Effects of anticoagulants on clotting with low platelets

AN *IN VITRO* MODEL TO EVALUATE THE EFFECTS OF ANTICOAGULANTS ON CLOT FORMATION IN THE PRESENCE OF LOW PLATELET COUNTS

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TITLE: An *In Vitro* Model to Evaluate the Effects of Anticoagulants on Clot Formation in the Presence of Low Platelet Counts

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Abstract

The management of thrombosis in the presence of thrombocytopenia is challenging because the inherent risk of bleeding associated with anticoagulant use may increase due to low platelet counts. Guidelines regarding anticoagulant use in this situation are based mainly on expert opinions and anecdotal data. We developed an *in-vitro* model to study the effect of anticoagulants on plasma clot formation in the presence of low platelet counts. We used thromboelastography (TEG) to measure global viscoelastic properties of clot formation and scanning electron microscopy (SEM) to observe and quantify changes in the fibrin clot structure. Experiments were conducted in plasma with varying platelet concentrations from $<10 – 150 \times 10^9$/L. Clotting was activated with tissue factor (TF) and calcium, in the presence of factor XIIa inhibitor, corn trypsin inhibitor. One of the following anticoagulants at therapeutic concentration was added to the mixture: unfractionated heparin (UFH), dalteparin, fondaparinux, rivaroxaban or dabigatran. We found clotting had different sensitivity to TF concentration depending on the anticoagulant present. Effects on TEG parameters varied at a fixed TF concentration with each anticoagulant. UFH had the greatest influence, delaying clotting significantly at low platelet counts. The factor-specific anticoagulants had the least impact on TEG parameters. SEM revealed that UFH had the greatest impact on clot structure. UFH caused significant increase in porosity and fibrin widths and had significantly less fibers when platelets decreased. In conclusion, this study may provide fundamental data to understand clot formation in the presence of anticoagulants at low platelet counts. At low platelets the anticoagulants can jeopardize clot formation, especially UFH. The mechanism of each anticoagulant may contribute to the variation in response to TF initiated clotting. AT-dependent anticoagulants compromised plasma clotting

more than the newer factor specific anticoagulants, possibly related to the multiple, non-specific inhibition of coagulation factors.

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Abbreviations

II: Prothrombin

IIa: Thrombin

α: Alpha, clot kinetics

ADP/ATP: Adenosine di/triphosphate

aPPT: activated partial thromboplastin time

AT: Antithrombin

AUC15: Area under the curve 15, clotting speed and strength

CTI: Corn trypsin inhibitor

DVT: Deep vein thrombosis

Fb(g): Fibrin(ogen)

GP: Glycoprotein

HIT: Heparin induced thrombocytopenia

HMWK: High molecular weight kininogen

K: Kallikrein

K(TEG): K value, clotting kinetics

LMWH: Low molecular weight heparin

MA: Maximum amplitude, clot strength

PAR 1/4: Protease-activated receptors 1 and 4

PE: Pulmonary Embolism

PPP: Platelet Poor Plasma

PRP: Platelet Rich Plasma

PSPC: Phosphatidylserine/phosphatidylcholine

PT/INR: Prothrombin time/ International normalized ratio

R: Reaction time, clot initiation

ROTEM: Rotational thromboelastometry

SEM: Scanning electron microscopy

TEG: Thromboelastography

TF: Tissue factor

TFPI: Tissue factor pathway inhibitor

TMA: Time to maximum amplitude, time to reach max strength

tPA: Tissue plasminogen activator

UFH: Unfractionated heparin

VTE: Venous thromboembolism

vWF: von Willibrand factor

WB: Whole blood

Declaration of Academic Achievement

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1. INTRODUCTION

1.1 Overview

Venous thromboembolism (VTE) is a leading cause of morbidity and mortality in many patients[1]. These patients usually require anticoagulant treatment to restrict further propagation of the thrombi[2]. Although anticoagulant use is associated with various risks, the most significant are bleeding complications[3]. Patients with certain types of cancer who are at higher risk for VTE may require anticoagulant treatment[3]. Moreover, these patients may also develop thrombocytopenia, which can also increase the risk of bleeding[3]. This scenario presents a dilemma for the managing clinician because the treatment of patients with thromboembolic disease and concomitant thrombocytopenia is a complicated balance. The guidelines for administering anticoagulants to these patients are based on expert opinions and anecdotal data. Therefore we developed an *in vitro* assay to evaluate the effect of anticoagulants on plasma clot formation in the presence of low platelet counts

1.2 Coagulation

Haemostasis is a physiological process that serves to maintain the integrity of the circulatory system[4]. Haemostasis requires a delicate balance between procoagulant activity and anticoagulant activity[5]. Any significant disruption to this system may lead to severe complications such as bleeding or thrombosis[5]. If there is injury to the vascular tissue, the procoagulant system is activated in order to prevent blood loss and eventually heal the injury. Several models have attempted to depict and explain the process of coagulation[6]. These models have gradually evolved over time to become more detailed and complex as our understanding of the coagulation system progressed. In the 1960's the cascade model depicted a series of steps

involving activation of enzymes from their cofactors, beginning with two separate pathways that eventually converged into a common pathway of activation (Fig. 1). The initial pathways were named extrinsic (tissue factor (TF) based) and intrinsic (contact) pathways[7]. The extrinsic pathway primarily involved subendothelial exposure of TF following vessel wall injury, and interaction of TF with factor VIIa to initiate coagulation[7;8]. The intrinsic pathway was initiated by contact activation of factor XII through negatively charged surfaces. Both pathways led to the activation of factor X to Xa, so that the Xa can then become incorporated in the prothrombinase complex (Xa + Va + anionic phospholipids + prothrombin (II) + calcium ions) to activate II to thrombin (IIa)[9;10]. IIa can then cleave fibrinogen into fibrin[7]. Fibrin is then polymerized into insoluble fibrin clots via cross-linking mediated by factor XIIIa[7].

While the cascade model provides useful information to further understand coagulation in a plasma-based *in vitro* environment, the model does not fully explain coagulation as it would occur *in vivo*[9]. For example, factor XII of the intrinsic pathway may not be clinically significant and may not be required to initiate coagulation *in vivo*[11]. Deficiencies in factor XII or XIIa are not associated with a tendency for bleeding in mice or humans[11;12]. However, patients with deficiencies in downstream factors XI and IX or extrinsic pathway factor VII, are all associated with bleeding complications[13-15]. A growing understanding of the involvement of various cellular interactions has led to a cell-based model of coagulation (Fig. 2). This cell-based model includes the important interactions between the coagulation proteins and suitable anionic phospholipid surfaces, such as the surface found on activated platelets[6]. This model is more accurate as it better represents the coagulation process *in vivo* and the roles that cells may play in the control of the duration, intensity and localization of coagulation[6;9]. The initiation of coagulation in this model is localized to TF-bearing cells[16]. TF is normally found in the subendothelium of intact

vessels or in the inactive state in TF-bearing cells[17]. The amount of TF in the blood stream has been a subject of debate[18]. Generally, when injury occurs and blood is exposed to TF, factor VII/VIIa in the blood will rapidly bind to TF to initiate coagulation[17]. The TF-Vlla will activate small amounts of factors IX to IXa and X to Xa[6]. In turn, IIa will be generated which results in activation of platelets and further progression of coagulation[9]. Activated platelets undergo morphological changes, one of which enables the exposure of phosphatidylserine from the inner membrane of the platelet to the outer surface[19]. This allows the formation of coagulation enzyme complexes that are required for further amplification of IIa generation[6].

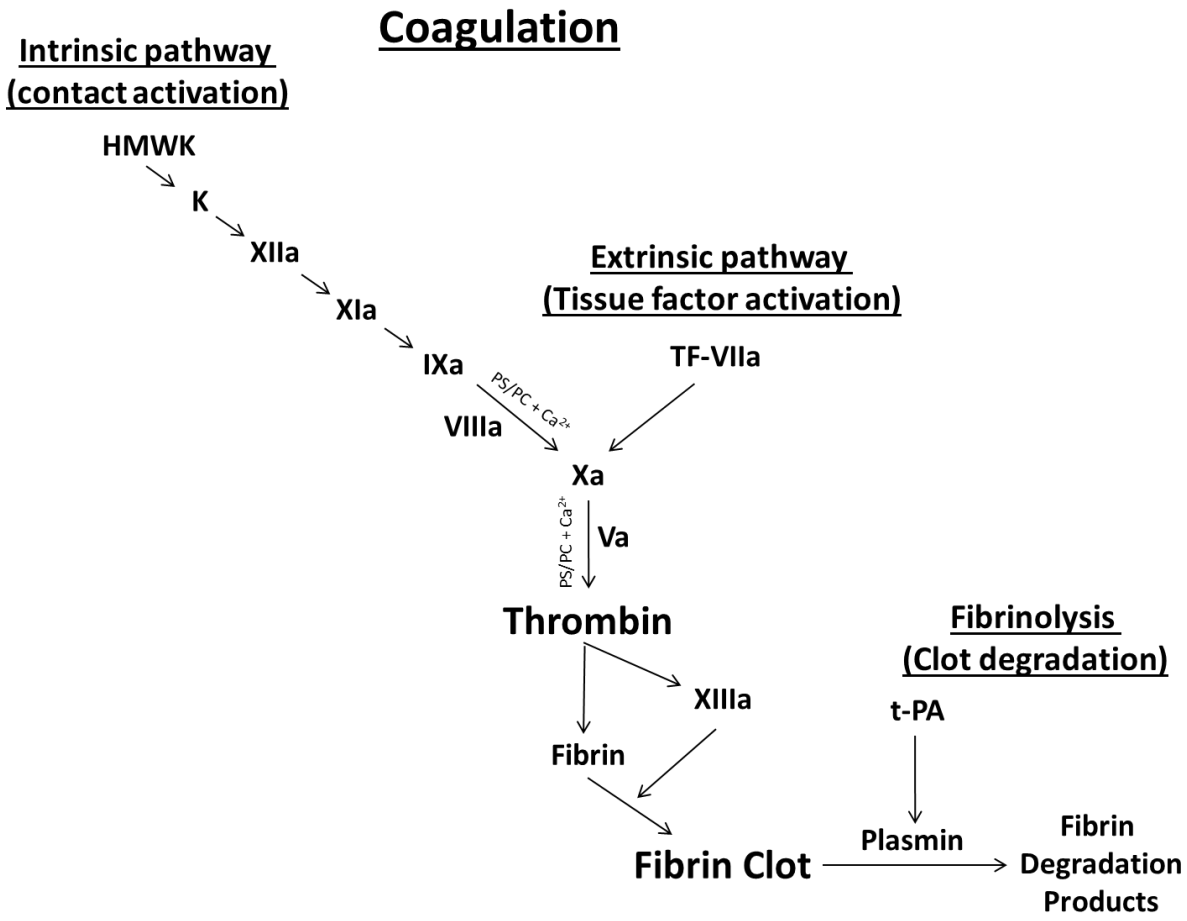

Figure 1. The classic coagulation cascade. The extrinsic tenase complex (tissue factor/VIIa) initiates the generation of small quantities of thrombin. Amplification/propagation of the cascade occurs through the formation of the tenase and prothrombinase complexes to generate additional thrombin required to convert soluble fibrinogen to the insoluble fibrin clot. Abbreviations: HMWK;high molecular weight kininogen, K; kallikrein, PSPC, phosphatidylserine/phosphatidylcholine, PS; protein S, TF; tissue factor, tPA; tissue plasminogen activator.

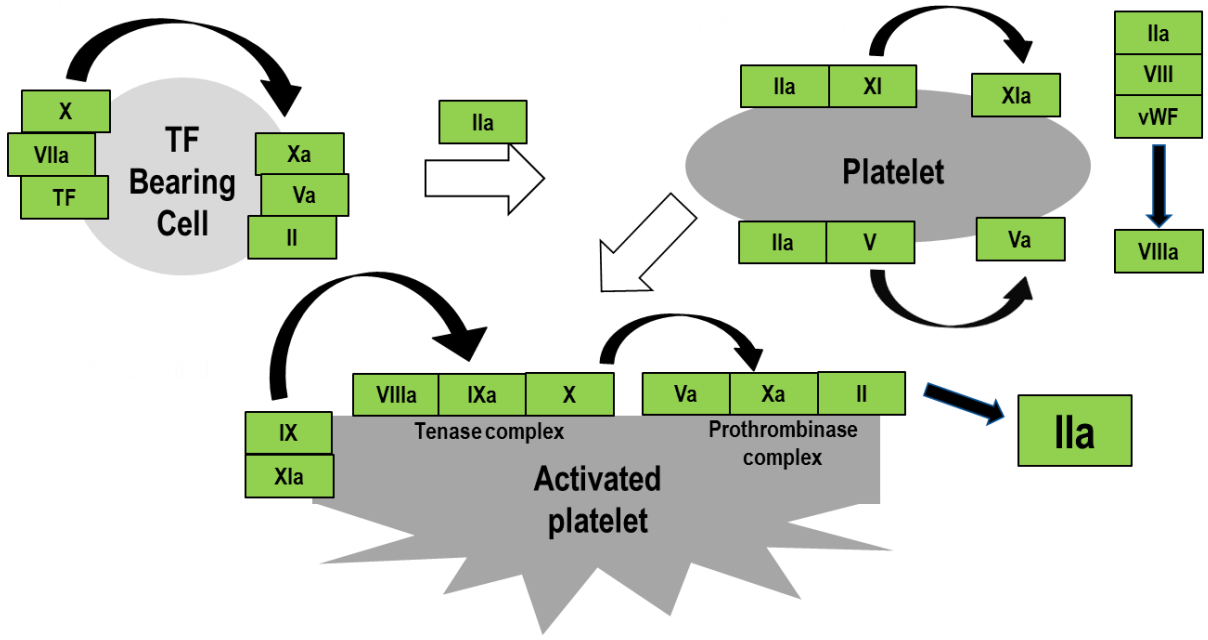

Figure 2. Initiation and amplification/propagation phases in the coagulation cascade. Small amounts of IIa are generated during exposure to TF-bearing cells. IIa will lead to the activation of platelets as well as the activation of various coagulation factors. Once the platelet is activated, it provides a phospholipid surface on which specific coagulation factor complexes can form leading to a larger burst in IIa generation, eventually leading to fibrin clot formation. Abbreviations: TF; tissue factor, vWF; von Willebrand Factor.

