IDENTIFICATION OF DIAGNOSTIC AND PROGNOSTIC INDICATORS OF HYPERCOAGULABILITY IN PATIENTS WITH PSEUDOEXFOLIATION SYNDROME

by

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Koç University

Graduate School of Health Sciences

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ABSTRACT

IDENTIFICATION OF DIAGNOSTIC AND PROGNOSTIC INDICATORS OF HYPERCOAGULABILITY IN PATIENTS WITH PSEUDOEXFOLIATION SYNDROME

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Patients diagnosed with Pseudoexfoliation (PEX) syndrome are highly detected in the group of people over 50 years old. The risk of thrombotic incidents increases in the PEX patients due to the impact of abnormal intraocular vascular deposition. This study aims to clarify the fundamental mechanism of possible hypercoagulability state in the PEX patients leading to venous thrombosis and secondary disease in this study group.

Eighteen ocular PEX syndrome cases (mean age 63.6 ± 6.5 years) attended as a study group and nineteen cases without PEX syndrome (mean age 69.7 ± 4.4 years) as a control group participated in this study. Blood samples were analyzed by ROTEM®, Complete Blood Count test and Flow cytometry. A one-tailed Mann-Whitney was performed for statistical assessment of the data presented in this study.

The mean values of the ROTEM analysis were significantly decreased in the FIBTEM clotting time (CT) (60.3 vs 56.9; $P < 0.05$) and increased FIBTEM alpha-angle (70.0 vs 73.3; $P < 0.05$) when comparing the control group with PEX patients. In flow cytometric measurement, data showed a considerable difference in the expression level of CD62P ($P < 0.0001$) and PAC-1 ($P < 0.05$) between the study groups. Antigen-binding capacity (ABC) analysis revealed Furthermore, a significant difference in the CD62p marker ($P < 0.05$). Complete Blood Count parameters of the PEX group did not show any significant differences compared to the controls.

The result from the study suggests the higher contribution of functional fibrinogen in clot formation and a high level of platelet activation markers that expressed in the PEX patients. These findings support the idea that PEX syndrome increase the chance of hypercoagulability in PEX patients. Further investigations are recommended to broaden our knowledge of the exact role of platelets and functional fibrinogen in the initiation or development of PEX syndrome.

Keywords: Hypercoagulability, ROTEM, Pseudoexfoliation syndrome, Coagulation

ÖZETÇE

Psödoeksfoliasyon Sendromu Olan Hastalarda Hiperkoagülabilitenin Tanısal Ve Prognostik Göstergelerinin Belirlenmesi

Ghazal Narimanfar

Hücresel ve Moleküler Tıp Yüksek Lisans

26 Ağustos, 2021

Psödoeksfoliasyon (PEX) sendromlu hastalar 50 yaş üzeri gruplarda sıklıkla görülmektedir. Anormal intraoküler vasküler birikimi etkisiyle trombotik olayların riski PEX hastalarında artmaktadır. Bu çalışmanın amacı venöz tromboz ve sekonder hastalığa neden olan muhtemel hiperkoagulasyonun PEX hastalarındaki ana mekanizmasını açıklamaktır.

Çalışma grubu olarak 18 oküler PEX sendromu vakası (ortalama 63.6 ± 6.5 yaş) ve kontrol grubu olarak 19 PEX sendromu olmayan vaka (ortalama 69.7 ± 4.4 yaş) bu çalışmaya katıldı. Kan örnekleri ROTEM®, Tam Kan Sayımı testi ve flow sitometri yöntemleri ile analiz edildi. Bu çalısmada sunulan verilerin istatistiksel değerlendirilmesi için one-tailed Mann-Whitney yapıldı.

ROTEM analizinin ortalama değerleri kontrol grubu ile PEX hastaları karşılaştırıldığında, FIBTEM pıhtılaşma süresi (CT) (60.3'e karşı 56.9; $P < 0.05$) önemli ölçüde azaldı ve FIBTEM alfa-angle (70.0'a 73.3; $P < 0.05$) arttı. Flow sitometri ölçümünde çalışma grupları arasındaki veriler, CD62P ($P < 0.0001$) ve PAC-1 ($P < 0.05$) ifade seviyelerinde önemli bir fark göstermiştir. Ayrıca, Antijen bağlama kapasitesi (ABC) analizi CD62p markerında önemli bir fark ortaya çıkardı ($P < 0.05$). PEX grubunun Tam Kan Sayımı parametreleri kontrollere kıyaslandığında anlamlı bir farklılık göstermedi.

Bu çalışmadaki sonuç fonksiyonel fibrinojenin pıhtı oluşumuna daha yüksek etkisini ve PEX hastalarında yüksek düzeyde trombosit aktivasyon markerlarının ifade edildiğini önermektedir. Bu bulgular, PEX sendromunun PEX hastalarında hiperkoagülasyon ihtimalini arttırdığı fikrini desteklemektedir. PEX sendromunun başlangıcında veya gelişiminde trombositlerin ve fonksiyonel fibrinojenin kesin rolü hakkındaki bilgimizi genişletmek için daha fazla araştırma yapılması önerilir.

Anahtar kelimeler: Hiperkoagülabilite, ROTEM, Psödoeksfoliasyon sendromu, Koagülasyon.

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ABBREVIATIONS

ADP	Adenosine Diphosphate
vWF	von Willebrand Factor
GP	Glycoprotein
ATP	Adenosine Triphosphate
Ca	Calcium
TEM	Transmission Electron Microscopy
ELISA	Enzyme-linked Immunosorbent Assay
PT	Prothrombin Time
PK	Prekallikrein
HMWK	High-molecular-weight Kininogen
aPTT	Activated Partial Thromboplastin Time
CCTs	Conventional Coagulation Tests
TCT	Thrombin Clotting Time
POC	Point Of Care
INR	International Normalized Ratio
ISI	International Sensitivity Index
MNPT	Mean Normal PT
PRP	Platelet reach plasma
PPP	Platelet Poor Plasma
VCTs	Viscoelastic Clotting Tests
ROTEM®	Rotational Thromboelastometer
ROTEM	Rotational Thromboelastometry
ROTEG®	Rotational Thromboelastographer
TEG	Thromboelastographer
EXTEM	Extrinsic-activated rotational thromboelastometry
INTEM	Intrinsic-activated rotational thromboelastometry
FIBTEM	Fibrin-based thromboelastometry
MCE	Maximum Clot Elasticity
PEX	Pseudoexfoliation

RVO	Retinal Vein Occlusion
BD	Behcet’s disease
TGF	Transforming Growth Factor
LOXL1	Lysyl oxidase-like 1
CLU	Clusterin
CNTNAP2	Contactin-associated protein-like 2
CT	Clotting Time
TNF α	Tumor necrosis factor-alpha
Hcy	Homocysteine
IOP	Intraocular Pressure
HT	Hypertension
DM	Diabetes mellitus
BRVO	Branch Retinal Vein Occlusion
CRVO	Central Retinal Vein Occlusion
nGnP	non-glaucoma non-PEX
CVDs	Cardiovascular diseases
CBC	Complete Blood Count
PLT	Platelet count
PCT	Plateletcrit
MPV	Mean Platelet Volume
PDW	Platelet Distribution Width
LMR	Lymphocyte/Monocyte count ratio
NLR	Neutrophil-Lymphocyte Count Ratio
LYMPH	Lymphocyte count
MONO	Monocyte count
NEUT	Neutrophil count
Sec	Second
mm	Millimeter
CFT	Clot Formation Time
MCF	Maximum Clot Firmness
A10	Amplitude at 10 min
A20	Amplitude at 20 min
LI30	lysis index at 30 minutes

INTRODUCTION

Systemic diseases are significant public health problems that involve many organs or the whole body which shows the necessity to clarify the causes and etiology of diseases. Many systemic diseases also affect the eyes. In fact, an eye examination sometimes leads to the first diagnosis of systemic diseases. In this study, we focused on Pseudoexfoliation (PEX) syndrome. This syndrome was first identified by Lindberg in 1920's by observing the deposited material on the pupillary border of the patients who were diagnosed with chronic glaucoma (Lindberg, 1917; John G Lindberg, 1989). Even today, the exact etiology of this syndrome remains unclear. The PEX syndrome is considered an age-related clinical condition since the frequency of the PEX syndrome increases with age. The PEX syndrome is commonly distinguished in people over 50 years old. This syndrome is described by the abnormality in connective tissue leading to irregular production and deposition of fibrillar-granular materials (Kanski & Bowling, 2011). These fibrillar deposits known as PEX materials are visible through slit lamp ophthalmology examination.

PEX syndrome needs special attention since this disease often causes complications in cataract surgery due to zonular weakness and poor pupillary dilation result into intraoperative or postoperative lens displacement (Sangal & Chen, 2014). In addition, PEX syndrome is considered not only a disease limited to the ocular tissues but also a systemic syndrome that may affect a vast area of the body including blood vessels of connective tissues (U. M. Schlotzer-Schrehardt, Koca, Naumann, & Volkholz, 1992). The risk of atherothrombotic vascular events, cardio and cerebrovascular disease, and venous thrombosis (especially retinal venous occlusion) is increased in patients with PEX syndrome, however, this association remains controversial (Andrikopoulos, Alexopoulos, & Gartaganis, 2014; Chung, Arora, Damji, & Weis, 2018; Karagiannis et al., 2015).

Although many factors such as homocysteine metabolism disorder, elastic microfibrillopathy, platelet dysfunction and endothelial dysfunction have been suggested in the etiopathogenesis of this hypercoagulability seen in PEX syndrome, the primary pathogenesis has not yet been fully elucidated (Andrikopoulos et al., 2014; Atalar et al.,

2006; Leibovitch et al., 2003; Turkcu et al., 2014). Study the hypercoagulability in PEX Patients considered to have an innovative perspective on the coagulation dysfunction of PEX cases.

The coagulation is a process by which blood tends to get solidified to respond to the abnormal conditions involving irregular interactions between coagulation and the fibrinolytic system as well as platelets and vessel walls. Normal physiology condition of the body preserves regular coagulation pathway in case of abnormal conditions like vessel wall damages. In the other word, coagulation pathway maintains an equilibrium between two dynamic pathways. Firstly, the pro-coagulant pathway which is responsible for the coagulation. secondly, the pathway which inhibits the exceeding of clot formation. An imbalance of the coagulation system may occur in any pathological scenario which leads to either hemorrhagic or thrombotic complications (Palta, Saroa, & Palta, 2014).

In pathological cases, including PEX syndrome, the blood tendency to thrombotic conditions due to increaseing vascular resistance has been observed. Vascular resistance can result in decreased blood flow and consequently lower shear rates that may lead to the unbridled aggregate formation and disturbance of the blood cycle (Hathcock, 2006). The histological studies were shown the accumulation of elastin and amyloid-P substances in the ocular and extraocular tissues due to a metabolism disorder in the connective tissues. Hence, it is considered that the structural alteration in the vascular endothelium tissue could lead to endothelial dysfunction and increase the risk of coagulation disorders (Turkcu et al., 2014).

To evaluate this hypothesis, we need to assess the coagulation states of favorable patients' group and the control group. The main limitation of coagulation assessment is the routine test known as Plasma-based conventional coagulation assays prothrombin time (PT), activated Partial Thromboplastin Time (aPTT), thrombin clotting time (TCT), and platelet count. These tests are not capable of providing information on cellular contribution to thrombotic events thus they are insufficient to determine hypercoagulability states. In recent years, considerable attention has been drawn to viscoelastic coagulation devices including the rotational thromboelastometry (ROTEM®) test. Viscoelastic coagulation tests not only evaluate the contribution of plasma protein in hypercoagulability states but also cellular contributions. Rotational Thromboelastometry (ROTEM) tests are the most used viscoelastic coagulation tests which provide a real-time methodology for the evaluation of all stages in hemostasis,

from the initiation point of clot formation leading to clot stabilization and finally fibrinolysis.

This study looked closely at the relationship between ROTEM tests (EXTEM, INTEM, and FIBTEM) results, platelet function analysis by flow cytometry and Complete Blood Count (CBC) data. Our research aims to improve knowledge and provide valuable information about differentially expressed platelet proteins among the groups of the controls and the PEX syndrome patients. The mechanism of the disease at the cellular level will lead to the identification of new biomarkers of the disease in clinical practice. It is believed that the findings of this study will predetermine the risk of eye-related vascular embolic diseases in PEX syndrome patients and help to develop new preventive treatment approaches.

Chapter 1:
INTRODUCTION TO BLOOD COAGULATION

1.1 Coagulation Mechanism

The coagulation is a tightly regulated process by which blood changes from fluid to a solid form to respond the vascular endothelium abnormalities or injuries. Internal part of blood vessels is covered by the endothelium tissue which provides an anticoagulant surface to maintain the blood flow. In case of any fractions in the endothelium, the subendothelial matrix is exposed to the blood and initiates the coagulation mechanism of hemostasis lead to the formation of a blood clot, composed primarily of platelets and fibrin.

The clotting mechanism involves vasoconstriction, platelets activation, adhesion, and aggregation. It is accompanied by deposition and maturation of fibrin while engaging series of proteins, enzymes, and ions participation to maintain blood flow. During primary hemostasis stage an unstable plug will form at the damaged site by aggregation of platelets. Secondary hemostasis consists of two main coagulation pathways know as extrinsic. These two pathways meet up at a point to initiate the common pathway leading to stabilization of the platelet plug which formed in primary hemostasis.

1.2 Primary Hemostasis

The body's earliest response to initiation of coagulation is commence by a vascular spasm leads to multiply the number of platelets in the injury site to form an unstable clot of aggregated platelets. The fundamental role in primary hemostasis is allocated to the platelets which are recognized as the smallest component of blood. Platelets originate from bone marrow; they are anucleate, colorless and survive in blood for 7–10 days (van der Meijden & Heemskerk, 2019). The essential role of platelets in thrombosis, mediating myocardial infarction, stroke, and venous thromboembolism gaining much attention due to the importance of these diseases (Koupenova, Clancy, Corkrey, & Freedman, 2018). The overview of primary hemostasis is illustrated in (Figure 1.1).

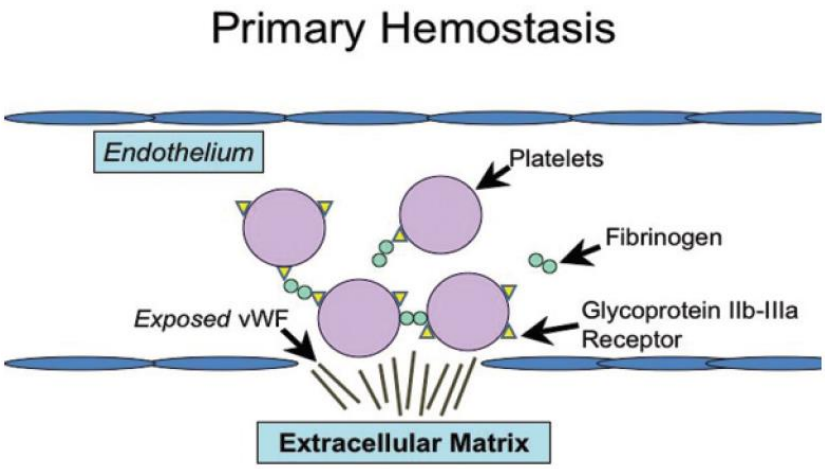

Figure 1. 1: Overview of the primary hemostasias. The initiation of the platelet plug formation begins with platelet clustering to constitute platelet plug due to the endothelial damage and exposure of extracellular matrix (made of proteins, such as von Willebrand factor (vWF)) to the glycoprotein (GP) receptors on the platelet surface and circulating fibrinogen (Mahadevan & Garmel, 2012).

1.2.1 Platelet's Role in Plug Formation

Platelet proceeds the plug formation through three processes including platelet adhesion, platelet activation, and platelet aggregation.

Platelet adhesion happens when the resting platelets are exposed to the subendothelial matrix and thrombogenic components including several types of collagens and laminin in the subendothelial layer and von Willebrand factor (vWF) (Clemetson, 2012; Ozaki, Suzuki-Inoue, & Inoue, 2013). This exposure leads to morphological alteration of resting platelets into a pseudopodal form, and releases the contents of their cytoplasmic granules, platelet-activating factors, and adenosine diphosphate (ADP).

VWF presents as blood glycoprotein in the plasma as well as in the immobilized state in the subendothelial matrix with a major role in Primary and secondary hemostasis (Bergmeier et al., 2006; Ruggeri, 2007). VWF plays a role in the critical process of slowing down the platelets and facilitate the binding of platelet receptor to collagen in primary hemostasis (Clemetson, 2012). Multiple receptors on platelet membranes including the glycoprotein (GP) Ib-V-IX receptor complex, GPVI and integrin α2β1 and

αIIbβ3 which are embedded in the phospholipid bilayer are involved in the initiation of platelet adhesion (Gibbins, 2004). Specifically, GPIbα which is one of the Gp Ib-IX receptor components on platelets surface binds to the A1 domain of vWF to form a bond between thrombogenic components and the platelet glycoprotein (GP) such as Ib-IX-V receptor on the platelet membrane (Garmo, Bajwa, & Burns, 2021; Kehrel et al., 1998; Nieswandt et al., 2001). This connection between receptors on platelets and vWF initiates the flow of events leading to third phase of primary hemostasis known as platelet activation (Figure 1.2).

It has been suggested that platelet activation occurs through a variety of stimuli; whereas, the Initiation of platelet activation requires adhesion proteins and soluble agonists to start the intracellular signal transductions (Estevez & Du, 2017). Platelet transformation begins with two specific incidents. First, the morphological transformation from smooth discs to multi-pseudopodal plugs shape via reorganization of the cortical actin cytoskeleton. The transformation of platelets shape is irreversibly and greatly increasing platelets surface area. Second, degranulation and releasing the cytoplasmic granules content including serotonin, platelet-activating factors, and ADP (Aslan, Itakura, Gertz, & McCarty, 2012; Estevez & Du, 2017; Periayah, Halim, & Mat Saad, 2017; Shin, Park, Noh, Lim, & Chung, 2017).

