THE PROCOAGULANT ROLE OF NUCLEIC ACIDS IN THE CONTACT SYSTEM

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By

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Abstract

Nucleic acids have been identified as procoagulant species in plasma and *in vivo* animal studies. It is believed that the nucleic acids act as physiological activators of the contact system. However, in order to obtain a better understanding of the role of nucleic acids in the activation of the contact system it is important to analyze and evaluate the individual proteins of the contact system that are stimulated by nucleic acids and identify accompanying proteins required to mediate the nucleic acid-stimulated activation.

Previous works suggested that nucleic acids stimulate the activation of factor XII (FXII) in the presence of prekallikrein (PK) and high molecular weight kininogen (HK). In this study we will confirm if both PK and HK are required for nucleic acid-stimulated activation of FXII. We will also study the role of nucleic acids in the activation of PK in the presence or absence of activated FXII (FXIIa) and HK. Previous works also identified that zinc (Zn^{2+}) accelerates surface-mediated activation of FXII by PK and HK, and PK by FXIIa and HK. We will be evaluating zinc's ability to enhance nucleic acid-stimulated activation of both FXII and PK.

We have found that nucleic acids stimulate activation of FXII in the presence of PK and this furthered upon addition of HK. Nucleic acids also stimulate the activation of PK in the presence of FXIIa and this is furthered upon addition of HK. Nucleic acids have stimulated activation of FXII and PK in a dose dependent manner in the presence of the aforementioned accompanying proteins. We have showed that Zn^{2+} enhances activation of the contact system. Zn^{2+} enhances nucleic acid-stimulated, PK/HK-mediated activation of FXII. It also enhances nucleic acid-stimulated, FXIIa/HK-mediated

activation of PK. These findings enrich our understanding of the role of nucleic acids and zinc in the contact system.

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List of Abbreviations

α1AT-	α_1-antitrypsin
α2AP-	α_2-antiplasmin
α2M-	α_2-macroglobulin
ANOVA-	Analysis of variance
APC-	Activated protein C
AT-	Antithrombin
C1-Inh-	C1-inhibitor
CTI-	Corn trypsin inhibitor
DNA-	Deoxyribonucleic acid
DNase-	Deoxyribonuclease
DVT-	Deep vein thrombosis
EGF-	Epidermal-growth-factor
EPCR-	Endothelial protein C receptor
ETP-	Endogenous thrombin potential
FVIIai-	Active site-blocked activated FVII
FVII-def-	FVII-deficient
FXa-	Activated FX
FXI-def-	FXI-deficient
FXII-	Factor XII
FXIIa-	Activated FXII
FXII-def-	FXII-deficient
HBS-	HEPES buffered saline
HK-	High molecular weight kininogen

HRG-	Histidine-rich glycoprotein
IVC-	Inferior vena cava
Kal-	Kallikrein
LK-	Low molecular weight kininogen
mRNA-	Messenger ribonucleic acid
NETs-	Neutrophil extracellular traps
NP-	Normal pooled platelet poor plasma
PAI-1-	Plasminogen activator inhibitor-1
PCI-	Protein C inhibitor
PK-	Prekallikrein
PK-dep-	PK-depleted
polyP-	Polyphosphates
PRCP-	Prolylcarboxypeptidase
pro-PK-	Pro-prekallikrein
RNA-	Ribonucleic acid
RNase-	Ribonuclease
rRNA-	Ribosomal ribonucleic acid
Serpin-	Serine protease inhibitor
STI-	Soybean trypsin inhibitor
TAE-	Tris, Acetic acid, Ethylenediaminetetraacetic acid
TF-	Tissue factor
TFPI-	Tissue factor pathway inhibitor
t-PA-	Tissue-plasminogen activator
tRNA-	Transfer ribonucleic acid

VWF-	von Willebrand factor

Chapter 1: Introduction

1.1 Hemostasis Overview

Hemostasis is a physiological equilibrium of the vasculature which keeps the blood in a fluid state and allows it to clot upon injury to blood vessels. Disruption of hemostasis can result in thrombotic or bleeding pathologies. Thrombosis, a major cause of mortality and morbidity, occurs in both the arterial and the venous system. Thrombosis leads to various well-known disorders such as ischemic stroke, myocardial infarction, deep vein thrombosis (DVT) and pulmonary embolism. These disorders are usually a result of temporary or permanent occlusion of a blood vessel. On the other hand, bleeding disorders occur as a result of defective hemostatic plug formation and inability to seal the vasculature (Weitz, 2013). Clinical manifestations, such as intracranial hemorrhaging and joint bleeds, can complicate bleeding disorders (Hayward, 2013). The severity of these pathologies highlights the importance of maintaining hemostasis.

Hemostasis can be divided into three stages: primary, secondary, and tertiary. Primary hemostasis is defined by the initial platelet plug formation. Platelets interact with various adhesive and aggregation proteins such as von Willebrand factor (VWF), collagen, and fibrinogen to form a platelet-rich plug that seals the leaks in the injured vascular wall. Secondary hemostasis, also known as coagulation, serves to produce a fibrin-hemostatic plug through activation of coagulation proteins. These coagulation proteins include procoagulant proteins, such as factors II, VII, VIII, IX, XI, and X, and regulatory anticoagulant proteins such as antithrombin, protein C, protein S, and tissue

factor pathway inhibitor. Tertiary hemostasis refers to the lysis and solubilization of fibrin clots in a process known as fibrinolysis. Fibrinolysis is carried out by plasmin, the active form of plasminogen generated by tissue-plasminogen activator (t-PA). Fibrinolysis also involves a variety of regulatory proteins, such as α_2-antiplasmin and plasminogen activator inhibitor 1, that prevent abnormal fibrinolysis (Lasne et al., 2006).

Appropriate regulation of these stages is important for the maintenance of hemostasis. The hemostatic equilibrium can be disturbed by many different causes which can be summarized by Virchow's triad. Virchow's triad attributes thrombosis to alterations in the vascular endothelium, defects in blood flow, and defects in blood components (Wolberg et al., 2012).

Under normal circumstances the endothelium acts to maintain a fluid vasculature. It maintains hemostasis by preventing activation and aggregation of platelets, restricting procoagulant pathways, and promoting fibrinolysis. With injury, the endothelium switches to a procoagulant state and various elements from the subendothelium, such as collagen, become exposed to the vasculature. This leads to platelet aggregation and subsequent fibrin production at the site of injury to produce fibrin-based platelet thrombi (Karsan and Harlan, 2013, Weitz, 2013). Defects in blood flow can also lead to disruption of hemostasis. Laminar blood flow is important in maintaining the endothelium in a quiescent state in the absence of vascular injury. Endothelial cells release nitric oxide and prostacyclin to prevent platelet adhesion (Lowe, 2003). However, platelets can become activated in a high shear environment. Platelets adhere to the

subendothelium and contribute to occlusive thrombus formation. Stasis due to prolonged immobility or plaques can both result in a low shear system. This system promotes an increase in blood viscosity and a reduction of blood flow leading to myocardial, cerebral, or limb infarction (Lowe, 2003, Wolberg *et al.*, 2012). Deficiencies in blood components also disrupt hemostasis. These disruptions can lead to either bleeding or hypercoagulable states by means of congenital or acquired disorders such as hemophilia and protein C deficiency (Anderson and Weitz, 2013, Weitz, 2013).

Under normal circumstances hemostasis actively maintains the dynamic equilibrium between coagulation and fibrinolysis. The coagulation system plays a key role in forming clots, and a deeper understanding of its constituents will further our understanding of the role that physiological species play in thrombosis (Weitz, 2013).

1.2 The Coagulation System

The coagulation system can be defined by two popular models: the cell-based model of hemostasis and the classical model of coagulation. The cell-based model of hemostasis describes three stages in which coagulation enzymes are sequentially generated to ultimately produce thrombin: initiation, amplification, and propagation. In the classical model of coagulation (Figure 1), the extrinsic and intrinsic pathways of coagulation converge into the common pathway for the production of thrombin. Thrombin can then execute downstream reactions involved in fibrin hemostatic plug formation and antithrombotic reactions (Hoffman and Monroe, III, 2001).

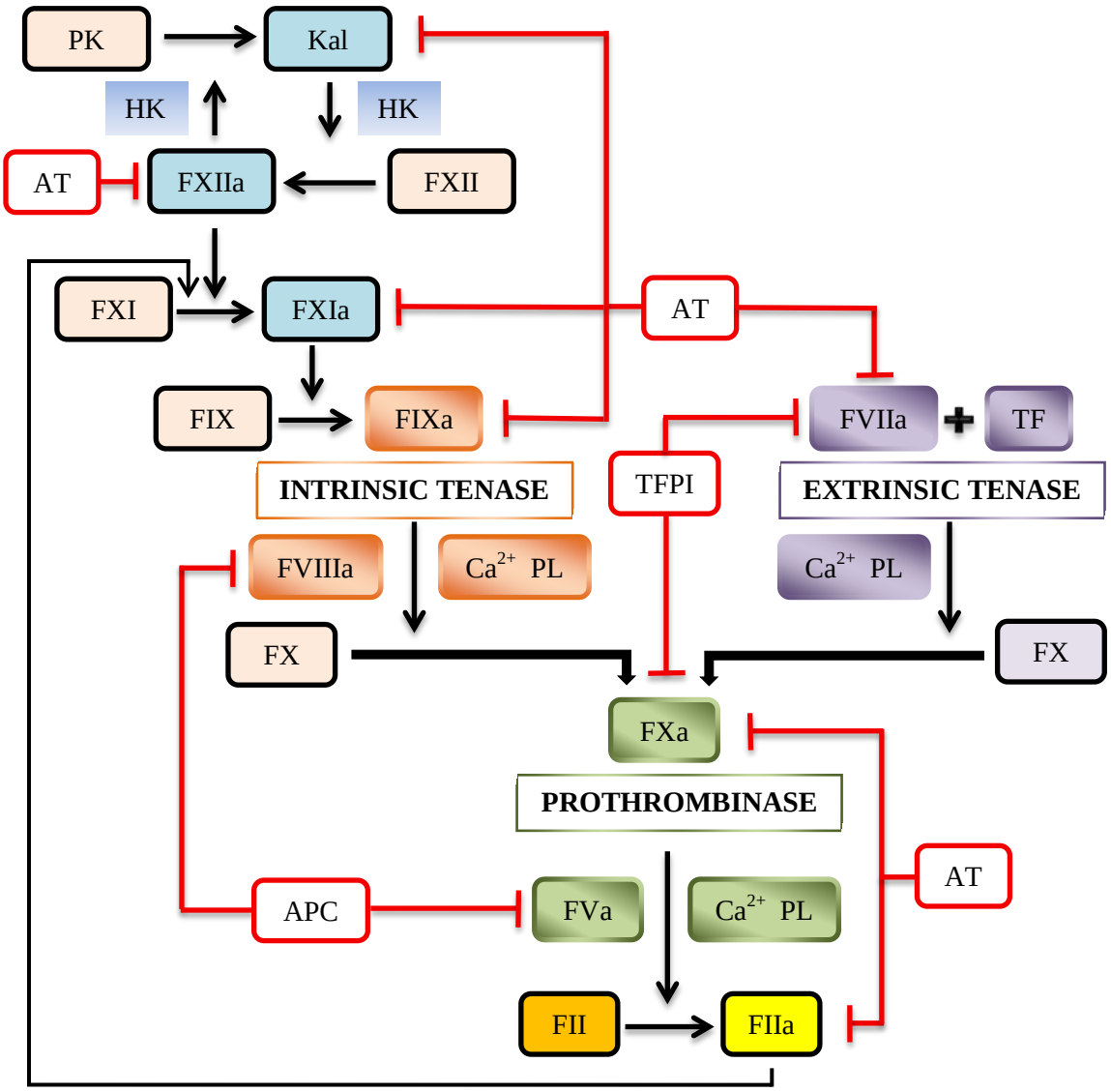

PK
Kal
HK
HK
AT
FXIIa
FXII
FXI
FXIa
AT
FIX
FIXa
FVIIa
TF
INTRINSIC TENASE
TFPI
EXTRINSIC TENASE
FVIIIa
Ca2+ PL
Ca2+ PL
FX
FX
FXa
PROTHROMBINASE
AT
APC
FVa
Ca2+ PL
FII
FIIa

Figure 1: The Classical Model of Coagulation.