1.3 Platelets

Platelets are a type of blood cell that is derived from megakaryocytes and play a vital role in primary haemostasis by forming the initial haemostatic plug at the sites of vascular injury[20]. Circulating platelets are kept in a non-reactive state partly by inhibitory compounds that disrupt cell signaling platelet activation, such as nitric oxide, which is secreted by endothelial cells[21]. The intact endothelium acts as a barrier separating platelets from adhesive substrates in the subendothelial connective tissue matrix[22]. When there is damage to the endothelium, collagen is exposed and binds to von Willibrand Factor (vWF) and platelet glycoproteins (GP) to activate

the platelets[22] (Fig. 3). The platelet GP-VI and GP-Ia/IIa (also known as integrin -$\alpha_2\beta_1$) interact with the collagen[23]. Rolling platelets will adhere to the site of injury through interaction of platelet GP-Ib/IX/V complex with subendothelial vWF[24]. Platelet activation by IIa interaction with specific protease-activated receptors 1 and 4 (PAR 1/4) may be facilitated by the GP-Ib/IX/V complex[25]. Activated platelets will interact with the fibrin meshwork to form a stable plug at the site of vessel wall injury[26]. Multiple events occur as a result of platelet activation. The release of platelet α-granules and dense granules results in additional platelet activation and aggregation[27]. Additionally, activated platelets release microparticles[28]. Microparticles and surface membrane alterations in activated platelets aid in accelerating thrombin generation[29]. Resting platelet membranes have an asymmetric distribution of phospholipids[19], where phosphatidylcholine is found mostly on the outer membrane leaflet while phosphatidylserine on the inner[30]. During platelet activation, this asymmetrical distribution of phospholipids is significantly altered by the enzyme scramblase, increasing phosphatidylserine exposure[19]. This leads to formation of tenase (VIIIa, IXa, X and Ca^{2+}) and prothrombinase (Va, Xa, II and Ca^{2+}) complexes on activated platelet surface[19]. Finally, platelet GP-IIb/IIIa (also known as integrin – αIIbβ3) is conformationally activated and exposed on the platelet surface[31]. GP-IIb/IIIa has enhanced affinity for fibrin(ogen) molecules[31], and the cross-linking of fibrin(ogen) to GP-IIb/IIa on adjacent platelets results in additional platelet aggregation[32;33]. Overall, platelets play an important role in hemostasis and impairment to their function or severely low abundance may lead to impaired clot formation.

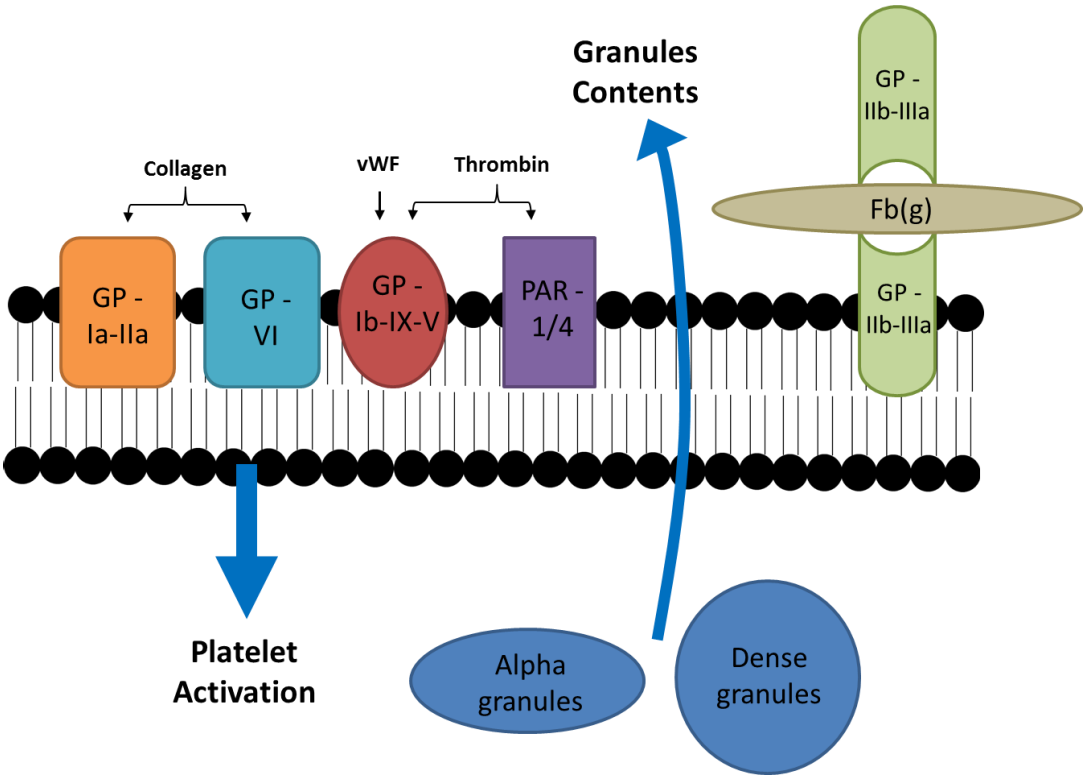

Figure 3. Platelet activation and aggregation. This figure depicts the interaction of most common receptors and their agonists/ligands responsible for platelet activation and aggregation. Abbreviations: ADP/ATP; adenosine di/triphosphate, Fb(g); fibrin(ogen), GP; glycoprotein, PAR-1/4; protease activated receptor 1 or 4[34].

1.4 Venous Thromboembolism

Venous thrombosis is the formation of a thrombus within the veins, which may result in obstruction of blood flow through the vessel. This can occur in any part of the venous system; however, thrombi most commonly occurs in the deep veins of the lower extremity[35]. Collectively this pathology is referred to as VTE, which includes deep vein thrombosis (DVT) and pulmonary embolism (PE)[5]. VTE has a high incidence rate and is responsible for a large proportion of morbidity and possibility of mortality in North America[35]. VTE may be due to hereditary or

acquired prothrombotic conditions[35;36]. Patients with identifiable risk factors generally have one or more of the components from Virchow's triad, which includes: hypercoagulability; endothelial injury; or stasis of blood flow[37]. Those without an identifiable risk factor for VTE may be considered to have an unknown origin, also known as unprovoked VTE[38]. Up to 25% of all patients with unprovoked VTE are found to have some form of cancer within the first 24 months of VTE diagnosis[36]. Approximately 20% of patients with cancer develop clinical manifestations of VTE but thromboembolism may be found in autopsy in up to 50%[1]. Cancer is a prothrombotic condition that causes an imbalance within the haemostatic system, although multiple mechanisms may be involved[39-41]. For example, procoagulants including TF, are expressed by tumor cells from several malignancies such as pancreatic and lung cancer[42]. In addition, the treatment of cancers with chemotheraputic agents and hormonal therapies are recognized as risk factors for VTE[43]. There have also been some cases where radiotherapy increases the risk of thrombosis[44]. Bedridden immobility or interventions such as central venous catheter placements and surgery may also contribute to an increased risk of VTE[45].

1.5 Anticoagulants

Anticoagulants are a class of drugs that are used in prevention of both pathological clot formation and the extension of existing clots within the blood for thromboembolic disease[46;47]. Anticoagulant intervention is necessary when there is a high risk of thrombosis[47]. Anticoagulants can be classified under different categories based on their mechanistic action[48]. The most significant complication associated with anticoagulant treatment is bleeding, which is potentially life-threatening.

Type	Anticoagulant	Main Target(s)	Administration	Half-life
AT-Dependent	UFH	IIa and Xa	IV	1-2.5 hrs
	LMWH	IIa < Xa	Subcutaneous	3-5 hrs
	fondaparinux	Xa	Subcutaneous	17-21 hrs
Direct factor-specific	rivaroxaban	Xa	Oral	5-10 hrs
	dabigatran	IIa	Oral	12-17 hrs

Table 1. Differential characteristics of anticoagulants studied[49].

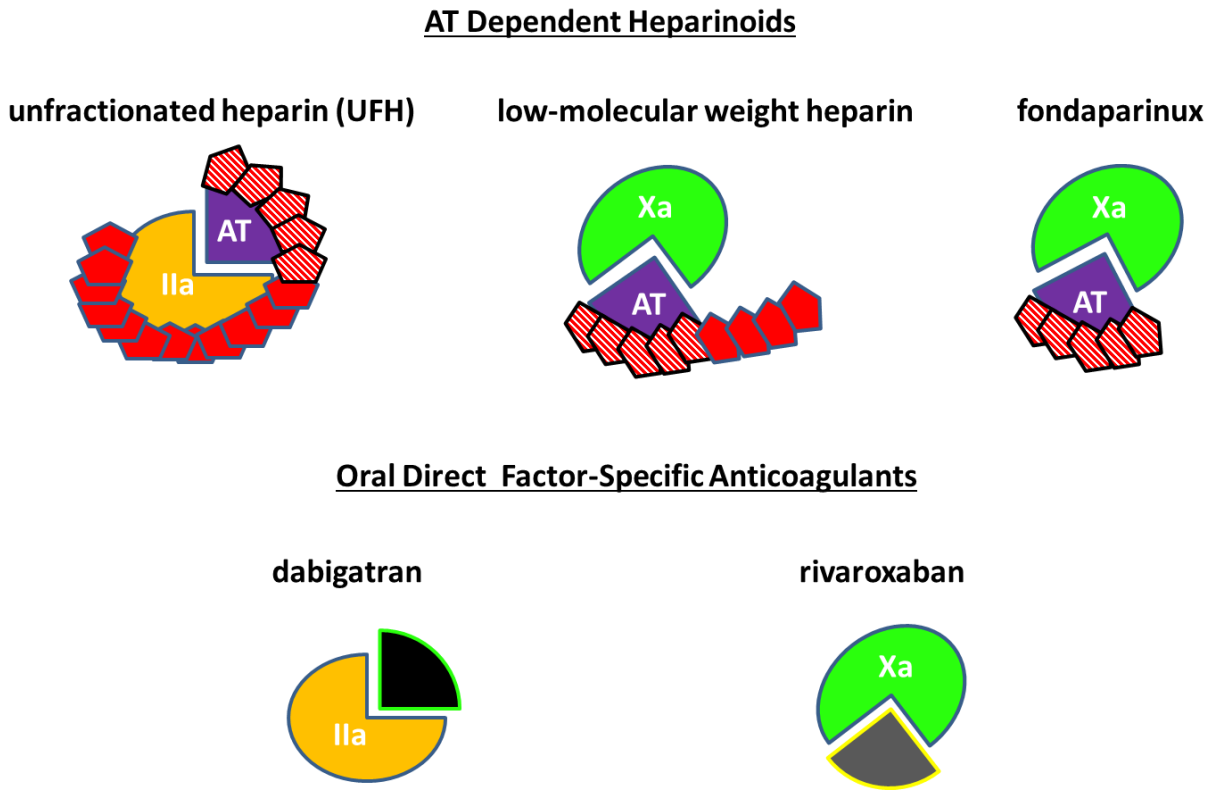

Figure 4. Simplified visual representation of the mechanisms for the 2 major classes of coagulation inhibitors studied. UFH requires interaction with both the AT molecule (specific pentasaccharide sequence) and factor IIa (excess saccharide chain) for efficient configuration (template mediated) to inhibit IIa, UFH also inhibits Xa which only requires AT binding. Low-molecular weight heparin (LMWH) has similar mechanism as UFH but has a greater preference to interact with Xa over IIa due to less than half of the molecules possessing an appropriate chain length required to inhibit IIa. The synthetic fondaparinux is made up of only the AT-interacting pentasaccharide sequence, making fondaparinux almost exclusively a Xa inhibitor. Dabigatran and rivaroxaban are direct inhibitors and do not require AT to inhibit coagulation factors. Dabigatran is specific only for IIa and rivaroxaban is specific for Xa.

1.5.1 Heparin

Unfractionated heparin (UFH) is an effective anticoagulant that has been widely used for prevention and treatment of both arterial and venous thromboembolism since 1930[50]. UFH is a very negatively charged and highly sulfated glycosaminoglycan that contains a specific pentasacharride sequence required for binding to antithrombin (AT)[50]. Since heparin is

dependent on the presence of AT to inhibit coagulation, it is known as an indirect inhibitor. Heparin bound to AT induces a conformational change in the AT causing an increased anticoagulant effect by exposing the reactive centre loop towards the protease, thus making it approximately 1000-fold more reactive with the serine proteases[50]. Heparin itself, interacts with exosite II on IIa[51]. Heparin helps to bridge AT to IIa and enhances AT inhibition of IIa through the template-mediated action[51]. Xa can be inhibited through the conformational activation of AT by heparin and does not require AT bridging to enhance its inhibitory activity[52]. UFH has a mean molecular weight of apporximatly15 k Da and ranges from 3 – 30 kDa[53]. The molecular weight corresponds to the chain length from 18-70 monosaccharide units[53]. However, only 1/3 of UFH preparations contain the AT-specific pentasaccharide sequence[54]. Interestingly, UFH has a short, dose-dependent intravenous half-life of 30-90 min, with relatively unpredictable pharmacokinetic properties[55]. Because of its large chains and highly negative charge, UFH can have unintended cellular interactions[56]. For example, UFH may interact with platelets, resulting in heparin induced thrombocytopenia (HIT)[57]. A major advantage of UFH is that it has a definitive antidote called protamine sulphate[58]. Protamine, a highly positive molecule, binds with UFH to form a stable biologically inactive complex[58]. The therapeutic range of UFH for treatment of VTE in plasma is 0.2-0.4units/ml[59].

1.5.2 Low Molecular Weight Heparin

Low-molecular-weight heparin (LMWH) is similar to UFH except it is composed of chain lengths that are much smaller[60]. Most LMWH retain the AT-specific pentasaccharide sequence that allows Xa inhibition, but loses most of its ability to enhance AT inhibition of IIa[61].

However a small percentage of LMWH may possess the minimum 18 saccharide chain length required to inhibit IIa[59]. The molecular weight range can vary from 3-8 kDa depending on the manufacturing methods[59]. LMWH are fragments of commercial grade UFH which are made by enzymatic or chemical depolymerisation[62].This shorter chain contributes to better pharmacokinetic predictability and lower occurrences of HIT[57]. However the UFH antidote, protamine is not as effective at reversing the anticoagulant effects of LMWH[63]. The therapeutic range of LMWH for treatment in plasma is 0.5-1.1IU/ml[64].

