A THROMBOELASTOGRAPHY STUDY ON THE EVALUATION OF CLOT FORMATION IN PLATELET-DEPLETED WHOLE BLOOD IN THE PRESENCE OF UNFRACTIONATED HEPARIN OR LOW-MOLECULAR WEIGHT HEPARIN

By

JASON CHUNG, B.Sc.

A Thesis

Submitted to the School of Graduate Studies

in Partial Fulfillment of the Requirements

for the Degree

Master of Science

McMaster University

© Copyright Jason Chung, 2015

MASTER OF SCIENCE (2015) McMaster University (Medical Sciences) Hamilton, ON

TITLE: A thromboelastography study on the evaluation of clot formation in platelet-depleted whole blood in the presence of unfractionated heparin or low-molecular weight heparin

AUTHOR: Jason Chung, B.Sc.

(Honours Life Science, McMaster University)

SUPERVISOR: Dr. Howard Chan

NUMBER OF PAGES: viii, 71

# ABSTRACT

The use of an appropriate anticoagulation regiment for the treatment of venous thromboembolism (VTE) in patients with concomitant thrombocytopenia is based on anecdotal evidence and the opinions of managing physicians. The current guidelines suggest that therapeutic levels of anticoagulants may be safely administered to patients who have a minimum platelet count of 50 x 109/L. However, it has recently been suggested that the minimal platelet threshold for safe anticoagulation treatment can be provided at a reduced platelet count of 30 x 109/L. Thus, in order evaluate these platelet threshold we used a thromboelastography (TEG) model to evaluate the clotting parameters of whole blood at predefined platelet counts in the presence of unfractionated heparin (UFH) and low-molecular weight heparin (LMWH). Due to the importance of red blood cells on hemostasis a whole blood TEG model was designed in order to mimic *in vivo* hemostasis. Clotting was initiated using different concentrations of tissue factor for each anticoagulant at therapeutic and prophylactic levels of UFH and LMWH at predefined platelet counts. In the presence of therapeutic concentrations of either UFH or LMWH, there were no significant differences in TEG parameters of whole blood clots between platelet counts of 30 x 109/L and 50 x 109/L when clotting was driven by the extrinsic pathway. At prophylactic levels of LMWH clot formation was less compromised. Furthermore, no significant difference was noted between platelet-depleted blood (PDB; <10 x 109/L) and 30 x 109/L with respect to r-time. This suggests LMWH at prophylactic levels has no significant bearing on clot formation at a lower platelet threshold versus therapeutic levels of LMWH. Overall, it shows that clot formation is similar for UFH and LMWH when platelet counts are reduced from 50 x 109/L to 30 x 109/L. This work provides insight on the potential for anticoagulation at a reduced platelet threshold in thrombocytopenic conditions.

# ACKNOWLEDGEMENTS

There are numerous people that I would like to thank for helping me to achieve this goal. My supervisor, Dr. Howard Chan, for the support and guidance he provided throughout my Master’s project. You have been an a great mentor and an awesome supervisor whose always willing to go out of their way to help during late hours and weekends and even on clinical duties to help me reach my goals. My committee members, Drs. Anthony Chan and Bill Sheffield for their valuable suggestions and direction with both clinical and basic sciences. Leslie Berry and Helen Atkinson for those late nights and chats in the lunchroom to help me to develop as a researcher and a person. My friend and mentor Dr. Ivan Stevic for always checking up on my progress and providing me with guidance all the way from Winnipeg. My fellow lab mates Jorell Gantioqui, Laura Calvert and Gabriela Chang for keeping me sane during those late night experiments and with all those technical assistances. Lastly, to my family and friends, who were always cheering me on and always asking me the exact same question “what is an anticoagulant?” to keep me focused. Thank you everyone for the support in helping me finish my degree.

# TABLE OF CONTENTS

[**ABSTRACT** ii](#_Toc419941871)

[**ACKNOWLEDGEMENTS** iii](#_Toc419941872)

[**TABLE** **OF** **CONTENTS** iv](#_Toc419941873)

[**List** **of** **Abbreviation** vii](#_Toc419941874)

[**1** **Introduction** 1](#_Toc419941875)

[1.1 **Overview** 1](#_Toc419941876)

[1.2 **Coagulation** **Cascade** 2](#_Toc419941877)

[1.2.1 Initiation 3](#_Toc419941878)

[1.2.2 Amplification 4](#_Toc419941879)

[1.2.3 Propagation 5](#_Toc419941880)

[1.2.4 Termination 6](#_Toc419941881)

[1.3 **Heparinoids** 7](#_Toc419941882)

[1.3.1 Unfractionated Heparin (UFH) 7](#_Toc419941883)

[1.3.2 Low-Molecular Weight Heparin (LMWH) 9](#_Toc419941884)

[1.3.3 Fondaparinux 10](#_Toc419941885)

[1.4 **Vitamin** **K** **Antagonist** 12](#_Toc419941886)

[1.4.1 Warfarin 12](#_Toc419941887)

[1.5 **Direct** **Factor** **Specific** **Anticoagulants** 14](#_Toc419941888)

[1.5.1 Dabigatran 14](#_Toc419941889)

[1.5.2 Rivaroxiban 16](#_Toc419941890)

[1.6 **Thromboelastography** 17](#_Toc419941891)

[1.7 **Role** **of** **FXII** ***In******Vivo*** 20](#_Toc419941892)

[1.8 **Overall** **Aim** 22](#_Toc419941893)

[1.9 **Hypothesis** 22](#_Toc419941894)

[1.10 **Rationale** 22](#_Toc419941895)

[1.11 **Specific** **Objectives** 23](#_Toc419941896)

[**2** **Experimental** **Procedures** 24](#_Toc419941897)

[2.1 **Materials** **and** **Methods** 24](#_Toc419941898)

[2.1.1 Reagents 24](#_Toc419941899)

[2.1.2 Blood Samples 24](#_Toc419941900)

[2.1.3 Thromboelastography 25](#_Toc419941901)

[2.1.4 Blood Column Dilution Optimization 25](#_Toc419941902)

[2.1.4.1 Bromophenol Blue as Blood Substitute 25](#_Toc419941903)

[2.1.4.2 Blood Volume Determination 26](#_Toc419941904)

[2.1.4.3 Phenolphthalein Marker for Blood in Column Dilution 26](#_Toc419941905)

[2.1.5 Optimization for Whole Blood Column Coating 27](#_Toc419941906)

[2.1.6 Determination of pH for CPDA-1 Chelated Whole Blood 27](#_Toc419941907)

[2.1.7 Determination on the Effects of Time on Citrate and CPDA-1 Chelated Blood 27](#_Toc419941908)

[2.1.8 Optimization of Platelet Depletion with Reduced Microbead Volume 28](#_Toc419941909)

[2.1.9 Optimization of Platelet-depleted Blood 29](#_Toc419941910)

[2.2 Optimization of PDB with Washed Microbeads 30](#_Toc419941911)

[2.2.1 Effect of Temperature on Blood 34](#_Toc419941912)

[2.2.2 Reduced Modified Microbead Incubation Period 34](#_Toc419941913)

[2.2.3 Platelet Count Percentile 35](#_Toc419941914)

[2.2.4 Hemodilution Effect 35](#_Toc419941915)

[2.2.5 Column Exposure (Microparticle Generation) 36](#_Toc419941916)

[2.2.6 Appropriate TF concentration for the Optimization of TEG 37](#_Toc419941917)

[2.2.14 Clotting Profiles at Predefined Platelet Counts in the Presence of Anticoagulants 37](#_Toc419941918)

[2.2.15 Statistical Analysis 38](#_Toc419941919)

[**3** **Results** 39](#_Toc419941920)

[3.1 Blood Column Dilution Optimization 39](#_Toc419941921)

[3.2 Albumin Coating 40](#_Toc419941922)

[3.3 pH of CPDA-1 Chelated Whole Blood 41](#_Toc419941923)

[3.4 Effect of time on Platelet Preservation 41](#_Toc419941924)

[3.5 Optimal CD 61 Microbead Volume 42](#_Toc419941925)

[3.6 Microparticle Generation and Platelet Functionality 43](#_Toc419941926)

[3.7 Effects of Temperature on Blood 44](#_Toc419941927)

[3.8 Modified Microbead Incubation Period 46](#_Toc419941928)

[3.9 Platelet Count Percentile 46](#_Toc419941929)

[3.10 Hemodilution Effect 47](#_Toc419941930)

[3.11 Column Exposure (Microparticle Generation) 48](#_Toc419941931)

[3.12 TF Optimization in the Presence of UFH and LMWH 49](#_Toc419941932)

[3.13 Clotting Profiles at Predefined Platelet Counts in the Presence of Anticoagulants 50](#_Toc419941933)

[3.13.1 R, Ma and AUC15 at Therapeutic and Prophylactic Levels of UFH 50](#_Toc419941934)

[3.13.2 R, Ma and AUC15 at Therapeutic and Prophylactic Levels of LMWH 53](#_Toc419941935)

[**4** **Discussion** 57](#_Toc419941936)

[4.1 Magnetic Column Chromotorgraphy Optimization 57](#_Toc419941937)

[4.2 Albumin Coating 59](#_Toc419941938)

[4.3 CPDA-1 Whole Blood Chelating Agent 59](#_Toc419941939)

[4.4 Preservation of Platelet Count Overtime 60](#_Toc419941940)

[4.5 Platelet Removal via CD 61 Antibody 60](#_Toc419941941)

[4.6 Microparticle Generation during Platelet Depletion 62](#_Toc419941942)

[4.7 Effect of Temperature on Whole Blood 63](#_Toc419941943)

[4.8 Modified Microbead Incubation Period 64](#_Toc419941944)

[4.9 Platelet Count Percentile 64](#_Toc419941945)

[4.10 Hemodilution Effect 65](#_Toc419941946)

[4.11 Column Exposure (Microparticle Generation) 65](#_Toc419941947)

[4.12 TF Optimization in the Presence of UFH and LMWH 66](#_Toc419941948)

[4.13 Clotting Profiles at Predefined Platelet Counts in the Presence of Anticoagulants 67](#_Toc419941949)

[4.14 Limitations 70](#_Toc419941950)

[4.15 Conclusions/Future Directions 70](#_Toc419941951)

[**5** **References** 71](#_Toc419941952)

# List of Abbreviation

|  |  |
| --- | --- |
| **Abbreviations** | **Terms** |
|  |  |
| aPTTAT | Activated Partial Thromboplastin TimeAntithrombin |
| CPDA-1 | Anhydrous Citric Acid (0.03M), Trisodium Citrate Dihydrate (0.18M), Sodium Phosphate Monohydrate (0.03M), Dextrose Monhydrate (0.3M) and Adenine (4.1 mM)  |
| DVT | Deep Vein Thrombosis |
|  |  |
| FV | Factor V |
|  |  |
| FVIII | Factor VIII |
|  |  |
| FVII | Factor VII |
|  |  |
| FIX | Factor IX |
|  |  |
| FX | Factor X |
|  |  |
| FXII | Factor XII |
|  |  |
| FXI | Factor XI |
|  |  |
| FVa | Activated Factor V |
|  |  |
| FVIIIa | Activated Factor VIII |
|  |  |
| FVIIa | Activated Factor VII |
|  |  |
| FIXa | Activated Factor IX |
|  |  |
| FXa | Activated Factor X |
|  |  |
| FXIIa | Activated Factor XII |
|  |  |
| FXIa | Activated FXI |
|  |  |
| HIT | Heparin-Induced Thrombocytopenia |
|  |  |
| LMWH | Low Molecular Weight Heparin |
|  |  |
| PBMC | Peripheral Blood Mononuclear Cell |
|  |  |
| PBS | Phosphate Buffered Saline |
|  |  |
| PDP | Platelet-Depleted Plasma |
|  |  |
| PDB | Platelet-Depleted Whole Blood |
|  |  |
| PF4 | Platelet Factor 4 |
|  |  |
| PPP | Platelet Poor Plasma |
|  |  |
| PRP | Platelet Rich Plasma |
|  |  |
| RMV | Recommended Microbead Volume |
|  |  |
| TEG | Thromboelastography |
|  |  |
| TF | Tissue Factor |
|  |  |
| TF-FVIIa | Tissue Factor-Activated Factor VII  |
|  |
| UFH | Unfractionated Heparin |
|  |  |
| VTE | Venous Thromboembolism |
|  |
| VKA | Vitamin K Antagonist |
|  |  |
| vWF | von Willebrand Factor |

# 1 Introduction

## 1.1 Overview

Hemostasis is the body’s ability to maintain the movement of blood in a fluid state while achieving a balance between coagulation, anticoagulation and fibrinolysis (Rosenberg & Aird, 1999). However, this delicate balance is easily perturbed in cancer patients when complicated by thrombocytopenia and venous thrombosis (Benjamin & Anderson, 2002). Thrombocytopenia is a condition characterized by abnormally low platelet counts (<150 x 109/L), which may increase the risk for bleeding complications (Strauss et al., 2002). This can happen in cancer patients either due to the disease itself or the adverse effects of treatments such as chemotherapy or bone marrow transplantation (Benjamin & Anderson, 2002). Platelets play an essential role in the blood clotting as they induce and enhance clot formation in order to stop bleeding (Ni & Freedman, 2003). Although cancer patients may develop thrombocytopenia that increases the risks of bleeding, these patients are paradoxically at risk for venous thromboembolism (VTE). Cancer patients may develop thrombosis due to chemotherapy or release of tissue factor (TF) from tumour cells (Gale & Gordon, 2001) or tissue injury. These patients may also suffer from other comorbidity such as bed-ridden conditions causing blood flow stasis. When thrombosis happens in cancer patients with thrombocytopenia, it is a challenge for the managing physicians to start anticoagulant because it further increases the risk of bleeding. Whereas some guidelines based on expert opinions suggest that anticoagulants may be safely administered to patients with a minimum platelet count of 50 x 109/L (Lyman et al., 2007), others suggest a lower platelet threshold of 30 x 109/L with or without dose reduction (Saccullo et al., 2013). None of these guidelines is based on good quality randomized clinical data. Hereby, we performed an *in vitro* study with thromboelastography (TEG) to evaluate the clot formation inhuman blood with low platelets (PDB) in the presence of anticoagulants. In this project, the perplexed interactions among blood cells, clotting proteins and anticoagulants will be measured with thromboelastography as a global assay. Therefore, the general concepts of the coagulation cascade will first be reviewed in the following sections.

## 1.2 Coagulation Cascade

When a vascular injury occurs, coagulation is initiated to stop blood loss. The coagulation cascade is a series of enzymatic reactions that involves the proteolysis of zymogen coagulation factors to their activated forms. Zymogen substrates upstream and downstream the cascade are cleaved by its respective enzyme to generate these activated forms required for the procession of the cascade (Smith, 2009). The rate at which these activated forms are generated increases with each subsequent step down the coagulation cascade (Gross et al., 2012). The cascade itself consists of two separate pathways that diverge to form the common pathway (Smith, 2009). These two separate pathways are known as the contact pathway and the TF-FVIIa mediated pathway (Smith, 2009). The ultimate goal of both pathways is for the generation of a highly regulated coagulation factor known as thrombin. Thrombin is an essential enzyme for establishing a hemostatic plug at the site of injury (Coughlin, 2000). Thus, the purpose of the coagulation cascade in clotting is to generate thrombin the enzyme required for preventing further blood loss. Historically the coagulation cascade was outlined in literature illustrating the series of steps involved in zymogen activation in an *in vitro* plasma system (Smith, 2009). However, with an *in vitro* model it is void of vascular walls and cell surfaces hence, leading to the development of the cell-based model of coagulation that introduces clot formation in a dynamic vascular system (Hoffman, 2003). The cell-based model will be discussed in greater detail below and categorised into the initiation, amplification, propagation and termination phases (Hoffman, 2003).

### 1.2.1 Initiation

 Upon vascular injury, the exposure of tissue factor (TF) allows further interaction with factor VII/VIIa to initiate the coagulation cascade *in vivo* (Butenas et al., 2005) (Giesen et al., 2000). TF is an integral membrane protein (46 kDa) primarily found in the subendothelium of blood vessels (Lockwood et al., 2000) (Stevic et al., 2011). A high concentration of TF are found within the vessel walls; however, soluble levels of unactivated TF (<20 fM) can be detected at physiological conditions (Butenas et al., 2005). TF acts as an anchor for circulating FVII/FVIIa to bind and form the TF-FVIIa complex (Rao & Rapaport, 1988). In circulation approximately 1% of FVII (only) can be found pre-activated at physiological levels (Morrissey et al., 1993). However, the majority of circulating FVII is found free from activation as its contact with TF is limited due to the greater localization of TF within the subendothelium (Stevic et al., 2011). Although a greater degree of TF exposure can be found with the disruption of the subendothelium it can also be detected on circulating cells, such as monocytes (TF-bearing cells) (Monroe et al., 1996). On the surface of these TF-bearing cells, the TF-FVIIa complex further auto-activates FVII to FVIIa (Yamamoto et al., 1992). These TF-FVIIa complexes are than involved in activating small amounts of FIX and FX to FIXa and FXa, respectively (Hoffman et al., 1995). The FXa becomes available for interaction with its co-factor FVa and calcium ions on the surface of phosphatidylserine-bearing cells to form the prothrombinase complex (Comfurius et al., 1994). Upon the assembly of the prothrombinase complex it can enzymatically activate prothrombin to generate minute amounts of thrombin on the surface of TF-bearing cells (Hoffman, 2003). With FIX activation via TF-FVIIa complex it is free to interact with co-factor VIIIa to form the tenase complex. The tenase complex can loop back into the coagulation cascade for further FXa generation ultimately, leading to increased prothrombin activation (Hoffman, 2003). The difference between FIXa and FXa upon generation in the initiation phase is FIXa is free to dissociate from the surface of phosphatidylserine-containing cells allowing for interaction with surrounding cells. Whereas FXa is limited to its site of activation as it is quickly inhibited by the tissue factor pathway inhibitor (TFPI) and by antithrombin (AT) (Hoffman, 2003). Overall, the initiation phase involves the activation of FVII to form the TF-FVIIa complex in order to activate FIX and FX for its subsequent role in the initial thrombin generation on the surface of TF-bearing cells.

### 1.2.2 Amplification

At sites of injury not only is TF exposed, but collagen and von Willebrand factor (vWF) are also exposed from the subendothelium allowing for initial platelet aggregation via Glycoprotein (GP) Ib/IX/V (Inoue et al., 2008). Further platelet aggregation can occur with the small amounts of thrombin generated in the initiation phase. These thrombin can diffuse to areas of platelet aggregation for further platelet activation via the protease associated receptors (PAR), PAR1 and PAR4 (Kahn et al., 1999). Upon thrombin binding with PAR1/4 it induces a morphological change in platelets by generating a procoagulant membrane as it mobilizes the phosphatidylserine (PS) from the inner leaflet to the outer leaflet of the platelet membrane (Boon et al., 2003). Activated platelets release procoagulant microparticles which further activates other resting platelets (Barry et al., 1997). Microparticles, composed of the negatively charged PS membranes vary from 0.2-1 μM (Piccin et al., 2007). Not only is thrombin involved in platelet activation it is a multifaceted enzyme that can activate FXI, FV and vWF bound FXIII to FXIa FVa and FXIIIa respectively (Dasgupta et al., 2007) (Soons et al., 1986). Thus, acting as a positive feedback to amplify its own thrombin generation (Mann et al., 2003). The vWF released upon FXIII activation is available for aiding in platelet activation and aggregation (Inoue et al., 2008) (Mann et al., 2003). In essence the amplification phase involves the activation of platelets via thrombin which fosters a procoagulant environment with the release of microparticles as well as the role of thrombin activating zymogen factors upstream within the cascade.

