 3: Thrombin generation potential, markers, and blood cellular indices in patients with acute PE

Aim 3: To understand the role of thrombin in cellular activation in patients with acute PE by analyzing the biomarkers of thrombin generation and its

potential, along with cellular indices and their relevance with disease progression (study from LUMC PERT program).

Sub-Aim 3a: To analyze the thrombin generation potential and thrombin generation biomarkers in patients with acute PE.

Sub-Aim 3b: To analyze the blood cellular indices as NLR, PLR and SII in patients with acute PE.

Sub-Aim 3c: To demonstrate the relationship between thrombin generation potential, and biomarkers with cellular indices in patients with acute PE.

Sub-Aim 3d: To stratify the thrombin generation potential, thrombin generation biomarkers, in patients with acute PE by low-risk, intermediate-risk, and high-risk PE, as determined by the European Society of Cardiology classification.

Sub-Aim 3e: To stratify blood cellular indices in patients with acute PE by low-risk, intermediate-risk, and high-risk PE, as determined by the European Society of Cardiology classification.

3.3.4. Specific Aim 4: Hemostatic activation, and thromboinflammatory biomarker profiling in patients with acute PE

Aim 4: To understand the molecular pathogenesis of pulmonary embolism by profiling the biomarkers of endothelial dysregulation, platelet activation, coagulation factor, fibrinolysis, and inflammation along with cellular indices in patients with acute PE and their relevance with disease progression and outcome as all-cause mortality within the first 30-day of diagnosis (study from LUMC PERT program).

Sub-Aim 4a: To measure the panel of biomarkers of endothelial dysregulation, platelet activation, coagulation factor, fibrinolysis, inflammation, and cellular indices in patients with acute PE.

Sub-Aim 4b: To analyze the levels of the biomarkers of endothelial dysregulation, platelet activation, coagulation factors, fibrinolysis, inflammation, and cellular indices in patients with acute PE, stratified by low-risk, intermediate-risk, and high-risk PE, as determined by the European Society of Cardiology classification.

Sub-Aim 4c: To demonstrate the relationship among the biomarkers of endothelial dysregulation, platelet activation, coagulation factors, fibrinolysis, inflammation, and cellular indices in patients with acute PE.

Sub-Aim 4d: To analyze the levels of the biomarkers of endothelial dysregulation, platelet activation, coagulation factors, fibrinolysis, inflammation, and cellular indices in patients with VTE for all-cause mortality within 30-day of diagnosis.

3.3.5. Specific Aim 5: Inflammatory biomarker profiling in patients with acute PE.

Aim 5: To understand the role of inflammation in patients with acute PE by profiling inflammatory cytokines and chemokines along with cellular indices and their relevance with disease progression and outcome as all-cause mortality within the 30-day of diagnosis (study from LUMC PERT program).

Sub-Aim 5a: To analyze the levels of cytokines and chemokines as inflammatory biomarkers with respect to cellular indices in patients with acute PE

Sub-Aim 5b: To analyze the levels of inflammatory cytokines, chemokines and blood cellular indices, stratified by low-risk, intermediate-risk, and high-risk PE, as determined by the European Society of Cardiology classification.

Sub-Aim 5c: To demonstrate the relationship between inflammatory cytokines and chemokines with cellular indices in patients with acute PE.

Sub-Aim 5d: To analyze the influence of inflammatory cytokines, chemokines and cellular indices for all-cause mortality within the 30-day of diagnosis in patients with acute PE.

IV – MATERIAL AND METHODS

IV - MATERIAL AND METHODS

4.1.1. RIETE Registry

The RIETE (Registro Informatizado Enfermedad TromboEmbólica) registry is an ongoing, multicenter, observational registry of consecutive patients with symptomatic, clinically confirmed acute VTE, with 209 collaborating centers in 29 countries (ClinicalTrials.gov identifier: NCT02832245).

The rationale and methodology of the RIETE registry have been reported. The aim of the registry is to compile a comprehensive database of patients with VTE and utilize it to understand patient presentation, treatment and outcomes, and to enhance the knowledge of VTE and improve related clinical practices. The protocol for patient enrollment was approved by the ethics committees at the participating sites and all patients (or their healthcare proxies) provided informed consent (appendix 2). Consecutive patients with acute VTE confirmed by objective tests (compression ultrasonography for suspected DVT; helical computed tomography [CT]-scan, ventilation-perfusion lung scintigraphy or contrast angiography for suspected PE) were recruited (table 9). Patients were excluded if they were currently participating in a blinded therapeutic clinical trial.

Table 9. Inclusion and exclusion criteria for RIETE Registry.

Inclusion Criteria	*Patients confirmed diagnosis of DVT, PE or both*
	3 months minimum follow-up
	Patients having 54 core variables (appendix)
Exclusion Criteria	*Patients participating in blinded clinical trial*
	Patients currently enrolled in other registries on VTE
	Patients with lack or no consent

Superficial vein thrombosis, splanchnic vein thrombosis (i.e. thrombosis involves thrombosis in the mesenteric, splenic, or portal veins), retinal vein thrombosis, and cerebral vein thrombosis have been separately enrolled.

The conduct of our study adhered to the ethical standards established in the 1975 Declaration of Helsinki and was approved by the relevant institutional review

boards. All patients (or their relatives) provided informed consent for participation in the registry, in accordance with the local ethics committee requirements.

4.1.2. LUMC PERT Project

The study included a cohort of patients with acute symptomatic PE recruited from the Pulmonary Embolism Response Team (PERT) program at Loyola University Medical Center, Gottlieb Memorial Hospital, and MacNeal Hospital. PERT was established to manage acute PE patients across the entire spectrum of PE risk.

The PERT program is an ongoing program which was initiated in 2017 and around 2,000 patients have been currently enrolled, the inclusion criteria was based on patients over 18 years old and admitted with confirmed diagnosis of PE (table 10). The data on clinical characteristic, presentation, hemodynamic data, imaging data (including echocardiographic and EKG), laboratory data, comorbidities, and outcome (including primary and secondary outcome) were collected from the chart. The study was approved by the Institutional Review (appendix 3).

Table 10. Inclusion and exclusion criteria for LUMC PERT Project.

Inclusion Criteria	*Patients age >18 years*
	Patients admitted with confirmed diagnosis of PE
Exclusion Criteria	*Patients do not meet inclusion criteria*

Blood samples were prospectively collected from Loyola University Medical Center and Gottlieb Memorial Hospital patients. PE diagnosis was confirmed using computed tomography (CT) scan, echocardiography, or angiography. Among this cohort, a sub-cohort of 500 patients, enrolled from 2018 to 2023, were consecutively selected for the biomarker profiling

4.2. STUDY DESIGN

This thesis integrates data from five distinct but related studies, each addressing specific aspects of the prognostic value of cellular indices and biomarkers in PE and VTE.

4.2.1. Study 1: Prognostic Value of NLR, PLR, and SII in VTE

An analysis was conducted using data from the prospective RIETE registry, encompassing 10,085 patients with acute VTE. The primary endpoints were VTE recurrence, major bleeding, and death. Multivariable analyses were performed to assess the prognostic value of NLR, PLR, and SII.

4.2.2. Study 2: Comparative Prognostic Value of NLR, PLR, and SII in PE

An analysis of 10,085 PE patients from RIETE was conducted to compare the prognostic value of NLR, PLR, and SII. The primary endpoint was 30-day mortality. Kaplan-Meier survival and multivariable analyses were used to evaluate the prognostic significance.

4.2.3. Study 3: Thrombin Generation Potential in PE Patients

A prospective cohort study was conducted involving 500 PE patients recruited from a PERT program. Thrombin generation parameters, thrombin generation biomarkers, and cellular indices were analyzed in relation to PE severity, according to the European Society of Cardiology (ESC) guidelines.

4.2.4. Study 4: Hemostatic Activation and Thromboinflammatory Biomarker Profiling in PE Patients

A prospective cohort study was conducted involving 500 PE patients from the Loyola Academic Center. A comprehensive panel of biomarkers related to endothelial dysfunction, coagulation, fibrinolysis, and inflammation was profiled. Correlations among biomarkers and their prognostic value for 30-day mortality were evaluated.

4.2.5. Study 5: Inflammatory Biomarker Profiling in PE Patients

A prospective cohort study was conducted involving 500 PE patients from the Loyola Academic Center. A comprehensive panel of inflammatory biomarkers including cytokines and chemokines was analyzed. Correlations among biomarkers and their prognostic value for 30-day mortality were evaluated.

4.3. PLASMA SAMPLES

4.3.1. Normal Human Plasma

A pool of normal human plasma (NHP) was used as internal quality control for the biomarker testing. Control citrated plasma from 25 healthy male and 25 healthy female volunteers (N=50) was purchased from George King Biomedical, Inc., (Overland Park, KS, USA.). All the control samples were free from any disease or medications.

4.3.2. LUMC PERT Project

Citrated blood samples (3.2% sodium citrate tubes) from 500 PE patients were collected within 24-72 hours of diagnosis for routine care under an IRB approved protocol (IRB# 209457032217) at Loyola University Medical Center. Samples were collected from 2-hospitals from the tertiary care unit, Loyola University Medical Center, and Gottlieb Memorial Hospital. All samples were de-identified, and platelet poor plasma was isolated by centrifugation at 3,000 rpm, aliquoted, and stored at -70 ^{O}C.

4.4. REAGENTS

4.4.1. Thrombin Generation Reagents

Reagents used in the assay included the FluCa-Kit (CAT# 86197), PPP-reagent high (CAT# 86195), and a thrombin calibrator (CAT# 86192) was obtained from Diagnostica Stago (Parsippany, NJ, US). FluCa-Kit contains fluo-buffer and fluo-substrate, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES at 20 mM and PH 7.35) and calcium chloride is the component of fluo-buffer while fluo-substrate contains thrombin-specific fluorogenic substrate (Z-Gly-Gly-Arg-AMC) solubilized in dimethyl sulfoxide (DMSO). PPP-reagent high is a mixture of human recombinant tissue factor and phospholipids and used as an activating agent for platelet poor plasma, while the concentration of tissue factor is high in the PPP-reagent. The thrombin generation assay was carried out in 96-well immulon 2HB transparent round bottom plates (cat# 3365) from Thermo Scientific™,

ThermoFisher Scientific Inc., (Maastricht, Netherlands). The manufacturer's protocol was used to analyze the thrombin generation potential.

4.4.2. Biomarkers

Biomarkers are biological indicators considered as signaling tools for various disease states which can be measured. Biomarkers can be used to diagnose as well as determine the progression of the disease. Biomarkers can be predictive and used to detect the possibility of developing a disease state. Biomarkers can also be used to identify a response to treatment and interventions.

All the biomarker studies were performed by utilizing assay kits that detect finite human proteins. Biomarkers were analyzed using commercially available enzyme-linked immunosorbent assays (ELISA) or sandwich chemiluminescence assays. All the assays were performed according to the standard manufacturer's protocol. Table 11 presents the list of the assays used and their manufacturer.

Table 11. List of biomarkers kits along with their vendors studied for human plasma proteins.

Marker	*Manufacturer*
Von Willebrand Factor (ZYMUTEST) *Factor VII (ZYMUTEST)* *Factor IX (ZYMUTEST)* *Factor X (ZYMUTEST)* *Factor XIII-A (ZYMUTEST)* *Tissue-plasminogen Activator (ZYMUTEST)* *Thrombin Activatable Factor Inhibitor-a (ZYMUTEST)* *Plasminogen Activator Inhibitor-1 antigen (ZYMUTEST)* *D-dimer (ZYMUTEST)* *C-Reactive Protein (ZYMUTEST)*	*Hyphen BioMed* *(Neuville-Sur-Oise, France)*
E-selectin *P-selectin* *Interleukin-6* *Tumor Necrosis Factor-α*	*R&D Systems* *(Minneapolis, MN, USA)*
Prothrombin Fragment 1+2 *Thrombin Anti-thrombin Complex*	*Siemens Healthcare Diagnostic Products* *(Marburg, Germany)*
Tissue Factor (IMUBIND)	*BioMedica Diagnostics* *(Windsor, Canada)*

Interleukin-2 *Interleukin-4* *Interleukin-6* *Interleukin-8* *Interleukin-10* *Vascular Endothelial Growth Factor* *Interferon-γ* *Tumor Necrosis Factor-α* *Interleukin-1α* *Interleukin-1β* *Monocyte Chemoattractant Protein-1* *Epidermal Growth Factor*	*Randox* *(London, UK)* *Cytokine High Sensitivity Array Kit*

4.5. INSTRUMENTS

4.5.1. Fluoroskan Ascent

Fluoroskan Ascent from Thermo Fisher Scientific (Massachusetts, US) along with Thrombinoscope™ software (version 5.0.0.708) from Thrombinoscope BV (Maastricht, Netherlands) was used for the thrombin generation assay.

4.5.2. SpectraMax Plus

SpectraMax Plus 384 microplate reader from Molecular Devices (San Jose, CA, US) in conjunction with Softmax Pro software (version 7.0) was used to measure the optical density of the biomarkers including vWF, FVII, FIX, FX, FXIII-A, tPA, TAFI, PAI-1, D-dimer, E-selectin, P-selectin, IL-6, TNF-α, F1+2, TAT, and TF using the sandwich ELISA method.

4.5.3. Randox Evidence Investigator

Randox Evidence Investigator EI-18534 from Randox Laboratories (London, UK) along with the EvInvest software (version 2.2.0) was used to measure cytokines and growth factors. The technology of this highly sensitive system uses a biochip microarray with capture of chemiluminescence signals.

4.6. ASSAYS

4.6.1. Thrombin Generation Assay

Thrombin generation potential studies were carried out by using the calibrated automated thrombogram (CAT) assay on a Fluoroskan Ascent fluorimeter from Diagnostica Stago (Paris, France). CAT is a functional test of the thrombotic-hemostatic system that measures the production of thrombin in plasma. Generation of thrombin is triggered by a mixture of phospholipid and tissue factor. The reaction is monitored over time, thus providing a kinetic response of thrombin generation. Thrombin that is produced, or generated, cleaves the fluorogenic substrate (Z-Gly-Gly-Arg-AMC) into the fluorophore amino-methyl coumarin (AMC, 7-amino-4-methuylcoumarin) that produces a fluorescent signal, which is measured by the fluorometer with an excitation at 390 nm and an emission at 460 nm. A thrombin calibrator is used to convert the fluorescence signals into thrombin units.

The thrombin generation potential was measured in terms of the peak thrombin concentration, lag time, and endogenous thrombin potential (ETP) /area under the curve (AUC). Lag time is the time required for the initiation of the clot formation in the plasma. Peak thrombin level measures the highest concentration of thrombin while ETP/AUC measures the overall thrombin generated for 90 minutes.

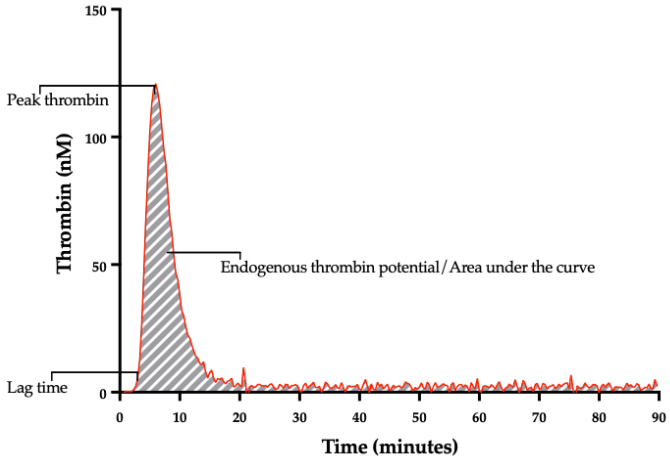


Figure 8. Thrombin generation parameters as peak thrombin, endogenous thrombin potential and lag time by using Calibrated Automated Thrombogram.

The assay was performed in a 96-well round bottom immulon 2HB transparent plate; 80 uL of the sample was added into the plate well along with 20 uL of the PPP-reagent high, 20 uL of thrombin calibrators was also added in their corresponding wells. The plate was placed into the instrument and incubated in the Fluorskan Fluorimeter for 10 minutes to warm the plate. FluCa was prepared by mixing the fluo-buffer (incubated for 10-15 minutes in the water bath at 37 ^{O}C) with fluo-substrate in the ratio of 1:40. Subsequent incubation, the dispenser was prepared to avoid air bubbles in the dispensing system; 10 ml of distilled water as washing solution was used and the dispenser was primed and emptied with the remaining water. FluCa was added and filled into the dispensing system; 20 uL was dispensed into each well by the analyzer. The lid on the instrument was closed as soon as the shaking began. The measurements started, continued at the set measuring interval, and the analyzer stopped automatically after 90 minutes.

4.6.2. Biomarker Analysis

The biomarkers quantified in this dissertation including vWF, E-selectin, P-selectin, FVII, FIX, FX, FXIII-A, tPA, TAFI, PAI-1, D-dimer, CRP, IL-6, TNF-α, F1+2, TAT, and TF were measured by commercially available sandwich ELISA methods. IL-2, IL-4, IL-6, IL-8, IL-10, VEGF, IFN-γ, TNF-α, IL-1α, IL-1β, MCP-1, and EGF were measured by the sandwich chemiluminescent biochip array technology.

4.6.2.1. ***Enzyme-linked Immunosorbent Assay***

Enzyme-linked immunosorbent assay (ELISA) is an immunoassay used to quantify the biological markers such as antigen, antibodies, hormones, and proteins. ELISA is a microplate-based immunoassay where antigen/antibody is immobilized on the solid phase. Based on the microplate coating, ELISA can be categorized into direct ELISA, indirect ELISA, competitive ELISA, and sandwich ELISA.

Direct ELISA utilizes a plate coated with an antigen and measures antibodies directly via enzyme-labelled primary antibody. Indirect ELISA plate is also coated with an antigen but can detect both antibodies or antigen but requires two antibodies (a primary antibody and an enzyme-labelled secondary antibody) having higher sensitivity compared to indirect ELISA. Competitive ELISA plate is

coated with antigen-specific antibody or antibody-specific antigen where sample antigen or antibody and enzyme-labelled antigen or antibody competes. Contrary to direct and indirect ELISA, sandwich ELISA plate is coated with the antibody and measured antigen. In this type of ELISA, antigen from the sample is 'sandwiched' between the captured and detection antibody, secondary enzyme-conjugated antibody is also used. A brief overview of the principal of sandwich ELISA is presented in the figure 9. Sandwich ELISA has comparatively higher sensitivity and specificity and can detect even low levels of antigen.

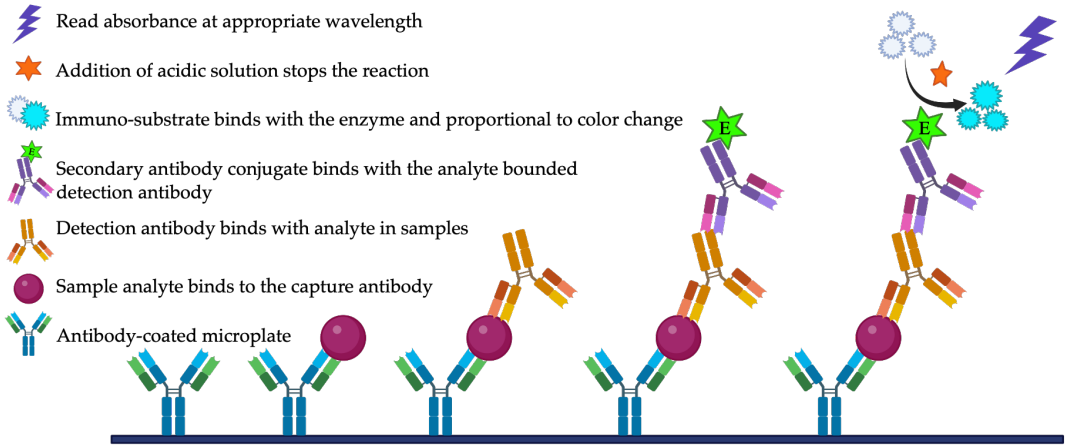


Figure 9. An overview of the principle of Sandwich ELISA assay.

All the biomarkers studied were measured by using the sandwich ELISA method, with a standardized protocol provided in the commercial kit used to detect the levels of each biomarker.

*4.6.2.2. **Biochip Array Technology***

Biochip Array Technology (BAT) is a chip-based chemiluminescence array. Light emitted from a chemical reaction is detected by the charge-coupled device (CCD) camera in Evidence Investigator™. BAT measures both immuno-assay and molecular assay in a multiplex system using a single plasma sample. Cytokine high-sensitivity array follows the sandwich immunoassay principle, where immobilized antibody in each of the discrete test regions (DTR's) of the biochip binds with the multiple analyte for multiple biomarkers, which binds with horseradish peroxidase (HRP)-labelled antibody. The addition of a signal reagent

emits the chemiluminescent signal at the DTR's which is detected by the CCD camera. CCD capture the images and quantifies the amount of light as a relative unit which is further transformed into analyte concentration using the known concentration of the analyte in the standards using an automated calibration curve generated by the RANDOX Evidence Investigator. The quantity of the analyte is based on the chemiluminescent signal and is directly proportional to the analyte concentration.

The BAT kit includes biochip carriers, calibrators, and reagents including assay diluent, conjugate, signal reagent-EV805 (luminol-EV805 and peroxide) and wash buffer. One kit has 6 biochip trays and each of them has 9 ceramic solid phase biochips to run 54 samples together with 9 controls. Controls are run once for all kits having the identical lot number.

Cytokine and growth factor high-sensitivity array was used to analyze IL-2, IL-4, IL-6, IL-8, IL-10, VEGF, IFN-γ, TNF-α, IL-1α, IL-1β, MCP-1, and EGF. All these analytes are measured simultaneously on one patient sample: each run measures 54 patient samples. Small volume of 100 uL of sample or controls were added into the trays together with 200 uL assay diluent and incubated at 37 ^{O}C for one hour in a thermoshaker at 370 rpm, then incubated overnight for 16-18 hours at 2-8 ^{O}C. This process allows for the binding of sample analyte with captured/immobilized antibody. Washing step was performed and 300 uL conjugate consisting of horse radish peroxidase-labeled, analyte-specific antibody was added and incubated for 1 hour at 37 ^{O}C at 370 rpm. Following the incubation another washing step was performed as per the protocol. Signal reagent was prepared by mixing luminol-EV701 and peroxide in a 1:1 ratio, placed on a roller to mix for 15 minutes prior to use. After the last wash, the wells were filled with wash buffer to soak until imaging. Each of the carriers was processed individually. Signal reagent (250 uL) was added into the tray, incubated for 2 minutes (±10 seconds), the carrier was placed into the Evidence Investigator™ and images were captured. Increased binding of bound cytokine led to the increased binding of the conjugate, resulting in a higher chemiluminescent signal upon activation of the signal reagent. The principal of assay is depicted in Figure 10.

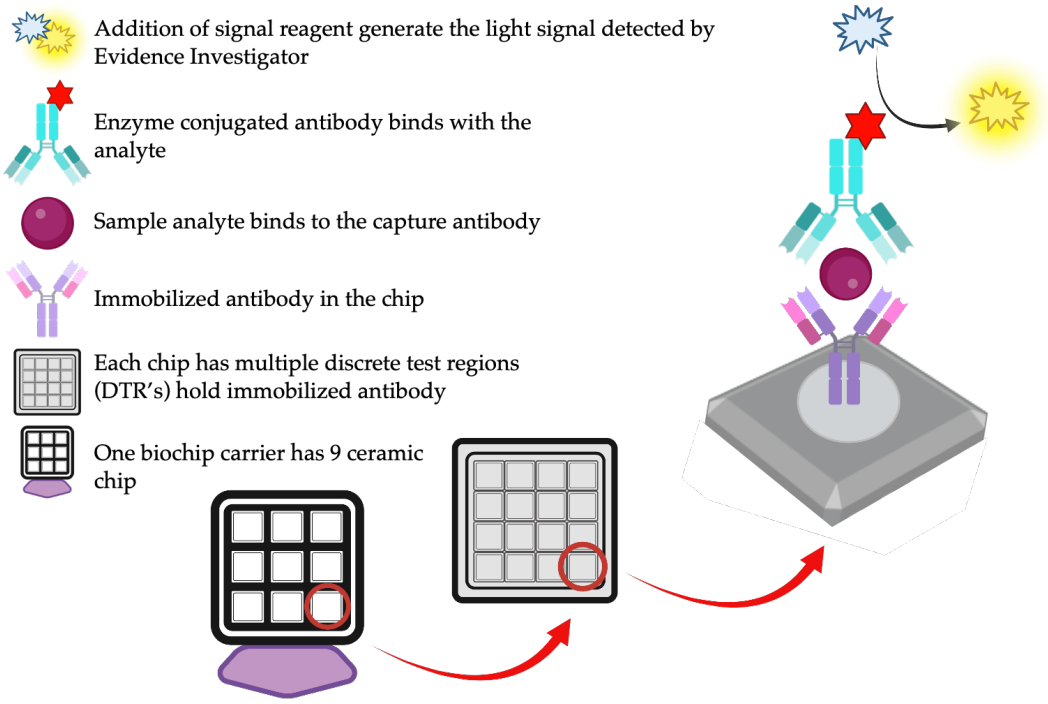


Figure 10. An overview of the principle of RANDOX Biochip Array.

4.7. STATISTICAL ANALYSIS

The clinical and experimental data collected in this dissertation were statistically analyzed and graphs were generated using GraphPad Prism Software (GraphPad Inc., La Jolla, CA) and IBM SPSS Statistics Software (IBM® Corporation, Armonk, NY). Depending upon the distribution pattern of the data, the best fitting analytic test was used for presentation and comparison purposes.

4.7.1. Descriptive Statistics

The statistical approach for summarizing, organizing, and presenting the data was selected to emphasize the core features of the study findings. Descriptive analysis used to measure the dispersion/distribution (skewness, and kurtosis), central tendency (mean, mode, and median), disparities (range, standard deviation, and interquartile range), and frequency, highlighted the essential features and patterns.

4.7.1.1. Mean, Median, Standard Deviation and Interquartile Range

Biomarker levels and clinical data including the demographics (age, weight, BMI), laboratory parameters and clinical presentation of the patients were presented as mean ± standard deviation (SD) or median with interquartile range (IQR). The presentation of the data depended upon the variability in the dataset. Data following the "Gaussian or bell-curve" dispersal, mean ± standard deviation (SD) was used. Mean was the arithmetic average of the values which measures the midpoint of the data, while standard deviation measures the magnitude of the variation in the dataset. The Skewed data with positive or negative transference or bimodal distribution was presented as median with interquartile range (IQR). Median is the midmost value in the data set and considered as central tendency in case of skewness whereas interquartile range was determined by the third quartile or 75^{th} percentile and first quartile or 25^{th} percentile in the dataset.

4.7.1.2. Frequency and Percentage

Data available in binary or categorical form such as demographics (gender, and ethnicity), comorbidities, and outcomes were presented as frequency and percentage in the dataset. Frequency measures the absolute number of the events that occur in the given time interval, while percentage was calculated using the frequency of the event in total.

4.7.2. Comparative Analysis

Statistical analysis was used to determine the differences between two or more variables in the study group. The relationship between two continuous variable were measured as parametric test as t-Test (independent, or paired t-Test) and analysis of variance (ANOVA, one-way or two-way ANOVA), non-parametric test as Mann Whitney T test, Wilcoxon Signed-Rank Test, and Kruskal-Wallis H Test, while the relationship between two categorical variables was determined by Chi-Square test.

4.7.2.1. Wilcoxon Mann Whitney Tests

The degree of association between the two continuous variables with skewed distribution was measured by the Wilcoxon Mann Whitney test. The test evaluates

the difference based on the mean rank of the data and used for testing the null hypothesis. P value less than 0.05 was considered statistically significant.

4.7.2.2. ***Chi-square Tests***

Chi-square test is used for the hypothesis testing for the categorical variables in the form of contingency table. P values less than 0.05 were considered significant to reject the null hypothesis. Odd's ratio (OR) with the confidence interval were noted. OR measures the association between the events and the outcome, or the odds for the outcome in the presence or the absence of the diseases/events or ratios of the likelihood for the occurrence of the outcome or not. Odds ratio equal to 1 demonstrates no relationship between the event and outcome, while >1 demonstrates the likelihood of the outcome to be appear in presence of event, while <1 demonstrates the likelihood of the outcome to decrease in presence of event. Confidence interval is the range of the odds where the population is expected to lie; a small range designates the higher accuracy of the odds, while a large range designates the lower accuracy of the odds ratio.

4.7.3. Correlation Analysis

Correlation analysis is the analysis used to measure the association between two or more variables, together with the potency and the direction of the relationship. In other words, it demonstrates that variation in one variable effects the other variables. Depending upon the Gaussian or bell-curve distribution pattern, correlation analysis was classified by Spearman (skewed data) or Pearson (bell-shape scattering) correlation.

4.7.3.1. ***Spearman Correlation Analysis***

Spearman correlation analysis measures the monotonic relationship between the two variables. Correlation coefficient ranges from scale +1 to -1, 0 demonstrates no relationship between the variable, >0 to 1 demonstrates a positive and direct relation, while <0 to -1 demonstrates a negative and inverse relationship between the variables. Correlation coefficient of 0 to 0.3 or 0 to -0.3 is considered as weak correlation, 0.3 to 0.6 and -0.3 to -0.6 considered as moderate, 0.7 to 1.0 and -0.7 to -1.0 considered as strong correlation

4.7.4. Prognostic Analysis

Statistical methods to predict the likelihood of future outcome or events established on existing data, is referred to as prognostic or predictive analysis.

4.7.4.1. ***Receiver Operating Characteristics Curve Analysis***

The receiver operating characteristics (ROC) curve analysis was performed to identify the optimal cutoff values for blood cellular indices and biomarker levels in predicting 30-day or 90-day mortality. The optimal cutoff values for blood cellular indices and biomarker levels were determined by identifying the point on the ROC curve that provided the optimal balance between sensitivity (true positive rate) and specificity (false positive rate) for predicting 30-day or 90-day mortality.

4.7.4.2. ***Stepwise Binary Logistic Regression Modelling***

Regression is a mathematical modelling used to determine the relationship between the dependent and one or more independent variable and to understand the effect of an independent variable towards the dependent variable. Regression analysis can be of four types: linear regression, logistic regression, polynomial regression or Ridge and Lasso regression. Linear regression measures the relationship between the dependent and independent variable in a straight line and is used for the continuous variable. Logistic regression analysis was used when the dependent variable was in a categorical form. Polynomial regression was used to measure a complex relationship by lengthening the linear regression into the polynomial equation of the data. Ridge and Lasso regression are variants of linear regression.

Logistic regression analysis was used to determine the association of dependent variables, which is a categorical variable with one or more independent variables and used to measure the probability of the outcome. The main type of regression analysis included binary logistic regression, multinomial logistic regression, and ordinal logistic regression. Binary logistic regression analysis was used when the dependent variable or the outcome was in the dichotomous form, while multinomial and ordinal logistic regression analysis was used when the outcome was more than three, however, the categorical variable was specified in the ordinal regression.

V – RESULTS

V - RESULTS

5.1. PROGNOSTIC VALUE OF BLOOD CELLULAR INDICES AS NLR, PLR, AND SII IN VTE PATIENTS

The cohort of 4,487 VTE patients consisted of 52.9% (n= 2,372) male and 47.1% (n= 2,115) female. The mean age of the patients was 65.2±16.6 years with mean weight 77.3±16.6 kg. Unprovoked VTE (46.1%), immobility (30.8%), active cancer (15.9%), and leg varicosities (15.5) were the most common risk factors in the cohort. The most common comorbid conditions included hypertension (45.1%), diabetes (15.4%), current smoker (10.5%), and chronic lung disease (10.1%). Clinically, 34.6% of the patients had anemia, while 27% of patients had creatinine clearance <60mL/min. Blood cellular indices for the cohort were as follows: NLR level was 5.8±7.1, PLR was 190.4±158.0, and SII was 1,458.6±2,028.3. During the first 90-day of diagnosis, 79 (1.8%) patients developed recurrent VTE, 152 (3.4%) suffered from major bleeding, and 484 (11%) died.

Blood cellular indices were analyzed with reference to the clinical characteristics of the patients (table 12). Mean NLR values were higher in men, in patients aged >70 years or weighing <70 kg, and in those with active cancer, recent immobility, chronic lung disease, diabetes, hypertension, anemia or renal insufficiency, but were lower in patients using estrogens, with unprovoked VTE, prior VTE, leg varicosities, or current smokers.

The mean PLR values were significantly higher in patients weighing <70 kg, and in those with cancer, recent surgery, immobility, or anemia. Conversely PLR values were significantly lower in patients who use estrogens, had unprovoked or prior VTE, leg varicosities, or are actively smoking.

The mean SII values were significantly higher in patients weighing <70 kg, with cancer, recent surgery, immobility, anemia, or renal insufficiency. Conversely SII values were significantly lower in patients using estrogens, had unprovoked VTE, prior VTE, or leg varicosities.

Table 12. Mean values at baseline of the 3 cellular indices, according to the clinical characteristics of the patients.

Variables	N	NLR	PLR	SII
		Mean±SD	Mean±SD	Mean±SD
Clinical Characteristics				
All patients	4,487	5.9±7.1	190±158	1,459±2,028
Female; sex	2,115	5.5±6.6‡	189±147	1,410±1,888
Age >70 years	1,956	6.3±6.9‡	195±154	1,470±1,831
Body weight <70 kg	1,425	6.3±7.8†	210±179‡	1,626±2,308‡
Risk Factors for VTE				
Active cancer	715	6.4±7.4*	230±196‡	1,687±2,402†
Recent surgery	381	5.6±5.2	222±164‡	1,687±1,844*
Immobility for ≥4 days	1,384	7.2±9.2‡	221±180‡	1,819±2,564‡
Estrogen intake	210	4.4±3.8‡	160±123‡	1,149±1,161‡
Pregnancy/Postpartum	37	5.6±3.6	197±137	1,738±1,682
Unprovoked	2,067	5.0±5.4‡	160±127‡	1,160±1,445‡
Prior VTE	524	4.9±5.3‡	161±120‡	1,134±1,415‡
Leg varicosities	695	5.3±5.9†	168±132‡	1,242±1,630‡
Concomitant Diseases				
Chronic heart failure	283	6.4±5.3	193±154	1,425±1,589
Chronic lung disease	453	6.7±7.9*	194±163	1,648±2,327
Diabetes	689	6.7±8.0†	202±172	1,575±1,866
Hypertension	2,025	6.3±7.4†	191±156	1,477±1,972
Current smoking	473	5.0±5.0‡	156±128‡	1,300±1,725
Prior myocardial infarction	234	6.4±6.9	205±183	1,579±2,310
Prior ischemic stroke	278	6.1±5.9	201±173	1,505±1,574
Peripheral artery disease	119	6.4±6.3	201±166	1,408±1,598
Recent major bleeding	119	7.0±7.0	207±157	1,746±1,918
Anemia	1,553	6.9±8.8‡	238±186‡	1,837±2,482‡
CrCl levels <60mL/min	1,210	6.9±7.6‡	200±164*	1,598±1,951†

*Comparisons between patients with- vs. without the variable: *p <0.05; †p <0.01; ‡p <0.001.*

Mean NLR values were highest in patients initially presenting with PE (either symptomatic or incidental), particularly in those with tachycardia or hypoxemia, and lowest in those with superficial vein thrombosis or retinal vein thrombosis (Table 13). Mean PLR values were also highest in patients initially presenting with PE, and lowest in patients with lower- limb DVT, superficial vein thrombosis or retinal vein thrombosis. Mean SII values were highest in patients with symptomatic PE (particularly in those with tachycardia) and lowest in patients with superficial vein thrombosis or retinal vein thrombosis.

Table 13. Mean values at baseline of the 3 cellular indices, according to the initial VTE presentation.

VTE	N	NLR	PLR	SII
		Mean±SD	Mean±SD	Mean±SD
All patients	4,487	5.9±7.1	190±158	1,459±2,028
Symptomatic PE	2,683	6.6±7.4‡	202±166‡	1,639±2,188‡
In patients with PE				
SBP <90 mm Hg	74	7.3±7.0	171±143	1,679±2,270
Heart rate >110 bpm	380	8.2±10.1‡	210±170	2,122±3,036‡
Sat O2 levels <90%	287	7.5±8.6†	214±183	1,764±2,053*
Incidental PE	283	6.2±7.2	214±160†	1,570±1,918
Lower-limb DVT	1,129	5.2±6.2	165±125	1,225±1,664
Proximal	905	5.1±5.6	161±120†	1,194±1,598
Distal	205	5.5±7.7	186±145†	1,304±1,687
Upper-extremity DVT	175	6.0±10	208±191	1,473±2,371
Splanchnic thrombosis	69	5.6±6.7	218±223	1,837±3,499
Superficial thrombosis	142	3.0±2.5‡	151±143†	760±818‡
Retinal vein thrombosis	20	2.1±0.8‡	109±34‡	466±202‡

*Comparisons between patients with- vs. without the variable: *p <0.05; †p <0.01; ‡p <0.001.*

5.1.1. Prognostic Value of NLR, PLR, and SII for All-cause Mortality

During the 90-day study period, 14.4% patients developed an outcome of note: 484 (11%) died, of those, 26 (5.4%) died with confirmed fatal PE while 26 (5.4%) died with confirmed fatal bleeding. The rest of the causes of death included various factors, such as cancer, renal insufficiency, heart failure, infection, cerebral ischemia, and multiorgan failure. Mean NLR, PLR and SII values were higher in patients who died rather than in those who did not die during the first 90 days (appendix 5, supplementary table 1).

The area under the ROC curves for each of the cellular indices and their association with the risk for all-cause mortality were performed (appendix 4, supplementary figure 1). The optimal cutoff values of each ratio were: NLR >4.96 for mortality with 66.9% sensitivity and 66.8% specificity, PLR >167.96 for mortality with 61.0% sensitivity and 60.8% specificity, and SII >1134.50 for mortality with 64.9% sensitivity and 64.9% specificity. The overall accuracy of NLR, PLR and SII for mortality events by 90 days were as follows; AUC: 0.730 (95% CI: 0.706-0.755; p value: <0.001), AUC: 0.632 (95% CI: 0.604-0.660; p value: <0.001), and AUC: 0.696 (95% CI: 0.670-0.772; p value: <0.001), respectively.

All the blood cellular indices along with the risk factors, concomitant disorder, and initial VTE presentations were analyzed using the univariate Chi-Square for 90-day of all-cause mortality (table 14). Adjusted Odd's ratio and 95% confidence interval were noted. Following chi-square test, all the significant variables were further incorporated into the binary logistic regression model.

Table 14. Univariate- and multivariate analyses for death within the first 3-months.

Variables	*Univariate*	*Multivariate*
	Odd's Ratio (95% CI)	*Odd's Ratio (95% CI)*
Cellular Indices		
NLR >4.96	*4.06 (3.33-4.97)*	*2.50 (1.83-3.42)*
PLR >167.96	*2.42 (1.99-2.93)*	*NS*
SII >1134.5	*3.41 (2.80-4.15)*	*1.52 (1.08-2.14)*
Clinical Characteristics		
Gender	*NS*	*-*
Age >70 years	*2.16 (1.78-2.62)*	*NS*
Weight <70 kg	*1.60 (1.32-1.94)*	*NS*
Risk Factors for VTE		
Active cancer	*5.09 (4.15-6.23)*	*4.87 (3.36-7.05)*
Recent immobility	*1.75 (1.44-2.12)*	*1.95 (1.38-2.75)*
Unprovoked VTE	*0.27 (0.22-0.34)*	*NS*
Prior VTE	*NS*	*-*
Leg varicosities	*NS*	*-*
Concomitant Diseases		
Chronic heart failure	*2.34 (1.73-3.17)*	*NS*
Chronic lung disease	*1.76 (1.34-2.30)*	*NS*
Diabetes	*1.99 (1.59-2.49)*	*1.47 (1.13-1.93)*
Hypertension	*1.57 (1.30-1.90)*	*NS*
Current smoking	*NS*	*-*
Prior myocardial infarction	*1.49 (1.03-2.16)*	*NS*
Prior ischemic stroke	*1.81 (1.30-2.50)*	*NS*
Recent major bleeding	*2.26 (1.44-3.56)*	*NS*
Anemia	*2.83 (2.34-3.43)*	*1.46 (1.16-1.84)*
CrCl levels <60 mL/min	*2.51 (2.07-3.05)*	*1.65 (1.28-2.14)*
Initial VTE Presentation		
Symptomatic PE	*6.56 (5.04-8.55)*	*2.46 (1.78-3.40)*
Incidental PE	*1.48 (1.05-2.09)*	*NS*
Lower limb DVT	*0.56 (0.46-0.69)*	*0.62 (0.50-0.78)*
Upper limb DVT	*NS*	*-*
Splanchnic vein thrombosis	*NS*	*-*
Superficial vein thrombosis	*0.06 (0.01-0.41)*	*0.11 (0.02-0.84)*
Retinal vein thrombosis	*NS*	*-*

On multivariable analysis, patients with NLR >4.96 were at increased risk for death (aOR: 2.50; 95% CI: 1.83-3.42). Patients with PLR >167.96 were not at increased risk for mortality. Patients with SII >1134.5 were at increased risk for death (aOR: 1.52; 95% CI: 1.08-2.14). Beside NLR and SII, patients with cancer, recent immobility, diabetes, anemia, and renal insufficiency were also at increased risk of death (table 14, figure 11).

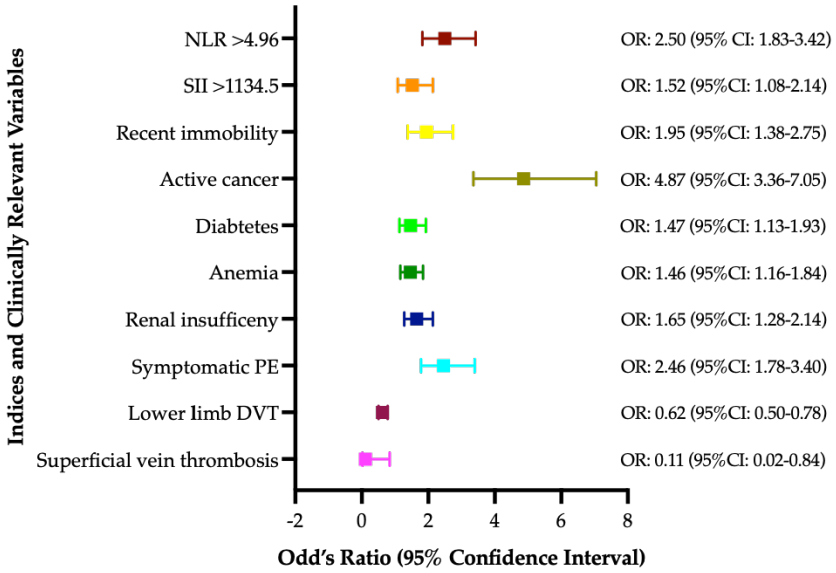


Figure 11. Multivariate regression analysis for cellular indices and clinically relevant variables in predicting 90-day mortality.

The results are different from the univariate analysis, where all three indices demonstrated an association with major bleeding in contrast to the multivariate analysis, where only NLR and SII exhibited this trend. NLR and SII both established the power to predict 90-day mortality in patients with VTE.

5.1.2. Prognostic Value of NLR, PLR, and SII for Major Bleeding

During the 90-day study period, 14.4% of the patients developed an outcome with 152 (3.4%) suffering a major bleeding. Mean NLR, PLR and SII values were higher in patients who bled than in those who did not during the first 90-days (appendix 5, supplementary table 1).

The area under ROC curves for each of the cellular indices and their association with the risk of major bleeding were performed (appendix 4, supplementary figure 2). The optimal cutoff values of each ratio based on sensitivity and specificity for major bleeding were: NLR >4.41 with 68.4% sensitivity and 68.0% specificity, PLR >166.47 with 60.5% sensitivity and 58.6% specificity, and SII >1154.81 with 62.5% sensitivity and 63.2% specificity. The overall accuracy of NLR, PLR and SII for major bleeding events by 90-days were as follows; AUC: 0.669 (95% CI: 0.623-0.715; p value: <0.001), AUC: 0.624 (95% CI: 0.577-0.671; p value: <0.001), and AUC: 0.659 (95% CI: 0.612-0.705; p value: <0.001), respectively.

All the blood cellular indices along with the risk factors, concomitant disorder, and initial VTE presentations were analyzed for the univariate Chi-Square for the 90-days of all-cause mortality (table 15). Adjusted Odd's ratio and 95% confidence interval were noted. The significant variables from Chi-square were integrated into the binary logistic regression model.

Table 15. Univariate- and multivariate analyses for major bleeding within the first 3-months.

Variables	*Univariate*	*Multivariate*
	Odd's Ratio (95% CI)	*Odd's Ratio (95% CI)*
Cellular Indices		
NLR >4.41	*2.99 (2.11-4.23)*	*1.73 (1.05-2.86)*
PLR >166.47	*2.17 (1.56-3.02)*	*NS*
SII >1154.81	*2.86 (2.05-4.00)*	*NS*
Clinical Characteristics		
Gender	*NS*	-
Age >70 years	*1.58 (1.14-2.19)*	*NS*
Weight <70 kg	*NS*	-
Risk Factors for VTE		
Active cancer	*1.73 (1.19-2.54)*	*NS*
Recent immobility	*1.72 (1.24-2.38)*	*NS*
Unprovoked VTE	*0.44 (0.30-0.62)*	*NS*
Prior VTE	*NS*	-
Leg varicosities	*NS*	-
Concomitant Diseases		
Chronic heart failure	*NS*	-
Chronic lung disease	*NS*	-
Diabetes	*NS*	-
Hypertension	*1.40 (1.01-1.94)*	*NS*
Current smoking	*NS*	-

Prior myocardial infarction	*NS*	*-*
Prior ischemic stroke	*NS*	*-*
Recent major bleeding	*3.05 (1.61-5.81)*	*NS*
Anemia	*2.41 (1.74-3.34)*	*1.56 (1.08-2.26)*
CrCl levels <60 mL/min	*2.14 (1.54-2.97)*	*1.68 (1.13-2.51)*
Initial VTE Presentation		
Symptomatic PE	*2.15 (1.52-3.06)*	*NS*
Incidental PE	*NS*	*-*
Lower limb DVT	*0.62 (0.44-0.87)*	*NS*
Upper limb DVT	*NS*	*-*
Splanchnic vein thrombosis	*NS*	*-*
Superficial vein thrombosis	*NS*	*-*
Retinal vein thrombosis	*NS*	*-*

On multivariable analysis, patients with NLR >4.41 were at increased risk for major bleeding (aOR: 1.73; 95% CI: 1.05-2.86). Patients with PLR >166.47 and SII >1154.81 were not at increased risk for major bleeding. Besides cellular indices, patients with anemia and renal insufficiency were also at increased risk of major bleeding (table 115, and figure 12).

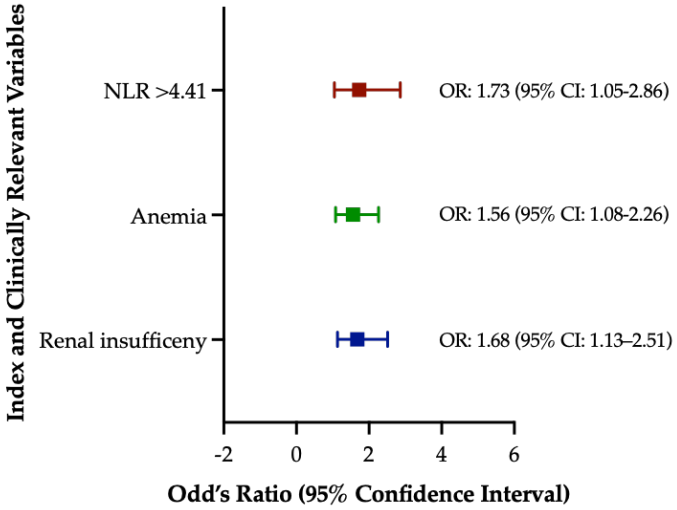


Figure 12. Multivariate regression analysis for cellular indices and clinically relevant variables in predicting 90-day major bleeding.

These results were different from the univariate analysis where all three indices demonstrated an association with major bleeding in contrast to the multivariate analysis, where only NLR exhibited this trend. NLR demonstrated prognostic values that predicted 90-day mortality in patients with VTE.

5.1.3. Prognostic Value of NLR, PLR, and SII for VTE Recurrence

During the 90-day study period, 14.4% of the patients developed a thrombotic outcome with 38 patients (0.8%) experiencing recurrent DVT and 45 (1.0%) with recurrent PE (appendix 5, supplementary table 1). Mean NLR, PLR and SII values were similar between patients developing DVT or PE recurrences and those who did not.

The area under ROC curves for each of the cellular indices and their association with the risk for VTE recurrences were performed (appendix 4, supplementary figure 3). No cellular indices were relevant to predict VTE recurrences (Figure 8). The optimal cutoff values of each ratio based on sensitivity and specificity for VTE recurrence were not calculated as the diagnostic strength of all the three cellular indices was poor, NLR with AUC: 0.480 (95% CI: 0.437-0.523; p value: 0.35), PLR with AUC: 0.431 (95% CI: 0.386-0.47; p value: <0.001), and SII with AUC: 0.461 (95% CI: 0.417-0.506; p value: 0.73).

Besides blood cellular indices, all the other clinically relevant factors including risk factor for VTE, concomitant disorder, and initial VTE presentations were analyzed for the univariate Chi-Square for the 90-days VTE recurrence (table 16). Adjusted Odd's ratio and 95% confidence interval were noted. The significant variables from Chi-square were integrated into the binary logistic regression model.

Table 16. Univariate- and multivariate analyses for VTE recurrence within the first 3 months.

Variables	*Univariate*	*Multivariate*
	Odd's Ratio (95% CI)	*Odd's Ratio (95% CI)*
Cellular Indices		
NLR >4.96	*NS*	-
PLR >167.96	*NS*	-
SII >1134.5	*NS*	-
Clinical Characteristics		
Gender	*NS*	-
Age >70 years	*NS*	-
Weight <70 kg	*NS*	-
Risk Factors for VTE		
Active cancer	*1.74 (1.23-2.46)*	*2.28 (1.26-4.15)*
Recent immobility	*0.47 (0.32-0.68)*	*NS*
Unprovoked VTE	*1.43 (1.06-1.92)*	*NS*

Prior VTE	*NS*	-
Leg varicosities	*1.98 (1.41-2.78)*	*1.73 (1.21-2.47)*
Concomitant Diseases		
Chronic heart failure	*NS*	-
Chronic lung disease	*1.85 (1.24-2.76)*	*1.67 (1.07-2.59)*
Diabetes	*NS*	-
Hypertension	*NS*	-
Current smoking	*NS*	-
Prior myocardial infarction	*NS*	-
Prior ischemic stroke	*NS*	-
Recent major bleeding	*NS*	-
Anemia	*NS*	-
CrCl levels <60 mL/min	*NS*	-
Initial VTE Presentation		
Symptomatic PE	*1.52 (1.12-2.06)*	*NS*
Incidental PE	*0.40 (0.16-0.99)*	*0.40 (0.16-0.99)*
Lower limb DVT	*NS*	-
Upper limb DVT	*NS*	-
Splanchnic vein thrombosis	*NS*	-
Superficial vein thrombosis	*2.67 (1.51-4.73)*	*2.06 (1.12-3.78)*
Retinal vein thrombosis	*NS*	-

On multivariable analysis, patients with active cancer, leg varicosities, and chronic lung disease were at increased risk of VTE recurrence (table 16, and figure 13). None of the blood cellular indices studied had a prognostic capability for 90-day VTE recurrence

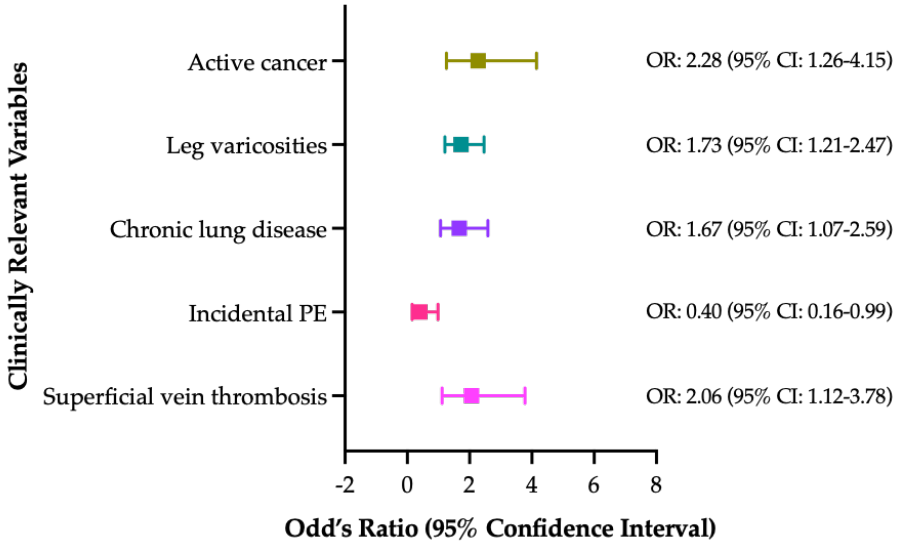


Figure 13. Multivariate regression analysis for clinically relevant variables in predicting 90-day VTE recurrence.