Platelets have three major storage granules: Alpha (α) Granules and dense granules, and Lysosomes. The main function of granules is to store a vast number of biologically active molecules which are specifically involved in the initiation of coagulation and recruiting other cells during inflammation (Ghoshal & Bhattacharyya, 2014). Granules differ in their fine-structure, cargo, generation, function and, kinetics of the exocytosis (Rumbaut & Thiagarajan, 2010; Sharda & Flaumenhaft, 2018).

Platelet α granules comprise approximately 10% of the platelet volume due to their large size (200–500 nm) and they are known to be the most abundant platelet granules (Blair & Flaumenhaft, 2009; Rumbaut & Thiagarajan, 2010). They contain the majority of platelet factors that release during platelet activation at the vascular endothelial damage sites. These platelet factors have a critical role in thrombosis, hemostasis, inflammation and, ultimate wound repair (Harrison & Cramer, 1993). The role of α granules is demonstrated more specifically in coagulation since they consist of coagulation factors including XIII, XI, V, P-selectin, fibronectin, von Willebrand factor, high molecular weight kininogens and, fibrinogen. Meanwhile, α-granules take part in other major

cascades including platelet aggregation, thrombosis, platelet adhesive upon release of their constituents (Palta et al., 2014; Rumbaut & Thiagarajan, 2010). The reduction or absence of platelet α-granules, known as gray platelet syndrome, leading to a mild to moderate bleeding disorder in which the primary defect is a deficiency in platelet alpha granules.

Platelet dense granules are known as the smallest platelet granules measured about 150 nm in diameter that found in lower numbers than α granules in platelets (White, 1998). Along with the platelet activation process, granules release their contents through exocytosis resulting in engaging additional platelets in the aggregation stage and participate to the local vasoconstriction stage of coagulation (Rumbaut & Thiagarajan, 2010). Dense granules are majorly composed of bioactive amines including serotonin, histamine, and adenine nucleotides including adenosine triphosphate (ATP), adenosine diphosphate (ADP)and cations, particularly Calcium (Ca) (Blair & Flaumenhaft, 2009; Sharda & Flaumenhaft, 2018). ADP plays a significant role in the amplification of platelet recruitment and activation, aggregation, and procoagulant responses to maintain the hemostasis and thrombosis (J. F. Hussain & Mahaut-Smith, 1999; Koupenova et al., 2018; Periayah et al., 2017)

Platelet lysosome has a similar morphology to α granules when studying with Transmission Electron Microscopy (TEM) (Rumbaut & Thiagarajan, 2010). They are mostly distinguishable by their acidic luminal pH (4.6–5.0) due to the presence of acid hydrolases and lysosomal membrane markers (Rumbaut & Thiagarajan, 2010; Trivedi, Bartlett, & Pulinilkunnil, 2020). Needs to be mentioned that the exact function of platelet lysosomes is not entirely understood when comparing with the other platelet granules. Lysosome cargo including acid phosphatase, arylsulfatase, glycosidases, acid proteases, β-glucuronidase and degradative enzymes (Bentfeld-Barker & Bainton, 1982; Estevez & Du, 2017; Jenne, Urrutia, & Kubes, 2013). This cargo can be released upon activation requiring more stimulation in comparison with other granules (Rumbaut & Thiagarajan, 2010). Exocytosis of lysosome content may have contributed to the regularization of thrombus formation as well as alteration of extracellular matrix functions (van der Meijden & Heemskerk, 2019).

Thrombin which is the strongest platelet agonist contributes to the platelet activation in response to vascular injuries. Thrombin mediates this response through two mechanisms leading to the conversion of fibrinogen into fibrin to stabilize the platelet

plugs (Tomaiuolo, Brass, & Stalker, 2017; Yun, Sim, Goh, Park, & Han, 2016). The first mechanism is when thrombin binding the protease-activated receptor via breaking down the peptide, this process leads to direct activation of platelet via thrombin. Second mechanism works via stimulation of platelet to release the content of their granules (Garmo et al., 2021). This process of platelet activation provides the circumstances to proceed to the further step in the primary hemostasis known as platelet aggregation.

Platelets activation markers routinely are studied by enzyme-linked immunosorbent assay (ELISA), Western blot, flow cytometer, or a combination of these techniques (Yun et al., 2016). Including old patients in a study, flow cytometry overtakes other techniques since it requires a small amount of blood samples. Flow cytometry enables the analysis of the platelet activation markers on single platelets, there is a low chance of platelet activation during sample preparation, and the output data is independent of platelet count (Clemetson, 2012; Mitsui et al., 2016). Activated platelets have the capacity to bind or regulate the expression of adhesive molecules on other platelets or blood cells. The most important receptors in platelet analysis via flow cytometry including: CD41a, CD42b, CD61, CD62p, and PAC-1. CD41a reacts with GP IIb and is expressed in normal platelet and used to distinguish platelets from other cells (Marquardt et al., 2002). CD42b (GPIb) is specifically expressed on the platelet membrane (Swanepoel, Stander, & Pretorius, 2013). CD61 (GPIIIa) is expressed on platelet membrane and in complex with CD41 are mediating the aggregation and adhesion (Fullard, 2004). CD62p (P-selectin) is a transmembrane protein that expresses on α -granules membrane when the platelet is in the resting phase. In contrast, in the platelet activation phase, P-selectin highly increased in platelet surface due to the fusion of α -granules membrane into platelet membrane (Lu & Malinauskas, 2011). PAC-1 is binding to activated GP IIb/IIIa due to its conformational changes and it is expressed in platelet and α-granules membrane(Schmitz et al., 1998). It has been reported that PAC-1 has a higher level of expression metabolic syndrome in comparison with vascular diseases(Serebruany, Malinin, Ong, & Atar, 2008). Although the flow cytometer is considered to be the best technique to study platelet function, it requires sophisticated equipment and highly trained experts.

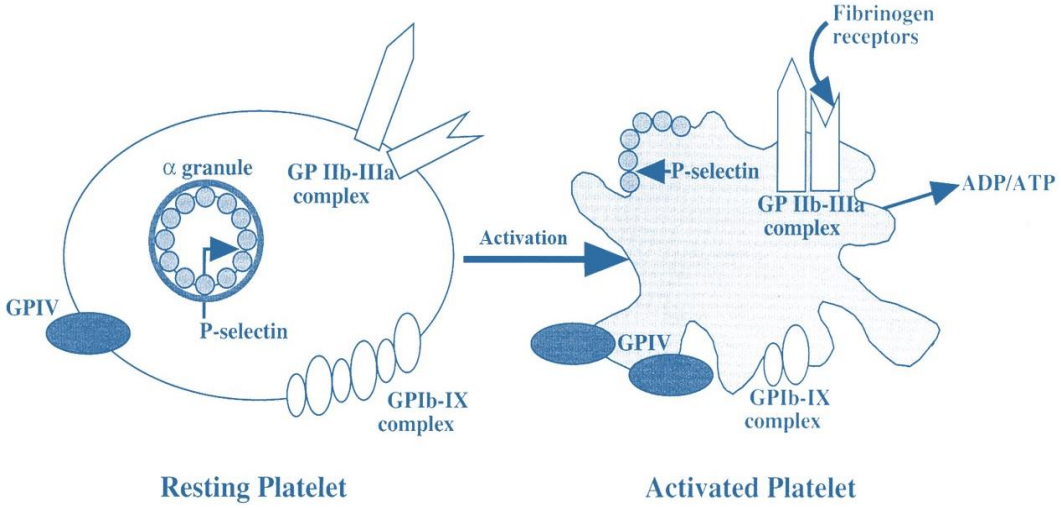

Figure 1. 2: comparison of resting platelet with activated platelet. Platelet surface markers and morphology will change upon activation. Changes in the number of GP receptors of the platelet membrane happens in the process of platelet activation. Expression of GPIIb-IIIa (CD41/CD61), GPIV (CD63) , and P-selectin (CD62p) increases with activation, whereas the expression of GPIb-IX decreases (Kestin et al., 1993).

Platelet aggregation begins once the Gp IIb/IIIa receptors adhere to vWF and fibrinogen. Fibrinogen is found in the blood circulation and manages the interconnection of platelets tough the Gp IIb/IIIa receptors of platelets. Eventually, a weak platelet plug will form to protect the damaged area from hemorrhage until further stabilization of fibrinogen to fibrin in secondary hemostasis (Garmo et al., 2021; Saracoglu & Saracoglu, 2018).

1.3 Secondary Hemostasis

In this stage, the weak platelet plugs which formed in primary hemostasis began to stabilize into a clot by the involvement of coagulation factors (Figure 1.3). These factors are categorized as β-Globulins which develop the fibrin network. The blood clotting process in secondary hemostasis initiate through one of two main pathways; first pathway is the contact activation pathway (intrinsic pathway) and second is the tissue factor

pathway (extrinsic pathway). Eventually, lead to the common pathway. Intrinsic and extrinsic pathways have an identical task to accomplish, despite their differences. These important tasks are including: 1- initiation of complex cascade that activates the coagulation factors and generates long strands of fibrin to strengthen the platelet plugs formed in primary hemostasis. 2- the transformation of prothrombin (PT) to thrombin. 3- transformation of fibrinogen to fibrin to enmeshed in the network with platelets, blood cells, and plasma component to form a steadier clot (Garmo et al., 2021; Periayah et al., 2017). It is important to remember that platelet accumulation at the place of damage is not only crucial in the primary hemostasis but also principally participate in cell-based thrombin generation of coagulation cascade (Hou et al., 2015; Monroe, Hoffman, & Roberts, 2002). Furthermore, the existence of calcium ions is necessary through the process of secondary hemostasis (Barmore, Bajwa, & Burns, 2020).

The responses of the coagulation system naturally balance between the clot formation and anticoagulant pathways that are critical for proper hemostasis of the body. Respectively, this balance maintains through pro-coagulant pathway and inhibition of the procoagulant forces. Dysfunctions in these processes can result into two condition which is hemorrhage (bleeding) or thrombosis (blood clot) (Chaudhry, Usama, & Babiker, 2020; Gale, 2011; Garmo et al., 2021)

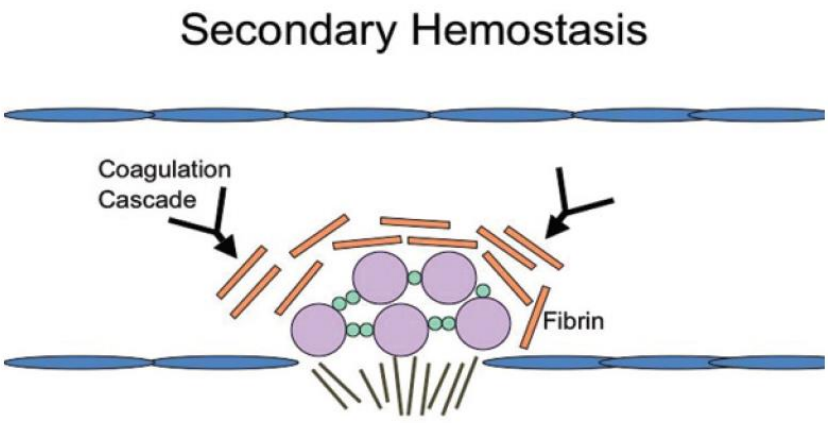

Figure 1. 3: Overview of the secondary hemostasis. Considering a final and stable fibrin clot, cross-linked fibrin contributes to stabilizing the platelet plug at the site of endothelial injury (Mahadevan & Garmel, 2012).

1.3.1 Intrinsic Pathway

The intrinsic pathway is slower than the extrinsic pathway. it involves factors including factor VIII, Christmas factor (IX), plasma thromboplastin factor (XI), and Hageman factor (XII) (Chaudhry et al., 2020; Garmo et al., 2021). The critical role of intrinsic pathway factors has been indicated in a study that targeted the elements of this pathway such as FXI (factor XI, inactivated serine protease), FXII, and PK (prekallikrein). They could show the alteration in each factor decrease thrombosis with no major effect on protective mechanism of hemostatic pathways (Grover & Mackman, 2019).

The cascade begins via direct exposure of FXII with subendothelial collagen which leads to a transformation of FXII to its active form (FXIIa). Plasma prekallikrein (PK) and High-molecular-weight kininogen (HMWK) are two proteins that facilitate this clotting process(Espana & Ratnoff, 1983). Afterward, FXIIa acts as a catalyst to activate factor FXI to FXIa and respectively FXIa activates FIX. The Factor FIXa works in combination with activated factor VIII (FVIIIa) and calcium ion to activate factor X. Once Factor X is activated by FIXa-FVIIIa complex, the cascade continues down the common pathway (Chaudhry et al., 2020; Garmo et al., 2021).

As the cascade progress further, the concentration of involved factors increases in the blood and their importance increases as well. Even though the deficiency of any component would disturb the flow and lead to abnormal incidents, it has been suggested that the role of each component of the intrinsic pathway are not equal and they result in different severity from mild to severe (Wheeler & Gailani, 2016). For instance, lack of FIX or its cofactor factor VIII (FVIII), causes the severe bleeding disorder, hemophilia. While the complete deficiency of factor FXII, causes the only prolongation of the activated Partial Thromboplastin Time (aPTT) (Kenne et al., 2015). aPTT is a parameter that clinically monitored the time required to form a clot by adding the kaolin to plasma (Furie & Furie, 1988). These observations suggesting that the classic intrinsic pathway does not accurately describe the involvement of components in hemostasis, thus it may require further revisions in the models that are more incompatible with clinical phenotypes (Wheeler & Gailani, 2016).

1.3.2 Extrinsic Pathway

The extrinsic pathway is a faster response to injury since it is more direct pathway comparison with the intrinsic pathway. The extrinsic pathway is initiated via exposure of blood to tissue factor (TF) that is expressed in the subendothelial tissue. When TF bind to FVII (known as Proconvertin), activates the FVII to factor VIIa (FVIIa) and forming a TF-FVIIa complex. Further, this complex activates factor X (FX) to FXa which is the initiation of a common pathway. On the other hand, TF-FVIIa complex has possibility to activate factor IX of the intrinsic pathway which shows the importance of the Extrinsic Pathway in the blood coagulation cascade since it can provide an alternative to the intrinsic pathway (Gale, 2011; Garmo et al., 2021; Kolde, 2004; Palta et al., 2014). The extrinsic pathway is clinically measured as the prothrombin time (PT) and Thrombin Clotting Time (TCT), which monitor the activity of the extrinsic and common coagulation pathways (Chaudhry et al., 2020).

1.3.3 Common Pathway

Whether coagulation cascade started with the intrinsic or extrinsic pathway, it will continue with common pathway. This pathway initiates via activation of Factor X and results in converting prothrombin to thrombin and ultimately forming the final clot (Periayah et al., 2017).

Factor Xa combines with prothrombinase complex including activated factor V (FVa) and calcium on the phospholipid surfaces. This, in turn, transform prothrombin into thrombin which activates factor XIIIa (FXIIIa) (Furie & Furie, 1988). FXIIIa crosslinks with soluble fibrinogen into fibrin monomers, which spontaneously polymerize to form protofibril strands that undergo linear extension, branching, and lateral association leading to the formation of a three-dimensional network of fibrin fibres to form the stabilized clot (Garmo et al., 2021; Green, 2006; Palta et al., 2014; Periayah et al., 2017). In order to evaluate the common pathway both PT and aPTT are effective, but the PT is a more sensitive screening test for common pathway problems (Jones, 2010).

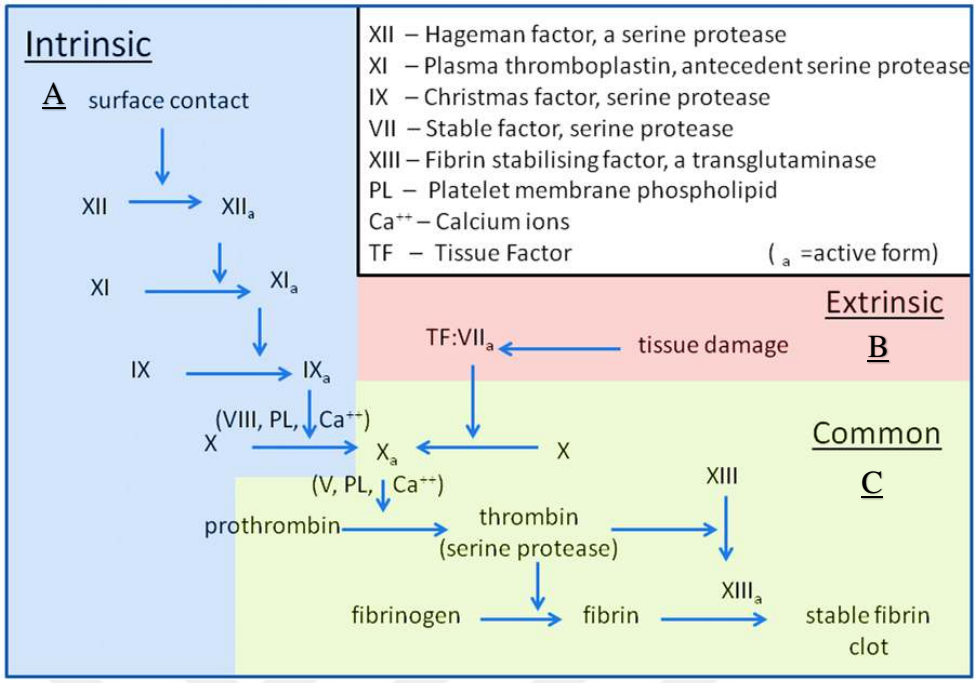

Figure 1. 4: Main models of the blood coagulation process. Two independent pathways; A) Intrinsic pathway and B) Extrinsic pathway which come together as a common pathway at the level of factor X. This model explains the factors involved in the stable clot fibrin formation ("Coagulation," 2020).