### 1.2.3 Propagation

 The microparticles released with the few platelets activated in the amplification phase allows for enhanced platelet aggregation via the release of dense granules at the site of injury (Israels et al., 1990). It is on the surface of these newly recruited platelets where the propagation phase occurs. The dense granule cargo contains ADP which aids in the activation of glycoprotein IIb/IIIa receptors from its resting state allowing for increased fibrinogen binding (Cattaneo, 2010) (Nachman & Leung, 1982). Fibrinogen is a soluble zymogen that is cleaved by thrombin to generate insoluble fibrin strands that polymerize (via FXIIIa) into a fibrin meshwork thus, stabilizing the haemostatic plug (Binnie & Lord, 1993) (Siebenlist et al., 2001). Fibrin formation within the propagation phase can occur upon thrombin generation. Thrombin generation on the surface of these activated platelets can occur when the diffused FIX from the TF-bearing cells in the initiation phase escapes to associate with the thrombin-activated FVIIIa to form the tenase complex (Monroe et al., 1994). The tenase complex is initially required for greatly enhancing FX activation for its use in the assembly of the prothrombinase complex on the surface of activated platelets (Monroe et al., 1994) (Tracy & Mann, 1983). In the initiation and amplification phase the initial source of coagulation factors are generated on the surface of TF-bearing cells that are to be used for generating thrombin on the surface of activated platelets. Therefore, at the site of vascular injury platelets activated by thrombin form the initial platelet plug, whereas the coagulation cascade further stabilizes the clot by generating a fibrin meshwork encompassing the platelet plug.

### 1.2.4 Termination

 Upon the establishment of a fibrin a clot it is essential for coagulation activation be limited to the site of injury. This is necessary to prevent further occlusion of the vessel walls. Thus, a natural anticoagulant process is also at hand to regulate the advancement of coagulation activation beyond the site of injury. Natural anticoagulants that can be found physiologically include: tissue factor pathway inhibitor (TFPI), activated protein C (APC), and antithrombin (AT) (Hoffman, 2003).

 TFPI is released from the endothelium and its anticoagulant properties are through inactivation of FXa and TF-VIIa complexes (Broze, 1995). On the other hand, APC acts on cofactors involved in the coagulation cascade by degrading FVa and FVIIIa, which ultimately prevents the formation of the prothrombinase and tenase complexes, respectively (Comp et al., 1984). APC deters FVa activity by cleaving FVa at R506, R306 and R679 (Kalafatis et al., 1995). Cleavage at site R506 results in a reduced co-factor activity (Kalafatis et al., 1995) (Rosendaal, 1999). However, the activity of FVa is completely lost with further cleavage at sites R306 and R679 (Kalafatis et al., 1995). APC is generated in response to the dual role of thrombin within the cell based model of coagulation as it can elicit an anticoagulant response (Esmon et al., 1982).In order for thrombin to elicit an effective anticoagulant response it must bind to a receptor on the endothelial surface known as thrombomodulin. Thrombomodulin-bound thrombin can activate protein C once it is bound to the endothelial protein C receptor (Esmon et al., 1982) (Wouwer et al., 2004). Activation of protein C is further amplified when it becomes associated with its cofactor, protein S (Franco & Reitsma, 2001). Lastly, AT is a serine protease inhibitor, that when it interacts with glycosaminoglycans, a conformational change in AT occurs that makes it more readily reactive for inhibiting the serine proteases (Huntington, 2003). Therefore, the anticoagulant properties that are also in play within the cell-based model can define coagulation as a fine balance between procoagulant and anticoagulant activity.

## 1.3 Heparinoids

### 1.3.1 Unfractionated Heparin (UFH)

UFH is a pharmaceutical reagent administered by clinicians to treat patients at risk for developing and preventing further thrombosis. UFH is a sulfonated glycosaminoglycan molecule with a mean molecular weight of 15000 Da (Andersson et al., 1979). Its anticoagulant properties are mediated through AT by inhibiting FIXa, FXa and thrombin (Rosenber & Bauer, 1994). Traditionally two models of inhibition are understood using UFH either template mediated inhibition or conformational activation of AT via UFH (Rezaie, 1998). In the case of conformational activation of AT, a unique pentasaccharide sequence that is found in one third of the commercially available heparin molecules shows a strong affinity towards AT (Lindahl et al., 1984). Once bound to AT a conformational change in the reactive centre loop occurs, which increases the interaction of AT with the serine protease (specifically FIXa and FXa) for inhibition (Huntington et al., 1996). This interaction is achieved with the exposure of the arginine residue located near the C-terminal, which interacts with the serine residue found on its target protease (Rosenberg, 1974). On the other hand, template mediated inhibition involves the inactivation of thrombin by forming a ternary complex with AT and thrombin (Rezaie, 1998). This reaction is catalyzed by UFH acting as a template to bridge the interaction of thrombin to AT (Rosenber & Bauer, 1994). However, this form of template mediated inactivation requires a minimum molecular mass of 5400 Da (Hirsh & Levine, 1992).

Although it seems the template mediated form of inactivation may be limited to thrombin it is suggested that UFH may act as a template for AT-FXa mediated inactivation in the presence of calcium (Rezaie, 1998). In the presence of calcium, the randomly arranged 11 gamma-carboxylated glutamic acid domain of FXa can fold to its proper conformation and thus, expressing its positively charged residue (Rezaie, 1998). In doing so this may prevent the Gla domain of FXa from antagonizing with UFH hence, allowing heparin to act as a template in AT-FXa interaction (Rezaie, 1998). However, in the absence of calcium the negatively charged Gla domain expressed due to improper folding may induce an electronegative repulsion from the UFH molecule by the Gla domain of FXa (Rezaie, 1998). Thus, in order to achieve template mediated inactivation of FXa calcium is required in proximity of AT-UFH association (Rezaie, 1998). Overall, UFH can exert its anticoagulant properties either through the conformational change of AT or via a template mediated model bridging AT and its target protease.

As outlined above the benefits of UFH is in its ability to inhibit active coagulation enzymes, but it is not without limitations. For instance, UFH has nonspecific interactions with various proteins found within plasma or endothelium (Hirsh, 1991), which results in variable bioavailability, unpredictable half-life and anticoagulant response (Young et al., 1992). Furthermore, patients undergoing UFH treatment are at risk for developing heparin-induced thrombocytopenia (HIT) (Warkentin et al., 1995). HIT can occur during platelet activation where the released alpha granules contain positively charged proteins known as platelet factor 4 (PF4) (Harrison & Martin Cramer, 1993). These PF4 molecules have a high affinity towards UFH as well, the frequency of PF4-UFH interaction is dependent on the length and the sulfonation of the UFH molecule (Horn & Hutchison, 1998) (Greinacher et al., 1995). Once bound to UFH, PF4 experiences a conformational change that allows for the expression of an epitope that become the target of autoantibodies (Ziporen et al., 1998). These autoantibodies have Fab domains that than can adhere to Fc receptors that are expressed on the surface of platelets (Ziporen et al., 1998). This leads to platelet activation followed by degranulation, and ultimately destruction of platelets (Chong et al., 1981). The newly generated PF4 upon degranulation are then free to associate with other UFH molecules and propagate the response (Chong et al., 1981). Furthermore, with this platelet activation/degranulation not only does it reduce platelet counts, but creates a hypercoagulable environment that causes a risk for thrombosis (Farner et al., 2001). Lastly, bleeding is a major characteristic that follows with UFH treatment (Morabia, 1986). Hence in order to counteract this limitation protamine sulfate (a polycationic protein) is introduced as an antidote to neutralize the effects of UFH (Carr & Silverman, 1999). In the end the major concern with UFH is its unpredictability following administration and thus, exploring other anticoagulants may serve to be beneficial in the prophylaxis and the treatment of thrombosis.

### 1.3.2 Low-Molecular Weight Heparin (LMWH)

LMWH is a glycosaminoglycan molecule that is derived from the fragmentation of UFH. The mean molecular weight of LMWH is 5000 Da (Hirsh & Levine, 1992). Its pharmacokinetic properties are similar to UFH as it binds with AT to induce a conformational change within the reactive centre loop of AT to enhance protease inhibition (Beeler et al., 1979). However, unlike UFH, due to the smaller size of the molecule, LMWH is not able to bridge and initiate template-mediated thrombin inhibition (Danielsson et al., 1986). As the size of the molecule is one third the size of UFH making its unique pentasaccharide sequence to be one fifth the size of the anticoagulant (Eikelboom et al., 2000). Utilizing smaller forms of heparin reduces the incidence of HIT, which is dependent on the length and sulfonation of the molecule (Greinacher et al., 1995). Furthermore, where non-specific binding to proteins was a concern with UFH administration the frequency of nonspecific interactions is minimized with LMWH thus, yielding a greater bioavailability and a more predictable dose response (Handeland et al., 1990). Not only is its concern with heparin binding protein minimized LMWH has a lower affinity towards vWF, which may ultimately reduce the risk for bleeding as well (Sobel et al., 1991).

In terms of route of elimination contrary to UFH the major clearance route for LMWH is via the renal system (Palm & Mattsson, 1987). Hence, with renal failure the half-life of the drug is elevated within the circulation which may lead to bleeding complications (Lim et al., 2006).

 Overall, the major advantage associated with LMWH therapy is that managing physicians may determine a more accurate dose response due to a greater half-life (Handeland et al., 1990). This allows for patients to be treated once daily without the constant monitoring that follows with UFH treatment (Hirsh & Levine, 1992). LMWH has been an effective drug in reducing the risk for major hemorrhaging (Mismetti et al., 2000). Lastly, when compared with UFH, patients are less likely to experience episodes of HIT with LMWH treatment (Warkentin et al., 1995). Although the benefits of LMWH are profound, there are still limitations with its use, and the cost of a single LMWH administration is ten-times the cost of UFH treatment (Wood & Weitz, 1997). Therefore, looking into alternative treatment methods that is more feasible is necessary.

### 1.3.3 Fondaparinux

 Based on the mechanistic characteristics of UFH and LMWH it is evident that the molecular size of the molecule affects the overall property of the drug in terms of anticoagulation and side-effects. The commonality between UFH and LMWH is that their anticoagulant properties are exerted through the affinity towards AT by binding at the pentassacharide sequence of the molecule. Thus, fondaparinux was developed as a synthetically derived pentassacharide sequence for selective inhibition of FXa (Herbert et al., 1997). The molecular weight of fondaparinux is 1728 Da hence, making it the smallest of the heparinoids discussed above (UFH>LMWH>Fondaparinux) (Walenga et al., 2002). Consistent with previous heparinoids, the affinity of the pentassacharide sequence towards the AT regulates the inhibition of FXa (Herbert et al., 1997). Once AT binds and inactivates FXa, the fondaparinux can dissociate from the AT without being consumed in the inactivation process (Bauer et al., 2002). Although fondaparinux is an FXa inhibitor once FXa is formed within the prothrombinase complex it is protected from inhibition by AT similar to UFH and LMWH (Brufatto et al., 2003). This indicates that FXa inactivation is only achieved with free soluble FXa and not complex-bound FXa.

 In further comparison with UFH and LMWH, this selective inhibitor achieves complete bioavailability following subcutaneous injections (Donat et al., 2002). Thus, its half-life ranges from 13 to 21 hours when given only a prophylactic dose of 2.5 mg/kg, suggesting that a daily dose is sufficient enough to achieve the anticoagulant effect (Turpie et al., 2003). Furthermore, where concerns lied with nonspecific UFH protein interaction an *in vitro,* analysis showed no evidence for PF4 interaction which may indicate the absence of HIT development following fondaparinux treatment (Warkentin et al., 2005). The low risk of PF4 interaction is the result of fondaparinux having a 94% affinity towards ATthus, minimizing any potential for nonspecific protein binding (Paolucci et al., 2002).

In terms of routes of elimination the major clearance route of fondaparinux is via the renal system (Donat et al., 2002). As well, for its interaction with cytochrome P450 based on an *in vitro* study, there seems to be no concrete evidence to indicate that fondaparinux inhibits cytochrome P450 enzymes, which suggest it does not affect the metabolism of other drugs via the liver (Lieu et al., 2002).

In terms of limitation with all anticoagulants including fondaparinux bleeding is a risk. However, when compared to LMWH there seems to be no significant difference in the incidence of bleeding following knee surgery (Bauer et al., 2001). Not only is there an indifference in bleeding with fondparinux, when given 3 mg/kg of fondaparniux in patients undergoing orthopedic surgery their risk for developing venous thromboembolism (VTE) was reduced to 1.7% which was an 82% reduction in VTE development when compared to LMWH (Turpie et al., 2001). Therefore, it seems that fondaparaniux is the most superior heparinoid for the prophylaxis of VTE compared to LMWH and UFH.

## 1.4 Vitamin K Antagonist

### 1.4.1 Warfarin

 The previously mentioned anticoagulants are given parenterally. However, with warfarin injection is no longer necessary. Warfarin is an indirect oral anticoagulant that acts upon the vitamin K cycle targeting vitamin K dependent coagulation factors (Friedman et al., 1977). Vitamin K dependent proteins include the clotting factors prothrombin (FII), FIX, FX, and FVII in addition to natural anticoagulants protein C and S (Furie & Furie, 1988). These proteins undergo post-translational modification which involves the gamma carboxylation of the glutamic acid residues (Gla domain) found on the clotting factors (Furie & Furie, 1988).

Through dietary means of vitamin K absorption, within the liver it is used as a cofactor via the vitamin K cycle (Kayata et al., 1989). Consumed vitamin K is initially converted to an accessible form called vitamin K quinone, by vitamin K epoxide reductase in the presence of dithiols (Greer, 2010). The quinone form is further reduced by the vitamin K quinone reductase enzyme in the presence of NADPH to generate vitamin K hydroquinone (Greer, 2010). The hydroquinone form acts as a cofactor in the presence of carbon dioxide and oxygen to create the Gla domain via gamma carboxylation of the glutamic acid residue by the glutamyl carboxylase enzyme (Greer, 2010). During this conversion, vitamin K hydroquinone is reduced to its epoxide form (Greer, 2010). Then vitamin K epoxide is converted back to its quinone form with vitamin K epoxide reductase enzyme (VKOR) in hand with dithiols (Greer, 2010). This in turn feeds vitamin K quinone back into the vitamin K cycle for further post translational modification of vitamin K dependent clotting proteins (Greer, 2010). It is to be noted that vitamin K epoxide generation is directly proportional to the number of gamma carboxylation (Stafford, 2005).

Gamma carboxylation is essential for vitamin K clotting proteins in order to generate their active forms (Greer, 2010). The Gla-domains aid in the calcium mediated interaction of clotting proteins with the negatively charged phospholipid surfaces (Nelsestuen, 1976). Hence, it is imperative for Gla-domains to be generated in order for effective clotting and inhibition via protein C and S. However, patients at risk for thrombosis can be administered warfarin, which inhibits VKOR. This in turn prevents the feedback of the vitamin K cycle in generating more cofactors (hydroquinone) required for gamma carboxylation (Hirsh et al., 2001). Although these clotting factors are still produced with the inhibition of VKOR, the proteins are inactive and non-functional due to their being either partially carboxylated or decarboxylated (Friedman et al., 1977).

Warfarin itself is composed of two active isomers that are portioned equally in either the R or S form (Breckenridge, 1978). It is found that the S- warfarin is more effective in the inhibition of VKOR when compared to the R isomer (Breckenridge et al., 1974).In terms of bioavailability of warfarin it is fairly high as it reaches optimal concentrations within the blood after 90 min (Hirsh et al., 2001).Its half-life upon circulation ranges from 36 to 42 hrs (Hirsh et al., 2001). As well within circulation prior to its delivery to the liver it circulates in blood bound to albumin (Hirsh et al., 2001).

When taking warfarin precautions must be taken as patients must control their dietary vitamin K intake. Increased vitamin K intake may hinder its anticoagulant properties (O’Reilly & Rytand, 1980). Thus monitoring its international normalized ratio (INR) levels are essential (Hirsh et al., 2001). The INR is a measurement calculated by dividing the patient’s prothrombin time by average normal prothrombin time (Bussey et al., 1992). Prothrombin time is generated by inducing clot formation of citrated whole blood with thromboplastin (TF and phospholipids) and calcium (Kamal et al., 2007). INR monitoring allows for clinicians to adjust for dosages to achieve optimal effective concentration as well as prevent any inherent form of bleeding. Like with all anticoagulants, bleeding is a major risk factor associated with warfarin therapy as well (Connolly et al., 2009). Thus, regular INR monitoring is necessary. Since constant monitoring is required for warfarin therapy, an alternative treatment option is necessary.

## 1.5 Direct Factor Specific Anticoagulants

### 1.5.1 Dabigatran

 The previously mentioned antithrombotic drugs targeted the coagulation factors indirectly. Whereas, dabigatran is an oral reversible and direct thrombin inhibitor that targets the active site of thrombin for inhibition (Connolly et al., 2009). Due to its mechanistic properties of targeting the active site the major benefit of dabigatran is that it can inhibit complex-bound thrombin in addition to the free thrombin (van Ryn et al., 2008). Thrombin itself contains an active site, exosite 1 (substrate docking site e.g. fibrin) and exosite 2 (UFH binding site), and it is this active site that is targeted by dabigatran (Huntington & Baglin, 2003). Thrombin exists in a ternary complex when bound to fibrin (at exosite 1) and UFH (at exosite 2) (Becker et al., 1999). The ternary complex prevents thrombin inhibition by UFH-AT due to exosite 2 being occupied by UFH (Becker et al., 1999). However, dabigatran is small molecule and is able to inhibit thrombin as it binds to the active site versus exosite 2 (van Ryn et al., 2008). Thus, adding another layer of anticoagulant properties by inhibiting clot bound thrombin versus UFH.

Dabigatran as mentioned previously is given orally, but in the form of a prodrug. The active dabigatran molecule itself cannot be absorbed when given orally due to its hydrophilic polar properties (Stangier et al., 1999). Thus, it is given in the form of a prodrug called dabigatran etexilate (Blech et al., 2008). Once the prodrug is consumed the bioavailability of dabigatran etexilate is approximately 6.5% (Stangier et al., 2007). Therefore, due to its low bioavailability in order for effective absorption in the GI tract it requires a low pH environment, hence it is packaged in tartaric acid to mimic this environment (Eisert et al., 2010).