1.5.3 Fondaparinux

Fondaparinux is a synthetic anticoagulant, based on the specific pentasaccharide component of UFH and LMWH that binds to AT. Fondaparinux almost exclusively inhibits factor Xa (Fig. 4) and not IIa[60]. This is because unlike LMWH, it lacks any additional saccharide chain beyond the pentasaccharide[65]. Its main advantage over UFH or LMWH is more predictable pharmacokinetics[66]. In patients suffering from HIT, fondaparinux can be used because of its very low associated risk of HIT [67]. However, protamine sulfate barely effects fondaparinux compared is UFH or LMWH[63]. The therapeutic range of fondaparinux for treatment of VTE in plasma is 1.20-1.24mg/l[66].

1.5.4 Rivaroxaban

Rivaroxaban is an direct oral anticoagulant that competitively inhibits Xa (k_i 0.4 nM) [68;69]. The selectivity for Xa is 10,000 fold greater when compared to any other serine protease[70]. Rivaroxaban is a small synthetic molecule (molecular mass: 435.88 g/mol) that does not require a cofactor to inhibit factor Xa[71] It is capable of reversible binding to free Xa, prothrombinase-bound Xa and clot-bound Xa[68;72]. Its half-life is approximately 5-12 hours and its clearance is

mainly through hepatic metabolism[73;74]. Rivaroxaban has been approved for the following indications: reducing stroke risk in nonvalvular atrial fibrillation, treatment of DVT, treatment of PE, risk reduction of reoccurrence of DVT and PE and prophylaxis of DVT after knee and hip replacement surgery[71]. Rivaroxaban has also been approved for DVT and PE treatment and risk reduction, as well as, prophylaxis of DVT after knee or hip replacement surgery[71]. With predictable, dose-dependent pharmacokinetics, wide therapeutic window, low drug-drug and food-drug interactions, rapid absorption, no routine monitoring, and oral administration, Rivaroxaban has many advantages over other anticoagulants[71]. Generally, rivaroxaban will have a plasma concentration of 100-150ng/ml[74]. There is no dependable reversal agent for rivaroxaban yet. However, there is an antidote in development using recombinant Xa lacking procoagulant activity that has been investigated in phase 1 and phase 2 studies[75].

1.5.5 Dabigatran

Dabigatran is an oral anticoagulant that directly inhibits factor IIa and has very high selectivity for IIa (k_i 4.5 nM)[76;77]. The selectivity for IIa is about 800 fold greater when compared to any other serine protease[77]. It is a small synthetic molecule (molecular mass: 471.51 g/mol) capable of inhibiting both free IIa and clot-bound IIa[78]. Dabigatran is approved for prevention of VTE after hip or knee replacement and for stroke and embolism prevention in patients with non-valvular atrial fibrillation[79]. Its half-life is approximately 14-17 hours and is mainly cleared by renal systems[78]. Generally dabigatran will have a plasma concentration of 64-340 ng/ml[78]. Overall, dabigatran provides many advantages over traditional anticoagulants, including more predictable anticoagulant effects, no food interactions, and convenient long-term administration without the need for regular anticoagulation monitoring[79]. Currently, there is no dependable

reversal agent of dabigatran. However, a monoclonal antibody called aDabi-Fab has shown promising results as antidote in animal models[80].

1.6 Thrombocytopenia

Thrombocytopenia is a condition where the circulating platelet count falls below the normal levels[81]. Normal platelet counts range from 150-400 × 10^9/L[81]. Platelet levels of < 150-100 × 10^9/L may be classified as mild thrombocytopenia, 50-100 ×10^9/L as moderate and platelet counts below 50 × 10^9/L as severe[82]. Typically, the risk for major bleeding does not increase until platelets are less than 10× 10^9/L[83]. Even if bleeding is not associated with observed low platelet counts, low counts will often influence patient management. This may prompt physicians to take precautions such as reducing anticoagulant dosing or platelet transfusion[84]. Although based on mostly anecdotal data and expert opinion, most guidelines suggest platelet counts should be above 30-50 × 10^9/L before initiating anticoagulant therapy to avoid major risks of bleeding[85;86] Typically, platelet transfusions are performed when the platelet count reaches < 10 × 10^9/L[82].

The pathophysiological mechanisms of thrombocytopenia can be divided into 3 major categories: 1) decreased platelet production due to marrow suppression, 2) increased platelet clearance or consumption and 3) sequestration within the spleen[87]. Observing the dynamics of platelet count changes over time is one approach used to aid in the identification of the potential cause of thrombocytopenia[84]. For example, a slow gradual fall in platelet counts developing over 5 to 7 days is most likely caused by consumptive coagulopathy or bone marrow failure[84]. A rapid decrease in platelet count after an initial increase 1-2 weeks after surgery may suggest increased platelet clearance stemming from HIT or immune thrombocytopenia[84]. Cancer therapy is a

known cause of thrombocytopenia, which may result from chemo- or radiotherapy use[88;89]. Chemotherapeutic agents may suppress cell production from the bone marrow and the toxicity from radiation may damage the bone marrow[89]. Cancers themselves such as leukemia or lymphoma can suppress platelet production by invading the bone marrow[90]. These cancers are also known to cause spleen enlargement, contributing to platelet sequestration[91]. Obtaining a complete blood count is a common method for establishing the presence and severity of thrombocytopenia[92]. Performing certain clotting tests that can assess cellular components may provide information regarding clot formation in the presence of lower platelet counts.

1.7 Thromboelastography

Traditionally, coagulation tests are used to assess haemostasis and its abnormalities. Commonly used tests include prothrombin time (PT) and activated partial thromboplastin time (aPTT)[93;94]. PT and aPTT are general measures of the plasma clotting times of the extrinsic and intrinsic coagulation pathways, respectively[95]. However, these tests are only performed on platelet poor plasma and cannot assess whole blood or interactions with cellular components[96]. These tests have helped to define some coagulation disorders such as hemophilia[97]. However, the value of these tests in acute perioperative settings has been in doubt[97]. This is due to long processing times between blood sampling and obtaining results[97]. A more rapid global haemostasis test would probably be more ideal in order to obtain a more complete picture of the coagulation process. Thromboelastography (TEG) is able to capture thrombin generation, platelet function and fibrin clot formation when compared to commonly employed tests[98]. TEG is gaining interest because of its ability to assess the effects of plasma factors, platelets and red cells in whole blood[97]. In addition, TEG is capable of measuring multiple phases of clot

formation including initiation, clot stability and clot break down during lysis[99]. TEG was developed in 1948 by Dr. Hartet; the technology has been updated since then but the basic concept remains the same[100-102]. TEG involves the use of a whole blood or plasma sample that is incubated in a TEG-specific plastic cup heated to 37^{o}C. The cup oscillates as the sample clots; the motion of the cup is transmitted to the sensor pin connected to a torsion wire (Fig. 5). An alternate tool that measures viscoelastic clotting properties is called rotational thromboelastometry (ROTEM)[103]. ROTEM is similar in concept but the cup and pin mechanism is reversed; where the cup is stationary while the pin rotates[103]. In either machine, the formation of the clot will generate a physical connection between the cup and sensor, which is recorded via a computer and translated into a diagram outputted on the screen depicting various parameters of the clot's dynamics from formation to dissolution (Fig. 6)[98;104]. ROTEM measures the same parameters, but with alternate terms[103].

Even though the TEG has existed for over 60 years, it has only recently been considered as a useful tool in the clinical setting, including in the management of trauma-induced coagulopathy, cardiopulmonary bypass, liver surgery and transfusion[102;105]. TEG is able to analyze citrated or native whole blood, platelet rich plasma and platelet poor plasma[98]. The coagulation initiators may include TF or kaolin, which depend on the focus of analysis[98]. Although the TEG is a versatile tool, there is still difficulty in establishing standardized protocols and normal reference ranges[105]. This may be the reason why TEG is not widely accepted in conventional clinical practice or considered as a reliable routine laboratory test and is only used in highly specific situations[105].

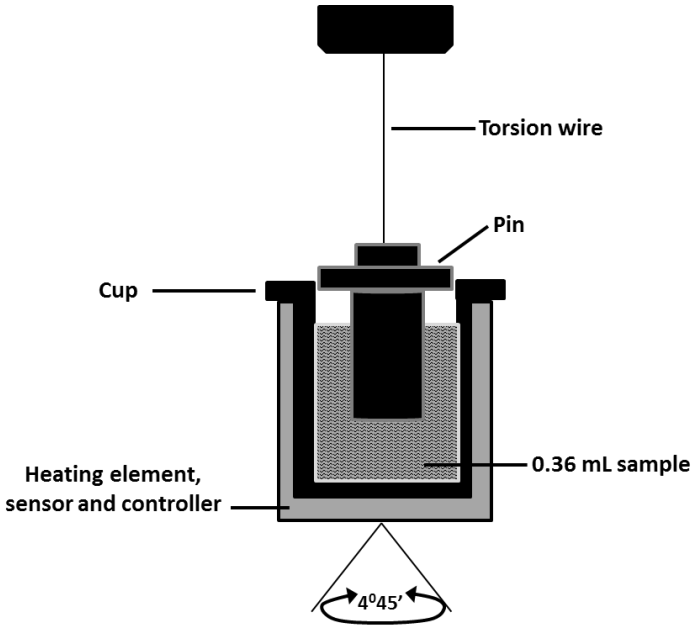

Figure 5: Illustration of TEG mechanism during clot formation.

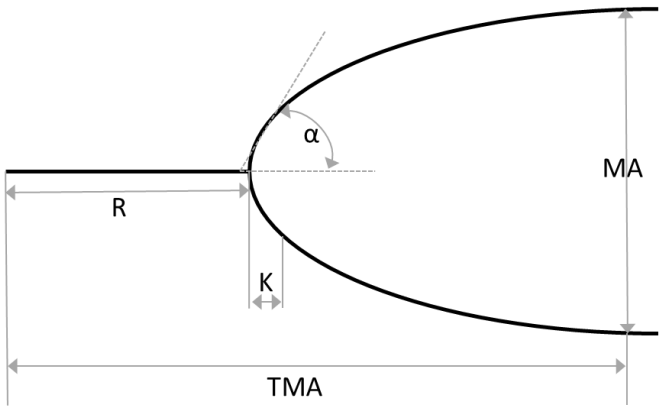

Figure 6: TEG clotting profile, displaying various important clotting parameters. Reaction time (R) represents the time elapsed before clot initiation is achieved. K value and alpha angle (α) represent clot kinetics, providing information regarding speed of clot formation. Maximum amplitude (MA) is the maximum strength achieved by the clot. Time to maximum amplitude (TMA) represents the time required to reach the clot's maximum strength.

2. OVERALL OBJECTIVE

The overall objective of this thesis project is to investigate the effects of anticoagulants on plasma clot formation in the presence of low platelets. More specifically, this experiment will examine the effect of anticoagulants on plasma enriched with varying suboptimal amounts of autologous platelets from healthy donors.

2.1 Hypothesis/Rationale

Clotting will be impaired when platelet counts fall below a certain concentration in the presence of anticoagulants. We may observe this because most guidelines for anticoagulant use in patients with thromboembolic disease, recommend withholding treatment when platelet counts are below 30-50 × 10^9/L. In addition, since each anticoagulant has a different mechanism, the

anticoagulants studied will have varying effects on clot formation when platelet counts are low, but similar at normal platelet levels.

3. MATERIALS & METHODS

3.1 Reagents

All chemical reagents were of analytical grade. Glutaraldehyde was obtained from VWR Int. (Ontario, Canada); osmium tetroxide from Sigma-Aldrich (Ontario, Canada); sodium cacodylate from Sigma-Aldrich (Ontario, Canada); imidazole from Alfa Aesar (Massachusetts, USA); corn trypsin inhibitor (CTI), a factor XIIa inhibitor, from Haematologic Technologies Inc (Vermont, USA); unfractionated heparin (UFH, Hepalean, 1000 units/mL) from Organon Pharmaceuticals (Ontario, Canada); dalteparin (fragmin, 15,000 units/0.6 mL) from Pfizer (New York, USA); fondaparinux (Arixtra 7.5 mg/0.6 mL) from GlaxoSmithKline Inc. (Ontario, Canada); rivaroxaban (Xarelto; 10mg) from Bayer Inc. (Ontario, Canada); and dabigatran etaxilate (Pradax; 75 mg) from Boehringer Ingelheim Canada (Ontario, Canada). Thromborel® S was sourced from Dade Behring Inc (Illinois, USA).

3.2 Preparation of rivaroxaban and dabigatran

(Rivaroxaban and dabigatran were purified and prepared by Dr. Mykhaylo Goncharenko)

Rivaroxaban was purified from a pharmaceutical reagent “Xarelto”. The final product was verified by mass spectrometry and Xa inhibition activity was determined by turbidimetric analysis in a plasma coagulation assay (data not shown).

Prodrug dabigatran etaxilate was extracted from the pharmaceutical agent "Pradax". The active dabigatran was prepared by chemical conversion of the prodrug. The structure of the purified product was verified by mass spectrometry and its activity was verified by testing against standards using the Hemoclot thrombin inhibitor kit (Aniara, West Chester, Ohio).

3.3 Blood Collection and Preparation

Approximately 20-30 mL of blood was freshly drawn from healthy normal volunteer donors each day using a 19 gauge butterfly needle and 30 mL syringe or 4.5 mL vacutainer containing 3.2% sodium citrate (10% v/v). The whole blood (WB) was then transferred into a 50mL polypropylene tube. Platelet rich plasma (PRP) was prepared by centrifuging WB for 15 min at 47 g to separate plasma and platelets from red cells. The PRP was carefully transferred to a 15mL polypropylene tube and placed on a swaying mixer at room temperature until used. Platelet poor plasma (PPP) was prepared by double centrifugation of PRP for 10 minutes at1530 g. The final PPP was transferred to a new fresh 15 ml polypropylene tube and placed on a swaying mixer at room temperature. Platelet counts were determined by a Coulter counter for each sample prior to use (provided by Hamilton General Hospital CORE lab). Plasma samples containing platelets at predefined concentrations (10, 20, 30,40,50,75,100,150 $\times 10^9$/L) were obtained by mixing PPP with PRP. PPP contains less than 10×10^9 platelets/L. The accuracy of platelet mixing was within ~90 % of the expected values when measured by a Coulter counter (data not shown).