5.2. COMPARATIVE PROGNOSTIC VALUE OF BLOOD CELLULAR INDICES AS NLR, PLR, AND SII IN ACUTE PE PATIENTS

This study included 10,085 patients with acute symptomatic PE from the RIETE registry, recruited between January 2021 and March 2023. Each patient had complete cellular index data, Additionally, for external validation, 700 patients from the LUMC PERT project were used.

The comparison of baseline clinical characteristics and laboratory parameters between the RIETE registry and LUMC PERT dataset is presented as mean±SD, and median (IQR) (table 17). The patient's cohort from the RIETE registry and LUMC PERT shows nearly equal distribution of males and females with 51.1% vs. 50.0% male, and 49.9% vs. 50.0% female in the RIETE registry and LUMC PERT cohort, respectively. The age of patients in both cohorts was similar with a modest increase in RIETE registry patients (67±16 years) compared to LUMC PERT patients (61±16 years). However, the body weight was markedly higher in the LUMC PERT cohort at 95±58 kg compared to 78±17 kg in the RIETE registry patients.

The LUMC PERT patients were also comparatively sicker with higher values in heart rate >110 bpm (39.6% vs. 16.6%), and SBP levels <90 mm Hg (17.9% vs 2.7%), respectively. The complete blood count data for both cohorts were quite similar. The RIETE cohort had a greater proportion of patients with low-risk PE compared to that of the LUMC cohort (32.5% vs. 16.9%, respectively), whereas the LUMC PERT cohort presented a greater proportion of patients with high-risk PE: 15.6%, as opposed to 2.7% in the RIETE cohort. These differences were further evident with RVDD/LVDD ratio >1.0 on TTE with 69.5% for LUMC PERT patients compared with 29.4% for RIETE registry patients.

Table 17. Baseline clinical characteristics, and laboratory parameters for PE patients in the RIETE Registry, and LUMC PERT Cohort.

Variables	*RIETE Cohort*	*LUMC PERT Cohort*
	N (%), Mean±SD, Median (IQR)	*N (%), Mean±SD, Median (IQR)*
Patients, N	*10,085*	*700*
Demographics		
Male sex	*5,154 (51.1%)*	*350 (50.0%)*
Age (years)	*67±16*	*61±16*
Body weight	*78±17*	*95±58*

Risk Factors for PE		
Active cancer	*1,482 (14.7%)*	*NA*
Recent surgery	*822 (8.2%)*	*NA*
Recent immobility	*2,710 (26.9%)*	*NA*
Pregnancy/postpartum	*57 (0.6%)*	*NA*
Hormonal use	*498 (5.1%)*	*NA*
None of the above (unprovoked)	*5,172 (51.3%)*	*NA*
Clinical Presentation		
Heart rate >110 bpm	*1,674 (16.6%)*	*227 (39.6%)*
SBP levels <90 mm Hg	*275 (2.7%)*	*125 (17.9%)*
Sat O_2 levels (%)	*93±7*	*92±7*
Laboratory Parameters		
Hemoglobin (g/dL)	*13.2±2.5*	*12.2±2.5*
Leukocytes (x 1000/mm^3)	*9.9±4.4*	*10.4±5.5*
Neutrophils (x 1000/mm^3)	*7.4±4.9*	*7.7±4.8*
Lymphocytes (x 1000/mm^3)	*1.7±1.7*	*1.6±1.1*
Platelets (x 1000/mm^3)	*243±100*	*243±114*
NLR (x 1000/mm^3)	*4.3 (2.7-7.2)*	*4.8 (2.6-8.7)*
PLR (x 1000/mm^3)	*151 (104-231)*	*166 (105-263)*
SII (x 1000/mm^3)	*963 (570-1,729)*	*1099 (526-2,182)*
Signs of RV Dysfunction		
RV hypokinesis on TTE	*734/3,788 (19.4%)*	*NA*
RVDD/LVDD ratio >1.0 on TTE	*216/734 (29.4%)*	*251/361 (69.5%)*
RVDD/LVDD ratio >1.0 on CT-scan	*449/971 (46.2%)*	*NA*
Risk Subgroups		
Low-risk PE	*3,276 (32.5%)*	*118 (16.9%)*
Intermediate-risk PE	*6,534 (64.8%)*	*473 (67.6%)*
High-risk PE	*275 (2.7%)*	*109 (15.6%)*
30-day Outcomes		
PE recurrences	*32 (0.3%)*	*NA*
DVT recurrences	*26 (0.3%)*	*NA*
Major bleeding	*209 (2.1%)*	*NA*
Death	*465 (4.6%)*	*58 (8.3%)*

Treatment strategies including initial and long-term therapy for the two cohorts, are presented in appendix 5 (supplementary table 2). Complete details with reference to the initial and long-term therapy in the LUMC PERT cohort are not available, which is due to the differences in methodology rationale and methodology between the LUMC PERT and RIETE registry cohorts. The RIETE registry patients were predominantly categorized as low-risk or intermediate-risk group, initial treatments included LMWH for 84.3% of patients followed by DOAC's for 7.7% and UFH 4.6% of patients. Conversely, due to the severe condition of patients in LUMC PERT cohort, initial treatments included

thrombolytics for 15.4% of patients, followed by insertion of an IVC filter for 9.1%, and surgical embolectomy 3.7%.

5.2.1. Prognostic Significance of Cellular Indices

The area under ROC curves for the cellular indices and their association with the risk of 30-day mortality in the RIETE cohort are presented in appendix 4 (supplementary figure 4). The optimal cutoff values were >7 for NLR, >220 for PLR, and >1600 for SII. The precision of these indices for predicting 30-day mortality is as follows: NLR has a precision of (AUC: 0.686; 95% CI: 0.660-0.713; p value: <0.001), PLR has a precision of (AUC: 0.611; 95% CI: 0.583-0.638; p value: <0.001), and SII has a precision of (AUC: 0.649; 95% CI: 0.622-0.676; p value: <0.001). The precision of NLR, PLR and SII for 30-day mortality are AUC: 0.686 (95% CI: 0.660-0.713, p value: <0.001), AUC: 0.611 (95% CI: 0.583-0.638; p value: <0.001), and AUC: 0.649 (95% CI: 0.622-0.676; p value: <0.001), respectively. Through optimal cutoff determination, the NLR, PLR, and SII are significant predictors of 30-day mortality.

A stepwise logistic regression analysis was performed for each ratio independently. To circumvent multicollinearity, separate models for each cellular index were developed. The analysis reflects that the patients with the NLR >7.0 (OR: 5.00; 95% CI: 4.12-6.06) were at increased risk of all-cause mortality in the RIETE cohort, followed by SII >1200 (OR: 3.56; 95% CI: 2.95-4.29), and PLR >220 (OR: 2.63; 95% CI: 2.18-3.18). To enhance the robustness of the analysis, regression analysis performed at adjusted levels for potential confounders included clinically relevant covariates (appendix 5, supplementary table 3). Specifically, an NLR >7.0 increased the risk of all-cause death in the RIETE cohort threefold (OR: 3.46; 95% CI: 2.60-4.60), surpassing the predictive strength of PLR >220 (OR: 2.36; 95% CI: 1.77-3.13), and SII >1600 (OR: 2.52; 95% CI: 1.90-3.33) (Figure 14). This comparative evaluation of NLR, PLR, and SII highlighted the NLR as the predominant index, demonstrating stronger correlations with patient outcomes, suggesting NLR as an independent predictor of 30-day mortality in PE patients.

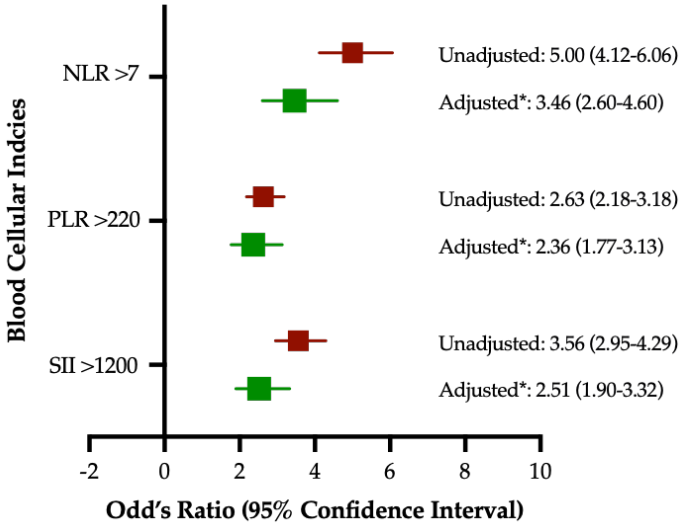


Figure 14. Association between cellular indices and 30-day mortality in unadjusted and adjusted analyses.

**Adjusted for patient's age, history of heart failure, dementia, recent major bleeding, transient risk factors for VTE, active cancer, use of corticosteroids, SBP levels <90 mm Hg, Sat O_2 levels <90%, and anemia.*

5.2.2. The prognostic Significance of Ratios Across PE Severity

The prognostic effectiveness of the NLR within the ESC risk categories of PE (low, intermediate, and high) was also evaluated (table 18). The 30-day all-cause mortality rate was 4.6% in the RIETE cohort and 8.3% in the LUMC PERT cohort. Upon risk stratification, mortality for low-risk PE patients was 1.1% in the RIETE cohort and 0.8% in the Loyola cohort. Intermediate-risk PE mortality was 5.8% for RIETE and 7.6% for Loyola, while the rates for high-risk PE patients were 16.7% and 19.3%, respectively. NLR demonstrated the higher predictive accuracy for low-risk patients (c-statistics: 0.78; 95% CI: 0.69-0.86) compared to intermediate-risk (c-statistics: 0.66; 95% CI: 0.63-0.69), or high-risk PE (c-statistics: 0.68; 95% CI: 0.59-0.76), as shown in table 18. This trend is consistent in the Loyola cohort.

Table 18. Prognostic utility of NLR among patients with different PE risk categories in the RIETE Registry, and LUMC PERT cohort.

Risk Stratification	*RIETE Cohort*	*LUMC PERT Cohort*
Patients, N	*10,085*	*700*
Low-risk PE		
Patients, N	*3,276*	*118*

30-day Mortality	*37 (1.13%)*	*1 (0.85%)*
NLR >7.0	*27/593 (4.55%)‡*	*1/20 (5.00%)*
NLR ≤7.0	*10/2,683 (0.37%)*	*0/98 (0%)*
c-statistics	*0.78 (0.69-0.86)*	*0.92 (0.82-1.00)*
Sensitivity	*73.0 (58.7-87.3)*	*100 (100-100)*
Specificity	*82.5 (81.2-83.8)*	*83.8 (77.1-90.4)*
PPV	*4.55 (2.88-6.23)*	*5.00 (0.00-14.6)*
NPV	*99.6 (99.4-99.9)*	*100 (100-100)*
Intermediate--risk PE		
Patients, N	*6,534*	*473*
30-day Mortality	*382 (5.85%)*	*36 (7.61%)*
NLR >7.0	*228/1,919 (11.9%)‡*	*23/165 (13.9%)‡*
NLR ≤7.0	*154/4,615 (3.34%)*	*13/308 (4.22%)*
c-statistics	*0.66 (0.63-0.69)*	*0.66 (0.56-0.75)*
Sensitivity	*59.7 (54.8-64.6)*	*63.9 (48.2-79.6)*
Specificity	*72.5 (71.4-73.6)*	*67.5 (63.1-71.9)*
PPV	*11.9 (10.4-13.3)*	*13.9 (8.65-19.2)*
NPV	*96.7 (96.1-97.2)*	*95.8 (93.5-98.0)*
High-risk PE		
Patients, N	*275*	*109*
30-day Mortality	*46 (16.7%)*	*21 (19.3%)*
NLR >7.0	*31/105 (29.5%)‡*	*8/47 (17.0%)*
NLR ≤7.0	*15/170 (8.82%)*	*13/62 (21.0%)*
c-statistics	*0.68 (0.59-0.76)*	*0.47 (0.33-0.61)*
Sensitivity	*67.4 (53.8-80.9)*	*38.1 (17.3-58.9)*
Specificity	*67.7 (61.6-73.7)*	*55.7 (45.3-66.1)*
PPV	*29.5 (20.8-38.3)*	*17.0 (6.28-27.8)*
NPV	*91.2 (86.9-95.4)*	*79 (68.9-89.2)*

Differences in mortality: ‡p <0.001.

The differences in mortality rate between patients with NLR >7.0 and those with NLR ≤7.0 became apparent shortly after the initial PE event (appendix 4, supplementary figure 5). At Day 5, the mortality rates were 1.0% vs. 0.1% in patients with low-risk PE; 3.6% vs. 0.7% in those with intermediate-risk PE; and 22.3% vs. 3.6% in those with high-risk PE. These differences persisted over time. At Day 30, the mortality rates were: 4.6% vs. 0.4% for low-risk PE, 12.1% vs. 3.4% for intermediate-risk PE, and 30.1% vs. 9.1% for high-risk PE, respectively (Figure 15). The early differentiation in mortality rates observed with NLR underscores its potential as an early prognostic marker.

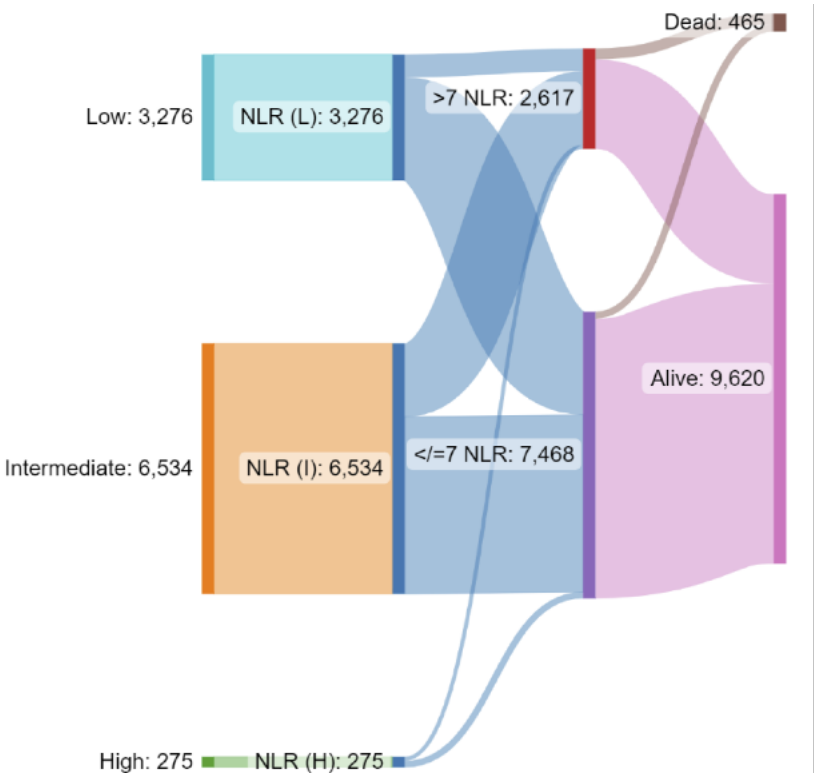


Figure 15. Thirty-day mortality rates according to different PE risk categories and NLR values at baseline.

The x-axis represents groups including Pulmonary Embolism risk (Low (L), Intermediate (I), High (H)), NLR risk segmentation, 30-day mortality. The height of the y-axis represents the full sample. The colored arcs represent the proportion of the sample transitioning from one node to the next.

5.2.3. Predictive Utility of Different Scoring Systems

NLR demonstrated to be the best predictor for low-risk PE patients. The predictive ability of NLR against sPESI score in patients categorized under the non-high-risk category (systolic blood pressure levels ≥90 mm Hg) was evaluated (table 19). Among 9,810 normotensive patients (SBP levels ≥90 mm Hg), 7,298 (74.4%) had an NLR ≤7.0, 4,236 (43.2%) scored zero points on the sPESI, and 3,432 (35.0%) scored 0 points on sPESI and had an NLR ≤7.0. The sPESI displayed superior sensitivity (87.1%) and NPV (98.7%) than a NLR ≤7.0 (60.9% and 97.8%, respectively) but integrating the NLR with sPESI reclassified 35.0% of patients, into the low-risk category, with corresponding sensitivity of 97.1% (95% CI: 95.5-98.7), and NPV of 99.7% (95% CI: 99.5-99.8). Although NLR alone revealed notable predictive power,

its integration with sPESI scores enhanced the accuracy in identifying patients at low risk for death.

Table 19. Prognostic value of the NLR, sPESI, and both scores integrated to predict 30-day mortality in normotensive patients in the RIETE Registry cohort.

	NLR alone	sPESI alone	NLR and sPESI
All patients, N	9,810	9,810	9,810
30-day Mortality	419 (4.3%)	419 (4.3%)	419 (4.3%)
Low-risk	NLR ≤7.0	sPESI=0	NLR ≤7.0 and sPESI=0
Patients, N	7,298	4,236	3,432
30-day mortality	164 (2.2%)	54 (1.3%)	12 (0.4%)
Non-low risk	NLR >7.0	sPESI >0	NLR >7.0 and/or sPESI >0
Patients, N	2,512	5,574	6,378
30-day mortality	255 (10.2%)‡	365 (6.5%)‡	407 (6.4%)
c-statistics	0.68 (0.66-0.71)	0.66 (0.64-0.68)	0.67 (0.65-0.69)
Sensitivity	60.9 (56.2-65.5)	87.1 (83.9-90.3)	97.1 (95.5-98.7)
Specificity	76.0 (75.1-76.8)	44.5 (43.5-45.5)	36.4 (35.4-37.4)
PPV	10.2 (8.97-11.3)	6.55 (5.90-7.20)	6.38 (5.78-6.98)
NPV	97.8 (97.4-98.1)	98.7 (98.4-99.1)	99.7 (99.5-99.8)

Differences in mortality: ‡p <0.001. Abbreviations: NLR, neutrophils to lymphocytes ratio; sPESI, simplified pulmonary embolism severity index; PPV, positive predictive value; NPV, negative predictive value.

5.3. THROMBIN GENERATION POTENTIAL, MARKERS, AND BLOOD CELLULAR INDICES IN ACUTE PE PATIENTS

Plasma samples were collected from PE patients involved in the PERT project adhering to the protocols outlined in the Materials and Methods section of this dissertation. Samples were collected from Loyola University Medical Center hospital (Chicago) and Gottlieb Memorial Hospital (Chicago) under IRB-approved protocols (appendix 3).

The cohort of 209 patients was randomly used to determine the thrombin generation potential in PE. Table 20 lists the basic demographic information, co-morbidities, clinical presentation, laboratory parameters, PE risk stratification, and mortality within the LUMC PERT cohort.

The cohort had an equal distribution between males and females (51.2% males vs. 48.8% female). The median of 63 years indicates a predominantly middle-aged population. However, the age range, spaninng from 16 to 95 years, reflects a

broad diversity in the entire study population. A median BMI of 30.2 classifies the patients as moderately obese, while 77% of the patients in the cohort were categorized as overweight or obese (BMI ≥25).

The most common presentations in the patients were dyspnea (89%), chest pain (29.2%), and syncope (12.4%), consistent with typical PE symptoms. Predictors of PE severity, such as right ventricular dysfunction, troponin and brain- natriuretic peptide levels, PESI and sPESI score were analyzed. Patients were categorized according to the ESC guidelines with 11.5% classified as low-risk, 80.9% as intermediate-risk, and 7.7% as high-risk PE patients.

The cohort included various comorbidities such as cancer, chronic lung disease, chronic heart failure, kidney disease, hypertension, coronary artery disease and others possibly affecting the coagulation cascade. The most predominant comorbidity in the cohort included hypertension (57.4%), cancer (20.6%), and chronic heart failure (14.8%). Mortality rates in the cohort increased over time, with 7.7% deaths within the first 30-days to 13.4% by 6-months, and 16.3% by 9-months. The increase in the mortality rate remained persistent through 12 months.

Table 20. Basic clinical characteristics, and laboratory parameters of the LUMC PERT Cohort.

Variables	*Counts*	*LUMC PERT Cohort*
		Median (IQR), N (%)
Patients	*209*	*---*
Demographics		
Age (years)	*209*	*63.0 (51.0-73.0)*
Male sex	*209*	*107 (51.2%)*
Weight (kg)	*209*	*87.8 (70.8-104.3)*
Body mass index	*209*	*30.2 (25.5-35.9)*
Clinical Presentation		
Systolic blood pressure (mmHg)	*209*	*116.0 (101.5-127.0)*
Heart rate (beats/minute)	*209*	*97.0 (82.0-110.0)*
Oxygen saturation (%)	*209*	*92.0 (90.0-95.0)*
Respiratory rate	*209*	*20.0 (18.0-24.5)*
Dyspnea	*209*	*186 (89.0%)*
Syncope	*209*	*26 (12.4%)*
Chest pain	*209*	*61 (29.2%)*
Predictors of PE Severity		
Right ventricular dysfunction	*169*	*0.9 (0.8-1.1)*
Troponin	*209*	*0.2 (0.1-0.2)*
Brain natriuretic peptide	*181*	*114.0 (40.0-385.0)*

PESI score	*209*	*107.0 (81.0-141.5)*
Risk Stratification		
Low-risk PE	*209*	*24 (11.5%)*
Intermediate-risk PE	*209*	*169 (80.9%)*
High-risk PE	*209*	*16 (7.7%)*
Comorbidities		
Active cancer	*209*	*43 (20.6%)*
Chronic heart failure	*209*	*37 (14.8%)*
Chronic lung disease	*209*	*24 (11.5%)*
Chronic kidney disease	*209*	*21 (10.0%)*
Hypertension	*209*	*120 (57.4%)*
Mortality		
30-day mortality	*209*	*16 (7.7%)*
3-months mortality	*209*	*28 (13.4%)*
6-months mortality	*209*	*34 (16.3%)*
Laboratory Parameters		
Hemoglobin (g/dl)	*209*	*12.4 (10.3-14.5)*
Neutrophils (x1000/mm³)	*209*	*6.9 (4.2-9.7)*
Lymphocytes (x,000/mm³)	*209*	*1.5 (0.9-2.1)*
Platelets (x1000/mm³)	*209*	*228.0 (165.5-320.5)*

5.3.1. Thrombin Generation Potential, and Biomarkers in PE Patients

Thrombin generation potential, and biomarker levels in PE patients compared to the healthy controls are presented in tables and figures. The data is presented as median (IQR), fold change, and significance level in table 21-22, while visual presentation among patients in the form of scatter plots is given in figure 16-17.

5.3.1.1. ***Thrombin Generation Potential***

In PE patients, significant variability was observed in thrombin generation potential as indicated by peak thrombin, ETP, and lag time (table 21). Thrombin and ETP levels showed a marked decrease in the levels compared to the controls. Conversely, lag time showed an increase among PE patients. The most pronounced decrease was noticed in thrombin levels by -0.56-fold compared to controls, while ETP levels were modestly decreased by -0.18-fold. Lag time exhibited a slight increase of 0.59-fold in PE patients.

Table 21. Comparison of thrombin generation potential in PE patients versus healthy controls.

Parameters	PE Samples		Controls		P value	Fold Change
	N	Median (IQR)	N	Median (IQR)		
Thrombin (nM)	209	61.3 (5.3-130.5)	50	138.4 (125.4-157.2)	<0.0001	-0.56
*ETP (nM*minutes)*	209	476.7 (119.2-738.3)	50	583.7 (529.8-658.3)	0.0126	-0.18
Lag time (minutes)	209	2.7 (2.0-3.7)	50	1.7 (1.7-2.0)	<0.0001	0.59

The data is also depicted in the scatter plot, which correspondingly showed a wide range in the levels across all the thrombin generation parameters in the patients (figure 16). Thrombin levels and ETP decreased while lag time increased. The graphical representation displays the distribution and variability in the levels for PE patients across all thrombin generation parameters studied reflecting the overall thrombin generation capacity in PE patients. In contrast to the control group that had a cluster of similar values, the PE patients had a wide range of values for ETP, peak thrombin, as well as lag time.

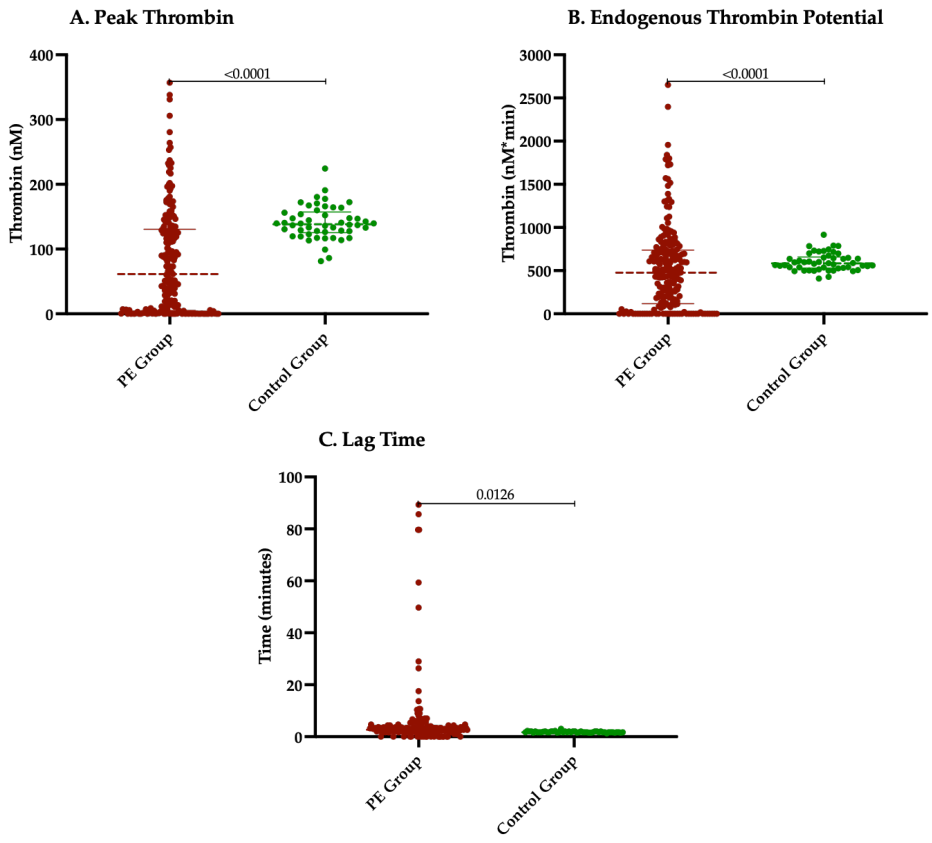


Figure 16. Scatter plot of peak thrombin, ETP, and lag time in PE patients versus healthy controls.

Points represent individual patient data with median and interquartile range (IQR). The biomarker levels between PE and control groups are compared by using Mann-Whitney U test, with a p value of <0.05 considered as the cutoff for significance.

*5.3.1.2. **Thrombin Generation Biomarkers***

Thrombin generation biomarkers including D-dimer, F1+2, TAT, and TF exhibited significant increases in PE patients compared to controls (table 22). In particular, D-dimer showed the most pronounced increase with a 48.86-fold elevation, followed by TAT and TF which showed a 3.89-fold and 1.48-fold increase, respectively. F1+2 demonstrated a modest increase of 0.85-fold. The increase in the levels highlights the increased thrombogenesis, along with enhanced coagulation processes (consumption of factors) and fibrinolysis in PE patients. Overall, these data reflect an elevated thrombotic and coagulation response.

Table 22. Comparison of thrombin generation biomarker in PE patients versus healthy controls.

Biomarkers	PE Samples		Controls		P value	Fold Change
	N	Median (IQR)	N	Median (IQR)		
D-dimer (ng/mL)	209	5400.0 (2550.6-10327.9)	50	108.3 (0.0-52.6)	<0.0001	48.86
F1+2 (pmol/L)	209	507.3 (286.6- 944.2)	50	274.9 (199.3-329.8)	<0.0001	0.85
TAT (μg/L)	209	13.7 (7.9-36.1)	50	2.8 (1.6-3.4)	<0.0001	3.89
TF (pg/mL)	198	111.7 (71.8-166.3)	50	45.1 (22.2-87.1)	<0.0001	1.48

The graphical presentation of the data in the scatter plot provides a comprehensive view of the variability in levels across all the thrombin generation biomarkers studied in the PE patients (figure 17). The scatter plot for D-dimer, F1+2, TAT, and TF illustrates the visual assessment of the distribution of individual patients, also the dispersion and central tendency of each biomarker, highlighting the heterogeneity in thrombin generation profiles among individuals with PE. Patients with PE demonstrated skewness in the data compared to the control with some high levels in D-dimer, F1+2, TAT, and TF levels.

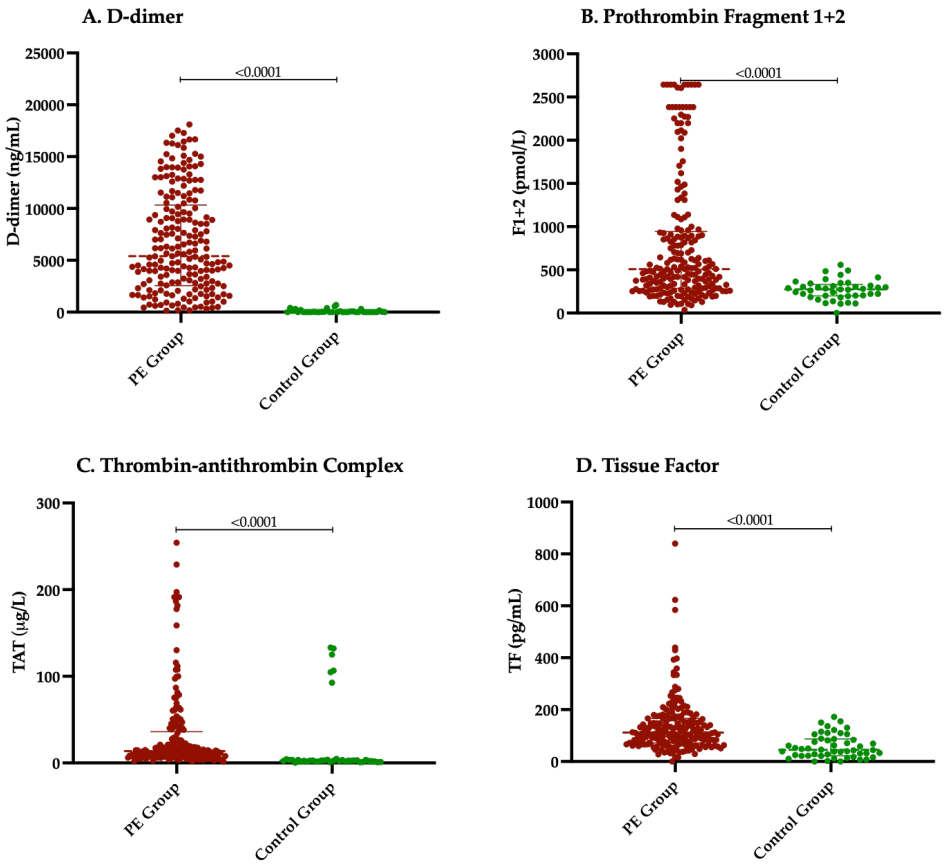


Figure 17. Scatter plot of D-dimer, F1+2, TAT, and TF levels in PE patients versus healthy controls.

Points represent individual patient data with median and interquartile range (IQR). The biomarker levels between PE and control groups are compared by using Mann-Whitney U test, with a p value of <0.05 considered as the cutoff for significance.

5.3.2. Blood Cellular Indices in PE Patients

Blood cellular indices demonstrated varying levels of increase in PE patients compared to the controls (table 23). NLR showed a marked increase with a 2.13-fold elevation, followed by SII with 1.64-fold and PLR with 0.33-fold elevation with reference to controls. The observed increase highlights the enhanced inflammatory and immune response, which would theoretically correspond with alterations in platelet and prothrombotic effects in PE patients.

Table 23. Comparison of blood cellular indices in PE patients versus healthy controls.

Cellular Indices	PE Samples		Controls		P value	Fold Change
	N	Median (IQR)	N	Median (IQR)		
NLR (x1000/mm³)	209	5.0 (2.7-7.6)	50	1.6 (1.2-2.0)	<0.0001	2.13
PLR (x1000/mm³)	209	174.4 (106.2-260.9)	50	131.6 (108.2-152.0)	0.0039	0.33
SII (x1000/mm³)	209	1175.3 (557.0-2042.7)	50	444.5 (311.0-537.5)	0.0004	1.64

The scatter plots for the ratios demonstrate the variability in PE patients compared to the controls (figure 18). The diagrammatic presentation of the patients for NLR, PLR and SII demonstrated a similar dispersion trend. Most of the patient's data were clustered with some high levels extending towards the upper limit which could be associated with disease severity and prognosis, while controls were mostly clustered.

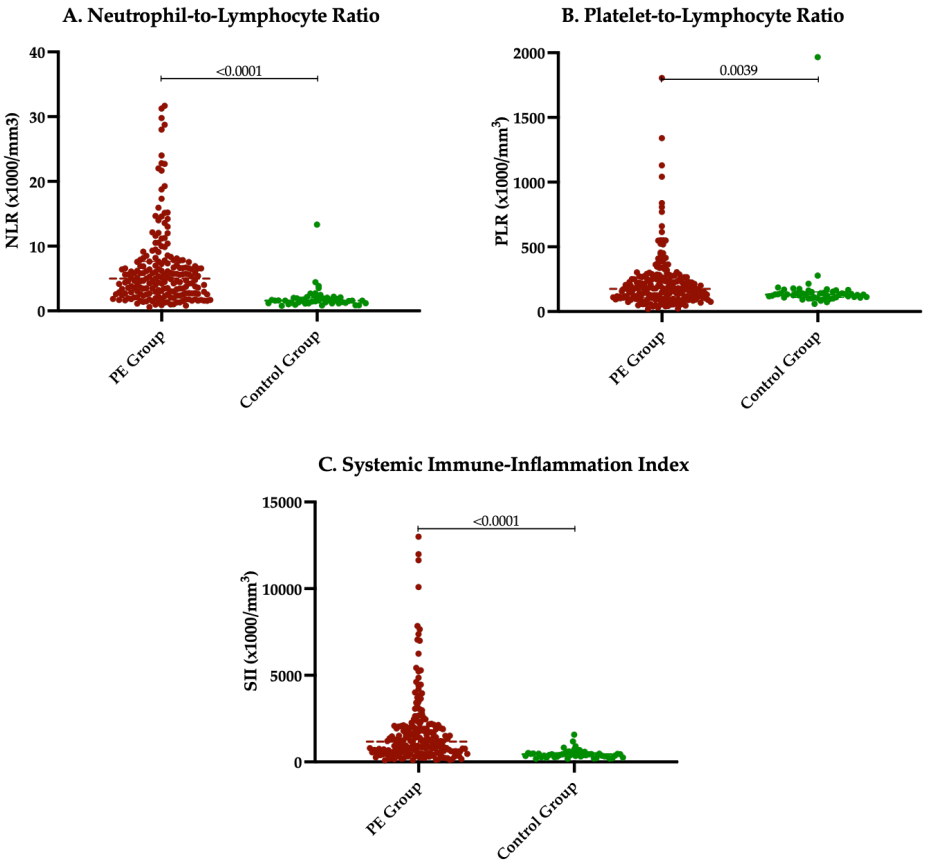


Figure 18. Scatter plot of NLR, PLR, and SII levels in PE patients versus healthy controls.

Points represent individual patient data with median and interquartile range (IQR). The biomarker levels between PE and control groups are compared by using Mann-Whitney U test, with a p value of <0.05 considered as the cutoff for significance.

5.3.3. Thrombin Generation Potential, and Biomarkers with Disease Severity

Thrombin generation potential and biomarker data was stratified according to ESC classifications of the PE patients. which is presented in tables and figures. The cohort of 209 PE patients was stratified into risk groups with 24 (11.5%) patients in the low-risk, 169 (80.9%) patients in the intermediate-risk, and 16 (7.7%) patients in the high-risk PE group. This stratification provides a detailed analysis of thrombin generation potential and biomarker levels across different risk categories highlighting thrombotic activity. The data is presented as a median (IQR) in table 24-25, while diagrammatic presentation among patients in the form of scatter plots is given in figure 19-20, along with the significance level.

5.3.3.1. Thrombin Generation Potential

Thrombin generation potential, characterized by thrombin levels and lag time exhibited variation across the PE severity (table 24). Thrombin levels decreased from 73.1 (44.0-144.4) nM in low-risk PE patients to 56.1 (4.7-131.4) nM in intermediate-risk PE patients. Conversely, thrombin levels increased to 65.0 (0.4-127.0) nM in high-risk PE patients. Lag time showed a modest change in levels across all groups. A consistent decreasing trend in ETP was observed with increasing PE severity: 590.0 (427.5-724.6) nM*min in low-risk PE, 453.1 (113.0-748.3) nM*min in intermediate-risk PE, and 370.5 (0.00-700.7) nM*min in high-risk PE. However, the variations among PE patients across disease severity was not statistically significant, which could be due to a disproportionate number of patients in each group.

Table 24. Comparison of thrombin generation potential in PE patients across different risk categories.

Parameters	Low-Risk PE		Intermediate-Risk PE		High-Risk PE	
	N	Median (IQR)	N	Median (IQR)	N	Median (IQR)
Thrombin (nM)	24	73.1 (44.0-144.4)	169	56.1 (4.7-131.4)	16	65.0 (0.4-127.0
ETP (nM*minutes)	24	590.0 (427.5-724.6)	169	453.1 (113.0-748.3)	16	370.5 (0.00-700.7)
Lag time (minutes)	24	2.8 (2.1-3.9)	169	2.7 (2.0-3.7)	16	2.7 (2.1-3.8)

The distribution pattern and variation among patients in the thrombin generation potential is presented in the form of scatter plots (figure 19). The scatter plots show the diversity in thrombin generation potential with wide dispersion in thrombin levels and ETP among patients, while lag time values were similarly clustered with delayed time in all patient groups.

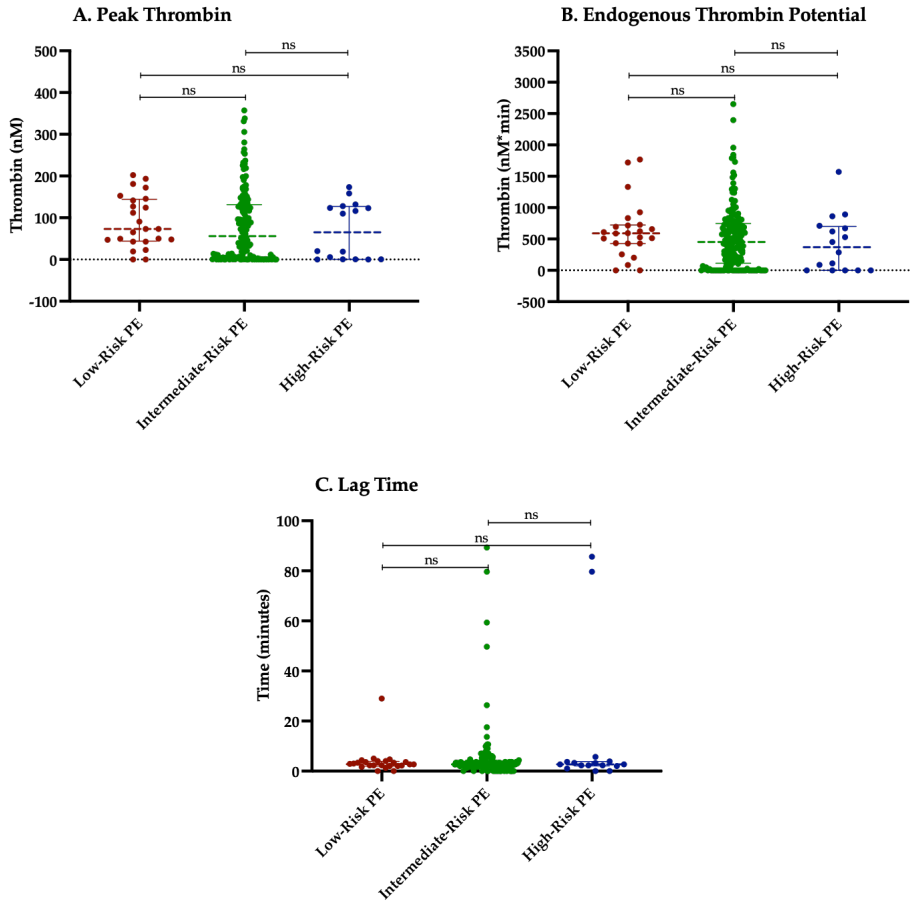


Figure 19. Scatter plot of peak thrombin, ETP, and lag time in PE patients across different risk categories.

Points represent individual patient data with median and interquartile range (IQR). The biomarker levels between PE severity groups are compared by using Mann-Whitney U test, with a p value of <0.05 considered as the cutoff for significance.

5.3.3.2. *Thrombin Generation Biomarkers*

Biomarker levels, including D-dimer, F1+2, and TAT generally increased with PE severity (table 25). This was reflected in the gradient of elevations across

low-risk, intermediate-risk, and high-risk PE groups. D-dimer levels significantly increased in high-risk PE patients with 13,338.2 (8581.2-15,976.6) ng/mL compared to 4407.6 (2319.0-7164.0) ng/mL for low-risk and 5135.4 (2169.3-10,100.5) ng/mL for intermediate-risk PE. The differences in D-dimer levels between low- and intermediate-risk PE were not significant. F1+2 also showed a consistent increase in levels with the severity of PE; however, the most significant increase was from low-risk PE 426.0 (268.1-580.8) pmol/L to high-risk PE 722.0 (411.4-1945.7) pmol/L. However, the difference between low-risk and intermediate-risk, as well as between intermediate-risk and high-risk PE was not statistically significant. TAT exhibited a similar trend to D-dimer, with significant increase in TAT levels for patients with high-risk PE 38.5 (15.0-59.1) μg/L compared to the low- and intermediate-risk PE with 11.5 (6.2-18.6) μg/L and 13.3 (7.9-36.1) μg/L, respectively. The change from low-risk to intermediate-risk PE was not significant. TF levels also increased with increase in the severity with 88.1 (55.6-134.6) pg/mL, 115.4 (71.8-173.6) pg/mL, and 117.3 (81.1-151.2) pg/mL, respectively; however, all the changes were not significant.

Table 25. Comparison of thrombin generation biomarker in PE patients across different risk categories.

Biomarkers	*Low-Risk PE*		*Intermediate-Risk PE*		*High-Risk PE*	
	N	*Median IQR)*	*N*	*Median (IQR)*	*N*	*Median (IQR)*
D-dimer (ng/mL)	*24*	*4407.6 (2319.0-716(4.0)*	*169*	*5135.4 (2169.3-10100.5)*	*16*	*13338.2 (8581.2-15976.6)*
F1+2 (pmol/L)	*24*	*426.0 (268.1-580.8)*	*169*	*507.3 (275.9-989.6)*	*16*	*722.0 (411.4-1945.7)*
TAT (μg/L)	*24*	*11.5 (6.2-18.6)*	*169*	*13.3 (7.9-36.1)*	*16*	*38.5 (15.0-59.1)*
TF (pg/mL)	*24*	*88.1 (55.6-134.6)*	*162*	*115.4 (71.8-173.6)*	*12*	*117.3 (81.1-151.2)*

The scatter plots represent the distribution pattern and variability of the thrombin generation biomarkers across the PE severity (figure 20). The scatter plots show the wide variations in biomarker levels with wide dispersion in D-dimer, F1+2 and TF among all groups of PE while TAT values were similarly clustered for all patient groups, but with more of a spread in values for the intermediate-risk PE.

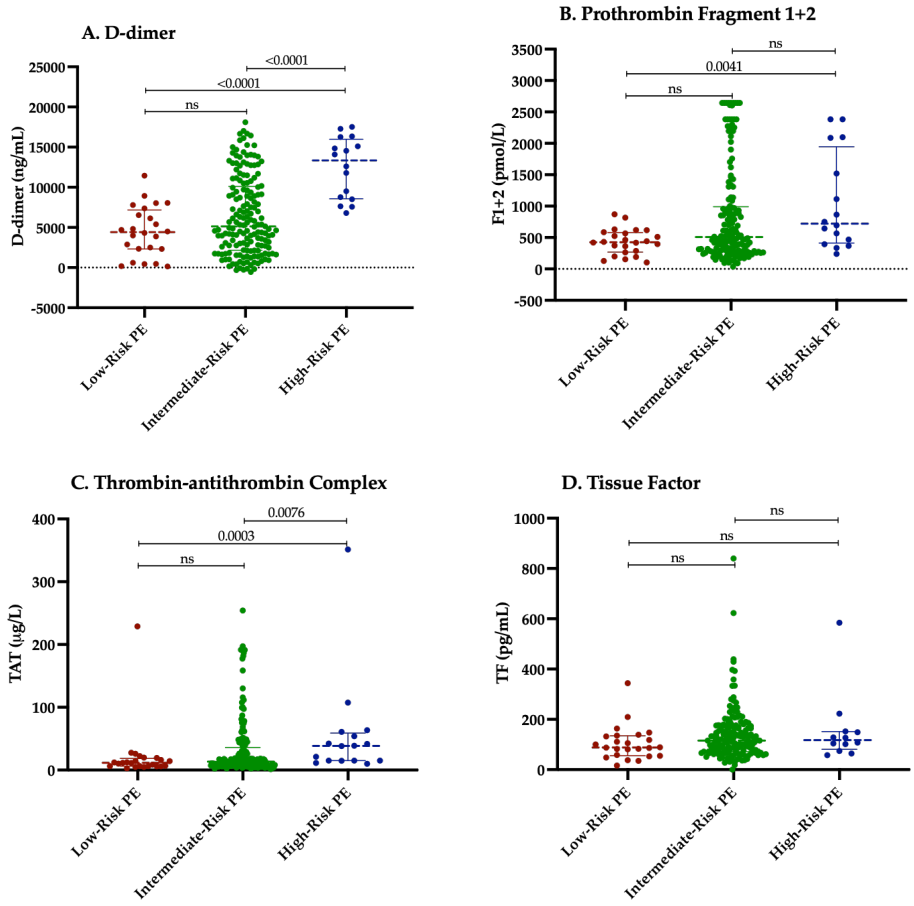


Figure 20. Scatter plot of D-dimer, F1+2, TAT, and TF levels in PE patients across different risk categories.

Points represent individual patient data with median and interquartile range (IQR). The biomarker levels between PE severity groups are compared by using Mann-Whitney U test, with a p value of <0.05 considered as the cutoff for significance.

5.3.4. Blood Cellular Indices with Disease Severity

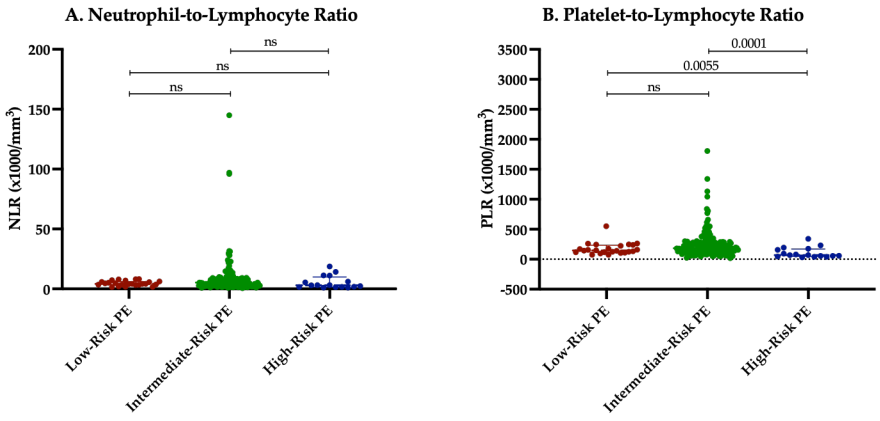
Blood cellular indices varied in relation to risk severity, with NLR and PLR levels increasing in intermediate-risk PE patients but decreasing in those with high-risk PE (table 26). The changes in the NLR levels were not significant; however, PLR showed a significant decrease in high-risk PE with 68.4 (47.7-170.8) x1000/mm^3 compared to low-risk 145.1 (117.2-234.7) x1000/mm^3 and intermediate-risk 183.3 (110.4-273.2) x1000/mm^3 PE. SII levels inversely correlated with the escalation of PE severity: 1233.1 (607.5-1653.0) x1000/mm^3 with low-risk patients, 1188.6 (600.4-

2164.5) x1000/mm³ with intermediate-risk patients and 545.3 (250.5-1654.7) x1000/mm³ with high-risk patients, with the decrease in the levels from low-risk to high-risk being statistically significant.

Table 26. Comparison of blood cellular indices in PE patients across different risk categories.

Cellular Indices	*Low-Risk PE*		*Intermediate-Risk PE*		*High-Risk PE*	
	N	*Median (IQR)*	*N*	*Median (IQR)*	*N*	*Median (IQR)*
NLR (x1000/mm³)	*24*	*4.2 (3.5-6.1)*	*169*	*5.3 (2.7-7.8)*	*16*	*3.1 (1.6-10.0)*
PLR (x1000/mm³)	*24*	*145.1 (117.2-234.7)*	*169*	*183.3 (110.4-273.2)*	*16*	*68.4 (47.7-170.8)*
SII (x1000/mm³)	*24*	*1233.1 (607.5-1653.0)*	*169*	*1188.6 (600.4-2164.5)*	*16*	*545.3 (250.5-1654.7)*

The scatter plots depict the distribution pattern of blood cellular indices across different PE risk groups (figure 21). They reveal substantial variability in biomarker levels, with significant dispersion observed in D-dimer and F1+2 across all PE groups. NLR, PLR and SII levels among patients across the severity were mostly clustered, while patients in the intermediate-risk PE showed some higher levels for all the cellular indices studied.



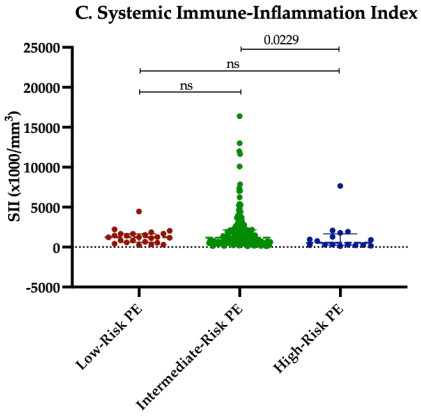


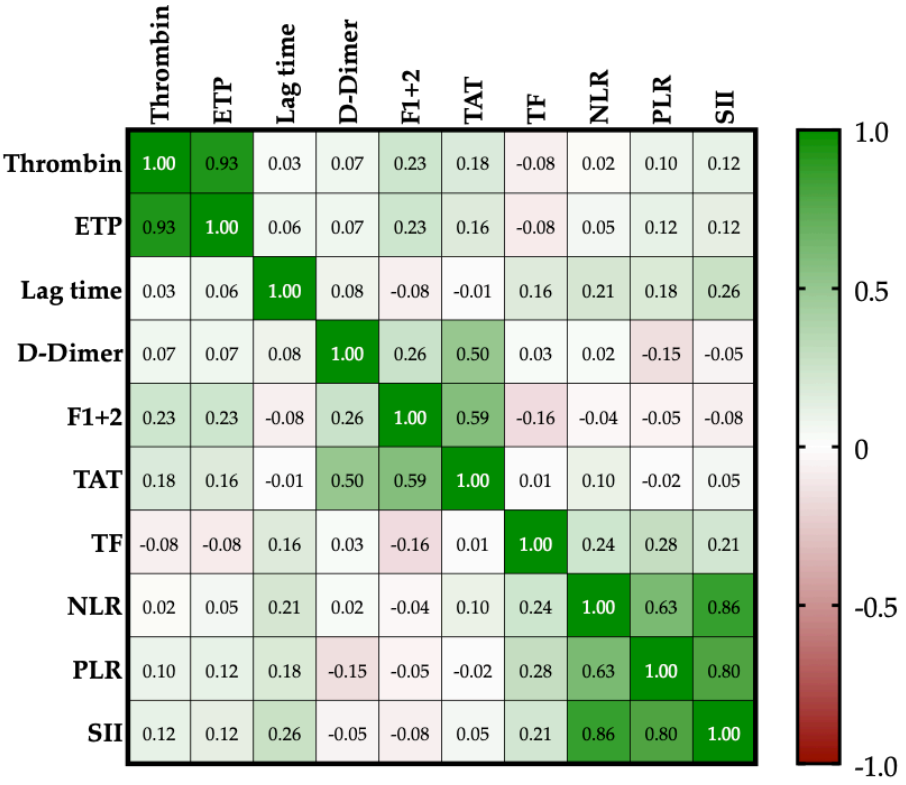
Figure 21. Scatter plot of NLR, PLR, and SII levels in PE patients across different risk categories.