Once Dabigatran etaxilate is consumed it is converted to its active form by a non-specific esterase enzyme that can be found in enterocytes, plasma and the liver (Eriksson et al., 2009). The half-life of this active dabigatran is roughly 7 to 9 h and doubled in geriatric patients (Stangier et al., 2007). As for its routes of clearance following oral consumption the majority of the molecule is excreted through the kidneys unchanged without leaving behind any active metabolites (Stangier et al., 2010). Additionally 20% of it can be removed through bowel movements (Golembiewski, 2011). Furthermore, due to the absence of its metabolism via the cytochrome P450 family of enzymes there are no active metabolites being produced (Blech et al., 2008). Lastly, in terms of its benefits dabigatran has been found to be linked with lower rates of haemorrhagic strokes in patients diagnosed with atrial fibrillation (Connolly et al., 2009). Compared to warfarin, dabigatran is superior in terms of no dietary precautions or routine monitoring requirement. Therefore, it can be viewed that dabigatran may be a safer oral alternative for anticoagulation therapy.

### 1.5.2 Rivaroxiban

 An alternative oral, direct factor specific anticoagulant is rivaroxiban, which has a mechanism of action different than dabigatran. Where dabigatran is a direct thrombin inhibitor rivaroxiban is a reversible potent FXa inhibitor (Tersteegen et al., 2007). Its selectivity towards FXa is increased by 100, 000-fold compared to other serine proteases (Perzborn et al., 2005). In terms of its mechanistic properties rivaroxiban targets the active site of FXa at pockets S1 and S4 (Roehrig et al., 2005). Specifically morpholinone residue of rivaroxaban interacts with S4 and its chlorothiophene moiety associates with the S1 pocket (Roehrig et al., 2005).

Following oral consumption rivaroxiban has roughly 60 to 80% bioavailability and it reaches its optimal plasma concentrations at 2.5 to 4 h (Kubitza et al., 2005). In comparison to dabigatran; rivaroxiban is not administered as a prodrug as it does not require conversion to its active form by the non-specific esterases (Gulseth et al., 2008). As well, it is also metabolized by cytochrome P450 (CYP3A4, CYP2J2), but it is to be noted that there is no detection of active metabolites (Weinz et al., 2004). However, similar to dabigatran, a majority of rivaroxiban (~66%) is excreted through the kidneys with minimal removal following bowel movements (Gross & Weitz, 2008). In terms of its limitation as with all anticoagulants bleeding is its major risk (Gulseth et al., 2008).

The major benefit of these direct factor specific anticoagulants lie in their ability to rapidly inhibit clot formation. Lastly, their convenience, no patient monitoring, predictable dose response, and ease of administration makes the factor specific anticoagulants far more superior compared to warfarin or the heparinoids. It is noteworthy that both dabigatran and rivaroxaban have no approved antidotes at this time.

## 1.6 Thromboelastography

The ability to accurately assess the hemostatic function heavily relies upon the results of numerous measurements and tests along the coagulation cascade. Many of these measurements (i.e. platelet count/function, clotting factor concentration, etc.) help to provide independent information regarding the clotting process in addition to their mechanisms. However, they may not be fully indicative of the actual real-time clotting process. To effectively examine whole blood coagulation, thromboelastography (TEG) is utilized as a tool to analyze kinetics and overall clotting profiles in whole blood. The TEG measures clotting by correlating the physico-elastic properties of clots with the oscillating motion of the torsion wire in contact with the sample being tested (Luddington, 2005). The clot’s strength depends on the ability of fibrin to accumulate in the cup surrounding the torsion wire, which is sensed by an electromagnetic transducer that relays the signal to a monitor for viewing (Whitten & Greilich, 2000). The resultant TEG tracing provides four specific parameters (R, K, α, and MA) and post-hoc (AUC15), as described in ***Table 1*** and ***Figure 1-2*.**

|  |  |
| --- | --- |
| Parameters | Description |
| Reactive time | The period of time it takes for fibrin formation to be detected. |
| Coagulation time  | Measures the time for the thickness of the fibres to reach 20mm following the end of r-time. It is indicative of the clot forming speed. |
| α- angle | Measures the instantaneous rate of clot development based on fibrin generation and cross-linking. This parameter is indicative of fibrinogen levels. |
| Maximum Amplitude (mm)  | Represents the final/overall strength of the clot and is a percentage ratio of platelet to fibrin based on its bonding via GPIIB/IIIa receptors. |
| Area Under the Curve 15  | Represents the combination of clot strength and speed of clot formation. Measuring the area under the curve from R-time+15 min of a TEG tracing (**Figure 2**) using Adobe Photoshop (Post hoc). Used as an alternative test for substituting clot kinetics k-time and α- angle in the presence of anticoagulants as MA becomes significantly delayed. |

***Table 1*:** Parameters analyzed following clot formation via TEG (Senzolo et al., 2007) (Swallow et al., 2006) (Hobson et al., 2007)



***Figure 1*:** TEG tracing displays four major clotting parameters, characterized by reaction time (R-time), coagulation time (K-time), alpha angle (α- angle) and maximum amplitude (MA). R-time represents the overall time elapsed for clot formation. K-time and α- angle displays information on the kinetics of clot formation regarding its speed and rate. MA defines the overall strength of the clot achieved.



***Figure 2*:** Post hoc analysis of TEG tracing via Adobe Photoshop in the quantification of Area under the curve 15 (AUC15). AUC15 combines the strength and speed of clot formation after 15 minutes of the r-time being established.

## 1.7 Role of FXII *In Vivo*

For decades, questions have been raised on the physiological significance of FXII. Although it plays a role in determining the activated partial thromboplastin time (aPTT) for diagnostic purposes, its deficiencies *in vivo* have not been linked to risks for bleeding (Meijers, 2014). However, in light of recent evidence it suggests the importance of FXII *in vivo* contrary to popular beliefs.

 FXII plays a role in the intrinsic pathway which involves the serine protease FXI, FIX and cofactor FVIII (Gailani & Renne, 2007). These serine proteases specifically FXI and FIX and cofactor FVIII can be activated under physiological conditions via thrombin and TF-FVIIa complex (Gailani & Renne, 2007). A deficiency in either FVIII or FIX are characterized as haemophilia A and B respectively with phenotypes ranging from mild to severe bleeding whereas, a deficiency in FXI is noted as haemophilia C characterized by minor increase in bleeding upon vascular injury (Muller et al., 2011) (Van den Berg et al., 2007). However, in terms of FXII it is suggested to be only activated upon contact with negatively charged surface such as an intravenous catheter (Cochrane & Griffin, 1982). In spite of this knowledge there seems to be a role for FXII *in vivo* at physiological conditions.

 It has been proposed that extracellular RNA may serve as the natural foreign surface in the activation of FXII *in vivo* (Kannemeier et al., 2007)*.* In response to damaged vessel walls extracellular RNA may be released from the damaged cells, hence providing a surface for FXII and FXI activation. Kannemeier et al. (2007) demonstrate that exogenous RNA was able to induce coagulation in an arterial thrombosis model. Moreover, when mice were treated with RNase antidote it was found to significantly delay thrombus formation. It was to be noted that when compared to DNA, the exogenous RNA had a more predominant role in a procoagulant response (Kannemeier et al., 2007). This may be due to the rapid exposure of RNA upon cell damage whereas DNA may still be complexed with histones in the nuclei, thus making it less readily accessible for coagulation factors (Kannemeier et al., 2007).

In addition to RNA, polyphosphates (PolyP), which are anionic polymers synthesized from ATP, may serve as negative compounds responsible for FXII activation (Morrissey et al., 2012). PolyP are secreted by activated platelets from dense granules (Ruiz et al., 2004). As PolyP is stored in dense granules, it has been found that patients with dense granule defects have platelet derived PolyP levels ten times lower than the normal level (Hernandadez-ruiz et al., 2009). PolyP itself is found to not only activate FXII, but also enhance the activation of cofactor FV, fibrin polymerization and FXI activation via thrombin feedback (Morrissey et al., 2012). For effective activation of these coagulation factors via PolyP, it is dependent on the size of the polymer itself (Smith et al., 2010). It has also been shown that shorter chained PolyP has a reduced procoagulant response compared to longer chained PolyP (Smith et al., 2010). However, shorter chained PolyP still serves its purpose in hemostasis as it is more specific towards increasing FV activation and inhibiting tissue factor pathway inhibitor (Smith et al., 2010). Despite these findings that suggest the role of PolyP in FXII activation *in vivo* recent evidence rebut the notion on the importance of PolyP in FXII activation, as Faxalv et al. (2013) found that PolyP is a poor activator of FXII (<10% activation) when compared to equal concentrations of Kaolin. However, the authors do not dispute that PolyP may play a hand in accelerating the activation of FV and FXI through thrombin.

Lastly as FXII deficiency has been shown to exhibit normal hemostasis, it has also been shown that reduction in FXII activity may affect the overall structure of the thrombi (Muller et al., 2011). Blockage of FXII activation with monoclonal antibodies resulted in altered fibrin formation and prolongation of aPTT (Matafonov et al., 2014). Although the clot continues to develop, inactivation of FXII may affect the overall strength and quality of clot formation, thus making it more vulnerable to dislodge, particularly during laminar shear stress. Overall, there seems to be a physiological agent *in vivo* that are also able to activate FXII thus, highlighting its potential importance in a physiological environment.

## 1.8 Overall Aim

 The overall purpose of the project is to compare the clotting profile of whole blood at platelet counts of 30 x 109/L and 50 x 109/L in the presence of anticoagulants.

## 1.9 Hypothesis

There will not be a statistical significant difference in the clotting profile of whole blood at platelet counts of 30 x 109/L versus 50 x 109/L in the presence of anticoagulants.

## 1.10 Rationale

It is currently accepted in clinical practice that a minimum platelet threshold of 50 x 109/L is required for safe administration of anticoagulants to thrombocytopenic patients at risk of thrombosis. Anecdotally, recent observations suggest a reduced platelet count of 30 x 109/L may be just as safe to administer anticoagulants to these patients.

## 1.11 Specific Objectives

1) Optimizing the throughput volume of the magnetic column chromatography

2) Determine the optimal volume of microbeads required to obtain platelet poor plasma (<10 x 109 platelets/L) while minimizing the effect of hemodilution

3) Determine the appropriate chelating agent to maintain the integrity of the platelets during venipuncture collection

4) Determine whether the eluted samples become procoagulant when exposed to the magnetic column

5) Optimize the appropriate percentage of hemodilution that does not impair the coagulant state of the eluted sample.

6) Determine the effects of whole blood sample preparation at 4oC versus 22oC (room temperature) via TEG clotting profiles.

7) Determine the optimal TF concentration for clot initiation in PDB in the presence of anticoagulants.

8) Analyze the clotting profiles at pre-defined platelet counts (PDB, 30, 50, 75, 100 and 150) in the presence of anticoagulants (UFH, LMWH) (therapeutic versus prophylactic dose) via TEG.

#

# 2 Experimental Procedures

## 2.1 Materials and Methods

### 2.1.1 Reagents

All reagents used were of analytical grade. Imidazole, 99% was purchased from Alfa Aesar (Heysham, Lancaster). Adenine, 99%, D-(+)-glucose were from Sigma (St Louis, Missouri). Trisodium citrate, citric acid, calcium chloride, sodium dihydrogen orthophosphate were from BDH (Toronto, Ontario). Sodium chloride and TRIS-HCL were from BioShop (Burlington, Ontario). Sodium phosphate, dibasic, anhydrous was from EMD (Darmstadt, Germany). Phenolphthalein was from Caledon (Georgetown, Ontario). Bromophenol Blue was from Fisher Scientific Company (Fair Lawn, New Jersey). CD 61 Microbeads was from MACS (Auburn, California). Albumin from Bovine Serum was from Sigma (St Louis, Missouri). Corn Trypsin Inhibitor was from Haematologic Technologies Inc. (Essex Junction, Vermont). Thromborel S was from Dade Behring (Newark, Delaware). Heparin was from Sandoz (Quebec, Canada) and Fragmin was from Pharmacia & Upjohn (Kalamazoo, Michigan).

### 2.1.2 Blood Samples

With the approval from the McMaster University Research Ethics Board, healthy volunteers who were not taking antiplatelet drugs for at least two weeks, were recruited to donate blood on the day of the experiment. Under aseptic conditions, 21 G butterfly needle was used to draw10 to 30 mL of blood from the antecubital vein with either 5 mL 3.2% sodium citrate (light blue top) vacutainers or 10 mL vacutainers containing 5% v/v citrate phosphate dextrose adenine (CPDA-1) buffer.

### 2.1.3 Thromboelastography

Two TEG Hemostasis Analyzers 5000 (Haemonetics, Braintree, MA) were used to analyze 4 samples for each experiment. Twenty µL of reaction mixture containing tissue factor (TF), 10 mM CaCl2 and either unfractionated heparin (UFH) or dalteparin (LMWH) were transferred to TEG cups and preincubated at 37oC for 5 min. The amount of TF was pre-determined to maximize the sensitivity of TEG (see section xxx). The clotting was initiated by mixing 340 µL blood samples with the reaction mixture for 10 sec inside the TEG cups with P1000 pipette. Clot formation was then monitored for a maximum of 180 min and the profile was characterised with four parameters: R (reaction time), K (coagulation time), α angle and TMA (time to reach maximum amplitude). Post hoc analysis of the TEG tracings was performed using Adobe Photoshop CS6 (Adobe, CA, USA) to determine AUC15 (dyn/cm2 × min) which was defined as the area under TEG curve within the first 15 min after clotting had started.

### 2.1.4 Blood Column Dilution Optimization

### 2.1.4.1 Bromophenol Blue as Blood Substitute

To determine the dilution of samples between the void volume and the reservoir volume within the column, initially the whole blood magnetic bead chromatography columns were washed with 600 μL of imidazole buffer pH 7.4 (0.15M). The throughput diluted volume was measured by obtaining the eluted 600 μL of imidazole buffer enclosed within the void volume by pipetting 1800 μL of bromophenol blue (substitute for blood) prepared in imidazole buffer pH 7.4 (0.15M). Single droplets of eluted samples were then collected in individual Costar microwells. The samples were then analyzed using the spectrophotometer at a wavelength of 600 nm.

### 2.1.4.2 Blood Volume Determination

 To determine the volume for a droplet of blood eluted from the column the mass for 20, 40, 60 and 80 μL of citrated blood was initially determined for use as a baseline. Then the mass for a droplet of blood eluted from the column were obtained. Using the determined mass of 20, 40, 60 and 80 μL of citrated blood, a baseline was determined by graphing the volume against its respective mass using Microsoft Excel. The equation of the graph was calculated and with the mean mass of a droplet of blood that is eluted from the column, the approximate volume for a single droplet of blood passing through the column was obtained using the calculated equation of the line.

### 2.1.4.3 Phenolphthalein Marker for Blood in Column Dilution

 In order to determine the volume of dilution between the void volume and the reservoir volume, untouched blood and phenolphthalein containing blood was used. The column was initially washed with 600 μL of imidazole buffer pH 7.4 (0.15M). The contents within the void volume was eluted and discarded by pipetting 2 mL of citrated blood. Following this step, 1800 μL of citrated blood+phenolphthalein prepared in 20% ethanol was introduced to the column where three droplets of the eluted sample were collected within the Costar microplate. The Costar microplate was centrifuged at 3000 RPM for 20 minutes to obtain the platelet poor plasma (PPP). Forty μL of the supernatant was pipetted into the empty microwells, where further centrifugation was performed at 3000 RPM for 15 minutes to remove unwanted red blood cells found within the PPP. The supernatant was removed and added into additional empty microwells then were treated with TRIS solution pH 13 into each wells until a stable pink solution was observed. The Costar microplate was analyzed using the spectrophotometer at a wavelength of 550 nm.

### 2.1.5 Optimization for Whole Blood Column Coating

In order to maximize platelet yield in the eluted samples following its passage through the column the columns were coated with albumin and compared against uncoated columns. **Control conditions:** At 4oC magnetic bead chromatography (attached to Midimacs separator) was washed with 1200 μL of imidazole buffer pH 7.4 (0.15M) + 0.32% citrate. The throughput volume was eluted with 3 mL of citrated blood and discarded. Another 500 μL of eluted sample was collected from the column following the addition of 600 μL of citrated whole blood. Platelet counts were determined at the core lab by medical laboratory technologists using the Coulter LH 750 analyzer. **Experimental conditions:** Magnetic chromatography, pipette tips, and collection tubes that have been coated in 0.32% citrate and 200 mg/100 mL BSA prepared in 0.15M imidazole buffer pH 7.4 (0.15M) overnight at 4oC were used. At 4oC whole blood magnetic chromatography was washed with 1800 μL of citrated blood and the eluted waste was discarded. Six hundred μL of citrated blood was introduced to the column where 500 μL of the eluted sample was collected and analyzed at the core lab for its platelet counts.

### 2.1.6 Determination of pH for CPDA-1 Chelated Whole Blood

In order to maximize the preservation of cells in blood citrate phosphate dextrose adenine (CPDA-1) chelating agent was used and its pH in blood was thus, determined. Four mL of CPDA-1 chelated blood 1:9 dilution was obtained from a healthy donor using a 5 mL syringe. The chelated blood was centrifuged at 3800 rpm for 20 minutes and the supernatant was obtained and analyzed using the Colorphast pH indicator strip to determine the pH of the sample.

### 2.1.7 Determination on the Effects of Time on Citrate and CPDA-1 Chelated Blood

 In order to determine the superior chelating agent for optimal preservation of platelets overtime citrated and CPDA-1 chelated blood was analyzed. The 3.2% citrated (1:9 dilution) and CPDA-1 (1:9 dilution) chelated blood was obtained from a single healthy donor. Following venipuncture samples were placed within an ice box during the delivery process to the core lab at the Hamilton General Hospital to determine its initial platelet counts. The samples were then brought back and incubated on the Barnstead rotisserie for 2 hours at 4oC in the cold room. After 2 hours the samples were placed back into the ice box and platelet counts were obtained once more at the core lab.

### 2.1.8 Optimization of Platelet Depletion with Reduced Microbead Volume

In order to minimize hemodilution with microbead addition while maximizing platelet depletion in blood different fractions of microbead was used. As outlined in section 2.1.2 two ten mL of CPDA-1 chelated blood was obtained. Following venipuncture samples were placed within an ice box and brought into the cold room (4oC). Within the cold room the blood samples were pooled together and divided into two Falcon Tubes (15mL). Two aliquots of 500 μL were obtained from either Falcon tube labelled 1 and 2. The Falcon tubes were placed on the Barnstead rotisserie and incubated at 4oC within the cold room. The two aliquots were placed in the ice box and delivered to the core lab for platelet count determination. Based on the platelet count acquired, the recommended microbead volume (RMV), required to remove a specific platelet count was then calculated. Based on the RMV the volume was reduced to 75% and 50% of the original RMV. Therefore, three different fractions of the RMVs were used, 100%, 75% and 50%. At 4oC, a 1500 μL aliquot of CPDA-1 chelated blood was taken from the Falcon tube that yielded the highest platelet count. The aliquot was incubated for 15 minutes with 100% of the RMV at 4oC. The latter two parameters (75% and 50% of the RMV) were also incubated at 4oC within 1500 μL of CPDA-1 chelated whole blood. After 15 minutes the whole blood magnetic chromatography column was rinsed with 1200 μL of PBS buffer pH 7.4. The column’s void volume was then displaced with 1800 μL of untouched blood. To deplete platelets from blood using the 100% RMV incubated blood, initially 800 μL of the 100% RMV incubated blood was added to the column and the eluted product was discarded. The remaining incubated sample was then added to the column and the eluted product was attained. To obtain the remaining PDB within the void volume 400 μL of untouched CPDA-1 chelated blood was added to the column and collected. This same protocol was carried out for the other two parameters (75% and 50% RMV). Once all samples have been collected, platelet counts were determined as described previously. All samples were placed back on the Barnstead rotisserie at 4oC.