Table 1. 1: Secondary hemostasis pathways, factors, and indicators.

Pathway	Clotting Factors	Pathway initiation
Intrinsic Pathway	Factors I (fibrinogen), II (prothrombin), IX (Christmas factor), X (Stuart-Prower factor), XI (plasma thromboplastin), and XII (Hageman factor)	Activated through exposed endothelial collagen
Extrinsic Pathway	Factors I, II, VII (stable factor), and X.	Extrinsic pathway is activated through tissue factor released by endothelial cells after external damage
Common Pathway	factors I, II, V, VIII, X	Factor X availability

1.4 Hypercoagulability and Hypocoagulability

There are a variety of hypercoagulable and hypocoagulable pathophysiologic conditions related to defects in the coagulation pathways. The natural coagulation pathway provides an equilibrium of the thrombotic mechanism that is responsible for clot formation and the antithrombotic mechanisms that inhibit the coagulation to control and localize these processes in the injury site (Figure 1.5) (De Caterina et al., 2013; Palta et al., 2014). Maintenance of coagulation is regulating at various levels, whether by enzyme inhibition or by cofactors activity modification. Imbalance of the coagulation system can be inherited or acquired which increase the tendency of either thrombosis or bleeding (Hollenhorst & Battinelli, 2016).

Hypercoagulability or thrombophilia defined as the enhanced tendency of blood to change from liquid form to solid form. This explains the pathological situation of excessive coagulation in the absence of bleeding that different constituent of the blood cooperates to create a thrombus (LaPelusa & Dave, 2019). Hypercoagulability is blood-clotting disorder that may be inherited, for example Factor V Leiden (the most common), prothrombin gene mutation, hyperhomocysteinemia, deficiencies of protein C and S (Senst, Tadi, Goyal, & Jan, 2020). Meanwhile, hypercoagulability may be diagnosed in case of operation and perioperative period, trauma, malignancies, ageing, pregnancy, immobilization, or chronic inflammatory condition (Senst et al., 2020).

Chronic inflammatory conditions are observed to give rise to vasculature inflammation and endothelial surface alteration which leads to platelet activation and thrombus generation (Garmo et al., 2021; Periayah et al., 2017). One of the chronic inflammatory conditions is Pseudoexfoliation syndrome that increases the chance of endothelial dysfunction and coagulation disorder (Andrikopoulos et al., 2014; Atalar et al., 2006).

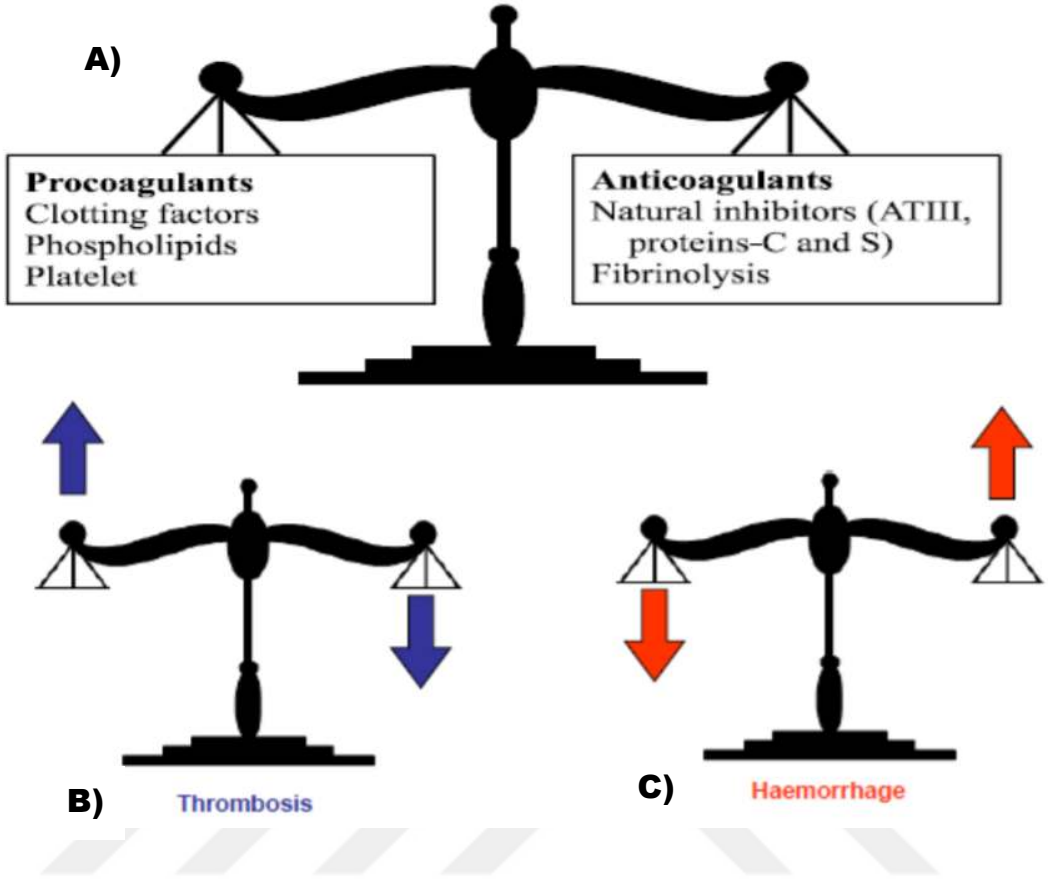

Figure 1. 5: Equilibrium of blood coagulation forces. A) Maintain of normal blood coagulation is in balance when procoagulant and anticoagulant forces are equal. Disturb in these forces lead to B) Thrombosis and C) Hemostasis.

1.5 Coagulation Assessment

Coagulation refers to a system of generation of blood clot (thrombus) which its balance always maintained under the natural physiology of the body. However, the pathophysiological conditions may disturb this balance to either hemorrhagic condition that led to abnormal bleeding or thrombotic complications that are known as abnormal clotting. Pathophysiological conditions may be due to the dysfunction in different parts of the clotting mechanism. Disorders related to primary hemostasis engage the factors including vWF, platelet defects, or receptor interference. And disorders related to secondary hemostasis happens due to the clotting factor defects, and small vessels flaws (Garmo et al., 2021).

Blood coagulation monitoring is critical to demonstrate the risk of bleeding during extensive surgery procedures, blood coagulation associated abnormalities, investigate the efficacy of anticoagulant drugs and hemostatic therapies. Blood coagulation monitoring traditionally performs via Conventional Coagulation Tests (CCTs) known as routine laboratory tests. Due to limitations of these tests, in recent years CTCs developed to a new phase which leads to creation of of devices with high degree of precision, powerful, and cost-efficient known as Point Of Care (POC) devices (Mohammadi Aria, Erten, & Yalcin, 2019).

1.6 Conventional Coagulation Tests (CCTs)

CCTs are using routinely in the laboratory to evaluates blood coagulation in vitro. These tests including PT/ international normalized ratio (INR), aPTT, TCT are the most useful tests which perform according to the significant diseases (M. Hussain, 2015; Kamal, Tefferi, & Pruthi, 2007; Yang et al., 2015). Several limitations of CCTs proposed such as, fail to entirely reflect coagulation system dysfunction in vivo, unable to detect hyperfibrinolysis, neglect the contribution of platelets in coagulation, obligation to be performed in the central laboratory and, time consuming (Schmidt, Israel, & Refaai, 2019; Weber & Zacharowski, 2012). Despite several limitations that have been suggested for CCTs, they are routinely performed in various groups of patients to conduct clinical decisions (Lloyd-Donald et al., 2020).

1.6.1 Prothrombin Time (PT)

In the history of PT, the assay has been used to analyze the prothrombin level. Nowadays, this assay applies to assess the adequacy of warfarin anticoagulation or allow the prompt diagnosis of coagulopathy disorders due to liver disease. The PT analyzes the extrinsic pathway as a tissue factor-dependent pathway via adding tissue factor to the patient's sample. Also, the common pathways can be analyzed by the PT. the PT values standardized by using INR value which calculated from INR = (patient PT/patient MNPT) ^{ISI}. INR is a factor that drives from PT which include reagent sensitivity and varying instrument to manage the patients in narrow therapeutic ranges. As mentioned in

formula, INR calculated from the MNPT which is the Mean Normal PT and, the ISI which is an International Sensitivity Index used to adjust instrument and reagent sensitivity differences (Mohammadi Aria et al., 2019).

1.6.2 Activated Partial Thromboplastin Time (aPTT)

The partial thromboplastin time (PTT) is utilized to diagnose patients with hemophilia A to evaluate the level of their antihemophilic factor VIII or evaluate factor IX in hemophilia B patients (Langdell, Wagner, & Brinkhous, 1953). The PTT assesses the intrinsic and common pathway of the coagulation system from citrated plasma samples. Since PTT assess the intrinsic coagulation pathway, it is suitable for inherited disease studies(Asaf et al., 2001). The aPTT test was invented with a same function of PTT but higher sensitivity. The aPTT test perform by adding an activator to accelerate the clotting time (CT) and results in a limited reference range. The aPPT test is utilized to evaluate the enzymatic reactions of intrinsic pathways like factor XIa, XIIa, and Xa (Martini, Cortez, Dubick, Park, & Holcomb, 2008).

1.6.3 Thrombin Clotting Time (TCT)

TCT is a coagulation assay which assess the fibrinogen. The Thrombin clotting Time evaluation is proceeded by adding Bovine or Human Thrombin to citrated Platelet Poor Plasma (PPP). Added thrombin enable the formation of firm clot and the time of this process is recorded in second and presented as TCT result. This test employed to distinguish acquired and congenital fibrinogen inadequacy (Ignjatovic, 2013). Thus, this test allows clinical researchers to demonstrate the conversion of fibrinogen to fibrin and also detect the presence of inhibitors factor like heparin in the plasma.

1.7 Point-of-Care (POC) for Coagulation Monitoring

Over the last few years, advanced technologies came together to overcome several limitations of TCTs through developing highly accurate, robust, rapid, and automated devices known as POC which measure the parameters of blood coagulation (Mohammadi Aria et al., 2019; Weber & Zacharowski, 2012). These innovative devices involved

techniques which take advantage of light transmission/scattering, electrical impedance, and viscoelastic properties (Brown, 1993; Ganter & Hofer, 2008; Sahli et al., 2020; Zhao, 1993). In this study we have used Viscoelastic Clotting Tests (VCTs) which used whole blood rather than the plasma and ensures that the entire of blood clotting cascade is monitored (Cohen, Haas, & Cushing, 2020).

Recently, Viscoelastic POC devices are commonly used in a variety of clinical scenarios since they provide critical information in case of guiding anti- and procoagulant therapies, massive hemorrhage, assessment of hyper- and hypocoagulable states, and in diagnosing surgical bleeding (Ganter & Hofer, 2008; Mohammadi Aria et al., 2019; Niederdockl et al., 2016). Analyzing the whole blood sample, allow the situation closer condition to in vivo to investigate the patient's coagulation status. Considering the interactions of plasma coagulation factors with red blood cells and platelets is essential since it may provide excessive data about platelet function (Ganter & Hofer, 2008; Stein, Kaserer, Spahn, & Spahn, 2017). In addition, viscoelastic tests measures the processes involving thrombin generation, clot formation and clot lysis, while the TCTs measure the entire process of generation of thrombin (Theusinger, Nurnberg, Asmis, Seifert, & Spahn, 2010). Mentioned privileges and recommendations of bleeding guidelines resulted in a rapid expansion of VCTs.

1.7.1 The Rotational Thromboelastometer (ROTEM®)

One of the most popular POC devices which use a viscoelastic hemostasis analyzer of whole blood is the ROTEM®.

The ROTEM® was introduced as a ROTEG® and later ROTEM® delta system by Helmut Hartert in 1948 and in the 1990s by Andreas Calatzis respectively (AN Calatzis et al., 1996; Hartert, 1948). ROTEM® device which has been developed from the thromboelastography (TEG®) system over the past decade, enables clinical and laboratory practitioners to evaluate clot formation and fibrinolysis in real-time. This device attracted attention as a user-friendly device since reduces intra- and inter-operator variability of test results, visually displays the results on the touch screen in real-time and a allow multiuser environment (Figure 1.6) (Haas et al., 2012; Mauch et al., 2011).

ROTEM® has been widely used in clinics as a most powerful method to monitor blood coagulation (Ganter & Hofer, 2008). ROTEM® enable monitoring the mechanical changes in clot formation under low shear stress conditions (approximately 0.1/s) equivalent to the environment present in areas of the venous part of the circulation, vena cava, large veins, and the arterial system (Figure 1.7) (Stanciakova et al., 2021; Whiting & DiNardo, 2014).

The ROTEM® delta system is equipped with four independent viscoelastic measurement channels which improves the diagnostic performance of the device since each channel is independently used for specific tests. Each channel consists of an oscillating pin with a ball bearing mechanism which rotates +/- 4 degrees 75' every 6 seconds, and a disposable cuvette cup which stabilized in the metal cup-holder and has a capacity of 340 µl of blood (Hunt et al., 2015; Whiting & DiNardo, 2014).

The ROTEM® measure the speed and strength of the formed clot at all stages of the coagulation process. Moreover, it can assess the quality of fibrinogen and platelets' contribution to the elasticity of formed clot (Ziegler, Solomon, Cadamuro, & Jones, 2015). Different reagents are used to perform different assays each of which targeted the different pathways and components of coagulation (Shaydakov, Sigmon, & Blebea, 2019). To investigate the contribution of the intrinsic pathway in coagulation, reagent such as ellagic acid/phospholipid (INTEM test) has been used to stimulate the pathway (Bock, Srinivasan, & Shore, 1981; Stanciakova et al., 2021). For extrinsic pathway evaluation, reagent such as tissue factor has been utilized (EXTEM test) and for fibrinolysis evaluation can be accessed via stimulation by the platelet inhibitors such as cytochalasin D (FIBTEM test) (Camerer, Kolsto, & Prydz, 1996; John, Mathews, & Nair; Nielsen, Geary, & Baird, 2000). The involvement of platelets in clot elasticity can be interpreted by the maximum clot elasticity (MCE) value. MCE is automatically calculated for each test by ROTEM® device (Solomon, Schochl, Ranucci, Schott, & Schlimp, 2015). The difference between the maximum clot elasticity (MCE) value of the EXTEM test and the MCE value of the FIBTEM test can show the quality of platelets' contribution to clot elasticity (Nielsen et al., 2000).

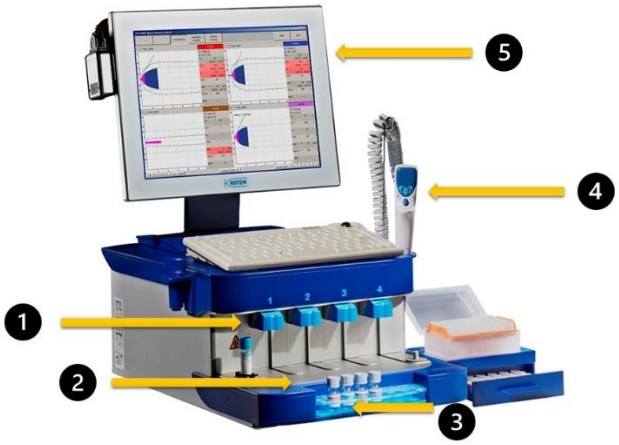

Figure 1. 6: ROTEM® delta device. This device consists of 1) a four-measurement unit with independent temperature-adjusted channels, 2) a pre-warming plate, 3) a reagent holder, 4) an automatic pipette, and 5) an integrated touch-screen computer.

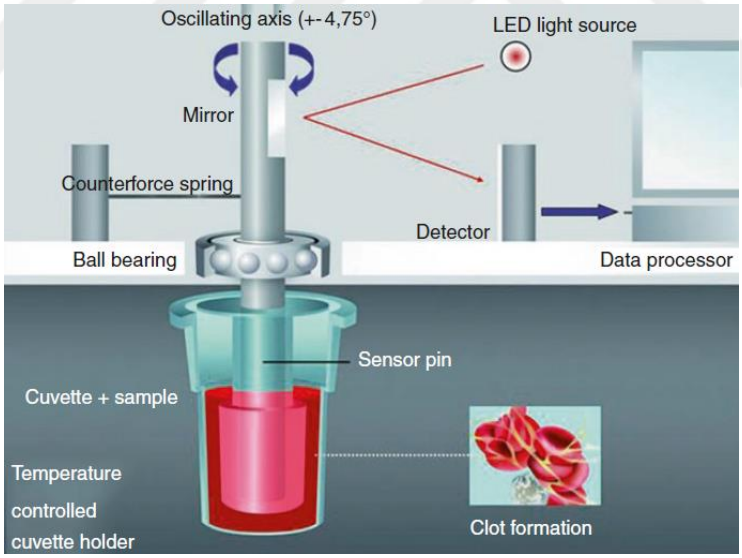

Figure 1. 7: Measuring principle of ROTEM® delta. The ROTEM® axis is rotating forth and back by 4° 75′ every 6 seconds. In the meantime, clot formation strands between pin and cup-wall are affecting the speed of pin rotation. These changes in the pin movement are constantly measured by a LED light source which reflect to the detector system. the result of light reflection is processed and transformed into a thromboelastometric graph displaying on the computer screen (Gจ๐rlinger, Dirkmann, & Hanke, 2016).