Points represent individual patient data with median and interquartile range (IQR). The biomarker levels between PE severity groups are compared by using Mann-Whitney U test, with a p value of <0.05 considered as the cutoff for significance.

5.3.5. Relationship between Thrombin Generation Potential, and Biomarkers with Cellular Indices

Figure 22 highlights the varying degrees of correlation between thrombin generation potential, biomarkers, and blood cellular indices. Thrombin levels strongly correlated with ETP (r: 0.93; p value: <0.0001) and showed some correlation with F1+2 (r: 0.23; p value: 0.0007). The correlation of thrombin levels with F1+2 (r: 0.23; p value: 0.0007) is logical as F1+2 is released when thrombin is produced. Lag time correlated with SII (r: 0.26; p value: <0.0001) highlighting the association between delayed thrombin generation and systemic inflammation. D-dimer showed moderate correlations with F1+2 (r: 0.26; p value: 0.0002), and TAT (r: 0.50; p value: <0.0001), F1+2 also showed moderate correlation with TAT (r: 0.59; p value: <0.0001) demonstrating an interconnection between thrombin generation (reflected in F1+2 levels) and thrombin activity as generated thrombin forms a complex with AT . TF correlated with NLR (r: 0.24; p value: 0.001), PLR (r: 0.28; p value: <0.0001), and SII (r: 0.21; p value: 0.003) suggests TF may signal systemic inflammation, revealing an interplay between inflammation and the coagulation processes. For the blood cellular indices, NLR showed a moderate to strong correlation with PLR (r: 0.63; p value: <0.0001), and SII (r: 0.86; p value: <0.0001), PLR also showed a strong correlation with SII (r: 0.80; p value: <0.0001) delineating

the interrelationship between the cellular indices and reflecting the inflammatory and immune status of patients.



	Thrombin	ETP	Lag time	D-Dimer	F1+2	TAT	TF	NLR	PLR	SII
Thrombin	1.00	0.93	0.03	0.07	0.23	0.18	-0.08	0.02	0.10	0.12
ETP	0.93	1.00	0.06	0.07	0.23	0.16	-0.08	0.05	0.12	0.12
Lag time	0.03	0.06	1.00	0.08	-0.08	-0.01	0.16	0.21	0.18	0.26
D-Dimer	0.07	0.07	0.08	1.00	0.26	0.50	0.03	0.02	-0.15	-0.05
F1+2	0.23	0.23	-0.08	0.26	1.00	0.59	-0.16	-0.04	-0.05	-0.08
TAT	0.18	0.16	-0.01	0.50	0.59	1.00	0.01	0.10	-0.02	0.05
TF	-0.08	-0.08	0.16	0.03	-0.16	0.01	1.00	0.24	0.28	0.21
NLR	0.02	0.05	0.21	0.02	-0.04	0.10	0.24	1.00	0.63	0.86
PLR	0.10	0.12	0.18	-0.15	-0.05	-0.02	0.28	0.63	1.00	0.80
SII	0.12	0.12	0.26	-0.05	-0.08	0.05	0.21	0.86	0.80	1.00

Figure 22. Spearman correlation matrix illustrating the relationships between thrombin generation potential, biomarkers, and cellular indices in patients with PE.

Correlation analysis displays the relationships among various biomarkers and cellular indices. Positive correlations are shown in green color indicating a direct association. while the negative correlations are depicted in maroon color representing an inverse association. The strength of these correlations is illustrated by the intensity of the color with darker shades denoting stronger correlations. Correlations with a p-value <0.05 considered as statistically significant.

The markers of severity including right ventricular dysfunction, troponin levels, brain-natriuretic peptide levels, and PESI score were significantly elevated while systolic blood pressure was significantly reduced across the severity group (appendix). Mortality rates also differed by risk category, with low-risk PE showing a 4.2% mortality rate extending from 3-months to 12-months. Intermediate-risk PE mortality increased from 7.7% at 30-days to 14.2% at 3-months and 17.8% at 6-

months, persisting through 12 months. High-risk PE maintained a constant mortality rate of 18.8% from 30 days to 12 months.

5.4. HEMOSTATIC ACTIVATION, AND THROMBOINFLAMMATORY BIOMARKER PROFILING IN ACUTE PE PATIENTS

Plasma samples were collected from PE patients involved in the PERT project adhering to the protocols outlined in the Materials and Methods section of this dissertation. Samples were collected from Loyola University Medical Center and Gottlieb Memorial Hospital under IRB-approved protocols (appendix 3).

This was a probe study where 500 patients were randomly used to assess biomarkers of endothelial dysregulation, platelet activation, coagulation factor, fibrinolysis, inflammatory process, and blood cellular indices in patients with PE. Table 27 details the basic demographic information, co-morbidities, clinical presentation, laboratory parameters, PE risk stratification, and mortality within the LUMC PERT cohort.

The cohort consisted of 209 patients, with almost equal distribution between males and females (52.1% males vs. 48.9% female). The median of 64 years indicates a predominantly middle-aged population; the broad range of patients spanning from 53 to 73 years reflects the diversity in the population. A median BMI of 30 classified the patients as moderately obese, while 74% of the patients in the cohort categorized as overweight or obese (BMI ≥25).

The most common symptoms were dyspnea (88.6%), chest pain (30.0%), and syncope (12.0%) consistent with typical PE symptoms. Predictors of the PE severity such as right ventricular dysfunction, troponin and brain- natriuretic peptide levels, PESI and sPESI score were also analyzed. Patients were categorized according to the ESC guidelines with 10.4% as low-risk, 80.9% as intermediate-risk, and 8.8% as high-risk PE patients.

The cohort included various comorbidities including cancer, chronic lung disease, chronic heart failure, kidney disease, hypertension, coronary artery disease and others possibly affecting the coagulation cascade. The most predominant comorbidity in the cohort included hypertension (57.0%), cancer (23.7%), chronic heart failure (12.8%), chronic lung disease (12.2%) and chronic kidney disease (10.8%). Mortality rates in the cohort increased overtime, with 9.7% of patients died

within the first 30-days to 14.8% by 3-months, and 18.7% by 6-months, persisted to 1-year.

Table 27. Basic clinical characteristics, and laboratory parameters in the LUMC PERT cohort.

Variables	*Counts*	*LUMC PERT Cohort*
		Median (IQR), N (%)
Patients	*500*	*---*
Demographics		
Age (years)	*493*	*64.0 (53.0-74.0)*
Male sex	*493*	*257 (52.1%)*
Weight (kg)	*492*	*87.5 (70.8-104.4)*
Body mass index	*492*	*29.7 (25.0-35.6)*
Clinical Presentation		
Systolic blood pressure (mmHg)	*493*	*117.0 (103.0-130.0)*
Heart rate (beats/minute)	*493*	*97.0 (85.0-112.0)*
Oxygen saturation (%)	*493*	*92.0 (89.0-95.0)*
Respiratory rate	*493*	*21.0 (18.0-26.0)*
Dyspnea	*493*	*437 (88.6%)*
Syncope	*493*	*59 (12.0%)*
Chest pain	*493*	*148 (30.0%)*
Predictors of PE Severity		
RV/LV ratio	*339*	*0.93 (0.80-1.17)*
Troponin	*493*	*0.05 (0.02-0.27)*
Brain natriuretic peptide	*449*	*129 (47-3,612)*
PESI score	*493*	*111 (82-148)*
Risk Stratification		
Low-risk PE	*491*	*51 (10.4%)*
Intermediate-risk PE	*491*	*397 (80.9%)*
High-risk PE	*491*	*44 (8.8%)*
Comorbidities		
Active cancer	*493*	*117 (23.7%)*
Chronic heart failure	*493*	*63 (12.8%)*
Chronic lung disease	*493*	*60 (12.2%)*
Chronic kidney disease	*493*	*53 (10.8%)*
Hypertension	*493*	*281 (57.0%)*
Mortality		
30-day mortality	*493*	*48 (9.7%)*
3-month mortality	*493*	*73 (14.8%)*
6-month mortality	*493*	*92 (18.7%)*
Laboratory Parameters		
Hemoglobin (g/dl)	*493*	*12.3 (10.1-13.9)*
Neutrophils (x1,000/mm^3)	*493*	*7.1 (4.7-9.8)*
Lymphocytes (x1,000/mm^3)	*493*	*1.4 (0.8-2.0)*
Platelets (x1,000/mm^3)	*493*	*228.0 (166.5-302.5)*

5.4.1. Biomarker Levels in PE Patients

The comparison of endothelial dysregulation, platelet activation, coagulation factors, marker of fibrinolysis, inflammatory processes, and blood cellular indices between patients with PE and controls are presented using median (IQR), fold changes and significance levels. Patients with PE showed significantly higher levels of biomarkers associated with endothelial dysfunction, platelet activation, coagulation, fibrinolysis, inflammation, and cellular indices compared to the controls, as detailed in Tables 28-32 and illustrated in the figure 23-27.

5.4.1.1. Endothelial Dysregulation Biomarkers

Markers of endothelial dysregulation including vWF, E-selectin, and TF were assessed in this thesis and compared to the controls (table 28). Both vWF and E-selectin levels were increased in patients with PE relative to controls with vWF increasing by 0.36-fold, E-selectin increasing by 0.09-fold, and TF increasing by 1.61-fold. The increase in vWF and TF levels were statistically significant, but the increase in E-selectins levels was not significant. These data provided insight into the critical role of endothelial dysfunction in the pathogenesis of PE. The marked increase in vWF highlights it as a distinguished marker of endothelial dysfunction in PE, reflecting a more pronounced or clinically relevant alteration in endothelial function. The increase in TF suggests the hypercoagulable state in PE.

Table 28. Comparison of endothelial dysregulation biomarkers in PE patients versus healthy controls.

Biomarkers	PE Samples		Controls		P value	Fold Change
	N	Median (IQR)	N	Median (IQR)		
vWF (%)	494	139.7 (128.4-147.4)	50	102.5 (78.7-115.0)	<0.0001	0.36
E-selectin (ng/mL)	470	31.6 (19.2-51.9)	50	29.0 (19.6-43.7)	NS	0.09
TF (pg/mL)	381	117.5 (75.2-171.4)	50	45.1 (22.2-87.1)	<0.0001	1.61

The scatter plots show the variability in the vWF, E-selectin, and TF levels among PE patients (figure 23). The scatter plot for all studied endothelial markers showed a skewed distribution. vWF levels for most patients clustered around a central range with values spread across both the upper and lower limits compared to the control that had more clustering. E-selectin and TF levels varied across both upper and lower limits in the PE group. The spread of values could be due to the multifaceted endothelial activation in PE.

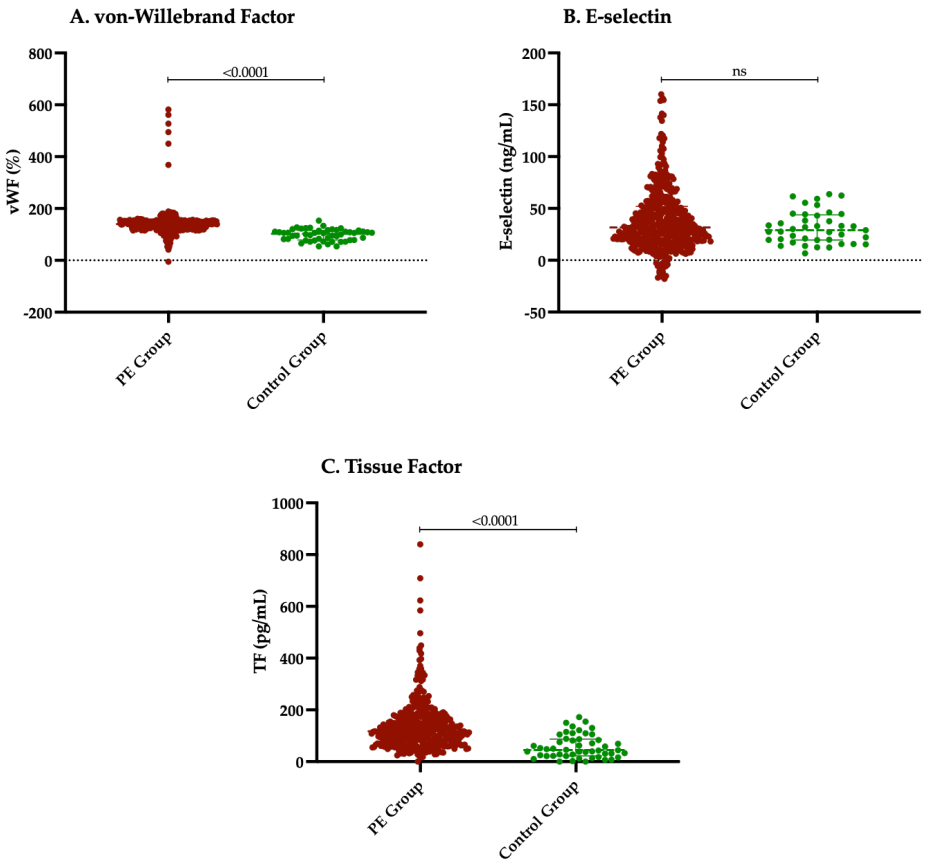


Figure 23. Scatter plot of vWF, E-selectin, and TF levels in PE patients versus healthy controls.

Points represent individual patient data with median and interquartile range (IQR). The biomarker levels between PE and control groups are compared by using Mann-Whitney U test, with a p value of <0.05 considered as the cutoff for significance.

5.4.1.2. *Platelet Activation Biomarker*

The platelet activation marker P-selectin was significantly higher in PE patients compared to the controls (table 29). P-selectin showed a 2.62-fold increase. The elevated level of P-selectin highlights an enhanced platelet response, indicating that platelet activation significantly contributes to thrombotic processes and most likely plays a role in the pathophysiology of PE.

Table 29. Comparison of platelet activation biomarker in PE patients versus healthy controls.

Biomarkers	PE Samples		Controls		P value	Fold Change
	N	Median (IQR)	N	Median (IQR)		
P-selectin (ng/mL)	494	91.6 (53.7-155.2)	50	25.3 (5.4-47.0)	<0.0001	2.62

The scatter plot for P-selection shows significant variability among patients compared to the controls (figure 24) with most of the values at the upper limit reflecting the disease severity or effect of therapeutics.

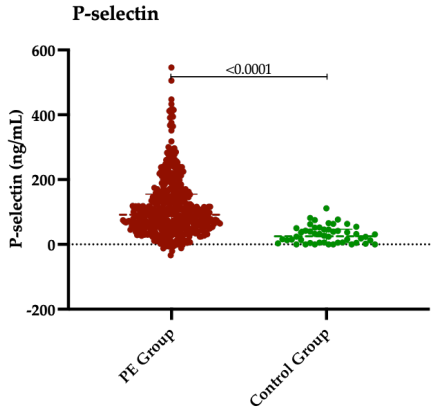


Figure 24. Scatter plot of P-selectin levels in PE patients versus healthy controls.

Points represent individual patient data with median and interquartile range (IQR). The biomarker levels between PE and control groups are compared by using Mann-Whitney U test, with a p value of <0.05 considered as the cutoff for significance.

5.4.1.3. *Coagulation Factors*

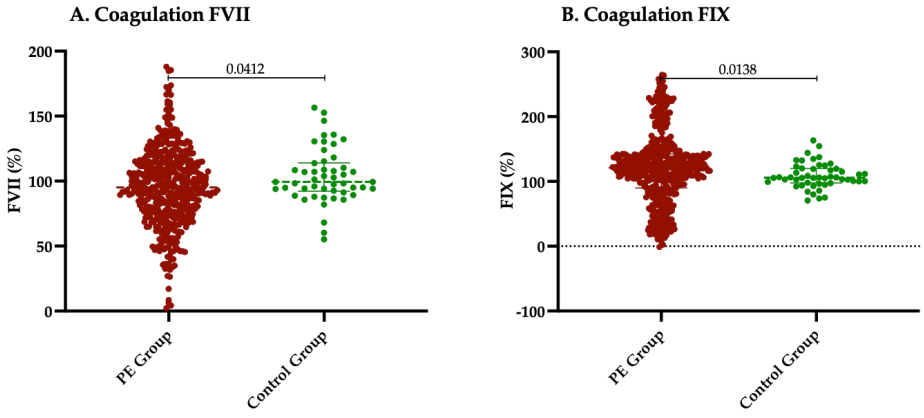
Coagulation factors including FVII, FIX, FX, and FXIII-A were measured by immunoassay and compared to controls (table 30). Varying levels of increase and decrease were noted in the factor levels. FIX showed a significant 0.15-fold increase in the levels, while FX levels increased with a 0.07-fold change compared to controls. Conversely, FVII and FXIII-A levels significantly decreased -0.04- and -0.16-fold, respectively. Profiling of coagulation factors provided new insights into the hypercoagulable state associated with PE. The perturbation in the clotting factor levels may also contribute to increased thrombogenesis PE. Decrease in FXIII-A reflects the crosslinking of fibrin, and the formation of stable clot leads to

the consumption of FXIII-A. FIX and FX may be upregulated due to the hypercoagulable state stimulation

Table 30. Comparison of coagulation factors in PE patients versus healthy controls.

Biomarkers	*PE Samples*		*Controls*		*P value*	*Fold Change*
	N	*Median (IQR)*	*N*	*Median (IQR)*		
FVII (%)	*488*	*95.2 (77.3-114.2)*	*50*	*99.4 (92.3-114.1)*	*0.0412*	*-0.04*
FIX (%)	*488*	*121.2 (90.1-142.5)*	*50*	*105.8 (97.8-119.7)*	*0.0138*	*0.15*
FX (%)	*488*	*95.4 (81.1-109.6)*	*50*	*89.1 (84.3-95.5)*	*0.0400*	*0.07*
FXIII-A (%)	*500*	*74.7 (55.3-94.9)*	*50*	*88.8 (75.1-101.7)*	*<0.0001*	*-0.16*

The graphical presentation of coagulation FVII, FIX, FX and FXIII-A in PE patients versus controls are also presented in the form of scatter plots (figure 25). PE patients demonstrated wide variability in levels which ranged from extremely high to very low levels. In contrast, controls also showed some variations among individuals; however, most cluster together with a less extreme range. This underscores the coagulation activation in PE patients.



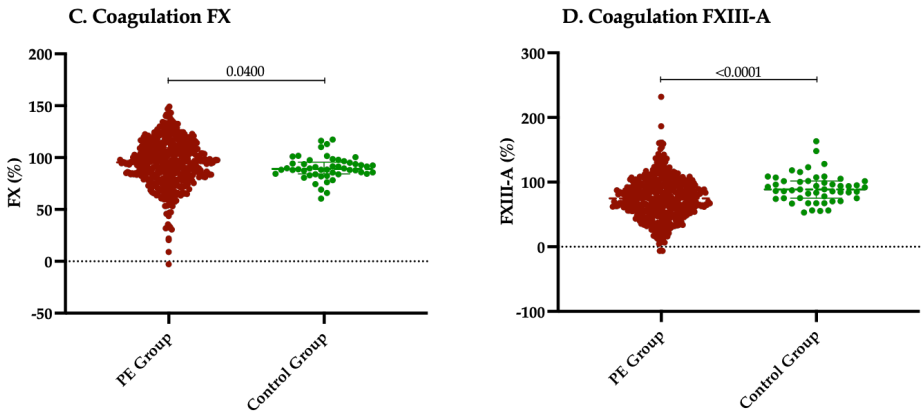


Figure 25. Scatter plot of coagulation FVII, FIX, FX, and FXIII-A levels in PE patients versus healthy controls.

Points represent individual patient data with median and interquartile range (IQR). The biomarker levels between PE and control groups are compared by using Mann-Whitney U test, with a p value of<0.05 considered as the cutoff for significance.

5.4.1.4. *Fibrinolysis Biomarkers*

Markers of fibrinolysis, including tPA, PAI-1, and D-dimer levels, exhibited significant increases in patients with PE compared to controls, except for TAFI (table 31). D-dimer showed the most substantial increase with a 48.97-fold elevation, followed by tPA and PAI-1, which increased by 1.71-fold and 1.08-fold, respectively. TAFI levels remained unchanged. The comprehensive profiling of fibrinolytic markers, especially the significant elevation in D-dimer, highlights a pronounced increase in fibrin formation and fibrin degradation. Elevation of tPA and PAI-1 levels demonstrated enhanced fibrinolysis activity judged by the increase in tPA that was more than the increase of its inhibitor PAI-1. Overall, these data provide a deeper understanding of the fibrinolytic activity in acute PE.

Table 31. Comparison of fibrinolytic biomarkers in PE patients versus healthy controls.

Biomarkers	*PE Samples*		*Controls*		*P value*	*Fold Change*
	N	*Median (IQR)*	*N*	*Median (IQR)*		
tPA (ng/mL)	*499*	*11.4 (7.2-18.1)*	*50*	*4.2 (1.4-6.3)*	*<0.0001*	*1.71*
PAI-1 (ng/mL)	*500*	*49.3 (34.4-66.9)*	*50*	*23.7 (8.3-30.5)*	*<0.0001*	*1.08*
TAFI (%)	*486*	*101.5 (92.1-112.0)*	*50*	*101.5 (93.7-106.2)*	*NS*	*0.00*
D-dimer (ng/mL)	*500*	*5412.6 (2820.1-10026.0)*	*50*	*108.3 (0.0-52.3)*	*<0.0001*	*48.97*

Biomarker levels among individual patients and controls are presented in the scatter plots (figure 26). The scatter plots for all the biomarkers including tPA, PAI-1, TAFI and D-dimer showed variability in the PE patients with skewness in both lower and higher levels. Controls samples also showed variation in PAI-1 (which is an acute phase reacting substance), and TAFI levels but to a lesser extent. The observed variation could be due to the complex nature of fibrinolysis process in PE and suggests its impact on the hemostatic mechanism may vary according to the extent of disease severity.

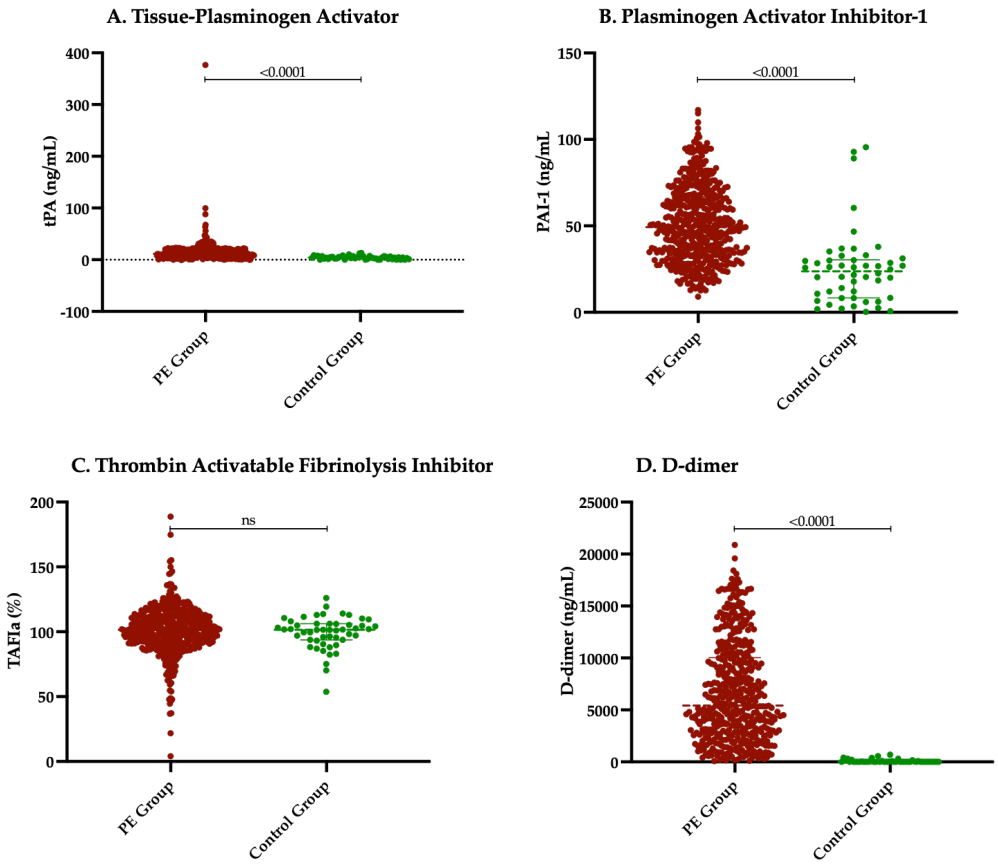


Figure 26. Scatter plot of tPA, PAI-1, TAFI, and D-dimer levels in PE patients versus healthy controls.

Points represent individual patient data with median and interquartile range (IQR). The biomarker levels between PE and control groups are compared by using Mann-Whitney U test, with a p value of <0.05 considered as the cutoff for significance.

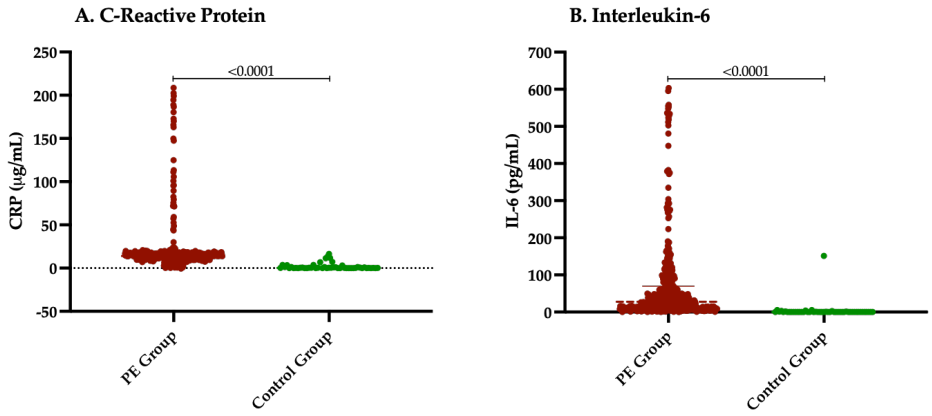
5.4.1.5. *Inflammatory Biomarkers*

Inflammatory markers including CRP, IL6 and TNF-α were upregulated in PE patients compared to controls (table 32). Of note, CRP showed the most pronounced increase in PE patients with a 45.33-fold compared to controls. IL-6 levels were increased with a 17.33-fold change, while TNF-α showed a modest increase with a 1.0-fold change. Increase in the inflammatory markers reinforces the central role of inflammation in PE pathogenesis, indicating that inflammatory processes are a key component of the disease mechanism.

Table 32. Comparison of inflammatory biomarkers in PE patients versus healthy controls.

Biomarkers	*PE Samples*		*Controls*		*P value*	*Fold Change*
	N	*Median (IQR)*	*N*	*Median (IQR)*		
CRP (μg/mL)	*500*	*13.9 (12.3-16.2)*	*50*	*0.3 (0.01-1.2)*	*<0.0001*	*45.33*
IL-6 (pg/mL)	*454*	*27.5 (10.5-69.9)*	*50*	*1.5 (0.0-0.3)*	*<0.0001*	*17.33*
TNF-α (pg/mL)	*475*	*1.2 (0.8-1.9)*	*50*	*0.6 (0.5-0.9)*	*<0.0001*	*1.0*

The graphical presentation for CRP, IL-6 and TNF-α among individual patients and controls are presented in the form of scatter plot (figure 27). The scatter plots for each of the inflammatory markers showed skewed distribution in PE patients especially with higher values reflecting an increased immune response.



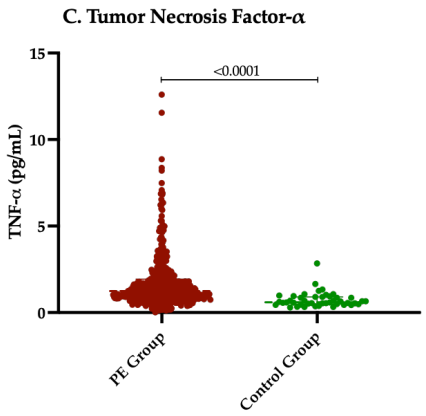


Figure 27. Scatter plot of CRP, IL-6, and TNF-α levels in PE patients versus healthy controls.

Points represent individual patient data with median and interquartile range (IQR). The biomarker levels between PE and control groups are compared by using Mann-Whitney U test, with a p value of <0.05 considered as the cutoff for significance.

*5.4.1.6. **Blood Cellular Indices***

Blood cellular indices such as NLR, PLR and SII were calculated by using the complete blood count data (table 33). Patients with PE exhibited elevated levels of NLR, PLR and SII levels compared to the controls, The most pronounced increase was noted for NLR with a 2.19-fold, followed by SII with a 1.69-fold, and PLR which showed modest increase with a 0.36-fold. The observed increase underscores the amplified inflammatory and immune response in patients with PE. This is consistent with similar alterations in platelet activity and prothrombotic effects.

Table 33. Comparison of blood cellular indices in PE patients versus healthy controls.

Cellular Indices	*PE Samples*		*Controls*		*P value*	*Fold Change*
	N	*Median (IQR)*	*N*	*Median (IQR)*		
NLR (x1000/mm³)	*493*	*5.1 (2.7-10.0)*	*50*	*1.6 (1.2-2.0)*	*<0.0001*	*2.19*
PLR (x1000/mm³)	*493*	*178.8 (106.8-277.2)*	*50*	*131.3 (108.2-149.7)*	*0.0003*	*0.36*
SII (x1000/mm³)	*493*	*1195.0 (595.1-2279.0)*	*50*	*444.5 (311.0-537.5)*	*<0.0001*	*1.69*

The scatter plots for the NLR, PLR, and SII showed a distribution pattern in PE patients compared to a tight cluster in the controls (figure 28). PE patients showed a wide variation in the levels with some very high levels. This reflects

marked activation of the immune response and intensified inflammation in PE patients highlighting the variability in disease severity.

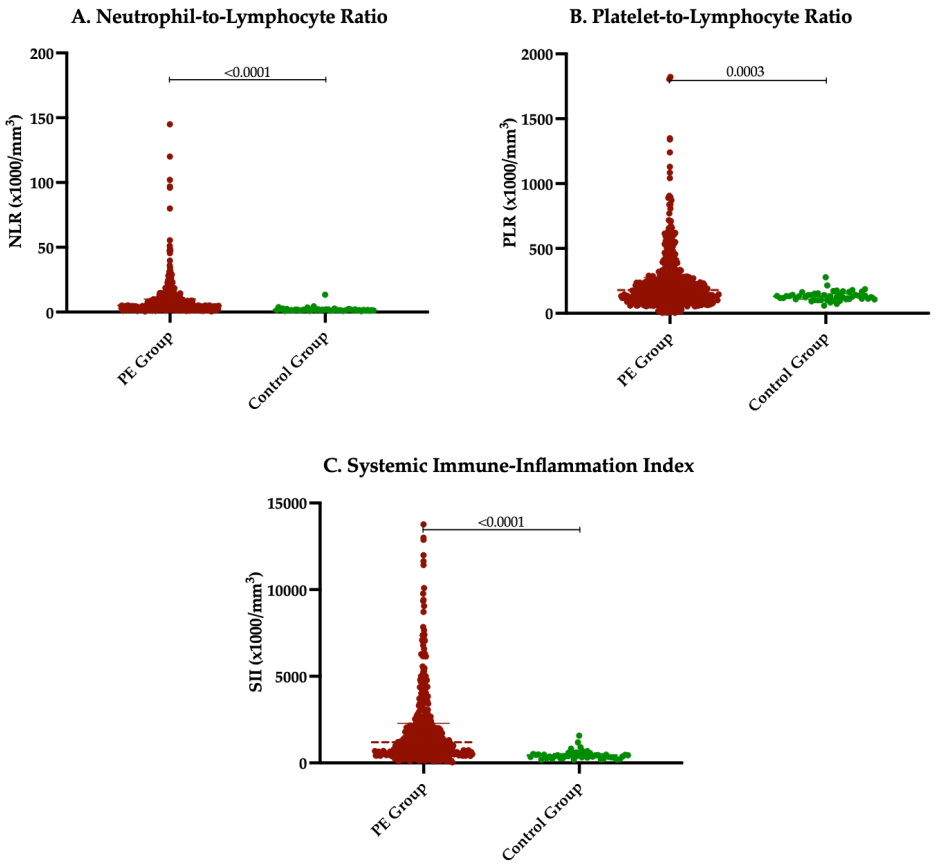


Figure 28. Scatter plot of NLR, PLR, and SII levels in PE patients versus healthy controls.

Points represent individual patient data with median and interquartile range (IQR). The biomarker levels between PE and control groups are compared by using Mann-Whitney U test, with a p value of <0.05 considered as the cutoff for significance.

5.4.2. Stratification of Biomarkers with Disease Severity

Biomarker levels along with cellular indices were also analyzed/stratified according to the ESC classification of PE is presented in tables and figures. In the cohort of 500 PE patients, 51 (10.4%) patients were in the low-risk, 397 (80.9%) patients in the intermediate-risk, and 44 (8.8%) in the high-risk PE group. This stratification provides a detailed analysis of the hemostatic and thrombo-

inflammatory biomarker and blood cellular indices levels across different risk categories and helps to identify individuals with varying levels of thrombotic activity. The data is presented as median (IQR) in table 34-38, while diagrammatic presentation among patients in the form of scatter plots are given in figure 29-33, along with significance level.

*5.4.2.1. **Endothelial Dysregulation Biomarkers***

Levels of markers of endothelial dysregulation such as vWF, E-selectin, and TF exhibited variations with PE severity (Table 34). vWF showed a consistent increase in the levels from low-risk to high-risk PE. The levels of vWF consistently increased from low-risk to high-risk PE, with significant increase from low-risk [131.5 (122.8-138.8) %] to intermediate-risk [140.4 (129.3-147.6) %] and high-risk PE [142.2 (121.7-152.5) %]. The change from intermediate- to high-risk PE was minimal. E-selectin levels showed a decrease in levels from low-risk PE to intermediate-risk and high-risk PE, also from intermediate-risk to high-risk PE. The levels of TF also increase with increase in the PE severity as 100.2 (58.6-135.4) pg/mL for low-risk PE 120.5 (76.2-176.2) pg/mL for intermediate-risk PE, and 121.8 (84.4-157.1) pg/mL for high-risk PE; however, the increase from low-risk to intermediate-risk PE was the only significant increase. This suggests that endothelium damage (as indicated by vWF levels) and coagulation activation (as indicated by TF) are intensified with increasing severity of PE.

Table 34. Comparison of endothelial dysregulation markers in PE patients across different risk categories.

Biomarkers	*Low-Risk PE*		*Intermediate-Risk PE*		*High-Risk PE*	
	N	*Median IQR)*	*N*	*Median (IQR)*	*N*	*Median (IQR)*
vWF (%)	*50*	*131.5 (122.8-138.8)*	*392*	*140.4 (129.3-147.6)*	*43*	*142.2 (121.7-152.5)*
E-selectin (ng/mL)	*50*	*36.6 (19.5-58.4)*	*373*	*31.2 (18.9-49.1)*	*40*	*31.8 (21.7-60.7)*
TF (pg/mL)	*47*	*100.2 (58.6-135.4)*	*304*	*120.5 (76.2-176.2)*	*28*	*121.8 (84.4-157.1)*

The scatter plots illustrate the distribution and variation of endothelial dysregulation markers across different severities of PE (figure 29). The scatter plot for the vWF reveals that most patients' levels are concentrated towards normal levels with only a few elevated levels. In contrast, the scatter plot for E-selectin and TF display a broad range of levels with considerable dispersion. The trend generally skews toward the upper limit across all PE severity groups, with

extremely elevated levels present in each group. This reflects the overall complexity of endothelial dysregulation along with a hypercoagulable state in PE patients, linked with the disease severity.

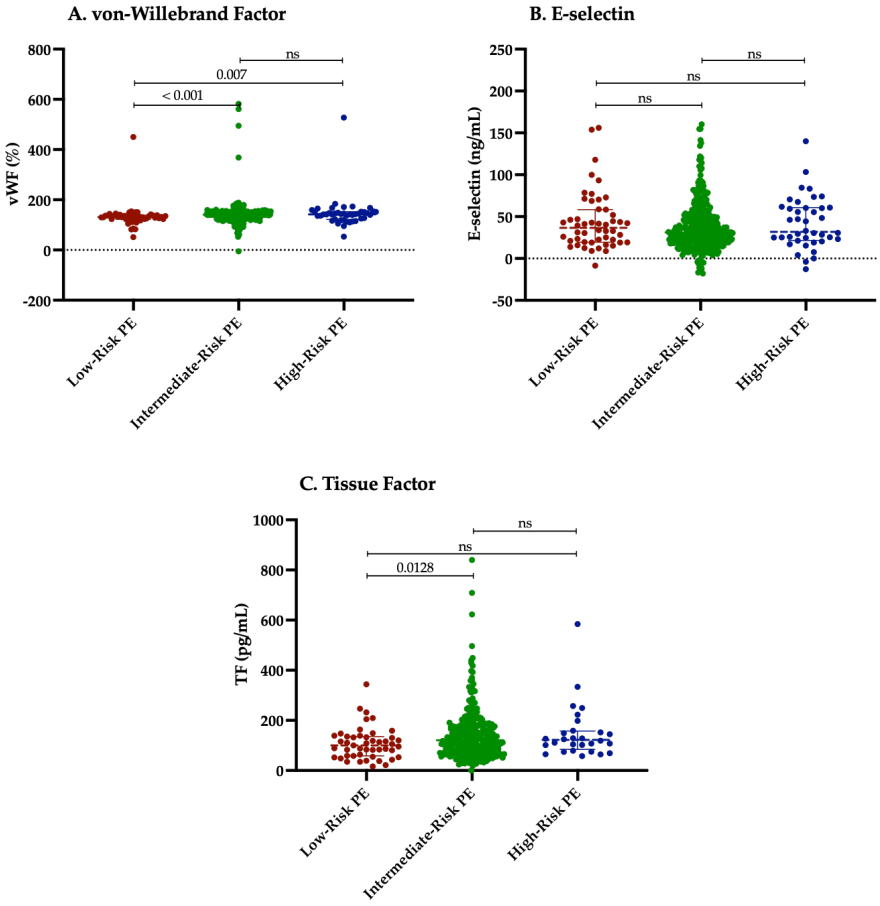


Figure 29. Scatter plot of vWF, E-selectin, and TF levels in PE patients across different risk categories.

Points represent individual patient data with median and interquartile range (IQR). The biomarker levels between PE severity groups are compared by using Mann-Whitney U test, with a p value of <0.05 considered as the cutoff for significance.

5.4.2.2. *Platelet Activation Biomarker*

The levels of P-selectin increased across the different risk groups (table 35). The increase in P-selectin levels from 81.0 (50.3-160.0) pg/mL in low-risk patients to 126.0 (86.9-201.1) pg/mL in high-risk patients and 89.9 (52.2-148.4) pg/mL in intermediate-risk patients to 126.0 (86.9-201.1) pg/mL in high-risk patients were

statistically significant. A modest increase in P-selectin levels was observed from low-risk to intermediate-risk PE. This could be due to the platelet activation and degranulation which increases with increased PE severity.

Table 35. Comparison of platelet activation marker in PE patients across different risk categories.

Biomarkers	*Low-Risk PE*		*Intermediate-Risk PE*		*High-Risk PE*	
	N	*Median IQR)*	*N*	*Median (IQR)*	*N*	*Median (IQR)*
P-selectin (ng/mL)	*51*	*81.0 (50.3-160.0)*	*391*	*89.9 (52.2-148.4)*	*43*	*126.0 (86.9-201.1)*

The graphical presentation of the data in PE patients with respect to severity is shown in the form of scatter plots (figure 20). Scatter plots demonstrate the variation in P-selectin levels across different severities of PE. P-selectin levels in all three groups of PE were clustered near the normal range with wide dispersal towards the upper limit, consistent with intermediate-risk and high-risk PE clinical presentations. This suggests that elevated P-selectin levels are associated with PE disease severity.

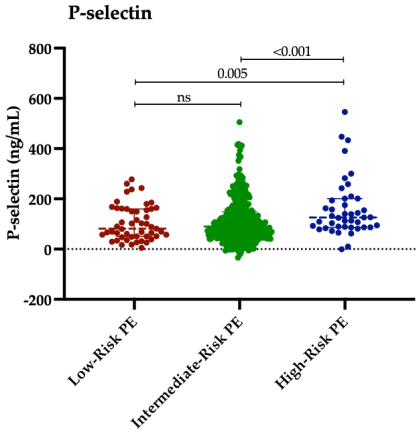


Figure 30. Scatter plot of P-selectin levels in PE patients across different risk categories.

Points represent individual patient data with median and interquartile range (IQR). The biomarker levels between PE severity groups are compared by using Mann-Whitney U test, with a p value of <0.05 considered as the cutoff for significance.

5.4.2.3. Coagulation Factors

Coagulation factor levels showed marked variations, FVII, FX and FXIII-A levels decreased with increased severity of PE, while levels of FIX revealed inconsistent values (table 36). FVII levels significantly decreased from low-risk

patients 99.8 (90.4-122.3) % to intermediate-risk and high-risk patients with 95.1 (76.7-113.8) % and 88.6 (56.1-112.1) %, respectively. The increase from intermediate-risk to high-risk was not significant. FIX showed increased levels from low-risk with 114.6 (73.2-142.4) % to intermediate-risk with 121.5 (93.0-143.8) %, while the level in the high-risk patients decreased with 111.9 (69.6-144.0) % & compared to both low-risk and intermediate-risk PE. All the changes in the FIX levels were not significant. FX demonstrated a consistent and significant decrease in levels with increased PE severity, 103.2 (90.9-117.0) %, 94.9 (81.8-109.2) %, and 83.9 (64.6-98.8) % respectively. FXIII-A followed the same trend as FX, with a consistent decrease in the levels 81.1 (62.3-95.6) %, 74.3 (54.5-95.1) %, and 70.0 (56.4-86.7) % for low-risk, intermediate-risk and high-risk PE. However, the change in the FXIII-A levels was not significant. These variations reflect the overall activation of coagulation cascade. From the above reported biomarkers, there is a known hypercoagulable state in the patients that increases with increasing severity of PE. Thrombosis can result in consumption of coagulation factors which may account for the decreased levels of some coagulation factors.

Table 36. Comparison of coagulation factors in PE patients across different risk categories.

Biomarkers	*Low-Risk PE*		*Intermediate-Risk PE*		*High-Risk PE*	
	N	*Median IQR)*	*N*	*Median (IQR)*	*N*	*Median (IQR)*
FVII (%)	*51*	*99.8 (90.4-122.3)*	*386*	*95.1 (76.7-113.8)*	*43*	*88.6 (56.1-112.1)*
FIX (%)	*51*	*114.6 (73.2-142.4)*	*386*	*121.5 (93.0-143.8)*	*42*	*111.9 (69.6-144.0)*
FX (%)	*51*	*103.2 (90.9-117.0)*	*386*	*94.9 (81.8-109.2)*	*42*	*83.9 (64.6-98.8)*
FXIII-A (%)	*51*	*81.1 (62.3-95.6)*	*397*	*74.3 (54.5-95.1)*	*43*	*70.0 (56.4-86.7)*

Levels of coagulation factors among patients with disease severity are graphically presented in the scatter plots (figure 31). The scatter plots for all the coagulation factors studied including FVII, FIX, FX, and FXIII-A illustrate a broad spectrum of levels among patients. Most patient values concentrated towards the normal range with variability extending to both the upper and lower limits across all the risk categories of PE, along with some values beyond these limits across the range of PE severity.

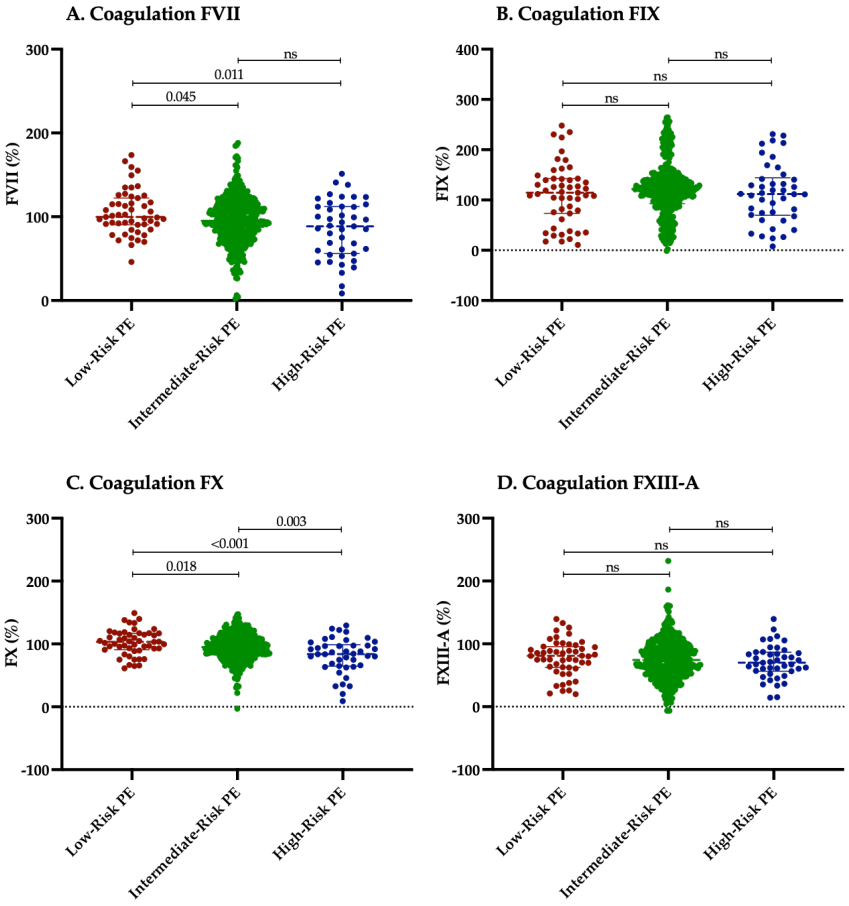


Figure 31. Scatter plot of coagulation FVII, FIX, FX, and FXIII-A levels in PE patients across different risk categories.

Points represent individual patient data with median and interquartile range (IQR). The biomarker levels between PE severity groups are compared by using Mann-Whitney U test, with a p value of <0.05 considered as the cutoff for significance.

5.4.2.4. *Fibrinolysis Biomarkers*

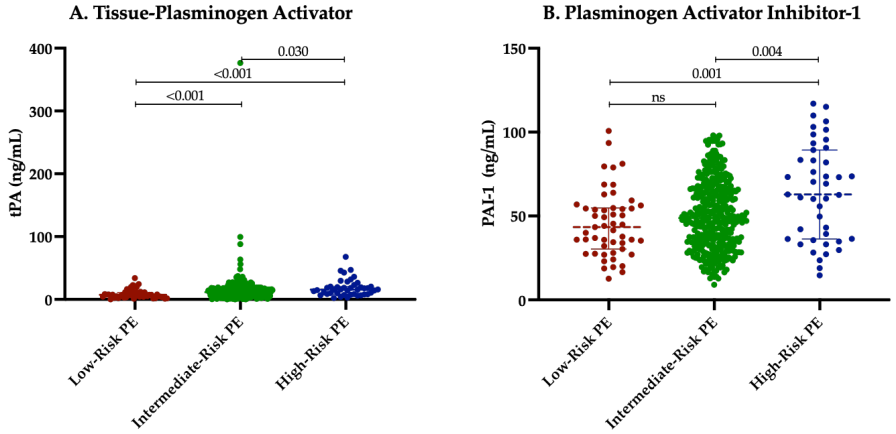
Fibrinolytic markers tPA, PAI-1, and D-dimer showed various degrees of significant elevation across PE severity, except for TAFI (table 37). The tPA levels significantly increased with the severity of PE, with 7.3 (4.2-10.9) ng/mL for low-risk, 11.7 (7.7-18.3) ng/mL for intermediate-risk, and 15.5 (8.3-20.5) ng/mL for high-risk PE, reflecting amplified fibrinolytic activity with PE severity. TAFI showed a modest increase from 101.3 (95.7-109.6) % in low-risk PE to 102.5 (92.1-112.2) % in intermediate-risk PE. However, TAFI levels significantly increased in high-risk PE

with 94.0 (84.8-106.2) % compared to both low-risk and intermediate-risk PE suggest altered fibrinolysis in high-risk PE patients. PAI-1 levels increased across PE severity, with a modest rise from 43.4 (30.3-54.8) ng/mL in low-risk to 48.8 (34.3-66.0) ng/mL in intermediate-risk PE. A significant increase was observed in high-risk PE compared to both low-risk and intermediate-risk PE highlighting impaired fibrinolysis. D-dimer levels elevated significantly with increasing severity with 3992.2 (1535.6-6511.6) ng/mL for low-risk, 5359.7 (2835.3-9675.3) ng/mL for intermediate-risk, and 13,263.8 (7566.8-16,358.3) ng/mL for high-risk PE, reflecting increased fibrin formation and clot degradation. This reflects the overall activation of fibrinolytic system along with the upregulation of fibrinolysis in PE patients

Table 37. Comparison of fibrinolysis markers in PE patients across different risk categories.

Biomarkers	*Low-Risk PE*		*Intermediate-Risk PE*		*High-Risk PE*	
	N	*Median IQR)*	*N*	*Median (IQR)*	*N*	*Median (IQR)*
tPA (ng/mL)	*51*	*7.3 (4.2-10.9)*	*396*	*11.7 (7.7-18.3)*	*43*	*15.5 (8.3-20.5)*
PAI-1 (ng/mL)	*51*	*43.4 (30.3-54.8)*	*397*	*48.8 (34.3-66.0)*	*43*	*62.9 (36.3-89.3)*
TAFI (%)	*50*	*101.3 (95.7-109.6)*	*386*	*102.5 (92.1-112.2)*	*41*	*94.0 (84.8-106.2)*
D-dimer (ng/mL)	*51*	*3992.2 (1535.6-6511.6)*	*397*	*5359.7 (2835.3-9675.3)*	*43*	*13263.8 (7566.8-16358.3)*

The scatter plots for fibrinolysis markers show a wide dispersion in the levels (figure 32). tPA levels across all the risk categories are mostly condensed around the normal range with a tendency of the patients to increase towards the upper end. PAI-1, TAFI, and D-dimer levels showed significant dispersion across different severity of PE, predominantly clustered in intermediate-risk PE with the skewness extending toward both the upper and lower limits.



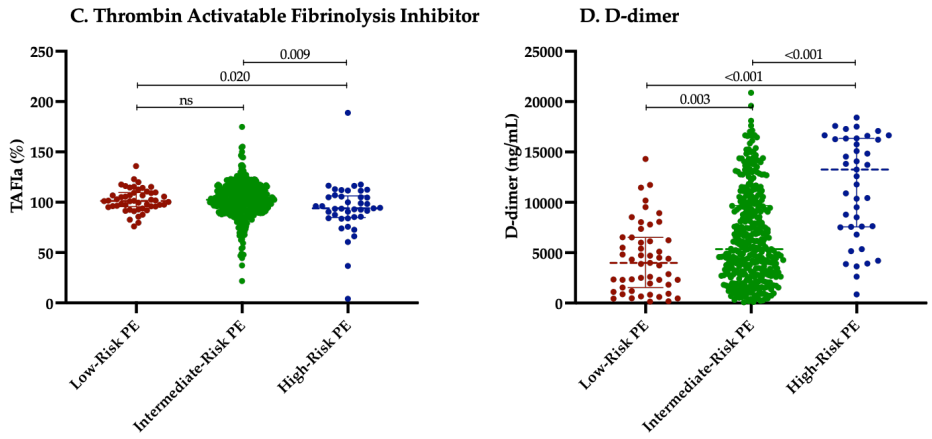


Figure 32. Scatter plot of tPA, PAI-1, TAFI, and D-dimer levels in PE patients across different risk categories.

Points represent individual patient data with median and interquartile range (IQR). The biomarker levels between PE severity groups are compared by using Mann-Whitney U test, with a p value of <0.05 considered as the cutoff for significance.

5.4.2.5. *Inflammatory Biomarkers*

Levels of CRP, IL-6 and TNF-α varied with the PE risk stratification (table 38). CRP showed a variation with a modest increase and decrease in the levels across the PE severity. Increase in IL-6 levels was consistent with the severity of PE, which significantly increased from low-risk PE with 13.7 (7.3-41.6) pg/mL, to intermediate-risk PE with 28.6 (10.5-73.7) pg/mL and high-risk PE with 31.8 (20.3-235.6) pg/mL. TNF-α also showed a variation in the levels across the risk categories, with a significant increase in levels from 1.0 (0.7-1.6) pg/mL in low-risk PE to 1.3 (0.9-2.0) pg/mL in intermediate-risk PE. The levels on TNF-α increased in high-risk PE compared to low-risk PE, while it decreased when compared with intermediate-risk PE. This suggests the role of IL-6 in highlighting the inflammatory process associated with the increased in PE severity.

Table 38. Comparison of inflammatory markers in PE patients across different risk categories.