3.4 TEG procedure

TEG coagulation analyzer (Model 5000) was obtained from Haemoscope Inc (Illinois, USA). Two separate TEG machines were utilized to perform four simultaneous TEG analyses. The temperature of each machine was preset to 37^{o}C. For the purposes of this experiment the standard non-coated plastic pins and cups were used throughout the whole series. Reactant solutions contained 1.2 pM TF, 10 mM $CaCl_2$, 30 µg/mL CTI and of the following anticoagulants at therapeutic concentrations (UFH 0.3 IU/mL, dalteparin 1.0 IU/mL, fondaparinux 1.0 IU/mL, rivaroxaban 150 ng/mL or dabigatran 180 ng/mL. The concentration of CTI was pre-optimized in PPP to achieve a plateau effect (Fig. 7). Plasma samples containing predefined platelet concentrations were prepared immediately before the experiments. Three hundred and thirty µL of the plasma sample were then mixed with 30 µL of the reactant solution within the TEG cups to initiate the clotting process. Five standard TEG parameters were monitored: R (time to initiate clot formation with a minimum strength of 2 mm), K (clot kinetics, time to achieve clot strength of 20 mm), α (angle, speed of clot development), MA (maximum strength the clot reaches) and TMA (time to achieve maximum amplitude) were monitored for a maximum of 180 min (Fig. 6). Another measurement known as area under the curve 15 (AUC15) which measures the area within the actual TEG tracing, 15 minutes after R has been established. It was used as a global parameter evaluating the speed and strength of clot formation (Fig. 9)[106].

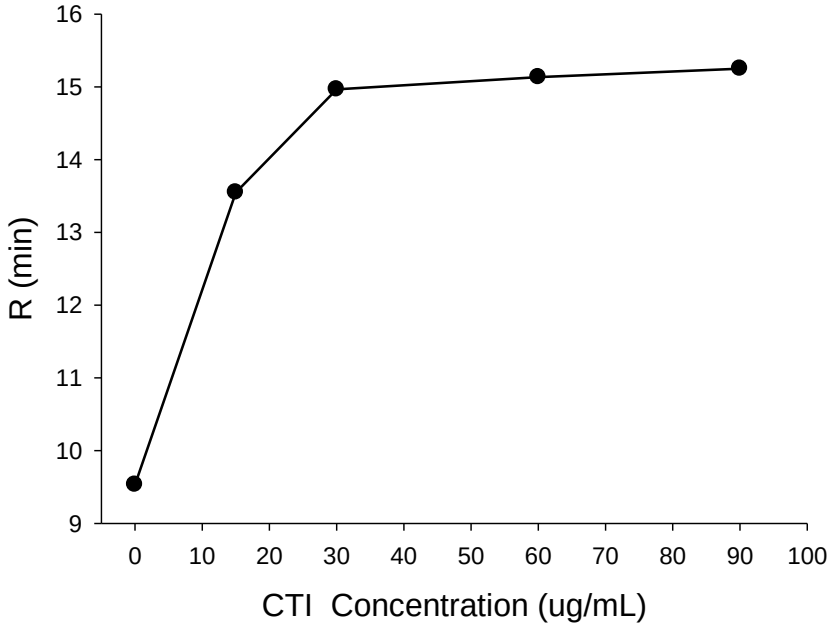

Figure 7: Optimization of CTI in PPP.

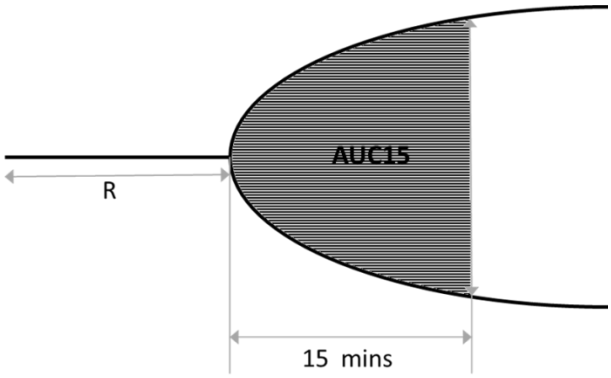

Figure 8: Area under the curve 15 (AUC15), measurement of area within TEG clot profile tracing 15 minutes after R has been established. AUC15 is a global measurement that combines the speed and strength of the forming clot.

3.5 Effects of Tissue Factor

TF is used to initiate coagulation in our assay. Our source of TF is from Thromborel S, a solution which contains TF, phospholipids and 10 mM calcium. The TF concentration of thromborel S (3140 pM) was determined with IMUBIND TF ELISA (American Diagnostica, Greenwich, CT, USA) (assay performed by Allen Stafford, TAaRI). To simulate a hypothetical clinical bleeding profile, the TF concentration used in our TEG experiments was first optimized so that the amount of TF was sufficient to clot plasma samples containing platelets at 150×10^9/L within a relatively low R time in the presence of an anticoagulant (Fig. 9). The TF concentration must also be low enough that R will be delayed to at least 120 minutes or not clot at all (R < 180 minutes). The optimal level of TF was determined by experiments using variable levels of TF (0-1.2 pM) mixed with 10 mM $CaCl_2$ in 0.15 M imidazole buffer (pH 7.4), 30 µg/mL CTI, as well as one of the anticoagulants at therapeutic concentrations.

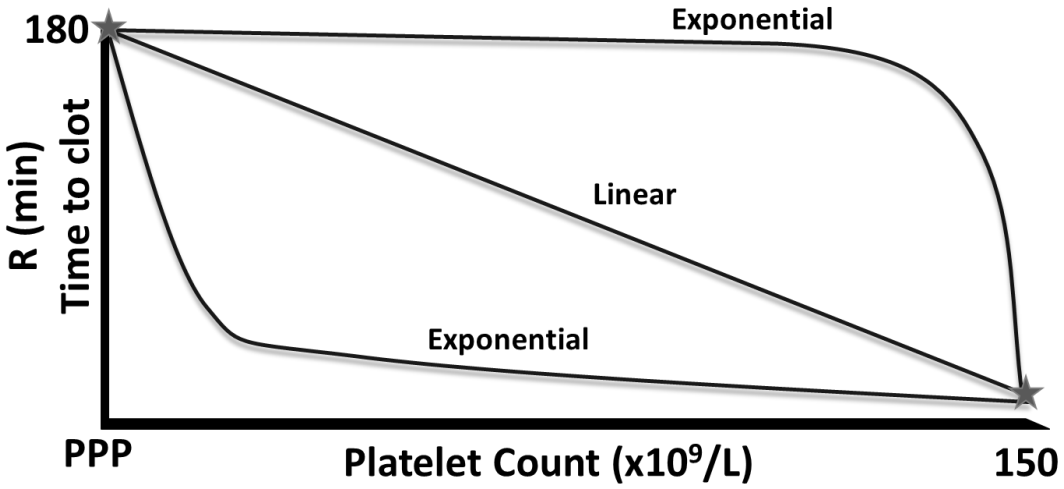

Figure 9: modelling the data to fulfill two criteria to mimic clinical observation. At 150×10^9/L in the presence of anticoagulants the plasma should clot with R times relatively close to the control (ie 9-24 mins experimentally) without anticoagulants as 150×10^9/L is the lowest normal platelet count and is clinically expected to clot normally. The second critical point is at PPP, when platelet counts fall below 10×10^9/L there is high risk of spontaneous bleeding and requires transfusion. We may assume that clotting is far beyond normal and will be considerably delayed or not form at all in the presence of anticoagulants (ie R = 120-180 mins). The profile of the curve or line across the platelet counts in between PPP and 150×10^9/L will reveal more information about the type of relationship between platelet count and anticoagulant in terms of the time it takes to initiate clot formation.

3.7 Scanning electronic microscopy (SEM) examination of fibrin clot

As the thromboelastographic profile for heparinoids was distinctively different from those of control or factor-specific new anticoagulants, SEM was performed to further characterize the clot structure. All samples were randomly coded and blinded to the individual performing the imaging and analysis to avoid bias. Clots were formed on a 0.025µm Millipore membrane containing platelet counts of 150×10^9/L , 30×10^9/L and PPP in the presence or absence of anticoagulants (0.3 IU/mL UFH, 1.0 IU/mL dalteparin, 1.0 IU/mL fondaparinux, 150 ng/mL rivaroxaban and 180 ng/mL dabigatran), 1.2 pM TF, 10 mM $CaCl_2$ and 30 µg/mL CTI. A 20 uL volume of plasma-reagent mix was placed on the Millipore membrane; the clots were

incubated in 100% humidity at 37^{o}C for 3 hours. After incubation, the clots were washed and magnetically stirred at 180 rpm in 0.1 M phosphate buffer (PB, pH 7.4). The clot samples were then fixed with 2% v/v glutaraldehyde in 0.1 M PB for at least 3 hours, further washed twice in PB, and then stained with 1% osmium tetroxide in 0.1 M sodium cacodylate buffer for 1 h. The samples were dehydrated with gradient ethanol series (50%, 70%, 95% and 100%), critical point dried, mounted onto aluminum stubs, and then sputter-coated with gold. To remove an unknown, randomly forming thick fibrin matte formation that covered and prevented visualization of underlying fibers, Parafilm® was placed on top of the sample after gold coating, and then peeled off to remove the loose fibrin matte. This step was repeated twice for each sample and gold sputter-coating was reapplied. SEM examination (TESCAN VEGA LSU scanning electron microscope, TESCAN a.s., Brno, Czech Republic) was conducted at 20,000 × magnification. Randomly selected areas were imaged. At least 3 representative images were obtained for each setting. Photoshop (CS5 Adobe, California, USA) was used to process and characterize the clot structure semi-quantitatively. Contrast and brightness of images were adjusted to provide full spectrum of grey scale from white to black. A 15 × 15 grid was applied to each image. Total pore area, defined as the number of pixels in the dark area between the fibrin strands of the superficial layer, was measured by the software. The total pixel count of the selected area was divided by the total pixel count of the entire image to provide a percentage of pore area. Fiber widths and numbers were quantified manually in the area confined by 5 of the 15 rows in the grid).

3.8 Statistical analysis

All TEG experiments were performed at least three times. A modified *t*-test, published by Williams *et al*, was used for comparisons between multiple variances and their corresponding control [107]. For comparisons between SEM fiber parameters, Student's *t*-test and ANOVA with Turkey post-Hoc were used. For all tests, $p < 0.05$ were considered to be statistically significant.

4. RESULTS

4.1 Effects of TF concentration on TEG profile

In the absence of anticoagulant, the time to initiate clotting (R) after addition of calcium was ~20 min for plasma containing 150×10^9/L platelets (Fig. 10A). Reducing the platelet concentration increased R slightly but the changes were not statistically significant ($p > 0.05$). Also, the addition of any concentration of TF decreased R across all platelet counts (Fig. 9A). In the presence of UFH at 0.3 IU/mL, PPP did not clot (R > 180 min) unless 1.2 pM TF was added to initiate clotting (Fig. 10B). In the presence of 1.0 IU/mL dalteparin PPP did not clot unless there was at least 1.2 pM TF (Fig. 10C). However, at 150×10^9/L platelets clotting occurred with at least 0.7 pM TF. In the presence of 1.0 IU/mL fondaparinux and no TF, the clot was not able to form across all platelet counts (Fig. 10D). Also, the addition of TF greater than 0.07 pM decreased R across all platelet count to less than 10 minutes. Rivaroxaban at 150 ng/mL was not able to delay R in PPP more than 20 min unless TF was removed (Fig. 10E). Dabigatran at 180 ng/mL delayed R in PPP to 40 min with 0.07 pM TF. R was further delayed to > 120 min by dabigatran when TF was removed (Fig. 10F). The optimal TF concentrations that allowed TEG R time to fall in line with the model's criteria were as follows: 1.2 pM (UFH), 0.7 pM

(dalteparin), 0.07 pM (fondaparinux) and no TF (dabigatran). For rivaroxaban, the PPP anchor point was not achievable even without any TF.

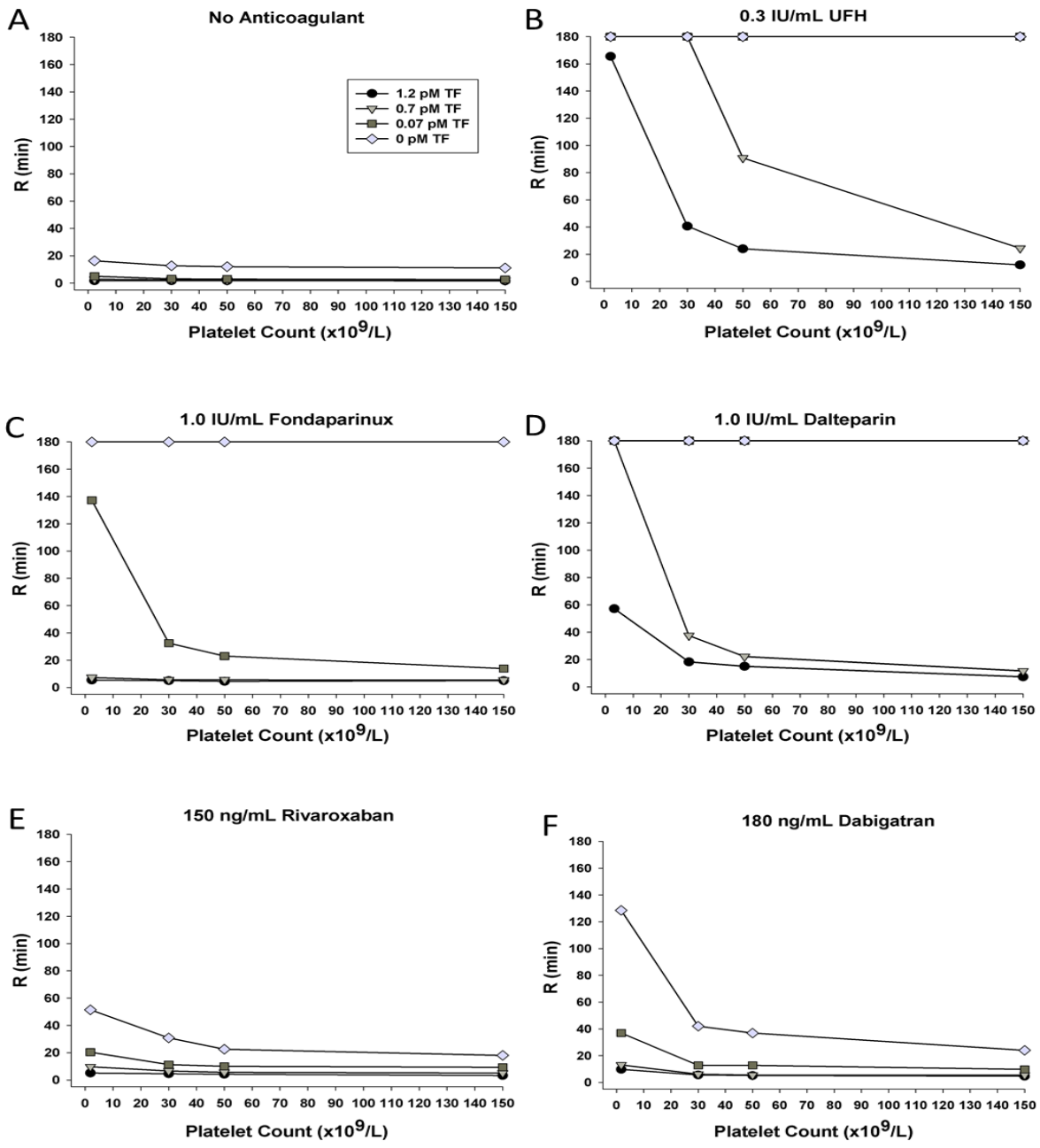

Figure 10: TF optimization experiments in which each anticoagulant was combined with varying amounts of TF and R was measured across the platelet range. Optimal TF was chosen based on fulfilling the two set R time criteria at 150×10^9/L and PPP. (A) setting with no anticoagulants present with 0-1.2 pM TF. The same experiment was performed for each anticoagulant individually at therapeutic plasma steady state concentrations (B) UFH, (C) dalteparin, (D) fondaparinux, (E) rivaroxaban, (F) dabigatran.