The classical model of coagulation depicts coagulation initiated by two pathways: intrinsic and extrinsic pathway of coagulation. The pathways represent a series of inactive zymogens that are activated leading to the generation of thrombin. The intrinsic pathway is initiated by FXII activation and leads to activation of downstream zymogens to produce intrinsic tenase. The extrinsic pathway is initiated by the TF-FVIIa complex and leads to the production of extrinsic tenase. Both tenases activate FX to generate FXa. Assembly of the prothrombinase complex ultimately leads to the activation of prothrombin to thrombin. In addition to FXIIa, FXI is also activated by thrombin by a feedback mechanism. The coagulation pathways are inhibited at various points by three main anticoagulants: tissue factor pathway inhibitor (TFPI), activated protein C (APC), and antithrombin (AT).

During the initiation phase, extravascular tissue factor (TF) becomes exposed to the blood at the site of injury (Hoffman and Monroe, 2007). TF, usually found on the surface of smooth muscle cells and fibroblasts, binds and activates FVII (Versteeg *et al.*, 2013). In the classical model of coagulation, the TF-FVIIa complex initiates the extrinsic pathway of coagulation. The extrinsic pathway of coagulation culminates into the common pathway with the activation of FX (Riddel, Jr. *et al.*, 2007).

The TF-FVIIa complex activates FIX and FX on the surface of TF-bearing cells. Activated FX (FXa) and FVa assemble on the cells and platelets to form the prothrombinase complex in the presence of anionic phospholipids and calcium (Hoffman and Monroe, III, 2001, Hoffman and Monroe, 2007, Jenny and Mann, 2001, Monroe *et al.*, 1996, Weitz, 2013). The prothrombinase complex activates prothrombin to generate the small amount of thrombin required to activate many different targets (Hoffman and Monroe, 2007).

The amplification phase of hemostasis involves activation of platelets and coagulation proteins for subsequent assembly of procoagulant complexes. Upon adhering to the subendothelial surface, platelets become activated at the site of injury. Initial thrombin generation augments platelet activation and triggers the release of FV. FV activation can be catalyzed by thrombin. Thrombin also liberates FVIII from the VWF-FVIII complex, bound to the platelet surface, and activates it to produce FVIIIa. Furthermore, thrombin also activates FXI which plays an important downstream role in tenase formation. Thus, the amplification phase activates and prepares platelets for

assembly of procoagulant complexes and the subsequent burst of thrombin generation (Hoffman and Monroe, III, 2001).

The propagation phase of hemostasis is defined by the formation of intrinsic tenase, composed of FIXa, FVIIIa, and calcium on a negatively charged membrane surface, and prothrombinase complexes and encapsulates many components of the intrinsic pathway required for large scale thrombin generation on the surface of activated platelets (Brummel-Ziedins and Mann, 2013, Hoffman and Monroe, III, 2001). The components of the intrinsic pathway of coagulation include FXII, prekallikrein (PK), high molecular weight kininogen (HK), FXI, FVIII, and FIX. FXII becomes activated to FXIIa in the presence of a negatively charged surface. FXIIa, along with its cofactor HK, can activate PK to kallikrein. Kallikrein, along with HK, can subsequently activate additional FXII in a positive feedback reaction. FXIIa proteolytically cleaves FXI to activate it to FXIa, which subsequently activates FIX (Riddel, Jr. *et al.*, 2007).

FIXa, produced on the surface of TF-bearing cells, relocates to the surface of activated platelets and forms the intrinsic tenase complex with FVIIIa in presence of calcium. FXIa, produced on the surface of TF-bearing cells, also diffuses to the platelet surface and enhances FIX activation. This process is augmented when FXI also binds to the surface of activated platelets and becomes activated by thrombin. The assembly of the intrinsic tenase complex on the activated platelet surface results in the activation of FX (Brummel-Ziedins and Mann, 2013, Butenas and Mann, 2002, Hoffman and Monroe, III, 2001). FXa and FVa then assemble together on the activated platelet surface to form the

prothrombinase complex in the presence of calcium. The formation of the prothrombinase complex indicates the culmination of the extrinsic and intrinsic pathways into the common pathway. The prothrombinase complex then generates large amounts of thrombin from prothrombin for subsequent fibrin clot formation (Hoffman and Monroe, III, 2001, Hoffman and Monroe, 2007, Jenny and Mann, 2001). Thrombin also activates FXIII to FXIIIa which mediates the cross-linking of fibrin polymers to form stable fibrin clots (Riddel, Jr. *et al.*, 2007).

1.2.1 Calibrated Thrombin Generation Assay

The calibrated thrombin generation assay is a useful tool for determining the amount of thrombin generated from platelet-poor or platelet-rich plasma (Lance, 2015). This assay has interesting clinical applications as it reveals increased thrombin generation in prothrombotic states due to antithrombin, protein C, or S deficiency and reveals decreased thrombin generation in hemorrhagic states of patients suffering from hemophilia. It is believed that the thrombin generation assays may be better than traditional coagulation tests (aPTT and PT) for evaluating bleeding risk especially in the case of hemophilia patients. It is also believed that thrombin generation assays can be used to better monitor the state of patients undergoing antithrombotic therapy to minimize the risk of bleeding (Lance, 2015).

This assay involves the use of a fluorogenic thrombin substrate (Z-Gly-Gly-Arg-AMC) to provide a fluorescence signal. The signal is then compared with fluorescence signals of known thrombin concentrations to determine the amount of thrombin generated

in the test sample. This assay generates a thrombin generation profile and provides various parameters that shape the profile (Lance, 2015). These parameters include lag time, time to peak thrombin concentration, peak thrombin concentration, velocity index, and the endogenous thrombin potential (ETP). The lag time refers to the time before the initial burst of thrombin generation while ETP refers to the total amount of thrombin generated (Brummel-Ziedins, 2013, Hemker *et al.*, 2006). These parameters can also be qualitatively assessed on the thrombin generation profile (Figure 2).

1.3 The Importance of the Extrinsic Pathway of Coagulation

The extrinsic pathway of coagulation, involving TF and FVII, plays an essential role in both maintaining hemostasis and promoting thrombosis. The fact that humans exhibiting TF deficiency have yet to be identified indicates the significance of TF in maintaining hemostasis. Results from various murine models, studying viability in the absence of TF or in presence of low levels of TF, support the notion that TF is essential for survival (Mackman *et al.*, 2007).

In mice, TF knockout results in embryonic lethality. The embryos exhibit hemorrhage into the yolk sac cavity and there is tissue necrosis due to disrupted vascular development (Bugge *et al.*, 1996). Low TF mice have been generated by knocking out murine TF and inserting a transgene for human TF. These low TF mice have TF expression levels of less than 2% compared with wild-type mice (Pawlinski *et al.*, 2004). Unlike $TF^{-/-}$ mice, low TF mice survive a normal vaginal birth. It is believed that low TF mice are rescued due to the presence of human TF, the only source of TF in these mice,

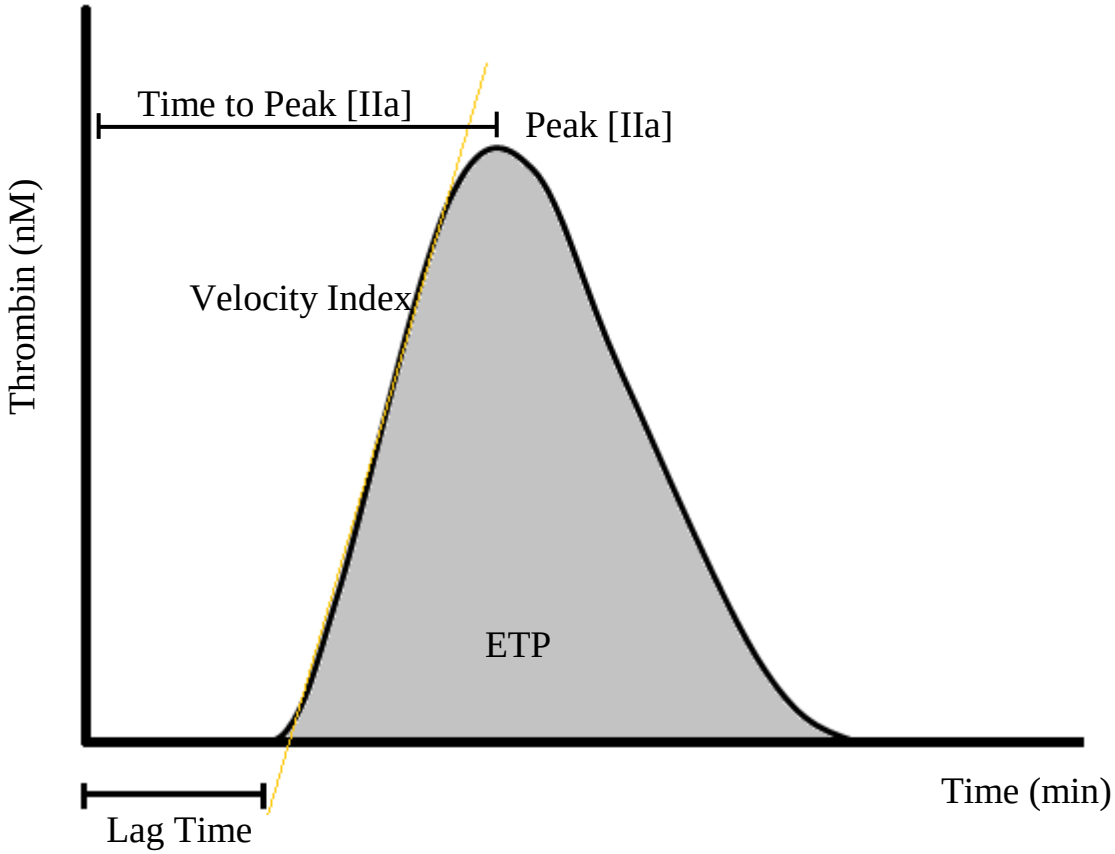


Figure 2: Thrombin generation profile.