1.8 Clinical usage of the ROTEM®

ROTEM® analysis of viscoelastic properties using whole blood, running several samples separately and in parallel, and investigating the clot formation in a real-time, make ROTEM® a remarkable device to be used in clinics. The ROTEM® is a viscoelastic-based device which empower researchers by invaluable data that conventional clotting tests like PT and aPTT are unable to provide. Specific data presented by ROTEM® device is including interaction coagulation factors, their inhibitors, anticoagulant drugs, blood cells, and specifically platelets. Given all potentials, this device is still used in limited clinical researches in comparison with CCTs (Cohen et al., 2020).

According to literature review, ROTEM tests are used in various conditions including Pseudoexfoliation syndrome (PEX), Retinal Vein Occlusion (RVO), Behcet's disease (BD), and malignancies.

In our previous study by ROTEM® on Pseudoexfoliation syndrome we could show the EXTEM CT decreased in PEX Syndrome patients as compared to control group. In other words, faster clot formation in PEX patients could be a valid sign of hypercoagulability. This result is capable to explain the increase of thrombotic events in PEX patients (Goren Sahin, Sahin, & Akay, 2016).

Moreover, on the other study by Goren Sahin et al. on RVO patients we could show the faster CT which means shorter clotting formation time In RVO patients. ROTEM® is enabled to detects the differences in clotting dynamics between study groups or individual cases. This feature can be a beneficial method to clarify the pathophysiology of diseases that involve hypercoagulable state such as RVO (Goren Sahin et al., 2019).

Yasar Bilge et al. benefited from the ROTEM test to examine the role of hemostatic mechanisms in the occurrence of thrombosis in BD. They could show that in the INTEM assay, Maximum Clot Firmness (MCF) increased and Clot Formation Tima (CFT) decreased in BD patients compared with the control group. In the EXTEM assay, they reported an increase in MCF value and a decrease in CFT value in BD patients compared with the control group. These significant differences in ROTEM parameters confirmed the tendency to hypercoagulation in BD patients. (Yasar Bilge, Akay, Kasifoglu, Kus, & Korkmaz, 2013).

Also, the ROTEM® has been used to detect thrombosis in malignancies. Informative studies have a vital importance for cancer patients since it is one of the major complications in this group of patients. The results of a the ROTEM® study which included 78 participants showed the considerable level of hypercoagulability in cases that diagnosed with solid tumors. Major drop in the CFT and increase in the MCF parameters in INTEM, EXTEM, and FIBTEM proved the hypercoagulable state in this patient group. In addition, they could show the engagement of platelets and fibrinogen to form a stable clot in this patients (Akay, 2018; Akay, Ustuner, Canturk, Mutlu, & Gulbas, 2009). In the other study, Larsen et al could show the patients who undergo chemotherapy have a lower ability of platelet aggregation. It can be explained by considering the damage made during chemotherapy to bone marrow leading to an insufficient number of platelets. It has been shown that the thrombopoiesis and platelet function may intervene with the active infection and antibiotic treatments as well as chemotherapy (Larsen et al., 2009)

Chapter 2:
PSEUDOEXFOLIATION (PEX) SYNDROME

2.1 PEX Syndrome

Pseudoexfoliation (PEX) syndrome was first identified by Lindberg in 1917, yet the exact etiology of this syndrome remains unclear (Anastasopoulos, Founti, & Topouzis, 2015; J. G. Lindberg, 1989). The PEX syndrome is mostly considered to be an age-related syndrome since this syndrome commonly emerge in individuals over 50 years of age. Also, the incidence of PEX increases with age. The PEX syndrome is characterized by the abnormality in connective tissue leading to irregular production and deposition of fibrillar-granular material in the anterior segment of the eye tissue including pupillary margin, follicle, lens zones, and the anterior vitreous (Figure 2.1) (Cursiefen, Hammer, Kuchle, Naumann, & Schlotzer-Schrehardt, 2001). These fibrillar deposits are known as PEX materials and they are visible through slit-lamp ophthalmology examination (Tarkkanen, 2018).

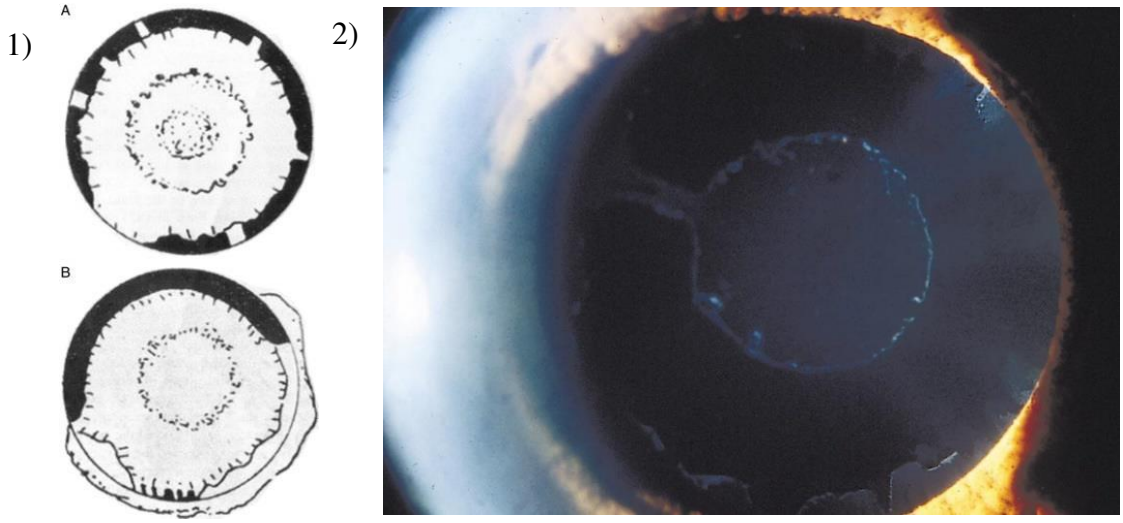

Figure 2. 1: Deposited PEX material in the eye. 1) Illustrated the hand-drawing that introduced in the Lindberg's thesis to display the typical appearance of exfoliation syndrome. 1A) Deposit on the anterior capsule. 1B) Exfoliation in the Central disc type.

2) Classic appearance of the eye lens with PEX material undergo slit-lamp ophthalmology examination. The relatively homogeneous central disk and peripheral zone are separated by a clear zone, which results from scraping of the PEX material by the iris (Ritch & Schlotzer-Schrehardt, 2001b).

2.2 Epidemiology of the Pseudoexfoliation Syndrome

The prevalence of PEX syndrome ranges from 10% to 20% in the world's general population over 40. In studies conducted in our country, PEX prevalence was reported by 11-13% (Yalaz et al., 1992). In the more recent study by Yildirim et al, 2017 cases from a total number of 2356 cases were randomly chosen as the study subjects. From the 2017 invited cases only 2009 of them were eligible for the study. The study population was based on the Turkish Statistical Agency. They could show the prevalence of the PEX syndrome in patients over 40 years old was 5.0%. In addition they reported the association of the PEX syndrome with glaucoma and cataract, hypertension, hearing loss, using drugs for cardiac and psychiatric diseases (Yildirim, Yasar, Gursoy, & Colak, 2017).

2.3 Origin and Composition of PEX Material

Abnormally deposited PEX materials have been described as a white fluffy undegradable microfibrillar material (Andrikopoulos et al., 2014; Ritch & Schlotzer-Schrehardt, 2001a). PEX deposits are found in various parts of ocular tissues but the site of production of PEX material is unclear. Several sites including the corneal endothelium, trabecular endothelium, iris, lens epithelium, or the ciliary epithelium have been suggested to be the origin of this material (Ritch & Schlotzer-Schrehardt, 2001a; U. Schlotzer-Schrehardt & Naumann, 2006). Mature PEX fibrils produced by aggregation of microfibrils consist of glycoprotein-proteoglycan complex (Figure 2.2) (Zenkel et al., 2010).

Histochemical studies illustrated that microfibrils comprised of elastic microfibrillar components containing fibrillin-1, fibronectin, elastin, tropoelastin, and transforming growth factor (TGF)-b-binding proteins (Anastasopoulos et al., 2015). The excessive accumulation of PEX material disturbs and destroy the normal basement membrane of

affective tissues specifically by depositing around blood vessels of connective tissue. It has been mentioned that PEX materials also have been found in tissues outside the eye such as the heart, liver, blood vessel walls, orbital connective tissue, and meninges via Electron microscopic and immunohistochemistry studies (Andrikopoulos et al., 2014; Elhawy, Kamthan, Dong, & Danias, 2012; Turkcu et al., 2014). Hence, PEX is considered not only to be an isolated ocular disease, but also a systemic syndrome that can affect all vascular structures.

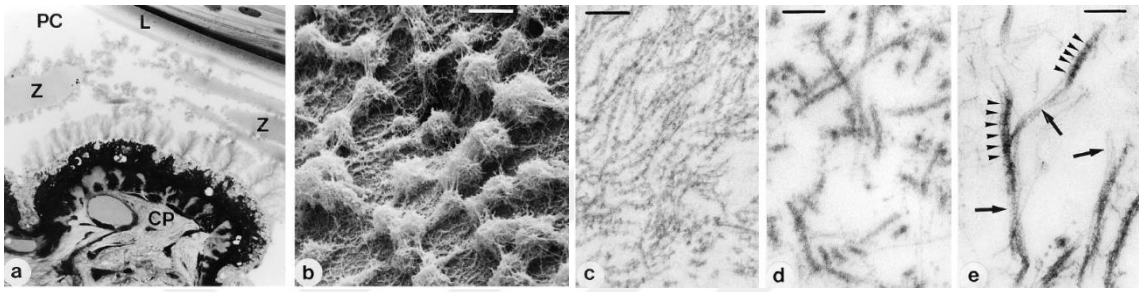

Figure 2. 2: Structure of PEX material. A) Light microscopic illustration of exfoliation deposits on a zonule (Z), ciliary process (PC= posterior chamber), and the lens equator (L); B) View of exfoliation deposits displayed by the Scanning electron microscopy deposited on the anterior lens capsule; C–E) Transmission electron micrographs (TEM) reveal the shape of exfoliation fibres deposited on the anterior lens capsule. E) The arrows pointing at cross-bands (Ritch & Schlotzer-Schrehardt, 2001b).

2.4 PEX Syndrome Etiology

Even though the etiology of the PEX disease is not fully understood, several genetic and environmental factors associated with PEX have been reported. Lysyl oxidase-like 1 (LOXL1) gene on chromosome 15q24.1 is responsible to express a member of the lysyl oxidase (Elhawy et al., 2012). The enzyme is necessary for cross-linking of collagen and elastin polymers and elastic fiber generation and maintenance (Elhawy et al., 2012; Liu et al., 2004). Mutation in LOXL1 can be a strong risk factor to progress PEX syndrome (Thorleifsson et al., 2007). However, in a study conducted by Zenkel et al. demonestrated that LOXL1 risk alleles have been carried out by around 50% of the normal population.

Several studies investigate the effect of other associated genetic variants including clusterin (CLU) gene, Glutathione transferase gene, contactin-associated protein-like 2

*(*CNTNAP2*)* gene, and tumor necrosis factor-alpha (TNF α) which shows the relatively small effects of these gens. The mentioned investigation suggested the high possibility of contribution of other genetic, epigenetic, or environmental factors leading to the pathogenesis and progressive deposition of PEX materials (Damji et al., 1998; Wiggs, 2008; Zenkel et al., 2011). Involvement of environmental factors including ultraviolet light, slow virus infection, autoimmunity, oxidative stress, or traumatic factors shows that PEX syndrome may be a multifactorial disorder (Damji et al., 1998; Elhawy et al., 2012; Luntz & Ballard, 1979; Ringvold, 1988; Zenkel et al., 2011).

2.5 Clinical Complications Caused by PEX Syndrome

PEX syndrome is the most common cause of secondary open-angle glaucoma, and it causes significant complications for intraoperative and postoperative cataract surgery. The risk of conversion of intraocular hypertension to glaucoma is twice as high in patients with PEX syndrome. One study showed that 7%–30% of patients diagnosed with PEX syndrome will develop glaucoma within 5 years (Anastasopoulos et al., 2015; Puska, 2002).

Sainz Gomez et al. reported the patients suffering heart failure have 3.5 times more chance to be diagnosed with PEX syndrome. However, several studies failed to show cardiovascular disease association with PEX (Ritland, Egge, Lydersen, Juul, & Semb, 2004; Sainz Gomez et al., 2003; Shrum, Hattenhauer, & Hodge, 2000). Moreover, there are several clinical conditions associated with PEX which shows the importance of investigation in the PEX pathology for ophthalmologists (Tekin, Inanc, & Elgin, 2019). Scientists could show in several studies that PEX syndrome increased the risk of severe diseases like Secondary Open-angle Glaucoma, RVO, and cardiovascular diseases.

Although deregulated homocysteine (Hcy) metabolism and elastic microfibrillopathy are two identified factors associated with PEX, there are very little data about the exact pathogenesis of the increased Thromboembolism and the Hypercoagulability seen in PEX patients (Goren Sahin et al., 2016; Roedl et al., 2007). It has been suggested that the PEX material deposits around blood vessels of connective tissues may describes the endothelial dysfunction and PEX syndrome functional role in thrombus formation.

2.5.1 Secondary Open-Angle Glaucoma

The PEX syndrome is strongly associated with the development of glaucoma in the eyes of PEX patients; specifically Secondary Open-angle Glaucoma. A glaucoma is a group of common eye diseases that can cause irreversible visual impairment and early detection is essential. It is a progressive optic neuropathy that results in damage to the optic nerve due to elevation of intraocular pressure (IOP) (Weinreb & Khaw, 2004). IOP is regulated by a balance between secretion and drainage of aqueous humor through trabecular meshwork where the iris and cornea join. Secondary Open-angle Glaucoma refers to a condition that glaucoma occurs as a consequence of another eye diseases. This type of glaucoma involves the blockage of the outflow of aqueous humor when the cornea and iris remain open, but the trabecular meshwork is blocked (Figure 2.3) (Bai, Yao, Wang, Jin, & Wang, 2009; D. A. Lee & Higginbotham, 2005).

As mentioned previously, PEX syndrome characterized is by the accumulation of pseudoexfoliative material. It has been considered a factor to increase the IOP levels due to the accumulation of PEX material lead to blockage in the trabecular meshwork (Plateroti, Plateroti, Abdolrahimzadeh, & Scuderi, 2015). The accumulation of PEX material highly results in the chronic Secondary Open-angle Glaucoma. Moreover it was reported that PEX material increase the chance of the lens dislocation, blood-aqueous barrier impairment, angle-closure glaucoma, cataract extraction complications like capsular rupture, zonular dialysis, and vitreous loss (Naumann, Schlotzer-Schrehardt, & Kuchle, 1998).

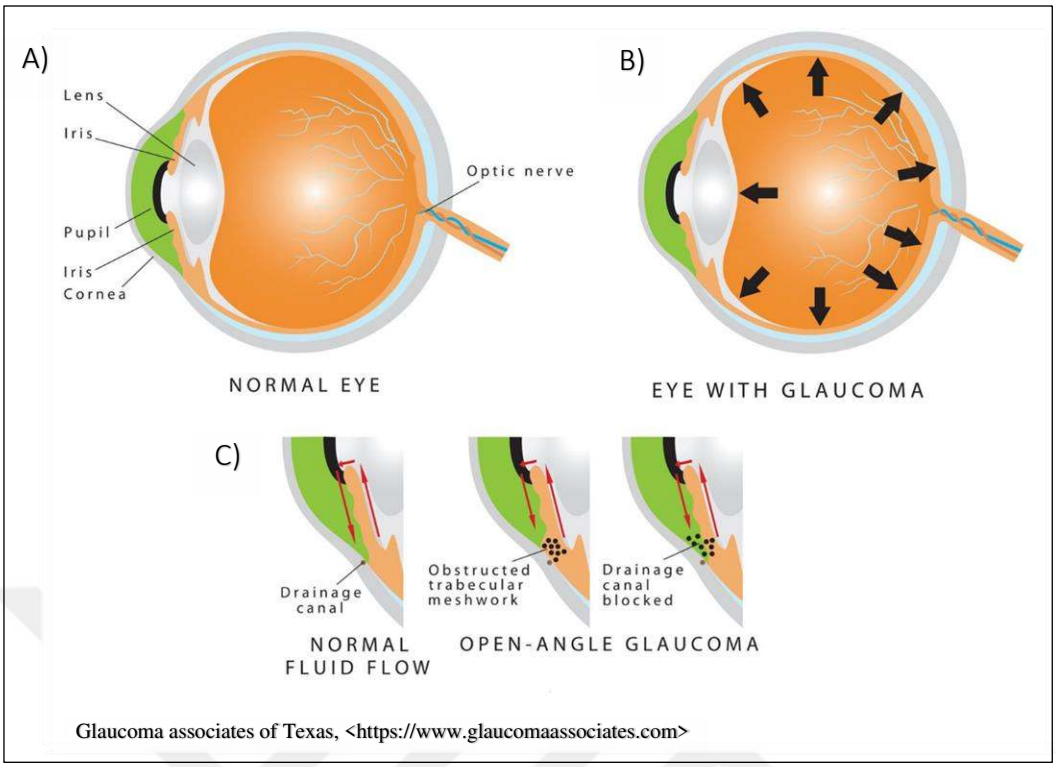

Figure 2. 3: Illustration of Secondary Open-angle Glaucoma. A) Normal eye Structure B) Eye with Glaucoma and high intraocular pressure which increase pressure in the optic nerve. C) The difference in the flow of fluid in normal eye versus Open-angle Glaucoma eye.

2.5.2 Retinal Vein Occlusion (RVO)

RVO happens due to the blockage of the small veins that drain blood away from the retina. RVO is known as the second most common vascular cause of vision loss (Karagiannis et al., 2015). The pathogenesis of RVO is not fully understood. However, it has been suggested that the etiology of RVO is multifactorial and based on a vascular abnormality (Deobhakta & Chang, 2013).