### 2.1.9 Optimization of Platelet-depleted Blood

To determine if PDB eluted from the column becomes procoagulant a turbidometric clotting assay was implored. The samples for this experiment was prepared as followed:

**Positive Control:** Using the other Falcon tube from section 2.1.8 PRP with proportional dilution as the experimental group, but using PBS buffer pH 7.4 as a substitute versus 100% of the RMV was attained via centrifugation at and RPM of 750 for 20 minutes.

**Negative Control:** Similar to the positive control the PPP with proportional dilution as the experimental group, but using PBS buffer pH 7.4 a substitute versus 100% of the RMV was attained via centrifugation with the microcentrifuge for 20 minutes.

**Experimental:** To determine if microparticles are generated, the PDB sample obtained from section 2.1.8 using 100% of RMV was centrifuged using the microcentrifuge for 20 minutes and its PPP was obtained. All of the preparations were then subjected to a turbidometric analysis.

**Turbidometric clotting assay:** The spectrophotometer was pre-warmed to achieve an internal temperature of 37 oC and parameters for a kinetics test were set at a wavelength of 350 nm that was read at 15 second intervals for a test period of 3 hours. Once the temperature was stable at 37oC 50 μL of PRP, PPP and PPP from PDB were added to wells A, B and C respectively. To initiate clotting, 50 μL of 10 mM CaCl2 was added to wells A, B and C, respectively **(*Figure 3*)**.

|  |  |
| --- | --- |
|  |  |
| **A** | **50 μL 10mM CaCl2+ PRP** |
| **B** | **50 μL 10mM CaCl2 + PPP** |
| **C** | **50 μL 10mM CaCl2 + PPP from PDB** |

***Figure 3*:** Costar microwell reaction setup for clot analysis within the spectrophotometer at 37oC

### 2.2 Optimization of PDB with Washed Microbeads

Effective platelet depletion was analyzed following the use of washed microbeads in addition to testing for microparticle generation following its exposure to the column. As outlined in section 2.1.2 thirty mL of CPDA-1 chelated blood was obtained. The platelet count and RMV for these samples were obtained as outlined in section 2.1.8 with additional RMV fractions of 25% and 10%. At 4oC microbeads were washed of EDTA by following the modified manufacturer’s protocol. Four different reaction volumes were used: 100%, 50%, 25% and 10% of the RMV. PDB and its platelet count was obtained with these RMV using the protocol outlined in section 2.1.8. To test for platelet functionality and column exposure samples were prepared as followed:

 **Positive Control:** Using the untouched CPDA-1 chelated blood with the other falcon tube PRP with equal dilution as the experimental group, but using PBS buffer pH 7.4 versus 100% of the RMV was attained via centrifugation at an RPM of 750 for 20 minutes.

**Negative Control:** Similar to the positive control the PPP with proportional dilution as the experimental group, but using PBS buffer pH 7.4 a substitute versus 100% of the RMV was attained via centrifugation at an RPM of 2400 for 20 minutes.

**Experimental:** To determine how the platelets in the PDB behave the PDB samples that used 100%, 50%, 25% and 10% RMV were centrifuged at an RPM of 750 for 20 minutes to obtain PRP from PDB. Prior to centrifugation the samples that used only 50%, 25%, and 10% of the RMV were given additional PBS buffer pH 7.4, the difference in RMV with respect to 100% was added to each sample to achieve equal dilution.

**Column Exposure:** With a new magnetic chromatography the column was rinsed with 1200 μL of PBS buffer pH 7.4 at 4oC. The PBS buffer pH 7.4 remaining within the void volume was flushed out with 1800 μL of untouched CPDA-1 chelated blood. Then 800 μL of CPDA-1 chelated blood diluted with PBS buffer pH 7.4 matching the 100% RMV dilution was added to the column and the eluted product was discarded. Following this step the remaining volume was added and approximately1500 μL were collected in two Falcon tubes. One test tube was centrifuged at 750 RPM for 20 minutes to obtain PRP and the latter was centrifuged at 2400 RPM to obtain PPP for20 minutes.

**Clotting Assay:** The spectrophotometer was pre-warmed to achieve an internal temperature of 37oC and parameters for running a kinetics test was set at a wavelength of 350 nm, read at 15 second intervals, for a test period of 3 hours. Once the temperature was stable at 37oC 50 μL of PRP, 10% RMV used PRP from PDB, 25% RMV used PRP from PDB, 50% RMV used PRP from PDB, 100% RMV used PRP from PDB and PPP were added to wells A1, B1, C1, D1, E1 and F1. Then in wells A5, B5, C5 and D5 PRP, column exposed PRP, PPP and column exposed PPP were added respectively. Clotting was initiated (***Figure 4***) in each well by adding 50 μL of 10 mM CaCl2.

|  |  |
| --- | --- |
|  |  |
| **A1** | **50 μL 10mM CaCl2+ PRP** |
| **B1** | **50 μL 10mM CaCl2 + 10% RMV used PRP from PDB** |
| **C1** | **50 μL 10mM CaCl2 + 25% RMV used PRP from PDB** |
| **D1** | **50 μL 10mM CaCl2 + 50% RMV used PRP from PDB** |
| **E1** | **50 μL 10mM CaCl2 + 100% RMV used PRP from PDB** |
| **F1** | **50 μL 10mM CaCl2 + PPP** |
| **A5** | **50 μL 10mM CaCl2+ PRP** |
| **B5** | **50 μL 10mM CaCl2+ Column exposed PRP** |
| **C5** | **50 μL 10mM CaCl2+ PPP** |
| **D5** | **50 μL 10mM CaCl2+ Column exposed PPP** |

***Figure 4*:** Costar microwell reaction setup for clot analysis within the spectrophotometer at 37oC

### 2.2.1 Effect of Temperature on Blood

In order to test the effects of varying temperature on platelet functionality the TEG was utilized. As outlined in section 2.1.2 ten ml of CPDA-1 chelated blood was obtained. An adjusted volume of blood was added to two separate mini Eppendorf tubes labeled either 22oC or 4 oC containing 30 μg/μL of Corn Trypsin Inhibitor (CTI) (Total volume 500 μL). The cold labeled samples were incubated in the cold room at 4 oC for a fixed time period (15, 40, 80, 120 and 160 minutes) on a rocker. The 22oC labeled samples were placed on a rocker at room temperature for the respective time period as the 4 oC labeled samples. Following the incubation periods in the cold room the samples were allowed to reach room temperature for 10 min. While the samples were equilibrating to room temperature the TEG was calibrated for use. Following the 10 min time period clotting was initiated using 20 μL of 10 mM CaCl2 for either samples (4oC versus 22oC) and monitored using the TEG. The clotting profiles determined from the TEG were then subjected to statistical analysis.

### 2.2.2 Reduced Modified Microbead Incubation Period

A reduced incubation period of 15 minutes with microbeads was tested to determine its efficiency in effective platelet depletion (>90%) in blood. As discussed in section 2.1.2 ten ml of CPDA-1 chelated blood was obtained and its platelet counts was determined. The appropriate volume of blood were separated into two mini Eppendorf tubes labeled either CD61 treated versus control (untreated) containing 30 μg/μL of CTI (Total volume 1200 μL). Then 50% of the washed RMV as outlined in section 2.1.8 was added to the “CD61 treated” labeled samples and using its proportional volume PBS buffer (pH 7.4) was added to the control (untreated) labeled sample. Then both samples were brought into the cold room and were briefly exposed /handled at 4 oC for 15 minutes. Within that 15 minute time frame the CD61 treated sample were incubated for 10 minutes on the Barnstead rotisserie. During this 10 minute period the whole blood chromatography column was prepared. The column was placed on the magnetic adapter and was rinsed with 1200 μL of PBS buffer pH 7.4. The void volume prefilled with the buffer (PBS) was displaced using 1800 μL of untouched blood. After 5 minute of the 10 minute incubation period 800 μL of the CD61 treated sample was placed within the column to displace the untouched blood within the void volume (all eluted samples were discarded). The “CD61 treated” labeled sample was then placed back on the Barnstead rotisserie for the remaining 10 minutes of the incubation period. Once the 10 min incubation period was completed using the remaining 5 minute within the 15 minute time frame the entire volume of the “CD61 treated” labeled sample was pipetted into the column and its eluted product was collected within a test tube. The remaining PDB within the void volume was displaced using 400 μL of untouched blood. Following the collection of the PDB the sample was placed back on the rotor at 22oC for 10 minutes to reach room temperature. After 10 minutes the platelet count of PDB was obtained at the core lab as described above.

### 2.2.3 Platelet Count Percentile

 In order to have a standard fixed hemodilution upon microbead addition with each experiments the general platelet count observed in a small population was determined. Previously determined untouched blood platelet counts from healthy volunteers were recorded onto a Microsoft Excel spread sheet. The following values were than used to create a percentile graph of the platelet counts obtained from healthy volunteers within a small population.

### 2.2.4 Hemodilution Effect

To determine the effects of hemodilution on clot formation blood samples with fixed hemodilution values were measured and analyzed using the TEG. Approximately ten ml of CPDA-1 chelated blood was obtained as outlined in section 2.1.2. Appropriate volume of PBS (pH 7.4) was added as a substitute for microbeads and 30 μg/μL of CTI (Total volume 1200 μL). The sample with the buffer addition was labeled either 26.7% total hemodilution or 18.8% total hemodilution and samples devoid of buffer were labeled control. Based on the findings from the platelet count percentile the platelet count was assumed to be 250 x 109/L in order to use a fixed standard CD61 microbead volume. Clotting was initiated using 20 μL of 10 mM CaCl2 and monitored using the TEG. Using the same sample clotting was initiated two more times for both undiluted and diluted samples.

Furthermore, after determining the appropriate volume of microbeads to use corresponding to the volume of blood required for a standard experiment the fixed total hemodilution in blood was calculated to be 21.8%. As outlined in section 2.1.2 three healthy volunteers were recruited and 10 ml of CPDA-1 chelated blood was obtained. Following the same procedure as described above hemodilution effect on clotting was analyzed at a total hemodilution of 21.8% via TEG. The TEG parameters were obtained for all three different volunteers and used for statistical analysis.

### 2.2.5 Column Exposure (Microparticle Generation)

In order to test the generation of microparticles upon contact with the whole blood column the PPP of PDB and untouched blood was analyzed using TEG. Thirty ml of CPDA-1 chelated blood was obtained as outlined in section 2.1.2. Then using two test tubes labeled either “CD61 treated” or “buffer substitute” 254.1 μL of either CD61 microbeads or PBS buffer (pH 7.4) were added respectively in addition to 30 μg/μL of CTI (Total volume 2200 μL). PDB was than obtained as outlined in section 2.2.2. Then with the PDB and the untouched blood both samples were centrifuged to obtain its PPP. The PPP obtained from each labeled samples were brought to the Core lab to obtain its platelet count. Than using both PPP from either the CD61 treated or the buffer substitute clotting was initiated using 20 mM CaCl2 and monitored with the TEG.

### 2.2.6 Appropriate TF concentration for the Optimization of TEG

In order to maximize the full sensitivity of the TEG PDB in the presence of anticoagulants were tested with various TF concentration to initiate clotting. Approximately ten ml of CPDA-1 chelated blood was obtained as outlined in section 2.1.2. Than PDB was prepared as outlined in section 2.2.2 using the fixed microbead volume from section 2.2.5.Than following the collection of the PDB (<10 x 109/L) it was placed back on the rotor at 22oC for a minimum of 10 minutes to reach room temperature. The newly acquired PDB samples were than brought to the core lab to determine it platelet counts. Then clotting was initiated for the PDB samples with 20 μL of 10 mM CaCl2and various TF concentrations in the presence of therapeutic levels of LMWH (1.0 IU/ml) or UFH (0.3 U/ml) and monitored via TEG. The following r-time derived was used to determine the TF concentration required to initiate bare minimal clotting (>120 min) of PDB at therapeutic levels of UFH and LMWH.

### 2.2.14 Clotting Profiles at Predefined Platelet Counts in the Presence of Anticoagulants

The clotting profiles at platelet counts of 30 x 109/L and 50 x 109/L in the presence of anticoagulants were tested via TEG. Approximately ten ml of CPDA-1 chelated blood was obtained as outlined in section 2.1.2. PDB was than prepared using the protocol outlined in section 2.2.6. The control and the newly acquired PDB samples were than brought to the core lab to determine it platelet counts upon reaching room temperature (10 minutes). Using the known platelet counts of PDB and its control they were mixed together at appropriate volumes to create blood with predefined platelet counts of 30, 50 and 150 x 109/L. The following four samples PDB, 30, 50 and 150 x 109/L were than subjected to clotting with 20 μL of 10 mM CaCl2and 2.05pM or 2.25pM of TF in the presence of either LMWH (0.1, 0.3, 0.5 or 1.0 IU/ml) or UFH (0.1 or 0.3 U/ml) respectively and monitored via TEG as outlined in section 2.1.3. The following TEG tracings and the values for R and MA derived upon clot formation was used for statistical analysis.

### 2.2.15 Statistical Analysis

All TEG experiments in the presence of therapeutic and prophylactic levels of anticoagulants were performed at least five times. For comparisons between samples, Student’s *t*-test and ANOVA with a post-hoc Tukey test was used. All TEG parameter values were subjected to a Grubbs test for potential outliers (no data was omitted). Values with a *p* ≤0.05 were considered to be statistically significant.

# 3 Results

## 3.1 Blood Column Dilution Optimization

The solution within the void volume accounts for 600 μL. Upon subsequent loading the initial void volume is displaced with the solution containing bromophenol blue. The total 600 μL of the original solution within the void volume eluted due to its displacement is not fully recovered as bromophenol blue is detected prior to the elution of the full 600 μL (***Figure 5***). Approximately 494 μL of the original solution is free from bromphenol blue contamination. After determining the “true void volume” is less than 600 μL blood mixed with phenolphthalein was used to determine the approximate recovery of blood within the void volume free from contamination upon subsequent loading. The approximate volume of blood recovered from the column was approximated by determining its mean volume per drop of blood (***Table 2***). Upon calculating the mean volume per drop of blood eluted, blood recovered from the void volume free from phenolphthalein detection is less than 600 μL (565 μL) as indicated by ***Figure 6***.

***Figure 5*:** Column dilution experiment identifying the level of dilution with the void volume and the reservoir volume upon subsequent sample loading using bromophenol blue as a marker for dilution detection in distilled water. Each symbol indicates a single absorbance value for eluted samples from the column. Red arrow identifies the initial change in absorbance value at a specific volume due to the presence of bromophenol blue detected by the spectrophotometer.

|  |  |
| --- | --- |
| Equation of the Line | Volume (μL) |
| y=1.013x +0.22 | **47.82** |
| y=0.9875x + 2.9 | **47.2** |
| y=0.9875x + 2.9 | **47.7** |
| Mean | **47.6** |

***Table 2*:** Determination for the volume of a single droplet of blood eluted from the column. Baseline equation for each healthy volunteer (N=3) with respect to the volume of blood (x) versus mass (y).

**Figure 6:** Column dilution experiment identifying the level of dilution with the void volume and the reservoir volume upon subsequent sample loading using phenolphthalein as a marker for dilution detection in blood. Each symbol indicates a single absorbance value for eluted samples from the column. Red arrow identifies the initial change in absorbance value due to the presence of phenolphthalein at a specific volume detected by the spectrophotometer.

## 3.2 Albumin Coating

Upon comparison of either non albumin coated versus albumin coated column blood recovered from the albumin coated column has a platelet recovery of less than 34% of its original starting platelet count (***Table 3***). There seems to not be a significant difference in platelet recovery upon using coated versus non coated columns with respect to its original platelet count.

|  |  |  |
| --- | --- | --- |
| Untouched Blood Platelet Count (x109/L) | Non-coated ColumnsPlatelet Count (x109/L) | Albumin Coated ColumnsPlatelet Count (x109/L) |
| 133.4 | **14.6** | **16.4** |
| 49.4 | **13.3** | **14.3** |
| 215.8 | **62.9** | **75.4** |

***Table 3*:** Table identifies donor platelet counts (N=3) pre and post exposure to non-coated or albumin coated columns.

## 3.3 pH of CPDA-1 Chelated Whole Blood

The pH of CPDA-1 solution seems to be ~5.5 and upon collection of blood in CPDA-1 the chelated blood exhibit a pH of ~7.5 when analyzed using a pH indicator (Figure 7).

***Figure 7*:** Image shows pH indicator strips with its colour corresponding pH values upon exposure to CPDA-1 solution and CPDA-1 chelated blood samples.

**CPDA-1** **CPDA-1+Blood**

## 3.4 Effect of time on Platelet Preservation

 With the use of CPDA-1 for blood collection platelet preservation is greater than 96% after 2 hours following venipuncture (Table 4). In citrated blood platelet preservation seems to be compromised due to platelet clumping. After 2 hours platelet counts diminish greater than 20% when collected in CPDA-1.

|  |  |  |
| --- | --- | --- |
| Time (hours) | CPDA-1 Blood Platelet Count (x109/L) | Citrated Blood Platelet Count (x109/L) |
| 0 | **155.9** | **17.6\*** |
| 2 | **150.6** | **N/A\*** |
| >2 | **120.4** | **N/A\*** |

***Table 4*:** Table compares platelet preservation overtime with different chelating agents (CPDA-1 versus Citrate). \*Platelet count compromised due to platelet clumping.

## 3.5 Optimal CD 61 Microbead Volume

The fraction of microbead volume used for platelet depletion at either 50% or 75% of the RMV shows a greater than 90% depletion in platelet count similar to using 100% of the RMV (***Table 5***). The PDB obtained from using 100% of the RMV once centrifuged to generate PPP did not exhibit a clotting time shorter than PRP or PPP of untouched blood rather a delay in clotting time was observed for PPP derived from PDB (***Table 6***).

|  |  |
| --- | --- |
| **Untouched Blood Platelet Count** **(x 109/L)** | **212.4** |
| **100% RMV Platelet Count** **(x 109/L)** | **6.7** |
| **75% RMV Platelet Count** **(x 109/L)** | **12.3** |
| **50% RMV Platelet Count** **(x 109/L)** | **15.6** |

 ***Table 5*:** Table indicates platelet values pre and post platelet depletion using various fractions of CD-61 microbead volumes.

|  |  |  |
| --- | --- | --- |
| Conditions Tested | Platelet Count (x109/L) | Time to ½ Maximum (secs) |
| PRP Control | **212.4** | **780.04** |
| PPP Control | **N/A** | **874.35** |
| PPP from PDB | **6.7** | **1858.6** |

***Table 6*:** Table identifies clotting times in a turbidometric assay for untouched plasma and plasma obtained from PDB.