The thrombin generation assay generates a thrombin generation profile with its many parameters contributing to its shape. Lag time refers to the time before the initial burst of thrombin. Peak thrombin concentration is the maximal concentration of thrombin and the time to peak thrombin concentration is the time it takes to reach peak thrombin concentration from the beginning of the assay. Velocity index is the slope of the thrombin generation from the initial burst to peak thrombin. ETP is the area under the curve and represents the total amount of thrombin generated in the assay. Figure adapted from Hemker et al., 2006.

allowing them to undergo successful embryogenesis (Parry *et al.*, 1998). The lack of identified TF-deficient humans, the embryonic lethality of $TF^{-/-}$ mice, and the survival of low TF mice all suggest that TF is indispensable for hemostasis.

Contrary to TF deficiency, severe FVII deficiency has been identified in humans but at a low frequency of 1 in 500,000. FVII-deficient patients exhibit bleeding that varies in severity from epistaxis to intracranial hemorrhage (Bartosh *et al.*, 2013). FVII knockout mice develop a normal blood vasculature and survive embryonic development. However during the perinatal stage after birth, FVII-deficient mice suffer from fatal intra-abdominal bleeding and intracranial hemorrhaging (Rosen *et al.*, 1997). Murine studies have also been conducted with "low-FVII" mice and it was found that they not only undergo successful embryonic development but also survive to adulthood. This suggests that there was sufficient FVII in the low-FVII mice to maintain hemostasis for survival to adulthood. Therefore, FVII plays an essential role in maintaining perinatal hemostasis (Rosen *et al.*, 1997, Rosen *et al.*, 2005).

The extrinsic pathway of coagulation has also been implicated in thrombosis. TF is suggested to be involved in atherosclerotic plaque-induced thrombosis. TF is found on smooth muscle cells and macrophages within atherosclerotic plaques of patients suffering from acute coronary syndrome (Ott, 2011). TF-induced thrombosis plays a significant role in various animal injury models. Carotid artery injury and stenosis in rabbits results in induction of thrombosis at the stenotic region. Upon intravenous injection of anti-rabbit TF monoclonal antibody, thrombosis is eliminated and normal blood flow is

restored (Pawashe *et al.*, 1994). Similar results are found in a rabbit balloon injury restenosis model where the aorta of the rabbit is rescued from thrombosis by injection of recombinant tissue factor pathway inhibitor (TFPI) following injury (Asada *et al.*, 1998). Inhibition of TF-associated activity either by anti-TF antibody or TFPI generates antithrombotic effects and implicates TF, a member of the extrinsic pathway, as a contributor of thrombosis.

Experiments with active site-blocked activated FVII, FVIIai, have also been conducted to assess the role of FVII in promoting thrombosis. FVIIai generates antithrombotic effects by competing with FVIIa for TF but is not able to activate downstream factors, FIX and FX, due to its blocked active site. Thrombosis, produced in a rabbit model of stenosis and injury of the carotid artery, is abolished upon infusion of FVIIai. Thrombosis is restored upon infusion of recombinant human FVIIa, thus emphasizing that the antithrombotic effect is driven by FVIIai (Golino *et al.*, 1998). Similar results are produced in baboon models of arterial thrombosis where the intravenous injection of FVIIai prevents thrombus formation (Soderstrom *et al.*, 2001). The antithrombotic effect generated by FVIIai suggests that FVII, as a part of the extrinsic pathway of coagulation, plays an essential role in thrombosis.

1.4 Regulation of Coagulation

In addition to the activation of procoagulant proteins, inhibition of coagulation is also an important part of hemostasis. Regulation of procoagulant proteins is achieved by

the natural anticoagulants, which include TFPI, antithrombin, and the protein C pathway (Figure 1) (Esmon and Esmon, 2013).

TFPI is a Kunitz-type protease inhibitor that inhibits FXa and the TF-FVIIa complex. The primary site of TFPI production is the vascular endothelium and it is primarily expressed on the luminal surface of endothelial cells. TFPI is also expressed at low concentrations by megakaryocytes, monocytes, lung fibroblasts, and synovial cells. In addition to the aforementioned sites, a small percentage of TFPI can be found circulating freely in the blood stream. TFPI consists of three Kunitz domains; the first and the second Kunitz domain bind and inhibit the TF-FVIIa complex and FXa, respectively (Kubier and O'Brien, 2012). TFPI inhibits its targets by mimicking the substrates of the protease. The TFPI-inhibited protease either cleaves TFPI at a slow rate or not at all (Versteeg *et al.*, 2013).

Initially, TFPI reversibly binds and inhibits FXa while forming the TFPI-FXa complex. The TFPI-FXa complex then binds irreversibly to FVIIa within the TF-FVIIa complex in a calcium-dependent manner. Inhibition of this complex shuts down further generation of FXa and FIXa (Kubier and O'Brien, 2012).

Antithrombin, also known as antithrombin III, is a glycoprotein serine protease inhibitor (serpin). It is synthesized in the liver and exists predominantly as the α-isoform of the two isoforms in plasma, α and β-isoform (Kubier and O'Brien, 2012). Antithrombin inhibits thrombin (FIIa), FVIIa, FIXa, FXa, kallikrein-kininogen complex, FXIa, and FXIIa (Bock, 2001). Antithrombin inhibits proteases by presenting a reactive

center loop to its targets. Proteases cleave this loop and form a covalent complex with antithrombin and become incorporated into the serpin. This results in distortion and subsequent inactivation of the protease (Versteeg *et al.*, 2013).

Heparin, an important cofactor for antithrombin, is administered as an anticoagulant. It is a heterogeneous mixture of glycosaminoglycans that is isolated from porcine intestinal mucosa or bovine lung mast cells (Bock, 2001). The physiological equivalence of heparin is heparan sulfate glycosaminoglycan, which has a similar structure and is anchored to the endothelium. Like heparin, heparan sulfate interacts with antithrombin via a unique pentasaccharide sequence (Weitz, 2003). This interaction induces a conformational change in antithrombin that allows proteases better access to the reactive center loop, resulting in a 1000-fold increase in antithrombin's inhibitory effect (Esmon and Esmon, 2013, Kubier and O'Brien, 2012). Aside from its role in inducing conformational changes, unfractionated heparin has the ability to bind to proteases and antithrombin. This bridges both the protease and antithrombin to allow for better inhibition. The ability to bridge the two components is necessary for the inhibition of thrombin but less important for FXa. Conversely, low-molecular-weight heparins are not able to bridge but are still able to mediate FXa inhibition as long as the pentasaccharide sequence is conserved (Esmon and Esmon, 2013, Hirsh *et al.*, 2001).

The protein C pathway consists of protein C, activated protein C (APC), thrombin, thrombomodulin, endothelial protein C receptor (EPCR), and protein S that act in concert to inactivate FVa and FVIIIa (Esmon, 2003). Protein C, a vitamin K-dependent

protein, is produced primarily in the liver and circulates in the vasculature as a single chain zymogen. The active form of protein C is APC, the primary product of the protein C pathway that inactivates FVa and FVIIIa by proteolysis in the presence of protein S, calcium, and phospholipids (Kubier and O'Brien, 2012, Wildhagen *et al.*, 2011). The inactivation of FVa and FVIIIa, important procoagulant proteins for thrombin generation, results in an anticoagulant response (Weitz, 2013).

The thrombin-thrombomodulin complex, which is responsible for the activation of protein C, forms on the surface of the endothelium. Thrombomodulin, a transmembrane endothelial protein, binds to exosite 1 on thrombin and shifts its specificity from a procoagulant to an anticoagulant protein. Although thrombin can activate protein C, it is a very inefficient activator until it binds to thrombomodulin (Esmon, 2003, Versteeg *et al.*, 2013).

There are high concentrations of thrombomodulin on the endothelial cells lining the microcirculation because of the high endothelial surface area-blood volume ratio. Consequently, APC generation is favoured. In contrast, large blood vessels have a lower concentration of thrombomodulin. To compensate for this, the large blood vessels rely on EPCR to support APC generation. EPCR is a transmembrane protein on the surface of the endothelium that serves as a receptor for protein C. By binding protein C and presenting it to the thrombin-thrombomodulin complex for activation, EPCR augments protein C activation by 20-fold (Versteeg *et al.*, 2013).

Upon activation of protein C, APC dissociates from EPCR and binds to its cofactor, protein S (Esmon, 2003). Protein S, a vitamin-K dependent plasma protein, is produced by the liver and endothelial cells. It is a non-enzymatic protein that is commonly found in endothelial cells and platelets. Protein S acts as a cofactor to augment APC-mediated inactivation of FVa and FVIIIa by interacting with APC and binding to phospholipid membranes (Kubier and O'Brien, 2012).

TFPI, antithrombin, and the protein C pathway form the natural anticoagulants of the body. They regulate the procoagulant pathway at various points and maintain hemostasis.

1.5 The Contact System

The contact system refers to the initial stages of the intrinsic pathway of coagulation (Figure 3). It involves the activation of FXII and PK in a positive feedback loop consisting of FXII, FXIIa, PK, kallikrein and HK (McMichael, 2012, Stavrou and Schmaier, 2010). Another important aspect of the contact system is that it is activated by interaction with surfaces; both artificial and physiological. The interaction with surfaces is what makes the contact system important and may explain its role in thrombosis. This includes thrombosis induced by interaction with medical devices such as heart valves and catheters and also physiological species such as DNA, RNA, neutrophil extracellular traps (NETs) and platelet-derived polyphosphates(Jaffer *et al.*, 2015, Kannemeier *et al.*, 2007, Long *et al.*, 2016, McMichael, 2012, Pavlov *et al.*, 2006, Smith *et al.*, 2015, Yau *et al.*, 2012).