Biomarkers	*Low-Risk PE*		*Intermediate-Risk PE*		*High-Risk PE*	
	N	*Median IQR)*	*N*	*Median (IQR)*	*N*	*Median (IQR)*
CRP (μg/mL)	*51*	*13.5 (11.4-14.5)*	*397*	*14.0 (12.3-16.4)*	*43*	*13.6 (12.7-15.8)*
IL-6 (pg/mL)	*47*	*13.7 (7.3-41.6)*	*360*	*28.6 (10.5-73.7)*	*38*	*31.8 (20.3-235.6)*
TNF-α (pg/mL)	*50*	*1.0 (0.7-1.6)*	*378*	*1.3 (0.9-2.0)*	*40*	*1.2 (0.8-1.9)*

The scatter plots for inflammatory markers across the PE severity displays broad variation in the levels among patients (figure 33). CRP levels for most of the patients in the low-risk and high-risk PE are clustered near the normal range, while there is a great dispersion in the intermediate-risk PE group. The levels of IL-6 in patients across all risk categories with a wide dispersion and tendency towards the upper limit, TNF-α level also shows considerable variation with wide skewness predominantly towards the upper end of the spectrum. Overall, the results reflect the heterogenous inflammatory response in PE patients with a more dynamic response observed in severe cases.

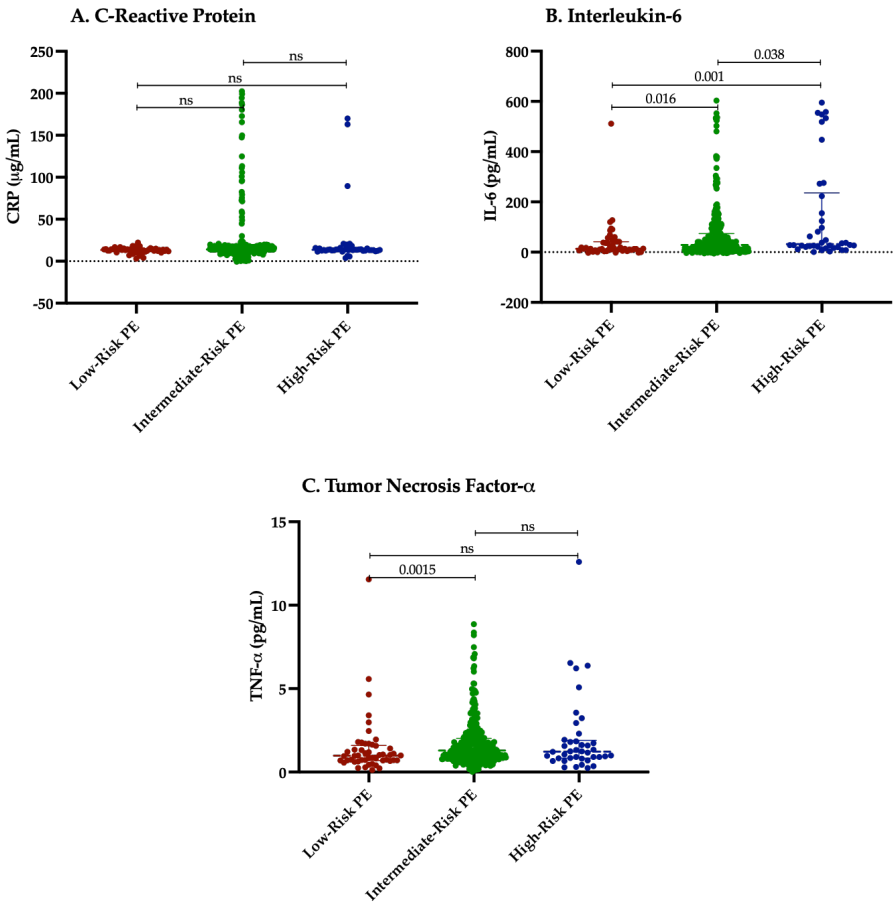


Figure 33. Scatter plot of CRP, IL-6, and TNF-α levels in PE patients across different risk categories.

Points represent individual patient data with median and interquartile range (IQR). The biomarker levels between PE severity groups are compared by using Mann-Whitney U test, with a p value of <0.05 considered as the cutoff for significance.

5.4.2.6. ***Blood Cellular Indices***

The levels of NLR, PLR and SII varied across the PE risk severity (table 39). NLR levels consistently increased from low-risk PE with 5.3 (2.8-10.6) x1000/mm^3 to intermediate-risk PE with 5.3 (2.8-10.6) x1000/mm^3 and high-risk PE with 6.0 (2.4-12.8) x1000/mm^3. Only the increase from low-risk to intermediate-risk PE was significant. PLR levels increased from 155.3 (115.0-246.4) x1000/mm^3 in low-risk PE to 185.5 (113.5-282.3) x1000/mm^3 in intermediate-risk PE but decreased to 106.5 (68.6-269.3) in high-risk PE. However, the decrease in the intermediate-risk subgroup to high-risk PE was the only significant change. SII levels also increased from 1142.0 (555.7-1819.0) x1000/mm^3 in low-risk PE to 2465.0 (612.4-2465.0) x1000/mm^3 in intermediate-risk PE and then decreased to 1212.0 (457.8-2223.0) x1000/mm^3 in high-risk PE. The variation in the levels suggest the complex interplay between the immune response and inflammation.

Table 39. Comparison of blood cellular indices in PE patients across different risk categories.

Cellular Indices	*Low-Risk PE*		*Intermediate-Risk PE*		*High-Risk PE*	
	N	*Median IQR)*	*N*	*Median (IQR)*	*N*	*Median (IQR)*
NLR (x1000/mm^3)	*51*	*4.2 (2.8-6.1)*	*397*	*5.3 (2.8-10.6)*	*43*	*6.0 (2.4-12.8)*
PLR (x 1000/mm^3)	*51*	*155.3 (115.0-246.4)*	*397*	*185.5 (113.5-282.3)*	*43*	*106.5 (68.6-269.3)*
SII (x 1000/mm^3)	*51*	*1142.0 (555.7-1819.0)*	*397*	*2465.0 (612.4-2465.0)*	*43*	*1212.0 (457.8-2223.0)*

The scatter plots for blood cellular indices across the PE severity shows variation (figure 34). NLR demonstrates a wide variation in the levels particularly for intermediate-risk and high-risk PE, while PLR and SII plots display dispersion among the patients in the upper limit, suggesting differential immune activation.

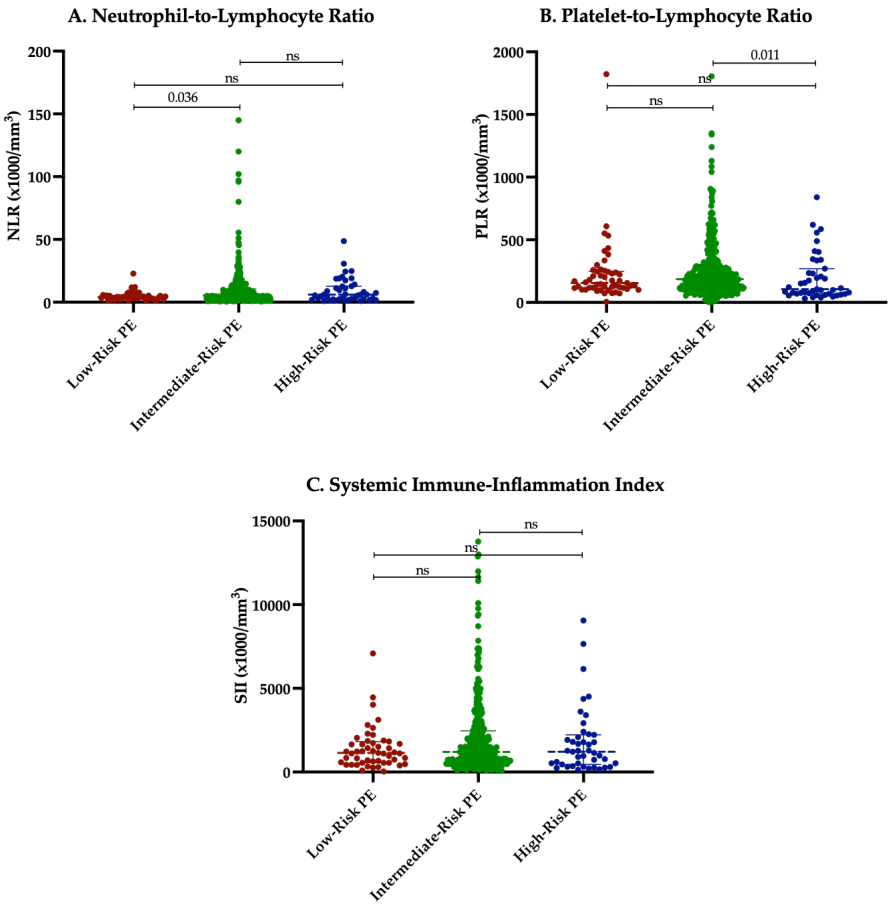


Figure 34. Scatter plot of NLR, PLR, and SII levels in PE patients across different risk categories.

Points represent individual patient data with median and interquartile range (IQR). The biomarker levels between PE severity groups are compared by using Mann-Whitney U test, with a p value of <0.05 considered as the cutoff for significance.

5.4.3. Relationship Among the Biomarkers and Cellular Indices

All the biomarkers along with cellular indices were analyzed to demonstrate their interrelationships. This comprehensive analysis elucidates how these biomarkers and cellular indices interact with one another, providing insights into their combined impact on the overall pathophysiology of PE. Notable correlations were observed between various biomarkers, highlighting the complex interplay in patients with acute PE (figure 35). vWF correlated with tPA (r: 0.32; p: <0.0001), and CRP (r: 0.27; p value: <0.0001); E-selectin vs TNF-α (r: 0.20; p value: <0.0001)

pointing to a link between endothelial dysregulation, fibrinolysis process, and inflammatory processes in patients with PE. P-selectin vs PAI-1 (r: 0.32; p value: <0.0001), and D-dimer (r: 0.34; p value: <0.0001) pointing to a link between platelet activation and fibrinolysis.

Fibrinolysis markers also correlated with various markers. Of note tPA correlated with PAI-1 (r: 0.31; p value: <0.0001), suggesting a balance between fibrinolysis and its inhibition; and tPA also correlated with TNF-α (r: 0.26; p value: <0.0001). PAI-1 associated with IL-6 (r: 26; p value: <0.0001) indicating a significant relationship between fibrinolytic activity and inflammatory response. These findings underscore the complex interplay between platelet activation, fibrinolysis, and inflammatory responses in the pathophysiology of PE.

Coagulation factors, such as FVII, significantly correlated with FX (r: 0.26; p value: <0.0001) and FXIII-A (r: 0.36; p value: <0.0001); FIX with FX (r: 0.39; p value: <0.0001); FX with CRP (r: 0.25; p value: <0.0001) and TNF-α (r: 0.20; p value: <0.0001). These findings suggest that the activity levels of coagulation factors FVII, FIX, and FX are inter-related, and that coagulation activation is also associated with the inflammatory responses in patients with PE.

For the inflammatory markers, CRP was significantly associated with IL-6 (r: 0.43; p value: <0.0001), and NLR (r: 0.22; p value: <0.0001), and SII (r: 0.24; p value: <0.0001). IL-6 correlated significantly with TNF-α (r: 0.36; p value: <0.0001), NLR (r: 0.36; p value: <0.0001), and SII (r: 0.35; p value: <0.0001). TNF-α also showed association with TF (r: 0.35; p value: <0.0001), NLR (r: 0.23; p value: <0.0001), and SII (r: 0.23; p value: <0.0001) reinforcing the central role of inflammation with increased coagulation activity. Notably also, higher NLR and SII levels were associated with elevated levels of several biomarkers, such as CRP, IL-6, TNF-α, and TF. These associations underscore the interconnected nature of inflammatory markers and their roles in both the inflammatory response and thrombotic states associated with PE. Elevated NLR and SII indicate a more pronounced inflammatory response, which correlates with higher levels of these biomarkers, which is correlated with higher levels of these biomarkers.

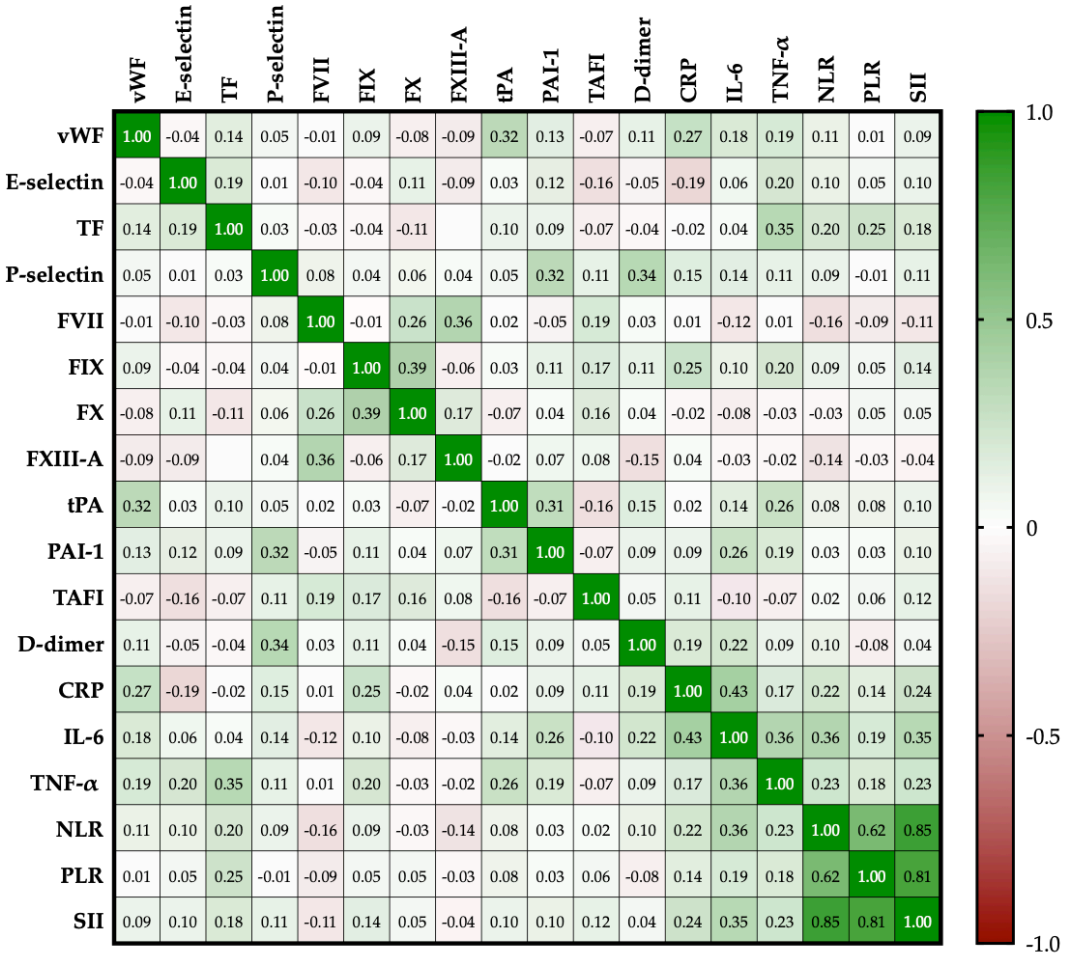


Figure 35. Spearman correlation matrix illustrating the relationships between hemostatic and thrombo-inflammatory biomarkers and cellular indices in patients with PE.

Correlation analysis displays the relationships among various biomarkers and cellular indices. Positive correlations are shown in green color indicating a direct association. while the negative correlations are depicted in maroon color representing an inverse association. The strength of these correlations is illustrated by the intensity of the color with darker shades denoting stronger correlations. Correlations with a p-value <0.05 considered as statistically significant.

5.4.4. Association of Biomarkers with Mortality

Biomarker levels along with cellular indices were also analyzed/stratified with respect to the 30-day mortality (table 40-44, figure 36-40). In the cohort of 500 patients, 48 patients (9.7%) died during the first 30-days. The biomarkers and indices may thus prove useful in predicting short-term outcomes.

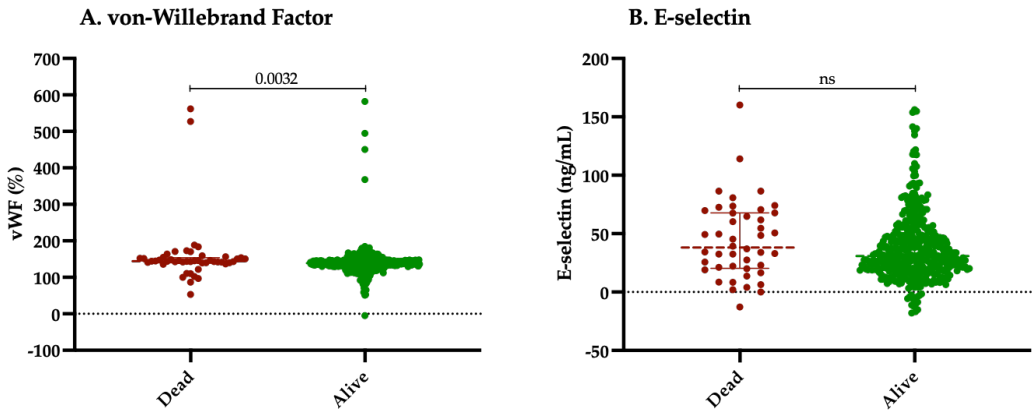
5.4.4.1. *Endothelial Dysregulation Biomarkers*

Endothelial marker including vWF, E-selectin and TF were increased in patients with PE who died within the first 30-days of diagnosis (table 40). The increase in the levels of vWF and TF was significantly associated with 30-day mortality, whereas increase in the E-selectin levels was not significant. This highlights the role of vWF and TF in reflecting disease severity and its association with mortality.

Table 40. Comparison of endothelial dysregulation biomarkers in PE patients by 30-day mortality status.

Biomarkers	*Dead*		*Alive*		*P value*
	N	*Median (IQR)*	*N*	*Median (IQR)*	
vWF (%)	*47*	*144.3 (140.0-153.7)*	*440*	*138.4 (128.1-147.2)*	*0.0032*
E-selectin (ng/mL)	*44*	*38.1 (20.3-67.7)*	*421*	*30.9 (19.1-49.0)*	*NS*
TF (pg/mL)	*22*	*189.3 (124.9-343.3)*	*358*	*115.4 (72.1-165.7)*	*<0.0001*

The graphical presentation of the levels of endothelial dysregulation markers based on the mortality are depicted in the form of scatter plots. (figure 36). Both groups exhibit comparable variability in biomarker levels with some individuals show exceptionally high or low levels. However, the change in vWF and TF were significant highlighting a marked difference between the groups.



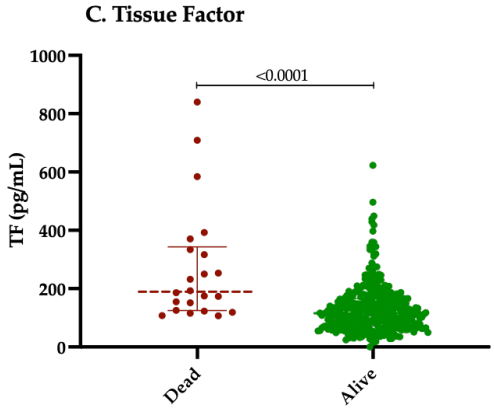


Figure 36. Scatter plot of vWF, and E-selectin levels in PE patients by 30-day mortality status.

Points represent individual patient data with median and interquartile range (IQR). The biomarker levels in PE patients across 30-day mortality are compared by using Mann-Whitney U test, with a p value of <0.05 considered as the cutoff for significance.

5.4.4.2. *Platelet Activation Biomarker*

P-selectin levels increased in patients who died within 30 days of PE (table 41) compared to those who survived, reflecting its role in thrombotic processes and its association with the severity and outcome of the condition.

Table 41. Comparison of platelet activation biomarker in PE patients by 30-day mortality status.

Biomarkers	*Dead*		*Alive*		*P value*
	N	*Median (IQR)*	*N*	*Median (IQR)*	
P-selectin (ng/mL)	*47*	*117.6 (71.6-219.3)*	*440*	*89.8 (52.6-149.6)*	*0.0181*

Scatter plot for P-selectin among individuals based on the mortality showed a varying levels of change (figure 37), while the patients who alive showed more cluster towards the center compared to the who died significant exhibit a significant increase in levels, indicating a marked variation associated with mortality.

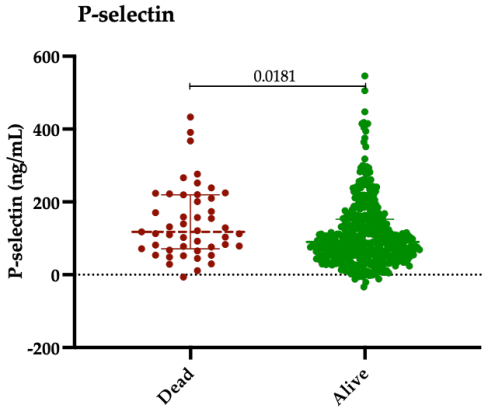


Figure 37. Scatter plot of P-selectin levels in PE patients by 30-day mortality status.

Points represent individual patient data with median and interquartile range (IQR). The biomarker levels in PE patients across 30-day mortality are compared by using Mann-Whitney U test, with a p value of <0.05 considered as the cutoff for significance.

5.4.4.3. *Coagulation Factors*

Varying levels of increase and decrease in the coagulation factors were observed in the patients who died within 30 days (table 42). Coagulation factors including FVII, FX, and FXIII-A were significantly decreased in patients with PE who died within the first 30 days of diagnosis, except for the FIX which increased slightly but, this change was not significant. This decrease in the factors may reflect a dysregulation in the coagulation cascade suggesting their potential role in the severity and prognosis of pulmonary embolism.

Table 42. Comparison of coagulation factors in PE patients by 30-day mortality status.

Biomarkers	*Dead*		*Alive*		*P value*
	N	*Median (IQR)*	*N*	*Median (IQR)*	
FVII (%)	*47*	*87.5 (55.1-97.9)*	*435*	*97.3 (78.8-115.1)*	*<0.0001*
FIX (%)	*47*	*124.7 (75.6-147.2)*	*434*	*121.1 (91.3-142.5)*	*NS*
FX (%)	*47*	*86.0 (63.6-104.1)*	*434*	*95.7 (83.4-110.8)*	*0.0022*
FXIII-A (%)	*48*	*57.5 (43.8-83.2)*	*445*	*76.0 (56.2-95.4)*	*0.0012*

The visual presentation of the coagulation factors is presented in the form of scatter plots (figure 38). All the factors showed wide variation among individuals in both the groups, while FVII, FX, and FXIII-A showed significant decrease in the levels among those who died.

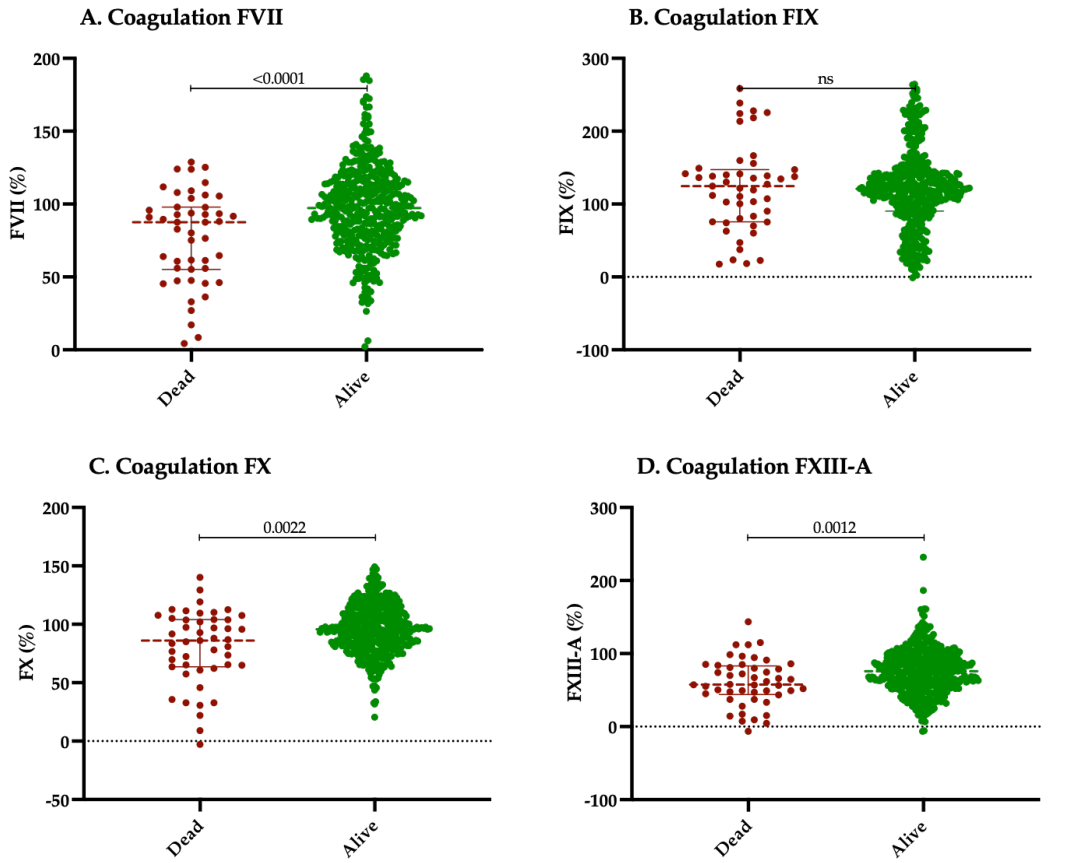


Figure 38. Scatter plot of coagulation FVII, FIX, FX, and FXIII-A levels in PE patients by 30-day mortality status.

Points represent individual patient data with median and interquartile range (IQR). The biomarker levels in PE patients across 30-day mortality are compared by using Mann-Whitney U test, with a p value of <0.05 considered as the cutoff for significance.

5.4.4.4. *Fibrinolysis Biomarkers*

Markers of fibrinolysis also showed variations in the levels among patients who died with 30-day mortality (table 43). Of note, tPA, TAFI, and D-dimer showed significant increase in the patients who died, whereas the PAI-1 levels demonstrated significant decrease. These findings suggest an imbalance in fibrinolysis activity leading to a fibrinolytic deficit which would contribute to the clinical outcome in patients with PE.

Table 43. Comparison of fibrinolysis biomarkers in PE patients by 30-day mortality status.

Biomarkers	Dead		Alive		P value
	N	Median (IQR)	N	Median (IQR)	
tPA (ng/mL)	48	14.9 (7.4-20.9)	444	11.2 (7.2-17.1)	0.0430
PAI-1 (ng/mL)	48	64.9 (40.6-85.5)	433	48.6 (33.9-65.3)	0.0303
TAFI (%)	46	94.5 (84.0-111.4)	445	102.0 (92.8-112.0)	0.0010
D-dimer (ng/mL)	48	7174.1 (3167.8-14283.6)	445	5392.0 (2814.8-9692.8)	0.0479

The levels of the markers of fibrinolysis process are also graphically presented in the form of scatter plots (figure 39). Markers including PAI-1, TAFI, and D-dimer are mostly clustered towards the normal range in both the groups, with some high and low values and skewness at both upper and lower end, while PAI-1 levels are mostly skewed towards the upper end.

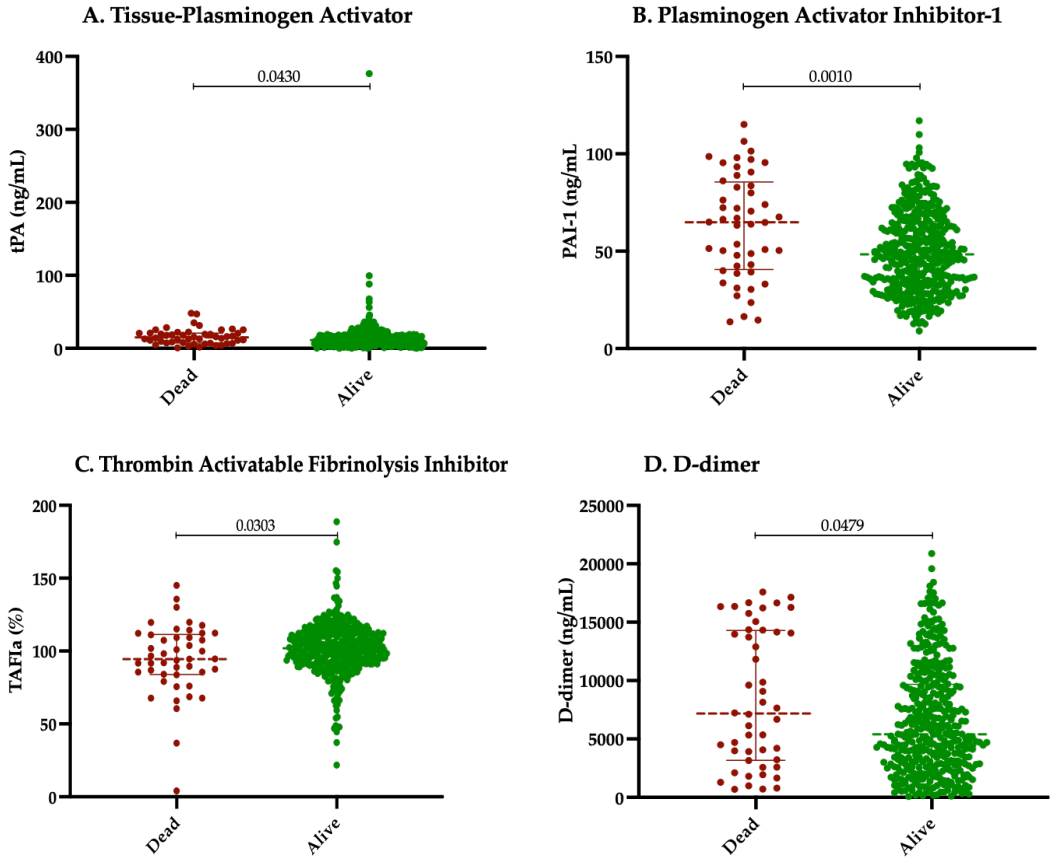


Figure 39. Scatter plot of tPA, PAI-1, TAFI, and D-dimer levels in PE patients by 30-day mortality status.

Points represent individual patient data with median and interquartile range (IQR). The biomarker levels in PE patients across 30-day mortality are compared by using Mann-Whitney U test, with a p value of <0.05 considered as the cutoff for significance.

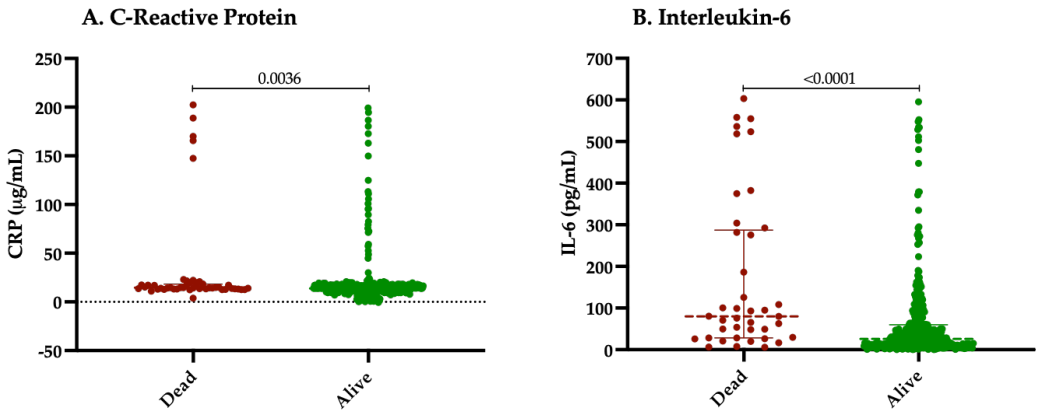
5.4.4.5. *Inflammatory Biomarkers*

All the inflammatory markers including CRP, IL-6 and TNF-α had increased levels in patients who died within the first 30 days of PE diagnosis (table 44). Among these, IL-6 exhibited the most pronounced increase in the levels. This reflects the significant role that inflammatory markers play in the severity and prognosis of pulmonary embolism.

Table 44. Comparison of inflammatory biomarkers in PE patients by 30-day mortality status.

Biomarkers	*Dead*		*Alive*		*P value*
	N	*Median (IQR)*	*N*	*Median (IQR)*	
CRP (μg/mL)	*48*	*14.6 (13.3-18.2)*	*445*	*13.9 (12.1-16.0)*	*0.0036*
IL-6 (pg/mL)	*41*	*79.9 (28.0-287.1)*	*406*	*25.5 (9.8-59.8)*	*<0.0001*
TNF-α (pg/mL)	*44*	*2.2 (1.0-3.6)*	*426*	*1.2 (0.8-1.8)*	*0.0005*

The graphical distribution for the inflammatory markers depicted in the form of scatter plots (figure 40). Scatter plot for CRP, IL-6, TNF-α show significant variation with some individuals in both groups exhibiting high values, while levels vary widely among those who died, suggesting a potential link between these biomarkers .



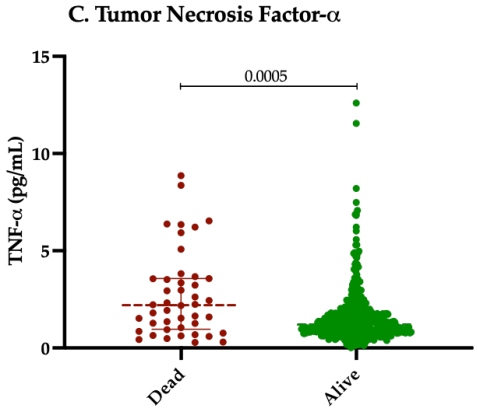


Figure 40. Scatter plot of CRP, IL-6, and TNF-α levels in PE patients by 30-day mortality status.

Points represent individual patient data with median and interquartile range (IQR). The biomarker levels in PE patients across 30-day mortality are compared by using Mann-Whitney U test, with a p value of <0.05 considered as the cutoff for significance.

5.4.4.6. *Blood Cellular Index*

NLR, PLR, and SII levels were significantly elevated in patients who died within the first 30 days after PE diagnosis (table 45), emphasizing its potential as an early indicator of mortality.

Table 45. Comparison of blood cellular indices in PE patients by 30-day mortality status.

Cellular Indices	*Dead*		*Alive*		*P value*
	N	*Median (IQR)*	*N*	*Median (IQR)*	
NLR (x1000/mm³)	*48*	*12.6 (6.1-23.2)*	*445*	*4.7 (2.7-8.4)*	*<0.0001*
PLR (x1000/mm³)	*48*	*284.8 (122.4-476.7)*	*445*	*173.1 (104.6-263.0)*	*0.0012*
SII (x1000/mm³)	*48*	*2583.0 (1277.0-2820.0)*	*445*	*1120.0 (567.3-2043.0)*	*<0.0001*

The graphical presentation for blood cellular indices including NLR, PLR, and SII in the form of scatter plots depicts substantial variability in the levels (figure 41). All the indices show a skewness with extremely high levels in both the upper and lower end indicating significant variability among individuals in both groups.

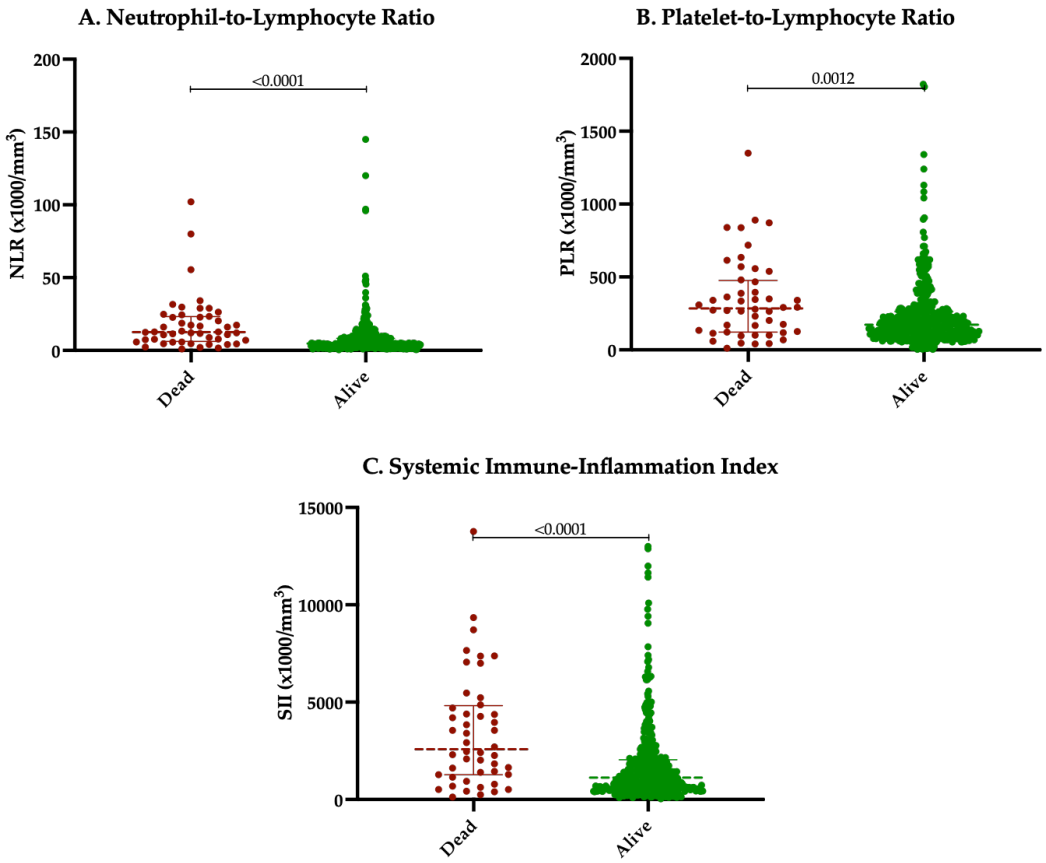


Figure 41. Scatter plot of NLR, PLR, and SII levels in PE patients by 30-day mortality status.

Points represent individual patient data with median and interquartile range (IQR). The biomarker levels in PE patients across 30-day mortality are compared by using Mann-Whitney U test, with a p value of <0.05 considered as the cutoff for significance.

All the biomarker levels were analyzed for 30-day mortality by using the ROC analysis. The area under the ROC curve for all the biomarkers and their association with 30-day mortality are presented in the appendix 4 (supplementary figure 6-11). The optimal cutoff values for the endothelial dysregulation and platelet activation markers were: vWF >140 % (AUC: 0.632; 95% CI: 0.544-0.720; p value: 0.003), E-selectin >48 pg/mL (AUC: 0.563; 95% CI: 0.466-0.659; p value: NS), TF >130 pg/mL (AUC: 0.790; 95% CI: 0.705-0.874; p value: 0.000), and P-selectin >110 pg/mL (AUC: 0.606; 95% CI: 0.522-0.691; p value: 0.014). Coagulation factors exhibited variability in association with 30-day mortality, with increase in FIX >135 % (AUC: 0.507; 95% CI: 0.414-0.600; p value:), while decrease in the FVII <65 % (AUC: 0.790; 95% CI: 0.589-0.754; p value: 0.000), FX <65 % (AUC: 0.636; 95% CI:

0.545-0.727; p value: 0.004), and FXIII-A <58 % (AUC: 0.642; 95% CI: 0.558-0.725; p value: 0.001) were associated with 30-day mortality in patients with PE. Fibrinolytic deficit markers, such tPA >15 ng/mL (AUC: 0.591; 95% CI: 0.502-0.680; p value: 0.046), PAI-1 >63 ng/mL (AUC: 0.643; 95% CI: 0.551-0.735; p value: 0.002), and D-dimer >13,688 ng/mL (AUC: 0.588; 95% CI: 0.494-0.681; p value: 0.007), were associated with 30-day mortality in patients with PE. Conversely, a decrease in TAFI <95 % (AUC: 0.599; 95% CI: 0.501-0.696; p value: 0.047) was also linked to increased 30-day mortality. Inflammatory markers including CRP >12 μg/mL (AUC: 0.626; 95% CI: 0.548-0.704; p value: 0.001), IL-6 >48 pg/mL (AUC: 0.727; 95% CI: 0.644-0.810; p value: 0.000), and TNF-α >2.0 pg/mL (AUC: 0.649; 95% CI: 0.541-0.756; p value: 0.007) were associated with 30-day mortality in patients with PE. Blood cellular indices including NLR >7 (AUC: 0.758; 95% CI: 0.683-0.834; p value: 0.000), PLR >220 (AUC: 0.642; 95% CI: 0.547-0.736; p value: 0.003), and SII (AUC: 0.706; 95% CI: 0.624-0.789; p value: 0.000) were associated with 30-day mortality in patients with PE.

All the biomarker levels were categorized based on their optimal cutoff values and transformed into a categorical variable. These were then analyzed for association with 30-day mortality using the Chi-Square test as part of a univariate analysis. On univariable analysis, elevated levels of vWF, E-selectin, P-selectin, tPA, PAI-I, D-dimer, CRP, IL-6, TNF-α, and NLR were significantly associated with 30-day mortality, decreased levels of FVII, FX, FXIII-A, and TAFI were also associated with increased mortality (table 46). Specifically, the highest odds ratios were observed for CRP >12 μg/mL (OR: 7.371; 95% CI: 1.760-30.869), NLR >7.0 x1000/mm^3 (OR: 6.401; 95% CI: 3.233-12.674), and IL-6 >48 pg/mL (OR: 5.370; 95% CI: 2.654-10.867). These findings underscore the significant prognostic value of inflammatory markers and NLR in predicting 30-day mortality in PE patients.

Table 46. Univariable Chi-Square analysis of biomarkers and cellular indices with 30-day mortality.

Biomarkers	*N (%)*	*Odds Ratio (95% CI)*	*P value*
vWF >140 (%)	*35 (74.5%)*	*3.374 (1.706-6.673)*	*<0.001*
E-selectin >48 (ng/mL)	*20 (45.5%)*	*2.327 (1.237-4.378)*	*0.007*
TF >130 (pg/mL)	*16 (72.7%)*	*3.917 (1.497-10.248)*	*0.003*
P-selectin >110 (ng/mL)	*26 (55.3 %)*	*1.948 (1.062-3.571)*	*0.029*
FVII <65 (%)	*19 (40.4 %)*	*5.602 (2.905-10.801)*	*<0.001*
FIX >135 (%)	*20 (42.6%)*	*1.507 (0.817-2.779)*	*NS*

FX <65 (%)	*13 (27.7%)*	*5.544 (2.632-11.679)*	*<0.001*
FXIII-A <58 (%)	*25 (52.1%)*	*3.083 (1.684-5.643)*	*<0.001*
tPA >15 (ng/mL)	*24 (50.0%)*	*2.241 (1.229-4.085)*	*0.007*
PAI-1 >63 (ng/mL)	*27 (56.3%)*	*3.443 (1.876-6.319)*	*<0.001*
TAFI <95 (%)	*24 (52.2%)*	*2.515 (1.361-4.646)*	*0.003*
D-dimer >13,688 (ng/mL)	*16 (33.3%)*	*4.557 (2.318-8.959)*	*<0.001*
CRP >12 (μg/mL)	*46 (95.8%)*	*7.371 (1.760-30.869)*	*0.001*
IL-6 >48 (pg/mL)	*29 (70.7%)*	*5.370 (2.654-10.867)*	*<0.001*
TNF-α >2.0 (pg/mL)	*23 (47.9%)*	*3.680 (1.995--6.787)*	*<0.001*
NLR >7.0 (x1000/mm³)	*36 (75.0%)*	*6.401 (3.233-12.674)*	*<0.001*
PLR >220 (x1000/mm³)	*30 (62.5%)*	*3.149 (1.701-5.832)*	*<0.001*
SII >1600 (x1000/mm³)	*33 (68.8%)*	*4.116 (2.169-7.812)*	*<0.001*

All the significant markers from the univariate analysis including vWF, E-selectin, P-selectin, FVII, FX, FXIII-A, tPA, PAI-1, TAFI, D-dimer, CRP, IL-6, TNF-α, and NLR along with clinical variables were modelled into the binary logistic regression analysis by using the backward stepwise method. On multivariable analysis, all tested biomarkers (except for P-selectin) were independently associated with 30-day all-cause death (in descending order): CRP >12 μg/mL (OR: 6.82; 95% CI: 1.62-28.70), NLR >7 x1000/mm³ (OR: 6.31; 95% CI: 3.15-12.70), IL-6 >48 pg/mL (OR: 5.13; 95% CI: 2.52-10.50), TF >130 pg/mL (OR: 5.07; 95% CI: 1.79-14.35), FVII <65 % (OR: 5.01; 95% CI: 2.54-9.88), FX <65 % (OR: 4.61; 95% CI: 2.12-10.10), SII >1600 x1000/mm³ (OR:4.47; 95% CI: 2.30-8.70), TNF-α >2 pg/mL (OR: 3.76; 95% CI: 1.99-7.09), PLR >220 x1000/mm³ (OR: 3.73; 95% CI: 1.95-7.14), D-dimer >13688 ng/mL (OR: 3.61; 95% CI: 1.71-7.60), FXIII-A <58 % (OR: 3.38; 95% CI: 1.81-6.31), vWF >140 % (OR: 3.13; 95% CI: 1.57-6.24), PAI-1 >63 ng/mL (OR: 2.98; 95% CI: 1.60-5.56), E-selectin >48 ng/mL (OR: 2.24; 95% CI: 1.17-4.30), TAFI <95 % (OR: 2.14; 95% CI: 1.14-4.03), and tPA >15 ng/mL (OR: 2.09; 95% CI: 1.13-3.89) (figure 42).

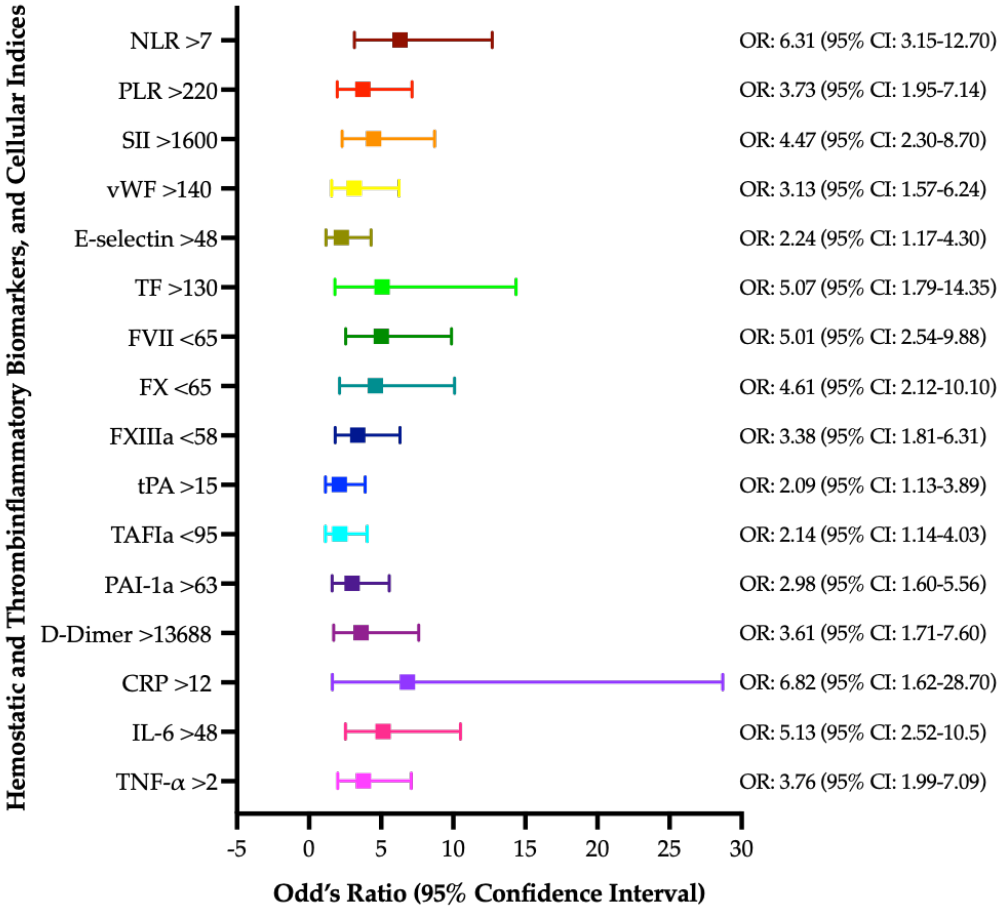


Figure 42. Multivariate regression analysis for hemostatic activation, and thrombo-inflammatory markers, and cellular indices in predicting 30-day mortality.

The model is adjusted for the ESC severity.

5.5. INFLAMMATORY BIOMARKER PROFILING IN ACUTE PE PATIENTS

Plasma samples were collected from PE patients enrolled in the PERT project following the protocols, adhering to the protocols outlined in the Materials and Methods section of this dissertation. Samples were collected from Loyola University Medical Center and Gottlieb Memorial Hospital under an IRB-approved protocols (appendix 3).

In this study, 193 patients were randomly used to measure the levels of inflammatory cytokines, chemokines and growth factors along with blood cellular

indices in patients with PE. Table 47 illustrates the basic demographic information, co-morbidities, clinical presentation, laboratory parameters, PE risk stratification, and mortality within the PERT cohort.

The cohort consisted of 193 patients, with almost equal distribution between males and females (52.1% males vs. 48.9% female). The median age of 65 years indicates a predominantly middle-aged population, with a broad range of age spanning from 56 to 75 years reflecting a diversity in the population. A median BMI of 29.8 classifies the patients as moderately obese, while 78% of the patients in the cohort were categorized as overweight or obese (BMI ≥25).

Most of the patients presented with dyspnea (92.7%), chest pain (31.1%), and/or syncope (10.9%), which is consistent with typical PE symptoms. Predictors of the PE severity such as right ventricular dysfunction, troponin and brain-natriuretic peptide levels, PESI and sPESI scores were also considered. Patients were categorized according to the ESC guidelines with 7.8% as low-risk, 80.8% as intermediate-risk, and 11.4% as high-risk PE patients.

Comorbidities were collected from a review of the patient's medical chart. Items included were cancer, chronic lung disease, chronic heart failure, kidney disease, hypertension, coronary artery disease and others possibly affecting the coagulation cascade. The most predominant comorbidity in the cohort includes hypertension (59.1%), cancer (28.0%), chronic heart failure (10.9%), chronic lung disease (14.0%) and chronic kidney disease (11.4%). Mortality rate in the cohort increased overtime, with 14.0% of patients dead within the first 30-days which gradually increased to 20.2% by 3-months, 24.9% by 6-months, 25.4% by 9-months, and persisted to 1-year.

Table 47. Basic clinical characteristics and laboratory parameters in the LUMC PERT cohort

Variables	*Counts*	*LUMC PERT Cohort*
		Median (IQR), N (%)
Patients	*193*	*---*
Demographics		
Age (years)	*193*	*65 (55.5-74.5)*
Male sex	*193*	*104 (53.9%)*
Weight (kg)	*193*	*88.9 (73.8-106.9)*
Body mass index	*193*	*29.8 (25.4-36.1)*
Clinical Presentation		

Systolic blood pressure (mmHg)	*193*	*117.0 (101.0-132.0)*
Heart rate (beats/minute)	*193*	*100.0 (85.5-113.0)*
Oxygen saturation (%)	*193*	*92.0 (88.0-92.0)*
Respiratory rate	*193*	*21.0 (18.0-26.0)*
Dyspnea	*193*	*179 (92.7%)*
Syncope	*193*	*21 (10.9%)*
Chest pain	*193*	*60 (31.1%)*
Predictors of PE Severity		
RV/LV ratio	*93*	*1.0 (0.8-1.3)*
Troponin	*193*	*0.07 (0.03-0.3)*
Brain natriuretic peptide	*188*	*158.5 (60.3-395.5)*
PESI Score	*193*	*109.0 (83.0-149.5)*
Risk Stratification		
Low-risk PE	*193*	*15 (7.8%)*
Intermediate-risk PE	*193*	*156 (80.8%)*
High-risk PE	*193*	*22 (11.4%)*
Comorbidities		
Active cancer	*193*	*54 (28.0%)*
Chronic heart failure	*193*	*21 (10.9%)*
Chronic lung disease	*193*	*27 (14.0%)*
Chronic kidney disease	*193*	*22 (11.4%)*
Hypertension	*193*	*114 (59.1%)*
Mortality		
30-day mortality	*193*	*27 (14.0%)*
3-month mortality	*193*	*39 (20.2%)*
6-month mortality	*193*	*48 (24.9%)*
Laboratory Parameters		
Hemoglobin (g/dl)	*193*	*12.3 (10.2-14.0)*
Neutrophils (x1,000/mm^3)	*193*	*7.2 (4.7-10.0)*
Lymphocytes (x1,000/mm^3)	*193*	*0.8 (1.3-2.0)*
Platelets (x1,000/mm^3)	*193*	*229.0 (178.0-281.5)*

5.5.1. Biomarker Levels in PE Patients

Inflammatory cytokine, chemokine and growth factor levels were measured in patients with PE and compared with the healthy controls. Most of the marker levels were significantly increased in PE patients. The data were presented with median (IQR), fold changes, together with significance levels. Patients with PE showed significantly higher levels for most of the biomarkers and cellular indices compared to the controls, as detailed in tables 48-51and depicted as figure 43-47.