In the nineteenth century, Rudolf Virchow described three critical factors that are important in the development of venous thrombosis. These factors know as Virchow's triad including venous stasis, abnormalities of the vessel wall and activation of blood coagulation states can explain the pathophysiology of RVO (Kushner, West, & Pillarisetty, 2019). Thus, any condition that led to increasing these factors can cause a retinal vein occlusion (Rogers et al., 2010). The main known risk factors affecting

Virchow's triad is not only advancing age but also hypertension (HT), diabetes mellitus (DM), hyperlipidemia and glaucoma (Antman, Keren, Kurtz, Rosenblatt, & Rachmiel, 2019; Koizumi, Ferrara, Brue, & Spaide, 2007; Kolar, 2014).

RVO mainly appears in two types. The first type is when blockage happens in one or more temporal or nasal branches of the central retinal vein known as Branch Retinal Vein Occlusion (BRVO). Second, when the central retinal vein that drains blood from the retina encounter partial or complete blockage. The meta-analysis by Rogers et al showed BRVO is 4 to 6 times more prevalent than Central Retinal Vein Occlusion (CRVO) among American, European, Asian, and Australian populations (Figure 2.4) (Rogers et al., 2010). In one study which investigates the association of RVO with PEX independently from glaucoma, it has been shown that PEX is a risk factor for the development of CRVO (Karagiannis et al., 2015). On the other hand, the retrospective cohort study by Antman et.al, determined the higher rate of CRVO in both PEX and non-PEX glaucoma groups compared to the non-glaucoma non-PEX (nGnP) group. This fact can be attributed to PEX, and glaucoma associated with RVOs, but PEX was not found to be an independent risk factor for CRVO (Antman et al., 2019). These incomprehensible data show the importance of the further investigation to explicit the associations and responsible mechanism for these clinical observations.

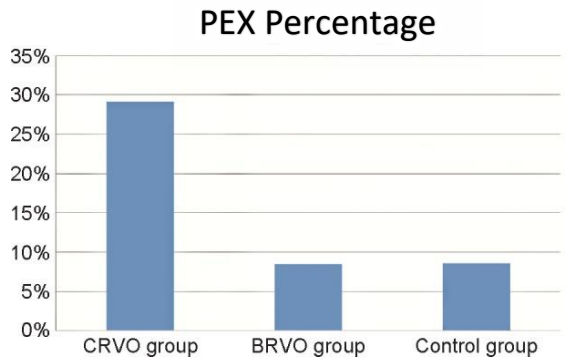

Figure 2. 4: Histogram representing the percentage of PEX Patients. Determine the incident of PEX syndrom among each of the CRVO, BRVO, and control groups (Karagiannis et al., 2015).

2.5.3 Cardiovascular Diseases

Cardiovascular diseases (CVDs) are known as the leading cause of death worldwide. There is increasing evidence in recent years that explore the association of the PEX syndrome with several cardiovascular disorders. A clinical study could show the prevalence of the CVDs in the PEX patients by analyzing the coronary angiography results (Citirik, Acaroglu, Batman, Yildiran, & Zilelioglu, 2007). The arterial endothelial dysfunction in PEX patients was specified in two independent studies (Atalar et al., 2006; Naji et al., 2008). In a study conducted by Atalar et al. arterial endothelial dysfunction was attributed to the accumulation of the abnormal extracellular fibrillar material in the vessel walls. Arterial endothelial dysfunction is considered as an independent factor to predict cardiovascular events (Andrikopoulos et al., 2014). In the study by Browne et al increased level of connective tissue growth factor in the PEX patients have been shown that associated with cardiac fibrosis (Browne et al., 2011; Dean et al., 2005). Altintas et al. could show the increase in the level of serum antiphospholipid antibody which is considered a risk factor for cardiovascular and cerebrovascular diseases in the PEX patients (Altintas et al., 2012).

The recently published meta-analysis by Chung et al. reviewed 20 different studies investigating the relationship of PEX with CVDs. These 20 studies were including a total of 9583 individuals and provided strong evidence that PEX is significantly associated with CVDs (Chung et al., 2018). In addition, the study that investigates the elevated level of plasma homocysteine in PEX patients may partly explain the increased risk of vascular disease among patients with PEX (Roedl et al., 2007; Tranchina et al., 2011). The vascular endothelium of PEX patients is threatened by high levels of plasma homocysteine. homocysteine creates an environment of hypercoagulability due to the releasing of free radicals lead to vessel wall alteration and injuries (Altintas et al., 2005).

Various mechanisms have been mentioned in studies that may explain the increased risk of cardiovascular events for PEX patients. Mechanisms like accumulation of PEX material in blood vessel walls, endothelial dysfunction, elevated levels of homocysteine, and other pro-inflammatory proteins may be involved in the pathogenesis of cardiovascular events. Nevertheless, more investigation is required but also it might be important to inform and examine the patients with PEX syndrome for cardiovascular events.

2.6 Hypercoagulability in PEX Patients

The coagulation process begins with primary hemostasis, continues with the activation of the secondary hemostasis also known as coagulation cascade, leading to the fibrinolytic system terminated by the mechanisms of melting the mediated clot. The formation of healthy clots requires the smooth process of polymerization of fibrin and fibrinolysis. Platelets and elastic microfibrils play an important role in this polymerization process (Halper & Kjaer, 2014). Since elastic microfibrillopathy (Krumbiegel et al., 2010) is seen in PEX syndrome, it has been suggested that there may also be pathological effects in clot formation. In a study by Cursiefen et al. there is no significant exfoliative vasculopathy in or just behind the lamina cribrosa has been observed in PEX patients who undergoes retinal vein occlusion (Cursiefen et al., 2001). It suggests that the platelet dysfunction and/or disorders in hemostasis in this group of patients may cause thrombotic events.

Although, there are numbers of factors such as disorder in homocysteine metabolism, platelet dysfunction, PEX material deposition, platelet volume, and endothelial dysfunction have been put forward in the etiopathogenesis of the hypercoagulation seen in PEX syndrome. There is limited knowledge about the mechanisms underlying the increased risk of thrombosis in PEX patients. In addition, a comprehensive evaluation of the hemostatic system, the path thorough clot formation to fibrinolys, and platelet functions have not been evaluated together in PEX cases.

2.7 Clinical Complications Caused by PEX Syndrome

Investigation in PEX disease through literature elucidated that several differences between the PEX patients and the control groups hematology examination exist.

Complete blood count (CBC) is a test that evaluates the cells that circulate in the blood. The CBC is a test for examining the general health of subjects also it can detect a variety of diseases and conditions, such as anemia, inflammation, and infection.

Several studies have shown an association between PEX syndrome and increased systemic vascular risk. In addition, scientists place a great value on the evaluation of inflammation and platelet properties in patients diagnosed with PEX syndrome. It has been reported that the factors such as total platelet count (PLT), plateletcrit (PCT), Mean platelet volume (MPV) and platelet distribution width (PDW) may have the potential to

be used as prognostic and diagnostic biomarkers. Platelet properties like PLT and MPV have an important role in evaluating the platelet activity, the larger platelets are biologically more active with stronger prothrombotic effects (Chu et al., 2010). Noticeably, MPV is a platelet function indicator that associated with a variety of prothrombotic and proinflammatory disease states (Korniluk, Koper-Lenkiewicz, Kaminska, Kemona, & Dymicka-Piekarska, 2019). The PCT is a highly informative parameter for platelet mass and platelet status which defined the volume occupied by platelets in the blood sample (Tvedten, Lilliehook, Hillstrom, & Haggstrom, 2008). The PDW measures the variability in platelet size. This parameter shows the increase in the number and size of platelet which undergoes platelet activation and morphologic changes (Vagdatli et al., 2010). In a study by Yazgan et al. the comparison of hemogram parameters have been studied in ocular PEX patients. It has been shown that PCT and PDW were significantly higher in patients with ocular PEX syndrome while no significant differenced in MPV has been reported(Yazgan, Celik, Kaldrm, Ayar, & Akdemir, 2016). However, In another study Turkcu et al could show that increased in MPV parameter is associated with PEX syndrome (Turkcu et al., 2014).

Lymphocyte/Monocyte count ratio (LMR) reflects the balance of immune response. LMR emerged as a new inflammatory biomarker in diseases like lung cancer (Li et al., 2017) colorectal cancer (Song, Wang, Zhang, & Zou, 2016), and diabetic retinopathy (Yue et al., 2015). In a study, Mirza et al. showed the lower LMR in PEX patients in comparison with healthy control. However, they could not show a significant difference (Mirza, Oltulu, Katipoglu, Mirza, & Ozkagnici, 2020).

Neutrophil-Lymphocyte Count Ratio (NLR) is a parameter which evaluates the effects of neutrophilia as an inflammation indicator and lymphopenia as a physiologic stress indicator (Gibson et al., 2010). The NLR demonstrate as a potential indicator of subclinical systemic inflammation. In a study by Ozgonul et al, it has been reported that the increase in the NLR indicates that inflammation plays a significant function to the initiation of the PEX syndrome. Noticeable ration of neutrophil-to-lymphocyte can be a practical indicator of inflammation (Ozgonul, Sertoglu, Mumcuoglu, Ozge, & Gokce, 2016). Their findings suggest ocular PEX syndrome as a part of a general disorder of the extracellular matrix and that patients with PEX may suffer from increased comorbidity.

Chapter 3:
AIM OF THE STUDY

The present thesis aimed to identify the diagnostic and prognostic biomarkers of hypercoagulability in PEX patient with focus on the differentially expressed platelet protein and coagulation states. Clarification of the mechanism of the PEX syndrome at the protein level will predetermine the risk of eye related vascular embolic diseases in PEX syndrome patients and help to develop new preventive treatment approaches.

Searching for the literature on hypercoagulability states in PEX syndrome, it is seen that most of the studies are clinical trials or disease-risk factor studies. Although the only study on ROTEM analysis in PEX syndrome in the literature is the one conducted by our group, as far as we know, there is no study yet that shows a holistic approach to the risk of thrombosis seen in PEX patients. In our study, it is aimed to examine the coagulation/fibrinolysis dynamics and platelet functions together in both the PEX group and control group. We investigated the relationship of the changes in coagulation state with ROTEM® device meanwhile, the platelet functions were studied via flow cytometry to evaluate the expression of CD41a, CD61, CD42b, CD62P, PAC-1, and the capacity of these receptors to connect relative antigens per cell. Furthermore, the hematology examination has been done for all patients who participated in this study. In hemogram analysis we evaluate the factors such as to PLT, PCT, MPV, PDW, LMR and NLR.

PEX syndrome often is without symptoms but as mentioned it is a risk factor for retinal vein thrombosis, cardiac diseases, and glaucoma. From a pathological point of view, the secondary open-angle glaucoma and vascular embolic diseases caused by pseudoexfoliation syndrome could be the cause of retina venous thrombosis which is a dangerous situation including blindness. Thus, it is essential to clarify the pathogenesis of hypercoagulability states in PEX patients and investigate the increased frequency of venous thrombosis events in these patients. The purpose of this study was to undertake ROTEM analysis to increase understanding of coagulopathy.

Chapter 4:
MATERIALS AND METHODS

4.1 Study Population and Screening Methodology

Patients of this study attended the Department of Ophthalmology, Koc University Hospital, Istanbul, Turkey, and Beyoğlu Eye Training and Research Hospital, Istanbul, Turkey. Informed consent was provided for all patients prior to sample collection, and the studies were approved by the local medical ethics committee the study and guidelines followed the tenets of the Declaration of Helsinki. Included patients' samples have been collected from September 2019 until March 2021.

The main characteristic to include a patient in a PEX group was the presence of PEX material on the anterior side of the lens. All study subjects underwent a comprehensive ophthalmic examination prior to the blood sample's collection. Exclusion criteria for the patients' group and control group were based on the following criteria:

1. Patient whose age is not between <40 and >75
2. Patients with Liver disease, Von Willebrand disease, Thrombocyte dysfunction, Diabetes mellitus.
3. Patients with Intraocular Pressure abnormalities
4. Patients using anticoagulant and antiplatelet drugs.

4.2 Exclusion Criteria

Exclusion criteria for the patients' group and control group are liver disease, Von Willebrand disease, thrombocyte dysfunction, diabetes mellitus, and patients using anticoagulant and antiplatelet drugs.

4.3 Sample Collection

Blood sample collection following informed consent from all the donors. All experiments adhered to the Declaration of Helsinki (IRB1.013/2017). One purple-capped

tube VACUETTE® TUBE 9 ml K3E K3EDTA was collected for the flow cytometry experiment, a 4.5ml blue-capped coagulation tube sodium citrate additive tube (BD, Franklin Lakes, NJ, USA) was used for ROTEM analysis, and a 3 ml purple-capped tube in K2E K2Edta tube (Disera A.S., Izmir, Turkey) for CBC analysis. This amount has been drawn from all patients who participated in this study. The collected sample underwent experiment within 90 minutes from sample collection time. Apyrase has been added to the blood sample collected for the flow cytometry at a final concentration of 0.02 U/ml as fast as the sample collected and before transfer to the laboratory.

4.4 Methods Overview

This experimental design was employed to investigate the causes of hypercoagulability in PEX syndrome patients because the primary pathogenesis has not yet been fully elucidated. Cellular contribution in hypercoagulability states is not detectable by routine CBC tests. We believe that the ROTEM test, which is the most used viscoelastic coagulation tests, is a new real-time methodology for the evaluation of all stages in hemostasis. ROTEM tests evaluate from the beginning of clot formation to the end of fibrinolysis thus it will clear up the difference between these stages of hemostasis between control and patient groups. In the preliminary study, we have proved that the extrinsic thromboelastometry clotting time (EXTEM CT) was significantly increased in cases with PEX syndrome compared to the healthy control group (Goren Sahin et al., 2016). It has been considered that ROTEM have an innovative perspective on the coagulation dysfunction of PEX cases. Our study was the first study in literature investigating ROTEM parameters in PEX cases and the findings suggest that thrombocyte functions and hemostasis should be evaluated together for the etiopathology of common thromboembolic events in PEX syndrome.

In this study, ROTEM parameters tested with different parameters have been performed. The evaluated tests including INTEM, EXTEM, and FIBTEM and the parameters like clotting time (CT), clot formation time (CFT), alpha angle, maximum clot firmness (MCF), amplitude at 10 min (A10), amplitude at 20 min (A20) and lysis index at 30 minutes (LI30) has been reported in (Table 4.1). Flow cytometry test investigated the platelet functions by evaluating the expression and binding capacity of CD 41a,

CD42b, CD61, CD62p, and PAC-1. Regarding the CBC test the parameters like total platelet mass (PLT), plateletcrit (PCT), mean platelet volume (MPV) and platelet distribution width (PDW). In addition, lymphocyte count (LYMPH), monocyte count (MONO), and neutrophil count (NEUT) has been measured due to evaluation of NLR and LMR.

There is no integrative study in the literature evaluating ROTEM parameters with platelet functions and complete blood count in this patient group. Thus, this study is designed to investigate the association of these parameters in PEX cases.

4.4.1 Principle of ROTEM®

ROTEM® (Pentapharm Gmbh, Munich, Germany) is a device that analyzes the whole blood viscoelastic hemostasis this device evolved from the system known as the TEG system.

The utilization of the ROTEM® delta system has dramatically increased in recent years. This device provides a real-time method that demonstrates the global hemostatic picture from the beginning of clot formation to the end of fibrinolysis. The ROTEM® device outputs have been used to detect and manage coagulopathy in hemorrhagic disorders including trauma, post-partum hemorrhage, intensive care, and surgery including liver transplantation. EXTEM and INTEM analyses the initiation, spread, and stabilization of clot formation while FIBTEM analysis is used for quantitative analysis of clot dissolution (fibrinolysis). EXTEM studies the extrinsic pathway by using tissue factor as an activator of the pathway. INTEM studies the intrinsic pathway and uses ellagic acid/phospholipids for contact activation. FIBTEM assesses the extent of fibrin polymerization by inhibiting platelet function with cytochalasin-D after tissue factor activations (Solomon et al., 2011).

The ROTEM® delta provides several indicators per test, in this study most important indicators are mentioned in the table below (Andreas Calatzis, Spannagl, & Vorwe-g, 2013).

Table 4. 1 ROTEM® delta parameters (Hunt et al., 2015)

Test Function	Acronym	Indicators	Unit	Definition
Coagulation activation and clot polymerization parameters	CT	Clotting Time	Sec	Required time until a clot firmness amplitude reaches to 2 mm from the beginning of the test.
	CFT	Clot Formation Time	Sec	Required time that clot firmness amplitude reaches from 2mm amplitude to 20 mm amplitude
	α-angle	Alpha Angle	degree (°)	This parameter displays between 30 to 40 seconds after the CT by evaluating the angle between the middle axis and the line which tangent to the curved surface at the 2mm amplitude point.
Clot firmness parameters	A10	Amplitude at 10 min	mm	Amplitude of clot firmness 10 min after CT
	A20	Amplitude at 20 min	mm	Amplitude of clot firmness 20 min after CT
	MCF	Maximum Clot Firmness	mm	Maximum amplitude of clot firmness reached during the run time
Clot Lysis Parameters	IL30	lysis index at 30 minutes	%	Percentage of Remaining of a clot firmness at 30 minutes after CT detection.