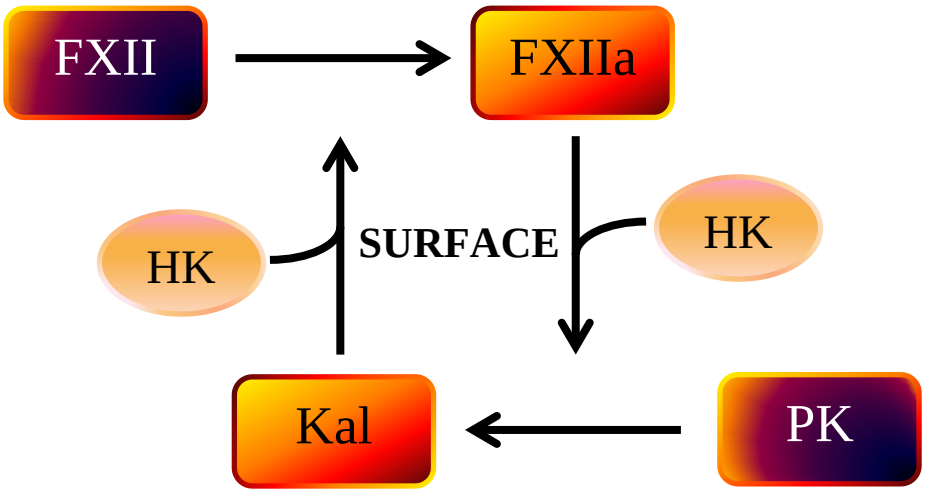


Figure 3: The Contact System.

The contact system consists of reactions that lead to the activation of FXII and PK in the presence of the cofactor HK and a negatively charged surface. The activation of FXII and PK occur in a positive feedback loop where a negatively charged surface activates FXII to FXIIa. FXIIa activates PK to generate Kallikrein (Kal) which activates FXII to complete the reciprocal activation of the contact system. Activation of the contact system leads to thrombin generation through the intrinsic pathway.

1.5.1 Structure and Function of FXII/FXIIa

FXII is encoded by the 12 kb *F12* gene located on chromosome 5q35.3. The gene is comprised of 13 introns and 14 exons. The FXII protein is a single chain polypeptide comprised of 596 amino acids. It is an 80 kDa zymogen that is produced in the liver and is found circulating in the plasma at a concentration of 375 nM (Schmaier, 2016, Smith *et al.*, 2015, Stavrou and Schmaier, 2010). FXII is composed of several domains including from N- to C-terminus the fibronectin type II domain, the epidermal-growth-factor (EGF)-like domain, the fibronectin type I domain, another EGF-like domain, the kringle domain, the proline-rich region, and the catalytic domain (Figure 4) (Stavrou and Schmaier, 2010).

A key functional role of FXII activation is to bind and activate FXI to carry out coagulation via the intrinsic pathway. FXII binds to FXI via its FXI binding site located at residues 3-19 in the fibronectin type II domain. Another key characteristic of FXII is its ability to activate upon interaction with negatively-charged surfaces. The ability to interact with surfaces is thought to be derived from specific regions that are known as artificial surface binding regions. There are two artificial surface binding regions that are located in the fibronectin type II (residues 1-28) and fibronectin type I (residues 134-153) domains. There are also two other proposed artificial surface binding sites located in the second EGF-like domain and the kringle domain (Stavrou and Schmaier, 2010).

In addition to FXII's ability to interact with negatively charged surfaces, FXII also binds zinc resulting in a conformational change that allows it to act as a better

Figure 4: Structure of FXII.

FXII is a single-chain zymogen composed of fibronectin type II, epidermal growth factor 1 (EGF1), fibronectin type I, epidermal growth factor 2 (EGF2), kringle, proline-rich region, and catalytic domain.

substrate for activation. Zinc also enhances the ability of FXII and its activated form, α-FXIIa, to bind to artificial surfaces such as dextran sulfate (Vu *et al.*, 2013). Four zinc binding sites have been identified on FXII; two are located in the fibronectin type II domain at residues 40-44 and 78-82 and the other two in each of the EGF-like domains at residues 94-131 and 174-176, respectively (Stavrou and Schmaier, 2010).

The largest domain of FXII is the catalytic domain and upon proteolysis of the catalytic domain at R353-V354, FXII is activated to α-FXIIa. FXII is activated to α-FXIIa in two ways: autoactivation by α-FXIIa and activation via a positive feedback loop in which α-FXIIa activates PK to kallikrein and kallikrein reciprocally activates FXII to α-FXIIa (Smith *et al.*, 2015, Stavrou and Schmaier, 2010). FXII, a single chain zymogen, converts into α-FXIIa, a two chain protein. The two chains of α-FXIIa are a 50 kDa heavy chain, consisting of 353 amino acids, and a 30 kDa light chain, consisting of 243 amino acids, which are linked by a disulfide bond. The active site of α-FXIIa, a serine protease, is comprised of H394, D442, and S544 which form the catalytic triad. It carries out coagulation through the intrinsic pathway by activating FXII, PK, and FXI. Further proteolysis of α-FXIIa by kallikrein yields β-FXIIa in which the disulfide bond is reduced. Due to the reduction of the disulfide bond, the 30 kDa light chain, which contains the catalytic domain, is freed from the heavy chain to generate β-FXIIa. Since the catalytic domain is preserved in β-FXIIa, it maintains the ability to activate its substrates and to propagate coagulation. However, because β-FXIIa lacks the heavy chain, it is unable to bind negatively charged surfaces and zinc ions. Conversely, the

presence of the heavy chain in α-FXIIa allows it to bind negatively charged surfaces and zinc ions (Stavrou and Schmaier, 2010).

1.5.2 Structure and Function of PK/Kallikrein

PK is encoded by the 22 kb *Klkb1* gene located on chromosome 4q34-35. This gene is comprised of 14 introns and 15 exons. Initially, the *Klkb1* gene codes for a 638 amino acid-long single chain polypeptide that contains a 19 amino acid signal sequence at the N-terminus and is known as pro-prekallikrein (pro-PK) (Bjorkqvist *et al.*, 2013, Schmaier, 2016). Cleavage of the signal peptide by signal peptidase results in the generation of PK, which consists of 619 amino acids. PK is a single chain zymogen that has two molecular weights of 85 and 88 kDa because of variability in its glycosylation. It is mainly produced in the liver and circulates in plasma at a concentration of approximately 580 nM. About 75-90% of PK circulates in complex with HK. PK and kallikrein both bind to HK with comparable affinity (Bjorkqvist *et al.*, 2013, Colman *et al.*, 1985).

PK is composed of five domains. Starting from the N-terminal, these include four apple domains followed by the catalytic domain. The apple 2 domain is responsible for its tight binding to HK. The apple 1 and apple 4 domains increase the affinity of HK-binding. The catalytic domain is responsible for the serine protease activity of kallikrein (Bjorkqvist *et al.*, 2013).

The activation of PK is primarily executed by FXIIa in the contact system to generate kallikrein that reciprocally activates more FXII in a positive feedback loop to

carry out coagulation through the intrinsic pathway. An alternate activator of PK is prolylcarboxypeptidase (PRCP). PRCP is an endothelium-associated activator of PK that is thought to activate PK bound to cells (Bjorkqvist *et al.*, 2013). Aside from its role in activating FXII, kallikrein also binds and cleaves HK to release bradykinin. Bradykinin is a peptide that serves as an inflammatory mediator and induces vasodilation of blood vessels. The ability of kallikrein to activate FXII and to release bradykinin from HK implicates kallikrein in both the contact system and the kallikrein-kinin system (Bjorkqvist *et al.*, 2013, Schmaier, 2016).

FXIIa activates PK by cleaving the R371-I372 bond to yield a two-chain serine protease known as α-kallikrein, which consists of a 52 kDa heavy chain composed of 371 amino acids and a light chain of either 36 or 38 kDa composed of 248 amino acids. The two chains are linked by a disulfide bond between the apple 4 domain and the catalytic domain. The heavy chain of α-kallikrein consists of the four apple domains while the light chain contains the serine protease domain. The catalytic triad of this serine protease consists of H415, D464, and S559 (Bjorkqvist *et al.*, 2013, Burger *et al.*, 1986, Schmaier, 2016, Smith *et al.*, 2015). With incubation, α-kallikrein slowly undergoes autocatalytic proteolysis with the generation of β-kallikrein by cleavage of the K140-A141 bond within the apple 2 domain (Bjorkqvist *et al.*, 2013). β-kallikrein has a three chain structure composed of the intact 36 or 38 kDa light chain variant bound to two cleaved products of the heavy chain by disulfide bonds with molecular weights of 30 and 20 kDa (Burger *et al.*, 1986). Both α-kallikrein and β-kallikrein activate FXII to FXIIa with similar rates as the light chain containing the serine protease remains intact in both variants. However, β-

kallikrein exhibits a markedly reduced rate of HK cleavage compared with α-kallikrein. This reduction is thought to be the result of the cleavage within the apple 2 domain of the heavy chain (Bjorkqvist *et al.*, 2013, Colman *et al.*, 1985), which disrupts the ability of β-kallikrein to bind and subsequently cleave HK. Overall, β-kallikrein has been found to have reduced coagulation activity compared with α -kallikrein due to disruption of the heavy chain and consequential reduction in the HK-binding ability of β-kallikrein(Colman *et al.*, 1985).

1.5.3 Structure and Function of HK

HK is encoded by the 27 kb *Kgn1* gene located on chromosome 3q26-qter. This gene consists of 11 exons and by alternative splicing, it codes for both HK and low molecular weight kininogen (LK). HK is a 120 kDa single chain protein composed of 626 amino acids (Colman and Schmaier, 1997, Pathak *et al.*, 2013, Weisel *et al.*, 1994). One of the major sources for HK production is the liver and HK circulates in plasma at a concentration of 670 nM. In plasma, HK circulates in complex with PK and FXI (Colman and Schmaier, 1997, Weisel *et al.*, 1994). HK is composed of six domains termed domains 1 through 6, from the N- to C-terminus. Domain 1 is known as the low-affinity binding site for calcium. It is also known to play a role in the inhibition of atrial natriuretic peptide. Domain 2 has been suggested to bind to endothelial C1q receptors while domain 3 binds to platelets, endothelial cells and neutrophils. Domain 4 contains bradykinin, an inflammatory peptide, which is released upon cleavage of HK. Domain 5 is a histidine-rich region that binds to anionic surfaces. Along with anionic surfaces,

domain 5 is also known to bind neutrophils, zinc, heparin, and endothelial cells. Domain 6 contains the binding sites for PK and FXI (Colman and Schmaier, 1997, Colman, 2006, Pathak *et al.*, 2013). Furthermore, the domains of HK are classified into three groups: the heavy chain, the bradykinin peptide, and the light chain. The heavy chain is composed of domains 1-3, the bradykinin peptide is the main component of domain 4, and the 56 kDa light chain is composed of domains 5 and 6 (Colman and Schmaier, 1997).