5.5.1.1. *Pro-inflammatory Cytokines*

Levels of pro-inflammatory cytokines including IL-1α, IL-1β, IL-2, IL-6, IL-8, TNF-α, and IFN-γ were measured in PE patients and compared to the controls (table 48). All the proinflammatory cytokines measured in the thesis were significantly elevated in the PE patients compared to the control group; however, the increase in the level of IFN-γ was not statistically significant. IL-6 showed the most pronounced increase of 21.11-fold, followed by TNF-α with 0.50-fold, and IL1-α with 0.40-fold increase compared to controls. The overall increase in the pro-inflammatory cytokines proposes a robust inflammatory response and systemic inflammation in PE patients which may contribute towards the pathophysiology of PE. The marked increase in the level of IL-6 suggests it as a major mediator of the inflammatory processes in PE.

Table 48. Comparison of pro-inflammatory cytokines in PE patients versus healthy controls.

Biomarkers	PE Samples		Controls		P value	Fold Change
	N	Median (IQR)	N	Median (IQR)		
IL-1α (pg/mL)	193	0.14 (0.1-0.2)	50	0.1 (0.0-0.1)	0.0121	0.40
IL-1β (pg/mL)	193	1.2 (0.9-2.0)	50	0.0 (0.0-1.2)	0.0070	NA
IL-2 (pg/mL)	193	1.0 (0.0-1.9)	50	0.0 (0.0-1.7)	0.0391	NA
IL-6 (pg/mL)	193	19.9 (6.1-49.2)	50	0.9 (0.5-1.5)	<0.0001	21.11
TNF-α (pg/mL)	193	2.7 (1.9-4.0)	50	1.8 (1.4-2.2)	<0.0001	0.50
IFN-γ (pg/mL)	193	0.3 (0.0-0.5)	50	0.0 (0.0-0.3)	0.0142	NA

NA: Fold change is not applicable due to number zero.

The graphical presentation of data for pro-inflammatory cytokines in PE patients versus healthy controls is presented in the form of scatter plots (figure 43). The scatter plots for all the pro-inflammatory cytokines including IL-1α, IL-1β, IL-2, IL-6, TNF-α, and IFN-γ in PE patients demonstrates a wide variation among individual patients with the marked increase in the levels towards the upper limit. This reflects the intense inflammatory response among individuals, which may be dependent on the variation in the patient's illness.

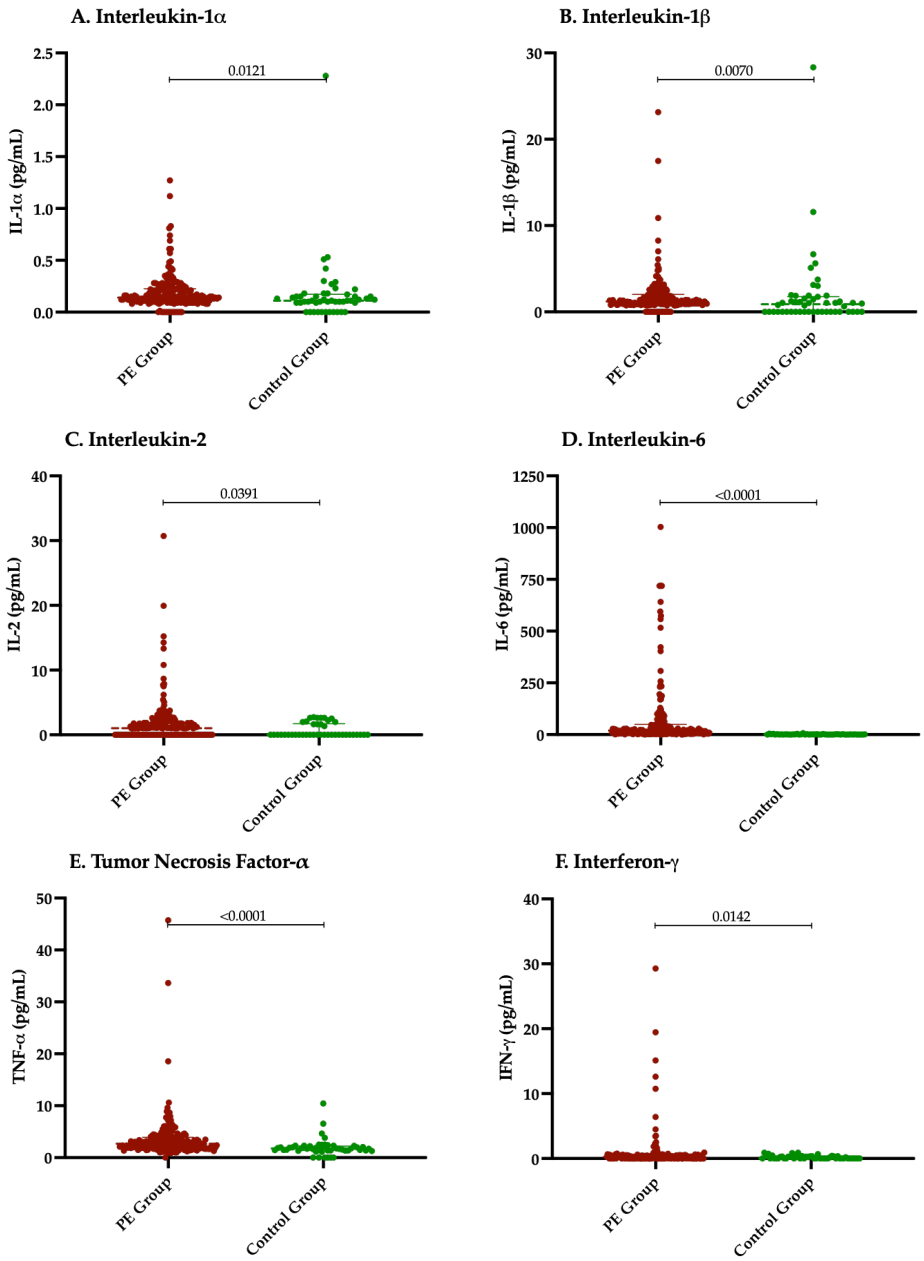


Figure 43. Scatter plot of IL-1α, IL-1β, IL-2, IL-6, TNF-α, and IFN-γ levels in PE patients versus healthy controls.

Points represent individual patient data with median and interquartile range (IQR). The biomarker levels between PE and control groups are compared by using Mann-Whitney U test, with a p value of <0.05 considered as the cutoff for significance.

5.5.1.2. *Anti- inflammatory Cytokines*

Anti-inflammatory cytokines including IL-4, and IL-10 were also measured in PE patients and compared to the controls (table 49). All these cytokines were significantly elevated in the PE patients compared to the control group. Both IL-4 and IL-10 exhibited a modest increase with a 0.13-fold and 0.60-fold relative to controls. The increase in the anti-inflammatory cytokines suggests an intricate inflammatory response, which may balance the inflammatory response we demonstrated in PE.

Table 49. Comparison of anti-inflammatory cytokines in PE patients versus healthy controls.

Biomarkers	PE Samples		Controls		P value	Fold Change
	N	Median (IQR)	N	Median (IQR)		
IL-4 (pg/mL)	193	1.8 (1.5-2.1)	50	1.6 (1.3-2.1)	0.0395	0.13
IL-10 (pg/mL)	193	0.8 (0.5-1.9)	50	0.5 (0.4-0.7)	<0.0001	0.60

The visual presentation of the anti-inflammatory cytokines for individual patients and controls is depicted in the scatter plots (figure 44). Scatter plot for the IL-4 and IL-10 in PE patients shows most of the patients are clustered towards the normal level with some extremely high levels which possibly are due to a severe or prolonged inflammation, depending on the individual condition or disease severity.

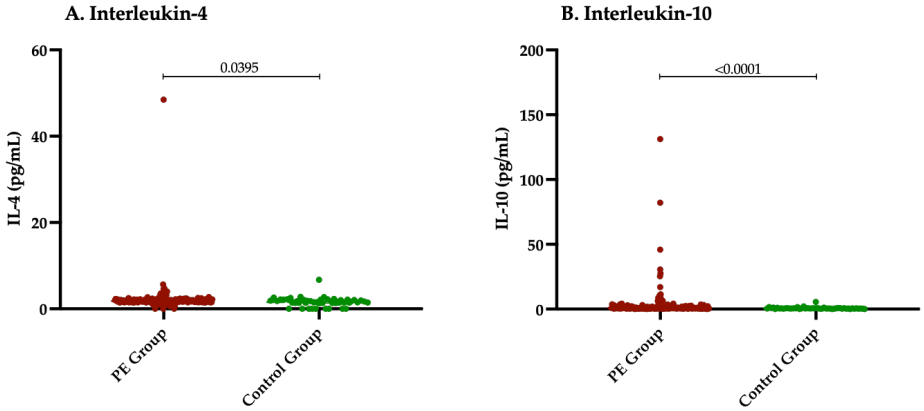


Figure 44. Scatter plot of IL-4, and IL-10 levels in PE patients versus healthy controls.

Points represent individual patient data with median and interquartile range (IQR). The biomarker levels between PE and control groups are compared by using Mann-Whitney U test, with a p value of <0.05 considered as the cutoff for significance.

5.5.1.3. *Chemokines*

Inflammatory chemokines including MCP-1 and IL-8 were significantly elevated in PE patients compared to the healthy controls (table 50). IL-8 showed a predominant increase in levels with 4.79-fold compared to control, MCP1 demonstrated a modest increase with a 0.35-fold. Increase in the chemokines in PE patients reflects an ongoing, active inflammation. The marked increase in IL-8 is associated with recruitment and activation of neutrophils, emphasizing the acute inflammation in PE.

Table 50. Comparison of inflammatory chemokines in PE patients versus healthy controls.

Biomarkers	*PE Samples*		*Controls*		*P value*	*Fold Change*
	N	*Median (IQR)*	*N*	*Median (IQR)*		
MCP-1 (pg/mL)	*193*	*133.5 (82.9-218.5)*	*50*	*98.7 (80.1-122.8)*	*0.0003*	*0.35*
IL-8 (pg/mL)	*193*	*16.2 (8.1-42.8)*	*50*	*2.8 (2.2-4.6)*	*<0.0001*	*4.79*

The scatter plots reflect the graphical presentation of the inflammatory chemokines for individual patients among the PE risk groups (figure 45). The plots for the MCP-1 and IL-8 in the PE patients depict the variation among individual patients especially towards the upper end. This could be due to a severe inflammatory response and active immune response in patients in the presence of comorbid conditions.

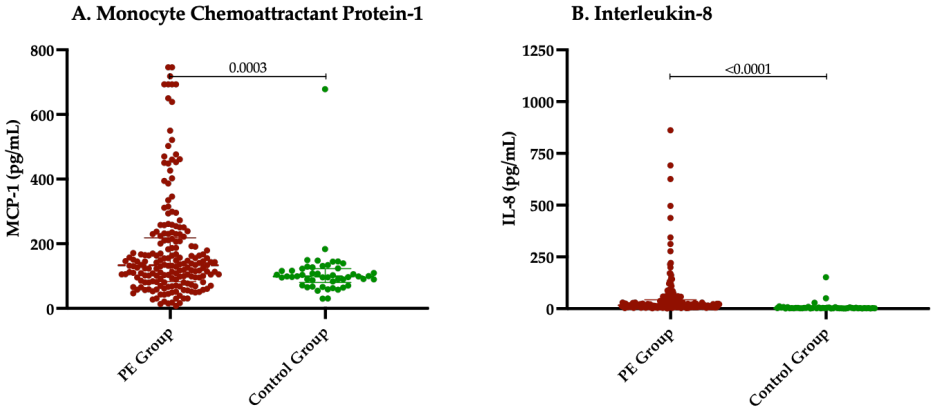


Figure 45. Scatter plot of MCP-1, and IL-8 levels in PE patients versus healthy controls.

Points represent individual patient data with median and interquartile range (IQR). The biomarker levels between PE and control groups are compared by using Mann-Whitney U test, with a p value of <0.05 considered as the cutoff for significance.

5.5.1.4. *Growth Factors*

Growth factors including the EGF and VEGF levels were significantly elevated in PE patients versus healthy controls (table 51). EGF levels were exceptionally high in PE patients with a 24.64-fold increase followed by VEGF with a 3.0-fold increase compared to the controls. Increase in these factors suggests the repair mechanism via tissue remodeling and angiogenesis in PE patients reflecting the damage, ischemia, hypoxia, and stress by the embolism and constant inflammation.

Table 51. Comparison of inflammatory cytokines in PE patients versus healthy controls.

Biomarkers	*PE Samples*		*Controls*		*P value*	*Fold Change*
	N	*Median (IQR)*	*N*	*Median (IQR)*		
EGF (pg/mL)	*193*	*35.9 (15.4-75.2)*	*50*	*1.4 (1.2-10.4)*	*<0.0001*	*24.64*
VEGF (pg/mL)	*193*	*25.2 (7.3-68.4)*	*50*	*6.3 (4.6-8.7)*	*<0.0001*	*3.0*

The graphical appearance of the data in the form of scatter plots for EGF and VEGF demonstrates variations among the patients (figure 46). Both EGF and VEGF are distributed widely with marked variations towards the high ends. The broad distribution reflects the variability among patients which could be due to the degree of the inflammatory response and the extent of tissue damage and repair.

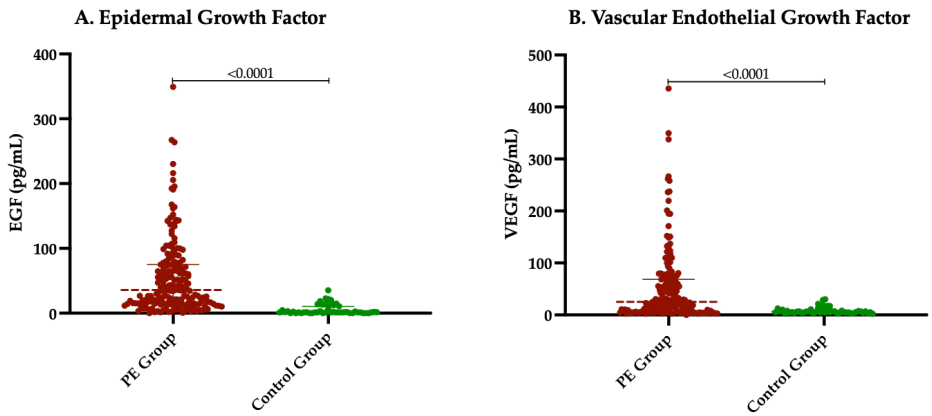


Figure 46. Scatter plot of EGF, and VEGF levels in PE patients versus healthy controls.

Points represent individual patient data with median and interquartile range (IQR). The biomarker levels between PE and control groups are compared by using Mann-Whitney U test, with a p value of <0.05 considered as the cutoff for significance.

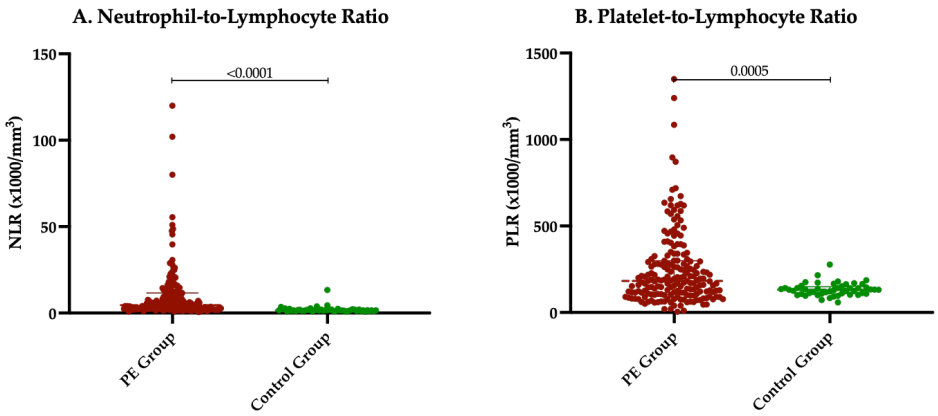
5.5.1.5. *Blood Cellular Indices*

Blood cellular indices including NLR, PLR, and SII were markedly increased in PE patients compared to controls (table 52). NLR demonstrated the most distinct alteration with a 1.81-fold increase compared to PLR with a 0.39-fold increase and SII with a 1.58-fold increase compared to controls. This suggests an overall systemic inflammation along with platelet activation in PE patients. Furthermore, NLR is considered to be a marker of systemic inflammation and immune response in PE patients.

Table 52. Comparison of blood cellular indices in PE patients versus healthy controls.

Cellular Indices	PE Samples		Controls		P value	Fold Change
	N	Median (IQR)	N	Median (IQR)		
NLR (x1000/mm³)	193	4.5 (2.6-11.7)	50	1.6 (1.2-2.0)	<0.0001	1.81
PLR (x1000/mm³)	193	182.7 (106.9-299.4)	50	131.3 (108.2-149.7)	0.0005	0.39
SII (x1000/mm³)	193	1147.5 (607.1-2633.0)	50	444.5 (311.0-537.5)	<0.0001	1.58

The scatter plots provide a graphical presentation of data in PE patients compared to the controls (figure 47). NLR, PLR, and SII all depict a similar distribution pattern with wide variability towards the upper limits in PE patients. These findings suggest substantial alterations in inflammatory and immune responses in PE patients and may be associated with PE severity or underlying inflammation.



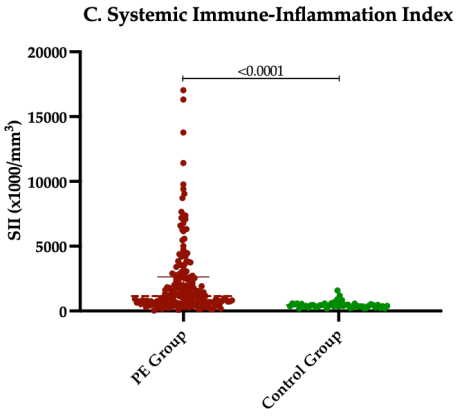


Figure 47. Scatter plot of NLR, PLR, and SII levels in PE patients versus healthy controls.

Points represent individual patient data with median and interquartile range (IQR). The biomarker levels between PE and control groups are compared by using Mann-Whitney U test, with a p value of <0.05 considered as the cutoff for significance.

5.5.2. Stratification of Biomarkers with Disease Severity

Plasma samples analyzed for the inflammatory cytokines, chemokines and growth factors were stratified and analyzed according to the ESC classification of PE which is presented in tables and figures. In the cohort of 193 PE patients, 15 (7.8%) patients were in the low-risk, 155 (80.7%) patients in the intermediate-risk, and 22 (11.5%) patients in the high-risk PE group. This stratification provides a detailed analysis of the pro-inflammatory cytokines, anti-inflammatory cytokines, chemokines, growth factors and blood cellular indices levels across different risk categories, which may help to identify individuals with varying levels of thrombotic activity in PE. The data is presented as median (IQR) in table 53-57, while diagrammatic presentation among patients in the form of scatter plots is given in figures 48-52, along with significance level.

5.5.2.1. Pro-inflammatory Cytokines

Pro-inflammatory cytokines including IL-1α, IL-1β, IL-2, IL-6, IL-8, TNF-α, and IFN-γ were stratified across PE severity (table 53). Wide variations for all the cytokines were noted between the risk groups. IL-1α levels modestly increased in low-risk PE with 0.19 (0.12-0.33) pg/mL compared to intermediate-risk with 0.14 (0.11-0.22) pg/mL and high-risk with 0.14 (0.09-0.22) pg/mL PE. IL-1β showed a

variation in the levels across the severity, with a decrease in intermediate-risk PE at 1.11 (0.81-1.82) pg/mL compared to low-risk PE with 1.47 (0.97-3.08) pg/mL but an increase for high-risk PE with 1.88 (1.04-2.71) pg/mL. IL-2 levels also increased with severity; however, all the changes were not significant. The levels of IL-6 also increased along with the increase in PE severity, with 13.30 (4.14-50.33) pg/mL for low-risk PE, 16.56 (5.75-36.87) pg/mL for intermediate-risk PE, and 186.49 (20.50-561.85) pg/mL for the high-risk PE, with a significant increase noted from low- to high-risk and intermediate- to high-risk PE groups. TNF-α levels varied across the different severities; the only significant increase was noted from intermediate-risk to high-risk PE with 2.63 (1.79-3.63) pg/mL and 3.06 (2.05-4.76) pg/mL respectively. IFN-γ demonstrated variations with an inverse relationship with PE severity, decreasing as the severity of PE increases. The observed variations underscore the complex interplay between the inflammation and disease progression.

Table 53. Comparison of pro-inflammatory cytokines in PE patients across different risk categories.

Biomarkers	*Low-risk PE*		*Intermediate-risk PE*		*High-risk PE*	
	N	*Median IQR)*	*N*	*Median (IQR)*	*N*	*Median (IQR)*
IL-1α (pg/mL)	*15*	*0.2 (0.1-0.3)*	*155*	*0.14 (0.1-0.2)*	*22*	*0.14 (0.1-0.2)*
IL-1β (pg/mL)	*15*	*1.5 (1.0-3.1)*	*155*	*1.1 (0.8-1.8)*	*22*	*1.9 (1.0-2.7)*
IL-2 (pg/mL)	*15*	*0.0 (0.0-2.9)*	*155*	*1.0 (0.0-1.8)*	*22*	*1.2 (0.0-2.8)*
IL-6 (pg/mL)	*15*	*13.0 (4.1-50.3)*	*155*	*16.7 (5.8-36.9)*	*22*	*186.5 (20.5-561.9)*
TNF-α (pg/mL)	*15*	*2.7 (1.8-5.8)*	*155*	*2.6 (1.8-3.6)*	*22*	*3.1 (2.1-4.8)*
IFN-γ (pg/mL)	*15*	*0.4 (0.0-0.8)*	*155*	*0.2 (0.0-0.4)*	*22*	*0.3 (0.0-0.6)*

The visual presentation of the data is presented in the scatter plots (figure 48). All the pro-inflammatory cytokines involving IL-1α, IL-1β, IL-2, IL-6, IL-8, TNF-α, and IFN-γ depict a similar distribution pattern among all the risk groups of PE. All the cytokines studied showed a wide scatter in the levels demonstrating variability among individuals, with some very high levels reflecting the heterogeneous nature of the inflammatory response in PE patients that could be due to other factors affecting the response.

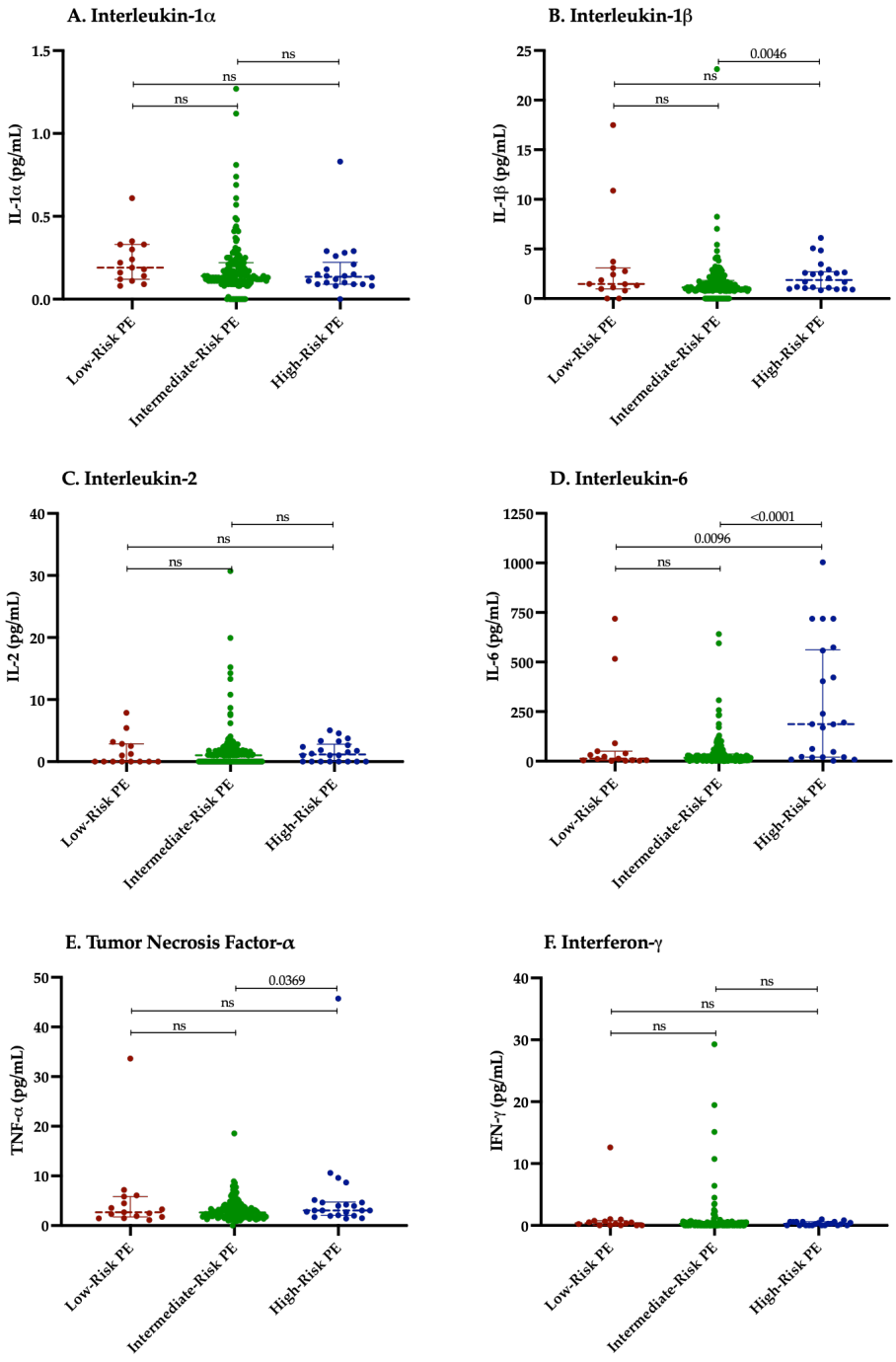


Figure 48. Scatter plot of IL-1α, IL-1β, IL-2, IL-6, TNF-α, and IFN-γ levels in PE patients across different risk categories.

Points represent individual patient data with median and interquartile range (IQR). The biomarker levels between PE severity groups are compared by using Mann-Whitney U test, with a p value of <0.05 considered as the cutoff for significance.

*5.5.2.2. **Anti-inflammatory Cytokines***

The levels of anti-inflammatory cytokines including IL-4 and IL-10 were stratified among PE severity groups (table 54). IL-4 levels decreased with increase in risk from low-risk PE 2.04 (1.57-2.17) pg/mL to intermediate-risk 1.72 (1.51-2.04) pg/mL and high-risk PE 2.02 (1.67-2.50) pg/mL but then increased significantly from intermediate-risk PE to high-risk PE. The change in the IL-10 levels across PE severity also varied, with a decrease in levels from low-risk PE 1.21 (0.86-2.22) pg/mL to intermediate-risk PE 0.79 (0.50-1.43) pg/mL, while there was an increase in high-risk PE 4.06 (0.61-28.23) pg/mL. However, the levels of IL-10 increased significantly in high-risk PE compared to the intermediate-risk PE . These variations suggest a possible reduction in the anti-inflammatory response which escalates in more severe cases of PE.

Table 54. Comparison of anti-inflammatory cytokines in PE patients across different risk categories.

Biomarkers	*Low-risk PE*		*Intermediate-risk PE*		*High-risk PE*	
	N	*Median IQR)*	*N*	*Median (IQR)*	*N*	*Median (IQR)*
IL-4 (pg/mL)	*15*	*2.0 (1.6-2.2)*	*155*	*1.7 (1.5-2.0)*	*22*	*2.0 (1.7-2.5)*
IL-10 (pg/mL)	*15*	*1.2 (0.9-2.2)*	*155*	*0.8 (0.5-1.4)*	*22*	*4.1 (0.6-28.2)*

The levels of cytokines are also graphically presented in the form of scatter plots (figure 49). Both IL-4, and IL-10 levels are mostly clustered towards the normal range in all PE risk groups, with some high values, reflecting the variability among individual patient responses.

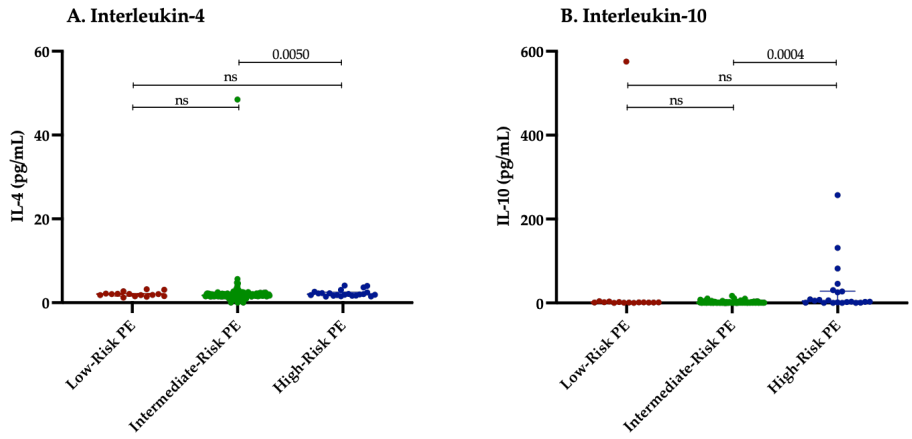


Figure 49. Scatter plot of IL-4, and IL-10 levels in PE patients across different risk categories.

Points represent individual patient data with median and interquartile range (IQR). The biomarker levels between PE severity groups are compared by using Mann-Whitney U test, with a p value of <0.05 considered as the cutoff for significance.

5.5.2.3. *Chemokines*

The inflammatory chemokine levels also showed a wide range across the PE severity (table 55). MCP-1 levels constantly increased among the risk group of PE with 124.0 (93.7-250.8) pg/mL for low-risk PE, 130.1 (80.8-183.2) pg/mL for intermediate, and 313.4 (126.3-641.7) pg/mL for high-risk PE, increasing from low-risk to high-risk PE and intermediate-risk to high-risk PE showed with significance. The levels for IL-8 varied with the risk with the levels decreased from low-risk PE 15.6 (11.79-37.2) pg/mL to intermediate-risk PE 14.7 (7.1-35.2) pg/mL, while it increased for the high-risk PE 33.8 (16.8-115.5) pg/mL group. Among all these changes only the increase from intermediate-risk to high-risk PE was significant.

Table 55. Comparison of inflammatory chemokines in PE patients across different risk categories.

Biomarkers	*Low-risk PE*		*Intermediate-risk PE*		*High-risk PE*	
	N	*Median IQR)*	*N*	*Median (IQR)*	*N*	*Median (IQR)*
MCP-1 (pg/mL)	*15*	*124.0 (93.7-250.8)*	*155*	*130.1 (80.8-183.2)*	*22*	*313.4 (126.3-641.7)*
IL-8 (pg/mL)	*15*	*15.6 (11.79-37.2)*	*155*	*14.7 (7.1-35.2)*	*22*	*33.8 (16.8-115.5)*

The chemokine levels among individual patients are represented as scatter plots (figure 50). The MCP-1 levels among individuals showed skewness across severity, with some low and high levels, in contrast IL-8 levels are mostly clustered

with some extremely elevated values in all risk groups. These variations reflect a diverse variability in inflammatory responses among patients underscoring the overall heterogenicity of the PE disease.

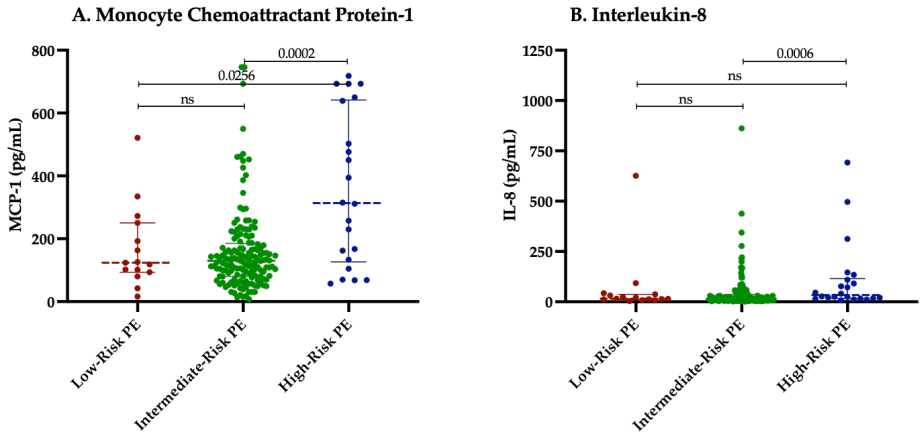


Figure 50. Scatter plot of MCP-1, and IL-8 levels in PE patients across different risk categories.

Points represent individual patient data with median and interquartile range (IQR). The biomarker levels between PE severity groups are compared by using Mann-Whitney U test, with a p value of <0.05 considered as the cutoff for significance.

5.5.2.4. *Growth Factors*

The levels of growth factors including EGF and VEGF were stratified across PE severity (table 56). The levels of EGF decreased with respect to the increase in severity of PE, with 61.9 (24.0-142.4) pg/mL, 34.96 (15.4-68.9) pg/mL, and 26.21 (12.4-80.8) pg/mL respectively. VEGF levels also decreased with increasing severity with 45.3 (20.1-72.4) pg/mL, 24.35 (6.3-66.3) pg/mL, 24.52 (6.2-103.5) pg/mL, respectively. All the changes in EGF and VEGF levels were not significant. The observed decrease in the levels of growth factors with increase in PE severity provides insight into their role in the pathophysiology of PE; however, the non-significant nature of these changes could be due to the sample size or distribution within each severity group.

Table 56. Comparison of growth factors in PE patients across different risk categories.

Biomarkers	*Low-risk PE*		*Intermediate-risk PE*		*High-risk PE*	
	N	*Median IQR)*	*N*	*Median (IQR)*	*N*	*Median (IQR)*

EGF (pg/mL)	*15*	*61.9 (24.0-142.4)*	*155*	*34.96 (15.4-68.9)*	*22*	*26.21 (12.4-80.8)*
VEGF (pg/mL)	*15*	*45.3 (20.1-72.4)*	*155*	*24.35 (6.3-66.3)*	*22*	*24.52 (6.2-103.5)*

The graphical demonstration of EGF and VEGF levels is presented in scatter plot (figure 51). The plot for both the EGF and VEGF showed a wide distribution pattern among all risk groups of PE with some individuals exhibiting extremely high and low levels in each of the risk groups. This variation reflects distinct responses among individual patients, which could be due to the influence of several factors affecting them.

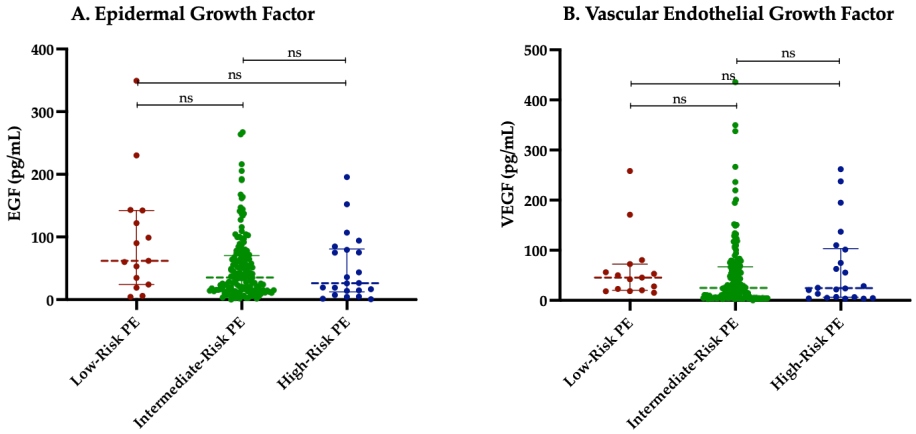


Figure 51. Scatter plot of EGF, and VEGF levels in PE patients across different risk categories.

Points represent individual patient data with median and interquartile range (IQR). The biomarker levels between PE severity groups are compared by using Mann-Whitney U test, with a p value of <0.05 considered as the cutoff for significance.

*5.5.2.5. **Blood Cellular Indices***

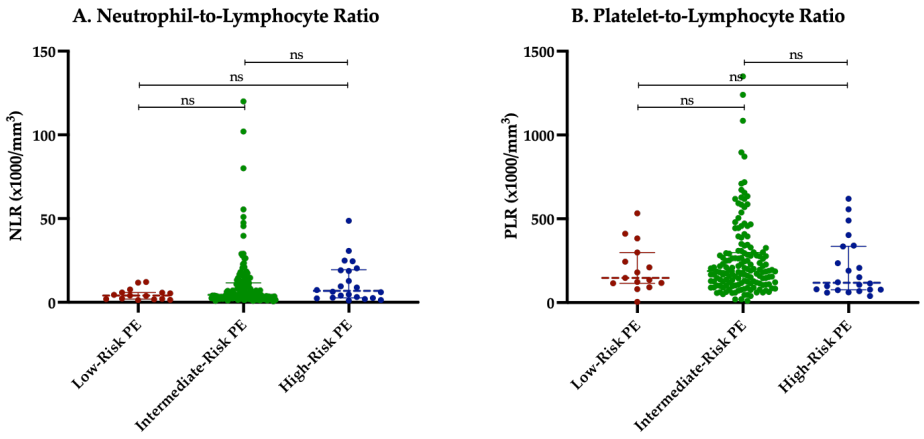
Blood cellular indices in this cohort of patients showed variations in the levels across risk groups (table 57). NLR levels increased with the PE groups, from 4.00 (1.96-5.85) x1000/mm^3 4.5 (2.7-11.6) x1000/mm^3 and 6.86 (2.7-19.4) x1000/mm^3 respectively. PLR levels also increased from low-risk PE 147.7 (115.0-298.8) x1000/mm^3 to intermediate-risk PE 189.1 (115.8-300.0) x1000/mm^3, which decreased for the high-risk PE 117.9 (76.4-336.3) x1000/mm^3 group. SII demonstrated a consistent increase in the levels with 962.9 (432.0-2643.2) x1000/mm^3, 1147.5 (628.5-2622.8) x1000/mm^3, and 1458.5 (582.1-3038.7) x1000/mm^3, respectively. However, all the changes did not reach to the level of significance. Although these indices

reflect the immune-inflammation response in PE, their utility in distinguishing risk severities is limited, possibly because of a low number of patients in low-risk and high-risk PE groups.

Table 57. Comparison of inflammatory markers in PE patients across different risk categories.

Biomarkers	Low-risk PE		Intermediate-risk PE		High-risk PE	
	N	Median IQR)	N	Median (IQR)	N	Median (IQR)
NLR (x1000/mm³)	15	4.00 (1.96-5.85)	155	4.5 (2.7-11.6)	22	6.86 (2.7-19.4)
PLR (x1000/mm³)	15	147.7 (115.0-298.8)	155	189.1 (115.8-300.0)	22	117.9 (76.4-336.3)
SII (x1000/mm³)	15	962.9 (432.0-2643.2)	155	1147.5 (628.5-2622.8)	22	1458.5 (582.1-3038.7)

The visual presentation of the NLR, PLR and SII levels among patients with different risk groups of PE is depicted in the scatter plots (figure 52). The plots reveal a broad range in the levels for all the indices with extreme values towards the upper ends in each of the risk groups. This variability highlights the varied immune and inflammatory response at the different stage of the conditions.



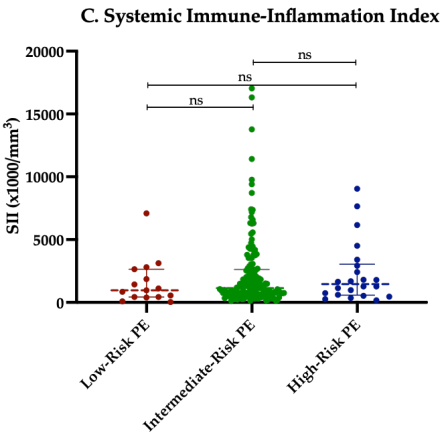


Figure 52. Scatter plot of NLR, PLR, and SII levels in PE patients across different risk categories.

Points represent individual patient data with median and interquartile range (IQR). The biomarker levels between PE severity groups are compared by using Mann-Whitney U test, with a p value of <0.05 considered as the cutoff for significance.

5.5.3. Relationship Among the Biomarker and Cellular Indices

All the inflammatory cytokines, chemokines, and growth factors along with cellular indices were analyzed to demonstrate their interrelationship. This comprehensive analysis elucidates how these inflammatory markers and cellular indices interact with one another, providing insight into their combined impact on the inflammatory response in the overall pathophysiology of PE. Notable correlations were observed between various cytokines, chemokines, and growth factors, highlighting the complex interplay in patients with acute PE (figure 53).

Pro-inflammatory cytokines demonstrated positive but varying levels of relationship between each of them. Of note, IL-1α correlated with IL-1β (r: 0.37; p value: <0.0001), and IL-2 (r: 0.22; p value: 0.0023); IL-1β relates with IL-2 (r: 0.37; p value: <0.0001), IL-6 (r: 0.21; p value: 0.0025), TNF-α (r: 0.39; p value: <0.0001), and IFN-γ (r: 0.25; p value: 0.0003); IL-2 with TNF-α (r: 0.25; p value: 0.0004), and IFN-γ (r: 0.28; p value: <0.0001); IL-6 moderately correlates with TNF-α (r: 0.30; p value: <0.0001); and TNF-α with IFN-γ (r: 0.35; p value: <0.0001). These relationships reflect an interconnected and a robust relationship between various pro-inflammatory cytokines in PE.

Pro-inflammatory cytokines demonstrated varying levels of correlation with anti-inflammatory cytokines. For instance, IL-1β correlated with IL-4 (r: 0.34; p value: <0.0001), and IL-10, (r: 0.33; p value: <0.0001); IL-2 showed weak correlation with IL-10 (r: 0.21; p value: 0.0032); IL-6 demonstrated moderate correlations with IL-10 (r: 0.40; p value: <0.0001); TNF-α with IL-4 (r: 0.26; p value: 0.0001), and IL-10 (r: 0.34; p value: <0.0001). All the correlations demonstrated an interrelationship between pro- and anti-inflammatory cytokines.

Inflammatory chemokines exhibited significant correlations with cytokines including both pro- and anti-inflammatory cytokines. Notable correlations were as follows, MCP-1 with IL-1β (r: 0.27; p value: 0.0001), IL-6 (r: 0.30; p value: <0.0001), TNF-α (r: 0.45; p value: <0.0001), IFN-γ (r: 0.23; p value: 0.0010), IL-4 (r: 0.24; p value: 0.0008), and IL-10 (r: 0.33; p value: <0.0001); IL-8 with IL-1β (r: 0.49; p value: <0.0001), IL-6 (r: 0.45; p value: <0.0001), TNF-α (r: 0.60; p value: <0.0001), and IL-10 (r: 0.45; p value: <0.0001). These findings explain the interrelatedness between the inflammatory cytokines and chemokines in PE, highlighting the integral role in determining the inflammatory milieu and the impact in progression of PE. MCP-1 correlated with IL-8 (r: 0.49; p value: <0.0001) demonstrating the recruitment and activation of immune cells in PE patients.

Growth factors also showed varying levels of association with inflammatory cytokines and chemokines. Markedly, EGF correlated with TNF-α (r: 0.23; p value: 0.0014), and IL-8 (r: 0.25; p value: 0.0003); VEGF correlated with IL-1β (r: 0.21, p value: 0.0035), TNF-α (r: 0.27; p value: 0.0001), and IL-8 (r: 0.30; p value: <0.0001). These findings reflect the role of growth factors in the inflammatory response in PE

Blood cellular indices demonstrated positive correlations with inflammatory cytokines and chemokines. NLR demonstrated a significant correlation with IL-6 (r: 0.44; p value: <0.0001), IL-10 (r: 0.41; p value: <0.0001), and IL-8 (r: 0.26; p value: 0.0003). SII relates with IL-6 (r: 0.43; p value: <0.0001), and IL-10 (r: 0.32; p value: <0.0001). The relationship between the cytokines and chemokines reflects their overall role in inflammation and systemic immune responses in PE.

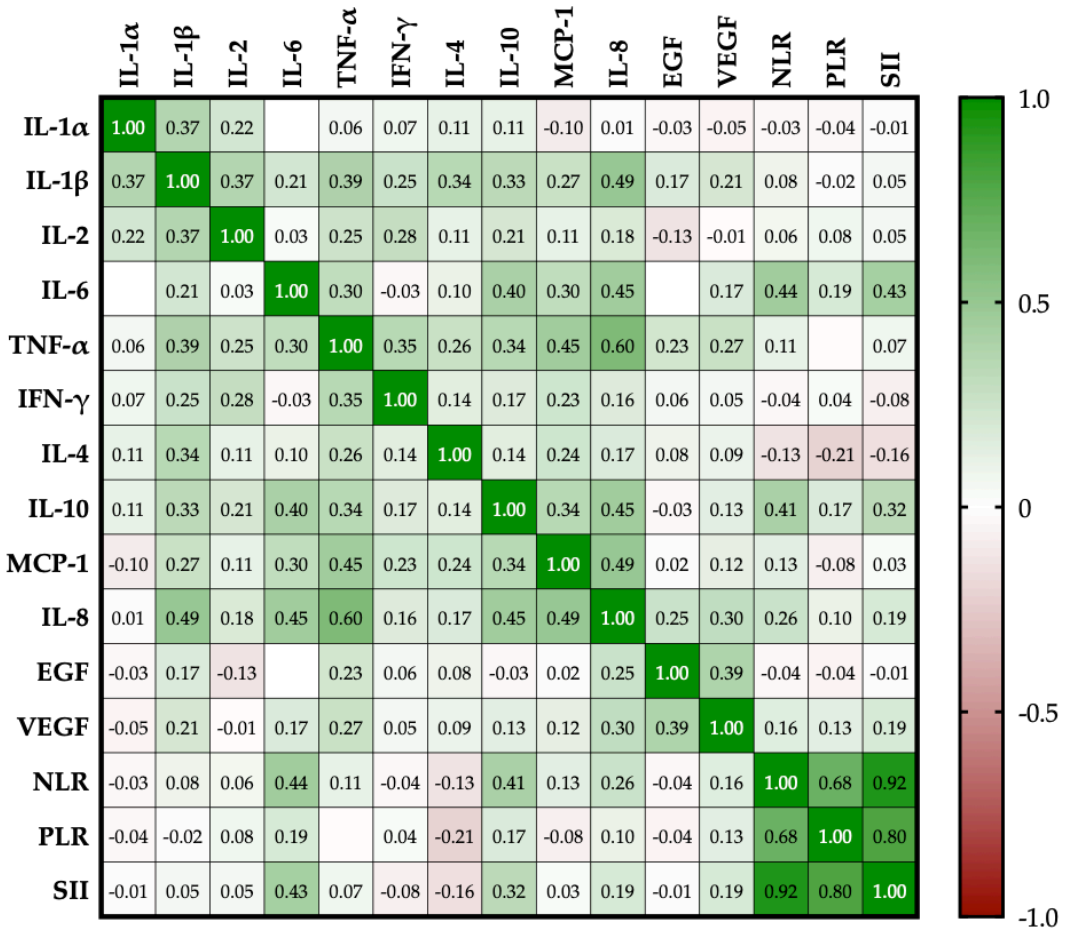


Figure 53. Spearman correlation matrix illustrating the relationship between inflammatory cytokines and chemokines, growth factors and cellular indices in patients with PE.

Correlation analysis displays the relationships among various biomarkers and cellular indices. Positive correlations are shown in green color indicating a direct association. while the negative correlations are depicted in maroon color representing an inverse association. The strength of these correlations is illustrated by the intensity of the color with darker shades denoting stronger correlations. Correlations with a p-value <0.05 considered as statistically significant.

5.5.4. Association of Biomarkers with Mortality

Inflammatory cytokine, chemokine and growth factor levels were further analyzed with reference to 30-day mortality. In the cohort of 193 patients, 27 patients (14.2%) died during the first 30-days. The stratification of data underscores the importance of these biomarkers and indices in predicting short-term outcomes. The data are presented with median (IQR), fold changes, together with significance

levels. Patients who died with in the first 30-days showed marked decreases in the levels as detailed in tables 58-62 and depicted as figure 54-58.

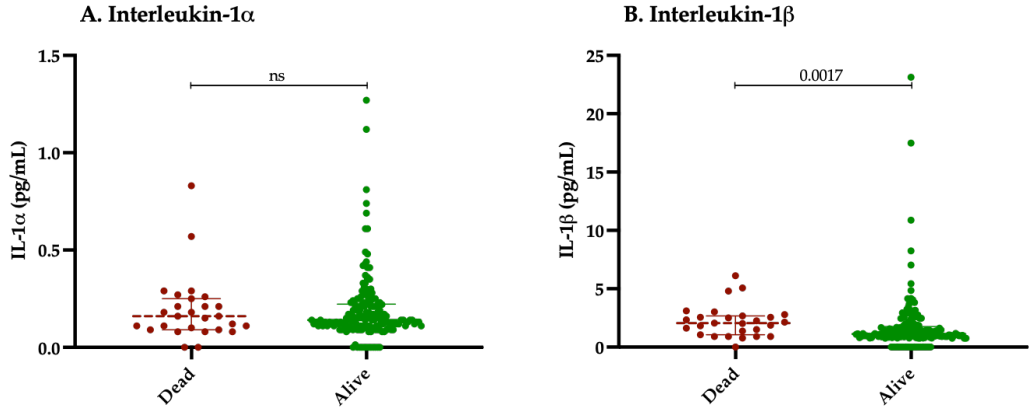
5.5.4.1. *Pro-inflammatory Cytokines*

The biomarkers of pro-inflammatory cytokines were elevated in PE patients who died within the first 30 days of diagnosis (table 58). The levels of IL-1β, IL-2, IL-6, TNF-α, and IFN-γ were significantly increased in these patients. The increase in the levels highlight the severe and intricate immune response in the patients who died.

Table 58. Comparison of pro-inflammatory cytokines in PE patients by 30-day mortality status.

Biomarkers	*Dead*		*Alive*		*P value*
	N	*Median (IQR)*	*N*	*Median (IQR)*	
IL-1α (pg/mL)	*27*	*0.2 (0.1-0.3)*	*166*	*0.14 (0.1-0.2)*	*NS*
IL-1β (pg/mL)	*27*	*2.1 (1.1-2.7)*	*166*	*1.13 (0.8-1.8)*	*0.0017*
IL-2 (pg/mL)	*27*	*1.8 (0.0-3.6)*	*166*	*0.99 (0.0-1.8)*	*0.0052*
IL-6 (pg/mL)	*27*	*73.5 (23.96-238.97)*	*166*	*15.3 (5.1-36.9)*	*<0.0001*
TNF-α (pg/mL)	*27*	*3.2 (2.3-5.96)*	*166*	*2.6 (1.8-3.8)*	*0.0063*
IFN-γ (pg/mL)	*27*	*0.3 (0.2-0.5)*	*166*	*0.2 (0.0-0.5)*	*NS*

The visual presentation for all the pro-inflmammatory cytokines are presented in the form of scatter plots (figure 54). PE patients who died within the first 30 days varied widely with some high levels of pro-inflammatory cytokines, in contrast the cytokine levels for patients who survived were skewed with a broader range



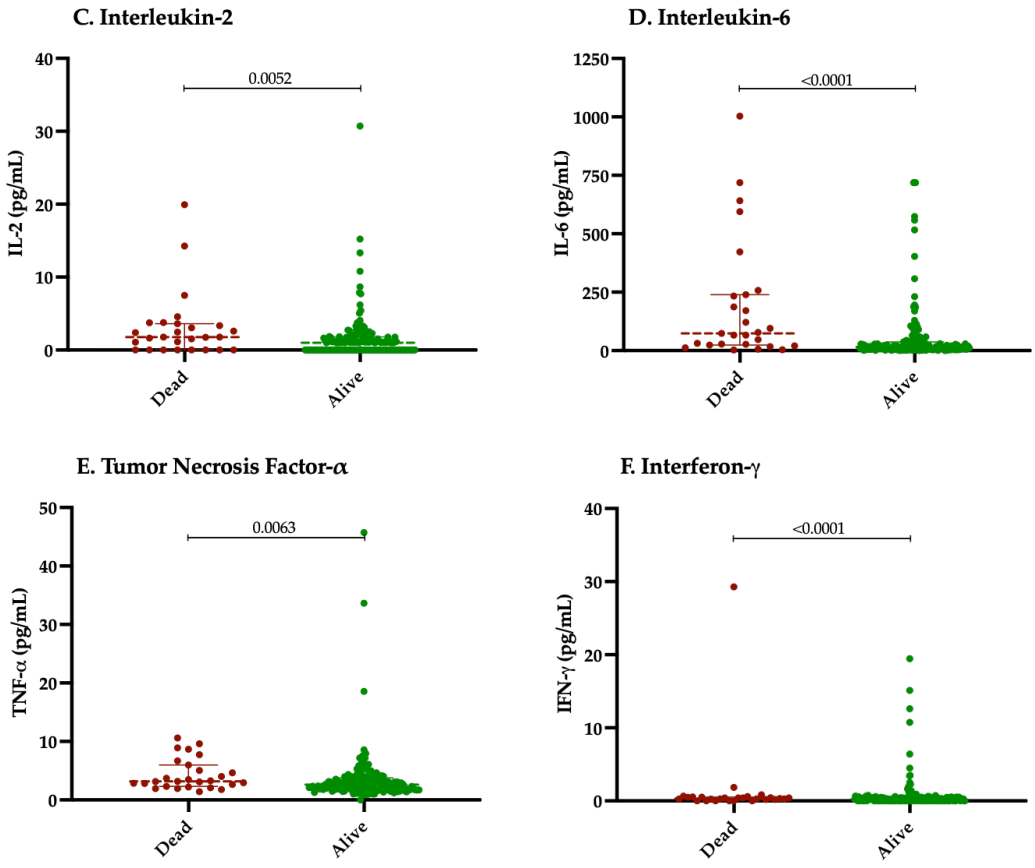


Figure 54. Scatter plot of IL-1α, IL-1β, IL-2, IL-6, TNF-α, and IFN-γ levels in PE patients by 30-day mortality status.