4.3 Modelling of TEG profile for anticoagulants with TF at concentration individualized for individual anticoagulant

After determining the optimal concentration of TF to initiate clotting, the effects of platelets on TEG profile was studied. Each anticoagulant required specific amount of TF, these experiments were not designed to compare different anticoagulants but to evaluate the optimal platelet counts at which clots could adequately form when compared with those with 150×10^9/L platelets (Fig.11). The chosen concentration of TF for UFH was 1.2 pM, R was not found significantly delayed until platelets were below 30×10^9/L relative to 150×10^9/L ($p > 0.05$) (Fig. 10A). For dalteparin the TF was 0.7 pM, R was not found significantly delayed until platelets were below 20×10^9/L relative to 150×10^9/L ($p < 0.05$) (Fig. 11B). In the presence of fondaparinux, 0.07 pM TF was chosen, R was not found significantly delayed until platelets were below 20×10^9/L relative to 150×10^9/L ($p < 0.05$) (Fig. 11C). When TF was not added into clots formed in the presence of either rivaroxaban or dabigatran, R was not found significantly delayed until PPP relative to 150×10^9/L ($p < 0.05$), therefore no TF was used for these anticoagulants (Fig. 11D,E).

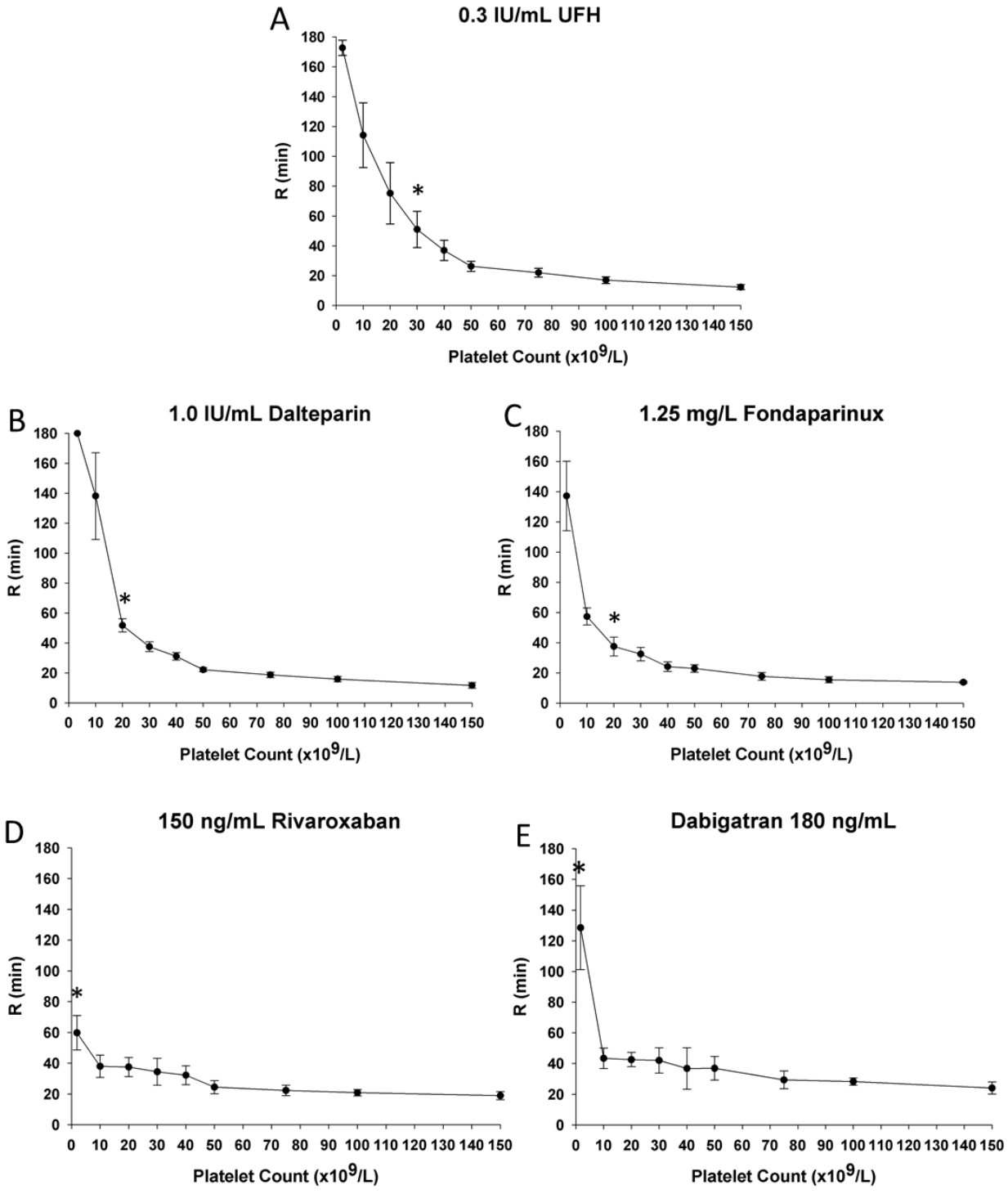

Figure 11: Modelling of TEG profile for anticoagulants with TF at concentration individualized for each individual anticoagulant. (A) UFH, (B) dalteparin, (C) fondaparinux, (D) rivaroxaban, (E) dabigatran. (*) significant ($p < 0.05$) relative platelet count of 150×10^9/L.

4.3 Comparison of TEG profile among anticoagulant using single concentration of TF

In the presence of any anticoagulant being evaluated in this study, tissue factor at 1.2 pM initiated clot formation in plasma containing 150×10^9/L platelets with similar R (Fig. 12). With UFH at 0.3 IU/mL, R progressively prolonged when platelet count was lower than 150×10^9/L and the increase in R was exponential when platelet count was lower than 50×10^9/L. Comparing with UFH, dalteparin induced less prolongation of R when platelet count was lower than 50×10^9/L. Both UFH and dalteparin delayed R significantly longer than fondaparinux, rivaroxaban and dabigatran when platelet count was lower than 100×10^9/L ($p < 0.05$). There was no significant difference in R between fondaparinux, rivaroxaban and dabigatran across all platelet counts($p > 0.05$).. However, all anticoagulants significantly prolonged R compared to the control at all platelet counts ($p < 0.05$).

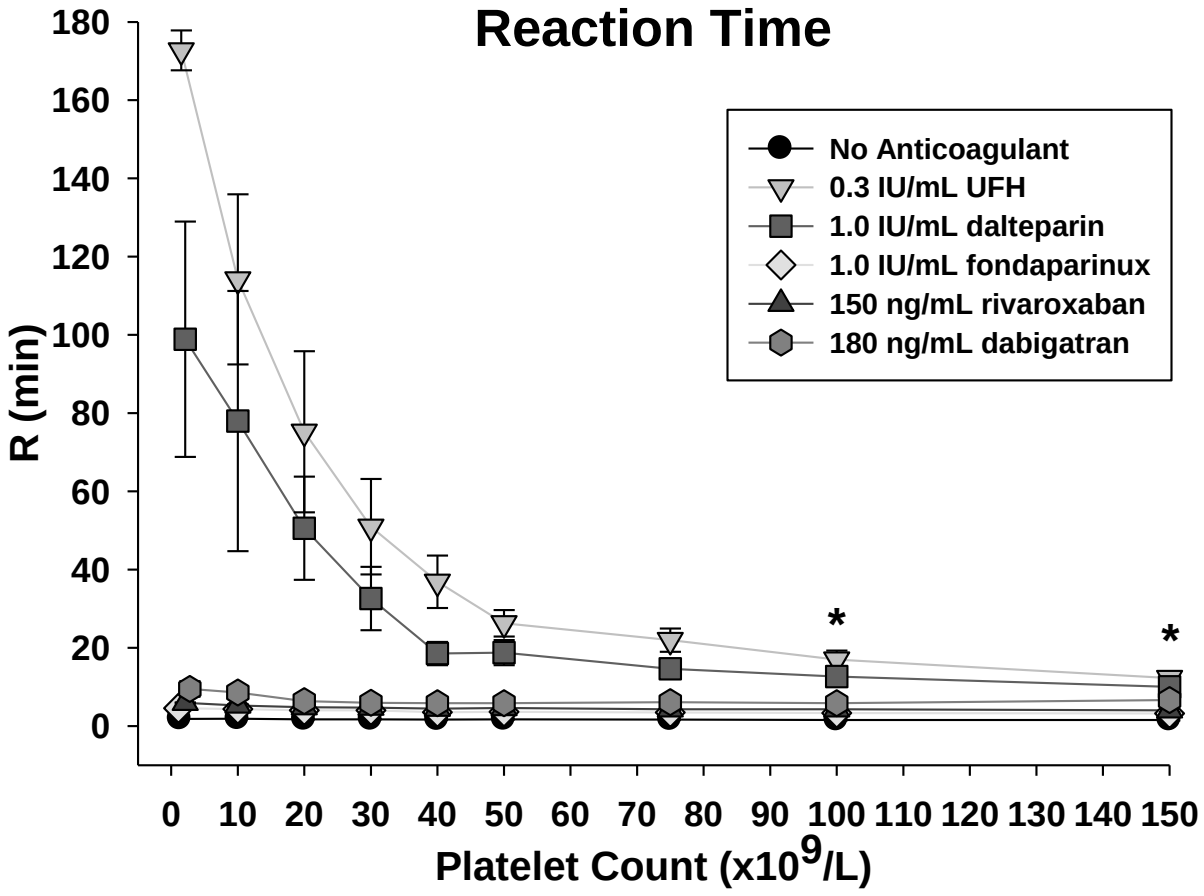

Figure 12: Direct comparison of anticoagulants effect on R at 1.2 pM TF. Control: no anticoagulant. (*) significant ($p < 0.05$).

4.4 TMA of plasma clotted in presence of anticoagulant and 1.2 pM TF

The profiles of the TMA results have a related trend to the R results, where fondaparinux, rivaroxaban and dabigatran were more similar to the control than UFH and dalteparin (Fig. 13). However, TMA values tended to be more variable overall. In the presence of anticoagulant, the TMA values across all platelet counts were significantly higher than the control ($p<0.05$). Comparing to the platelet count at 150×10^9/L, there is a significant increase in TMA when platelet counts fall below 10×10^9/L for UFH and dabigatran and below 30×10^9/L for

dabigatran ($p<0.05$). There was no significant difference noticed across all platelet counts in the presence of dalteparin and fondaparinux.

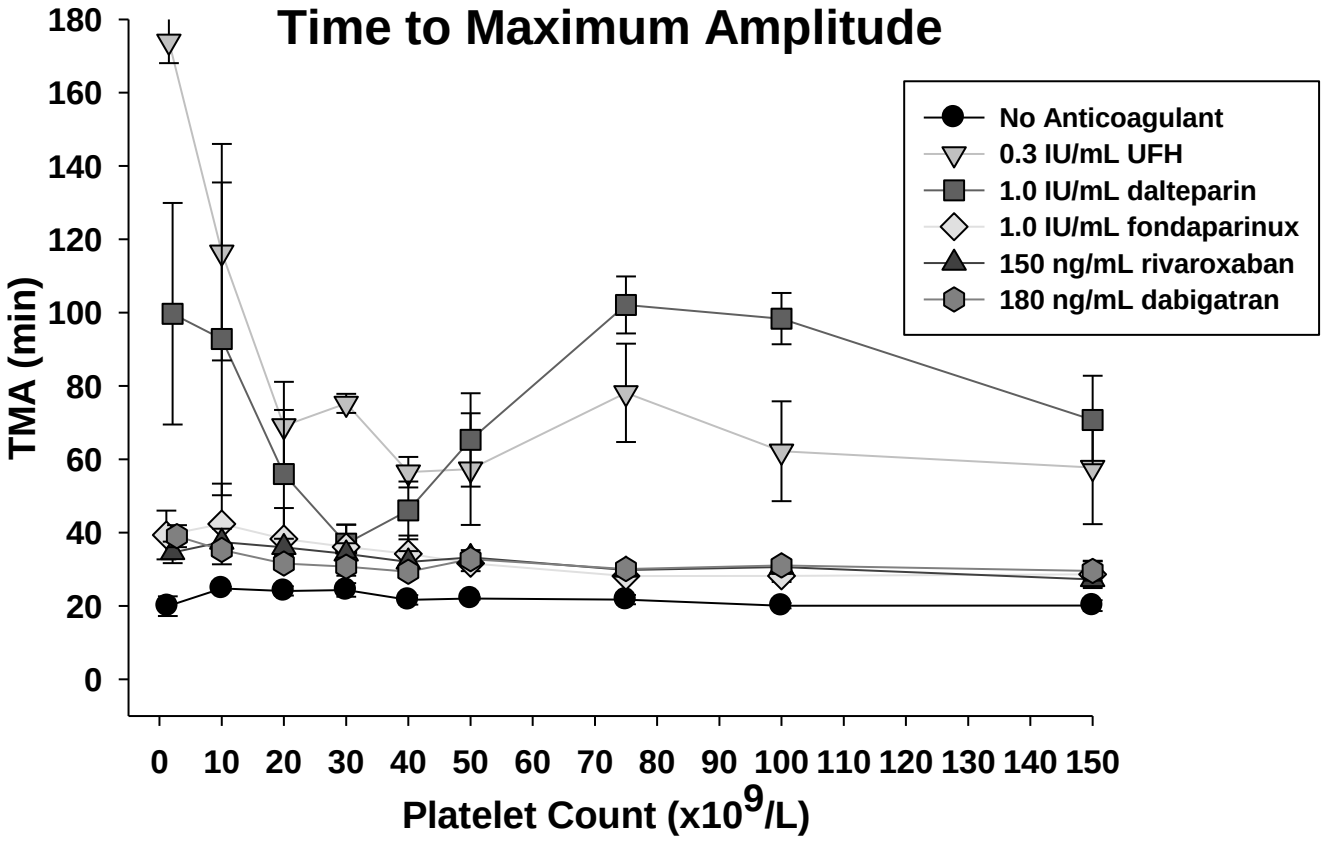

Figure 13: Direct comparison of anticoagulants effect on TMA at 1.2 pM TF.