Cleavage of HK by kallikrein occurs at the K362-R363 and R371-S372 bonds and results in the release of the bradykinin peptide. Bradykinin is an inflammatory peptide that affects blood pressure and plays a role in nitric oxide and prostacyclin production (Colman and Schmaier, 1997, Zhang *et al.*, 2000). Along with the release of the bradykinin peptide, cleavage of HK also results in the production of a two-chain protein termed HKa. Structurally, the heavy and light chains of HKa are held together by a disulfide bond between domains 1 and 6. Conversion of HK to HKa also results in a conformational change that increases domain 5 exposure. This change is thought to be responsible for the ability of HKa to inhibit angiogenesis and induce apoptosis of endothelial cells (Merkulov *et al.*, 2008, Zhang *et al.*, 2000).

Apart from its role in the release of bradykinin, HK is also a procoagulant player in the contact system (Wu, 2015). The procoagulant activity of HK depends on its ability to bind to negatively charged surfaces via its histidine-rich region within domain 5 and its ability to bind PK and FXI via domain 6 (Colman and Schmaier, 1997). FXII, PK, and HK can assemble to activate the contact system and downstream proteins such as FXI.

The ability of FXII to bind negatively charged surfaces is decreased in the absence of HK, PK and FXI. HK's ability to bind to negatively charged surfaces allows PK and FXI to assemble together with FXII to activate the contact system. FXII autoactivates upon interaction with negatively charged surfaces and subsequently activates PK bound to HK. Kallikrein can then reciprocally activate more FXII in a feedback loop to activate the contact system (Weisel *et al.*, 1994, Wu, 2015).

1.5.4 Inhibitors of the Contact System

There are several inhibitors that target one or both proteins of the contact system: FXIIa and kallikrein (McMichael, 2012). C1 inhibitor, α_2-macroglobulin, and antithrombin serve as inhibitors for both FXIIa and kallikrein. Other inhibitors of FXIIa include α_2-antiplasmin, histidine-rich glycoprotein (HRG), and corn trypsin inhibitor (CTI). Additional inhibitors of kallikrein include α_1-antitrypsin, plasminogen activator inhibitor-1, protein C inhibitor, and soybean trypsin inhibitor (STI) (Colman and Schmaier, 1997, MacQuarrie *et al.*, 2011, Nolly *et al.*, 1994, Schmaier and McCrae, 2007, Yau *et al.*, 2012) (Figure 5).

C1 inhibitor is a major inhibitor of the contact system that inhibits both FXIIa and kallikrein. It is encoded by a 17kb gene located on chromosome 11. The C1 inhibitor gene consists of 8 exons and 7 introns. C1 inhibitor is a 105 kDa protein composed of 478 amino acids, which circulates in plasma at a concentration of 1.8µM. It is synthesized by many different cells including hepatocytes, fibroblasts, and endothelial cells (Caliezi *et al.*, 2000, Smith *et al.*, 2015). It is also found in the alpha granules of

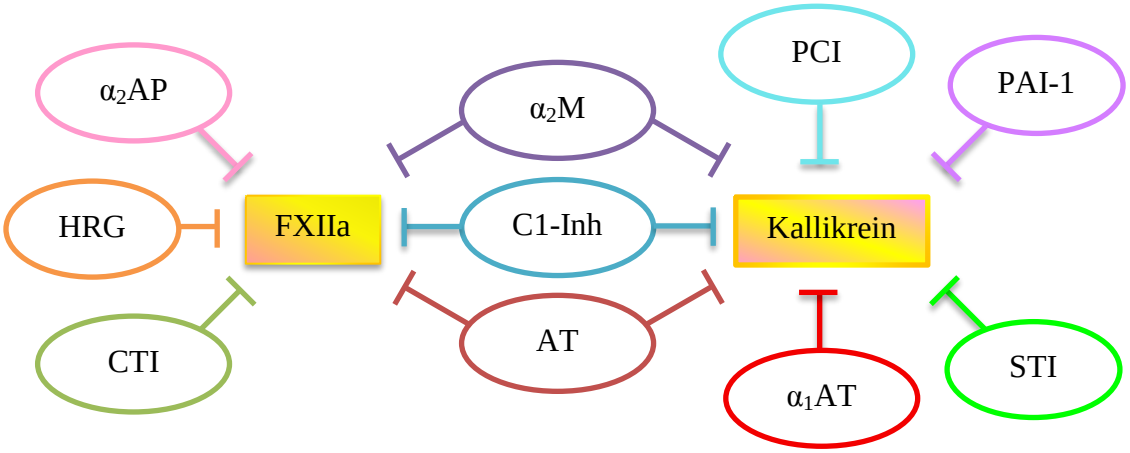


Figure 5: The Inhibitors of the Contact System.

The inhibitors of the contact system inhibit FXIIa and/or kallikrein. C1-inhibitor (C1-Inh), α_2-macroglobulin (α_2M), and antithrombin (AT) inhibit both FXIIa and kallikrein. α_2-antiplasmin (α_2AP), histidine-rich glycoprotein (HRG), and corn trypsin inhibitor (CTI) exclusively inhibit FXIIa. Kallikrein is also inhibited by α_1-antitrypsin (α_1AT), plasminogen activator inhibitor-1 (PAI-1), protein C inhibitor (PCI), and soybean trypsin inhibitor (STI).

platelets and upon the activation of platelets it is exposed on the surface of platelets. C1 inhibitor inhibits its targets by revealing the reactive centre loop containing the scissile bond at R444-T445. The cleavage of this bond results in the inactivation of the serine protease (Caliezi *et al.*, 2000).

Alongside C1 inhibitor, α_2-macroglobulin also plays a key role in inactivating FXIIa and kallikrein. α_2-macroglobulin forms a 1:1 stoichiometric complex with kallikrein. Antithrombin has also been identified as an inhibitor of FXIIa and kallikrein. In comparison with C1 inhibitor, the inactivation of FXIIa or kallikrein by antithrombin is slow (Caliezi *et al.*, 2000, Colman and Schmaier, 1986).

HRG is encoded by an 11kb gene located on chromosome 3q28-29 and is composed of 8 exons and 7 introns. HRG is a 507 amino acid single chain glycoprotein with a molecular weight of 75 kDa. It is synthesized in the liver and circulates in plasma at a concentration of 2µM. HRG is also reported to be stored in the alpha granules of platelets and is released upon activation of platelets. HRG is composed of 2 N-terminal cystatin domains, a histidine-rich region, and a C-terminal domain. These domains help identify key properties and ligands of HRG (Jones *et al.*, 2005, MacQuarrie *et al.*, 2011).

HRG has been suggested to be an inhibitor of the contact system due to its ability to bind to anionic surfaces with its histidine-rich region. In an effort to inhibit FXII activation, HRG has been shown to bind DNA and RNA and thus prevent activation of the contact system that would otherwise be activated by binding to negatively charged surfaces (MacQuarrie *et al.*, 2011, Vu *et al.*, 2016). HRG is known to bind FXIIa and

inhibit it from autoactivating FXII or activating FXI. Furthermore, HRG also inhibits the activation of PK mediated by FXIIa. However, this inhibition is lessened in the presence of HK. It has also been suggested that zinc binds to HRG via its histidine-rich region and then modifies HRG to increase its affinity for FXIIa (MacQuarrie *et al.*, 2011).

The location of the binding of FXIIa on HRG is not very clear. It has been suggested that HRG binds to FXIIa on a site other than the catalytic domain because HRG does not bind β-FXIIa. Furthermore, the inability of HRG to bind to FXII zymogen indicates that the conformational change that occurs upon activation of FXII is required to expose the region necessary for HRG binding (MacQuarrie *et al.*, 2011).

These findings have suggested that HRG plays a major role in regulating the contact system. In comparison to HRG, C1 inhibitor, which is considered to be a major inhibitor of FXIIa, is a slow inhibitor of FXIIa and is not able to inhibit FXIIa when it is bound to anionic surfaces. However, HRG is able to inhibit FXIIa bound to nucleic acids and promote dissociation of nucleic acids from FXIIa. The ability of HRG to bind FXIIa and negatively charged surfaces that stimulate activation of FXII establishes it as an important inhibitor of the contact system (MacQuarrie *et al.*, 2011, Vu *et al.*, 2016).

CTI and STI are commonly used inhibitors in various assays. CTI is a 127 amino acid serine protease inhibitor with a molecular weight of 13.6 kDa. It is isolated from corn seeds and inhibits FXIIa by reversibly binding to its active site. The inhibition of FXIIa by CTI involves cleavage of the scissile bond of CTI at R34-L35 by FXIIa (Behnke *et al.*, 1998, Korneeva *et al.*, 2014, Yau *et al.*, 2012). STI is an inhibitor of

plasma kallikrein. It is a 20.1 kDa single chain polypeptide composed of 181 amino acids. Inhibition of kallikrein by STI involves the cleavage of STI's scissile bond at R63-I64 by kallikrein (Koide and Ikenaka, 1973, Nolly *et al.*, 1994, Song and Suh, 1998).

1.5.5 The Role of Zinc in the Contact System

Zinc is one of the most abundant ions found in plasma. Most of the Zn^{2+} found in plasma is bound to albumin but 0.1-2μM of Zn^{2+} circulates freely. Zn^{2+} is also found to be stored in platelets and released from activated platelets. Accumulation of activated platelets at site of injury results in higher local Zn^{2+} concentration which can promote the contact system (Henderson *et al.*, 2016, Vu *et al.*, 2013).

Zinc is an important ion for the contact system due to its ability to bind to FXII. There are 4 zinc binding sites for FXII, 3 zinc binding sites for α-FXIIa but none for β-FXIIa. These findings have suggested that the zinc binding regions are located on the heavy chain of FXII. It has been suggested that FXII is able to undergo two conformational changes: the first upon binding to negatively charged surfaces and the second upon binding of zinc. Furthermore, surface-independent and surface-dependent autoactivation of FXII are both augmented upon interaction with zinc (Vu *et al.*, 2013).

The conformational change induced upon binding to zinc augments FXII's ability to bind to surfaces and to act as a better substrate for activation by FXIIa and kallikrein. Zinc augments kallikrein-mediated activation of FXII in the presence of HK and negatively charged surfaces (Vu *et al.*, 2013). Zinc also augments FXIIa-mediated activation of PK in the presence of HK and a sulfatide surface. Importance of HK is also

highlighted in these reactions as acceleration by zinc requires HK. This leads to the suggestion that zinc may interact with HK to enhance the effect of the surface on FXII and PK. However, kinetic analyses have revealed that zinc predominantly acts on FXII in the contact system compared to PK (Shimada *et al.*, 1987, Vu *et al.*, 2013).