## 3.6 Microparticle Generation and Platelet Functionality

The platelets of the PDB seems to be functional in its ability to clot before PPP (***Table 7***). With reduced fraction of microbead volume used more platelets are available and its clotting time nears the clotting time of PRP. Either in the presence of column or in the absence of column PRP and PPP shows delayed clotting time when obtained in the presence of column (***Table 8***).

|  |  |  |
| --- | --- | --- |
| Conditions Tested | Platelet Count (x109/L) | Time to ½ Maximum (secs) |
| PRP Control | **241.4** | **691** |
| PRP from PDB (10% RMV) | **97.4** | **768** |
| PRP from PDB (25% RMV) | **54.1** | **641** |
| PRP from PDB (50% RMV) | **5.9** | **884** |
| PRP from PDB (100% RMV) | **7.1** | **998** |
| PPP | **N/A** | **1154** |

***Table 7*:** Table identifies clotting times in a turbidometric assay for untouched plasma and plasma obtained from PDB using different fraction of microbead volume.

|  |  |
| --- | --- |
| Conditions Tested |  Time to ½ Maximum (secs) |
| PRP (-Column) | **691** |
| PRP (+Column) | **967** |
| PPP (-Column) | **747** |
| PPP (+Column) | **1002** |

***Table 8*:** Table identifies clotting times in a turbidometric assay for PRP and PPP pre and post column exposure.

## 3.7 Effects of Temperature on Blood

 For optimal platelet depletion microbeads are to be incubated and mixed in blood at 4oC. Upon comparison of blood handled at room temperature (22oC) versus 4oC the clotting profile of blood based on the four common TEG parameters does not seem to be significantly compromised with an exposure as great as 40 minutes (*p* ≤0.05) (***Figure 8 A-D***). With prolonged exposure (>40 minutes) of blood at 4oC the r-time is statistically significant when compared to blood incubated at 22oC becoming hypercoagulable (***Figure 8 A***). Although not statistically significant there seems to be a greater fluctuation in k-time and MA with prolonged exposure (>120 minutes) of blood to 4oC.

***Figure 8*:** Graphs showing the effects of temperature (22oC versus 4oC) on blood at various incubation periods represented by four common TEG parameters. **(A)** Reactive-time \* statistical significant difference between 22oC and 4oC at incubation periods greater than 80 minutes (*p* ≤0.05). No statistical significant difference for **(B)** Coagulation time, **(C)** alpha angle and **(D)** maximum amplitude at all incubation periods. (N=3, 15 minutes & N=4, 40-160 minutes)

## 3.8 Modified Microbead Incubation Period

 After determining the optimal microbead incubation period at 4oC its ability for effective platelet depletion was measured as it is less than the recommended manufacturer incubation period of 30 minutes. Blood incubated with CD 61 microbeads and handled at 4oC within a 15 minute time frame shows a greater than 90% platelet depletion (***Table 9***). Furthermore, PBMC loss due to the use of CD61 microbeads found no significant difference in the percentage of monocytes observed post platelet depletion (***Table 10****).*

|  |  |  |
| --- | --- | --- |
|  Platelet Counts(x109/L) | Post Platelet Counts(x109/L) | % Difference |
| 265.3 | **17.1** | **93.5** |
| 151 | **7.7** | **94.9** |

***Table 9*:** Modified incubation/handling period of 15 minutes for platelet depletion experiment. Values show pre and post platelet counts following platelet depletion. (N=2)

|  |  |  |
| --- | --- | --- |
|  | Monocyte % | Post Monocyte % |
|  | 0.0663 | 0.0523 |
|  | 0.0566 | 0.0569 |
|  | 0.0693 | 0.0564 |
| Mean |  **0.0641** | **0.0522** |

***Table 10*:** Table looks at pre and post monocyte % following platelet depletion to determine change in PBMC. Values obtained from core lab CBC results. (N=3)

## 3.9 Platelet Count Percentile

It seems within a small population of blood donated the greater frequency of platelet counts observed falls below the 80th percentile (***Figure 9***). There seems to be a greater occurrence of platelet counts between 203 x 109/L and 248 x 109/L.

***Figure 9*:** Histogram percentile showing platelet count distribution in a small population upon donation from healthy volunteers. 20% of platelet counts lie between 113.1-158.1 x 109/L, 80% of platelet counts fall below 248.1 x 109/L and greater than 80% of platelet counts were observed in the range of 248.1-293.1 x 109/L. (N=6)

## 3.10 Hemodilution Effect

Following the addition of microbeads hemodilution is expected in blood and its ability to foster a hypocoagulable environment is assessed using the four common TEG parameters. With a final total hemodilution of 26.7% no significant difference was noted when compared to the control and a reduced hemodilution of 18.8% (***Figure 10 A***). When hemodilution of 21.8% was compared in three healthy volunteers a hypocoagulable environment did not exist at all four TEG parameters as no significant difference was noted (***Figure 10 B***).

 

***Figure 10*:** Graphs illustrate the effects of hemodilution represented by four common TEG parameters R, k,  and MA. **(A)** Compares control to 18.8% and 26.7% final hemodilution (N=1, 3 replications). **(B)** Compares control to 21.8% final hemodilution (N=3).

## 3.11 Column Exposure (Microparticle Generation)

An indirect detection of microparticles generated in response to column exposure of blood is measured in TEG. Increase in r-time for PPP from PDB (+Column) versus PPP obtained via centrifugation was observed (***Table 10***).

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| Conditions | R | k |  | MA |
| PPP  | **26.8** | **N/A** | **11.9** | **17.5** |
| PPP from PDB | **47.6** | **N/A** | **23.7** | **18.5** |

***Table 11*:** Table comparing the TEG parameters of R, k, and

## 3.12 TF Optimization in the Presence of UFH and LMWH

 Reducing the platelet count to <10 x 109/L (PDB) shows a compromise in the ability to clot at therapeutic levels of UFH and LMWH. Clotting seems to be further delayed with reduced TF concentration and improved with increasing TF concentration. An inverse relationship seems to exist with TF concentration and r-time. With increasing TF concentration r-time diminishes and with lower TF concentration r-time increases. It seems bare minimal clotting is best achieved in PDB for 0.3 U/ml UFH at a TF concentration of 2.25 pM (***Figure 11 A***). At therapeutic levels of LMWH (1 IU/ml) bare minimal clotting is optimally achieved at a lower TF concentration versus UFH at 2.05 pM (***Figure 11 B***).



***Figure 11*:** TF optimization experiments in PDB at therapeutic levels of anticoagulant where R was measured with varying amounts of TF. Optimal TF concentration was chosen based on achieving bare minimal clotting (120-160 minutes) in PDB. (A) UFH (N=2 1.2, 2.5 and 8pM N=1 2.25 and 4pM N=3 2pM) (B) LMWH (N=1 2.05 pM, N=3 2.1-2.2 pM).

## 3.13 Clotting Profiles at Predefined Platelet Counts in the Presence of Anticoagulants

### 3.13.1 R, Ma and AUC15 at Therapeutic and Prophylactic Levels of UFH

After determining the optimal TF concentration for each individual anticoagulant the effects of platelets in blood was studied. Statistical significant difference was not found when comparing platelet counts 30 x 109/L and 50 x 109/L at therapeutic and prophylactic levels of UFH with respect to r-time (***Figure 12 A and B***). However, a statistical significant in the overall strength of the clot identified by MA is found at prophylactic levels of UFH (*p* ≤0.05) (***Figure 13*** ***B***). There seems to be an inverse relationship between r-time and MA when comparing therapeutic and prophylactic levels of UFH. The quality of the clot (MA) seems to diminish with increasing levels of UFH. Overall a significant difference was noted when comparing the r-time of PDB or 150 x 109/L to the other predefined platelet counts. In terms of the speed and strength of clot formation represented by AUC15 at therapeutic levels of UFHshow no significant difference at platelet counts of 30 x 109/L and 50 x 109/L (***Figure 14 A***). However, similar to the MA for prophylactic levels of UFH a difference is noted at these specific platelet counts (*p* ≤0.05) (***Figure 14 B***). Furthermore, a significant difference is noted at therapeutic levels of UFH when comparing platelet counts of 150 x 109/L to the other reduced platelet level. In terms of prophylactic levels of UFH, AUC15 seems to be compromised significantly in a linear trend as platelet counts are further reduced. Interestingly the AUC15 of PDB at prophylactic levels of UFH is found to be similar statistically to the AUC15 at platelet counts of 150 x 109/L at therapeutic levels of UFH.



***Figure 12*:** Modelling of TEG parameter r-time at therapeutic and prophylactic levels of UFH with TF concentration optimized for UFH (2.25 pM). **(A)** Therapeutic levels of UFH (0.3 U/ml) \*PDB compared to 30 x 109/L, 50 x 109/L, and 150 x 109/L \*\* 150 x 109/L compared to PDB, 30 x 109/L and 50 x 109/L statistical significant difference exist (*p* ≤0.05). **(B)** Prophylactic levels of UFH (0.1 U/ml) \*PDB compared to 150 x 109/L and \*\* 150 x 109/L compared to PDB, 30 x 109/L and 50 x 109/L statistical significant difference exist (*p* ≤0.05). (N=5)



***Figure 13*:** Modelling of TEG parameter MA at therapeutic and prophylactic levels of UFH with TF concentration optimized for UFH (2.25 pM). **(A)** Therapeutic levels of UFH (0.3 U/ml) \*PDB compared to 150 x 109/L and \*\* 150 x 109/L compared to PDB, 30 x 109/L and 50 x 109/L statistical significant difference exist (*p* ≤0.05). **(B)** Prophylactic levels of UFH (0.1 U/ml) \*PDB compared to 30 x 109/L, 50 x 109/L and 150 x 109/L, \*\* 30 x 109/L compared to 50 x 109/L and \*\*\* 150 x 109/L compared to PDB, 30 x 109/L and 50 x 109/L statistical significant difference exist (*p* ≤0.05). (N=5)



****

***Figure 14*:** Post-hoc TEG tracing analysis for AUC15 at therapeutic and prophylactic levels of UFH with TF concentration optimized for UFH (2.25 pM). **(A)** Therapeutic levels of UFH (0.3 U/ml) \*PDB compared to 150 x 109/L and \*\* 150 x 109/L compared to PDB, 30 x 109/L and 50 x 109/L statistical significant difference exist (*p* ≤0.05). **(B)** Prophylactic levels of UFH (0.1 U/ml) \*PDB compared to 30 x 109/L, 50 x 109/L and 150 x 109/L, \*\* 30 x 109/L compared to 50 x 109/L and \*\*\* 150 x 109/L compared to PDB, 30 x 109/L and 50 x 109/L statistical significant difference exist (*p* ≤0.05). (N=5)

### 3.13.2 R, Ma and AUC15 at Therapeutic and Prophylactic Levels of LMWH

Using the optimal TF concentration for LMWH the clotting profile of platelets in blood was studied using TEG similar to UFH. At upper and lower therapeutic levels of LMWH at platelet counts of 30 x 109/L and 50 x 109/L are not found to be statistically significant with respect to r-time and MA (***Figure 15 A and B***) (***Figure 16 A and B***). However, the r-time is significantly prolonged at upper therapeutic levels of LMWH when compared to its lower therapeutic levels at platelet counts of 30 x 109/L and 50 x 109/L (*p* ≤0.05). Furthermore, it seems the r-time bar graph at a platelet count of 150 x 109/L at 1.0 IU/ml of LMWH seems similar to the r-time bar graph at a platelet count of 30 x 109/L in the presence of 0.3 IU/ml of LMWH (***Figure 15 A and C***). With respect to MA in a similar fashion the quality/strength of the clot improves at lower levels of LMWH. At upper and lower prophylactic levels of LMWH the r-times of 30 x 109/L and 50 x 109/L do not show any statistical significant difference (***Figure 15 C and D***). While no statistical significant difference seems to exist for MA at lower prophylactic levels of LMWH for 30 x 109/L and 50 x 109/L a change is noted at higher prophylactic levels of LMWH (*p* ≤0.05) (***Figure 16 C and D***). Interestingly at higher and lower prophylactic levels of LMWH no difference in terms of r-time can be seen amongst all predefined platelet counts. Thus, PDB in comparison to 30 x 109/L for both upper and lower prophylactic levels of LMWH show no significant difference. In terms of the strength and speed of clot formation represented by AUC15 is not compromised at platelet counts of 30 x 109/L and 50 x 109/L for therapeutic and prophylactic levels of LMWH (upper or lower). It seems AUC15 improves linearly at all platelet levels with reducing levels of LMWH concentrations.



***Figure 15*:** Modelling of TEG parameter r-time at therapeutic and prophylactic levels of LMWH with TF concentration optimized for LMWH (2.05 pM). **(A)** Upper therapeutic levels of LMWH (1 IU/ml) \*PDB compared to 30 x 109/L, 50 x 109/L, and 150 x 109/L and \*\* 150 x 109/L compared to PDB, 30 x 109/L and 50 x 109/L statistical significant difference exist (*p* ≤0.05). **(B)** Lower therapeutic levels of LMWH (0.5 IU/ml) \*PDB compared to 30 x 109/L, 50 x 109/L, and 150 x 109/L \*\* 150 x 109/L compared to PDB statistical significant difference exist (*p* ≤0.05). **(C)** Upper prophylactic levels of LMWH (0.3 IU/ml). **(D)** Lower prophylactic levels of LMWH (0.1 IU/ml). (N=5)



***Figure 16*:** Modelling of TEG parameter MA at therapeutic and prophylactic levels of LMWH with TF concentration optimized for LMWH (2.05 pM). **(A)** Upper therapeutic levels of LMWH (1 IU/ml) \*PDB compared to 150 x 109/L and \*\* 150 x 109/L compared to PDB, 30 x 109/L and 50 x 109/L statistical significant difference exist (*p* ≤0.05). **(B)** Lower therapeutic levels of LMWH (0.5 IU/ml) \*PDB compared to 50 x 109/L and 150 x 109/L \*\* 150 x 109/L compared to PDB, 30 x 109/L and 50 x 109/L statistical significant difference exist (*p* ≤0.05). **(C)** Upper prophylactic levels of LMWH (0.3 IU/ml) \*PDB compared to 50 x 109/L and 150 x 109/L, \*\* 30 x 109/L compared to 50 x 109/L and \*\*\*150 x 109/L compared to PDB, 30 x 109/L and 50 x 109/L statistical significant difference exist (*p* ≤0.05). **(D)** Lower prophylactic levels of LMWH (0.1 IU/ml) \*PDB compared to 30, 50 and 150 x 109/L \*\* 150 x 109/L compared to PDB, 30 x 109/L and 50 x 109/L statistical significant difference exist (*p* ≤0.05). (N=5)



*****Figure 17*:** Post-hoc TEG tracing analysis for AUC15 at therapeutic and prophylactic levels of LMWH with TF concentration optimized for LMWH (2.05 pM). **(A)** Upper therapeutic levels of LMWH (1 IU/ml) \*PDB compared to 150 x 109/L and \*\* 150 x 109/L compared to PDB, 30 x 109/L and 50 x 109/L statistical significant difference exist (*p* ≤0.05). **(B)** Lower therapeutic levels of LMWH (0.5 IU/ml) \*PDB compared to 150 x 109/L \*\* 150 x 109/L compared to PDB, 30 x 109/L and 50 x 109/L statistical significant difference exist (*p* ≤0.05). **(C)** Upper prophylactic levels of LMWH (0.3 IU/ml) \*PDB compared to 150 x 109/L and \*\*150 x 109/L compared to PDB, 30 x 109/L and 50 x 109/L statistical significant difference exist (*p* ≤0.05). **(D)** Lower prophylactic levels of LMWH (0.1 IU/ml) \*PDB compared to 30, 50 and 150 x 109/L \*\* 150 x 109/L compared to PDB, 30 x 109/L and 50 x 109/L statistical significant difference exist (*p* ≤0.05). (N=5)

# 4 Discussion

The anticoagulation regiment for the management of VTE and concomitant thrombocytopenia is still quite controversial. The platelet threshold at which managing physicians must hold off on anticoagulant treatments is still debated. Previous literature suggested platelet counts should be elevated to a minimal platelet threshold of 50 x 109/L before any anticoagulation therapy may be provided (Lee, 2009). However, Arnold & Lim (2011) suggest anticoagulation therapy can be provided at a reduced platelet level of 30 x 109/L when given prophylactic doses. In the most recent literature it suggest that anticoagulants may be provided at a reduced platelet count of 30 x 109/L even at therapeutic levels (Athale et al., 2012). Therefore, due to these contradictory suggestions this *in vitro* study explores the clotting profiles in blood at predefined platelet counts in the presence of UFH and LMWH specifically looking at platelet counts of 30 x 109/L and 50 x 109/L. Initially due to the novelty in generating thrombocytopenic blood using CD 61 microbeads with the magnetic bead column optimization protocols are necessary before generating and analyzing thrombocytopenic blood in the presence of UFH and LMWH.

##  4.1 Magnetic Column Chromotorgraphy Optimization

The mechanistic design of the magnetic bead column involves the removal of molecules from samples pre-incubated with monoclonal antibodies (CD 61 microbeads). These samples pass through the column and the monoclonal bound molecules adhere to the iron spheres layered within the column as it is subjected to a high gradient magnetic force. The iron sphered portion of the column itself has a void volume of 600 μL. Thus, the loading of further volumes to the column will displace the initial 600 μL sample within the column. However, contamination may occur between the samples if the void volume is not taken into consideration between subsequent sample loadings. Therefore, in order to test for the degree of contamination (dilution) with subsequent loading bromophenol blue was used as a surrogate marker of contamination. Its purpose was to determine the initial contamination of samples within the void volume following subsequent displacement of the void volume with further sample addition. Following this experiment it was determined that the “true void volume” is not 600 μL (suggested by manufacturer), but rather ~494 μL which is the approximate volume free of bromophenol blue detection at the initial contact between the void volume and the reservoir volume. Hence, adding in approximately 800 μL of the subsequent sample to the column should remove any unwanted contamination from the prior run. As well with additional volumes added to the column it is evident that the samples are not further diluted with bromophenol blue (whole blood substitute) based on the observed plateau effect from the absorbance readings (***Figure 5***).This suggests that there are no further dilutions beyond the “true void volume”, which may infer that whole blood is not compromised by additional loading of samples.

However, the limiting factor with this experiment is the use of bromophenol blue as a substitute for whole blood. This assumes that each single droplet accounts for a density of 1 kg/m3 (equivalent of water). However, the density of whole blood is not identical to water. Thus, with determining the mean volume for a single droplet of blood as ~47.6 μL (***Table*** ***2***) (passing through column) it provided an estimate for at which volume of phenolphthalein mixed blood can be detected upon elution from the column. It is clear that in both cases the throughput volume free of dilution is not 600 μL (***Figure*** ***6***), hence by adding 800 uL (additional 200 μL) it would displace the contaminated portion within the void volume. In this experiment phenolphthalein was used as the dye to determine whether any contamination occurred versus bromophenol blue due to the latter inducing hemolysis. Steigmann & Dyniewicz (1944) determined that the resistance of red blood cells was not altered in the blood of rats administered with phenolphthalein, which suggests that it does not induce hemolysis, making it the appropriate dye to be added in blood. Furthermore phenolphthalein was dissolved in 20% ethanol due to its inability to dissolve in water, which was found to not be hemolytic at that particular concentration. After determining the volume required to avoid contamination between subsequent loading column coatings was optimized as a way to reduce platelet loss during sample loading.