4.5 MA of plasma clotted in presence of anticoagulant and 1.2 pM TF

There is an almost linear relationship between the platelet count and MA in all settings overall, as the platelet count decreases, the MA also decreases (Fig. 14). In the control setting and in presence of fondaparinux, rivaroxaban and dabigatran respectively, there is no significant difference in MA until platelet count decrease to 50×10^9/L comparing to 150×10^9/L ($p<0.01$). When platelet counts where at 75×10^9/L and 100×10^9/L, clots formed with UFH and

dalteparin respectively, had MA values that were significantly lower than at platelet counts of 150×10^9/L (p<0.01). There was no significant difference in MA across all platelet counts between clots that were formed in the presence of no anticoagulants, fondaparinux, rivaroxaban and dabigatran. The UFH and dalteparin settings showed no significant difference between each other across all platelet counts. However, the UFH and dalteparin settings both had significantly lower MA across all platelet counts compared to the control, fondaparinux, rivaroxaban and dabigatran settings (p<0.01).

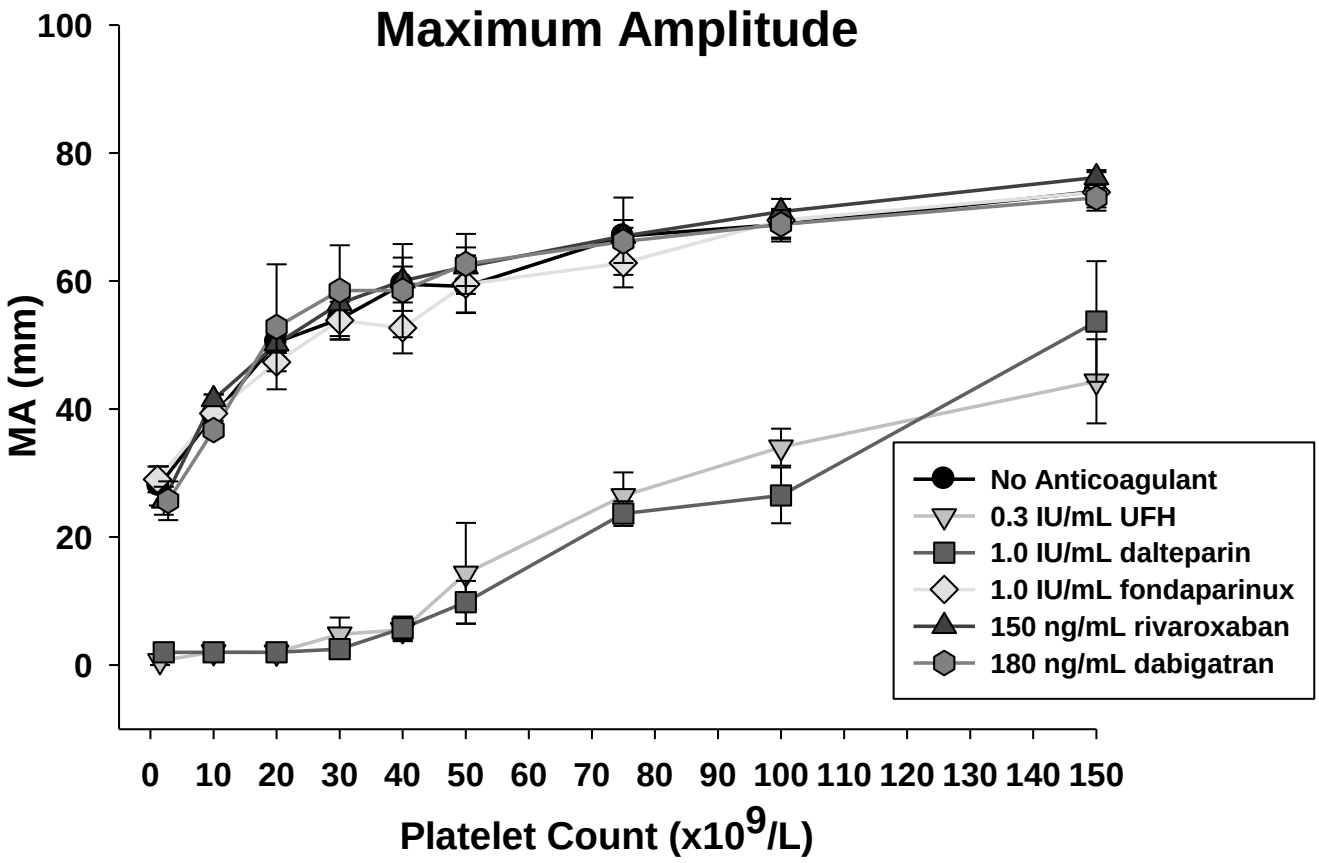

Figure 14: Direct comparison of anticoagulants effect on MA at 1.2 pM TF.

4.6 AUC15 of plasma clotted in presence of anticoagulant and 1.2 pM

Similar to the MA measurements, the AUC15 data shows that there are two distinct groups of almost linear data (Fig. 15). One group is clustered near the control, which includes fondaparinux, rivaroxaban and dabigatran. UFH and dalteparin have significantly lower AUC15 measurements ($p<0.01$) across all platelet counts compared to the other settings. Compared to platelet count at 150×10^9/L, there is a significantly lower AUC15 value when platelet counts decrease to 75×10^9/L in all settings with and without anticoagulant.

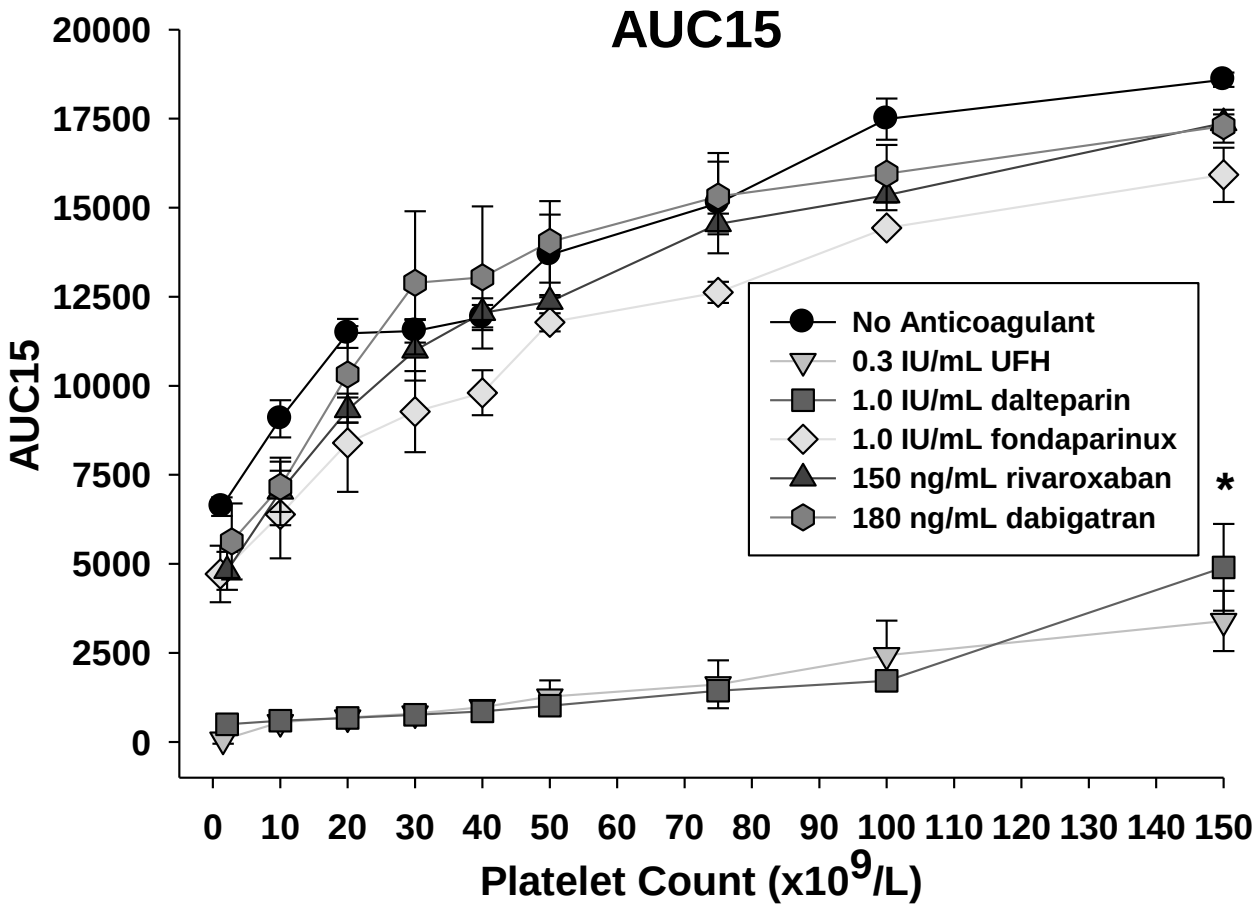

Figure 15: Direct comparison of anticoagulants effect on AUC15 at 1.2 pM TF. (*) significant ($p < 0.05$).

4.7 SEM of plasma fibrin clot structures in the presence of anticoagulants

SEM images were taken of clots formed in a static system on a Millipore membrane surface. One representative image of several taken of clots at each anticoagulant setting with different platelet counts was placed in an overall summary figure (Fig. 16). Quantitatively, the pore area was shown to increase significantly as platelet count decreased from150 x 10^9 /L to PPP in the presence of UFH, dalteparin and dabigatran ($p<0.05$) (Fig.17). In the presence of either fondaparinux, rivaroxaban or no anticoagulant, there was no significant change in pore area when the platelet count is altered. However, as platelet count decreases from 150 x 10^9 /L to PPP the number of fibers in all anticoagulant setting decrease significantly ($p<0.05$) (Fig. 18). Clots formed in the presence of UFH and dalteparin have the most fibers at 150 x 10^9 platelet/L, but show a drastic decrease in fiber number as platelets decrease relative to the other anticoagulant settings. In the presence of UFH and dalteparin, the fibrin widths of the clot had a larger abundance of thicker fibers than all anticoagulant setting at PPP (Fig. 19). Even at 30 x 10^9 platelets/L, UFH showed an increases in the distribution of thicker fibers compared to a platelet count of 150 x 10^9 /L. Dabigatran also showed a minor increase in fiber width at PPP. The distribution of fiber width did not seem to change in the presence of fondaparinux, rivaroxaban or the no anticoagulant control when platelet count is decreased.

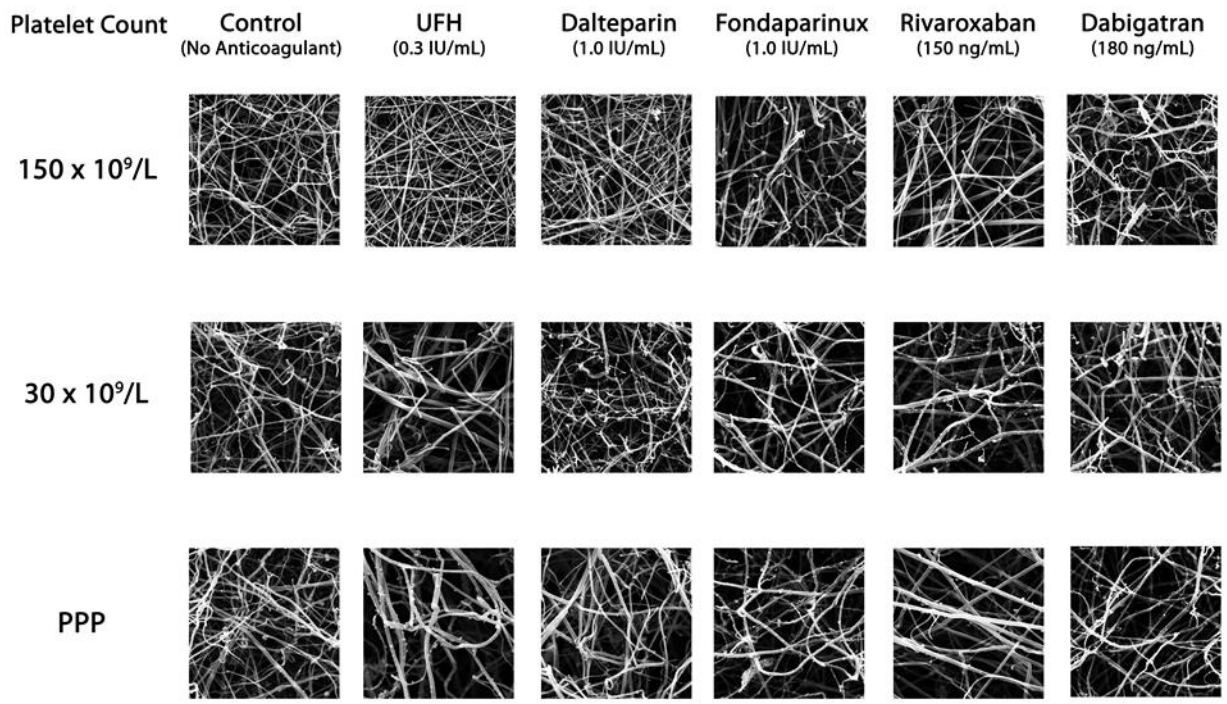

Figure 16: SEM micrograph comparison of anticoagulants effect on clot structure at 1.2 pM TF. Representative image at each anticoagulant setting with 150, 30 x 10^9 platelets/L and PPP. Magnification at 20, 000 ×, observing clot formation after 3hour incubation on Millipore membrane.