1.6 Physiological Activators of the Contact System

Until recently, only non-physiological activators of the contact system, such as kaolin, glass, ellagic acid, and high molecular weight dextran sulfate were identified. Recently several laboratories have identified a variety of biological substances that serve as platforms for FXII autoactivation, including exposed vessel collagen, DNA and RNA, aggregated proteins, and long chain polyphosphates (Long *et al.*, 2016, Smith *et al.*, 2015). Collagen is a physiological activator of the contact system because of its ability to bind and activate FXII. It has been found to augment thrombin generation and reduce plasma clotting times in a FXII-dependent manner (van der Meijden *et al.*, 2009, Wu, 2015). Heparin derived from mast cells is also known to autoactivate FXII and stimulates FXIIa-mediated activation of PK but not FXI. Activation of PK subsequently results in cleavage of HK and release of bradykinin (Brunnee *et al.*, 1997, Long *et al.*, 2016, Smith *et al.*, 2015).

Similarly, misfolded proteins are also known to activate FXII without leading to the activation of FXI. This results in FXIIa-mediated activation of kallikrein which subsequently results in the cleavage of HK and generation of bradykinin. Bradykinin production has been detected in the cerebrospinal fluid of patients suffering from

Alzheimer's disease and it has been suggested that amyloid β peptide aggregates promote FXII autoactivation. Furthermore, activation of the kallikrein-kinin system through FXII has also been suggested in patients suffering from systemic amyloidosis without leading to the activation of FXI (Maas *et al.*, 2011, Smith *et al.*, 2015).

Polyphosphates (polyP) have also been suggested as physiological activators of the contact system. Polyphosphates are anionic chains of linearly linked orthophosphates of various chain lengths. Polyphosphates derived from microbial sources are longer in chain length, from hundreds to thousands of phosphate units, compared to those released from dense granules of activated platelets, which range from 60-100 phosphate units. In addition to their ability to activate the contact system, polyphosphates accelerate activation of FV by thrombin and FXa, limit the activity of TFPI, increase the turbidity of fibrin clots, and increase feedback activation of FXI by thrombin. However, the capacity of polyphosphates to activate the contact system depends on their chain length. In comparison to platelet-derived polyphosphates, long polyphosphate polymers have been shown to be significantly more potent activators of the contact system. This finding is supported by the ability of longer polyphosphate polymers to bind FXII with high affinity (Morrissey *et al.*, 2012, Smith *et al.*, 2010). It should also be noted that by promoting FXII activation, polyphosphates enhance thrombin and bradykinin generation by activating FXI and the kallikrein-kinin system, respectively (Maas *et al.*, 2011). *In vivo* mouse studies have also revealed the thrombogenic nature of polyphosphates. Administration of platelet polyphosphates to wild-type mice resulted in death due to pulmonary embolism. However, FXII-deficient mice undergoing the same treatment

survived. Other experiments showed similar findings regarding the thrombogenic nature of polyphosphates. Agonist-mediated activation of platelets resulted in death by pulmonary embolism in most wild-type mice. However, most wild-type mice pretreated with phosphatases and most FXII-deficient mice did not develop pulmonary embolism and survived. Administration of phosphatases to degrade polyphosphates eliminated the surface required for FXII activation and the lack of FXII in FXII-deficient mice eliminated the target of polyphosphates to promote thrombosis. These findings suggest that polyphosphates can promote thrombosis *in vivo* (Morrissey *et al.*, 2012, Muller *et al.*, 2009). Although platelet-derived polyphosphates are limited in their capacity to promote contact activation, they are still able to accelerate feedback activation of FXI by thrombin, accelerate activation of FV by thrombin and FXa, and limit the activity of TFPI (Morrissey *et al.*, 2012, Smith *et al.*, 2010).

Extracellular nucleic acids have also been identified as physiological activators of the contact system. Binding studies using surface plasmon resonance demonstrated that DNA and RNA bind to FXII, FXI, and HK. Although nucleic acids do not bind to PK, it has been previously suggested that HK may serve as an assembly platform for PK and nucleic acids (Vu *et al.*, 2016, Wu, 2015). DNA and RNA are procoagulant and augment thrombin generation through the intrinsic pathway. The procoagulant activity of nucleic acids has been demonstrated in platelet poor plasma using the thrombin generation assay. Nucleic acids enhance thrombin generation in normal plasma or in plasma deficient in FVII. In contrast, their procoagulant activity is lost in plasma deficient in FXII or FXI.

Therefore, these findings indicate that the procoagulant activity of nucleic acids is mediated via the contact pathway (Vu *et al.*, 2016).

Nucleic acids are released from apoptotic and necrotic cells. Furthermore, DNA is also released as a component of neutrophil extracellular traps (NETs) in a process known as NETosis. NETs are composed of DNA, histones, and antimicrobial proteins and serve as a component of innate immunity to entrap and kill bacteria. Although NETs are released from neutrophils undergoing a unique cell death program, they can also be released through pathways that do not result in neutrophil death (Long *et al.*, 2016, Smith *et al.*, 2015).

The composition of NETs endows them with procoagulant activities. It has been suggested that NETs provide a negatively charged surface, due to the presence of DNA in NETs, for FXII activation (von Bruhl *et al.*, 2012). Furthermore, NETs have also been shown to generate thrombin in platelet-rich and platelet-poor environments. In platelet poor plasma, NETs have been shown to enhance thrombin generation through the intrinsic pathway. Also, upon addition of DNase, and not RNase, thrombin generated as a result of stimulation by NETs is reduced. This highlights the role of DNA in enhancing thrombin generation through the intrinsic pathway in platelet poor plasma. NETs also enhance thrombin generation in platelet rich plasma which is thought to be mediated by histones. Histones have been suggested to activate and induce subsequent aggregation of platelets through toll-like receptors 2 and 4 on platelets. Addition of DNase to platelet rich plasma containing NETs results in an interesting finding. Upon addition of DNase,

there is an increase in thrombin generation suggesting that the addition of DNase may liberate histones and allow it to better interact with platelets (Geddings and Mackman, 2014, Gould *et al.*, 2014, Gould *et al.*, 2015). The presence of NETs has also been identified in venous thrombi in various animal models. Furthermore, treatment with DNase protects mice from venous thrombosis presumably by degrading the DNA in the NETs scaffold (Geddings and Mackman, 2014).

In vitro assays have also revealed the ability of both DNA and RNA to augment activation of FXII in the presence of PK and HK (Kannemeier *et al.*, 2007, Vu *et al.*, 2016). *In vitro* assays have shown an increase in FXIIa-mediated activation of FXI in the presence of DNA or RNA. Furthermore, nucleic acids also enhance thrombin-mediated activation of FXI (Vu *et al.*, 2016).

Studies conducted with murine arterial thrombosis models have also suggested that RNA is an important procoagulant. Unlike DNA, extracellular RNA has been found associated to the thrombi of mice that have undergone $FeCl_3$-induced thrombosis of the carotid artery. Treatment with RNase prior to the application of the $FeCl_3$ results in a prolonged time to occlusion. In a different study, RNase treatment did not result in occlusion 30 minutes after $FeCl_3$ application. However, treatment with DNase did not produce the same result. Treatment with DNase had a time to occlusion similar to that of wild-type mice administered saline. These results suggests that RNA mediates $FeCl_3$-induced thrombosis of the carotid artery (Kannemeier *et al.*, 2007, Vu *et al.*, 2015). Other *in vivo* studies have also established the role of DNA in thrombosis. Baboons and mice

have been reported to form deep vein thrombi comprised of DNA, histones, and NET-like structures. Several mice undergoing inferior vena cava (IVC) stenosis develop a thrombus but those injected with DNase after surgery were protected in most cases. This suggests that DVT induced by IVC stenosis is reduced by the administration of DNase which degrades the DNA component of the thrombus (Brill *et al.*, 2012).

1.7 The Importance of the Intrinsic Pathway of Coagulation

Similar to the extrinsic pathway of coagulation, the intrinsic pathway also plays an important role in promoting thrombosis, however, only some of its components are essential for maintaining hemostasis. The major components of the intrinsic pathway that are important for hemostasis are FVIII, FIX, and FXI. The deficiencies of FVIII and FIX result in hemophilia A and B, respectively. Severe hemophilia is characterized by hemorrhaging in joints and muscles and can also result in intracranial hemorrhaging (Carcao *et al.*, 2013, Gailani and Renne, 2007). In contrast to deficiency of FVIII or FIX, FXI deficiency, also known as hemophilia C, results in a milder bleeding disorder. Patients with FXI deficiency may experience significant bleeding after trauma but rarely experience spontaneous bleeding (Gailani and Neff, 2013, Gailani and Renne, 2007). In contrast, deficiencies of FXII, PK, or HK are not associated with bleeding, despite the prolonged aPTT (van Montfoort and Meijers, 2014). Murine models yield results similar to those in humans. Mice deficient in FVIII or FIX have increased bleeding while those deficient in FXII, FXI, PK, or HK do not (Gailani and Renne, 2007, Merkulov *et al.*,

2008, Revenko *et al.*, 2011). These findings indicate that the contact system does not play a role in hemostasis, whereas FVIII and FIX are essential for hemostasis (Wu, 2015).

The intrinsic pathway of coagulation also plays a role in thrombosis. Elevated FVIII, FIX, and FXI levels have been associated with venous thromboembolism. Furthermore, there is an increased risk for recurrent thrombosis for patients with elevated FVIII levels (Bertina, 2003, Jenkins *et al.*, 2012). Animal studies have also shown that partial inhibition of FVIII with a monoclonal antibody results in reduced thrombus formation in a mouse inferior vena cava stenosis model and in baboon arteriovenous shunt model. These findings confirm that FVIII plays an important role in thrombosis because the risk of thrombosis is increased with elevated FVIII levels and thrombosis is attenuated when FVIII is inhibited (Jacquemin *et al.*, 2009, Singh *et al.*, 2002). Along with FVIII, FIX is also thought to play an important role in thrombosis. Elevated FIX levels increase the risk for developing venous thrombosis by 2- to 3-fold (van, V *et al.*, 2000).

FXI has also been established as a contributor in thrombosis. Various animal model studies have established that inhibition of FXI leads to a protective effect against thrombosis. Ferric chloride and vena cava ligation murine thrombosis models have been used to show that inhibition of FXI by inhibitory antibodies leads to protection from arterial and venous thrombosis (van Montfoort and Meijers, 2014). Studies have also been performed with primates where knockdown of FXI by antisense oligonucleotides in vascular graft occlusion models leads to an antithrombotic effect. These results led to a

study using FXI antisense oligonucleotides in patients undergoing total knee replacement (Crosby *et al.*, 2013, van Montfoort and Meijers, 2014). This study revealed a preventive effect on postoperative venous thromboembolism with a low risk of bleeding (Buller *et al.*, 2015).