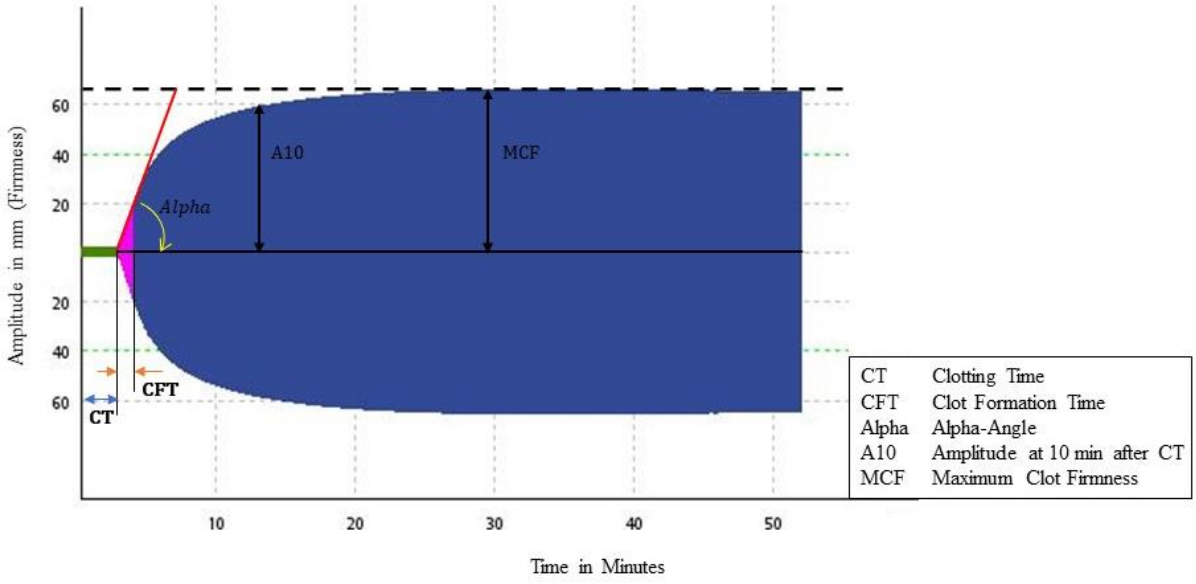

Figure 4. 1: Rotational thromboelastometry (ROTEM) Graphics. This graph shows clot initiation (CT), clot dynamics (alpha angle, clot formation time) and clot firmness (MCF) and Amplitude at 10 (A10) minutes after CT.

Thromboelastographic Analysis of the ROTEM®

The ROTEM test was performed using freshly collected blood sample (the ROTEM test has been performed maximum 2 hours after sample collection). A sample of citrated whole blood is collected in blue-capped tube (containing 3.2% Sodium Citrate) that is placed into space available in the device to keep the blood sample at 37°C. In advance, the ROTEM reagents which store at +4°C were brought to room temperature including star-tem® reagent (REF 503-10), ex-tem® reagent (REF 503-05), in-tem® reagent (REF 503-02), and fib-tem® reagent (REF 503-06) (Tem Innovations GMBH Munich, Germany) (Figure 4.2). Disposable cups and pins (Tem Innovations GMBH; Munich, Germany), cup holder, electronic pipette and pipette tips got prepared and used during ROTEM performance. According to the manufacturer's instructions, all the processes are carried on at 37°C. Each test has been performed in one measurement channel and three different tests were run for each blood sample. In all tests, first a new disposable pin and cup were positioned on the sensor pin of the measurement channel and a cup was placed into the pre-warmed cup holder. Future, the automated pipette is programmed to take a specific amount of the related reagents and added to the disposable cup. Then 300 µl of

citrated whole blood was added to reagents and pipetted up and down and immediately slid into the ROTEM measurement channel. ROTEM measurements were recorded at least for 50 minutes and a maximum within 90 minutes after the blood draw.

Table 4. 2: Rotational Thromboelastometry (ROTEM) Tests (Korpallova et al., 2018)

Test	Activator/Inhibitor	Information Provided	Liquid Reagents	Reagents
EXTEM	Tissue factor activation	Fast assessment of clot formation, fibrin polymerization, and fibrinolysis via the extrinsic pathway	Startem and Ex-tem	$CaCl_2$ + recombinant tissue factor + polybrene
INTEM	Contact activation	Fast assessment of clot formation, fibrin polymerization, and fibrinolysis via the intrinsic pathway	Startem and In-them	$CaCl_2$ + ellagic acid
FIBTEM	Tissue factor activation + platelet inhibition	ROTEM analysis without platelets: qualitative assessment of fibrinogen status	Ex-tem and Fibtem	$CaCl_2$ + recombinant tissue factor + cytochalasin D + polybrene

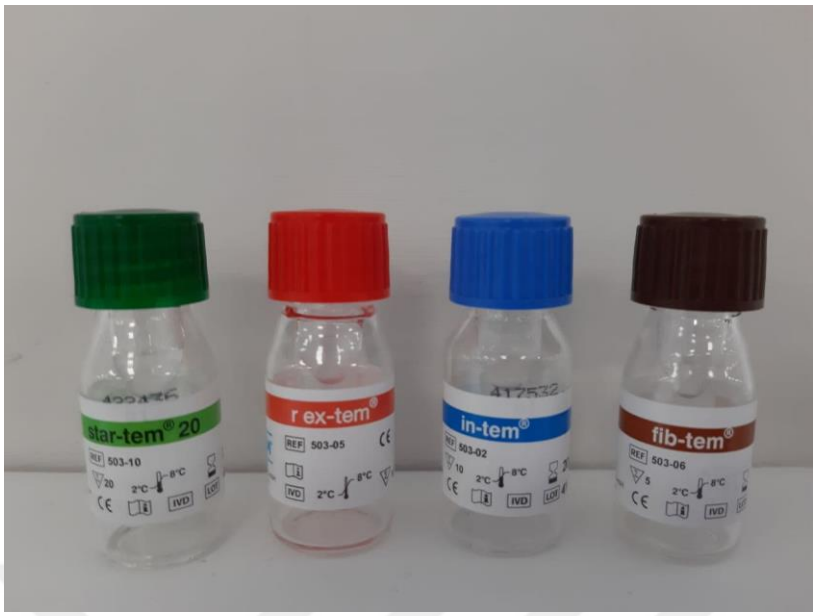

Figure 4. 2: Utilized reagents in ROTEM study. Reagent's star-tem®, ex-tem®, in-tem® and, fib-tem® for ROTEM® analyzer tests.

4.4.2 Principle of Flow Cytometry

Flow cytometry is a broadly used technique that was established a long time ago. This technique enables scientists to investigate the fluorescently labeled population of cells at a single-cell level. Although flow cytometry is a common and reliable technique in laboratories, it is a complex and time-consuming procedure which acquires expertise. In this study flow cytometry technique was employed to evaluate the platelets based on the percentage of expression of the desirable marker and the antibody binding capacity (ABC). Suspension of targeted cells, in our study platelets, are labeled with fluorescence and injected into the flow cytometry device. This fluid is passed through a focused laser beam on single cell basis and provide invaluable data about the physical and chemical characteristics of a population of cells.

In this study flow cytometry was performed by Accuri® C6 flow cytometer (Becton Dickinson, Franklin Lakes, NJ, USA) to quantify the expression of platelet surface receptors and ABC level. Regarding the analysis of expressed receptors, antibodies are chosen to specifically bind to the proteins of interest on the platelet surface (Krueger, Barnard, Frelinger, Furman, & Michelson, 2002). The antibodies used in this study including PE-labeled anti-human CD41a (Cat. No. 555467), PE-labeled anti-human

CD42b (Cat. No. 555473), PE-labeled anti-human CD61 (Cat. No. 555754) and PE-labeled anti-human CD62P (Cat. No. 550561) were purchased from BD Pharmingen (NJ, USA) and anti-PAC-1 antibody (Cat. No. sc-32776) was purchased from Santa Cruz Biotech (CA, USA). Furthermore, the platelet isolation a technique has been optimized for FITC-labeled anti-human CD45 (Cat. No. 555482) from BD Pharmingen to show that isolation technique has successfully isolated high percentage of platelets.

Flow cytometry sample preparation

To study the surface expression of platelet markers and ABC via flow cytometry, platelet isolated from citrated whole blood was based on the procedure proposed by Krueger et al (Krueger et al., 2002). Citrated whole blood samples were collected in the purple-capped tube (containing K3 EDTA anticoagulant). To create platelet-rich plasma (PRP) blood sample was withdrawn to sterile polypropylene 15 ml centrifuge tubes (Sarstedt, Numbrecht, Germany) and centrifuged at 150xg for 15 min at room temperature (± 22 °C) using Allegra X-12R centrifuge (Beckman Coulter, Fullerton, California, USA) without any break. The top 2/3 of platelet reach plasma (PRP) was collected to a new polypropylene tube to minimize white blood cell contamination. In this step, apyrase (Cat. No. A6237, Sigma-Aldrich) was added to PRP at a final concentration of 0.02 U/ml and centrifuged at 200xg for 10 min at room temperature in Allegra X-12R centrifuge without any break. For a second time, the top 2/3 of PRP was collected to an Eppendorf tube and centrifuge at 1500xg for 10 minutes at room temperature in Microfuge 20R Centrifuge (Beckman Coulter) without any break. The PPP (supernatant) discarded and 1 ml of pre-warmed (at 37 °C) citrate wash buffer was added to the platelet pellet. Citrate wash buffer used to eliminate the extra cells than platelets. The materials including 11 mM glucose (Cat. No. G8270), 4.3 mM NaH2PO4 (Cat. No. S8282), 4.8 mM sodium citrate tribasic dihydrate (Cat. No. C7254), 2.4 mM citric acid monohydrate (Cat. No. C7129) and 0.35% Bovine serum albumin (BSA) (Cat. No. A4919) were purchased from Sigma–Aldrich (St. Louis, MO). In addition, 128 mM sodium chloride (Cat. No. 969.036) from ISOLAB Laborgeräte GmbH (Eschau, DE) and 7.5 mM sodium phosphate dibasic (Cat. No. 600-085-LG) from WISENT Inc (Saint-Jean-Baptiste, QC) were purchased. These materials were added to 100ml deionized, distilled water and mix till it gets homogeneous. The PH was adjusted to 6.5 with 0.1 M NaOH and store at -20 °C (Krueger et al., 2002). In this step, apyrase was added in a final concentration of 0.02 U/ml and the pellet was

resuspended slowly (platelet pellet may not be visible to naked eye) and centrifuged at 1200xg for 10 minutes at room temperature in Microfuge 20R centrifuge without any break. Supernatant was discarded and 1 ml of pre-warmed (at 37 °C) Citrate Wash Buffer was added for the second time. Apyrase was added in a final concentration of 0.02 U/ml and the pellet was resuspended slowly and centrifuged at 1500xg for 10 minutes at room temperature in Microfuge 20R centrifuge without any break. Isolation protocol has been proceeded to discard the supernatant and the platelet pellet was suspended in DPBS (1x) (PAN Biotech, Aidenbach, Germany) and antibodies were added to separated tubes according to the dilution range provided by antibody manuals (Table 4.3). 50 ul of platelet sample was divided into the flow cytometry centrifuge tubes (Sarstedt, Numbrecht, Germany) and one control tube was prepared without antibody. Tubes were incubated for 20 minutes at dark in room temperature. Further, 2 ml of DPBS (1x) was added to each tube and centrifuged at 3600xg for 4 minutes in Allegra X-12R centrifuge. The supernatant was discarded and 300 ul DPBS (1x) was added to each tube. Flow cytometry data analyses were performed using FlowJo v10.4.1, and data were exported to GraphPad Prism version 8.00 (GraphPad Software, La Jolla, CA, USA) for further analysis.

Table 4. 3: Details of monoclonal antibodies

Antibody	Antibody amount per test	Clone / Fluorophore	Isotype
PE-labeled anti-human CD41a	20 µl	HIP8	Mouse IgG1, κ
PE-labeled anti-human CD42b	20 µl	Hip1	Mouse IgG1, κ
PE-labeled anti-human CD61	20 µl	VI-PL2	Mouse IgG1, κ
PE-labeled anti-human CD62	20 µl	AC1.2	Mouse (BALB/c) IgG1, κ
FITC-labeled anti-human CD45	20 µl	HI30	Mouse IgG1, κ
anti-PAC-1 antibody	20 µl *	None	Mouse IgG2a, κ

* This amount optimized in the laboratory by researchers.

Flow Cytometry Gating Strategy for Platelets

In this study, platelets are isolated from PRP thus flow cytometric data display only one population in pseudo color graph which belongs to platelets. Forward scatter and side scatter laser were both displayed logarithmically during platelet analysis (Figures 4.3 A). The control gating of the flow cytometry analysis mentioned in (Figures 4.3 B) Gating strategies for sorting are described in Figures (Figure 4.4).

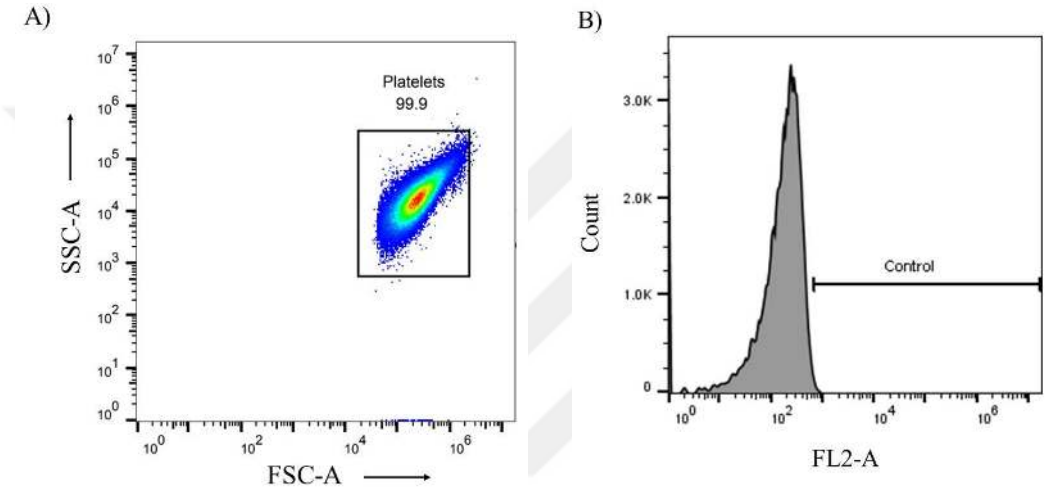

Figure 4. 3: Gating strategy was used in flow cytometry analysis. In this study platelet population was the target of study. A) forward light scatter (FSC) / side light scatter (SSC) to define the populations and to exclude; B) cell count / FL2-A to define the control gating in the flow cytometry.

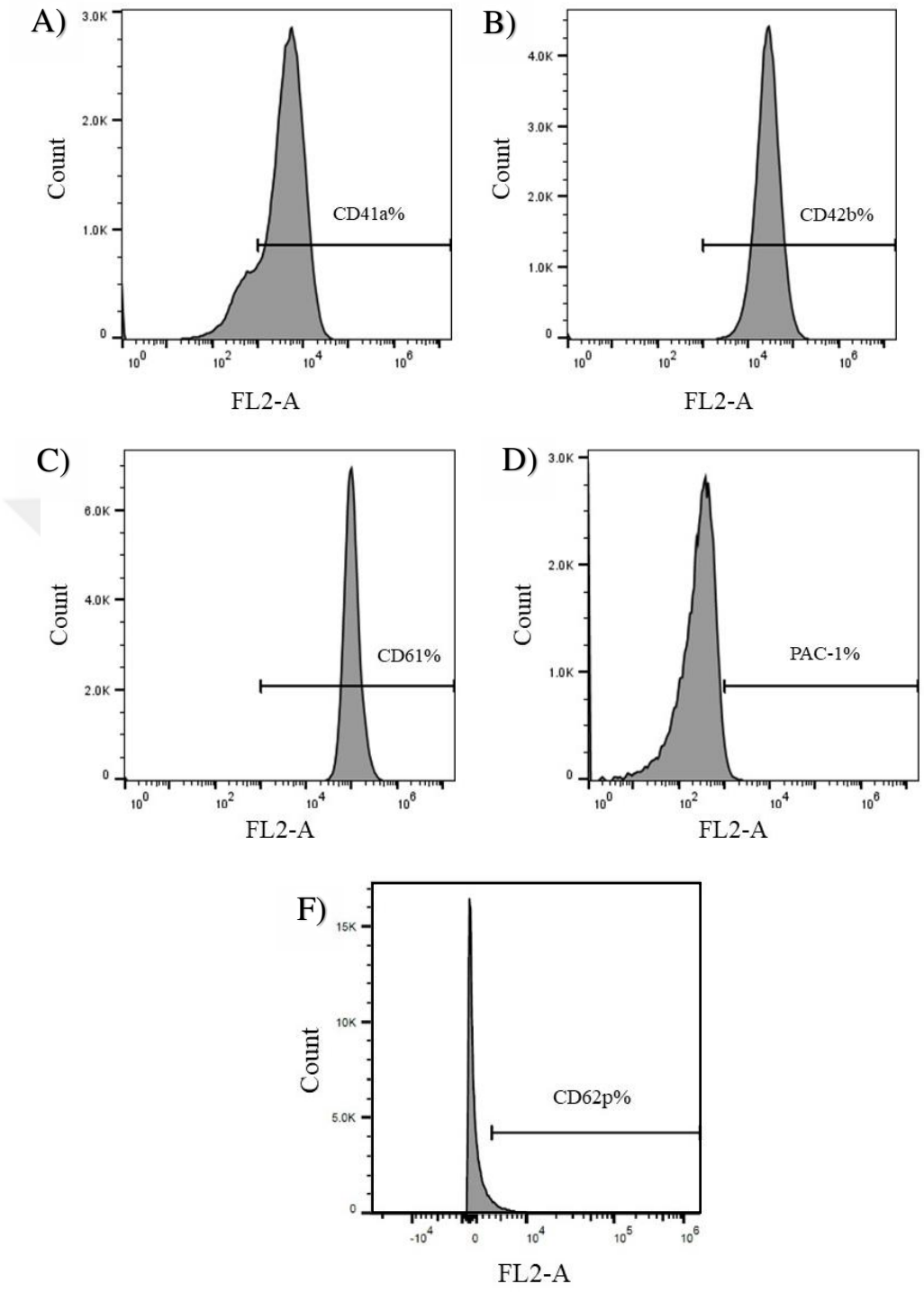

Figure 4. 4 Expression of platelet markers in inactivated platelet. These graph shows the percentage of expressed markers in inactivated platelets. Flow cytometric analysis of platelet function and activation was performed as described in the "Materials and Methods" section. Platelets stained for A) CD41a, B) CD42b, C) CD61, D) PAC-1, F) CD62 to analyse the properties of platelet population.