## 4.2 Albumin Coating

 The initial purpose of using magnetic chromatography is to control for platelet depletion with varying CD 61 microbeads as blood passes through the column. However, it is suggested that when foreign surfaces such as the column comes in contact with whole blood, platelets are the first to adhere (Broberg et al., 2002). Thus, it was suggested that albumin coating of columns may reduce the adhesion of proteins and platelet aggregation to the surface of walls (Kinnari et al., 2002) (Jorgensen & Stoffersen, 1979). Upon passing blood through coated columns our results showed albumin coating yielded <10% platelet count versus non-coated columns (***Table*** ***3***). Additionally, there was significant loss in platelets when passing through either the coated or non-coated columns. Therefore, it is evident that coating of columns is not necessary as it does not preserve platelet counts versus non-coated columns.

## 4.3 CPDA-1 Whole Blood Chelating Agent

It is necessary for this study to preserve platelet counts untouched blood prior to mixing with thrombocytopenic blood. Therefore, CPDA-1 chelating was used for collecting blood due to its superior properties in platelet preservation. Initially it is imperative to determine the pH of CPDA-1 chelated blood in order to preserve the physiological pH of blood samples. Maintaining the pH balance is essential for the normal hemostatic response and to reduce hemolysis. Upon analysis it was determined that the pH of CPDA-1chelated whole blood was 7.5, which suggests the buffer is able to maintain the physiological pH of blood (***Figure 7***). Furthermore, the pH is consistent with the study of Moore et al. (1981) which shows the pH of whole blood stored in CPDA-1 buffer to be 7.6 +/- 0.13.

## 4.4 Preservation of Platelet Count Overtime

As stated in section 4.3 CPDA-1 was used as it has been noted to increase the shelf life of whole blood which suggests the components of whole blood are better preserved (Beutler & West, 1979). As well, in transfusion medicine, CPDA-1 is used as the primary chelating agent when prolonging whole blood storage from 28 to 35 days (Moore et al., 1981). In order to confirm whether or not CPDA-1 has any effect on stored whole blood, it was compared against a commonly used chelating agent, sodium citrate. Initially whole blood was stored at 4oC as it better preserves platelet functionality versus 37oC, which showed reduced platelet function (Kattlove et al., 1972). However, it was determined that citrated whole blood stored in an ice box initiated platelet clumping, which significantly reduced platelet count. Whereas, the platelets of CPDA-1 chelated whole blood incubated at 4oC did not experience clumping, and in addition, CPDA-1 preserved the platelet count by 96.6% compared to the control after 2 hours post venipuncture (***Table 4***). This suggests that CPDA-1 is an effective reagent in preserving the stability of platelets in whole blood overtime. This result provides the optimal time for using whole blood stored in CPDA-1 thus, we can determine an effective time frame for using whole blood during our experiments.

## 4.5 Platelet Removal via CD 61 Antibody

The optimization of volume of microbeads used is essential in order to achieve effective platelet depletion from whole blood as well as reducing hemodilution. It is important to note that peripheral blood mononuclear cell (PBMC) found in whole blood competes the removal of platelets effectively as PBMC’s are also targeted by anti-CD 61 antibodies. Therefore, it is inevitable that not all platelets are bound by the magnetic force when subjected to the column because not all CD 61 antibodies would bind the platelets equally. However, it is essential that PBMC not be removed from the whole blood as the focus of this study was to mimic *in vivo* thrombocytopenia. As well, PBMC may play a role in coagulation via the cell based model of hemostasis as it may act as a TF-bearing cell (Monroe et al., 1996).

Upon CBC reports obtained following platelet depletion it was found that PBMC was not reduced significantly versus the control (***Table 10***).Furthermore CBC reports showed approximately 94 % of platelets were depleted from whole blood when a minimal RMV of 50% was used. This was essential as platelet counts must be significantly reduced for this study prior to testing with TEG. Prior to this work, the goal of the optimization was to control for platelet depletion via anti-CD 61 antibodies. However, the preservation of platelets using albumin coating was not predictable enough for the project to proceed. Hence, an alternative approach was initiated which involves removing 90% of the original platelet count. By greatly reducing the platelet count, we can then introduce and mix untouched whole blood at various volumes to achieve a specific platelet count. Most importantly, by reducing the volume of antibodies introduced, it minimizes hemodilution. By adding 50% of CD 61 antibodies it will increase the hemodilution to 22.5%. Based on clotting tests using the TEG, hemodilution of up to 50% does not affect the clotting properties of whole blood when compared to its control (undiluted) (Tobias, Wambold, Pilla, & Greer, 1998). Therefore, the dilution of whole blood up to 50% should be acceptable as clot formation is not significantly affected.

## 4.6 Microparticle Generation during Platelet Depletion

The microbeads being used were intended to be devoid of EDTA and sodium azide as EDTA itself may alter the clotting assay by further preventing clotting once the activator (calcium chloride) is introduced into the sample. Also, using two different chelating agents may further deter coagulation as samples are initially collected in CPDA-1. Moreover, it is imperative that EDTA be removed from the microbead solution as EDTA added to CPDA-1 chelated whole blood has been shown to increase mean platelet volume (MPV) (McShine, Das, Smit Sibinga, & Brozovic, 1991). Elevated levels of MPV have been linked to activated platelets, thus this may suggest that platelets can become prone to activation in the presence of EDTA (Park, Schoene, & Harris, 2002). As well, the removal of sodium azide is necessary because of its ability to induce hemolysis (Inns, Cecchini, & Mattoni, 1989). The concern that may exist with the removal of EDTA and sodium azide does not compromise the intended purpose of the beads. This was evident by the >90% platelet depletion following the use of washed beads at 100% and 50% of the RMV (***Table 5***). In addition, evaluating the functionality of platelets following the use of washed microbeads is imperative as it contradicts any notion that the platelet depletion process affects normal platelet function. In the clotting assay, it was determined that platelets were functional after the centrifugation of PDB to PRP as evident by its ability to clot (***Table 6***). Furthermore, the platelets from the whole blood of 100% and 50% of the RMV treated samples (reduced platelet counts) clotted within the half-maximum time of the untouched PPP whereas the 10% and 25% of the RMV treated samples experienced a shorter clotting time than the untouched PPP (***Table 7***). The clotting assay also determined that the concentrations of platelets within the PDB were not found to be in a procaogulant state. In the event platelets would be activated, degranulation would occur, which would result in the release of platelet-derived microparticles, which would subsequently cause an elevated procoagulant state within the whole blood. However, this was not the case as the half-max time for clot formation for platelets within PDB was not found to be similar to the positive control. As well platelets were not further activated when in contact with the magnetic chromatography as the PRP and PPP of the column exposed group did not quicken clot formation before the control group (***Table 8***). This further suggests that the exposure of the column does not induce platelet activation thus, not leading to a greater procoagulant state of whole blood.

## 4.7 Effect of Temperature on Whole Blood

The morphology of unstimulated platelets in circulation tends to be in the form of a flat disc (White & Krivit, 1967). Upon platelet activation this morphology is lost and platelets begin developing pseudopods as the shape transitions from a flat disc to an irregular shape (White & Krivit, 1967). This modification is necessary for platelets to adhere and aggregate at sites of injuries (White & Krivit, 1967). However, White and Krivit (1967) have found that when platelets are exposed to cold temperatures (4oC) their morphology changes in the same manner as when platelets become activated. The platelets seems to lose its marginal bundles of microtubules as it deviates from a flat disc to a more discoid shape (White & Krivit, 1967). But it is important to note that the authors have found that the original morphology can be rescued with prolonged incubation at 37oC (White & Krivit, 1967). The importance of this background is that within this project in order for the antibodies (CD61 microbeads) to work optimally it must be incubated in whole blood at 4oC. Therefore, this form of platelet cooling may cause the platelets within the whole blood to become procoagulant, hence affecting the overall clotting profile. When comparing whole blood stored at room temperature (22oC) versus 4oC the TEG parameters (r, k, alpha and MA) were not statistically different up to an incubation period of 40 minutes (***Figure 8 A-D***), which suggests that the platelets were not compromised during this experiment. At incubation periods greater than 80 min, there was a statically significant difference in the r-time, which suggests the platelets may have become compromised during this prolonged period of chilling. Thus, the whole blood exposed at 4oC over prolonged incubation periods (>40 min) experience a shortened r-time suggesting a more procoagulant condition. Overall, it seems that a chilling period within 40 minutes does not affect the clotting profiles when compared to whole blood stored at 22oC via TEG.

## 4.8 Modified Microbead Incubation Period

 MACs microbead company standard protocol for the removal of platelets require beads to be incubated within whole blood for approximately 30 miuntes at 4oC. Although our previous work found no statistical significant difference in TEG parameters up to a cooling period of 40 minutes, a smaller *p* value (0.103) was noted at 40 minutes with a reduced r-time. However, a chilling period of 15 minute yielded a higher *p* value (0.85) with near identical TEG parameters when compared to samples incubated at room temperature (22oC) suggesting less statistical error. Therefore, a minimum incubation period of 15 minute was implemented as a new standard protocol. Furthermore, it is imperative to confirm whether or not a brief 15 minute exposure of whole blood with microbeads at 4oC can optimally deplete platelets in whole blood to less than 10 x 109/L. The results confirm a platelet depletion of > 90% was achieved with a 15 minute exposure of anti-CD61 microbeads to whole blood at 4oC (***Table 9***). Therefore, a 15 minute incubation period at 4oC is effective in creating PDB.

## 4.9 Platelet Count Percentile

 Based on company standard protocol the volume of microbeads required to effectively remove >90% of the original platelet count is dependent on the initial platelet count of the healthy volunteer. Thus, the volume of microbeads used may vary with different volunteers. This will cause a dilution variation amongst samples which may affect the clotting profiles. Hence, by pooling previous platelet counts from healthy volunteers it was determined that 80% of platelet counts fall below 250 x 109/L (***Figure 9***). Therefore, a platelet count of 250 x 109/L was assumed in order to achieve equal hemodilution amongst all samples for all experiments.

## 4.10 Hemodilution Effect

 Hemodilution has been found to delay prothrombin time (PT) and aPTT by inducing a hypocoagulable state (Tobias, Wambold, Pilla, & Greer, 1998). As our protocol requires the addition of anti-CD 61 microbeads to the whole blood, the added volume of the sample may induce a hypocoagulable state. Hence, it was necessary to optimize the minimum hemodilution acceptable without affecting the overall hemostasis of the sample. Our results found no statistical significant difference in terms of TEG parameters (r, k, alpha and MA) at normal platelet counts (>150 x 109/L to 400 x 109/L) up to a hemodilution of 26.7% versus the control (<10%) when recalcified [N of 1 (3 times)] (***Figure 10 A***). Based on the volume of whole blood and microbeads required for each experiment the final hemodilution upon calculation is determined to be 21.8%. Thus, when the work was repeated with n of 3 at a final hemodilution of 21.8% there was no significant difference at all 4 TEG parameters as well (***Figure 10 B***). Therefore, a final hemodilution of 21.8% does not induce a hypocoagulable state when conducting experiments using the TEG for all testing purposes.

## 4.11 Column Exposure (Microparticle Generation)

 As mentioned in the introduction, platelet activation may trigger the release of microparticles. Platelets may become activated when in contact with foreign surfaces, thus enhancing platelet activation and aggregation (microparticle release) (Packham, 1988) (White et al., 1989). Hence, it is important to confirm whether or not microparticles are generated during the platelet depletion process, which may produce a procoagulant sample. When centrifuging the PDB to obtain PPP the r-time was prolonged versus the r-time of untouched whole blood’s PPP (not exposed to column) (***Table 11***). This suggests no microparticles were generated within the eluted product. The reason for the delayed clotting may be due to the superiority of the platelet depletion process using the column as the anti-CD61 antibodies can also target microparticles (Piccin et al., 2007). Therefore, the column may be removing platelets in addition to microparticles generated within the process. In terms of the PPP from the untouched whole blood, microparticles were more than likely generated due to the centrifugation process (Piccin et al., 2007).

## 4.12 TF Optimization in the Presence of UFH and LMWH

 As the mechanism for UFH and LMWH are different the concentration of TF required was optimized in order to fully maximize the sensitivity of the TEG for each individual anticoagulants. For these experiments as clotting is induced in the presence of anticoagulants at extremely low platelet counts recalcification alone may not trigger clotting hence, the exogenous addition of TF is necessary. Furthermore, due to the limitations of the device (TEG) clotting profiles can only be monitored within a 3 h time frame. Therefore, maximizing the r-time at extremely low platelet counts (PDB) at therapeutic levels of anticoagulants is necessary in order to gain a better depiction of the r-time at higher platelet counts within the 3 h time frame. Our results find that bare minimal clotting (>120 min) is achieved at a TF concentration of 2.25 and 2.05 in the presence of UFH and LMWH respectively (***Figure 11 A and B***). The higher TF concentration required for UFH may be due to its ability to be a more potent anticoagulant as it targets both FXa and FII versus LMWH.

## 4.13 Clotting Profiles at Predefined Platelet Counts in the Presence of Anticoagulants

Due to the contradictory guidelines from different literature as described above this study evaluated the clotting profiles of platelets counts of 30 x 109/L and 50 x 109/L in the presence of UFH or LMWH in blood. In our work the therapeutic and prophylactic levels for UFH were within the anti-Xa levels of 0.3-0.7 U/ml and 0.1-0.3 U/ml respectively (Eikelboom & Hirsh, 2006) (Monagle et al., 2008). Due to the different routes of administration with LMWH (subcutaneous injection) its therapeutic and prophylactic levels were evaluated at upper and lower levels. The therapeutic and prophylactic anti-Xa levels of LMWH were 0.5-1.0 IU/ml and 0.1-0.4 IU/ml respectively (Duhl et al., 2007).

Following a TEG analysis at therapeutic and prophylactic levels of UFH no statistically significant difference was noted at platelet counts of 30 x 109/L and 50 x 109/L with respect to r-time (***Figure 12 A***). However, when platelet levels were reduced below 10 x 109/L (PDB) a significant difference was found when compared to platelet levels of 30 x 109/L. This may correlate with the risk for spontaneous bleeding when platelet counts fall below 20 x 109/L (Stasi, 2012). Furthermore, a statistically significant difference in the overall strength of the clot represented by MA at platelet counts of 30 x 109/L and 50 x 109/L is found at prophylactic levels of UFH (*p* ≤0.05) (***Figure 13 B***) specifically at 0.1 U/ml of UFH. As r-time is dependent on the enzymatic properties, whereas MA is represented by platelet counts and fibrinogen levels, the reduced platelet levels may be the contributing factor for its significant difference at low UFH concentrations. An inverse relationship between r-time and MA is noted when comparing therapeutic and prophylactic levels of UFH. With elevated UFH levels r-time is prolonged as platelet counts are lowered, on the other hand as UFH levels decrease the strength of the clot improves with increased platelet counts. As well, the speed and strength of clot formation, represented by AUC15, at therapeutic levels of UFHshow no significant difference at platelet counts of 30 x 109/L and 50 x 109/L (***Figure 14 A***). However, similar to the MA for prophylactic levels of UFH a difference is noted at these specific platelet counts (*p* ≤0.05) (***Figure 14 B***) at reduced levels of UFH. It seems the prophylactic levels of UFH are having a less profound effect on clot formation at lower platelet counts. The more surfaces available for coagulation complexes to form and generate fibrinogen at varying platelet levels does not seem to be significantly deterred at prophylactic levels of UFH. However, at therapeutic levels of UFH the increased UFH compounds may be inhibiting thrombin at a greater degree thus, leading to reduced platelet activation, hence the reason for no significant difference in MA and AUC15 at therapeutic levels of UFH. Additionally, a significant difference was noted at therapeutic levels of UFH when comparing platelet counts of 150 x 109/L to the other reduced platelet levels. As platelet counts fall below the normal range it may be expected that clotting times defer with respect to normal platelet counts in the presence of anticoagulants. Moreover, the AUC15 of PDB at prophylactic levels of UFH is found to be similar statistically to the AUC15 at platelet counts of 150 x 109/L at therapeutic levels of UFH. This suggest that platelet counts below 10 x 109/L in the presence of prophylactic levels of UFH the strength and speed of clot formation is not any more deterred than at platelet counts of 150 x 109/L at therapeutic levels of UFH.

Similar to UFH at therapeutic upper and lower levels of LMWH no statistically significant difference was found at platelet counts of 30 x 109/L and 50 x 109/L with respect to r-time (***Figure 15 A-D***). This was also noted at prophylactic upper and lower levels of LMWH. However, the r-time was significantly prolonged at upper levels of LMWH when compared to its lower levels (*p* ≤0.05). It seems LMWH is most effective at reduced platelet counts at upper levels. As LMWH levels decrease the r-time shortens as it gets closer to the clotting time of normal platelet counts 150 x 109/L. Interestingly at lower prophylactic levels of LMWH no difference in terms of r-time can be seen at all predefined platelet counts. This suggest that at low platelet counts in the presence of prophylactic lower levels of LMWH a delay in clotting is non-existent as clotting time is similar to normal platelet counts. Furthermore, at prophylactic levels of LMWH PDB in comparison to 30 x 109/L for both upper and lower levels show no significant difference. Although platelet counts are less than 10 x 109/L for PDB which may suggest spontaneous bleeding, the risk for major bleeding where it signals for hematological emergency may only be in the event where platelet counts fall below 5 x 109/L coupled with a prior medical condition (Gauer and Braun, 2012) (Slichter, 2004). However, a significant difference between PDB and 30 x 109/L was evident at therapeutic upper and lower levels which may be attributed to the increased abundance of LMWH compound coupled with extremely low platelet counts. In terms of the overall quality of the clot a difference was only noted at prophylactic upper levels of LMWH when comparing platelet thresholds of 30 x 109/L and 50 x 109/L (***Figure 16 C***). As well, it seems the quality of the clot improves with decreasing levels of LMWH which is similar to UFH. Furthermore, the strength and speed of clot formation is compromised neither at platelet counts of 30 x 109/L nor 50 x 109/L for therapeutic and prophylactic levels of LMWH (upper & lower). Ultimately, it seems at prophylactic levels of UFH and LMWH its effect on clotting time is less effective at lower platelet counts thus, supporting the platelet threshold beyond 30 x 109/L for prophylactic use. Lastly, for therapeutic use the platelet threshold seems to exist at 30 x 109/L for UFH and LMWH hence, rejecting the hypothesis as there are no differences at platelet counts of 30 x 109/L and 50 x 109/L on the clotting profiles as measured by TEG.