Along with FXI, the contact system is also believed to play an important role in thrombosis. There have been many animal studies conducted using various thrombosis models, targeting FXII, that have shown a protective effect against thrombosis. FXII-deficient mice have been shown to be protected from arterial and venous thrombosis in various artificially-induced thrombosis models, without an effect on hemostasis indicated by normal bleeding times (van Montfoort and Meijers, 2014). Specifically, FXII-deficient mice undergoing injury of the cerebral artery revealed reduced ischemic injury following reperfusion of the occluded artery compared with wild-type mice. Ferric chloride injury of the carotid artery in FXII-deficient mice also revealed reduced occlusive thrombus formation (Gailani and Renne, 2007). Animal studies have also been conducted using FXII-inhibiting antibodies. FXII-inhibiting antibodies reduced platelet deposition and fibrin accumulation in arteriovenous shunts in baboons. This indicates an antithrombotic effect of FXII inhibition (Matafonov *et al.*, 2014). Another method for FXII inhibition is by the use of antisense oligonucleotides. Mice treated with FXII antisense oligonucleotides exhibit reduced thrombosis in arterial and venous thrombosis models. The ferric chloride injury model of the mesenteric artery revealed that mice treated with FXII antisense oligonucleotides are protected against arterial occlusion compared with control. Thrombosis of the inferior vena cava, induced by ferric chloride application, also

revealed a reduction in thrombus size compared with control. Thrombosis induced by stenosis of the inferior vena cava also led to similar results in mice treated with FXII antisense oligonucleotides. It should also be noted that FXII inhibition does not result in bleeding thereby validating the concept that it does not play a role in hemostasis (Revenko *et al.*, 2011). Across various methods of FXII inhibition in arterial and venous thrombosis models it has been found that FXII inhibition results in an antithrombotic effect and thus implicates FXII as an important contributor in thrombosis.

Other contact system proteins that play important roles in thrombosis are prekallikrein and high molecular weight kininogen. Effects of PK depletion have been studied in mice using antisense oligonucleotides. These studies have been conducted in the arterial and venous system and have shown an antithrombotic effect. Using a mesenteric arterial thrombosis model, PK-depletion prevented platelet aggregation, fibrin formation, and occlusion of the artery (Revenko *et al.*, 2011) . Ferric chloride injury and stenosis-induced thrombosis models in the venous system revealed similar findings in PK-depleted mice. These models revealed a reduction in thrombus formation and weight (Revenko *et al.*, 2011). The importance of PK for thrombosis has also been established by the use of the ferric chloride carotid artery injury model in PK-deficient mice. The antithrombotic effect was also present in this scenario as PK-deficient mice had reduced occlusion of the carotid artery compared with wild-type mice (Kokoye *et al.*, 2016).

HK is also important for thrombosis. The ferric chloride carotid artery injury model, conducted with HK-deficient mice, revealed reduced occlusion of the artery

similar to the results obtained by PK-deficient mice (Kokoye *et al.*, 2016). The Rose Bengal laser injury model also revealed an antithrombotic effect in HK-deficient mice. Time to occlusion of the carotid artery has been shown to be increased in the HK-deficient mice compared to wild-type. It is also important to note that although there is an antithrombotic effect produced by HK-deficient mice, hemostasis of these mice is not jeopardized as indicated by normal tail bleeding times compared to wild-type mice (Merkulov *et al.*, 2008). Ischemic stroke studies have also been conducted with HK-deficient mice. These studies also revealed an antithrombotic protective effect with reduced thrombus formation and less severe neurological defects in HK-deficient mice (Langhauser *et al.*, 2012). The collective findings establish HK as a prothrombotic protein and its deficiency leads to antithrombotic effects without compromising hemostasis.

Along with the role of the contact system in thrombosis, the discovery of physiological activators such as DNA, RNA, and polyP confirms the importance of the contact system in physiology and it may serve as a therapeutic target (Long *et al.*, 2016).

1.8 Overview of the Thesis and Rationale

Although the contact system is not important for hemostasis, there is mounting evidence that it is critical for thrombus stabilization and growth (Vu *et al.*, 2015). The contact system consists of FXII, PK, and HK and receives its name because the system is activated when blood comes in contact with anionic surfaces (Colman and Schmaier, 1997, Vu *et al.*, 2016).

Recently, DNA and RNA have been identified as potential physiological activators of the contact system. The relevance of nucleic acids is especially apparent upon consideration of the elevated cell-free DNA levels in patients suffering from sepsis or cancer (Dwivedi *et al.,* 2012, Hawes *et al.,* 2015). These findings raise interesting questions about the role of nucleic acids in activation of both FXII and PK in the contact system and the positive feedback loop that generates FXIIa and kallikrein.

Previous work in the literature has suggested that nucleic acids promote thrombin generation via the intrinsic pathway (Vu *et al.,* 2016). Studies in animals have also individually implicated DNA and RNA as procoagulant (Brill *et al.*, 2012, Kannemeier *et al.*, 2007, Vu *et al.*, 2015). Furthermore, nucleic acids have been shown to promote FXII activation in buffer systems (Kannemeier *et al.,* 2007, Vu *et al.,* 2016). This study will identify specific reactions within the contact system that are influenced by nucleic acids and investigate which coagulation factors are essential for these reactions. Specifically, the capacity of nucleic acids to activate prekallikrein will be studied, which has not been previously studied and the effect of Zn^{2+} on the activation of both FXII and PK.

The purpose of these studies were to (a) confirm that nucleic acids promote thrombin generation in plasma via the intrinsic pathway, (b) compare the procoagulant activity of DNA and RNA, (c) use purified reagents to identify the contact system reactions that are influenced by nucleic acids, and (d) determine the role of zinc in enhancement of these reactions.

1.8.1 Hypothesis

We hypothesized that DNA and RNA promote thrombin generation via the intrinsic pathway by stimulating the reciprocal activation of FXII and PK. We also hypothesize that zinc promotes this reciprocal activation by nucleic acids because of its capacity to bind to FXII and possibly other contact pathway components.

1.8.2 Objective and Specific Aims

The overall objective of this study is to attain a better understanding of the procoagulant effect of nucleic acids on the individual reactions of the contact system and to delineate the role of Zn^{2+} in these reactions. There are 3 specific aims.

1. To compare the capacities of DNA and RNA to promote thrombin generation in human plasma and to confirm that such promotion occurs via the intrinsic pathway.
2. To use purified reagents to identify the contact pathway reactions promoted by DNA and RNA.
3. To determine the role of zinc in nucleic acid-mediated promotion of the contact pathway reactions.

Chapter 2: Materials and Methods

2.1 Materials

A549 non-small cell lung cancer cells were a gift from Dr. P. Liaw (ATCC, Manassas, VA). Dextran sulfate sodium salt with a molecular weight of 500 kDa was obtained from GE Healthcare (Burlington, ON).

Flat bottom, non-coated, 96-well polystyrene plates were from Thermo Fisher Scientific (Waltham, MA). Thrombin generation assays were conducted in round bottom, black 96-well polystyrene plates from Corning Incorporated (Corning, NY). FXII, FXIIa, PK, kallikrein, HK, and CTI were obtained from Enzyme Research Laboratories (South Bend, IN). Soybean trypsin inhibitor (STI) was from Sigma-Aldrich (St. Louis, MO). Plasma-deficient in FXII, FXI, or FVII was obtained from George King Bio-Medical (Overland Park, KS). PK-depleted plasma was obtained from Affinity Biologicals (Ancaster, ON).

2.2 Methods

2.2.1 Nucleic Acid Isolation

A549 cells were cultured in Gibco RPMI 1640 medium containing 10% Fetal Bovine Serum and 1% Penicillin-Streptomycin (Life Technologies, Burlington, ON) at 37°C and 5% CO_2. DNA and RNA were isolated from these cells using DNeasy Blood & Tissue and RNeasy Plus Universal Mini kits, respectively (Qiagen, Toronto, ON). DNA and RNA concentrations were determined by measuring absorbance at 260nm using the

SpectraMax spectrophotometer (Molecular Devices, Sunnyvale, CA). The size and purity of the nucleic acids were assessed on an agarose gel. Nucleic acids were incubated with either 50µg/mL RNase-free DNase I (Thermo Fisher Scientific, Waltham, MA) or 50µg/mL DNase and protease-free RNase A (Thermo Fisher Scientific). A 2% agarose gel was prepared by dissolving agarose (BioShop, Burlington, ON) into TAE buffer consisting of 40mM Tris, 20mM acetic acid, and 1mM EDTA. After staining the dissolved agarose with 0.6µg/mL ethidium bromide (Sigma-Aldrich, St. Louis, MO) and allowing it to settle for 45min, intact or degraded nucleic acids were loaded on to the gel along with 1kb and 100bp molecular DNA ladders (Norgen Biotek, Thorold, ON) and RiboRuler High Range RNA ladder (Thermo Fisher Scientific, Waltham, MA). Nucleic acids were separated based on their molecular weight by running the gel for 45-60min at 110 volts.

2.2.2 Preparation of Platelet Poor Pooled Plasma

Platelet poor plasma was prepared by pooling blood from 10-15 individuals into tubes containing citrate with a final concentration of 0.32% citrate. The blood was centrifuged twice at 4000 x g for 20min each to separate the cells from the plasma. The platelet poor plasma was then frozen in aliquots at -80°C.

2.2.3 Thrombin Generation Assay

Thrombin generation assays were performed in platelet poor plasma using DNA, RNA or aPTT SP reagent (Instrumentation Laboratory, Bedford, MA), diluted in 125mM imidazole, pH 7.0, added to wells of a round bottom 96-well plate each containing 40µL

of plasma for 5min at 37°C. This was followed by the addition of 25µL of a solution containing 40mM $CaCl_2$ and 2mM Z-Gly-Gly-Arg-AMC (Bachem, Torrance, CA) and substrate hydrolysis was monitored using a SpectraMax M3 microplate reader (Molecular Devices, Sunnyvale, CA) with excitation, emission, and cutoff filter wavelengths of 360, 460, and 455nm, respectively. The final concentrations of $CaCl_2$ and Z-Gly-Gly-Arg-AMC were 10mM and 0.5mM, respectively in the final 100µL volume. Technoclone TGA software (Technoclone, Vienna, Austria) was used to analyze the thrombin generation data.

2.2.4 FXIIa Generation Assay

FXIIa generation assays were performed in wells of a 96-well plate and reagents were diluted in HEPES Buffered Saline (HBS) consisting of 10mM HEPES, 150mM NaCl), pH 7.4 containing 0.005% Tween 20 and, in some studies 10µM $ZnCl_2$. FXII (150nM) was incubated with 15nM PK and 15nM HK in the presence or absence of 0.05µg/mL dextran sulfate or 5 to 60µg/mL A549-derived DNA or RNA. The $ZnCl_2$ concentration was chosen based on a dose response study. After 60min at 37°C, 100µg/mL STI was added to each well to inhibit kallikrein activity and after an additional 5min incubation at 37°C, 400µM S2302 (Aniara Diagnostica, West Chester, OH) was added and hydrolysis was monitored at 405nm in a SpectraMax 340PC384 microplate reader (Molecular Devices, Sunnyvale, CA). The amount of FXIIa generated was then determined based on the comparison to a standard curve of FXIIa-mediated cleavage of

S2302 with predetermined concentrations of FXIIa. This assay was designed based on previously published work (Kannemeier *et al.*, 2007, Vu *et al.*, 2016).