4.4.3 Hematological Parameters

Blood samples were collected in K_2 EDTA tubes (Purple capped) were analysed directly using the COULTER® AcT diff 2™ (Beckman Coulter, Inc., Brea, CA). The analyser was calibrated daily using the manufacturer's controls and blood samples were analysed according to the operator's guide protocol. K_2 EDTA tubes were put on a rocker for continuous mixing and hematological parameters were performed directly in the analyser at room temperature within 60 minutes of sample collection. Reported parameters in this study including: PLT, MPV, PDW, NLR and LMR.

4.5 Statistical Analyses

The statistical analyses of this study were performed using GraphPad Prism version 8.00 (GraphPad Software, La Jolla, CA, USA). The Mann-Whitney nonparametric test was used due to the small number of study cases to compare numeric variables between the study groups. The output of the analysis is reported as mean ± standard deviation. Statistically significant value was considered at a 5% level (P-value <0.05).

Chapter 5:
RESULTS

5.1 Patient Characteristics

The details of the patient population that participated in this study were reported in Table 5.1. A total of 18 PEX patients including 10 male and 8 female with the mean age of 69.7 ± 4.4 were included in this study. The second group was 19 participants including 11 male and 8 female with the mean age of 63.6 ± 6.5 that considered as control group. Statistical analysis showed a significant difference in the age of participants of two study groups ($P < 0.05$). The participants age distribution demonstrated in (Figure 5.1). Statistical analysis of demographic data showed no statistical difference in the mean value of the platelet number and visual acuity in two study groups. While the mean value of intraocular pressure between groups has been reported statistically different ($P < 0.05$). The demographic information of participants is demonstrated (Table 5.1).

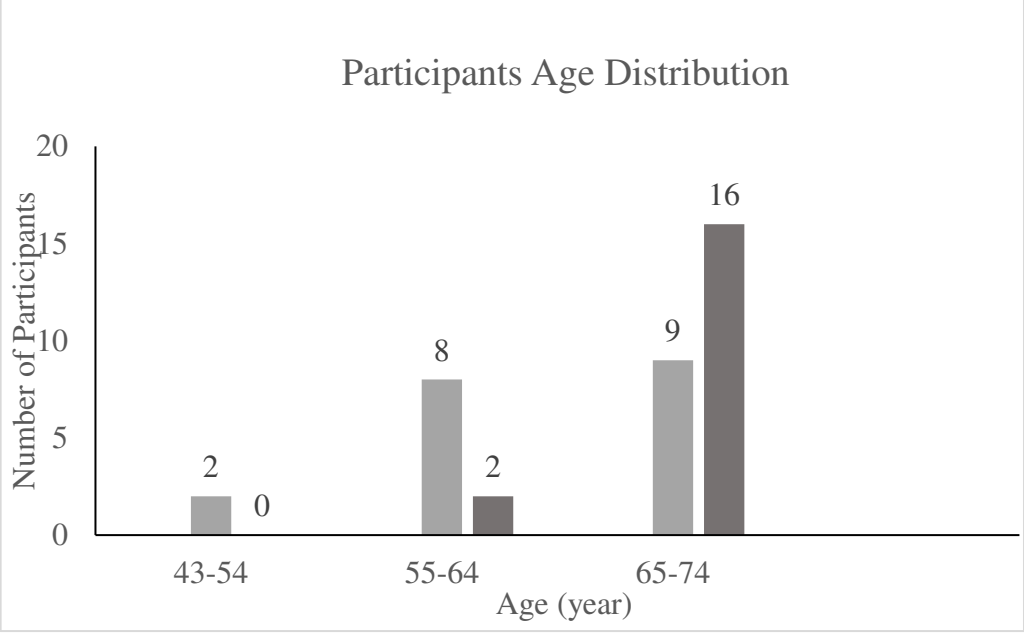

Figure 5. 1: Participant's age distribution. Patients are divided to tree different groups according to their age. This graph considerably shows the incident of PEX disease is increasing by age.

Table 5. 1: The Demographics of the Groups

Characteristic	Control Group (n=19)	PEX Group (n=18)
SEX (Male)	11 (58%)	10 (56%)
Age (Year)*	63.6 ± 6.5	69.7 ± 4.4
Platelet Number (x103/µl) *	249.6 ± 53.4	267.9 ± 80.8
Visual Acuity (log MAR) *	0.030 ± 0.046	0.032 ± 0.047
Intraocular Pressure (mmHg) *	14.8 ± 1.9	16.2 ± 2.1
Hypertension	3 (16%)	4 (22%)
Glaucoma	1 (5%)	1 (6%)
Macular Degeneration	1 (5%)	1 (6%)
Fuchs uveitis syndrome	1 (5%)	-
Coronary artery disease	-	1 (6%)
Benign prostate hyperplasia	1 (5%)	2 (11%)
Brown syndrome	1 (5%)	1 (6%)
Asthma	-	2 (11%)
Hepatitis C	-	1 (6%)
Epilepsy	-	1 (6%)
Hypothyroidism	1 (5%)	-
Laryngeal carcinoma	1 (5%)	-
Ovarian carcinoma	1 (5%)	

*These data are presented as mean (SD)

5.2. ROTEM Data Analysis

The result of the ROTEM analysis of extrinsic-activated rotational thromboelastometry (EXTEM), intrinsic-activated rotational thromboelastometry (INTEM) and fibrin-based thromboelastometry (FIBTEM) were compared between two study groups as shown in (Table 5.2).

Analysis of FIBTEM test showed the significant difference in FIBTEM CT (60.3±5.0 vs. 56.9 ± 5.9, $P < 0.05$) and FIBTEM alpha-angle (70.0± 5.0 vs. 73.3 ± 4.1, $P < 0.05$) when compare the control group with PEX patients. The data have been statistically analyzed and reported as box plot graphs (Figure 5.2). No significant differences were observed in the other ROTEM parameters. Furthermore, we have calculated the

maximum clot elasticity (MCE) for EXTEM, INTEM, and FIBTEM using the MCF×100/100−MCF formula and the data reported in the Table 5.3. However, the statistical analysis did not confirm any significant differences between groups, the FIBTEM MCE value showed 36.5% increase in PEX patients. The MCE values are used to calculate the platelet component in studies to estimate the platelet function and platelet/fibrinogen contribution to the whole blood clot by subtracting the FIBTEM MCE value from EXTEM MCE (ΔMCE = EXTEM MCE - FIBTEM MCE)(Solomon, Ranucci, Hochleitner, Schochl, & Schlimp, 2015; Ziegler et al., 2015). In the assessment of ΔMCE of the control group with the PEX group (168.16 ± 30.1 vs. 176.16 ± 32.1, P = 0.7018) no statistical difference has been found (Table 5.3).

Table 5. 2: ROTEM test results of patients and control

Parameters	Control Group (n=19)	PEX Group (n=18)	Manufacturer's Reference Ranges	P value
		EXTEM		
CT (s)	62.7±9.2	59 ± 7.9	38-79	0.1847
CFT (s)	75.0±17.5	70.6 ± 14.4	34-159	0.3734
Alpha-angle (°)	74.9±3.3	75.8 ± 3.1	63-83	0.3224
A10 (mm)	58.1±5.2	60.5 ± 4.5	43-65	0.1535
A20 (mm)	64.2±4.6	65.8 ± 4.0	50-71	0.2552
MCF (mm)	65.3±4.4	66.9 ± 4.1	50-72	0.2269
LI30 %	99.7±0.7	100 ± 0.0	94-100	0.2297
		INTEM		
CT (s)	184.0±38.1	173.9 ± 35.0	100-240	0.181
CFT (s)	77.7±16.7	72.9 ± 16.0	30-110	0.1654
Alpha-angle (°)	74.4±3.3	75.4 ± 3.1	70-83	0.1605
A10 (mm)	55.8±4.5	58.3 ± 4.4	44-66	0.0709
A20 (mm)	61.7±4.3	63.2 ± 4.5	50-71	0.1921
MCF (mm)	62.6±4.4	63.9 ± 4.4	50-72	0.2093
LI30 %	99.8±0.4	99.8 ± 0.4	94-100	>0.9999
		FIBTEM		
A10 (mm)	18.3±3.9	20.5 ± 6.6	7-23	0.2139
A20 (mm)	19.1±4.0	22.1 ± 7.5	8-24	0.1499
MCF (mm)	19.1±4.0	22.7 ± 8.0	9-25	0.1296
LI30 %	99.9±0.3	99.9 ± 0.3	n.d	>0.9999

Data are presented as mean (SD). CT: clotting time. CFT: clot formation time. A10 and A20: amplitude (firmness) at 10 and 20 minutes. MCF: maximum clot firmness. LI30: lysis index at 30 minutes. Manufactures reference range is based on a study by Calatzis et al. (Andreas Calatzis et al., 2013)

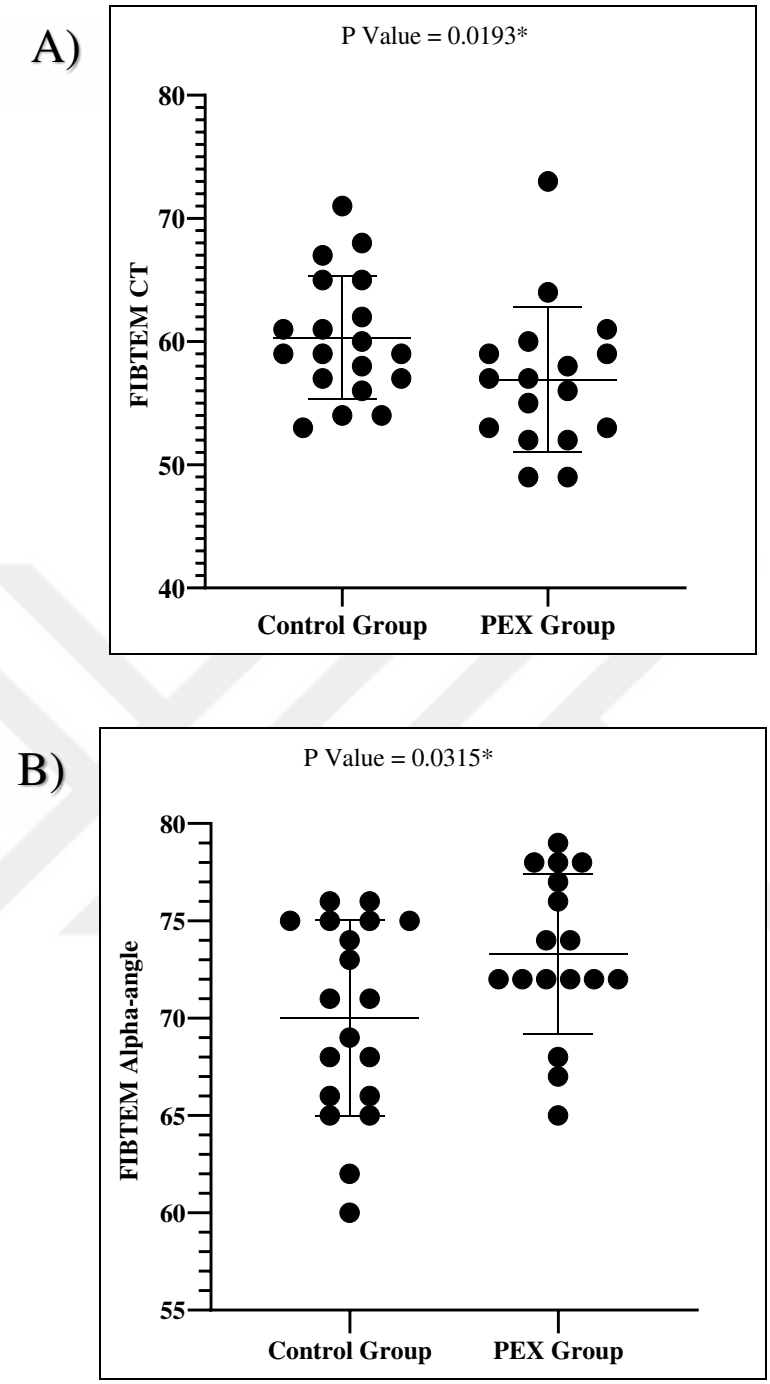

Figure 5. 2 Box dot plot analysis of FIBTEM CT and Alpha-angle. A) The Control group indicated significantly prolonged FIBTEM CT in compare with Control group (60.3 vs. 56.9; p = 0.0193); B) The control group indicated significantly shorter Alpha-angle than the PEX group (70.0 vs 73.3; P =0.0315). A one-tailed Mann-Whitney test was performed to analyze the data. Statistically Significant considered P-value <0.05.

Table 5. 3: Maximal clot elasticity (MCE) calculated for tests in two study groups

Test Type	Control	PEX Patients	P value
EXTEM MCE	192.1±35.3	207.2 ± 40.7	0.3829
INTEM MCE	170.8±32.5	181.1 ± 36.1	0.4185
FIBTEM MCE	22.7 ± 8.0	31.0 ±17.6	0.2592

All parameters are presented as mean (SD). The P value considered statistically significant in this study was <0.05.

5.2 Flow Cytometric Analysis of Platelet Markers

In this study, we have observed a significantly high percentage of expression of CD62p (P< 0.0001) and PAC-1 (P < 0.05) in PEX patients (Figure 5.3). Surface expression of CD41a, CD42b and CD61 has been mentioned in (Figure 5.4).

Analysis of CD62p antibody binding capacity (ABC) has been significantly different in PEX patients when comparing with control group (P < 0.05) (Figure 5.5). Statistical analysis of ABC of other antibodies did not show any significant difference. The surface expression of CD41a, CD42b and CD61 has been mentioned in Figure 5.4.

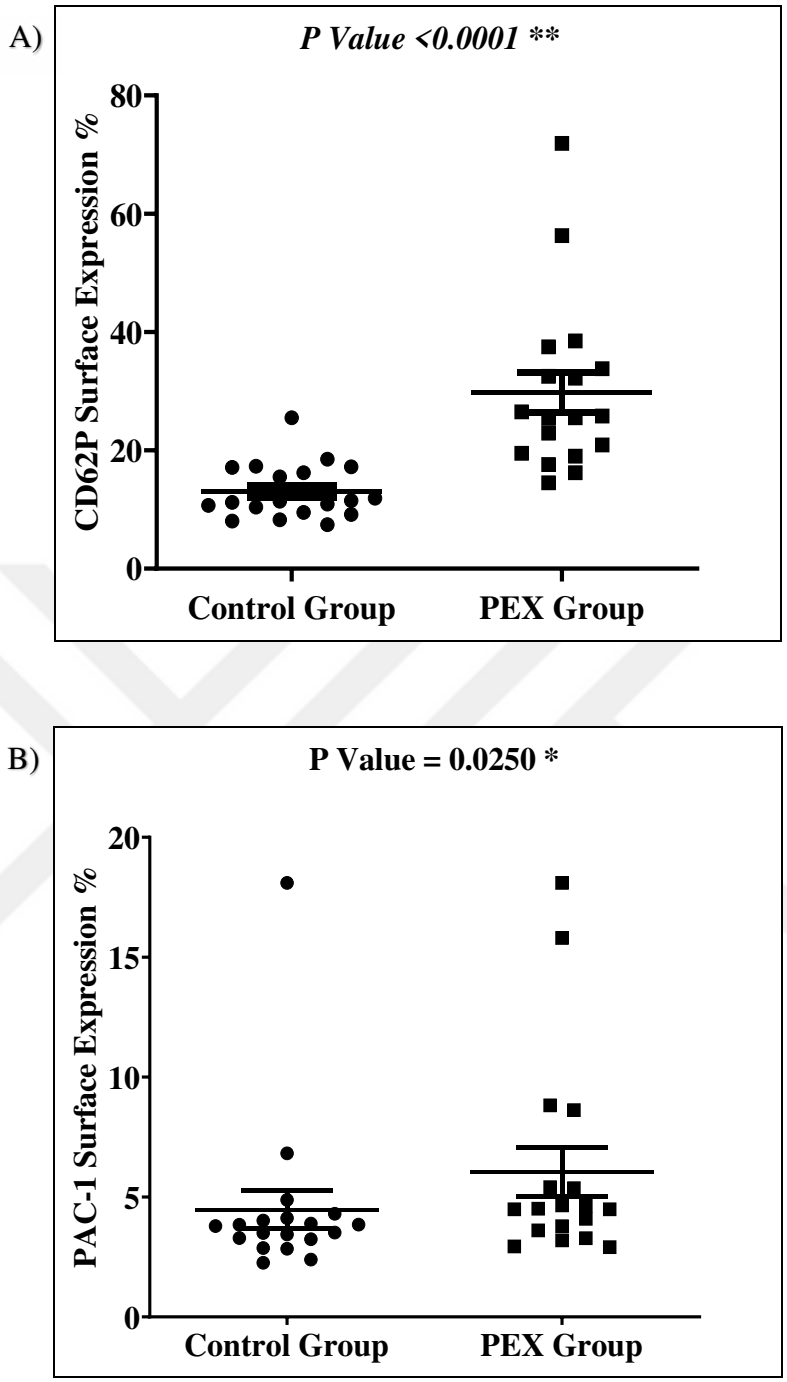

Figure 5. 3: Box dot plot analysis of CD62P and PAC-1. Illustrate the expression of platelet surface markers in percentage. Platelet Activation marker was evaluated with A) CD62p, the expression of this marker increased significantly in PEX group (P<0001) and B) PAC-1, the expression of this marker also increased significantly in the PEX group (P<0.025). These graphs interpret as a high level of platelet activation in the PEX group. A one-tailed Mann-Whitney test was performed to analyze the data. Statistically Significant considered P-value <0.05.