Points represent individual patient data with median and interquartile range (IQR). The biomarker levels in PE patients across 30-day mortality are compared by using Mann-Whitney U test, with a p value of <0.05 considered as the cutoff for significance.

5.5.4.2. *Anti-inflammatory Cytokines*

Anti-inflammatory cytokines also increased in patients who died within the first 30 days of diagnosis of PE (table 59). IL-4 demonstrated a modest increase in the levels while IL-10 showed significant and marked increase in the levels in patients who died. This indicates the considerable role of IL-10 towards the progression and adverse outcomes in PE patients, highlighting that inflammation and immune response are critical components in the pathophysiology of PE.

Table 59. Comparison inflammatory cytokines in PE patients by 30-day mortality status.

Biomarkers	*Dead*		*Alive*		*P value*
	N	*Median (IQR)*	*N*	*Median (IQR)*	
IL-4 (pg/mL)	*27*	*1.9 (1.5-2.3)*	*166*	*1.8 (1.5-2.1)*	*NS*
IL-10 (pg/mL)	*27*	*3.0 (1.0-10.0)*	*166*	*0.8 (0.5-1.5)*	*<0.0001*

The graphical distribution for the data is depicted in the form of scatter plots (figure 55). Scatter plots for both IL-4 and IL-10 show a central tendence among individuals with some high values for those who died. This suggests a possible link between the biomarkers and mortality.

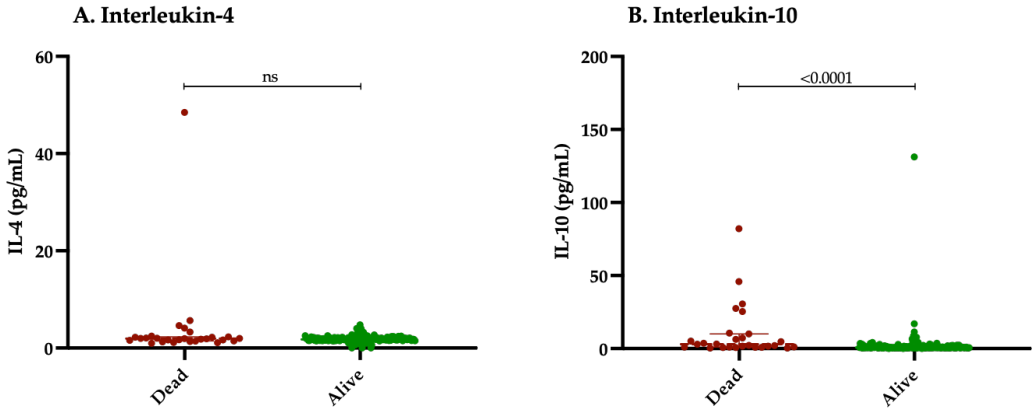


Figure 55. Scatter plot of IL-4, and IL-10 levels in PE patients by 30-day mortality status.

Points represent individual patient data with median and interquartile range (IQR). The biomarker levels in PE patients across 30-day mortality are compared by using Mann-Whitney U test, with a p value of <0.05 considered as the cutoff for significance.

*5.5.4.3. **Chemokines***

Inflammatory chemokines including MCP-1, and IL-8 levels significantly decreased in PE patients who died within the first 30 days of diagnosis (table 60). The marked increase in the levels suggest a significant inflammatory response which may be associated with disease severity and potentially contribute to the adverse outcome in PE patients.

Table 60. Comparison of inflammatory chemokines in PE patients by 30-day mortality status.

Biomarkers	*Dead*	*Alive*	*P value*

	N	Median (IQR)	N	Median (IQR)	
MCP-1 (pg/mL)	27	229.9 (147.1-502.4)	166	123.0 (79.2-187.9)	<0.0001
IL-8 (pg/mL)	27	57.6 (25.1-146.4)	166	14.2 (7.3-30.9)	<0.0001

The graphical presentation for the MCP-1 and IL-8 in the form of scatter plots depicts wide variations in the levels (figure 56). MCP-1 levels show a skewness with extremely high levels in both the upper and lower end indicating significant variability among individuals. IL-8 levels among patients are uniform and are mostly centered towards the normal range with some high levels.

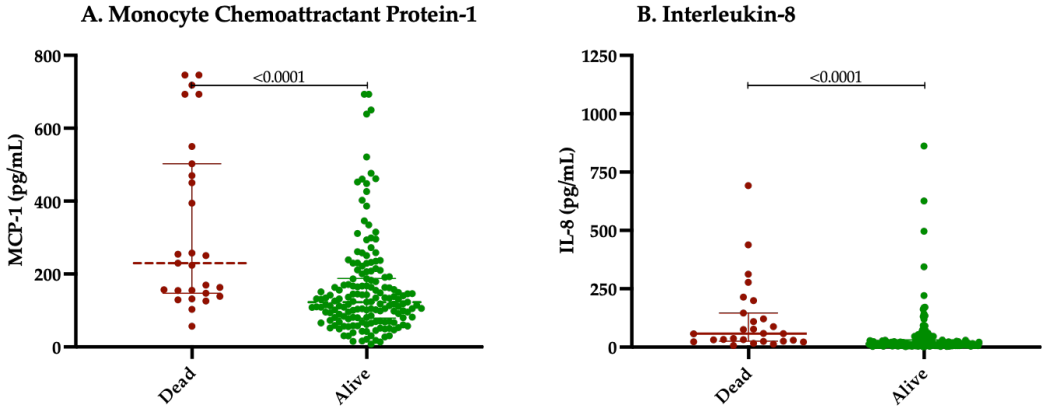


Figure 56. Scatter plot of MCP-1, and IL-8 levels in PE patients by 30-day mortality status.

Points represent individual patient data with median and interquartile range (IQR). The biomarker levels in PE patients across 30-day mortality are compared by using Mann-Whitney U test, with a p value of <0.05 considered as the cutoff for significance.

5.5.4.4. *Growth Factors*

The growth factor levels also increased in the patients who died within the first 30 days of PE diagnosis (table 61), but the increase was not significant. Even though the increase in the levels did not reach significance, the increase in the levels demonstrated the progression of the disease.

Table 61. Comparison of growth factors in PE patients by 30-day mortality status.

Biomarkers	Dead		Alive		P value
	N	Median (IQR)	N	Median (IQR)	
EGF (pg/mL)	27	34.2 (14.0-98.0)	166	36.7 (15.5-73.9)	NS
VEGF (pg/mL)	27	48.2 (10.6-121.7)	166	24.6 (6.3-63.5)	NS

The scatter plots for the EGF and VEGF showed variations in both the survivor and non-survivor groups (figure 57). EGF and VEGF both showed skewness in the data especially towards the upper end.

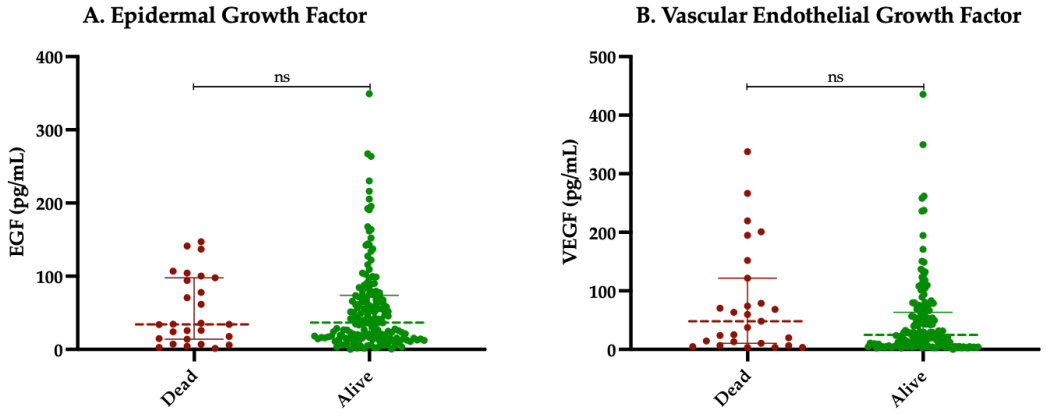


Figure 57. Scatter plot of EGF, and VEGF levels in PE patients by 30-day mortality status.

Points represent individual patient data with median and interquartile range (IQR). The biomarker levels in PE patients across 30-day mortality are compared by using Mann-Whitney U test, with a p value of <0.05 considered as the cutoff for significance.

5.5.4.5. *Blood Cellular Indices*

All the cellular indices studied were significantly high in the patients who died within the first 30-day of diagnosis of PE (table 62). Elevated ratios suggest an intensified inflammatory and immune response, reflecting their potential critical role as a prognostic marker.

Table 62. Comparison of blood cellular indices in PE patients by 30-day mortality status.

Cellular Indices	*Dead*		*Alive*		*P value*
	N	*Median (IQR)*	*N*	*Median (IQR)*	
NLR (x1000/mm³)	*27*	*16.0 (8.9-23.4)*	*166*	*4.2 (2.5-9.3)*	*<0.0001*
PLR (x1000/mm³)	*27*	*308.0 (167.4-480.0)*	*166*	*172.0 (102.1-277.6)*	*0.0081*
SII (x1000/mm³)	*27*	*2464.0 (1641.5-4270.0)*	*166*	*971.3 (536.2-2025.0)*	*0.0022*

The graphical representation of the individual patients is presented in the scatter plots (figure 58). All the cellular indices exhibited a similar distribution pattern, with patients who survived showing a tendency towards normal values

compared to those who died who presented with skewness, indicating differences in the inflammatory and immune responses between the two groups.

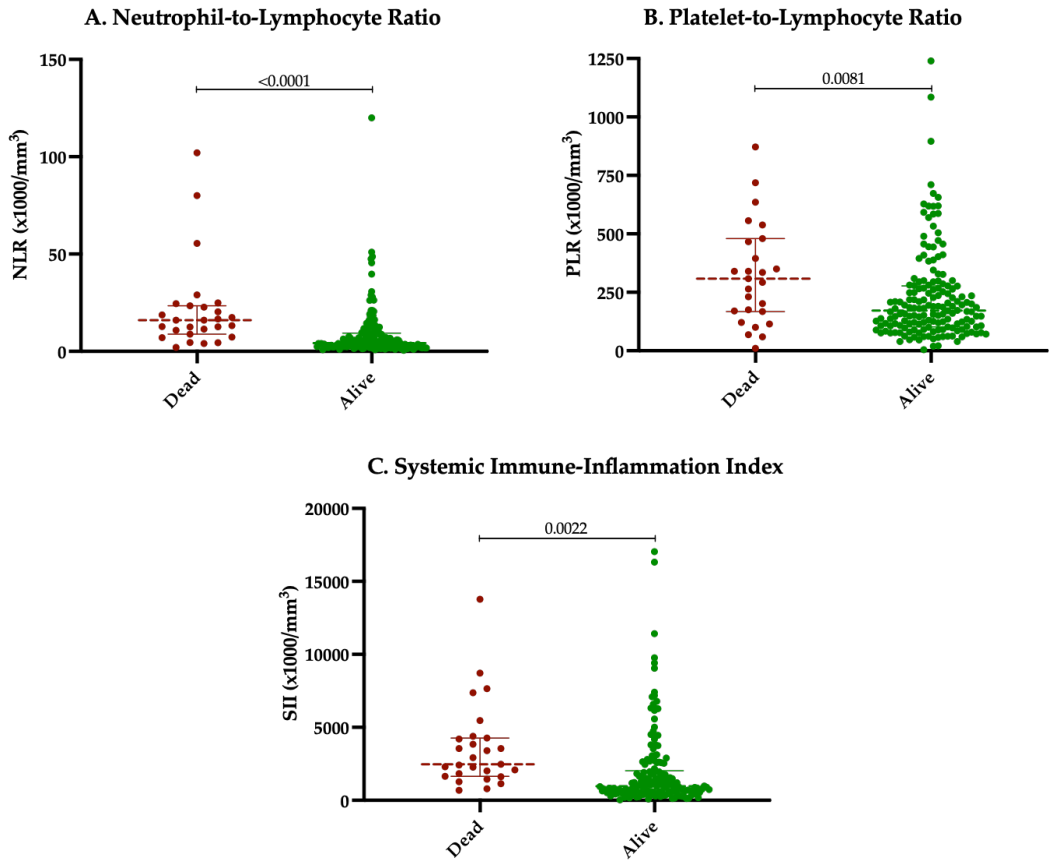


Figure 58. Scatter plot of NLR, PLR, and SII levels in PE patients by 30-day mortality status.

Points represent individual patient data with median and interquartile range (IQR). The biomarker levels in PE patients across 30-day mortality are compared by using Mann-Whitney U test, with a p value of <0.05 considered as the cutoff for significance.

All the cytokines, chemokines, and growth factors were analyzed for 30-day mortality by using the ROC curve analysis. The area under the ROC curve for all the biomarkers and their association with 30-day mortality are presented in the appendix 5 (supplementary figure 12-16).The optimal cutoff values for the pro-inflammatory cytokines associated with 30-day mortality were: IL-1β >1.4 pg/mL (AUC: 0.685; 95% CI: 0.579-0.792; p value: 0.001), IL-2 >1.4 pg/mL (AUC: 0.659; 95% CI: 0.539-0.779; p value: 0.010), IL-6 >65 pg/mL (AUC: 0.752; 95% CI: 0.649-0.855; p

value: 0.000), TNF-α >2.0 pg/mL (AUC: 0.663; 95% CI: 0.557-0.768; p value: 0.003). Exceptions were IL-1α (AUC: 0.492; 95% CI: 0.367-0.617; p value: NS) and IFN-γ (AUC: 0.593; 95% CI: 0.485-0.701; p value: NS) that were not associated with 30-day mortality. Anti-inflammatory cytokines such IL-10 >1.6 pg/mL (AUC: 0.760; 95% CI: 0.656-0.865; p value: 0.000) were linked with 30-day mortality while IL-4 (AUC: 0.566; 95% CI: 0.436-0.696; p value: NS) was not related with 30-day mortality. Chemokines including MCP-1 >125.0 pg/mL (AUC: 0.764; 95% CI: 0.673-0.855; p value: 0.000), and IL-8 >22.0 pg/mL (AUC: 0.810; 95% CI: 0.734-0.886; p value: 0.000) were associated with 30-day mortality in patients with PE. Both studied growth factors EGF (AUC: 0.494; 95% CI: 0.368-0.620; p value: NS), and VEGF (AUC: 0.593; 95% CI: 0.469-0.718; p value: NS) were not linked with mortality. Blood cellular indices including NLR >7 (AUC: 0.813; 95% CI: 0.734-0.892; p value: 0.000), PLR >220 (AUC: 0.658; 95% CI: 0.538-0.778; p value: 0.010), and SII (AUC: 0.772; 95% CI: 0.697-0.847; p value: 0.000) were associated with 30-day mortality in patients with PE.

Using the optimal cutoff values from the ROC curve analysis with the sensitivity and specificity, all the significant markers were categorized and transformed into a categorical variable. Univariate analysis using the Chi-square test, the association of markers with 30-day mortality were performed. On univariable analysis, elevated levels of cytokines and chemokines including IL-1β, IL-2, IL-6, TNF-α, IL-10, MCP-1, and IL-8 and blood cellular indies including NLR, PLR, and SII were significantly associated with 30-day mortality (table 63). Specifically, the highest odds ratios are observed for IL-8 >22 pg/mL (OR: 15.714; 95% CI: 4.536-54.444), MCP-1 >125 pg/mL (OR: 13.438; 95% CI: 3.083-58.566), NLR >7.0 x1000/mm^3 (OR: 12.606; 95% CI: 4.149-38.300), and SII >1600 x1000/mm^3 (OR: 9.381; 95% CI: 3.368-26.130). These findings underscore the substantial prognostic value of inflammatory cytokines, chemokines, and blood cellular indices in predicting 30-day mortality in PE patients.

Table 63. Univariable Chi-Square analysis of cytokines, chemokines, and cellular index with 30-day mortality.

Biomarkers	*N (%)*	*Odds Ratio (95% CI)*	*P value*
IL-1β >1.4 (pg/mL)	*19 (70.4%)*	*4.542 (1.873-11.015)*	*<0.001*
IL-2 >1.4 (pg/mL)	*17 (63.0%)*	*3.944 (1.688-9.214)*	*<0.001*
IL-6 >48.0 (pg/mL)	*12 (16.0%)*	*3.453 (1.318-9.052)*	*0.009*
TNF-α >2.0 (pg/mL)	*11 (40.4%)*	*2.669 (1.135-6.277)*	*0.021*

IL-10 >1.6 (pg/mL)	*18 (66.7%)*	*7.222 (2.992-17.433)*	*<0.001*
MCP-1 >125 (pg/mL)	*25 (92.6%)*	*13.438 (3.083-58.566)*	*<0.001*
IL-8 >22.0 (pg/mL)	*24 (88.9%)*	*15.714 (4.536-54.444)*	*<0.001*
NLR >7.0 (x1000/mm³)	*23 (85.2%)*	*12.606 (4.149-38.300)*	*<0.001*
PLR >220.0 (x1000/mm³)	*17 (63.0%)*	*3.166 (1.361-7.360)*	*0.006*
SII >1600.0 (x1000/mm³)	*22 (81.5%)*	*9.381 (3.368-26.130)*	*<0.001*

All the significant markers from the univariate analysis including IL-1β, IL-2, IL-6, TNF-α, IL-10, MCP-1, IL-8 and cellular indices along with clinical variables were modelled into the binary logistic regression analysis by using the backward stepwise method. On multivariable analysis, all tested cytokines, chemokines and cellular indices were independently associated with 30-day all-cause death (figure 59). The highest odd's ratio noted are as follows: IL-8 >22 pg/mL (OR: 12.86; 95% CI: 3.71-44.60) MCP-1 >125 pg/mL (OR: 11.61; 95% CI: 2.64-51.11), NLR >7 (OR: 11.85; 95% CI: 3.84-35.56), SII >1600 (OR: 11.57 (95% CI: 3.75-35.71), and IL-10 >1.6 pg/mL (OR: 6.82; 95% CI: 2.81-16.58).

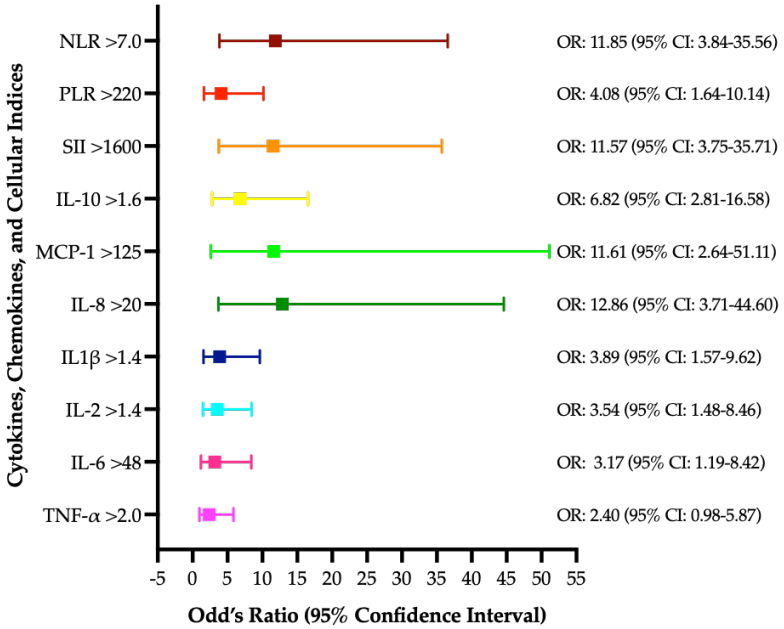


Figure 59. Multivariate regression analysis for cytokines, chemokines, and cellular indices in predicting 30-day mortality.

The model is adjusted for the ESC severity.

VI – DISCUSSION

VI - DISCUSSION

6.1. DISCUSSION OVERVIEW

Acute VTE, including DVT and PE, is a common disease and the third leading cause of cardiovascular death globally [429]. Despite significant advancements in its management, VTE remains a major public health concern due to the substantial burden and costs associated with hospitalizations, imaging, and treatment options. In addition to mortality risks, morbidity, and long-term complication risks continue to pose important challenges for VTE. For this reason, it is critically important to understand the causes and risk factors of this serious disease, incorporate preventive measures, and use accurate diagnostic methods, effective risk stratification, and appropriate treatment options. The complete eradication of the disease remains difficult.

To achieve this goal, we adopted an integrated approach that combines a comprehensive set of biomarkers, blood cellular indices, and registry data in our studies. When used together, biomarkers and blood cellular indices are instrumental in early detection and diagnosis, identifying populations at-risk, personalizing treatment, and monitoring disease progression bringing molecular insights into disease mechanisms. They also provide critical insights into the various disease states, guide treatment decisions, monitor therapeutic responses, and predict outcomes. Registries enable large-scale data analysis, inform public health strategies, and support research and evidence-based practices at the population health level. Collectively, these elements created a robust framework for achieving the specific aims of our studies.

In our studies, we used plasma samples from diagnostically confirmed pulmonary embolism (PE) patients and healthy controls to evaluate biomarkers in a relevant clinical context. These samples were collected from the Pulmonary Embolism Response Team (PERT) program at our institution, which handles a high volume of PE cases, with diagnoses confirmed by highly skilled specialist physicians. We employed highly sensitive methods to quantify biomarker levels, including ELISA, thrombin generation assays, and biochip array technologies,

which provide accurate measurements and are easily adaptable to clinical settings. The integrated analysis of biomarkers and cellular indices was performed following the validation of blood cellular indices in two cohorts: the large-scale RIETE Registry and the LUMC PERT Project.

In this context, alternative biomarkers that reflect diverse pathophysiological signals—such as endothelial dysregulation, platelet activation, coagulation factors, fibrinolysis, thrombin generation, and inflammation along with various cytokines, chemokines, and growth factors, or those specific to pathobiology within subsets of VTE, would be extremely valuable. These biomarkers could aid in differentiating between VTE subtypes, including severity and mortality, and enable more personalized and targeted patient management. To address this, we selected a comprehensive set of biomarkers in our studies to represent key pathophysiological pathways of VTE and tested them in a large cohort of PE patients from the PERT project compared to healthy controls. We believe this approach enhanced the significance of our results.

Complete blood count with differential is a cost-effective and routinely performed laboratory test used throughout the clinical course of all patients, including those with VTE. Studies have shown that the inflammatory response plays a crucial role in the pathogenesis of VTE, with cellular indices reflecting this response may serve as potential blood markers [430]. Among the components of blood cellular indices, both platelets and leukocytes play pivotal roles in thrombosis development [431].

Cellular indices—being inexpensive and readily accessible—can be valuable indicators for diagnosing and risk stratifying VTE and predicting mortality. They may have the potential to enhance the specificity of VTE detection tools and aid in identifying high-risk patients with poor prognosis. However, previous studies have produced diverse and often conflicting results. Most of these studies are single-center investigations that focus on a limited number of cellular indices without comparison. Furthermore, they vary widely in case characteristics, sample sizes, and study objectives. As a result, establishing consensus conclusions on the importance of cellular indices in VTE remains challenging.

To address these challenges, we conducted our analysis using two large cohorts: the RIETE registry and the LUMC PERT project. Both cohorts provided comprehensive data, including neutrophil-to-lymphocyte ratio (NLR), platelet-to-

lymphocyte ratio (PLR), and systemic immune/inflammatory index (SII), along with detailed demographic and clinical information. This allowed us to assess the predictive and prognostic value of each cellular index across both cohorts. We also had access to complete clinical information, including disease severity and adverse outcomes such as mortality. Additionally, we supported our findings with multiple investigative methods, including biomarker testing using ELISA, thrombin generation assays, and chip-based array technologies. Therefore, our findings hold significant importance in the context of VTE research and patient management.

It is important to note that none of the registries have previously assessed the impact of blood cellular indices, such as NLR, PLR, or SII, as inflammatory markers. Only the RIETE and COMMAND VTE registries have explored associations between hematological parameters like white blood cell count, platelet count and anemia with adverse outcomes, including mortality and major bleeding.

In the RIETE registry, several studies have evaluated the predictive value of various hematological parameters. One study confirmed that elevated white blood cell (WBC) count in cancer patients with acute VTE was independently associated with an increased incidence of recurrent VTE, major bleeding, and mortality through multivariate analysis [432]. Another study investigated the impact of platelet count on VTE recurrence, major bleeding, fatal bleeding, and mortality, revealing that patients with very low, low, high, or very high platelet counts had an increased risk of major and fatal bleeding compared to those with normal platelet counts [433]. Additionally, a study focused on patients with abnormal platelet counts receiving vitamin K antagonists (VKAs) for acute VTE treatment found a nonlinear 'U-shaped' relationship between abnormal platelet count at baseline and the risk of major bleeding during VKA therapy [434].

The RIETE registry also assessed the influence of anemia on bleeding risk, demonstrating that anemia independently predicted the risk for major bleeding in patients with and without cancer [435]. In patients without anemia, the rate of major bleeding was lower compared to VTE recurrences. Another study examined the clinical characteristics, treatment, and early outcomes (at 10 and 30 days) of cancer-associated VTE patients based on baseline platelet count [436]. It was found that while mortality rates decreased with increasing platelet counts, major bleeding rates did not. Severe thrombocytopenia was associated with a similar risk for major

bleeding at both 10 and 30 days, while mild thrombocytopenia increased the risk at both time points.

In the COMMAND VTE registry, hematological parameters were assessed in one study, which evaluated the influence of thrombocytopenia on long-term clinical outcomes [436]. The cumulative 5-year incidence of major bleeding was significantly higher in patients with moderate or severe thrombocytopenia. Even after adjusting for confounders, the risk of major bleeding and mortality remained significant for those with moderate or severe thrombocytopenia compared to those without thrombocytopenia.

As observed, these analyses focused on single parameters without comparing them to other hematological parameters or cellular indices. Furthermore, we were unable to identify any other registries that specifically focus on the impact of hematological parameters on VTE outcomes. Given that most prior studies on cellular indices were conducted in single centers with a limited number of indices evaluated, our study stands out by assessing the influence of all three cellular indices within a registry dataset like the RIETE registry, thereby highlighting the significance of our research. Moreover, our integrated analysis of the RIETE registry findings with the LUMC PERT project, coupled with the support of biomarker studies, further underscores the importance of our work.

This dissertation represents five comprehensive studies based on cellular indices and a detailed analysis of thrombo-inflammatory biomarkers in an integrated fashion. The results of the RIETE registry are analyzed to determine the value of cellular indices in the context of VTE outcomes. The second study has compared two independent cohorts in terms of outcomes which are conducted as multicenter RIETE registry and monocentric PERT project. The design of this study is carefully planned, and the differences are highlighted in the following discussions. The profiling of thrombin generation is detailed in terms of thrombogenesis, and biomarkers generated in the PERT study and its relevance to cellular indices is established. Additionally, a comprehensive biomarker profiling including thrombo-inflammatory biomarkers utilizing different techniques including high throughput biochip array technology. The role of tissue factor is highlighted in both the thrombin generation studies and its relevance to thrombo-inflammatory biomarkers and cellular indices. The following is a detailed discussion in a sequential manner.

6.2. CELLULAR INDICES AND THEIR VALUE IN THE DIAGNOSIS OF VTE

Blood cellular elements have long been considered as an index of pathophysiological transitions. Recently the emerging role of blood cellular indices has been targeted to investigate pathophysiologic transitions in VTE. The RIETE registry comprised of a large database which has been collected at different centers provides the validation of the value of blood cellular indices in the diagnosis and risk stratification of VTE. This multinational trial also represents a large database to predict the outcomes. The RIETE registry is ongoing and expected to expand at a global level and will have major impact in the diagnosis and management of VTE. Based on this registry certain other blood cellular indices are also calculated which will provide complimentary data to augment the value of cellular indices.

Reported data, obtained from a large series of consecutive patients with acute VTE, reveal that the cellular indices may help to predict adverse outcomes at 90 days, thus supporting the role of the immune/inflammatory response in the pathogenesis of VTE. In this cohort, none of the three cellular indices predicted the risk for VTE recurrences within the first 90 days, but patients with NLR >4.41, >4.96 were at increased risk for major bleeding (68.4% sensitivity and 68.0% specificity) or death (66.9% sensitivity and 66.8% specificity), and those with SII >1134.5 were at an increased risk of death (64.9% sensitivity and 64.9% specificity). One in every 30 patients (3.4%) suffered major bleeding within the first 90 days, and one in every four such patients died of bleeding. Thus, the clinical relevance of these indices at baseline should not be underestimated. On univariate analysis, all 3 cellular indices were associated to a higher risk for major bleeding, as were older age, cancer, recent immobility, hypertension, recent major bleeding, anemia, renal insufficiency or initial VTE presentation as PE. Most of these clinical variables have been identified and validated in the literature as independent risk factors for bleeding in patients with VTE. Interestingly however, only NLR, anemia and renal insufficiency independently predicted the risk for major bleeding. Moreover, both NLR and SII independently predicted the risk of death.

A meta-analysis including 2,323 patients from 7 retrospective studies found the NLR and the PLR to independently predict the risk of death (in the short- and in the long term) in patients with acute PE [437]. The prognostic role of the SII (including neutrophils, lymphocytes and platelets) is currently less known,

although a retrospective report involving 442 patients with PE suggested its association with worse outcomes, including hemodynamic compromise and in-hospital mortality [270]. In another study on patients with acute PE, NLR and PLR correlated with the severity of PE evaluated by the PESI or simplified PESI scores. In contrast, high NLR values were not associated with more severe risk profiles in a more recent small cohort of less than 100 patients with PE [438]. This study reports t PE patients with tachycardia or hypoxemia to have high NLR and SII values. Thus, the prognostic role of these cellular indices in patients with acute PE needs to be further investigated.

In this study, the value of cellular indices in predicting outcome in patients with acute venous thromboembolism has been addressed as novel biomarkers based on the utilization of blood counts. The molecular basis of these relationships requires detailed molecular and cellular investigations. Thrombo-inflammatory biomarkers have been extensively investigated and reported previously from a previous study, from our group [367]. A clear relationship between upregulation of inflammatory cytokines has been demonstrated in this comprehensive study. This study also provides an opportunity to demonstrate that, the variations in thrombo-inflammatory biomarkers may be relevant to the changes in the cellular indices, such as NLR, PLR and SII. The RIETE Registry, cohort did not address thrombo-inflammatory biomarkers except for the D-dimer, however the cellular indices were easily retrievable. Such a relationship between the cellular indices and the thrombo-inflammatory biomarkers will provide interesting insight in the understanding of the pathogenesis of VTE. In another study, Darwish et al, have also reported on the dysregulation of hemostatic and inflammatory biomarkers with reference to adhesion molecules TNF-a, and PAI-1. Ongoing studies on the cellular indices in this group and their correlation with the biomarkers investigated is being addressed. In a lymphoma cohort, some of the cellular indices and thrombo-inflammatory biomarkers have been simultaneously measured. Such measurements have provided useful data in stratifying risk and outcomes in cancer patients [439]. Future studies, to complement cellular indices data, with such biomarkers as inflammatory cytokines, tissue factor, and microparticles will provide additional information on the pathogenesis of VTE.

The role of microparticles in the understanding of the pathogenesis of venous thromboembolism is well known [440]. Microparticles are drive mainly from

platelets, white cells, and endothelial lining. These microparticles are pro-coagulant in nature, and profiling of these microparticles has been addressed in several studies. In conjunction with the PERT trial, we measured TF antigen which was upregulated in the PERT cohort. However, in the current study, the main focus is on demonstrating value of cellular indices in conjunction with a large registry. This registry, thus also offer an opportunity to quantify microparticles, however the current study is mainly focused on cellular indices and correlations with cellular microparticles can be subject of future studies. The role on inflammation in inducing hemorrhage in thrombocytopenia has been reported previously. Platelets play an important role in inflammatory responses. Inflammatory processes may result in hemorrhagic response to multiple pathways, where the cytokines play a role including the integrins [441]. In this study, recent bleeding was defined as a major bleed less than 30 days prior to VTE. In-terms of the association of cellular indices with bleeding risk, the NLR showed an association with bleeding risk in the multivariate analysis, whereas the PLR and SII did not reveal any relevance. Additional data, on a larger cohort will provide further insights. Therefore, the RIETE Registry provides a unique opportunity to validate the diagnostic and prognostic significance of these indices.

This study has several limitations to consider. First, reported findings should be treated with caution considering the limitations of observational studies to infer causality. This study did not include a control group. This study did not address the prognostic relevance of repeated measurements of cellular indices, which may have a higher accuracy than a single one. Reported study only represents the outcomes appearing during the first 90 days. Additionally, this study did not completely consider the clinical characteristics of the patients while including in this study. Increased levels of blood cellular indices is detected in several other illnesses (particularly in cancer patients) other than VTE. Moreover, reported study did not compare other inflammatory biomarkers (such as CRP, D-dimer) with the selected variables. Finally, although clinicians might not perceive that this is readily available, and easily calculated indicators with blood cell counts.

This analysis also has some strengths. To the best of our knowledge, this is the largest cohort study evaluating the prognostic value of three cellular indices simultaneously in patients with acute VTE. Prior publications concerning the prognostic role of these immune/inflammatory indicators were single-center

studies. Our data provides a multicenter perspective, which increases the generalizability of the results. In the current literature, there is no specific cut-off values of NLR, PLR or SII for mortality. This study has obtained different optimal cut-off points of the cellular indices for major bleeding outcomes. Thus, reported study offers a new perspective of the problem, and notable data.

In summary, the cellular indices represent a consistent and powerful predictor of major bleeding or death within the first 90 days in patients with acute VTE. This should lead to increased awareness about these indicators, particularly NLR. Nonetheless, the prognostic role of these tests in patients with different risk profiles (i.e., active cancer, normotensive acute PE, etc.) is still underexplored and warrants further investigations.

This study also confirms the value of the cellular indices in the diagnosis of VTE in a large cohort of over 4,000 patients. The value of NLR is established in the diagnosis and risk stratification of VTE. Moreover, this RIETE registry will provide additional data on the bleeding and other complications. The value of the cellular indices can be established in other diseases such as stroke, atrial fibrillation, sepsis and auto-immune diseases. Additional information is needed for the establishment.

6.3. COMPARATIVE PROGNOSTIC VALUE OF CELLULAR INDICES IN ACUTE PE PATIENTS IN TWO COHORTS

The prognostic value of cellular indices is also validated in 2 separate independent cohorts. the first cohort represented the RIETE registry which is conducted primarily in European countries and the second cohort comprised of mainly pulmonary embolism patients and is carried out in conjunction with the PERT consortium. The prognostic value of cellular indices in both trials have been validated, although there are some obvious differences in the recruitment criteria and type of patients included. The RIETE registry represents the inclusion of patients with less severe VTE and PE whereas the PERT trial included more severe patients which require acute intervention. While the RIETE registry comprised of over 50,000 patients, the Loyola cohort for the PERT trial included only a few hundred patients. Thus, this dissertation compared the value of cellular indices in

prediction of the outcome in two independent trials. Such comparisons will be very useful in the future to establish the standards, and the data can be integrated. Our study therefore represents the first ever attempt to compare the value of cellular indices in the outcome in terms of mortality and severity. Both the RIETE registry and PERT program are ongoing and will continue to provide additional data to demonstrate the values of cellular indices in the diagnosis of VTE.

Our study into the prognostic utility of cellular indices in PE patients has yielded significant insights into risk stratification for patients with acute PE. Our comprehensive analysis of over 10,000 patients from the RIETE registry and a validation cohort from the LUMC database underscores the significance of the NLR as a predictor of short-term mortality in this patient population. This sequential approach, using two distinct patient populations, enhances the robustness and generalizability of our findings. Notably, the discrepancy in the prevalence of low-risk and high-risk PE patients between the two cohorts may reflect demographic or clinical practice differences, which warrants further investigation. Notwithstanding these differences, the NLR consistently emerged as significant predictor of mortality, irrespective of the cohort. This reinforces the premise that systemic inflammation plays a pivotal role in the pathophysiology and prognosis of PE.

The application of NLR as a prognostic tool has significant implications for the risk stratification of patients with acute PE, particularly when considering the potential reclassification of risk categories. In our RIETE cohort, where a predominant number of patients were initially classified as low-risk, the NLR was instrumental in uncovering a subset of patients with a higher actual risk. This reclassification capability is critical, as it suggests that additional factors captured by the NLR can contribute to a more nuanced assessment beyond traditional risk stratification tools like sPESI

The early differentiation in mortality rates highlighted by NLR measurements indicates its potential utility as an early prognostic marker. This could be instrumental in triaging patients more effectively, optimizing the allocation of healthcare resources, and potentially guiding decisions regarding the intensity of surveillance or intervention. Importantly, the superior performance of NLR in low-risk PE patients offers a valuable indicator for identifying patients who

could be safely managed on an outpatient basis or with abbreviated hospital stays, a consideration that holds particular weight in resource-limited settings.

While the NLR alone displayed noteworthy predictive power, its integration with sPESI scores further refined the identification of patients at low risk for death. Our findings suggest the potential of such combined indices in enhancing risk stratification, although for patients identified as high-risk, the NLR showed diminished prognostic value. Therefore, it should be emphasized that while the NLR is a robust marker, it is not a panacea and should be integrated into a multifaceted assessment strategy, particularly when stratifying very low-risk patients.

Acknowledging the limitations of our study is crucial. The NLR, as a single marker, may not encapsulate the full clinical picture for PE patients. Further investigation is needed to elucidate the mechanisms linking cellular indices like NLR to PE outcomes, and more comprehensive studies encompassing a broader spectrum of severe PE cases could refine risk prediction through multifactorial analyses, including clinical, biomarker, and imaging parameters.

In this study the RIETE trial is compared to the PERT trial which is conducted independently. While the differences in the trail design were noted, the NLR, has increased value with outcome. There are some significant differences in both trials. The PERT trial represented more of a heterogenous group of patients with severe PE, whereas the RIETE trial were more homogenous nature with less severe symptoms. Both trials validated the hypothesis that cellular indices are valuable in the diagnosis of PE. This information will be better for therapeutic interventions and prediction of the outcomes.

6.4. THROMBIN GENERATION POTENTIAL, MARKERS, AND CELLULAR INDICES IN ACUTE PE PATIENTS

Thrombogenesis plays a pivotal role in the pathogenesis of VTE. An integrated analysis of thrombogenesis and thrombin generation biomarkers has not been available currently. In this dissertation thrombin generation utilizing a highly sensitive kinetic method has shown to be associated with increased biomarker generation such as F1.2 and thrombin anti-thrombin complex (TAT). This study also demonstrated a decreased thrombin generation in patients with PE. Since

prothrombin complex is consumed during the generation of biomarkers an inverse relationship between the biomarkers is established. Thrombin plays a key role in regulating blood coagulation, fibrinolysis and platelet and endothelial function. Moreover, blood cells are activated due to receptor modulation. The study demonstrated a relevance between the blood cellular indices and thrombin generation biomarkers and the process of thrombogenesis. Since tissue factor plays a role in the process of thrombogenesis, this investigation demonstrated an upregulation of tissue factor in PE patients. Although it was difficult to collect blood samples in conjunction with the RIETE trial, we have analyzed mainly samples from the PERT trial. The outcome in terms of severity and mortality have been linked with both cellular indices and thrombin generation biomarkers.

This study provides a comprehensive analysis of thrombin generation potential, biomarkers, and cellular indices in a well-defined cohort of PE patients. Our findings underscore the heterogeneity of PE pathophysiology, as evidenced by the wide variations in thrombin generation potential between patients and healthy controls. The significant downregulation of peak thrombin and endogenous thrombin potential (ETP), alongside the prolongation of lag time in PE patients, indicates a distinct thrombin generation profile that is associated with the disease. Importantly, the upsurge in lag time suggests a delayed initiation of coagulation, potentially contributing to the progression of thromboembolic events.

The marked elevation of biomarkers, especially D-dimer, in PE patients points to an ongoing hypercoagulable state. The strong positive correlation between D-dimer levels and thrombin generation potential underlines the clinical utility of these markers in assessing disease severity and the effectiveness of therapeutic interventions. Notably, the biomarkers studied, including F1+2 and TAT, have demonstrated their prognostic relevance, which could be integrated in risk stratification models to improve patient management and outcomes.

The stratification of patients based on the European Society of Cardiology (ESC) guidelines revealed a gradient of thrombin generation potential and biomarker levels in accordance with the severity of PE. This stratification not only confirms the escalated thrombo-inflammatory response in higher-risk categories but also reflects the potential for these markers to guide therapeutic decisions. For instance, the observed decrease in ETP with increasing PE severity may offer insights into the intensity of anticoagulant therapy required.

The study further delves into the role of blood cellular indices such as the NLR, PLR, and SII in PE. The association of elevated NLR and PLR with intermediate-risk PE could point to their involvement in thrombo-inflammation and their potential as early markers for acute PE. These findings are in line with emerging literature that suggests a link between inflammation and thrombosis in the pathogenesis of PE This may involve NETosis and related extra vesicle formation along with degranulation and release of histones and damage-associated molecular patterns (DAMPs) [86, 87]. Moreover, microparticles derived from monocytes and platelets may trigger procoagulant and proinflammatory responses.

Our data also highlight the prognostic value of cellular indices in mortality rates, which were shown to be consistently higher in patients with higher NLR, PLR, and SII. The significant increase in mortality rates observed in the CAT subgroup necessitates a closer examination of these indices as potential predictors of adverse outcomes. The utility of these indices in clinical practice, however, requires further validation in larger, prospective studies to determine their role in the routine evaluation of PE patients.

The findings of this study may also have some important clinical implications for PE management. Firstly, the normalization of thrombin generation potential, thrombin generation biomarkers and blood cellular indices may be more precise predictors of optimal treatment response in PE patients. Additionally, these parameters can also be used for better risk stratification of PE patients. Although not all of them are available for routine clinical practice, blood cellular indices can easily be incorporated into risk assessment models to help identify high-risk patients who require closer monitoring and more aggressive treatment. Additionally, our results in CAT patients clearly demonstrates a more prominent downregulation of thrombin generation potential with elevation of thrombin generation biomarkers and blood cellular indices which may be helpful to explain the increased rates of VTE and bleeding complications observed in this patient population.

This study has several limitations. The study is based on a small cohort of only 209 patients with diverse patient population. In addition, the time for the sample collection varied from 24-72 hours after the onset of PE and moreover the samples were stored for prolong time. The study based on single sample analysis,

and sequential samples were not collected. In terms of thrombin generation markers, D-dimer, F1+2, and TAT was analyzed while other markers such as tissue factor, fibrinopeptide A, fibrinopeptide B, and soluble monomer were not studied. One of the major limitation is that we did not study prothrombinase complex and its contribution towards thrombin generation and the biomarkers. The patient distribution with respect to the PE severity was disproportional as 24 patients in low-risk, 166 in intermediate-risk, and 16 patients in high-risk PE. History and type of drugs and anticoagulation management is not taken into the account as they were used immediately after the diagnosis. Other considerations including the diagnostic procedures and use of intravenous fluids and factor contributing to hemodilution are not consider. Despite these limitations, this study has validated that increase thrombin generation biomarkers are associated with pulmonary embolism with the simultaneous decrease in thrombin generation parameters.

To summarize, the relationships between thrombin generation potential, biomarkers, and cellular indices not only reinforce their pathophysiological relevance in PE but also emphasize the complexity of the disease. The integration of these markers into clinical practice could enhance the precision of PE risk stratification and management, ultimately improving patient care. The current study included only 209 patients and because of the complex nature of pulmonary embolism, a larger cohort may further strengthen these findings, thus a multi-center trial may be helpful before any recommendations offered to the clinicians. Future studies should focus on the longitudinal assessment of these markers to validate their predictive value and to refine the therapeutic strategies for PE.

Thrombogenesis is an important step in the progression of VTE. Both thrombin generation and thrombin generation biomarkers including F1.2, thrombin anti-thrombin complex and D-dimer are profiled. We hypothesize that thrombin generation is decreased in the VTE patients whereas thrombin generation markers are increased. Consistent to this hypothesis our results validated this hypothesis. To date there is no study in which thrombin generation decrease is compared to the increase in biomarkers. Due to the limitation of samples size, the study is not comprehensive which may have included other thrombin generation biomarkers like FPA and fibrin monomers. This study has also validated the value of cellular indices, and the relevance established between these biomarkers of thrombin generation with these cellular indices.

6.5. HEMOSTATIC ACTIVATION AND THROMBOINFLAMMATORY BIOMARKERS IN ACUTE PE PATIENTS

This dissertation also addressed an extensive biomarker profiling representing coagulation, fibrinolysis, platelet activation and inflammatory biomarkers. This comprehensive biomarker profile provided molecular insights and probed the mechanisms involved in the pathogenesis of VTE. Both the profiling and their value in the diagnosis and risk stratification models are established. Thrombo-inflammation is hallmark of VTE. Such comprehensive biomarker screening has not been conducted to date. The LUMC PERT cohort provided a unique opportunity to carry out the comprehensive biomarker profiling in which the role of thrombo-inflammatory biomarkers has emerged. Such biomarkers as D-dimer, PAI-1, TNF-α, and CRP along with interleukins provides a fingerprint for the pathogenesis for this complex syndrome. More interestingly several of the biomarkers demonstrated the relevance of the cellular indices will be utilized for risk prediction and outcome. Although the role of complement pathways and immunologic activation has not been addressed, this integrated data will form the basis of more comprehensive analysis utilizing molecular biology and cellular science advancements in the prognosis of VTE.

The primary findings of this study can be summarized as follows: 1) Levels of all the markers of endothelial dysfunction, platelet activation, coagulation abnormalities, fibrinolysis, and inflammation along cellular indices were significantly elevated in patients compared to healthy controls. 2) There was a significant relationship between endothelial dysfunction, platelet activation, coagulation abnormalities, fibrinolysis, and inflammation and cellular indices. 3) The increases in endothelial dysfunction, platelet activation, coagulation abnormalities, fibrinolysis, and inflammation along cellular indices were notable, correlating with the severity of PE and associated 30-day mortality. These findings underscore the substantial prognostic value of these biomarkers and blood cellular indices in predicting 30-day mortality in PE patients

This study provides a broad-spectrum analysis of various biomarkers related to endothelial dysfunction, platelet activation, coagulation abnormalities, fibrinolysis, and inflammation in patients with acute PE. The most significant findings include the markedly elevated levels of vWF, P-selectin, tPA, PAI-1, D-

dimer, CRP, IL-6, TNF-α, and NLR in PE patients compared to controls. These biomarkers not only reflect the underlying pathophysiological mechanisms of PE but also correlate strongly with disease severity and 30-day mortality. vWF, TF, P-selectin, tPA, PAI-1, D-dimer, IL-6 and TNF-α along with cellular indices NLR, and PLR showed varying levels of increase with respect to the disease severity, while TAFI, FV and FX levels showed decrease. Most of the biomarkers including vWF, TF, P-selectin, tPA, TAFI, D-dimer, CRP, IL-6 and TNF-α along with cellular indices NLR, PLR, and SII showed significant increase while FVII, FX, FXIII-A, and PAI-1 decrease with the 30-day mortality. The observed correlations among different biomarkers further highlight the complex interplay between coagulation, inflammation, and platelet activation in the pathogenesis of PE.

One of the key findings of this study is the pronounced elevation of vWF levels and its correlation with PE severity. Previous studies have acknowledged the role of endothelial injury in PE, but the specific quantification and correlation of vWF levels with disease severity presented here add a new dimension to our understanding of how endothelial damage contributes to PE progression. The alterations in coagulation factors, such as the increased levels of FIX and FX and the decreased levels of FVII and FXIII-A, provide new insights into the hypercoagulable state associated with PE. The perturbation in the clotting factor levels may also contribute to increase thrombogenesis and fibrinolytic deficit in PE. Since, the levels of these factors were measured by immunologic method's which are prone to epitopic modification by interactions with other proteins, these changes need further validation by functional assays and the nature of binding proteins. The comprehensive profiling of fibrinolytic markers, especially the significant elevation in D-dimer and tPA levels, provides a deeper understanding of the fibrinolysis activity in acute PE. Inflammatory markers such as CRP, IL-6, and TNF-α were also significantly associated with PE severity and 30-day mortality, reinforcing the central role of inflammation in PE pathogenesis. On multivariable analysis, all tested biomarkers (except for P-selectin) were independently associated with 30-day all-cause death, especially CRP >12, NLR >7, IL-6 >48, TF >130, and FVII <65 demonstrated the highest odds ratios highlighting their predictive value for 30-day mortality. These results emphasize the potential prognostic utility of these biomarkers in assessing short-term outcomes in PE.

Notable correlations were observed between various biomarkers, highlighting the complex interplay in patients with acute PE. For instance, D-dimer correlated with IL-6, indicating a connection between coagulation and inflammation; D-dimer also correlated with P-selectin, pointing a link between coagulation and platelet activation; and PAI-1 correlated with tPA, suggesting a balance between fibrinolysis and its inhibition. As a result, the cumulative pathophysiology leads to fibrinolytic deficit due to interplay between these mediators. Additionally, CRP and IL-6 showed strong associations with vWF, FIX and NLR, illustrating the broad impact of inflammation. IL-6 also correlated significantly with TNF-α, and NLR, reinforcing the central role of inflammation. Notably also, higher NLR levels were associated with elevated levels of several biomarkers, such as CRP, IL-6, and D-dimer. This relationship underscores the significant role that NLR plays in reflecting the inflammatory and thrombotic state of patients with acute PE. Elevated NLR indicates a more pronounced inflammatory response, which correlates with higher levels of other biomarkers, thus reinforcing its value as a prognostic marker.

Following our recent findings from the RIETE registry, which highlighted the prognostic utility of the NLR in predicting 30-day mortality in acute PE, this study further elucidates the role of the NLR in the pathogenesis of PE relative to other biomarkers. Elevated NLR indicates a more pronounced inflammatory response, which correlates with higher levels of other biomarkers, thus reinforcing its value as a prognostic marker. The NLR's ability to predict 30-day mortality after adjusting for PE severity underscores its robustness as a prognostic tool. While most biomarkers studied also predicted 30-day mortality, the NLR had the second highest odds ratio (only after CRP). This emphasizes the practical advantage of NLR, being both cost-effective and readily available, thus making it valuable for routine clinical use in risk stratification and monitoring.

The strength of this study lies in its comprehensive profiling of a wide array of biomarkers and cellular index in a large cohort of PE patients. This extensive dataset allows for a robust analysis of the complex interplay between different pathophysiological pathways in PE. Additionally, the use of well-defined patient stratification criteria and advanced statistical methods enhances the reliability of the findings. However, the study also has certain limitations. The observational design precludes establishing causality between the observed biomarker changes

and PE outcomes. The single-center nature of the study may limit the generalizability of the findings to other populations. Furthermore, while the study included a large sample size, the relatively small number of high-risk PE patients might limit the power of the analysis in this subgroup. Despite these limitations, the study provides a new direction in the diagnosis and management of PE patients, where integrated cellular index and biomarkers are considered.

This research introduces several novel findings to the existing body of knowledge on PE, including detailed biomarker profiling and correlation analyses that enhance our understanding of the disease's pathophysiology. The identification of key biomarkers associated with endothelial dysfunction, coagulation abnormalities, fibrinolysis process, and inflammation, enhances our ability to stratify risk and tailor therapeutic interventions.