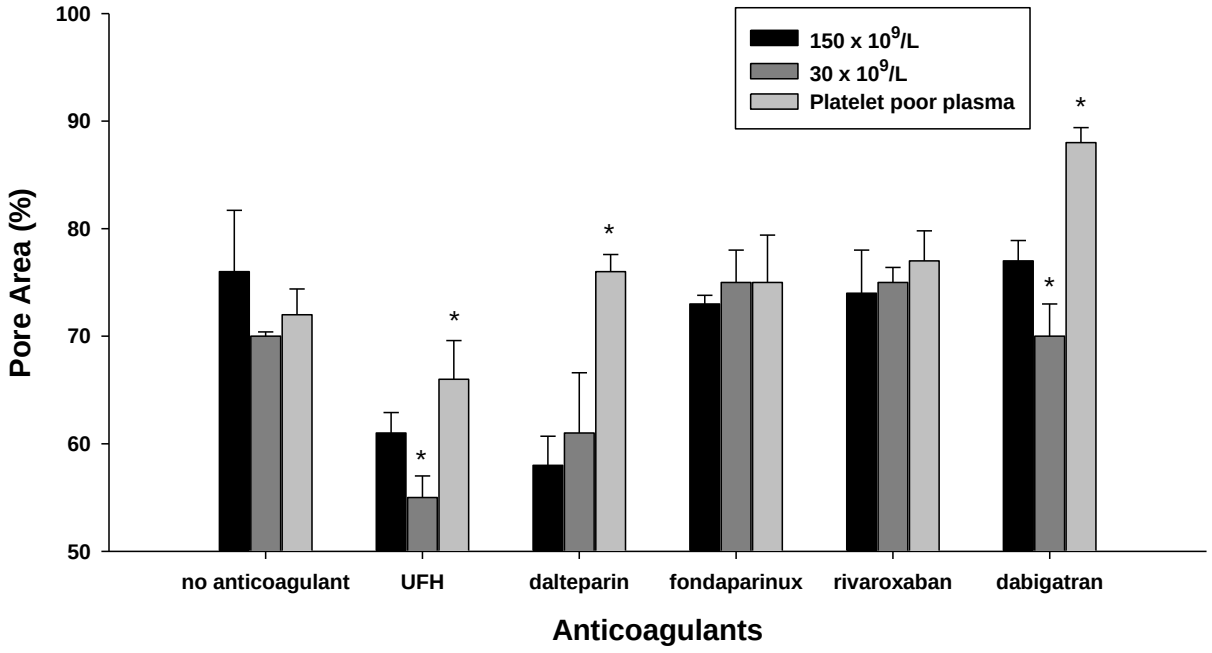

Figure 17: SEM quantification summary of results for pore area. (*) represents significance of at least (p<0.05) compared to values at platelet count of 150.

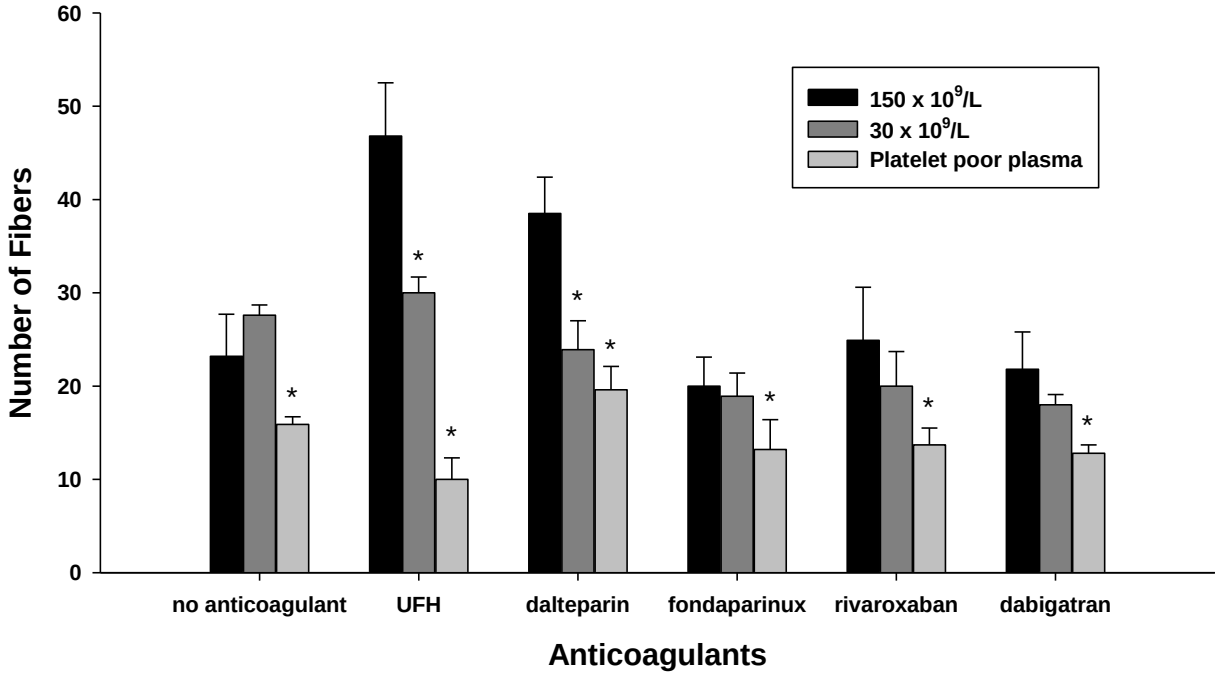

Figure 18: SEM quantification summary of results for fiber count. (*) represents significance of at least (p<0.05) compared to values at platelet count of 150.

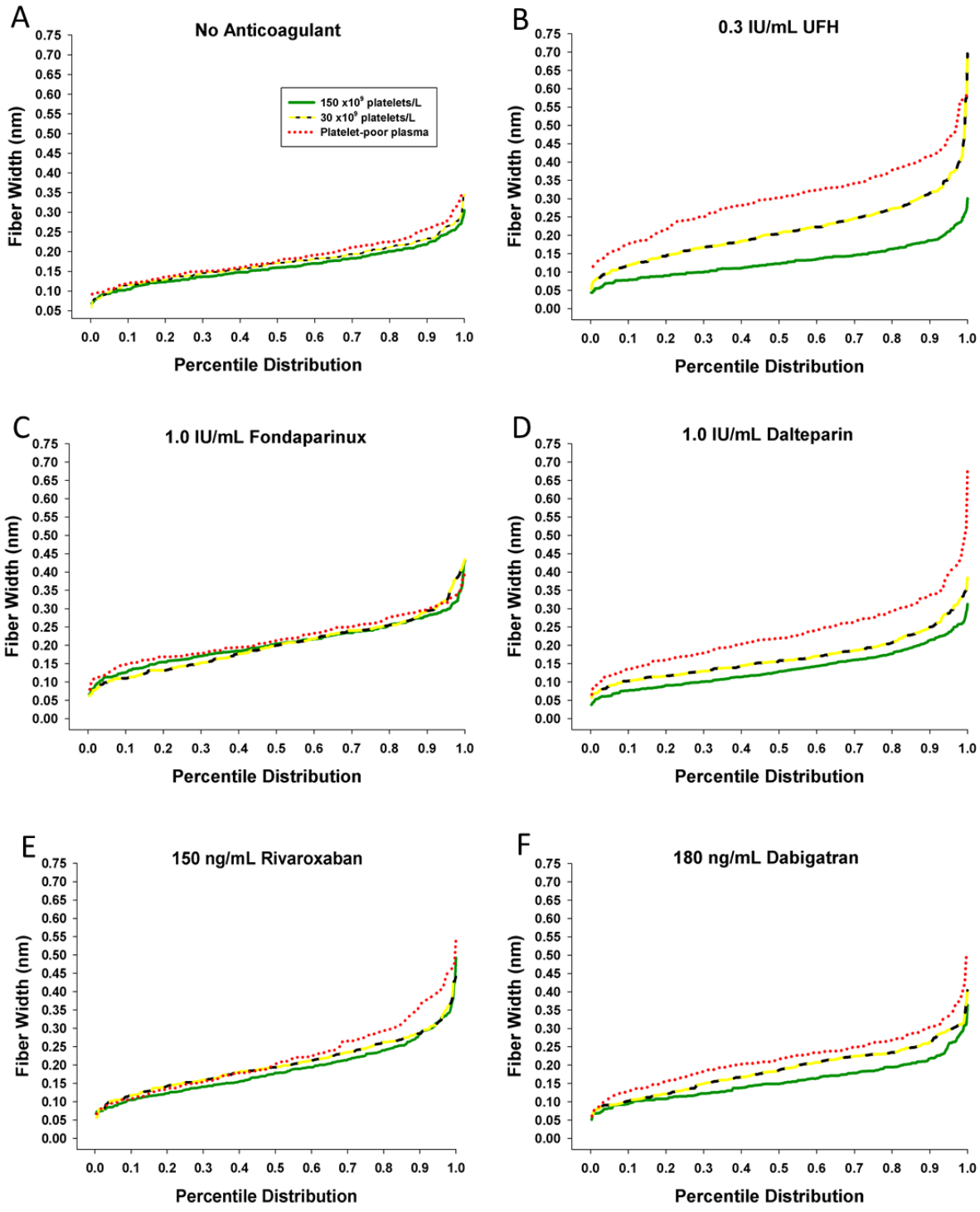

Figure 19: Quantification of SEM micrographs for fiber width distribution at 150×10^9, 30×10^9 and platelet poor plasma. (A) No anticoagulant control, (B) 0.3 IU/mL UFH, (C) 1.0 IU/mL fondaparinux, (D) 1.0 IU/mL dalteparin, (E) 150 ng/mL rivaroxaban, (F) 180 ng/mL dabigatran.

5. Discussion

Guidelines for anticoagulant use during antithrombotic therapy in patients with concomitant thrombocytopenia are based on expert opinion and mainly extrapolated from case reports or small retrospective studies in the literature[108]. In the past, 50×10^9 platelets/L was considered the safety threshold for anticoagulant use [109;110]. However, more recent guidelines suggest that antithrombotic therapy may be safe unless platelets are less than 30×10^9/L[111]. However, there is an absence in good quality data to support these practices. The purpose of this study is to evaluate the clot formation in plasma with platelets that are less than 150×10^9/L. Although the results of this study cannot directly be extrapolated to validate the current clinical practice, the information may provide insight regarding the effect of various anticoagulants on the clotting process of plasma in the presence of suboptimal platelet counts. Our results have shown that UFH compromised TEG clotting parameters the most out of all the anticoagulants, particularly at very low platelet counts. In addition, the SEM results revealed that the greatest effect of platelets on clot structure occurred in the presence of UFH.

The effects on clotting measured by TEG in our assay displayed differences among each of the anticoagulants, especially when platelet counts reached critically low levels. Each anticoagulant has its own unique mechanism of action. UFH anticoagulant function relies on its ability to bind to and enhance AT activity[112]. A specific pentasaccharide sequence within the UFH is required to bind to the AT[112]. UFH-enhanced AT is capable of inhibiting mainly IIa and Xa equally, in addition to IXa, XIa and XIIa[46]. IIa inhibition by UFH and AT is dependent on having UFH molecules with a minimum chain length of 18 saccharides, which serves as a template that also binds IIa, increasing inhibition 1,000-fold faster[112]. The ability of UFH and AT to inhibit multiple coagulation factors, especially the key factors IIa and Xa, within the

coagulation cascade may help to explain why we see such a potent anticoagulant effect in our *in-vitro* plasma assay. When both IIa and Xa are suppressed, this prevents downstream propagation of coagulation including furthering IIa generation, fibrinogen to fibrin conversion and IIa-mediated platelet activation[17]. Moreover, UFH is able to reversibly interact with AT, thus when the AT – heparin complex inhibits a clotting factor, the AT forms a covalent bond with that factor [113]. The heparin component can then dissociate from the AT ternary complex and activate another AT resulting in successive inhibition reactions dependent on the amount of AT available within the plasma[113]. The tested concentrations of TF less than 1.2 pM in this assay resulted in no clot formation in the presence of UFH at all platelet counts. This suggests that when UFH is present, a minimum concentration of TF may be critical to initiate clotting. Possibly, if a TF concentration threshold is not met, there will not be enough coagulation factors activated through the extrinsic pathway[17]. A probable reason for this competitive threshold effect between UFH and TF to either shut down or initiate clotting could be due the fact that UFH and TF are mixed into the plasma at the same time in this assay. If TF was added first, it is probable that the plasma would clot. However if UFH was allowed to incubate with the plasma, it can be speculated that even adding the 1.2 pM TF afterwards, may not initiate the clotting process. Clotting measured by the TEG in the presence of UFH also showed significant ($p < 0.01$) delay in R when platelets were below 30×10^9 /L at 1.2 pM TF relative to 150×10^9 /L. It seems that in addition to requiring a certain amount of TF in order to clot, there is also a threshold for platelet count to maintain R values. Platelets play a vital role within coagulation and have shown to have an impact on most TEG parameters beyond MA, especially at lower concentrations [114]. Clotting in the presence of UFH required the highest TF concentration. At 1.2 pM TF, the UFH conditions required platelet counts above 30×10^9 /L to maintain R values similar to that of 150×10^9 /L,

which is the highest platelet amount used for all anticoagulant conditions. This fact reveals the potency of UFH within an *in-vitro* plasma setting to disrupt clotting. Therefore, UFH shows the greatest impact on clotting parameters which may be attributed to the dependency on TF and platelet concentration.

LMWH such as dalteparin, are smaller fractions of heparin molecules[57]. AT binding with LMWH still requires the specific pentasaccharide sequence[115]. LMWH has decreased affinity for IIa inhibition compared with UFH at an estimated ratio around 3:1[115]. Fondaparinux has even more specificity towards Xa inhibition. Fondaparinux is small synthetic heparin molecule that contains the AT-specific pentasaccharide sequence with no additional saccharide side-chain. Therefore, fondaparinux has almost exclusive inhibition of Xa over IIa compared with UFH or LMWH[115]. The decreasing IIa-inhibitory activity of LMWH and fondaparinux may provide support for our finding that clot formation at 150×10^9 platelets/L required a lower concentration of TF than UFH. Fondaparinux, required far less, compared to both UFH and LMWH. Any IIa generated from the extrinsic pathway may not be efficiently inhibited by either LMWH or fondaparinux, thus IIa may still propagate downstream action within coagulation. At 1.2 pM TF, clots formed in the presence of dalteparin, with R values that were significantly delayed when platelet counts were below 20×10^9 /L, while in the presence of fondaparinux significant delay was only seen in PPP. These platelet counts were both lower than what were observed in the presence of UFH. This further shows that UFH has a greater impact on clotting in this assay because LMWH was able to clot at 150×10^9 /L with a lower TF concentration and required a lower platelet threshold at 1.2 pM TF. Out of the heparinoids, fondaparinux had the least impact on clotting parameters.