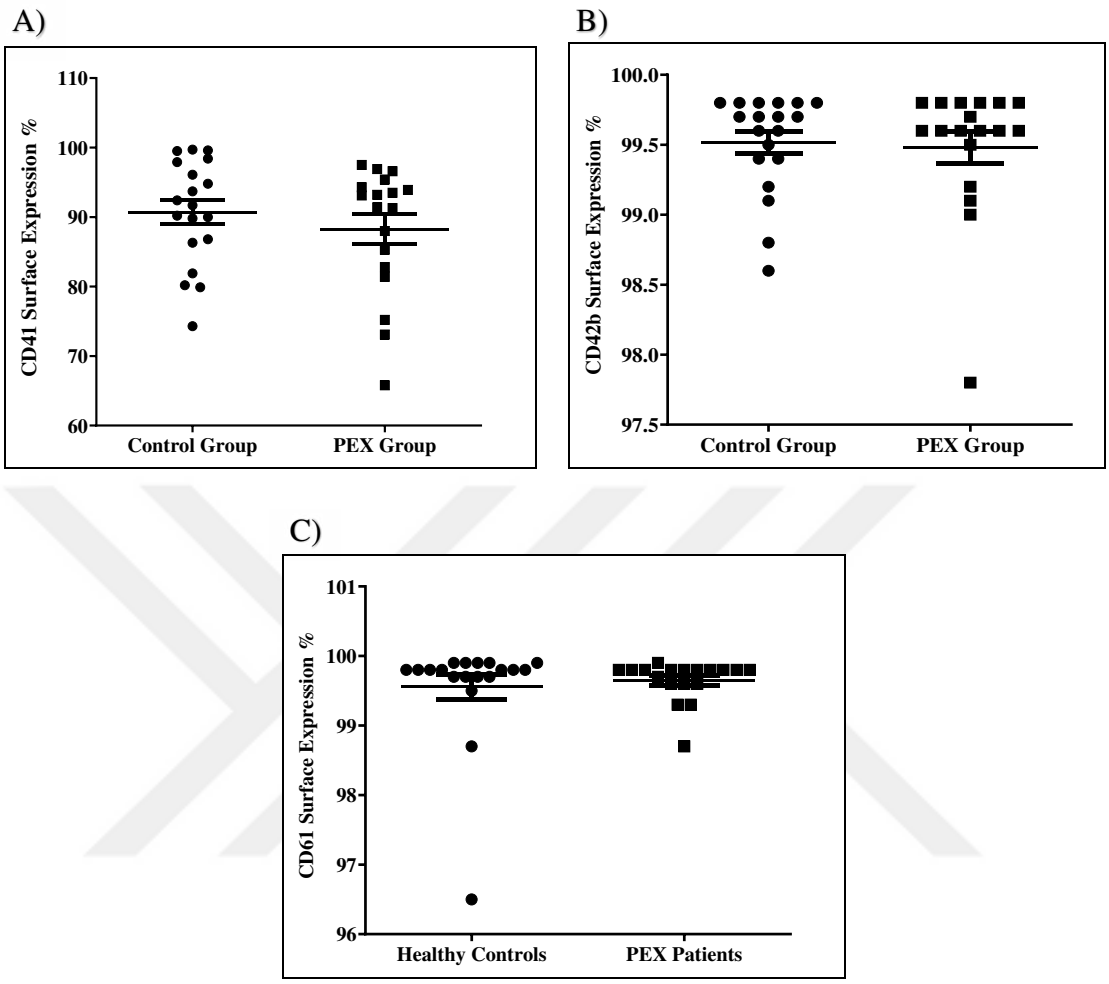

Figure 5. 4: Box dot plot analysis of platelet surface expression markers. Two groups have been studied for resting to evaluate the platelet function A) CD41a, B) CD42b and C) CD61. There was no significant difference in the expression level of studied markers. A one-tailed Mann-Whitney test was performed to analyze the data. Statistically Significant considered P <0.05.

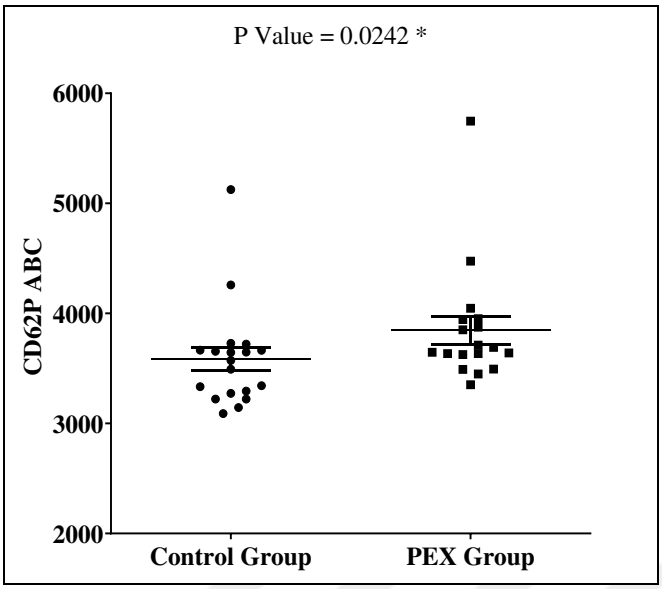

Figure 5. 5: Box dot plot analysis of Antibody Binding Capacity (ABC). ABC value showed a significant difference in CD62p marker (P=0.0242). A one-tailed Mann-Whitney test was performed to analyze the data. Statistically Significant considered P-value <0.05.

5.3 Complete Blood Count (CBC) Analysis

In this study CBC test was obtained for each participant to evaluate blood parameters with a focus on platelet properties and infection signs. For each set of experiments, the results were calculated as mean ± standard deviation. Several parameters including: PLT, PCT, MPV, PDW, NEUT, LYMPH and, MONO parameters were checked within sixty minutes of blood collection. Meanwhile, NLR and LMR have been computed from the CBC data. No statistically significant difference was found in the parameters of CBC test analysis. The result is reported with mean ± standard deviation values for each set of experiments in Table 5.4.

Table 5. 4: Complete blood count parameters of two study groups

Parameters	Control Group	PEX Group	P value
PLT (10^3/μL)	249.58 ± 53.40	267.89 ± 80.80	0.3234
PCT (%)	0.25 ± 0.05	0.26 ± 0.06	0.3501
MPV (fL)	10.37 ± 0.97	10.04 ± 0.96	0.1432
PDW (fL)	12.15 ± 2.39	11.21 ± 1.98	0.124
NLR	2.17 ± 0.76	2.46 ± 1.04	0.1975
LMR	4.07 ± 1.47	4.57 ± 2.61	0.3678

PLT, platelet count; PCT, plateletcrit; MPV, mean platelet volume; PDW, platelet distribution width; NLR, neutrophil/lymphocyte ratio; LMR, Lymphocyte/Monocyte count ratio. A one-tailed Mann-Whitney test was performed to calculate the P value. Statistically Significant considered P-value <0.05.

Chapter 6:
DISCUSSION

The main goal of this research was to provide critical information to indicate diagnostic and prognostic markers of hypercoagulability in PEX patients through investigation of the blood at the cellular level. Therefore, blood sample analysis has been prioritized to assess the platelet function and property via platelet markers analysis and the viscoelastic properties of whole blood. In addition, the CBC test was performed to provide general insight into the patients' health and blood quality. It was hypothesized that PEX syndrome associates with hypercoagulable disorders, due to the endothelial dysfunction and alterations in platelet function. This study was designed to examine the patients' blood samples via ROTEM®, flow cytometry, and CBC test.

This study is important since it aimed to shed light on the pathogenesis and diagnostic of PEX syndrome. The blood sample of PEX syndrome patients was studied with a focus on clot formation kinetics platelet activation, platelet function and CBC analysis. To the best of my knowledge, this is the first time that platelet characteristics accompanying ROTEM analysis in PEX patients. In addition, it is the first time that fibrinogen-related coagulopathy is studied via FIBTEM test in PEX patients.

The FIBTEM test is used as an efficient method to prevent thrombocytes' contribution in clot formation and fibrin polymerization with the aim of assessing the fibrin function in clot firmness (Biolik et al., 2017). Our ROTEM result in the FIBTEM test showed a decrease in the FIBTEM CT and an increase in the FIBTEM Alpha-angle parameter in PEX patients when comparing with the control group. The FIBTEM CT result demonstrates the higher contribution of fibrin in the initiation of clotting and clot polymerization via fibrin/fibrinogen involvement in PEX patients. FIBTEM CT decrease shows that the necessary time to reach 2 mm clot amplitude got shorten in PEX patients. This result point to the fact that abnormal involvement of fibrin in coagulation increases the risk of thrombotic disorders (Undas, 2014). In addition, rise in the FIBTEM alpha-angle parameter determine as the speed of clot formation indicates the thromboembolic complications in patients. This result is in line with the earlier findings on the association of fibrin dysfunction with thrombosis disorder in PEX patients (Goren Sahin et al., 2016).

The FIBTEM CT and the FIBTEM alpha-angle parameters are not typically reported thus, there is no normal range provided by the manufacturer for these two parameters. The FIBTEM A10 and FIBTEM A20 parameters display a higher level when in PEX group. The higher amplitudes in PEX group interprets as increased hypercoagulability. Meanwhile, the FIBTEM MCF result interpret as higher overall clot firmness on the basis of fibrin contribution in PEX group. In this study, fibrinogen level test was not performed. It is suggested to perform the fibrinogen level test simultaneously with FIBTEM test since this test had inadequate accuracy in predicting the fibrinogen level (Khunakanan, Akaraborworn, Sangthong, & Thongkhao, 2019).

All the studied parameters of the EXTEM analysis were focused on extrinsic pathway analysis, fall into the normal range provided by the manufacturer. No statistically significant difference was detected from the EXTEM data analysis. This study has not confirmed previous research by Sahin et al. in the EXTEM CT parameter (Goren Sahin et al., 2016). However, we could show a trend toward significance ($P = 0.1847$) which suggests the tendency of faster clot formation in the PEX patients. This result support the lower level of the EXTEM CFT and higher level of the EXTEM MCF in the PEX patients. The EXTEM IL30 shows a higher percentage of remaining clot firmness in the PEX patients.

Analysis of INTEM data which is focused on intrinsic pathway analysis lies in the normal range provided by the manufacturer. None of the reported INTEM parameters were statistically significant. However, INTEM A10 ($P = 0.0709$) shows a clear tendency to a significant difference. This shows the amplitude of clot firmness at the 10 minutes after CT in PEX patients is higher in comparison with the control group. A review article by Akay indicates that INTEM A10 was the best parameter to predict the thromboembolic complications from a clinical point of view (Akay, 2018). In our study we could show that the INTEM A10 result has been supported with a decrease in the INTEM CT and the INTEM CFT values when comparing the control group with the PEX patients' group. These results indicate the excessive blood clotting risk in the PEX group. Furthermore, an increase in the INTEM MCF suggest the elevated maximum clot firmness in patients' group. Overall, the mean of ROTEM parameters in this study is in line with a study by Lee et al. which include a higher number of participants (J. Lee et al., 2020).

The same trends have been identified in the ROTEM parameters of control group versus patient group. This correlation supports the hypothesis that the PEX patients have

tendency to hypercoagulability. There is still considerable uncertainty which can be solved by increasing the number of participants. Since this study has great focus on platelet function and activation, the MCE has been calculated to assess the contribution of platelets activity to clot elasticity by excluding the impact of fibrinogen in EXTEM and FIBTEM tests. In a study by Ziegler et al., the MCE was evaluated to show the positive effect of platelet concentrates transfusion in coagulation patterns (Ziegler et al., 2015). In the other study, the MCE value has been reported in a study by Lauridsen et al. to correlate the increase in clot strength in spontaneous subarachnoid hemorrhage (SAH) by incising in the MCE value (Lauridsen et al., 2019). This finding could not pass the statistical tests. Our finding is in line with Lauridsen et al. since we could not find any significant differences between MCE nor ΔMCE values between two groups. Still a slight increase in MCE and ΔMCE value has been reported in the PEX group.

Platelet function was examined using flow cytometry to detect the expression and ABC of CD41a (GP IIb), CD61(GPIIIa), CD42b (GPIb), CD62p (P-selectin), and PAC-1 (activated GP IIb/IIIa). Association of hypercoagulability and platelet activation has been assessed by flow cytometry in patients with early primary biliary cholangitis and cardiovascular events associated with platelet activation in type 2 diabetes patients (Pretorius et al., 2018; Vlachogiannakos et al., 2021).

We found a high percentage of surface expression of CD62p and PAC-1 in PEX patients when compared to the control group. Platelet has been isolated immediately and without fixation to avoid platelet activation during isolation thus, high level of CD62p and PAC-1 expression are associated with significant platelet activation and hypercoagulability in the PEX patients. These values have been found to be significantly involved in the physiological processes of thrombosis and hemostasis disorders (Lu & Malinauskas, 2011; Schmitz et al., 1998). In a study by Vlachogiannakos et al., elevation of CD62p expression was in asscociation with ROTEM alpha-angle and MCF parameters as evidence of hypercoagulability in the patient group (Vlachogiannakos et al., 2021). Meanwhile, Cambien & Wagner could show the importance of P-selectin expression in fibrin deposit regulation (Cambien & Wagner, 2004). Our result in elevation of PAC-1 (activated GPIIb/llla receptor) is consistent with a study by Tantry et al (Tantry et al., 2010). They revealed a connection between the severity of coronary artery disease with hypercoagulation and platelet activation.

Furthermore, we found a significant increase in the ABC level of CD62p in the PEX group. ABC was used as the quantitative evaluation of antigen, and it shows increase in the CD62p density and proves the changes in CD62p expression in the PEX group. Animal studies shows that CD62p, in the other word P-selectin has a critical role in the pathology of inflammatory disorders. In a study by Birner et al., chronic arthritis in the rat, inhibition of the P-selectin lead to prevention of the neutrophil accumulation (Birner, Issekutz, & Issekutz, 1999). The result of this study significantly shows the potential of hypercoagulation due to platelet activation. It is suggested to consider performing the additional tests including ELISA test to provide supplementary data of P-selectin expression.

CBC test analysis attracted the great attention of researchers who studied PEX syndrome. In this study, a CBC test was evaluated to provide information on not only the general health of each participant but also to assess the platelet status examine the signs of systemic inflammation and their association with the PEX syndrome. In this study, no statistically significant difference between the means of the two study groups has been found. Unlike other research carried out in the analysis of CBC data, we did not find a significant correlation between these parameters and PEX disease.

In a study by Yazgan et al., the PCT and PDW were significantly elevated in PEX patients which was interpreted as high platelet activity in PEX patients (Yazgan et al., 2016). They also could show the higher level of PLT in the PEX patients. Our findings appear to be well substantiated with PCT and PLT values since we could show the increas in both parameters. Turkcu et al. revealed the noticeable increase in MPV parameters in PEX patients. Increase in MPV value is an indicator of amplification of platelet reactivity in correlation with prothrombotic and proinflammatory agents of platelets (Turkcu et al., 2014). Unexpectedly, our study could not show the increase in MPV value in PEX patients. In the other study by Ozgonul et al., the NLR value was evaluated to investigate the systemic inflammation in PEX patients (Ozgonul et al., 2016). They could show a significant increase in the NLR value in the PEX patients, and they believe NLR can predict the prognosis of inflammatory conditions in combination with other inflammatory parameters. In the current study, we could also show the increase in the NLR value in the PEX patients. The LMR value has been studied by Mirza et al. which demonstrated the decrease in the LMR value in the PEX group however, it could not pass the statistical test (Mirza et al., 2020). The LMR has been received research attention as an inflammatory

biomarker. Since the decrease in LMR values is significantly associated with survival rate in lung cancer patients, the author suggests that maybe it is a better predictive indicator in patients with malignancy (Li et al., 2017). Our experiments confirm the previous results provided by Mirza et al. it is speculated that the weakness in either study is the small number size of participants.

As we know, changes in the platelet indicators specifically PDW and MPV are potential markers of thrombotic events (Vagdatli et al., 2010). Although it was expected to observe the increase in PDW and MPV in my study, our findings in PDW and MPV were not in line with the previous studies and our flow cytometry results. It is suggested that the unexpected result is due to the small study population or related to the other diseases that the control group may have and it impacted their platelets characteristics.

We are aware that the present study may have limitations that influenced the obtained results. The first is a limited number of participants and significant diffrence of age between the study groups. This study could not reach the targeted number of participants due to the limited duration of my master's program. In addition, the study population limitted to the elderlies. We faced difficulties to find suitable donors as most of the patients who were diagnosed with PEX syndrome were also diagnosed by diabetes and/or taken anti-coagulant medications. Moreover, limited time prevented me from performing supplementary techniques like ELISA, Western Blot, Proteomics to analyze the alteration of platelet properties and activation markers. The second limtation was the lack of information about the fibrinogen level of participants which interfered with the accurate analysis of the FIBTEM test in the ROTEM study.

In conclusion, The evidence from this study tends towards the idea that PEX syndrome increase the hypercoagulability in patients. This study has highlighted the enhancement of fibrin involvement in clot initiation and polymerization followed by thromboembolic disorder and platelet activation in the PEX patients. Besides, the parameters which evaluate the extrinsic and intrinsic pathways determine the trends toward hypercoagulability in this patient group. Further work needs to be carried out with larger sample size and evaluating the fibrinogen level to confirm our findings. It will allow to discovery of specific biomarker/s with a diagnostic and prognostic value to predict the PEX syndrome and secondary diseases including RVO in this study group.

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