In summary, our study advocates for incorporating the NLR into clinical toolkit for managing PE, underscoring its utility, especially for patients at the lower end of the risk spectrum. The simplicity and cost-effectiveness of this biomarker make it an appealing addition to current management strategies. Further research should aim to validate the applicability of our findings across various clinical contexts and to explore the biological underpinnings that tie the NLR to PE-related mortality. Such efforts will undoubtedly enhance our understanding and improve patient satisfaction and care.

A comprehensive analysis of hemostatic activation and thromboinflammatory biomarkers using immune-enzymatic methods. This comprehensive biomarker analysis included coagulation, fibrinolysis, platelet and cellular activation biomarkers including inflammatory cytokines are profiled in conjunction with the PERT trial. It would have been beneficial to include the RIETE trials cohort in this analysis. However, the data included in this trial validated the importance of biomarker profiling in VTE. Furthermore, the relevance of cellular indices with biomarkers are also noted. Such comprehensive screening of biomarkers will not only be helpful in the diagnosis and risk stratification of patients with VTE but will lead to develop therapeutic strategies of VTE.

6.6. INFLAMMATORY BIOMARKERS IN ACUTE PE PATIENTS

To further profile the various inflammatory mediators, we also employed a dedicated bio-chip array method which analyzed simultaneously twelve relevant parameters. This is a high through-put method with highly sensitive immunofluorescence probe requiring only small amount of plasma for analysis [442]. Such profiling of multiple mediators has been useful in other studies, such as sepsis and DIC [443].

The primary findings of this study can be summarized as follows: 1) Levels of inflammatory cytokines, chemokines, growth factors, and cellular indices were significantly elevated in patients compared to healthy controls. 2) There was a significant relationship between inflammatory cytokines, chemokines, growth factors and cellular indices. 3) The increases in inflammatory cytokines, chemokines, growth factors and cellular indices were notable, correlating with the severity of PE and associated 30-day mortality. These findings underscore the substantial prognostic value of these inflammatory markers and blood cellular indices in predicting 30-day mortality in PE patients.

This study provides a broad-spectrum analysis of various inflammatory biomarkers in patients with acute PE. The most significant findings include the markedly elevated levels of pro-inflammatory cytokines including IL-1α, IL-1β, IL-2, IL-6, IL-8, TNF-a, and IFN- γ; anti-inflammatory cytokines including IL-4, and IL-10; chemokines including MCP-1 and IL-8; growth factors including the EGF and VEGF; and blood cellular indices including NLR, PLR, and SII showed significant increase in PE patients compared to controls. The most pronounced increase was noted for EGF, IL-6, IL-8, VEGF. Only three studies have previously analyzed all these inflammatory markers together in the setting of VTE, where two studies compared with non-VTE (controls). And our results are consistent with both the studies [367, 369]. However, a population-based study reported an IL-1β, IL-6, IL-8 and TNF-α are not associated with the risk of VTE [342]. This significant difference between these two studies could be due to the demographics and the methods used for the assay.

In addition to these findings, we were able to stratify all tested parameters based on PE severity and 30-day mortality. In this analysis TNF-α, IL-1β, IL-4, IL-6, IL-8, IL-10, MCP-1 showed various levels of significant associations with the

severity of PE, while cellular indices did not. This data is also consistent and supported with the previous findings which also showed elevation of IL-4, IL-6, IL-8, IL-10, MCP-1 and IL-1β [367]. It is interesting to note that we also found elevated levels of TNF-α with severity.

Our study demonstrated significant positive correlations between cellular indices, particularly NLR with IL-6, IL-8, and IL-10, and SII with IL-6 and IL-10. Interestingly, PLR did not show significant correlations with any of the cytokines, chemokines, or growth factors, but did correlate with other cellular indices. Our data is also supported by the previous study with some minor changes in correlations [367].

Moreover, all the tested parameters including inflammatory cytokines, chemokines, growth factors and cellular indices were significantly higher associated with 30-day mortality of PE. Of these, IL-1β, IL-2, IL-6, TNF-α, IL-8, IL-10, MCP-1, along with NLR, PLR, and SII were significantly elevated with 30-day mortality. This is the first study to compare the levels of all inflammatory markers and cellular indices in relation to short-term mortality; however, the association between only IL-6 and mortality has been reported [444] .In multivariate analysis, NLR >7, SII >1600, IL-8 >20, IL-10 >1.6, and MCP-1 >125 demonstrated the highest odds ratios highlighting their predictive value for 30-day mortality. These results emphasize the potential prognostic utility of these biomarkers in assessing short-term outcomes in PE.

This study has several strengths. We profiled a comprehensive set of inflammatory cytokines, chemokines, and growth factors, in addition to cellular indices. This approach allowed us to examine their relationships in detail using stratification and statistical inference methodologies. Certain limitations of this study should also be acknowledged. The observational design of the study precludes establishing direct causality between changes in tested parameters and outcomes of PE. Additionally, the single-center nature of the study may limit the generalizability of the results to other populations or healthcare settings. The lack of follow-up samples restricts our ability to assess the progression of parameter levels over time or the impact of repeated measurements on clinical outcomes. Despite these limitations, the study provides valuable insights into the role of cellular indices, inflammatory cytokines, chemokines, and growth factors in the management of PE.

In summary, this study highlights the significant impact of cellular indices, particularly NLR, on the prognosis of acute PE. NLR demonstrated notable relationships with inflammatory cytokines, chemokines, and growth factors, underscoring its prognostic value. Utilizing an NLR cutoff of >7 could enhance risk scoring systems and improve the accuracy of outcome predictions in acute PE.

6.7. INTEGRATED SYNTHESIS OF MULTI-STUDY FINDINGS

This dissertation encompassed multi-layered objectives, with specific aims designed to investigate the pathophysiology of VTE in a comprehensive manner. Central to this investigation was the identification of novel biomarkers, including blood cellular indices and thrombo-inflammatory mediators, and validating their roles in PE. Through an in-depth analysis of cellular and protease networks by emphasizing thrombin generation potential and associated biomarkers, as well as the regulatory roles of the endothelium, platelet activation, fibrinolysis, coagulation, and inflammation, these studies aimed to enhance management strategies for VTE. The integrative synthesis of these findings addresses important knowledge gaps, establishing a comprehensive framework to enhance the care of VTE.

The treatment of VTE involves anticoagulant therapy in addition to various interventional or surgical approaches in selected patient groups [225, 445]. Despite effective treatment options, VTE and particularly its most severe form of acute PE continues to carry substantial short- and long-term risks such as mortality, major bleeding and VTE recurrence [257, 258]. The short-term prognosis is typically defined as a three-month period, during which patients are expected to achieve complete physical and functional recovery and return to normal life. While short term prognosis is largely determined by the hemodynamic impact of the thromboembolic event, bleeding complications related to anticoagulation therapy and the likelihood of short-term recurrence, long-term outcomes are typically shaped by the presence of underlying comorbidities. However, the prognosis for VTE patients varies widely, certain comorbidities may also impact short-term outcomes. This variability necessitates individualized risk assessment and tailored management strategies.

Considering these factors, our first study involved an analysis of three major 90-day outcomes as mortality, major bleeding, and VTE recurrence—using data from the RIETE registry [266]. We evaluated three key blood cellular indices: NLR, PLR, and SII, using a comprehensive dataset of clinical information. These cell indices were among the most studied and promising ones in the context of acute VTE.

The results of the multivariate analysis have shown that NLR, SII (but not PLR), active cancer, recent immobilization, diabetes, anemia, renal insufficiency, symptomatic PE, lower limb DVT, and superficial DVT were independent predictors of 90-day mortality. Numerous studies in the literature have explored potential risk factors to better define the prognosis of PE patients, incorporating clinical findings, biomarkers, clinical prediction rules, and imaging tests. Notably, while these parameters have been frequently assessed in prior studies, our study uniquely evaluated them comparatively, incorporating cellular indices within a large, registry-based database, ensuring the robustness and accuracy of the findings. Two recent meta-analyses reported similar findings. NLR was identified as the most significant predictor of mortality in patients with PE [446]. In contrast, pooled analysis did not demonstrate PLR as a predictor of mortality in this patient population. While not outperforming NLR, SII, however, was shown to be a significant predictor for PE diagnosis, severity, and mortality in PE [447].

Furthermore, NLR, along with anemia and renal insufficiency (but not SII or PLR), emerged as an independent predictor of major bleeding in this study. None of the cellular indices predicted recurrent VTE; however, active cancer, leg varicosities, chronic lung disease, and incidental PE were identified as independent predictors of VTE recurrence. It is important to note that no previous studies have evaluated cellular indices in the context of bleeding and VTE recurrence, highlighting the significance of these findings. Lastly, these results suggest that NLR may be a more promising cellular index for further investigation in the setting of VTE.

To further evaluate cellular indices, our second study analyzed three key blood cellular indices; NLR, PLR, and SII in relation to 30-day mortality outcomes using both the RIETE registry and the LUMC PERT cohort [448]. The results showed that, in multivariable analysis, an elevated NLR (>7.0) was significantly associated with increased 30-day mortality (adjusted odds ratio [aOR]: 3.46; 95%

CI: 2.60-4.60), outperforming PLR >220 (aOR: 2.36; 95% CI: 1.77–3.13) and SII >1600 (aOR: 2.52; 95% CI: 1.90–3.33). NLR demonstrated the highest predictive accuracy for low-risk patients (c-statistic: 0.78; 95% CI: 0.69–0.86) compared to intermediate-risk (c-statistic: 0.66; 95% CI: 0.63–0.69) or high-risk PE (c-statistic: 0.68; 95% CI: 0.59–0.76), establishing NLR as the best predictor for low-risk PE patients. And this trend was also consistent in the LUMC PERT cohort.

We also compared the predictive ability of NLR with the simplified Pulmonary Embolism Severity Index (sPESI) in normotensive, non-high-risk patients. The sPESI demonstrated superior sensitivity (87.1%) and negative predictive value (NPV) (98.7%) compared to NLR ≤7.0 (60.9% sensitivity and 97.8% NPV). However, integrating NLR with sPESI improved sensitivity to 97.1% (95% CI: 95.5–98.7) and NPV to 99.7% (95% CI: 99.5–99.8). Although NLR alone showed significant predictive power, its integration with sPESI scores further enhanced the accuracy in identifying low-risk patients.

The sPESI score, designed with six variables, offers clinicians a streamlined and efficient method for predicting short-term mortality risk in acute PE. This score has demonstrated similar prognostic accuracy to the PESI score for predicting 30-day mortality and has been evaluated for other PE outcomes [262, 449].

Several studies have aimed to enhance the sPESI score for risk prediction in PE. For example, incorporating serum sodium and bicarbonate into sPESI scores improved the accuracy of in-hospital mortality predictions following PE [450].Another study that added D-dimer to sPESI significantly increased the specificity of in-hospital death prediction [451]. Additionally, an analysis from the RIETE registry found that including acute kidney injury status in the sPESI score improved the prediction of 30-day mortality [452].

Another RIETE study identified four additional predictors (high-risk sPESI, immobilization, coexisting DVT, and plasma creatinine >2 mg/dL) which, when added to sPESI, enhanced the prediction of 30-day mortality in elderly PE patients [453]. These findings were validated in both the RIETE derivation cohort and the COMMAND VTE validation cohort. One study from the Loyola group has explored incorporating cellular indices to improve sPESI score. This study demonstrated that adding red cell distribution width, hematocrit, and neutrophil-lymphocyte ratio to sPESI score provided better predictive ability for overall mortality (AUC: 0.852 vs. 0.754) compared to sPESI alone [454]. It is important to

note that blood cellular indices may offer an additional inflammatory and hemodynamic perspective not currently included in sPESI. The complete blood count (CBC) with differential is a widely used, low-cost test that could enhance current risk stratification tools including sPESI score for prognosis in acute PE patients.

Thrombin exerts a multifaceted role in the pathophysiology of VTE, influencing not only clot formation and stabilization but also the activation of platelets, monocytes, macrophages, and endothelial cells [17, 455, 456]. Activated cells release products that can induce localized effects, such as vasoconstriction and blood flow impedance. The aim of our third study was to elucidate the relationship between thrombin generation potential, thrombin generation biomarkers and cellular indices, enhancing our understanding of thrombin's role in cellular activation within the context of VTE [457].

In this study, thrombin generation potential parameters exhibited significant differences when compared to healthy controls. Specifically, peak thrombin and endogenous thrombin potential (ETP) were lower in patients with PE compared to healthy controls, resulting in a prolonged lag time for thrombin generation in PE patients. Additionally, elevated levels of thrombin generation biomarkers such as D-dimer, F1+2, and thrombin-antithrombin (TAT) indicated increased thrombogenesis and enhanced fibrinolysis in PE patients. Moreover, all cellular indices tested were elevated in PE patients compared to healthy controls, highlighting the influence of thrombo-inflammation on thrombin generation potential and biomarkers in PE patients.

Stratification of parameters for PE severity revealed significant differences in D-dimer, F1+2, TAT, PLR, and SII levels, whereas thrombin generation potential parameters did not show any significant difference in PE severity. There were significant correlations between thrombin generation potential, biomarkers and cellular indices, specifically NLR and SII were correlated with lag time. NLR, PLR and SII correlated with TF levels as well. These results collectively explain the paradoxical imbalance observed in PE patients, characterized by increased thrombogenesis alongside dampened thrombin generation potential and over-activated fibrinolysis. The influence of thrombo-inflammation, as reflected by cellular indices, further highlights this imbalance. Given the use of anticoagulants

for treatment, this imbalance may also contribute to an increased risk of bleeding complications in these patients.

Related to these findings, several studies in the literature have utilized thrombin generation potential to assess VTE. One study evaluated the reactivity of thrombin to thrombomodulin (TM) by measuring the TM-bound thrombin to total thrombin generation (TMBT ratio) in patients with deep vein thrombosis (DVT) compared to healthy controls [458]. The TMBT ratio was identified as an independent predictor of VTE, suggesting its potential as a marker for DVT. In trauma patients, two studies assessed symptomatic VTE [459, 460]. One found that markers of thrombin generation were significantly elevated within 24 hours of injury, remained persistently high for 5 days, and declined by day 14. However, no coagulation markers were independently predictive of VTE. The other study, however, found that time to peak thrombin (tPeak) was an independent predictor of symptomatic VTE within 92 days post-trauma. Thrombin generation potential has also been evaluated in cancer patients, where elevated peak thrombin was associated with a higher risk of cancer-related VTE [461]. The cumulative probability of VTE over six months was significantly greater in patients with elevated peak thrombin. These findings suggested that measuring thrombin generation may help identify cancer patients at high risk for VTE.

Several studies have evaluated thrombin generation potential in relation to recurrent VTE. One study found that ETP was the only parameter significantly associated with unprovoked recurrent venous thrombosis in multivariate analysis, with patients exhibiting higher ETP having a significantly increased recurrence rate [462]. Similarly, another study demonstrated that elevated ETP conferred a 1.6-fold increased risk of recurrent VTE and was an independent predictor of recurrence [463]. In a study that measured thrombin generation markers during an acute VTE event, patients with recurrent VTE exhibited significantly elevated thrombin generation, whereas those without recurrence had higher peak thrombin concentrations [464]. Another investigation of recurrent DVT demonstrated altered plasma clot properties, increasing the risk of recurrence, although peak thrombin levels were not significantly different in PTS patients [464, 465].

As observed, none of these studies incorporated cellular indices in conjunction with thrombin generation potential and biomarkers. Therefore, the findings of this study are of major importance, as they offer novel insights into the

interplay between cellular activation and thrombin generation in the context of VTE.

In our fourth study, we investigated a comprehensive panel of biomarkers related to endothelial dysfunction, platelet activation, coagulation, fibrinolysis, and inflammation in PE, providing valuable insights into the complex interplay among these networks in VTE. Key findings included significantly elevated endothelial markers (VWF, and TF) and platelet activation biomarker (P-selectin) in PE patients, with a mixed coagulation profile showing increased factors IX and X and decreased factors VII and XIII. Elevated fibrinolysis markers (D-dimer, tPA, and PAI-1) and inflammatory markers (CRP, IL-6, and TNF-α) further underscored the dysregulation of these systems in PE. Cellular indices (NLR, PLR, SII) were also higher, reinforcing the link between inflammation and VTE pathophysiology.

Correlations between cellular indices and inflammatory/coagulation biomarkers were comprehensive, with NLR showing significant associations with TNF-α, CRP, IL-6, and TF, while PLR correlated with TF and SII with TNF-α, CRP, and IL-6. Stratifying biomarkers by severity and mortality revealed that endothelial, platelet activation, coagulation, and inflammatory markers, as well as cellular indices, were significantly linked to disease severity and mortality.

Multivariate analysis identified specific biomarkers as independent predictors of mortality, including VWF >140 %, E-selectin >48 ng/mL, TF >130 pg/mL, decreased coagulation factors (FVII <65 %, FX <65 %, FXIII-A <58 %), elevated fibrinolytic markers (D-dimer >13,688 ng/mL, tPA >15 ng/mL, PAI-1 >63 ng/mL, TAFIa <95 ng/mL), and inflammatory markers (CRP >12 μg/mL, IL-6 >48 pg/mL, TNF-α >2 pg/mL), along with higher NLR, PLR, and SII. These findings highlight the significant roles of these biomarkers in both reflecting disease severity and predicting mortality, offering potential tools for improving risk stratification in PE patients.

This study holds particular significance because, while many of these biomarkers and cellular indices have been tested individually in numerous studies, they have never been examined comparatively in such a large patient cohort. By leveraging the LUMC PERT cohort, we were able to comprehensively analyze these markers in the context of VTE. Notably, the results demonstrated that the NLR >7, a readily available tool in clinical practice, represented a higher risk of mortality compared to other biomarkers. The other cellular indices, PLR and SII also had

significant associations with mortality. This finding highlights the utility of NLR as a simple, cost-effective marker for risk stratification in VTE patients, underscoring its potential value in guiding clinical decision-making.

This study highlighted the valuable role of biomarkers and cellular indices in predicting severity and mortality in VTE, providing valuable insights into the interplay among endothelial function, platelet activation, coagulation, fibrinolysis, and inflammation. Further research is needed to explore these findings in other clinical settings, such as bleeding complications; particularly major bleeding, cancer-associated thrombosis, coronary artery syndrome, postoperative outcomes, infections, autoimmune/inflammatory diseases, and chronic kidney disease in the setting of VTE. These investigations could enhance our understanding and improve the clinical application of these markers.

In our fifth study, we assessed inflammatory cytokines, chemokines, growth factors, and cellular indices in PE patients and healthy controls to further elucidate the role of inflammation in VTE. In this study, all the tested parameters were significantly elevated in PE patients compared to healthy controls. Notably, TNF-α, IL-1β, IL-4, IL-6, IL-8, IL-10, and MCP-1 were significantly associated with PE severity, while cellular indices did not show direct associations. Correlation analysis highlighted the role of additional inflammatory cytokines, with NLR correlating with IL-6, IL-8, and IL-10, and SII correlating with IL-6 and IL-10. PLR was correlated with IL-4, and all cellular indices were interrelated. All parameters were significantly associated with 30-day mortality. Multivariate analysis identified TNF-α >2.0, IL-1β >1.4, IL-2 >1.4, IL-6 >48, IL-8 >20, IL-10 >1.6, MCP-1 >125, NLR >7, PLR > 220, and SII >1600 as significant predictors of 30-day mortality, with NLR >7, SII >1600, IL-8 >20, and MCP-1 >125 showing the highest prognostic value.

This study provided significant insights into the inflammatory landscape of VTE. The relationship of inflammatory cytokines, chemokines, and growth factors with NLR were notable. These findings highlighted the potential of NLR >7 as a valuable tool leading more precise and effective management strategies in VTE.

Despite the independent design of the RIETE and PERT trials, both trials have similar parameters. The neutrophil count is comparable. The lymphocyte count also is comparable and platelet count is indistinguishable. Therefore, the ratios such as NLR range from 2.7-7.2 with a mean value of 4.3 for the RIETE trial and 2.6-

8.7 with a mean value of 4.8 for the PERT trial. The PLR ranged from 104-231 with a mean value of 151 for the RIETE trial and 151-263 for the PERT trial. The SII ranged from 570-1729 with a mean value of 961 for RIETE trial, whereas 526-2089 with a mean value of 1099 for PERT. The patient numbers in the RIETE trial were 10085 and the PERT trial they were 700. The gender and age were balanced, however in the RIETE trial the prevalence of cancer was 14.7% and was 23.7% in PERT. The 30-day mortality rate was higher in the PERT trial at 8.8% in comparison to 4.4% in RIETE. There were some differences in the PERT trial compared to the RIETE study in terms of risk stratification. The PERT cohort represented Loyola in house patients which included mostly pulmonary embolism patients which were referred from other hospitals. The demographic parameters included cellular population which were similar and while the number of patients recruited were different.

The comprehensive synthesis of the five studies presented in this thesis offers a robust and multilayered approach to understand VTE and its associated complications. The first study's evaluation of blood cellular indices (NLR, PLR, and SII) provided valuable insights into their role as predictors of mortality, and bleeding, highlighting the utility of NLR particularly. The second study reinforced these findings by demonstrating NLR's superior predictive accuracy for 30-day mortality compared to other indices and its enhanced performance when integrated with the sPESI scores. The third study expanded on these results by elucidating the complex interplay between thrombin generation potential with biomarkers and cellular indices, revealing the paradoxical balance between thrombogenesis and fibrinolysis in PE patients. The fourth study's extensive analysis of biomarkers related to endothelial function, platelet activation, coagulation, and inflammation further underscored the complex networks involved in VTE pathophysiology and provided novel insights into biomarkers for predicting severity and mortality. Finally, the fifth study's focus on inflammatory cytokines, chemokines and growth factors with cellular indices highlighted their significant associations with VTE severity and mortality, reinforcing the potential of these indices for improving risk stratification and management strategies. Collectively, these studies address critical knowledge gaps and establish a

comprehensive framework for enhancing VTE care, emphasizing the need for further research to refine diagnostic and prognostic tools in diverse clinical settings.

To conclude, this dissertation successfully fulfilled its primary hypothesis that cellular indices, including NLR, PLR, and SII, along with thromboinflammatory biomarkers serve as significant predictors of clinical outcomes in VTE patients. By achieving all specific aims, we demonstrated the multifaceted role of these indices in predicting VTE prognosis and provided new insights into their utility in clinical practice. Our studies encompassed a comprehensive evaluation of the prognostic value of blood cellular indices, elucidated the complex relationship between thrombin generation potential, thrombo-inflammatory biomarkers, and cellular indices in acute PE, and highlighted their comparative roles. Additionally, we conducted extensive biomarker profiling, encompassing both endothelial and inflammatory markers, which underscored the complex interplay of these factors in VTE. Collectively, these findings substantiate the clinical relevance of cellular indices and thromboinflammatory biomarkers in risk stratification models and therapeutic and interventional strategies of VTE, thereby advancing our understanding and potentially guiding future management strategies.

VII – CONCLUSION

VII - CONCLUSION

This dissertation has validated the prognostic values of blood cellular indices and the profiling of biomarkers in VTE in 2 large cohorts of independent studies. This is the first study in which the integrated role of cellular indices and thromboinflammatory biomarkers is investigated.

Consistent with first aim, we were able to determine the prognostic value of blood cellular indices of NLR, PLR, and SII in patients with VTE and their relevance with the 90-day outcomes for all-cause mortality, major bleeding and VTE recurrence. In conjunction with the RIETE registry, the importance of the cellular indices such as NLR, PLR, and SII were validated. Predictive analysis on patient's results was carried out. NLR was found to be more reliable in predicting outcomes.

Second aim was focused on the comparative prognostic value of blood cellular indices in patients with acute PE in two independent cohorts namely the RIETE registry and LUMC PERT cohort. Of all the cellular indices, NLR was found to be the best predictor for 30-day mortality in PE patients and demonstrated to have highest predictive accuracy in low-risk patients. Furthermore, the incorporation of NLR in sPESI score enhanced the predictive power for identifying low-risk PE patients at increased risk of death.

The RIETE registry is ongoing and continues to recruit patients and additional analysis can lead to more information regarding the cellular contribution to pathophysiology of VTE. Sub-analysis in terms of genetic predisposition and other demographics will reveal additional information. The cellular indices offer the ready availability of the data, cost and additional benefits which can be included in standard of care for the patients.

Third aim addressed the thrombogenesis in reference to decreases in thrombin generation potential and simultaneous increase of biomarkers of thrombin generation in PE patients, such as D-dimer, TAT, F1+2, and TF. Combining the cellular indices with thrombin generation profile amplified the predictive value in PE. The relationships between thrombin generation potential, biomarkers, and cellular indices not only reinforce their pathophysiological

relevance in PE but also emphasize the complexity of the disease including its severity.

Fourth aim were designed to understand the molecular pathogenesis of PE by profiling the biomarkers of hemostatic activation and thromboinflammation in patients with acute PE and their relevance with disease progression and outcome as all-cause mortality within the 30-days of diagnosis. To accomplish this, biomarkers originating from both blood cellular elements and endothelial cells were considered. In this integrated analysis, the hemostatic activation, and thromboinflammatory biomarkers including coagulation activation, fibrinolysis process, and platelet function was profiled in 500 PE patients. Most of the biomarkers along with cellular indices were significantly elevated, consistent with PE severity, and 30-day mortality suggesting that combined use of cellular indices parameters with biomarkers data may improve risk stratification for outcome in these patients.

Since TF plays an important role in the regulation of thrombo-inflammatory biomarkers and their mediation with thrombin, the PERT study also included TF. The data is correlated with the cellular indices in an integrated manner. The relevance of cellular activation in VTE in terms of biomarker generation is established. The Loyola cohort also served as the validation for the RIETE registry in an independent manner.

The fifth aim was focused to understand the role of inflammation in patients with acute PE by profiling inflammatory cytokines, and chemokines along with cellular indices and their relevance with disease progression. To accomplish this, we profile the mediators of inflammation utilizing the biochip array for cytokines, chemokines, and growth factors. In addition, the generated data was stratified with severity, and outcome as all-cause mortality within the 30 days of diagnosis**.** The data is correlated with the cellular indices in an integrated manner.

The central role of thromboinflammation is widely known in the mediation of VTE, however the role of cellular components and the value of cellular indices remains to be investigated, it is likely that the activation of blood and endothelial cells results in the mediation of the generation of additional biomarkers of thromboinflammation. Moreover, the role of TF is central in the process of the pathogenesis of VTE. Additional identification of the cellular activation biomarkers and the recent findings regarding the role of mRNA warrants further investigation.

A comprehensive molecular reassessment of the complex pathogenesis involving VTE, the interdependence of cellular activation and the biomarkers generated will require further understanding on the molecular mechanisms using advance molecular biological techniques.

Overall, this dissertation focused on the prediction of the adverse outcomes based on cellular indices and various biomarkers. Numerous risk models were proposed and demonstrated their relevance to the cellular modulation and biomarker changes in plasma.

Together with the RIETE registry and PERT program at Loyola, the data presented in this dissertation demonstrated a unique approach in the molecular and cellular diagnosis of VTE. Not only risk stratification, and outcomes predicted, but these findings may also have major implications on improvement of individualized therapy, and development of novel therapeutic interventions in VTE. Based on these investigations, sub-clinical diagnosis, and risk prediction with outcomes will be possible. This thesis therefore represents a unique approach which can be tested for molecular, and cellular diagnosis of VTE. Based on specific biomarker profiles, by using biosensor technology, newer diagnostic modalities can also be developed.

VIII – FUTURE CONSIDERATIONS

VII - FUTURE CONSIDERATIONS

The RIETE registry provides a large cohort of patients with VTE, which made it possible to validate the value of cellular indices and their predictive value for outcome. Such registries will play a role in validating cellular indices and future instrumentation will include these indices for prognostic usage. Owing to the simplicity and the ease of data retrieval, cellular indices can be tabulated and computed. These can be computed automatically, and results can be available along with the complete blood count.

Beside the blood cellular indices, circulating endothelial cells may be factored in and included in the analysis along with the blood cellular population. Thus, endothelial cells and distribution could be part of cellular indices calculations in the future. Fluctuations in cellular indices may reflect the pathologic alterations and drug modulation and interactions between the cells.

It will be interesting to measure the cellular indices during normal and abnormal hematopoiesis. This will be useful in the diagnosis of bone marrow health. It would be interesting to evaluate blood cellular indices in other inflammatory states particularly the chronic conditions. Blood cells are integral to the physiological reaction to inflammation. Inflammatory biomarkers associated with blood cellular indices could potentially be a more robust indicator of clinical disease than blood cell indices alone.

The integration of multi-omics technologies, including genomics, proteomics, and metabolomics with blood cellular indices and biomarker profiling holds significant promise for a comprehensive understanding of VTE. Future studies can aim to correlate these biomarkers with genetic and proteomic profiles, offering deeper understanding into variations in different pathways leading to development of precision medicine approaches and personalized therapeutic strategies.

Most studies have focused on single time point measurements of blood cellular indices and biomarker profiles. Future research should prioritize longitudinal studies to monitor these biomarkers over time particularly in response

to treatment and disease progression. Such studies can clarify dynamic changes in biomarker levels, enhancing our knowledge to predict outcomes and modify treatments more precisely. Interventional studies are also needed to determine treatment effects on biomarkers. Clinical trials can explore the therapeutic implications of targeting cellular indices and biomarkers especially in high-risk populations such as patients with cardiovascular diseases or cancer.

Mounting evidence suggests that microbiome may influence VTE pathogenesis. Future research should investigate the interactions between cellular indices and biomarker profiles and microbiome. This will uncover novel therapeutic targets for VTE.

Comprehensive biomarker profiling will provide molecular dissection for the pathophysiologic transition and drug induced modification of cellular indices and will help not only in the diagnosis but therapeutic management of ongoing VTE. The panel profiling can provide computerized algorithms for contributions to cellular populations and their impact on biomarkers. Such analysis on cellular indices, biomarker profiling through biochip technology will be carried out on microreader samples of drops of blood. Additional and highly sensitive biomarkers will be developed, and microRNA platforms can be used for the analysis using dedicated instruments. Additional studies including epigenetics will be integrated in these biomarkers of cellular indices profiling of large registries enabling the vast explanation for pharmacovigilance and drug discovery programs. Large databases from NIH, NCI and other organizations will be very useful in further validating the high throughput technology. Artificial intelligence, machine learning and hybrid technology will play a role in future studies. There will be uniformity at international levels and disease panels can be generated.

Non-invasive technology will also play a role in sensitive biosensor including microimaging analysis for blood cellular and endothelial indices and biomarkers of thromboinflammation may be developed. The integrated cellular and biomarker analysis with some imaging techniques will be the future of risk prediction and management of optimal care of VTE patients. The data provided in this dissertation lends itself to the foundation of advanced technologies to be used, not only in the management of VTE but also other diseases such as cancer, auto-immune diseases, infections, sepsis, COVID-19, and long COVID syndrome.

IX – SIGNIFICANCE

VIII - SIGNIFICANCE

This research on the value of cellular indices and biomarker profiling in VTE in two large independent cohorts provides an opportunity to validate the hypothesis that cellular indices in biomarker profiling may provide independent signals to identify high risk patients and aid in the prognosis of VTE. RIETE is a large registry which addresses thrombosis, and in one subset the impact of cancer on the diagnosis and treatment of VTE. RIETE provided a distinctly unique opportunity to investigate and validate the predictive value of cellular indices in our chosen cohort of patients with PE. Such data is helpful in the development of guidelines for VTE management. The simplicity and ready availability of blood count parameters make it an affordable and practical medical management advancement.

The Loyola study represents the largest profile in 500 patients with confirmed PE for the most extensive profiling of biomarkers including blood cellular and endothelial markers along with protease regulation and the cleavage biomarkers which are generated because of pathologic regulation. The biomarkers of this study provide a comprehensive signal as they complement coagulation, fibrinolysis, organ-based proteases, and related cleavage biomarkers. A unique feature of this study is the integration of the biomarkers and the cellular indices. This is the first time an attempt has been made to delineate the role of cellular activation and its impact on biomarker discovery. This data lends itself to improve the comprehension of the molecular pathophysiology of the complex mechanisms involved in PE. Since biomarkers are highly sensitive measurable analytes, the pathophysiology of VTE can be investigated at a subclinical state. Additionally, and importantly, the generation of biomarkers is proportional to the extent of severity of VTE. Thus, the current studies, taken together, provide a unique approach where the integrated value of cellular biomarkers and cellular indices is considered. The information gained is most useful in developing mathematical models which profile the disease state by identifying specific deficits within the pathologic mechanisms. The multiple correlations between cellular indices and biomarkers sheds light on the interdependence of the mechanisms involved.

Therefore, this is the first study where cellular indices and biomarker findings, obtained from large database research, will be available to develop clinically effective predictive modellings with artificial intelligence and machine learning tools.

Both the RIETE and PERT study will continue to provide additional information on the mechanisms involved in the pathogenesis of VTE. Additional advances and molecular and cellular sciences will result in the discovery of new biomarkers which can be included in the improvement of diagnosis of VTE. The information generated can be banked in large database such as World Health Organization, and Center for Disease Control. This information will also be useful in developing new therapeutic strategies and device management of thrombotic diseases and other cardiovascular, and cerebrovascular disorders. The overarching outcome of this project will benefit health care globally.

X – REFERENCES

IX - REFERENCES

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XI – APPENDICES

X - APPENDICES

Appendix 1. UCAM Approval

UCAM
UNIVERSIDAD CATÓLICA SAN ANTONIO

COMITÉ DE ÉTICA DE LA UCAM

Universidad Católica San Antonio. Vicerrectorado de Investigación. Salida nº 8011 29/04/2022 17:36:13

DATOS DEL PROYECTO

Título:	"Utilidad clínica de los contadores celulares en pacientes con enfermedad tromboembólica venosa"	
Investigador Principal	**Nombre**	**Correo-e**
Dr.	Manuel Monreal Bosch	manuelmonrealbosch@gmail.com

INFORME DEL COMITÉ

Fecha	**29/04/2022**	**Código**	**CE042203**

Tipo de Experimentación

Investigación experimental clínica con seres humanos	
Investigación experimental no clínica con seres humanos	
Utilización de tejidos humanos procedentes de pacientes, personas sanas, tejidos embrionarios o fetales	
Utilización de tejidos humanos, tejidos embrionarios o fetales procedentes de bancos de muestras o tejidos	
Investigación observacional, psicológica o comportamental en humanos	X
Uso de datos personales, información genética, etc.	X
Experimentación animal	
Utilización de agentes biológicos de riesgo para la salud humana, animal o las plantas	
Uso de organismos modificados genéticamente (OMGs)	

Comentarios Respecto al Tipo de Experimentación
Nada Obsta

Comentarios Respecto a la Metodología de Experimentación
Nada Obsta

UNIVERSIDAD CATÓLICA SAN ANTONIO · UCAM · COMITÉ DE ÉTICA · MURCIA ·

UCAM
UNIVERSIDAD CATÓLICA SAN ANTONIO

COMITÉ DE ÉTICA DE LA UCAM

Sugerencias al Investigador

A la vista de la solicitud de informe adjunto por el Investigador y de las recomendaciones anteriormente expuestas el dictamen del Comité es:

Emitir Informe Favorable	X
Emitir Informe Desfavorable	
Emitir Informe Favorable condicionado a Subsanación	

MOTIVACIÓN
Incrementará conocimientos en su área

Vº Bº El Presidente,

Fdo.: José Alberto Cánovas Sánchez

UNIVERSIDAD CATÓLICA SAN ANTONIO · UCAM · COMITÉ DE ÉTICA · MURCIA ·

El Secretario,

Fdo.: José Alarcón Teruel

Appendix 2. RIETE Registry IRB approval

Germans Trias i Pujol
Hospital

Comitè d'Ètica de la Investigació
Crta. De Canyet, s/n - 08916 Badalona
Tel. 93 497.89.56 Fax 93 497.89.74
E-mail: ceic.germanstrias@gencat.cat

Institut Català de la Salut

INFORME DEL COMITÉ DE ÉTICA DE LA INVESTIGACIÓN

El Dr. Magí Farré Albaladejo, Presidente del Comité de Ética de la Investigación del Hospital Universitario Germans Trias i Pujol,

CERTIFICA

Que este Comité ha evaluado la propuesta del **Dr. Manel Monreal Bosch (Servei de Medicina Interna - HUGTiP)** para que se realice el proyecto de investigación, código de protocolo **RIETE** titulado:

"Registro informatizado de pacientes con enfermedad tromboembólica (RIETE)." Versión: **3 (17/03/2017)**

Hoja de Información al Paciente y Consentimiento Informado Versión: **3 (17/03/2017)**

y considera que:

Se cumplen los requisitos necesarios de idoneidad del protocolo en relación con los objetivos del estudio y que el estudio cumple con los aspectos que se recogen en la Ley de Investigación Biomédica 14/2007.

El procedimiento para obtener el consentimiento informado, incluyendo la hoja de información para los sujetos y el plan de reclutamiento de sujetos previstos son adecuados.

La capacidad del investigador y sus colaboradores, así como los medios disponibles son apropiados para llevar a cabo el estudio.

Y que este Comité acepta que dicho estudio sea realizado en el Hospital Universitario Germans Trias i Pujol por el **Dr. Manel Monreal Bosch** del Servicio de Medicina Interna como investigador principal.

Lo que firmo en Badalona a 7 de abril de 2017

Firmado:

Generalitat de Catalunya
Departament de Salut

Germans Trias i Pujol
Hospital

Comitè d'Ètica de la Investigació

Dr. Magí Farré Albaladejo

REF. CEI: PI-17-053

Appendix 3. Loyola University Medical Center IRB approval.

LOYOLA UNIVERSITY CHICAGO
Health Sciences Division
Institutional Review Board for the Protection of Human Subjects
2160 South First Avenue
Maywood, IL 60153

11/09/2021

NOTICE OF FULL APPROVAL OF A RESEARCH PROJECT

Investigator	**Darki, Amir**
LU Number	**209457**
Title	**Experience with the Pulmonary Embolism Response Team at LUMC**
Date of Initial Review	**03/22/2017**
Type of Review	**Expedited**
Action of Initial Review	**Full Approval**

IRB Findings

1. The study is of minimal risk and qualifies for expedited review 45CFR46.110, b-1, HHS Secretary Category # 5).

2. The requirement for consent is waived:

a) the research could not practicably be done without the waiver;

b) the waiver does not adversely affect the rights of the individual;

c) the research involves no more than minimal risk;

d) there is no information that would need to be provided to the individual.

(45CFR46.116, d1-4).

3. The IRB finds that:

(A) The use or disclosure of protected health information involves no more than minimal risk to the individuals;

(B) The alteration or waiver will not adversely affect the privacy rights and the welfare of the individuals;

(C) The research could not practicably be conducted without the alteration or waiver;

(D) The research could not practicably be conducted without access to and use of the protected health information;

(E) The privacy risks to individuals whose protected health information is to be used or disclosed are reasonable in relation to the anticipated benefits if any to the individuals, and the importance of the knowledge that may reasonably be expected to result from the research.

(F) There is an adequate plan to protect the identifiers from improper use and disclosure;

(G) There is an adequate plan to destroy the identifiers at the earliest opportunity consistent with conduct of the research, unless there is a health or research justification for retaining the identifiers, or such retention is otherwise required by law;

(H) There are adequate written assurances that the protected health information will not be reused or disclosed to any other person or entity, except as required by law, for authorized oversight of the research project, or for other research for which the use or disclosure of protected health information would be permitted by this subpart.

4. The IRB finds that in # 3 above and the conditions of approval that the requirements detailed in 45CFR164.512i2 are met and the requirement for patient authorization waiver is met.

5. This letter should be shown to medical records to allow the retrospective chart review to begin.

6. Refer to conditions of approval.

Informed Consent Document required? **NO**

Reason ICD not required	**45CFR46.116, (d1-d4)**
# of Participants	**0**
Participants Compensated?	**NO**

IRB Number	**209457032217**
Date of Approval	**03/22/2017**
Frequency of Review	**Annual**
Date of First Review	**03/06/2018**

Conditions of Approval **Conditions of Approval:**

1. Please review the information section of this approval letter. You are required to review and adhere to the conditions of approval as stated in this letter (refer to project summary) as well as the LUMC P-6 policy regarding access to and release of patient information for the purpose of research. For questions you may contact the IRB office at extension 64608.

2. Institutional policy requires that patient identifiable research data must be collected and stored in a secured location. The medical center provides a centralized research server for this purpose. If you have not already done so, please e-mail Joseph Koral (x67904, jkoral@lumc.edu) and request an account for this server. In the request, please include all domain IDs of the individuals that require access to this resource. If you have questions regarding information system security please contact Dan Smith (x68207, or dasmith@lumc.edu).

3. You are bound by the usual and customary medical, legal and ethical considerations governing the confidentiality of the medical record.

4. The data you collect may not be sold or given to any third party outside the scope of this submission unless it is to a journal for publication. Data to a journal for publication must be de-identified.

5. The chart reviewed is to be identified on the data collection form by a unique code number and the master list kept under lock and key. If you are not collecting data elements that can directly or indirectly identify the chart reviewed then this requirement is not relevant.

6. The link of the patient to the project is to be destroyed when it is no longer necessary.

ITEMS SUBMITTED FOR REVIEW
- **01/09/2017 Document- uploaded 01/09/2017**

YOU HAVE FULL APPROVAL AND YOUR PROJECT MAY BEGIN.

The following is for your information and will help you meet local and federal IRB requirements.

1. You must use the final IRB-approved version of the Consent Document. Spelling and grammatical changes may be made as necessary, but any other changes require prior review and approval.

2. You are required to maintain complete records of this project. Any changes in the protocol and the Consent Document must receive prior IRB approval. Use the online Research Portal's Project Amendment form to report changes. A change to the protocol necessary for the immediate safety and welfare of a research participant may be implemented prior to IRB review and approval.

3. Federal Regulations require that projects undergo periodic review of research activity at least once a year. This review must be substantive. The frequency of review and next scheduled date of periodic review for your project can be found under the "Annual Review" tab in the Research Portal's IRB section. You will receive notification 4-8 weeks prior to the scheduled date of review. At that time, you must provide information regarding the status of the project. If the information is not received, the project will be suspended. It is important that you not let approval lapse.

4. The IRB must be notified any time that the project temporarily or permanently stops enrolling participants along with the reason. Use the online Closure form to submit these notifications.

5. Any notices or advertisements soliciting participation must receive prior IRB approval. Use the online Amendment reporting form.

6. The IRB must be notified PROMPTLY of all serious and any unanticipated adverse events associated with the project (or the device or the drug). This includes any notification received of adverse events occurring at other performance sites. Further guidance on adverse event reporting may be found at the Office for Human Research Protections web site; http://www.hhs.gov/ohrp/policy/AdvEvntGuid.htm#Q5

Reportable events include, but are not limited to:
a) a serious adverse event (including events that produce injury or death, an event leading to hospitalization or lead to prolongation of a current hospital stay);
b) the enrollment of a patient on a study that is no longer enrolling participants;
c) pregnancy occurring on the study where the study excludes pregnancy;
d) any patient reporting a billing problem as a result of project participation;
e) any participant who has voiced a complaint about some aspect of the project or the consent document;
f) any unanticipated, untoward, or unexpected adverse event not covered above including rare adverse events or adverse events that occur at an unexpected rate
g) protocol deviations
h) investigational drug/device brochures, revisions

Adverse Protocol Events are reported through the online Research Portal.

7. The IRB may suspend the project to new participant enrollment or may suspend the participation of current subjects if there is a perceived safety and/or regulatory issue.

8. Prospective consent must be obtained from all research participants.

9. The IRB may review your records relating to this project, including signed consent documents.

10. The Institutional Review Board of Loyola University Medical Center is appropriately constituted and has been granted Federal Wide Assurance Number FWA00017487.

11. If you are unsure of your reporting requirements or of what is expected of you during the conduct of this research, please call the IRB Office (708-216-4608) or Dr. Kenneth Micetich (708-327-3144).

12. The Loyola Institutional Review Board is appropriately constituted as stipulated in 45cfr46 and is compliance with Good Clinical Practice Guidelines insofar as those guidelines are consistent with the U.S. Food and Drug Administration regulations (21 CFR Parts 50 and 56) and the Department of Health and Human Services regulations (45 CFR 46) pertaining to the protection of human subjects in research.

Kenneth Craig Micetich, M.D.
Chairman
Institutional Review Board for the Protection of Human Subjects
Loyola University Health Sciences Division

LOYOLA UNIVERSITY CHICAGO
Health Sciences Division
Institutional Review Board for the Protection of Human Subjects
2160 South First Avenue
Maywood, IL 60153

09/23/2024

PROJECT AMENDMENT: NOTICE OF FULL APPROVAL

Investigator	**Darki, Amir**
LU Number	**209457**
Title	**Experience with the Pulmonary Embolism Response Team at LUMC**
IRB Number	**209457032217**
AMENDMENT #7 :	**Please add Mit Patel and Fakiha Siddiqui to the study. They have completed the citi training.**
Type of Change	**Administrative**
Change in Patient Risk	**No Change**
Change to ICD?	**NO**
Inform Past or Current Patients?	**NO**
Review Date	**12/06/2021**
Review Type	**Expedited**
Action	**Full Approval**
Comments	**None.**
DATE OF APPROVAL	**12/06/2021**

This Amendment Approval has been granted through an Expedited Review.
The Full Board will review the Amendment and/or changes to the
Informed Consent Document on 12/15/2021.

If the Board does not reaffirm this expedited decision,
you will be notified by 12/22/2021.

Kenneth Craig Micetich, M.D.
Chairman
Institutional Review Board for the Protection of Human Subjects
Loyola University Health Sciences Division

LOYOLA UNIVERSITY CHICAGO
Health Sciences Division
Institutional Review Board for the Protection of Human Subjects
2160 South First Avenue
Maywood, IL 60153

11/09/2021

CONTINUING REVIEW OF A RESEARCH PROJECT: NOTICE OF BOARD ACTION

Investigator	**Darki, Amir**
LU Number	**209457**
Title	**Experience with the Pulmonary Embolism Response Team at LUMC**
Date of Initial Approval	**03/22/2017**
IRB Number	**209457032217**
ITEMS SUBMITTED FOR REVIEW	• **02/28/2018 Protocol ver 1/07/2017**
Date of Continuing/Annual Review	**03/06/2018**
Date of Board Meeting	**03/21/2018**
Review Action	**Approved**
Comments	**This project continuing re-review is conducted via expedited mechanism; 45CFR46.110 category 8. The information in your report is reviewed. This is a retrospective chart review study. There are no research related interventions and no additional risks have been identified. Your project may continue.**

The Approval Date of this Continuing/Annual Review (#1) is 03/06/2018.

The scheduled date of the next continuing review of this Project is 03/06/2019.

Note: If the project was suspended and then subsequently approved, the Comments section will indicate the dates of the suspension.

Angela Carrico

LOYOLA UNIVERSITY CHICAGO
Health Sciences Division
Institutional Review Board for the Protection of Human Subjects
2160 South First Avenue
Maywood, IL 60153

11/09/2021

CONTINUING REVIEW OF A RESEARCH PROJECT: NOTICE OF BOARD ACTION

Investigator	**Darki, Amir**
LU Number	**209457**
Title	**Experience with the Pulmonary Embolism Response Team at LUMC**
Date of Initial Approval	**03/22/2017**
IRB Number	**209457032217**
ITEMS SUBMITTED FOR REVIEW	• **01/24/2019 protocol**
Date of Continuing/Annual Review	**01/29/2019**
Date of Board Meeting	**02/20/2019**
Review Action	
Comments	**This project continuing re-review is conducted via expedited mechanism; 45CFR46.110 category 8. The information in your report is reviewed. This is a retrospective chart review study. There are no research related interventions and no additional risks have been identified. Your project may continue.**

The Approval Date of this Continuing/Annual Review (#2) is 01/29/2019.
The scheduled date of the next continuing review of this Project is 01/29/2020.

Note: If the project was suspended and then subsequently approved, the Comments section will indicate the dates of the suspension.

Angela Carries

LOYOLA UNIVERSITY CHICAGO
Health Sciences Division
Institutional Review Board for the Protection of Human Subjects
2160 South First Avenue
Maywood, IL 60153

11/09/2021

CONTINUING REVIEW OF A RESEARCH PROJECT: NOTICE OF BOARD ACTION

Investigator	**Darki, Amir**
LU Number	**209457**
Title	**Experience with the Pulmonary Embolism Response Team at LUMC**
Date of Initial Approval	**03/22/2017**
IRB Number	**209457032217**
ITEMS SUBMITTED FOR REVIEW	• **11/24/2020 Protocol ver4 8/18/20**
Date of Continuing/Annual Review	**12/07/2020**
Date of Board Meeting	**12/16/2020**
Review Action	
Comments	**This project continuing re-review is conducted via expedited mechanism; 45CFR46.110 category 8. The information in your report is reviewed. This is a retrospective chart review study. There are no research related interventions and no additional risks have been identified. Your project may continue.**

The Approval Date of this Continuing/Annual Review (#4) is 12/07/2020.
The scheduled date of the next continuing review of this Project is 12/07/2021.

Note: If the project was suspended and then subsequently approved, the Comments section will indicate the dates of the suspension.

Angela Carrico

LOYOLA UNIVERSITY CHICAGO
Health Sciences Division
Institutional Review Board for the Protection of Human Subjects
2160 South First Avenue
Maywood, IL 60153

11/09/2021

CONTINUING REVIEW OF A RESEARCH PROJECT: NOTICE OF BOARD ACTION

Investigator	**Darki, Amir**
LU Number	**209457**
Title	**Experience with the Pulmonary Embolism Response Team at LUMC**
Date of Initial Approval	**03/22/2017**
IRB Number	**209457032217**
ITEMS SUBMITTED FOR REVIEW	• **10/26/2021 Protocol 08/18/2020 version 4**
Date of Continuing/Annual Review	**11/02/2021**
Date of Board Meeting	**11/17/2021**
Review Action	
Comments	**This project continuing re-review is conducted via expedited mechanism; 45CFR46.110 category 8. The information in your report is reviewed. This is a retrospective chart review study. There are no research related interventions and no additional risks have been identified. Your project may continue.**

The Approval Date of this Continuing/Annual Review (#5) is 11/02/2021.
The scheduled date of the next continuing review of this Project is 11/02/2022.

Note: If the project was suspended and then subsequently approved, the Comments section will indicate the dates of the suspension.

Kenneth Craig Micetich, M.D.
Chairman
Institutional Review Board for the Protection of Human Subjects
Loyola University Health Sciences Division

LOYOLA UNIVERSITY CHICAGO
Health Sciences Division
Institutional Review Board for the Protection of Human Subjects
2160 South First Avenue
Maywood, IL 60153

10/07/2022

CONTINUING REVIEW OF A RESEARCH PROJECT: NOTICE OF BOARD ACTION

Investigator	**Darki, Amir**
LU Number	**209457**
Title	**Experience with the Pulmonary Embolism Response Team at LUMC**
Date of Initial Approval	**03/22/2017**
IRB Number	**209457032217**
ITEMS SUBMITTED FOR REVIEW	• **10/06/2022 Protocol 09/27/22 version 6**
Date of Continuing/Annual Review	**10/07/2022**
Date of Board Meeting	**10/19/2022**
Review Action	
Comments	**This project continuing re-review is conducted via expedited mechanism; 45CFR46.110 category 8. The information in your report is reviewed. This is a retrospective chart review study. There are no research related interventions and no additional risks have been identified. Your project may continue.**

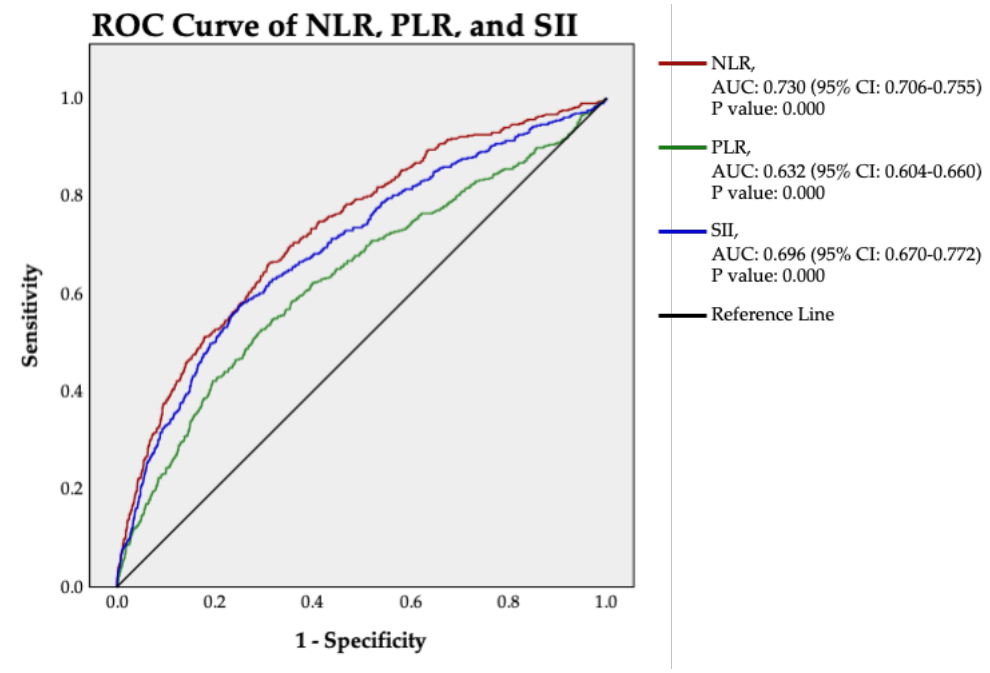
The Approval Date of this Continuing/Annual Review (#6) is 10/07/2022.