## 4.14 Limitations

 Our study evaluates experimental data for thrombocytopenic blood clot formation in the presence of anticoagulants. However, our *in vitro* model contains some limitations. One limitation of this work is that currently an algorithm does not exist for the translation of r-time to PT or aPTT. Furthermore, CD61 microbeads are not specifically selective for platelets as CD 61 is found on other cells. As well our use of CTI to inhibit FXIIs limits our understanding of clot formation to the extrinsic pathway. This is important with recent evidence supporting the role of FXII *in vivo*. Furthermore, our work evaluates clot formation within a TEG cup rather than on an endothelial surface. This is important as the endothelial surface plays a major contribution into understanding the cell based model of coagulation. Lastly, our use of different TF concentration amongst different anticoagulants limits our understanding of clot formation to within each anticoagulants. Although we find no statistical significant difference at platelet counts of 30 x 109/L and 50 x 109/L with respect to R it is important to stress that statistical correlation does not lead to a clinical outcome. Therefore, future work looking at clotting profiles of thrombocytopenic blood obtained from thrombocytopenic patients is worth evaluating.

## 4.15 Conclusions/Future Directions

In conclusion, this study showed that, clot formation is not more deterred or significantly different at a platelet threshold of 30 x 109/L versus 50 x 109/L in the presence of anticoagulants. For both UFH and LMWH it seems at prophylactic levels clot formation is less compromised when evaluated by TEG. Ultimately, it seems anticoagulants in this study elicit no statistical significant difference in clot formation at a reduced platelet threshold of 30 x 109/L. Future work may include the evaluation of clot formation in the presence of direct factor specific anticoagulant as wells as clotting induced through the intrinsic pathway.

# 5 References

1. Andersson, L. O., Barrowcliffe, T. W., Holmer, E., Johnson, E. A., & Söderström, G. (1979). Molecular weight dependency of the heparin potentiated inhibition of thrombin and activated factor X. Effect of heparin neutralization in plasma. *Thrombosis research*, *15*(3), 531-541.
2. Arnold, D. M., & Lim, W. (2011). A rational approach to the diagnosis and management of thrombocytopenia in the hospitalized patient. *Seminars in hematology* *48*(4), 251-258.
3. Athale, U., Yang, J., & Chan, A. (2012). Thromboembolism in Children with Cancer.

*Emergencies in Pediatric Oncology*, 71-81.

1. Barry, O. P., Praticò, D., Lawson, J. A., & FitzGerald, G. A. (1997). Transcellular activation of platelets and endothelial cells by bioactive lipids in platelet microparticles. *The Journal of clinical investigation*, *99*(9), 2118.
2. Bauer, K. A., Eriksson, B. I., Lassen, M. R., & Turpie, A. G. (2001). Fondaparinux compared with enoxaparin for the prevention of venous thromboembolism after elective major knee surgery. *New England Journal of Medicine*, *345*(18), 1305-1310.
3. Bauer, K. A., Hawkins, D. W., Peters, P. C., Petitou, M., Herbert, J. M., Boeckel, C. A., & Meuleman, D. G. (2002). Fondaparinux, a synthetic pentasaccharide: the first in a new class of antithrombotic agents—the selective factor Xa inhibitors. *Cardiovascular drug reviews*, *20*(1), 37-52.
4. Becker, D. L., Fredenburgh, J. C., Stafford, A. R., & Weitz, J. I. (1999). Exosites 1 and 2 are essential for protection of fibrin-bound thrombin from heparin-catalyzed inhibition by antithrombin and heparin cofactor II. *Journal of Biological Chemistry*, *274*(10), 6226-6233.
5. Beeler, D., Rosenberg, R., & Jordan, R. (1979). Fractionation of low molecular weight heparin species and their interaction with antithrombin. *Journal of Biological Chemistry*, *254*(8), 2902-2913.
6. Beutler, E., & West, C. (1979). The storage of hard-packed red blood cells in citrate-phosphate-dextrose (CPD) and CPD-adenine (CPDA-1). *Blood*, *54*(1), 280-284.
7. Benjamin, R. J., & Anderson, K. C. (2002). What is the proper threshold for platelet transfusion in patients with chemotherapy-induced thrombocytopenia?.*Critical Reviews in Oncology/Hematology*, *42*(2), 163-171.
8. Binnie, C. G., & Lord, S. T. (1993). The fibrinogen sequences that interact with thrombin. *Blood*, *81*(12), 3186-3192.
9. Blech, S., Ebner, T., Ludwig-Schwellinger, E., Stangier, J., & Roth, W. (2008). The metabolism and disposition of the oral direct thrombin inhibitor, dabigatran, in humans. *Drug Metabolism and Disposition*, *36*(2), 386-399.
10. Breckenridge, A. (1978, January). Oral anticoagulant drugs: pharmacokinetic aspects. *Seminars in Hematology*, *15*(1), 19.
11. Breckenridge, A., Orme, M., Wesseling, H., Lewis, R. J., & Gibbons, R. (1974). Pharmacokinetics and pharmacodynamics of the enantiomers of warfarin in man. *Clinical pharmacology and therapeutics*, *15*(4), 424.
12. Broberg, M., Eriksson, C., & Nygren, H. (2002). GpIIb/IIIa is the main receptor for initial platelet adhesion to glass and titanium surfaces in contact with whole blood. *Journal of Laboratory and Clinical Medicine*, *139*(3), 163-172.
13. Broze Jr, G. J. (1995). Tissue factor pathway inhibitor and the current concept of blood coagulation. *Blood coagulation & fibrinolysis*, *6*, S7-S13.
14. Brufatto, N., Ward, A., & Nesheim, M. E. (2003). Factor Xa is highly protected from antithrombin–fondaparinux and antithrombin–enoxaparin when incorporated into the prothrombinase complex. *Journal of Thrombosis and Haemostasis*, *1*(6), 1258-1263.
15. Boon, J. M., Lambert, T. N., Sisson, A. L., Davis, A. P., & Smith, B. D. (2003). Facilitated phosphatidylserine (PS) flip-flop and thrombin activation using a synthetic PS scramblase. *Journal of the American Chemical Society*, *125*(27), 8195-8201.
16. Bussey, H. I., Force, R. W., Bianco, T. M., & Leonard, A. D. (1992). Reliance on prothrombin time ratios causes significant errors in anticoagulation therapy. *Archives of Internal Medicine*, *152*(2), 278.
17. Butenas, S., Bouchard, B. A., Brummel-Ziedins, K. E., Parhami-Seren, B., & Mann, K. G. (2005). Tissue factor activity in whole blood. *Blood*, *105*(7), 2764-2770.
18. Carr, J. A., & Silverman, N. (1999). The heparin-protamine interaction. A review. *The Journal of cardiovascular surgery*, *40*(5), 659-666.
19. Cattaneo, M. (2010). New P2Y12 inhibitors. *Circulation*, *121*(1), 171-179.
20. Chong, B. H., Grace, C. S., & Rozenberg, M. C. (1981). Heparin‐induced Thrombocytopenia: Effect of Heparin Platelet Antibody on Platelets. *British journal of haematology*, *49*(4), 531-540.
21. Cochrane, C. G., & Griffin, J. H. (1982). The biochemistry and pathophysiology of the

contact system of plasma. *Advances in Immunology*, *33*, 241.

1. Comfurius, P., Smeets, E. F., Willems, G. M., Bevers, E. M., & Zwaal, R. F. A. (1994). Assembly of the prothrombinase complex on lipid vesicles depends on the stereochemical configuration of the polar head group of phosphatidylserine. *Biochemistry*, *33*(34), 10319-10324.
2. Comp, C. C., Nixon, R. R., Cooper, M. R., & Esmon, C. T. (1984). Familial protein S deficiency is associated with recurrent thrombosis. *Journal of Clinical Investigation*, *74*(6), 2082.
3. Connolly, S. J., Ezekowitz, M. D., Yusuf, S., Eikelboom, J., Oldgren, J., Parekh, A., Pogue, J., Reilly, P.A., Themeles, E., Varrone, J., Wang, S., Alings, M., Xavier, D., Zhu, J., Diaz, R., Lewis, B.S., Darious, H., Diener, H., Joyner, C.D., & Wallentin, L. (2009). Dabigatran versus warfarin in patients with atrial fibrillation. *New England Journal of Medicine*, *361*(12), 1139-1151.
4. Coughlin, S. R. (2000). Thrombin signalling and protease-activated receptors. *Nature*, *407*(6801), 258-264.
5. Danielsson, A., Raub, E., Lindahl, U., & Björk, I. (1986). Role of ternary complexes, in which heparin binds both antithrombin and proteinase, in the acceleration of the reactions between antithrombin and thrombin or factor Xa. *Journal of Biological Chemistry*, *261*(33), 15467-15473.
6. Dasgupta, S., Repessé, Y., Bayry, J., Navarrete, A. M., Wootla, B., Delignat, S., Irinopoulou, T., Kamate, C., Saint-Remy, J., Jacquemin, M., Lenting, P.J., Borel-Derlonn A., Kaveri, S.V., & Lacroix-Desmazes, S. (2007). VWF protects FVIII from endocytosis by dendritic cells and subsequent presentation to immune effectors. *Blood*, *109*(2), 610-612.
7. Donat, F., Duret, J. P., Santoni, A., Cariou, R., Necciari, J., Magnani, H., & de Greef, R. (2002). The pharmacokinetics of fondaparinux sodium in healthy volunteers. *Clinical pharmacokinetics*, *41*(2), 1-9.
8. Duhl, A. J., Paidas, M. J., Ural, S. H., Branch, W., Casele, H., Cox-Gill, J., Hamersley, S.L., Hyers, T.M., Katz, V., Kuhlmann, R., Nutescu, E.A., Thorp, J.A., Zehnder, J.L., & Pregnancy and Thrombosis Working Group. (2007). Antithrombotic therapy and pregnancy: consensus report and recommendations for prevention and treatment of venous thromboembolism and adverse pregnancy outcomes. *American journal of obstetrics and gynecology*, *197*(5), 457-e1.
9. Eikelboom, J. W., Anand, S. S., Malmberg, K., Weitz, J. I., Ginsberg, J. S., & Yusuf, S. (2000). Unfractionated heparin and low-molecular-weight heparin in acute coronary syndrome without ST elevation: a meta-analysis. *The Lancet*, *355*(9219), 1936-1942.
10. Eikelboom, J. W., & Hirsh, J. (2006). Monitoring unfractionated heparin with the aPTT:

time for a fresh look. *Thromb Haemost*, *96*(5), 547-552.

1. Eisert, W. G., Hauel, N., Stangier, J., Wienen, W., Clemens, A., & van Ryn, J. (2010). Dabigatran: an oral novel potent reversible nonpeptide inhibitor of thrombin. *Arteriosclerosis, thrombosis, and vascular biology*, *30*(10), 1885-1889.
2. Eriksson, B. I., Quinlan, D. J., & Weitz, J. I. (2009). Comparative pharmacodynamics and pharmacokinetics of oral direct thrombin and factor Xa inhibitors in development. *Clinical pharmacokinetics*, *48*(1), 1-22.
3. Esmon, C. T., Esmon, N. L., & Harris, K. W. (1982). Complex formation between thrombin and thrombomodulin inhibits both thrombin-catalyzed fibrin formation and factor V activation. *Journal of Biological Chemistry*, *257*(14), 7944-7947.
4. Farner, B., Eichler, P., Kroll, H., & Greinacher, A. (2001). A comparison of danaparoid and lepirudin in heparin-induced thrombocytopenia. *Thrombosis and Haemostasis-Stuttgart-*, *85*(6), 950-957.
5. Faxälv, L., Boknäs, N., Ström, J. O., Tengvall, P., Theodorsson, E., Ramström, S., &

Lindahl, T. L. (2013). Putting polyphosphates to the test: evidence against platelet-

induced activation of factor XII. *Blood*, *122*(23), 3818-3824.

1. Franco, R. F., & Reitsma, P. H. (2001). Genetic risk factors of venous thrombosis. *Human genetics*, *109*(4), 369-384.
2. Friedman, P. A., Rosenberg, R. D., Hauschka, P. V., & Fitz-James, A. (1977). A spectrum of partially carboxylated prothrombins in the plasmas of coumarin-treated patients. *Biochimica et Biophysica Acta*, *494*(1), 271-276.
3. Furie, B., & Furie, B. C. (1988). The molecular basis of blood coagulation. *Cell*, *53*(4), 505-518.
4. Gailani, D., & Renné, T. (2007). Intrinsic pathway of coagulation and arterial

thrombosis. *Arteriosclerosis, thrombosis, and vascular biology*, *27*(12), 2507-2513.

1. Gale, A. J., & Gordon, S. G. (2001). Update on tumor cell procoagulant factors. *Acta Haematologica*, *106*(1-2), 25-32.
2. Gauer, R. L., & Braun, M. M. (2012). Thrombocytopenia. *American family*

*physician*, *85*(6), 612-622.

1. Giesen, P. L., Fyfe, B. S., Fallon, J. T., Roque, M., Mendlowitz, M., Rossikhina, M., Guha, A., Badimon, J.J., Nemerson, Y., & Taubman, M. B. (2000). Intimal tissue factor activity is released from the arterial wall after injury. *Thrombosis and Haemostasis-Stuttgart-*, *83*(4), 622-628.
2. Golembiewski, J. A. (2011). Dabigatran: A New Oral Anticoagulant. *Journal of PeriAnesthesia Nursing*, *26*(6), 420-423.
3. Greer, F. R. (2010). Vitamin K the basics—What's new?. *Early Human Development*, *86*(1), 43-47
4. Greinacher, A., Alban, S., Dummel, V., Franz, G., & Mueller-Eckhardt, C. (1995). Characterization of the structural requirements for a carbohydrate based anticoagulant with a reduced risk of inducing the immunological type of heparin-associated thrombocytopenia. *Thrombosis and haemostasis*, *74*(3), 886-892.
5. Gross, P.L., Murray, R.K., & Rand, M.L. (2012). Hemostasis and thrombosis. *Harper’s Iluustrated Biochemistry*, 29ed, Ch51.
6. Gross, P. L., & Weitz, J. I. (2008). New anticoagulants for treatment of venous thromboembolism. *Arteriosclerosis, Thrombosis, and Vascular Biology*, *28*(3), 380-386.
7. Gulseth, M. P., Michaud, J., & Nutescu, E. A. (2008). Rivaroxaban: An oral direct inhibitor of factor Xa. *American Journal of Health-System Pharmacy*, *65*(16).
8. Handeland, G. F., Abildgaard, U., Holm, H. A., & Arnesen, K. E. (1990). Dose adjusted heparin treatment of deep venous thrombosis: a comparison of unfractionated and low molecular weight heparin. *European Journal of Clinical Pharmacology*, *39*(2), 107-112.
9. Harrison, P., & Martin Cramer, E. (1993). Platelet α-granules. *Blood reviews*, *7*(1), 52-62.
10. Herbert, J. M., Petitou, M., Lormeau, J. C., Cariou, R., Necciari, J., Magnani, H. N., Zandberg, P., van Amsterdam, R.G.M., van Boeckel, C.A.A., & Meuleman, D. G. (1997). SR 90107A/Org 31540, a Novel Anti‐Factor Xa Antithrombotic Agent. *Cardiovascular Drug Reviews*, *15*(1), 1-26.
11. Hernández-ruiz, L., Sáez-benito, A., Pujol-moix, N., Rodríguez-martorell, J., & Ruiz,

F.A. (2009). Platelet inorganic polyphosphate decreases in patients with delta storage

pool disease. *Journal of Thrombosis and Haemostasis*, *7*(2), 361-363.

1. Hirsh, J. (1991). Heparin. *New England Journal of Medicine*, *324*(22), 1565-1574.
2. Hirsh, J., Dalen, J. E., Anderson, D. R., Poller, L., Bussey, H., Ansell, J., & Deykin, D. (2001). Oral anticoagulants: mechanism of action, clinical effectiveness, and optimal therapeutic range. *Chest Journal*, *119*(1\_suppl), 8S-21S.
3. Hirsh, J., & Levine, M. N. (1992). Low molecular weight heparin. *Blood*, *79*(1), 1-17.
4. Hobson, A. R., Petley, G. W., Dawkins, K. D., & Curzen, N. (2007). A novel fifteen minute test for assessment of individual time-dependent clotting responses to aspirin and clopidogrel using modified thrombelastography. *Platelets*, *18*(7), 497-505.
5. Hoffman, M. (2003). Remodeling the blood coagulation cascade. *Journal of Thrombosis and Thrombolysis*, *16*(1-2), 17-20.
6. Hoffman, M., Monroe, D. M., Oliver, J. A., & Roberts, H. R. (1995). Factors IXa and Xa play distinct roles in tissue factor-dependent initiation of coagulation. *Blood*, *86*(5), 1794-1801.
7. Horne, M. K., & Hutchison, K. J. (1998). Simultaneous binding of heparin and platelet factor‐4 to platelets: Further insights into the mechanism of heparin‐induced thrombocytopenia. *American Journal of Hematology*, *58*(1), 24-30.
8. Huntington, J. A. (2003). Mechanisms of glycosaminoglycan activation of the serpins in hemostasis. *Journal of Thrombosis and Haemostasis*, *1*(7), 1535-1549.
9. Huntington, J. A., & Baglin, T. P. (2003). Targeting thrombin–rational drug design from natural mechanisms. *Trends in Pharmacological Sciences*, *24*(11), 589-595.
10. Huntington, J. A., Olson, S. T., Fan, B., & Gettins, P. G. (1996). Mechanism of heparin activation of antithrombin. Evidence for reactive center loop preinsertion with expulsion upon heparin binding. *Biochemistry*, *35*(26), 8495-8503.
11. Inns, J. H., Cecchini, G., & Mattoni, M. (1989). The effect of anticoagulant, storage time and temperature, and sodium azide on blood progesterone concentrations. *International Livestock Centre for Africa Bulletin*, *33*.
12. Inoue, O., Suzuki-Inoue, K., & Ozaki, Y. (2008). Redundant mechanism of platelet adhesion to laminin and collagen under flow involvement of von willebrand factor and glycoprotein Ib-IX-V. *Journal of Biological Chemistry*, *283*(24), 16279-16282.
13. Israels, S. J., McNicol, A., Robertson, C., & Gerrard, J. M. (1990). Platelet storage pool deficiency: diagnosis in patients with prolonged bleeding times and normal platelet aggregation. *British Journal of Haematology*, *75*(1), 118-121.
14. Jørgensen, K. A., & Stoffersen, E. (1980). On the inhibitory effect of albumin on platelet aggregation. *Thrombosis Research*, *17*(1), 13-18.
15. Kahn, M. L., Nakanishi-Matsui, M., Shapiro, M. J., Ishihara, H., & Coughlin, S. R. (1999). Protease-activated receptors 1 and 4 mediate activation of human platelets by thrombin. *Journal of Clinical Investigation*, *103*(6), 879-887.
16. Kinnari, T. J., Salonen, E. M., & Jero, J. (2003). Durability of the binding inhibition of albumin coating on tympanostomy tubes. *International Journal of Pediatric Otorhinolaryngology*, *67*(2), 157-164.
17. Kalafatis, M., Bertina, R. M., Rand, M. D., & Mann, K. G. (1995). Characterization of the molecular defect in factor V. *Journal of Biological Chemistry*, *270*(8), 4053-4057.
18. Kamal, A. H., Tefferi, A., & Pruthi, R. K. (2007, July). How to interpret and pursue an abnormal prothrombin time, activated partial thromboplastin time, and bleeding time in adults. *Mayo Clinic Proceedings*, 82(7), 864-873.
19. Kannemeier, C., Shibamiya, A., Nakazawa, F., Trusheim, H., Ruppert, C., Markart, &

Preissner, K. T. (2007). Extracellular RNA constitutes a natural procoagulant cofactor

in blood coagulation. *Proceedings of the National Academy of Sciences*, *104*(15), 6388-

6393.