Since UFH, LMWH and fondaparinux require AT for their anticoagulant action, they are defined as indirect inhibitors. In addition, fondaparinux can be described as an indirect factor-specific anticoagulant, a small amount of IXa can be inhibited by fondaparinux as well[116]. This is in contrast to the direct factor-specific anticoagulants such as rivaroxaban and dabigatran. The factor specific anticoagulants specifically bind and inhibit a single coagulation factor. Rivaroxaban inhibits Xa, while dabigatran inhibits IIa[117;118]. We observed that clotting was initiated without the use of exogenous TF in settings that contained rivaroxaban and dabigatran. These settings were highly influenced to any concentration of TF used in this assay, and clotting was rapid across all platelet counts. A possible explanation for the profound effect of TF may be due to the anticoagulants ability to inhibit only a single coagulation factor. This might allow the extrinsic pathway to more easily by-pass the anticoagulant effect, if there are any free unbound IIa or Xa. In addition, the heparinoids are able to dissociate from the ternary AT complex once bound to the coagulation factor, able to form a new complex with free AT[113]. Therefore anticoagulation is limited by the amount of rivaroxaban or dabigatran present with direct factor specific anticoagulants, while the heparinoids' limiting factor is AT availability. All together this suggests that in our *in-vitro* model, increasing the specificity of an anticoagulant, result in an increased effect by TF initiation. However, in contrast to our study, clinical uses of these anticoagulants have very similar result. There may be more complex interactions that may influence the effectiveness or sensitivity of these anticoagulants to TF, including the presence of the endothelium. Also, how these anticoagulants react in actual thromboembolic patients compared to the normal healthy donor plasma used in this assay may contribute to differences, which requires additional investigation.

To mimic clotting according to the cell-based coagulation pathway, TF was used to initiate the clotting process[16]. The amount of TF circulating in blood has been documented in literature. Many studies have used immunoassays to detect both active and inactive TF and have reported a plasma concentration of approximately 0.5 pM[18]. In contrast, activity-based assays have shown that active TF was also present in the circulation at a concentration of approximately 0.02 pM[18]. This study also showed that activated platelets and resting monocytes in the circulating blood did not contribute TF for the initiation of clotting, which may lead to underestimation of the true circulating TF levels. On the other hand, others have shown that specific situations such certain malignancies, which may actually increase circulating TF levels through increased surface expression of TF on activated platelets and monocytes[119;120]. Normally, healthy endothelium prevents local TF initiation by expressing TF-pathway inhibitor (TFPI)[121]. However, at the site of trauma, the amount of active TF and TF bearing microparticles may increase and can overwhelm the TFPI mechanism[121]. Other examples of increased sources of TF include disease states such as sepsis which can cause increased TF expression on monocytes, or cancerous cells express TF in almost all types[122;123]. Formation of microparticles from these cells will make the TF more available in the circulation[122]. In our study, we used TF concentrations between 0.07-1.2 pM to initiate clotting in the presence of UFH, dalteparin and fondaparinux. No additional exogenous TF was required for rivaroxaban and dabigatran. In PPP, the clot formation was relatively preserved despite the presence of rivaroxaban and dabigatran because R was not delayed more than 100 minutes while the other parameters (TMA, MA, and AUC15) remained similar to 150×10^9/L. However, when the PPP was filtered using a 0.2 um Millipore membrane; these anticoagulants inhibited clotting beyond 180 min. This may suggest that TF provided by microparticle formation was sufficient to override the inhibition by these

factor specific anticoagulants and initiate the clotting process. In order to directly compare the anticoagulants at estimated therapeutic plasma steady state levels, it was essential to fix the TF concentration at a particular concentration. We chose 1.2 pM as this allowed UFH to still clot at 150×10^9/L. The R time, TMA, MA and AUC 15 were the chosen parameters used as they provided the most consistent measurements.

The R parameter is one of the more significant and widely used of the TEG parameters to represent clotting time. Although similar in concept, TEG R has shown a weak correlation with traditional diagnostic clotting assays such as prothrombin time, partial thromboplastin time and activated clotting time[124]. However, TEG is able to use cellular components of blood, such as platelets, to further recapitulate *in-vivo* conditions, something the other tests are unable to do. In addition, the type and amount of initiators may vary with different assays and this may also contribute to the weak correlations between the TEG and other tests. Based on our study, we determined that the R value was significantly compromised when platelet counts were $< 30 \times 10^9$/L for clots formed in the presence of UFH. The R for dalteparin was impaired when platelet levels were $\leq 20 \times 10^9$/L. Fondaparinux, rivaroxaban and dabigatran had little effect on the clotting time relative to 150×10^9/L except at PPP. There was also a slight delay in R that was significant ($p < 0.05$) relative to the no anticoagulant control across all platelet counts. TMA shows a similar profile to R values but had much higher variability, particularly at the lower platelet counts.

MA represents the overall strength of the clot, closely influenced by fibrin content and platelets. In the literature, it has been shown that MA is linearly correlated to platelet count. As platelet count decreases, the MA will also decrease. This is consistent with our study for all anticoagulant and control settings[125]. UFH and dalteparin each caused MA values to be more

than 50% lower than MA's found in clots formed with fondaparinux, rivaroxaban, dabigatran and no anticoagulant at all platelet counts. The AUC15 measures the physical area of the TEG tracing profile 15 minutes after R has been achieved[126]. AUC15 was developed to rapidly measure individual patient response to anti-platelet drugs. There appears to be no difference between taking measurements of the AUC at 60 min versus at 15 min[126]. Since platelet function is correlated with MA, finalizing the MA value could take a considerable amount of time in the presence of anti-platelet drugs[127]. AUC15 is proposed to help mitigate this delay in obtaining information and also the inclusion of slightly more information over MA itself, as it takes into account the speed of clot strengthening as well. Regardless, AUC15 had a comparable trend in this study to the MA, where there were two distinct groups of data. One set that followed closely to the no anticoagulant control results across all platelet counts, and a second group which was significantly lower than the control for its respective anticoagulant. This second group included samples with UFH and dalteparin. UFH and dalteparin had the most distinct effect on AUC15 and MA, severely compromising the clotting strength compared to the control even at 150 × 10^9/L. In addition to severely delayed R values, UFH and dalteparin settings had very weak clot strengths at lower platelet counts.

The K and alpha (α) values were not included in this study. The K and α values represent clot kinetics and provide information regarding the speed of clot formation. The K measures the amount of time required for the clot to reach certain strength. While α measures the angle created between the R and K values. In many cases during these experiments, K was not obtainable when the strength of the clot did not meet the specific absolute value. In addition, since α is dependent of K, α may also be an unreliable measurement.

Having shown that platelets reduced viscoelastic strength of clots, in a mostly linear fashion in the presence of an anticoagulant, we next examined the surface architecture of these clots. Millipore membranes were used as a mounting medium, allowing firm adhesion of the clots to the membrane surface during the washing procedure to remove the coaggregation of proteins[128]. Different pore sized membranes were tested but 0.025 μm was found to be optimal for the clot formation without distorting the shape of the clot and maximizing adhesion to membrane. Despite extensive washing steps, a matte-like layer occasionally formed on the surface of the clot and this was a well-known issue when preparing SEM samples (personal communication). We incorporated an extra step to gently peel off the matte–like layer with Parafilm™ that was peeled to the samples with consistent pressure among the various experimental settings. To avoid subjective bias, the investigator in charge of imaging and analysis was blinded to each of the settings.

The three characteristics of the clots surface architecture chosen to quantify included porosity, fiber number and fiber width. These are fairly well established parameters for characterizing fibrin clot formations; however the techniques used to quantify these measurements vary. Porosity has been measured visually using imaging programs or physically through flow experiments measuring permeability[129].. Porosity may give an indication of the clot structure's susceptibility to fibrinolysis[130]. Fiber number and fiber width may give more information regarding the actual structure of the clot. Thrombin converts fibrinogen to fibrin, as such, the amount of IIa available has a large influence on how the final fibrin strands and ultimately the whole structure of the clot develops[131]. When there is less IIa, the fibrin strands tend to become thicker and form a less dense network as there are smaller abundance of actual strands[131]. If there is an increase in IIa present, the fibrin strands become thinner, more abundant

and form a denser network. There are also other factors that can affect the architecture of the fibrin clot such as pH and blood flow[132].

Although we acknowledge that fibrin network characteristics are different under flow compared to a static system, there is still important information that we can compare between the effects of each anticoagulant relative to the assay being used [133]. The analysis of the SEM samples revealed that there were minimal changes to the control setting as platelet count decreased except for the number of fibers. In all other sample settings, as platelet count decreased the number of fibers also decreased as well, however the major difference from the control was that at PPP the fibers were more noticeably thicker than the control. As the platelet count drops, there may be a reduction in available surface to generate vast amounts of thrombin. Less available thrombin results in fibers which are thicker in addition to having a less dense fibrin network[130]. The opposite is true when there are excessive amounts of thrombin. Thus in the presence of anticoagulants, combined with the low platelet count, may contribute to the higher distribution of thicker fibers in the presence of anticoagulants compared to the control. UFH and dalteparin showed the greatest effect on the clot compared to all other settings. UFH had the highest number of fibers at a platelet count of $150x10^9$/L but the lowest at PPP and had a higher distribution of thicker fibers also at PPP. Dalteparin showed a similar effect but to a lesser degree. Fondaparinux, rivaroxaban and dabigatran were more similar to each other than to UFH or dalteparin. The degree to which each anticoagulant affects different characteristics of the clot in SEM is similar in nature to the varying effect of each anticoagulant on TEG clotting parameters. UFH had the most noticeable effect on clotting, followed by dalteparin, while fondaparinux, rivaroxaban and dabigatran more closely resembled each other and shared some characteristics to the control. This may support the noticeable trends seen in terms of differences

between each anticoagulant's effects on clotting in TEG. There are two observations within the SEM study that may not have been noticed in the TEG work. First is that in the UFH and dalteparin groups at 150×10^9/L had the highest number of fibers out of all settings. Second, the anticoagulants capable of IIa inhibition (UFH, dalteparin and dabigatran), showed significant change in pore area as platelet count decreased. In the first observation, although UFH and dalteparin were the more potent anticoagulants, at 150×10^9/L, they seemed to form clots with characteristics of a procoagulant clot[134]. Since this SEM study was performed using a static assay, opposed to a more dynamic system in the TEG, the results may not be directly comparable. However, what actually contributes to the increased fiber number and denser network seen in this observation in the presence of UFH and dalteparin is not well understood and may require additional studies. The second observation showed significant increase in pore area as platelet count decreased relative to 150×10^9/L in the presence of UFH, dalteparin and dabigatran. Rivaroxaban, fondaparinux and the no anticoagulant control did not have any significant changes in pore area across all platelet counts. Coincidently, UFH, dalteparin and dabigatran have IIa inhibitory capabilities. Therefore in these settings, IIa could be a determining factor responsible for changing the pore area. A comparison of thrombin generation in future studies may clarify this.

While the R-value is influenced mainly by the activation of coagulation factors and the initial fibrin build up, α and MA parameters are mostly influenced by the fibrin meshwork and platelet levels[135]. Also, since the AUC15 incorporates both α and MA, it is expected that the AUC15 will also be influenced by fibrin and platelets[126]. In addition, fibrin strand structure is altered in the presence of anticoagulants. Anticoagulated clots are characterized by a loose conformation and less branched fibrin meshwork, and the strands become thicker and more

porous. As a result, the structure will be more susceptible to fibrinolysis[136]. The results gained from TEG experiments, as well as our SEM analysis, could be explained by the efficiency of heparionds, particularly UFH and dalteparin to reduce thrombin generation, which in turn reduces adequate fibrin monomer formation, factor XIIIa-dependent fibrin-crosslinking, and thrombin-PAR1/4-mediated platelet activation[137]. When platelet activation is affected, it reduces the availability of the phospholipid surface for clotting, which reduces formation of coagulation complexes and subsequently coagulation propagation. It also decreases thrombin-dependent PAR-1 mediated activation of GP-IIb/IIIa, which is responsible for fibrin(ogen) interaction and clot growth[137].

5.1 Limitations/Future Directions

Our study provides experimental data for plasma clot formation with low platelets and the presence of anticoagulants. However, our *in-vitro* model contains some limitations. For example, the model does not account for the interaction of endothelium, red blood cells and higher shear forces found in the blood stream. Yet, this study isolated and examined the interaction between the two most important factors in hemostasis, plasma clotting proteins and platelets in the presence of anticoagulants. Moreover, the concentrations of anticoagulants examined in our study were in the therapeutic range. The effects of anticoagulants at prophylactic intensity on the plasma clotting with subnormal platelets await further investigation in the future. Another aspect that we were not able to account for at the time was the actual contributions of the intrinsic pathway to our clotting system. Even though we inhibited FXIIa activity with CTI in order to focus on TF/extrinsic pathway activation, downstream factors in the intrinsic pathway such as IXa may still affect the various clotting parameters. One way to solely focus on the TF pathway and systematically find out the relative contribution of each

downstream intrinsic factor is to obtain plasma that is deficient in a particular factor. Another issue to consider is that although citrated blood offers consistent and stable blood samples that can be used in various assays, it may also adversely affect platelet GPIIb/IIIa found on the surface of platelet membranes, which may adversely affect platelet activation/aggregation[138;139]. Therefore, there may be a need in the future to use different chelating agents that reduce the adverse effects on cellular components during clotting.

5.2 Conclusion

In conclusion, this study showed that, in the presence of low platelets, anticoagulants can jeopardize clot formation. The platelet threshold where clot formation is impaired depends on the type of anticoagulant. Based on this TF-initiated clotting model, AT-dependent anticoagulants compromised plasma clotting more than the newer factor specific anticoagulants, possibly related to the multiple, non-specific inhibition of factors within the coagulation pathway. When platelets were critically low, the impact of thrombin inhibition may be more potent than FXa inhibition.

6. Reference List

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