The scheduled date of the next continuing review of this Project is 10/07/2023.

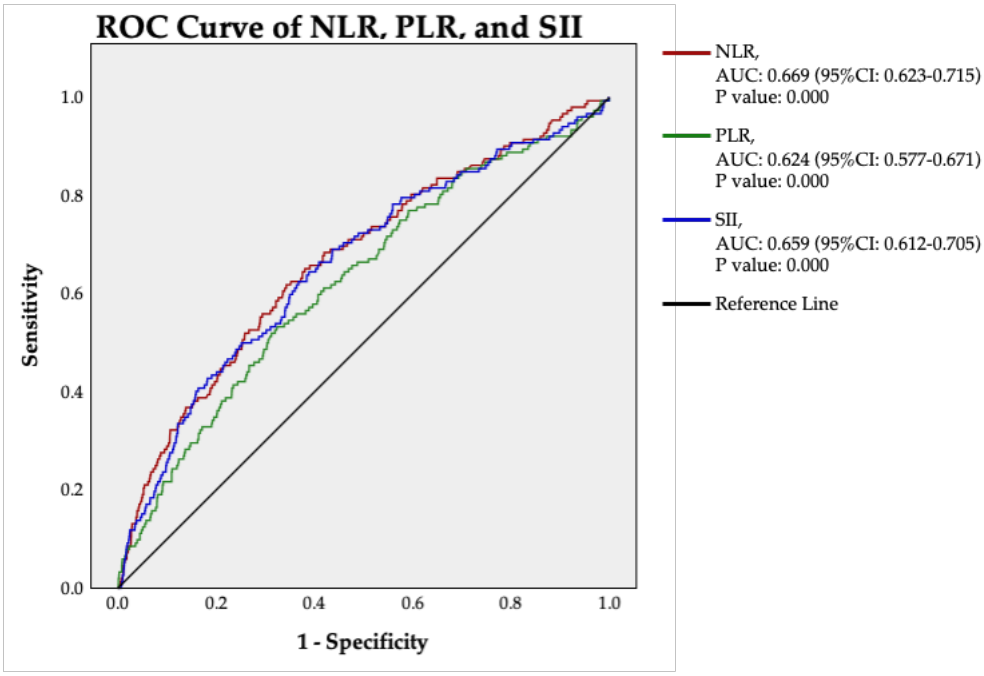
Note: If the project was suspended and then subsequently approved, the Comments section will indicate the dates of the suspension.

Angela Carrico

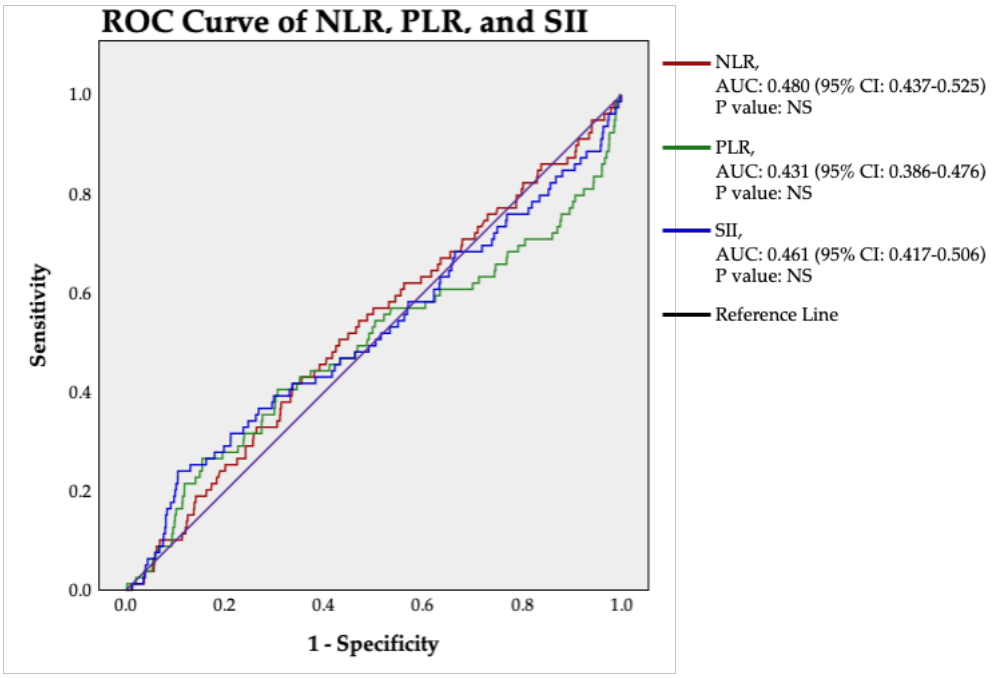
Appendix 4. Supplementary Figures



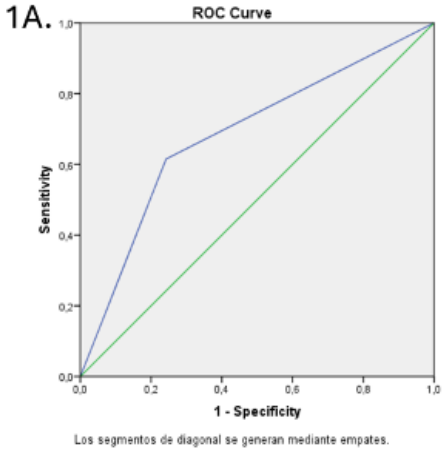
Supplementary Figure 1. ROC curve of NLR, PLR, and SII for 90-day mortality.



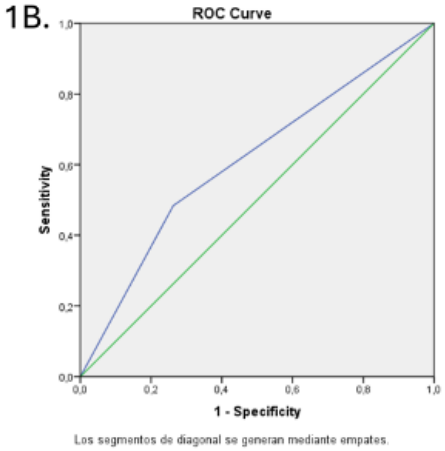
Supplementary Figure 2. ROC curve of NLR, PLR, and SII for 90-day major bleeding.



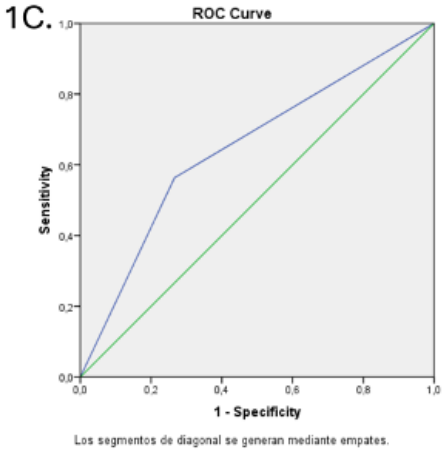
Supplementary Figure 3. ROC curve of NLR, PLR, and SII for 90-day VTE recurrence.



NLR cut-off point=7
AUC= 0.686 (0.660-0.713)
Standard error=0.013
p-value <0.001

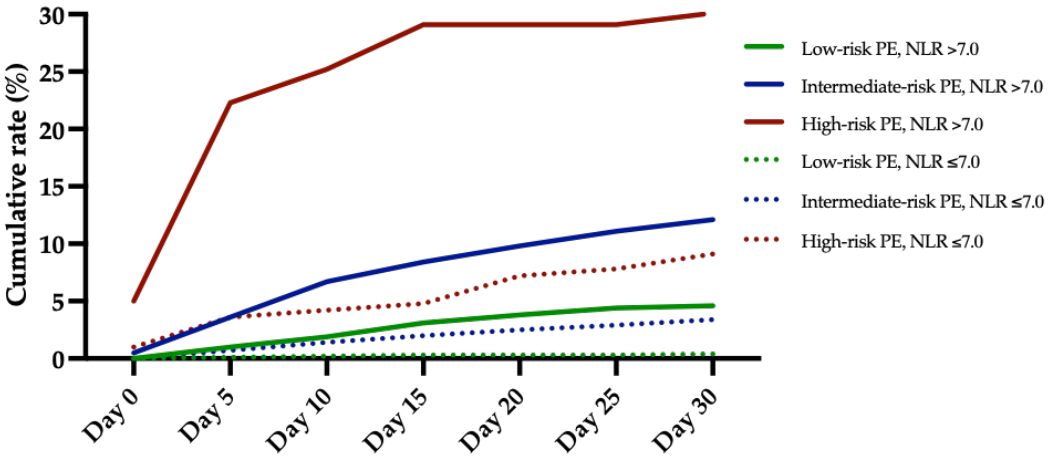


PLR cut-off point=220
AUC= 0.611 (0.583-0.638)
Standard error=0.014
p-value <0.001



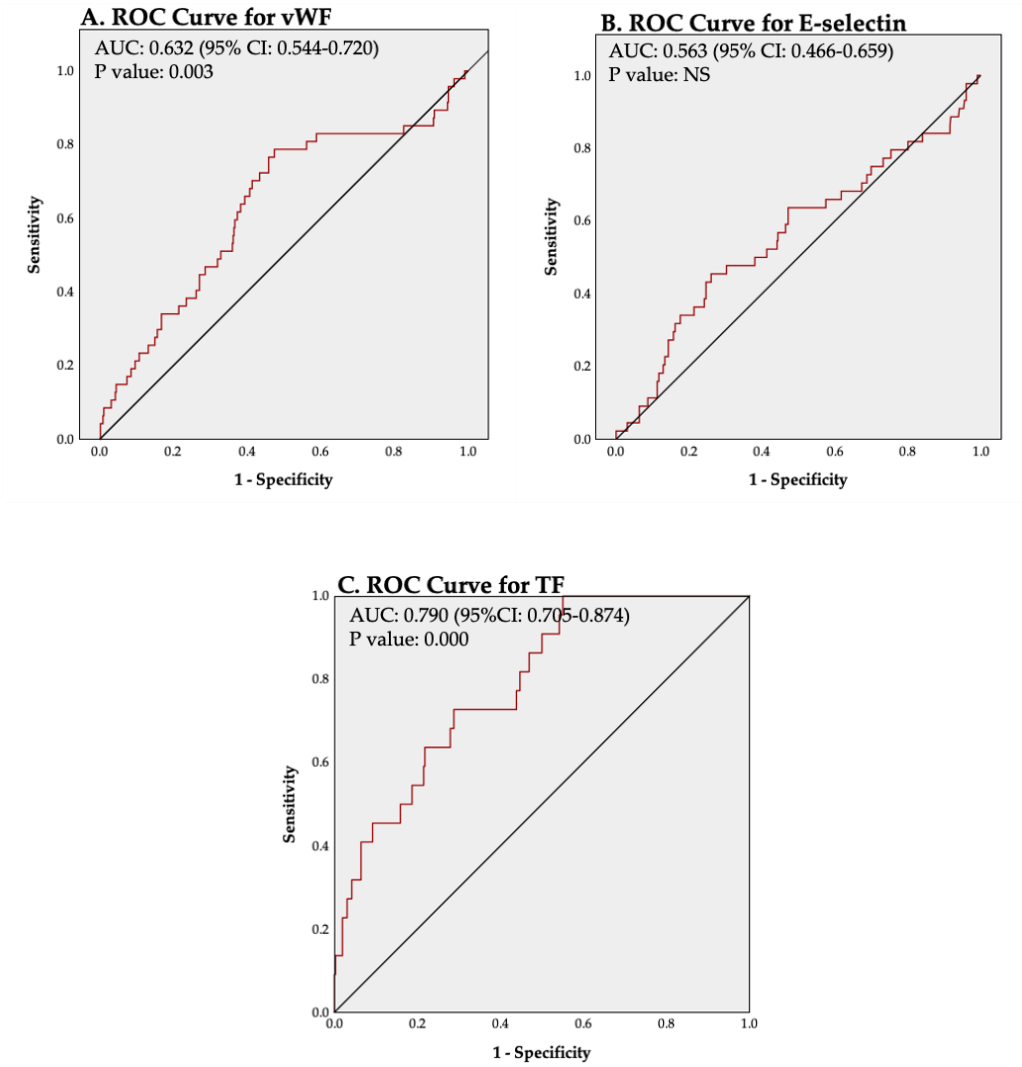
SII cut-off point=1,600
AUC= 0.649 (0.622-0.676)
Standard error=0.014
p-value <0.001

Supplementary Figure 4. ROC curve of NLR, PLR, and SII for 30-day mortality.

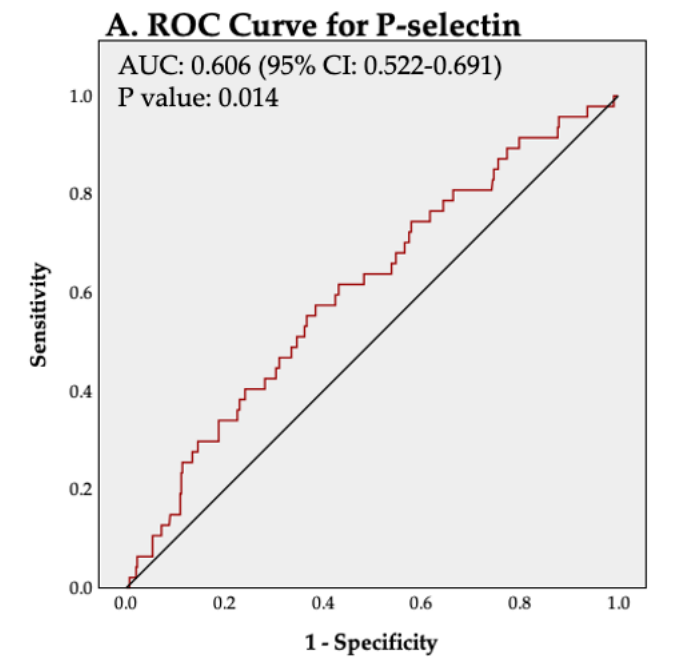


NLR with PE Risk group		*Day 5*	*Day 10*	*Day 15*	*Day 20*	*Day 25*	*Day 30*
Low-risk PE	***NLR >7.0***	*6 (1.0%)*	*11 (1.9%)*	*18 (3.1%)*	*22 (3.8%)*	*26 (4.4%)*	*27 (4.6%)*
	NLR ≤7.0	*3 (0.1%)*	*5 (0.2%)*	*7 (0.3%)*	*9 (0.3%)*	*9 (0.3%)*	*10 (0.4%)*
Intermediate-risk PE	***NLR >7.0***	*68 (3.6%)*	*127 (6.7%)*	*160 (8.4%)*	*185 (9.8%)*	*210 (11.1%)*	*228 (12.1%)*
	NLR ≤7.0	*33 (0.7%)*	*63 (1.4%)*	*92 (2.0%)*	*115 (2.5%)*	*132 (2.9%)*	*154 (3.4%)*
High-risk PE	***NLR >7.0***	*23 (22.3%)*	*26 (25.2%)*	*30 (29.1%)*	*30 (29.1%)*	*30 (29.1%)*	*31 (30.1%)*
	NLR ≤7.0	*6 (3.6%)*	*7 (4.2%)*	*8 (4.8%)*	*12 (7.2%)*	*13 (7.8%)*	*15 (9.1%)*

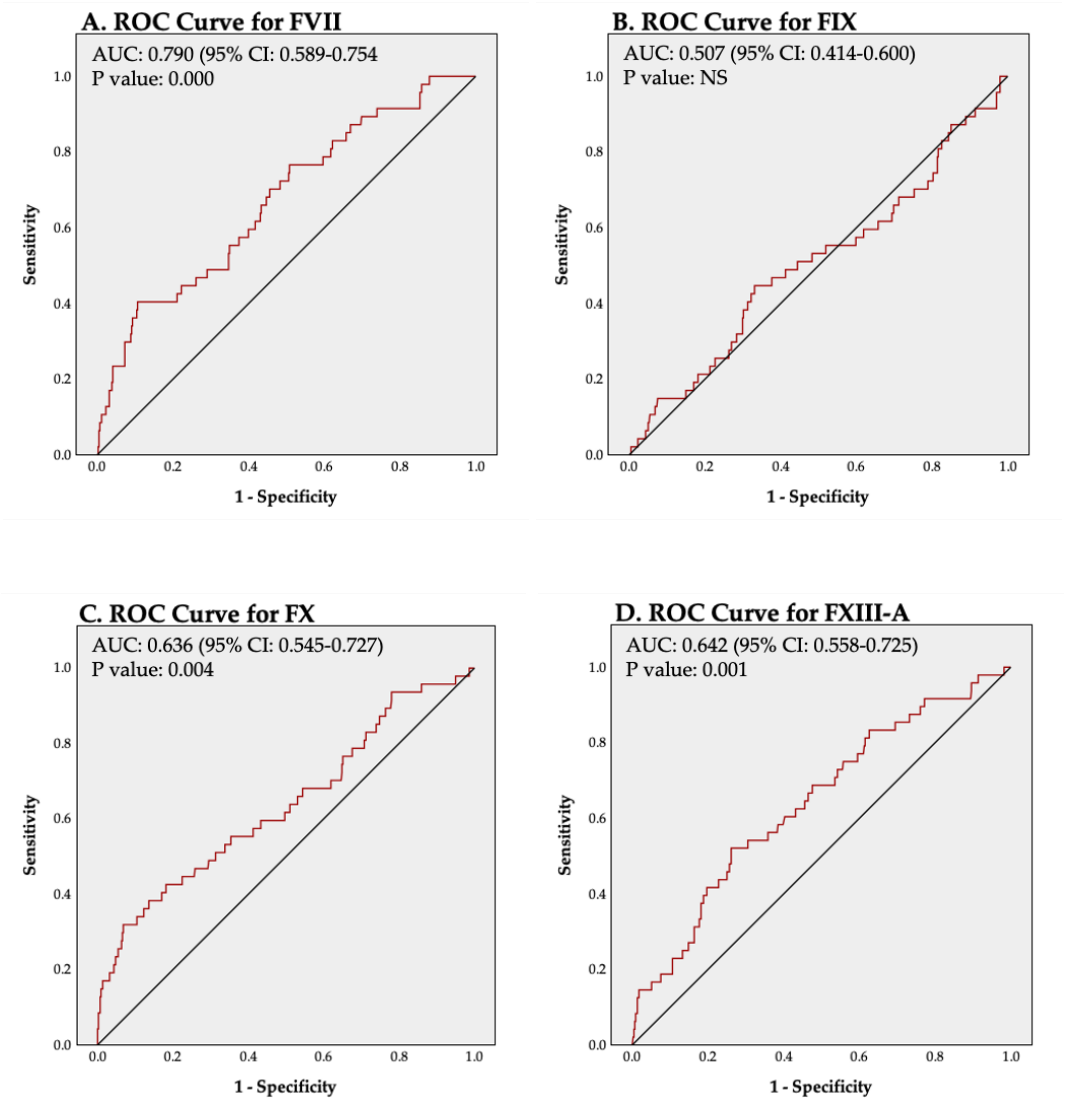
Supplementary Figure 5. Cumulative mortality rates according to NLR values in patients with different PE risk categories in the RIETE cohort.



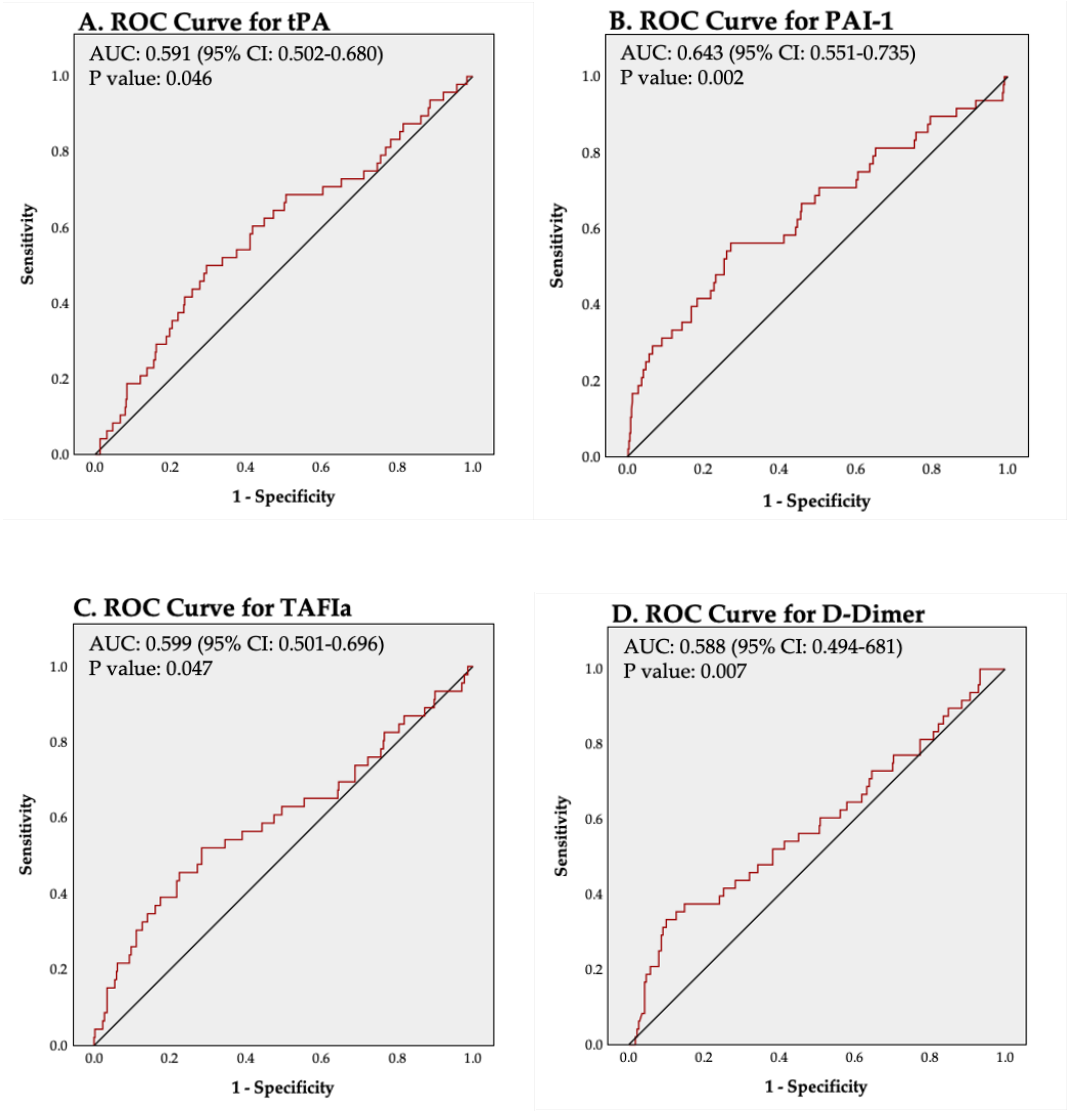
Supplementary Figure 6. ROC curves of 30-day mortality for markers of endothelial dysregulation.



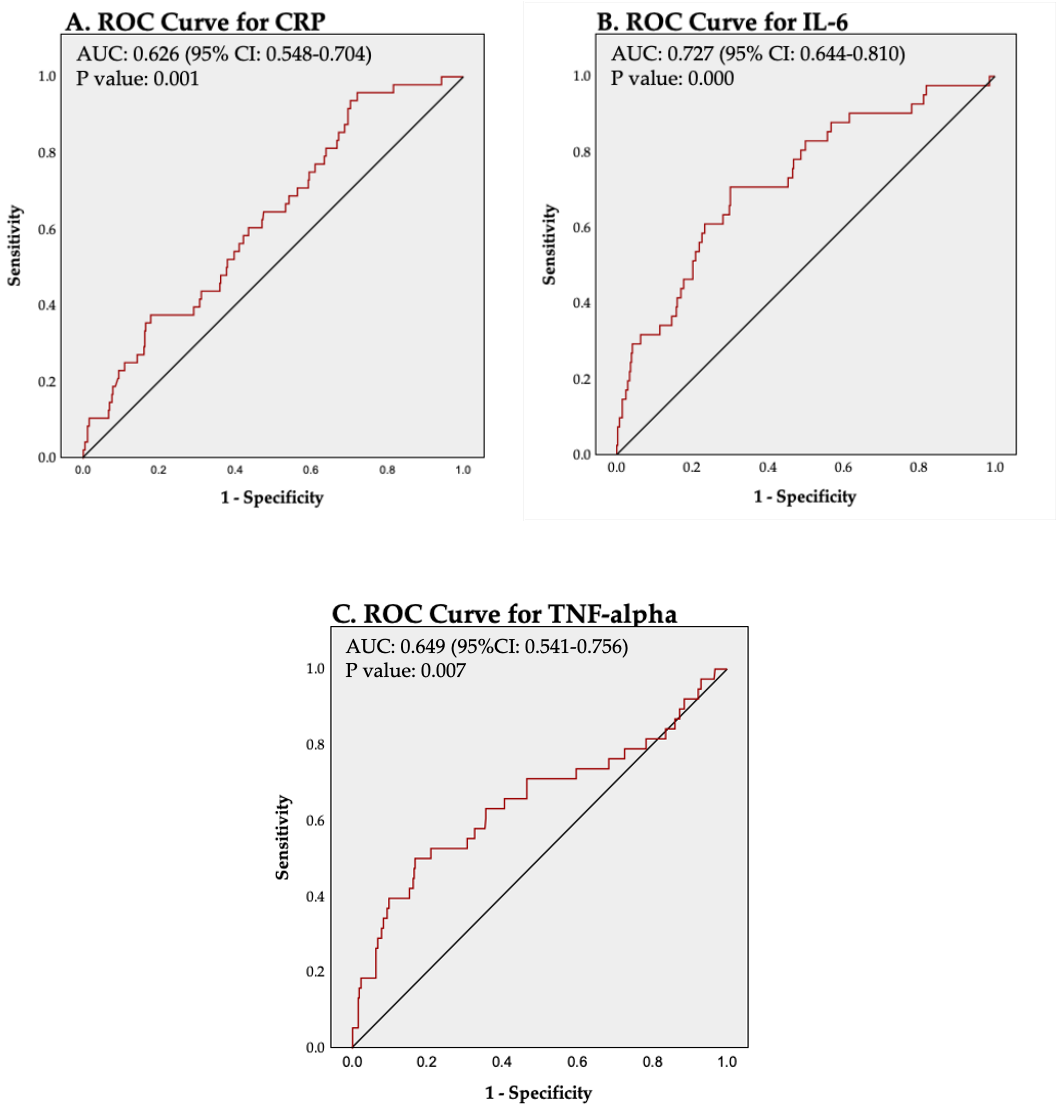
Supplementary Figure 7. ROC curve of 30-day mortality for marker of platelet activation.



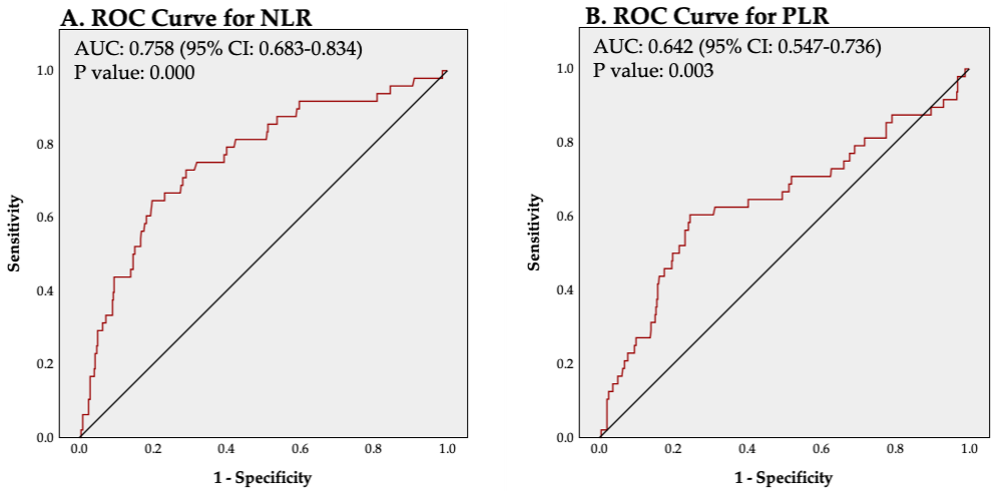
Supplementary Figure 8. ROC curve of 30-day mortality for coagulation factors.

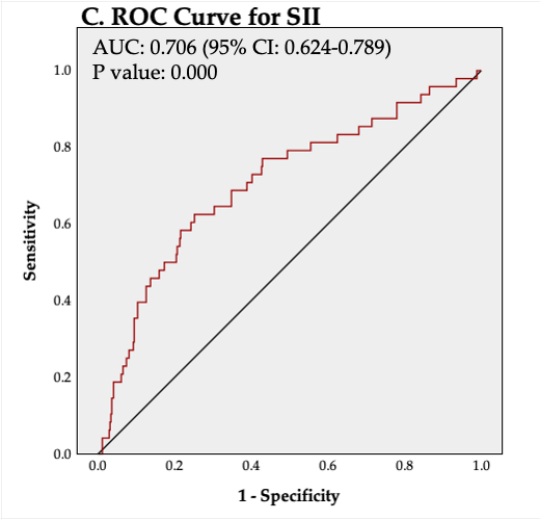


Supplementary Figure 9. ROC curve of 30-day mortality for markers of fibrinolysis.

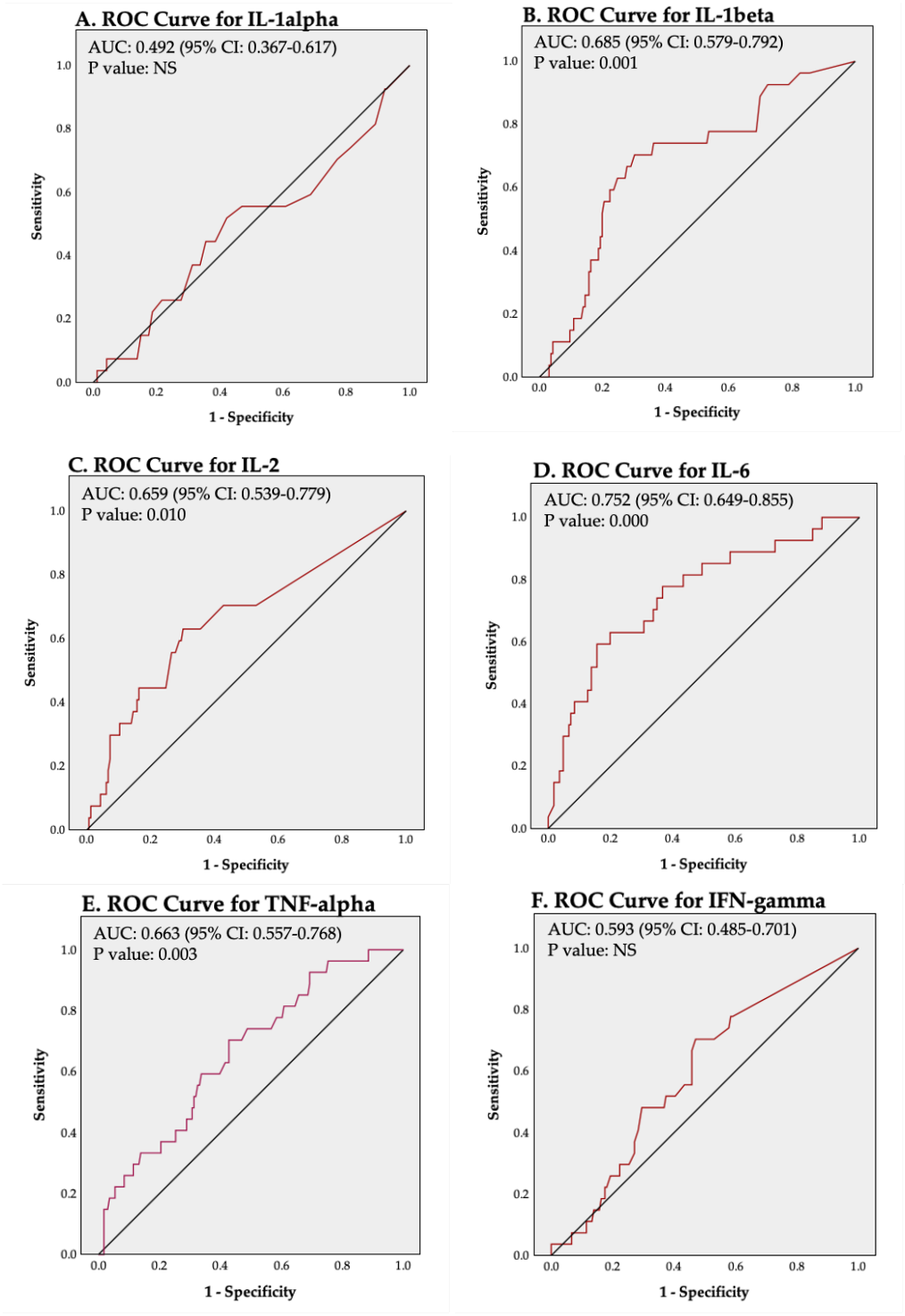


Supplementary Figure 10. ROC curve of 30-day mortality for inflammatory markers.

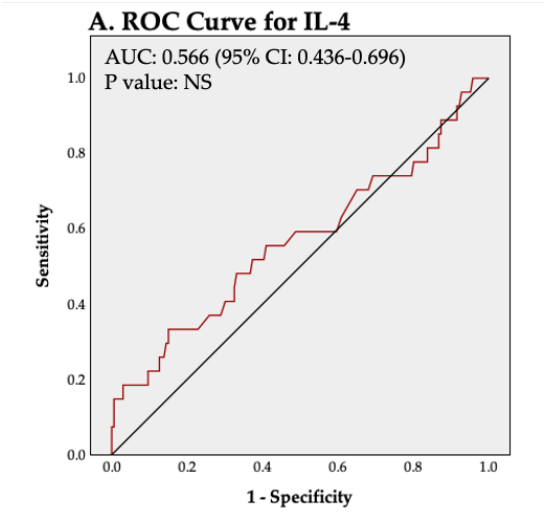


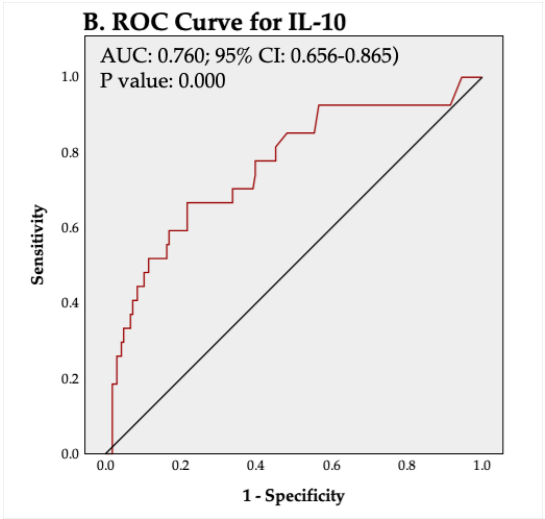


Supplementary Figure 11. ROC curve of 30-day mortality for blood cellular indices.

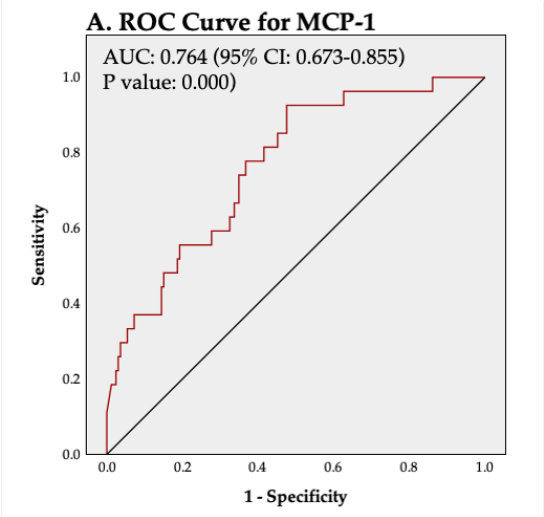


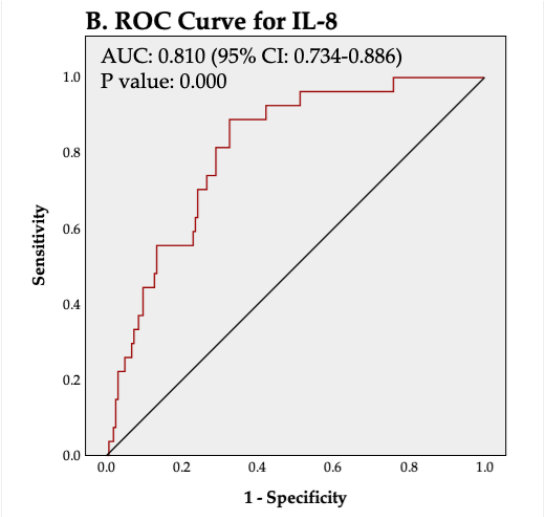
Supplementary Figure 12. ROC curve of 30-day mortality for pro-inflammatory cytokines.



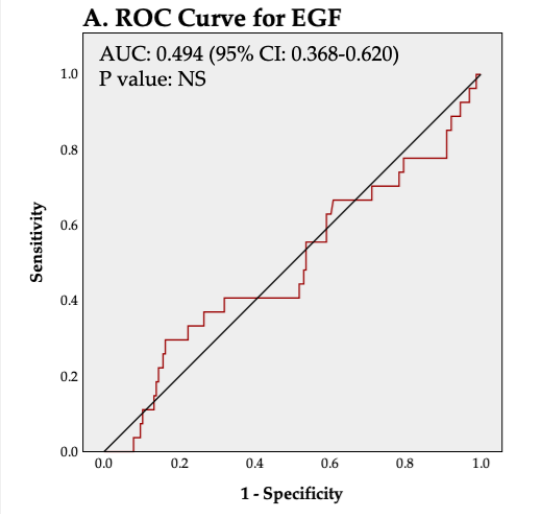


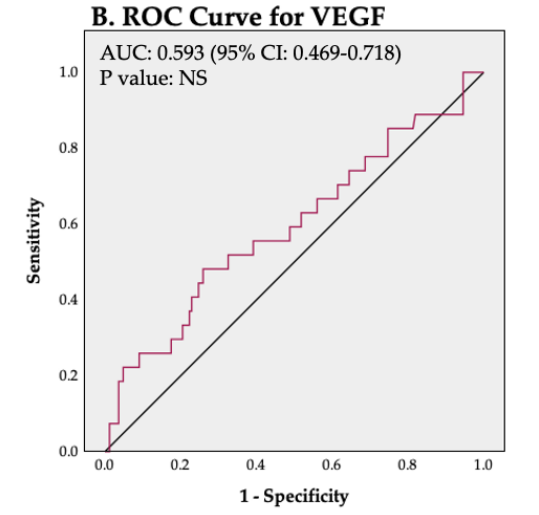
Supplementary Figure 13. ROC curve of 30-day mortality for anti-inflammatory cytokines.



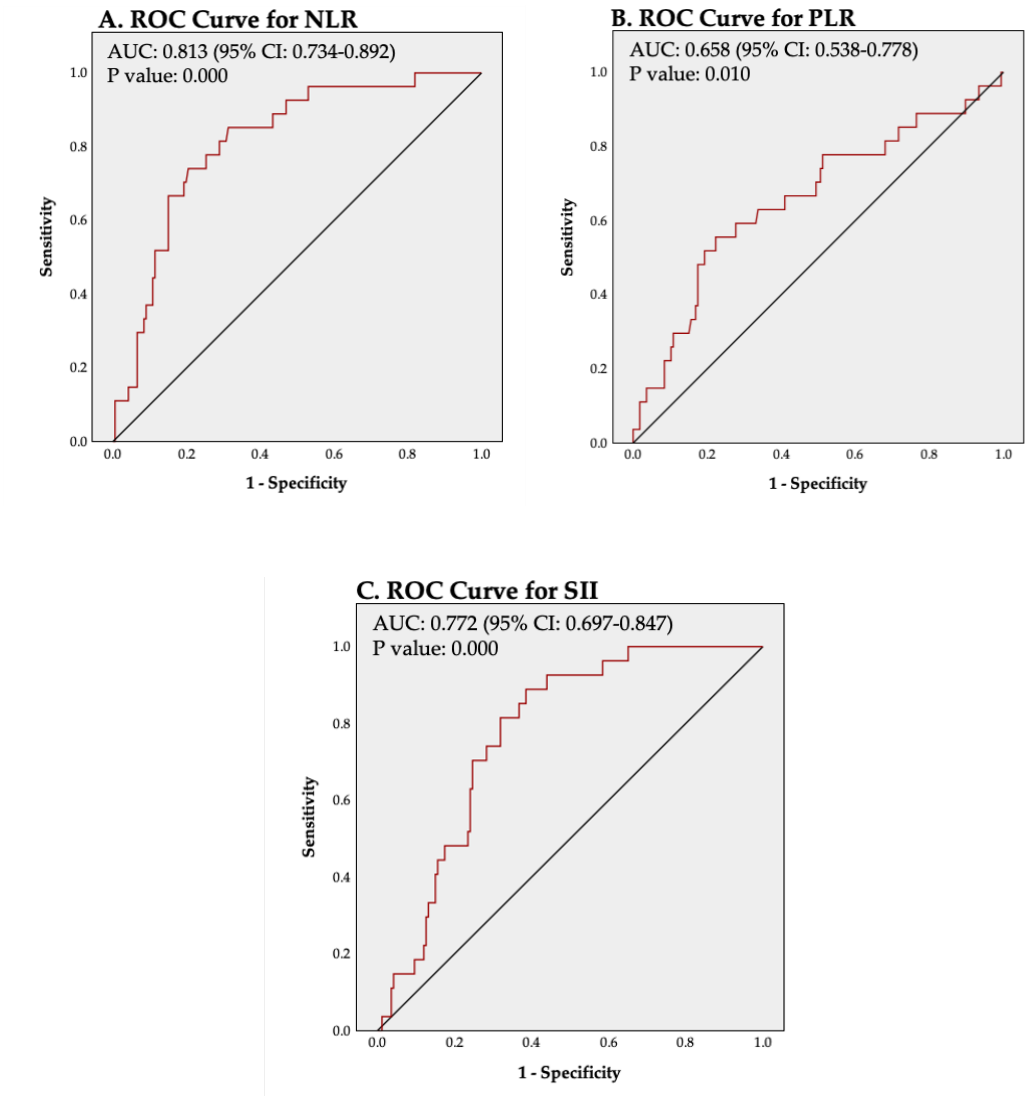


Supplementary Figure 14. ROC curve of 30-day morality for chemokines.





Supplementary Figure 15. ROC curve of 30-day morality for growth factors.



Supplementary Figure 16. ROC curve of 30-day morality for blood cellular indices.

Appendix 5. Supplementary Tables

Supplementary Table 1. Mean values at baseline of the 3 cellular indices, according to the 90-day outcomes.

Outcomes	N	NLR	PLR	SII
		Mean±SD	Mean±SD	Mean±SD
All patients	4,487	5.9±7.1	190±158	1,459±2,028
DVT recurrences	38	5.0±3.8	183±138	1,384±1,224
PE recurrences	45	6.3±6.5	198±219	1,631±1,865
VTE recurrences	79	6.0±5.5	197±188	1,581±1,613
Major bleeding	152	9.9±9.4‡	271±252‡	2,408±2,388‡
All-cause death	484	11.5±13‡	265±223‡	2,807±3,638‡
Any of the above	645	10.5±12‡	257±221‡	2,582±3,337‡
None of the above	3,842	5.1±5.6‡	179±142‡	1,270±1,640‡

*Comparisons between patients with- vs. without the variable: *p <0.05; †p <0.01; ‡p <0.001.*

Supplementary Table 2. Treatment strategies, and 30-day outcomes in the two cohorts.

Variables	*RIETE Cohort*	*LUMC Cohort*
	N (%), Mean±SD	*N (%), Mean±SD*
Patients, N	*10,085*	*700*
Initial Therapy		
Low-molecular-weight heparin	*8,503 (84.3%)*	*NA*
LMWH doses (IU/kg/day)	*169±46*	*NA*
Unfractionated heparin	*461 (4.6%)*	*NA*
Direct oral anticoagulants	*779 (7.7%)*	*NA*
Fondaparinux	*79 (0.8%)*	*NA*
Thrombolytic drugs	*185 (1.8%)*	*108 (15.4%)*
Insertion of an IVC filter	*238 (2.4%)*	*64 (9.1%)*
Surgical embolectomy	*31 (0.3%)*	*26 (3.7%)*
ECMO	*21 (0.2%)*	*NA*
Long-term Therapy		
Direct oral anticoagulants	*4,040 (40.1%)*	*NA*
Vitamin K antagonists	*2,810 (27.9%)*	*NA*
Low-molecular-weight heparin	*2,715 (26.9%)*	*NA*
LMWH doses (IU/kg/day)	*151±45*	*NA*
Fondaparinux	*32 (0.3%)*	*NA*
30-day Outcomes		
PE recurrences	*32 (0.3%)*	*NA*
DVT recurrences	*26 (0.3%)*	*NA*
Major bleeding	*209 (2.1%)*	*NA*
Death	*465 (4.6%)*	*58 (8.3%)*
*Low-risk PE patients**	*37/3,276 (1.1%)*	*1/118 (0.8%)*
*Intermediate-risk PE patients**	*382/6,534 (5.8%)*	*36/473 (7.6%)*
*High-risk PE patients**	*46/275 (16.7%)*	*21/109 (19.3%)*

**Risk categories are based on the European Society of Cardiology Criteria*

Supplementary Table 3. Multivariate analyses for the risk of all-cause death within 30-days in the RIETE cohort.

Variables	*NLR*	*PLR*	*SII*
Age >80 years	*1.57 (1.14-2.16)*	*1.67 (1.21-2.30)*	*1.62 (1.18-2.24)*
Chronic heart failure	*2.17 (1.44-3.25)*	*2.29 (1.53-3.43)*	*2.28 (1.52-3.42)*
Dementia	*2.70 (1.79-4.06)*	*2.75 (1.83-4.13)*	*2.59 (1.72-3.90)*
Recent major bleeding	*1.03 (0.47-2.24)*	*1.05 (0.49-2.26)*	*1.08 (0.50-2.33)*
Transient risk factors	*2.22 (1.56-3.17)*	*2.16 (1.51-3.08)*	*2.22 (1.55-3.16)*
Active cancer	*4.99 (3.40-7.32)*	*5.17 (3.54-7.56)*	*5.17 (3.53-7.56)*
Corticosteroids	*2.09 (1.48-2.94)*	*2.37 (1.70-3.32)*	*2.28 (1.63-3.20)*
SBP levels <90 mm Hg	*2.64 (1.55-4.50)*	*2.94 (1.74-4.98)*	*2.90 (1.72-4.90)*
Sat O_2 levels <90%	*1.67 (1.23-2.26)*	*1.73 (1.28-2.34)*	*1.69 (1.25-2.30)*
Anemia	*1.33 (0.99-1.78)*	*1.22 (0.90-1.64)*	*1.30 (0.97-1.75)*
NLR >7.0	*3.46 (2.60-4.60)*	-	-
PLR >220	-	*2.36 (1.77-3.13)*	-
SII >1,600	-	-	*2.52 (1.90-3.33)*

BIOGRAPHY

The author Fakiha Siddiqui was born on February 15, 1990, in Karachi Pakistan. She is the youngest daughter of Mr. Ghulam Muhammad Siddiqui (late) and Mrs. Khurshida Begum. In 2013 Fakiha completed her Bachelor of Dental Surgery (BDS) from Hamdard College of Medicine & Dentistry, Hamdard University, Karachi, Pakistan. Later (October 2013 - September 2015) she joined Hamdard University Dental Hospital, Karachi, Pakistan as Senior Dental Officer in the Department of Oral & Maxillofacial Surgery and Radiology Department. Due to her research interest Fakiha joined Bioinformatics & Molecular Medicine Laboratory of Dow University of Health Sciences Karachi under Dr. Mushtaq Hussain's supervision and worked as Research Fellow from February 2015 to February 2016.

From May 2017 Fakiha joined the Loyola University Chicago, Department of Pathology & Laboratory Medicine, IL, USA. Under the Supervision of Prof. Dr. Jawed Fareed. She was she was appointed as research assistant and then in March 2018, she was appointed as a research associate at the same Department. In April 2019, Fakiha also qualified National Board of Dental Examination (USA). Further to pursue her career in research she joined the joint PhD program under the supervision of Professor Manuel Monreal, and Professor Jawed Fareed at Universidad Católica San Antonio de Murcia, Murcia, Spain and Loyola University Chicago, Department of Pathology & Laboratory Medicine, IL, USA. Her major research area is based on biomarker profiling relevant to thrombosis, pulmonary embolism, cancer biology, cancer-associated thrombosis, end stage renal disease (ESRD), sepsis, and cardiovascular disorders.

During her academic and research career she has been awarded Summa cum laude position and scholarship, award in her first professional year in the dental school. Fakiha presented her research work in multiple national and international scientific forums which includes 10 oral and 28 poster presentations. She won the best oral presentation award on "A factor Xa inhibitor antidote, andexanet alfa differentially reverses the effects of apixaban, betrixaban, edoxaban, and rivaroxaban" at the 26th Anniversary International Congress on Thrombosis,

Athens, Greece, in June 2019. Fakiha also won several travel grants including CVRI Mini-grant, Cardiovascular Research Institute - Loyola University Chicago Health Science Division, IL, USA (2018, 2019, 2023). European Venous Forum, European Venous Foundation, Travel Grant, Greenford, Middx, UK (2022). By attending different national and international conferences, she has published 46 abstracts as a first author and more than 100 abstracts as co-author. Fakiha has also published 10 peer reviewed articles as a first author and more than 35 manuscripts as co-author in highly reputable peer reviewed journals. She mentored and trained more than 20 medical and undergraduate students at the Department of Pathology & Laboratory Medicine, Loyola University Chicago. She was also engaged in multiple outreach programs and were actively participated in community services at Pakistan and in USA.

Selected Published Papers:

Siddiqui, F., Tafur, A., Darki, A., Kantarcioglu, B., Krupa, E., Hoppensteadt, D., Jawed, F., Monreal, M. (2024). Decreased Thrombin Generation is Associated with Increased Thrombin Generation Biomarkers and Blood Cellular Indices in Pulmonary Embolism. Clinical and Applied Thrombosis/Hemostasis. DOI: 10.1177/10760296241261076

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Tavares, IR., Caffaro, RA., Portugal, MF., Ribeiro CM, da Silva, VS., Krupa, E., Nikolovski, S., de Britto, KF., Gomes Pereira Petisco, AC., Miranda, MC., Gomes de Souza Santos, S., da Silva Dourado, M., Siqueira, PV., Siddiqui, F., Fareed, J., Ramacciotti, E. (2024). Biomarkers Profile in Provoked Versus Unprovoked Deep Venous Thrombosis. Clinical and Applied Thrombosis/Hemostasis. DOI: 10.1177/10760296241238211

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Siddiqui, F., García-Ortega, A., Kantarcioglu, B., Sinacore, J., Tafur, A., Demelo-Rodríguez, P., Antonio Nieto, J., Usandizaga, E., Fareed, J., Monreal, M., & The Riete Investigators (2022). Cellular Indices and Outcome in Patients with Acute Venous Thromboembolism. Clinical and applied thrombosis/hemostasis. DOI: 10.1177/10760296221113346

Kantarcioglu, B., Darki, A., Siddiqui, F., Krupa, E., Vural, M., Kacmaz, M., Hoppensteadt, D., Iqbal, O., Jeske, W., Walenga, J., Adiguzel, C., & Fareed, J. (2022). Predictive Role of Blood Cellular Indices and Their Relationship with Endogenous Glycosaminoglycans as Determinants of Inflammatory Biomarkers in Pulmonary Embolism. Clinical and applied thrombosis/hemostasis. DOI: 10.1177/10760296221104801

Kantarcioglu, B., Darki, A., Siddiqui, F., Hoppensteadt, D., Lewis, J., Krämer, R., Adiguzel, C., & Fareed, J. (2022). The Relevance of Anti-PF4 Antibody Isotypes and Endogenous Glycosaminoglycans and their Relationship with Inflammatory Biomarkers in Pulmonary Embolism Patients. Clinical and applied thrombosis/hemostasis. DOI: 10.1177/10760296221091770

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