1. Kattlove, H. E., Alexander, B., & White, F. (1972). The effect of cold on platelets. II. Platelet function after short-term storage at cold temperatures. *Blood*, *40*(5), 688-696.
2. Kubitza, D., Becka, M., Voith, B., Zuehlsdorf, M., & Wensing, G. (2005). Safety, pharmacodynamics, and pharmacokinetics of single doses of BAY 59-7939, an oral, direct factor Xa inhibitor. *Clinical Pharmacology & Therapeutics*, *78*(4), 412-421.
3. Kayata, S., Kindberg, C., Greer, F. R., & Suttie, J. W. (1989). Vitamin K1 and K2 in infant human liver. *Journal of Pediatric Gastroenterology and Nutrition*, *8*(3), 304-307.
4. Lee, A. Y. (2009). Anticoagulation in the treatment of established venous thromboembolism in patients with cancer. *Journal of Clinical Oncology*, *27*(29), 4895-4901.
5. Lieu, C., Shi, J., Donat, F., Van Horn, R., Brian, W., Newton, J., Delbressine, L., & Vos, R. (2002). Fondaparinux sodium is not metabolised in mammalian liver fractions and does not inhibit cytochrome P450-mediated metabolism of concomitant drugs. *Clinical Pharmacokinetics*, *41*(2), 19-26.
6. Lim, W., Dentali, F., Eikelboom, J. W., & Crowther, M. A. (2006). Meta-analysis: low-molecular-weight heparin and bleeding in patients with severe renal insufficiency. *Annals of Internal Medicine*, *144*(9), 673-684.
7. Lindahl, U., Thunberg, L., Bäckström, G., Riesenfeld, J., Nordling, K., & Björk, I. (1984). Extension and structural variability of the antithrombin-binding sequence in heparin. *Journal of Biological Chemistry*, *259*(20), 12368-12376.
8. Lockwood, C. J., Runic, R., Wan, L., Krikun, G., Demopolous, R., & Schatz, F. (2000). The role of tissue factor in regulating endometrial haemostasis: implications for progestin-only contraception. *Human Reproduction*, *15*(suppl 3), 144-151.
9. Luddington, R. J. (2005). Thrombelastography/thromboelastometry. *Clinical & Laboratory Haematology*, *27*(2), 81-90.
10. Lyman, G. H., Khorana, A. A., Falanga, A., Clarke-Pearson, D., Flowers, C., Jahanzeb, M., Kakkar, A., Kuderer, N.M., Levine, M.N., Liebman, H., Mendelson, D., Raskob, G., Somerfield, M.R., Thodiyil, P., Trent, D., & Francis, C. W. (2007). American Society of Clinical Oncology guideline: recommendations for venous thromboembolism prophylaxis and treatment in patients with cancer. *Journal of Clinical Oncology*, *25*(34), 5490-5505.
11. Mann, K. G., Brummel, K., & Butenas, S. (2003). What is all that thrombin for?. *Journal of Thrombosis and Haemostasis*, *1*(7), 1504-1514.
12. Matafonov, A., Leung, P. Y., Gailani, A. E., Grach, S. L., Puy, C., Cheng, Q., Sun, M.,

McCarty, O., Tucker, E., Kataoka, H., Renne, T., Morrissey, J., Gruber, A., & Gailani,

D. (2014). Factor XII inhibition reduces thrombus formation in a primate thrombosis

model. *Blood*, *123*(11), 1739-1746.

1. McShine, R. L., Das, P. C., Sibinga, C. T., & Brozović, B. (1991). Effect of EDTA on Platelet Count and Other Platelet Parameters in Blood and Blood Components Collected with CPDA‐1. *Vox Sanguinis*, *61*(2), 84-89.
2. Meijers, J. C. (2014). No contact, no thrombosis?. *Blood*, *123*(11), 1629-1629.
3. Mismetti, P., Laporte-Simitsidis, S., Tardy, B., Cucherat, M., Buchmuller, A., Juillard-Delsart, D., & Decousus, H. (2000). Prevention of venous thromboembolism in internal medicine with unfractionated or low-molecular-weight heparins: a meta-analysis of randomised clinical trials. *Thrombosis and Haemostasis-Stuttgart-*, *83*(1), 14-19.
4. Monagle, P., Chalmers, E., Chan, A., Kirkham, F., Massicotte, P., & Michelson, A. D. (2008). Antithrombotic therapy in neonates and children: American College of Chest Physicians evidence-based clinical practice guidelines. *CHEST Journal*, *133*(6\_suppl), 887S-968S.
5. Monroe, D. M., Hoffman, M., & Roberts, H. R. (1996). Transmission of a procoagulant signal from tissue factor-bearing cells to platelets. *Blood Coagulation & Fibrinolysis*, *7*(4), 459-464.
6. Monroe, D. M., Roberts, H. R., & Hoffman, M. (1994). Platelet procoagulant complex assembly in a tissue factor‐initiated system. *British Journal of Haematology*, *88*(2), 364-371.
7. Moore, G. L., Peck, C. C., Sohmer, P. R., & Zuck, T. F. (1981). Some Properties of Blood Stored in Anticoagulant CPDA‐1 Solution. A Brief Summary. *Transfusion*, *21*(2), 135-137.
8. Morabia, A. (1986). Heparin doses and major bleedings. *The Lancet*, *327*(8492), 1278-1279.
9. Morrissey, J. H., Choi, S. H., & Smith, S. A. (2012). Polyphosphate: an ancient

molecule that links platelets, coagulation, and inflammation. *Blood*, *119*(25), 5972-

5979.

1. Morrissey, J. H., Macik, B. G., & Neuenschwander, P. F. (1993). Quantitation of activated factor VII levels in plasma using a tissue factor mutant selectively deficient in promoting factor VII activation. *Blood*, *81*(3), 734-744.
2. Müller, F., Gailani, D., & Renne, T. (2011). Factor XI and XII as antithrombotic targets.

*Current Opinion in Hematology*, *18*(5), 349-355.

1. Nachman, R. L., & Leung, L. L. (1982). Complex formation of platelet membrane glycoproteins IIb and IIIa with fibrinogen. *Journal of Clinical Investigation*, *69*(2), 263.
2. Nelsestuen, G. L. (1976). Role of gamma-carboxyglutamic acid. An unusual protein transition required for the calcium-dependent binding of prothrombin to phospholipid. *Journal of Biological Chemistry*, *251*(18), 5648-5656.
3. Ni, H., & Freedman, J. (2003). Platelets in hemostasis and thrombosis: role of integrins and their ligands. *Transfusion and apheresis science*, *28*(3), 257-264.
4. O'Reilly, R. A., & Rytand, D. A. (1980). “Resistance" to warfarin due to unrecognized vitamin K supplementation. *The New England Journal of Medicine*, *303*(3), 160.
5. Packham, M. A. (1988). The behavior of platelets at foreign surfaces. *Experimental Biology and Medicine*, *189*(3), 261-274.
6. Palm, M., & Mattsson, C. H. (1987). Pharmacokinetics of heparin and low molecular weight heparin fragment (Fragmin) in rabbits with impaired renal or metabolic clearance. *Thrombosis and Haemostasis*, *58*(3), 932-935.
7. Paolucci, F., Claviés, M. C., Donat, F., & Necciari, J. (2002). Fondaparinux Sodium Mechanism of Action. *Clinical Pharmacokinetics*, *41*(2), 11-18.
8. Park, Y., Schoene, N., & Harris, W. (2002). Mean platelet volume as an indicator of platelet activation: methodological issues. *Platelets*, *13*(5-6), 301-306.
9. Perzborn, E., Strassburger, J., Wilmen, A., Pohlmann, J., Roehrig, S., Schlemmer, K. H., & Straub, A. (2005). In vitro and in vivo studies of the novel antithrombotic agent BAY 59‐7939—an oral, direct Factor Xa inhibitor. *Journal of Thrombosis and Haemostasis*, *3*(3), 514-521.
10. Piccin, A., Murphy, W. G., & Smith, O. P. (2007). Circulating microparticles: pathophysiology and clinical implications. *Blood Reviews*, *21*(3), 157-171.
11. Rao, L. V., & Rapaport, S. I. (1988). Activation of factor VII bound to tissue factor: a key early step in the tissue factor pathway of blood coagulation. *Proceedings of the National Academy of Sciences*, *85*(18), 6687-6691.
12. Rezaie, A. R. (1998). Calcium enhances heparin catalysis of the antithrombin-factor Xa reaction by a template mechanism evidence that calcium alleviates Gla domain antagonism of heparin binding to factor Xa. *Journal of Biological Chemistry*, *273*(27), 16824-16827.
13. Roehrig, S., Straub, A., Pohlmann, J., Lampe, T., Pernerstorfer, J., Schlemmer, K. H., Reinemer, P., & Perzborn, E. (2005). Discovery of the novel antithrombotic agent 5-chloro-N-({(5 S)-2-oxo-3-[4-(3-oxomorpholin-4-yl) phenyl]-1, 3-oxazolidin-5-yl} methyl) thiophene-2-carboxamide (BAY 59-7939): an oral, direct factor Xa inhibitor. *Journal of Medicinal Chemistry*, *48*(19), 5900-5908.
14. Rosenberg, R. D. (1974). Heparin action. *Circulation*, *49*(4), 603-605.
15. Rosenberg, R. D., & Aird, W. C. (1999). Vascular-bed–specific hemostasis and hypercoagulable states. *New England Journal of Medicine*, *340*(20), 1555-1564.
16. Rosenberg, R. D., & Bauer, K. A. (1994). The heparin-antithrombin system: a natural anticoagulant mechanism. *Hemostasis and Thrombosis: Basic Principles and Clinical Practice. 3rd ed. Philadelphia, PA: JB Lippincott*, 837-860.
17. Rosendaal, F. R. (1999). Venous thrombosis: a multicausal disease. *The Lancet*, *353*(9159), 1167-1173.
18. Ruiz, F. A., Lea, C. R., Oldfield, E., & Docampo, R. (2004). Human platelet

dense granules contain polyphosphate and are similar to acidocalcisomes of bacteria and

unicellular eukaryotes. *Journal of Biological Chemistry*, *279*(43), 44250-44257.

1. Saccullo, G., Marietta, M., Carpenedo, M., De Stefano, V., Falanga, A., Federici, A. B., Rodeghiero, F.,Tosetto, A., & Siragusa, S.(2013). Platelet cut-off for anticoagulant therapy in cancer patients with venous thromboembolism and thrombocytopenia: an expert opinion based on rand/ucla appropriateness method (ram). *Blood*, *122*(21), 581-581.
2. Senzolo, M., Coppell, J., Cholongitas, E., Riddell, A., Triantos, C. K., Perry, D., & Burroughs, A. K. (2007). The effects of glycosaminoglycans on coagulation: a thromboelastographic study. *Blood Coagulation & Fibrinolysis*, *18*(3), 227-236.
3. Smith, S. A. (2009). The cell‐based model of coagulation. *Journal of Veterinary Emergency and Critical Care*, *19*(1), 3-10.
4. Slichter, S. J. (2004). Relationship between platelet count and bleeding risk in

thrombocytopenic patients. *Transfusion medicine reviews*, *18*(3), 153-167.

1. Smith, S. A., Choi, S. H., Davis-Harrison, R., Huyck, J., Boettcher, J., Rienstra,

C. M., & Morrissey, J. H. (2010). Polyphosphate exerts differential effects on blood

clotting, depending on polymer size. *Blood*, *116*(20), 4353-4359.

1. Stafford, D. W. (2005). The vitamin K cycle. *Journal of Thrombosis and Haemostasis*, *3*(8), 1873-1878.
2. Stangier, J., Rathgen, K., Stähle, H., Gansser, D., & Roth, W. (2007). The pharmacokinetics, pharmacodynamics and tolerability of dabigatran etexilate, a new oral direct thrombin inhibitor, in healthy male subjects. *British Journal of Clinical Pharmacology*, *64*(3), 292-303.
3. Stangier, J., Rathgen, K., Stähle, H., & Mazur, D. (2010). Influence of renal impairment on the pharmacokinetics and pharmacodynamics of oral dabigatran etexilate. *Clinical Pharmacokinetics*, *49*(4), 259-268.
4. Stasi, R. (2012). How to approach thrombocytopenia. *ASH Education Program Book*, *2012*(1), 191-197.
5. Steigmann, F., & Dyniewicz, J. M. (1944). The influence of phenolphthalein ingestion on red blood cell resistance to hemolysis. *The American Journal of Digestive Diseases*, *11*(9), 279-281.
6. Stevic, I., Chan, H. H., & Chan, A. K. (2011). Carotid artery dissections: thrombosis of the false lumen. *Thrombosis Research*, *128*(4), 317-324.
7. Strauss, R., Wehler, M., Mehler, K., Kreutzer, D., Koebnick, C., & Hahn, E. G. (2002). Thrombocytopenia in patients in the medical intensive care unit: Bleeding prevalence, transfusion requirements, and outcome\*. *Critical Care Medicine*, *30*(8), 1765-1771.
8. Siebenlist, K. R., Meh, D. A., & Mosesson, M. W. (2001). Protransglutaminase (factor XIII) mediated crosslinking of fibrinogen and fibrin. *Thrombosis and Haemostasis*, 86, 1221-1228.
9. Sobel, M., McNeill, P. M., Carlson, P. L., Kermode, J. C., Adelman, B., Conroy, R., & Marques, D. (1991). Heparin inhibition of von Willebrand factor-dependent platelet function in vitro and in vivo. *Journal of Clinical Investigation*, *87*(5), 1787.
10. Soons, H., Janssen-Claessen, T., Hemker, H. C., & Tans, G. (1986). The effect of platelets in the activation of human blood coagulation factor IX by factor XIa. *Blood*, *68*(1), 140-148.
11. Swallow, R. A., Agarwala, R. A., Dawkins, K. D., & Curzen, N. P. (2006). Thromboelastography: potential bedside tool to assess the effects of antiplatelet therapy?. *Platelets*, *17*(6), 385-392.
12. Tersteegen, A., Schmidt, S., & Burkhardt, N. (2007). Rivaroxaban—an oral, direct Factor Xa inhibitor—binds rapidly to Factor Xa. *Journal of Thrombosis and Haemostasis*, *5*(2 Suppl).
13. Tobias, M. D., Wambold, D., Pilla, M. A., & Greer, F. (1998). Differential effects of serial hemodilution with hydroxyethyl starch, albumin, and 0.9% saline on whole blood coagulation. *Journal of Clinical Anesthesia-Stoneham-*, *10*, 366-371.
14. Tracy, P. B., & Mann, K. G. (1983). Prothrombinase complex assembly on the platelet surface is mediated through the 74,000-dalton component of factor Va. *Proceedings of the National Academy of Sciences*, *80*(8), 2380-2384.
15. Turpie, A. G., Eriksson, B. I., Lassen, M. R., & Bauer, K. A. (2003). Fondaparinux, the first selective factor Xa inhibitor. *Current Opinion in Hematology*, *10*(5), 327-332.
16. Turpie, A. G., Gallus, A. S., & Hoek, J. A. (2001). A synthetic pentasaccharide for the prevention of deep-vein thrombosis after total hip replacement. *New England Journal of Medicine*, *344*(9), 619-625.
17. Van den Berg, H. M., De Groot, P. H. G., & Fischer, K. (2007). Phenotypic

heterogeneity in severe hemophilia. *Journal of Thrombosis and Haemostasis*, *5*(s1),

151-156.

1. van Ryn, J., Hauel, N., Waldmann, L., & Wienen, W. (2008). Dabigatran inhibits both clot-bound and fluid-phase thrombin in vitro: comparison to heparin and hirudin. *Arteriosclerosis Thrombosis and Vascular Biology*, 28(6), 136-137.
2. Walenga, J. M., Jeske, W. P., Frapaise, F. X., Bick, R. L., Fareed, J., & Samama, M. M. (2002). Fondaparinux: a synthetic heparin pentasaccharide as a new antithrombotic agent. *Expert Opinion on Investigational Drugs*, *11*(3), 397-407.
3. Warkentin, T. E., Cook, R. J., Marder, V. J., Sheppard, J. A. I., Moore, J. C., Eriksson, B. I., ... & Kelton, J. G. (2005). Anti–platelet factor 4/heparin antibodies in orthopedic surgery patients receiving antithrombotic prophylaxis with fondaparinux or enoxaparin. *Blood*, *106*(12), 3791-3796.
4. Wood, A.J.J., & Weitz, J. I. (1997). Low-molecular-weight heparins. *New England Journal of Medicine*, *337*(10), 688-699.
5. Warkentin, T. E., Levine, M. N., Hirsh, J., Horsewood, P., Roberts, R. S., Gent, M., & Kelton, J. G. (1995). Heparin-induced thrombocytopenia in patients treated with low-molecular-weight heparin or unfractionated heparin. *New England Journal of Medicine*, *332*(20), 1330-1336.
6. Weinz, C., Radtke, M., Schmeer, K., Kern, A., & Pleiss, U. (2004, August). In vitro metabolism of BAY 59-7939-an oral, direct Factor Xa inhibitor. In *Drug Metabolism Reviews*, 36, 98-98.
7. White, J. G., & Krivit, W. (1967). An ultrastructural basis for the shape changes induced in platelets by chilling. *Blood*, *30*(5), 625-635.
8. White, J. G., Leistikow, E. L., & Escolar, G. (1989). Platelet membrane responses to surface and suspension activation. *Blood cells*, *16*(1), 43-70.
9. Whitten, C. W., & Greilich, P. E. (2000). Thromboelastography: Past, Present, and Future. *Anesthesiology*, *92*(5), 1226.
10. Wouwer, M., Collen, D., & Conway, E. M. (2004). Thrombomodulin-protein C-EPCR system integrated to regulate coagulation and inflammation. *Arteriosclerosis, Thrombosis, and Vascular Biology*, *24*(8), 1374-1383.
11. Yamamoto, M., Nakagaki, T., & Kisiel, W. (1992). Tissue factor-dependent autoactivation of human blood coagulation factor VII. *Journal of Biological Chemistry*, *267*(27), 19089-19094.
12. Young, E., Prins, M., Levine, M. N., & Hirsh, J. (1992). Heparin binding to plasma proteins, an important mechanism for heparin resistance. *Thrombosis and Haemostasis*, *67*(6), 639-643.
13. Ziporen, L., Li, Z. Q., Park, K. S., Sabnekar, P., Liu, W. Y., Arepally, G., Kieber-Emmons, T., Clines, D.B., & Poncz, M. (1998). Defining an antigenic epitope on platelet factor 4 associated with heparin-induced thrombocytopenia. *Blood*, *92*(9), 3250-3259.