2.2.5 Kallikrein Generation Assay

The kallikrein generation assay was performed in wells of a 96-well plate and reagents were diluted in HBS containing 0.005% Tween 20 and, in some studies, 10 µM $ZnCl_2$. PK (5nM) was incubated with 0.1µg/mL dextran sulfate or 40µg/mL DNA or RNA in the absence or presence of 5nM HK for 5 minutes at 37°C. FXIIa was added and 1 minute later, 30nM CTI was added to inhibit FXIIa. After 5min incubation, 400µM S2302 was added and hydrolysis was monitored at 405nm as described above. This assay was designed based on the FXIIa generation assay.

2.2.6 Statistical Analysis

Data are presented as mean ± standard deviation. Comparisons were made using ANOVA for grouped data and the Mann-Whitney rank sum test for paired comparisons. For all analyses, p-values < 0.05 were considered statistically significant. Graphs and statistical analysis were completed using SigmaPlot (v.12, San Jose, CA). Linear regression analyses comparing slope and y-intercepts were completed using GraphPad Prism (v.4, La Jolla, CA).

Chapter 3: Results

3.1 Thrombin Generation Assay

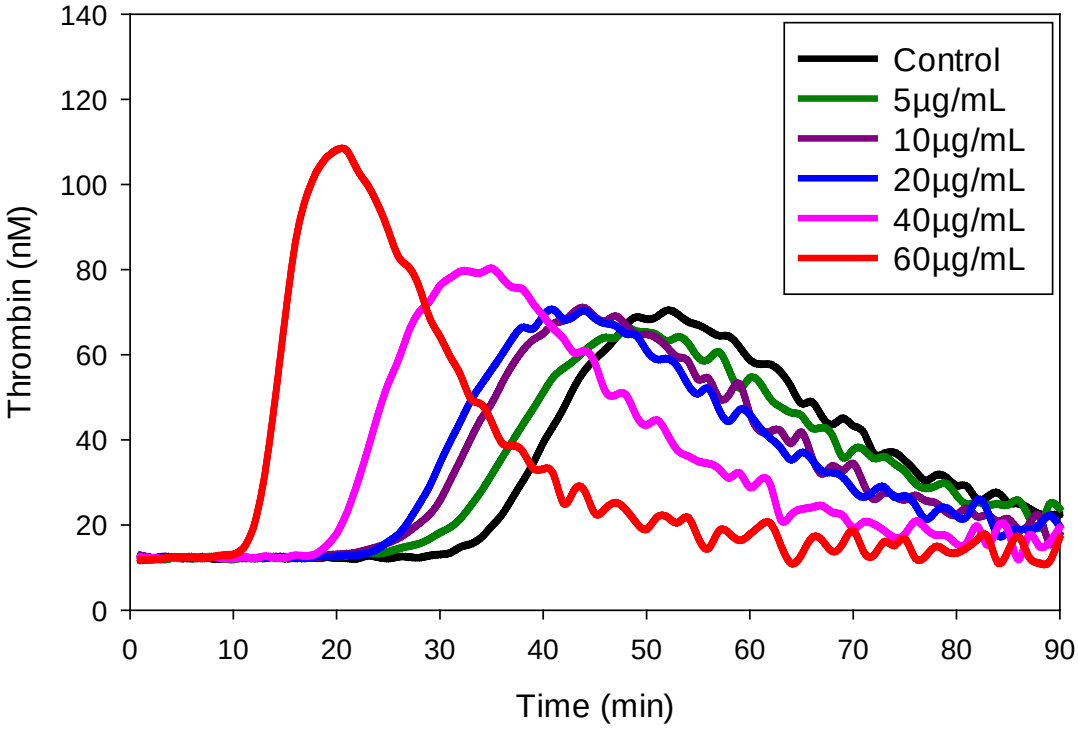
3.1.1 Increasing Concentrations of Nucleic Acids in Thrombin Generation Assay

DNA and RNA have been reported to have procoagulant activity (Kannemeier *et al.*, 2007, Vu *et al.*, 2016). As a first step, we set out to confirm this and to determine their potency. For this purpose, the effect of increasing concentrations of DNA or RNA was examined using a thrombin generation assay in normal pooled, platelet-poor plasma (NP). Plasma was incubated for 5 minutes with 0 to 60µg/mL of A549 DNA or RNA before initiating the assay with the addition of $CaCl_2$ and fluorogenic thrombin substrate.

Thrombin generation profiles revealed that increasing concentrations of nucleic acids led to increased thrombin generation (Figure 6). This can be assessed by analyzing individual parameters of the thrombin generation. Individual parameters of the thrombin generation assay can be visualized by the shape and shifts of the thrombin generation curves. Leftward shifts of the curve indicate a reduction in lag time and the increasing peaks of the curve indicate increased peak thrombin concentrations with increasing concentrations of DNA or RNA.

Quantitative analysis revealed a statistically significant ($p<0.05$) reduction in lag time compared to control upon addition of 40 or 60µg/mL of DNA or RNA. Lag time was reduced from 26.4 ± 6.1 min to 17.5 ± 1.9 and 12.7 ± 1.6 min upon addition of 40 and 60µg/mL of DNA, respectively (Table 1A). Similarly, the addition of 40 or 60µg/mL of RNA reduced the lag time to 14.0 ± 2.0 and 11.7 ± 1.4 min, respectively (Table 1B).

A


Thrombin (nM)
Time (min)
Control
5µg/mL
10µg/mL
20µg/mL
40µg/mL
60µg/mL
0
20
40
60
80
100
120
140
0
10
20
30
40
50
60
70
80
90

B

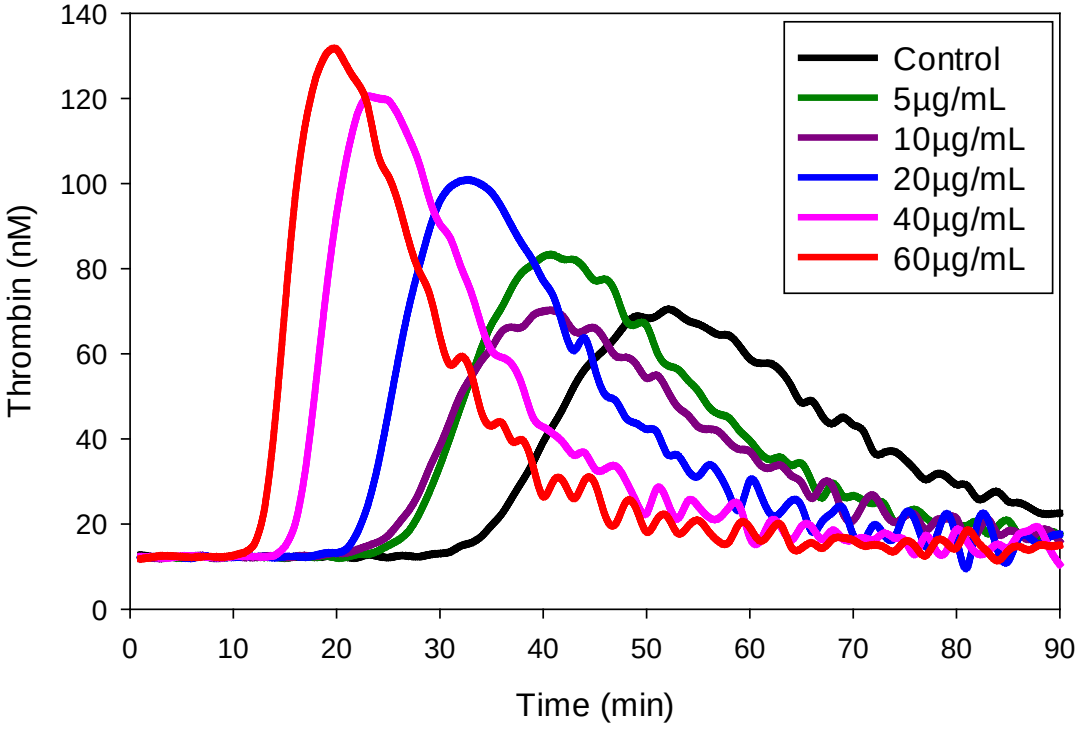

Thrombin (nM)
Time (min)
Control
5µg/mL
10µg/mL
20µg/mL
40µg/mL
60µg/mL
0
20
40
60
80
100
120
140
0
10
20
30
40
50
60
70
80
90

Figure 6: The Effect of Nucleic Acids on Thrombin Generation.

Normal plasma was incubated with 0 to 60µg/mL of (**A**) A549 DNA or (**B**) RNA before thrombin generation was initiated with the addition of 40mM $CaCl_2$ and 2mM Z-Gly-Gly-Arg-AMC. Data are displayed by representative thrombin generation profiles of the various concentrations of nucleic acids

This represents a 34-56% reduction in lag time upon addition of 40 to 60µg/mL of DNA or RNA.

Compared to control, peak thrombin concentration is increased with statistical significance ($p<0.05$) upon addition of 40 or 60µg/mL of DNA or 20 to 60µg/mL of RNA. Peak thrombin concentration was increased from 75.0 ± 9.1 nM to 87.1 ± 5.1 and 113.3 ± 7.3 nM upon addition of 40 and 60µg/mL of DNA, respectively (Table 1A). The addition of 20, 40 or 60µg/mL RNA resulted in increased peak thrombin concentration to 92.2 ± 13.2, 120.8 ± 4.7, and 124.3 ± 7.7 nM, respectively (Table 1B). This represents a 16-51% increase in peak thrombin concentration upon addition of 40 to 60µg/mL DNA and 23-66% increase upon addition of 20 to 60µg/mL RNA.

In addition to lag time and peak thrombin concentration, time to peak thrombin was found to be reduced and the endogenous thrombin potential (ETP) was found to be augmented with increasing concentrations of nucleic acids (Table 1).

In this study, DNA and RNA increased thrombin generation in a dose dependent manner. At equal concentrations, the procoagulant effect of RNA was sometimes found to be slightly more pronounced compared with DNA in normal plasma. This is particularly apparent when analyzing peak thrombin concentration ($p<0.05$) in the thrombin generation assay (Figure 7). Both DNA and RNA modified all of the parameters of the thrombin generation assay with statistical significance ($p<0.05$) starting at 40µg/mL. This concentration of nucleic acids was used for additional studies in factor-deficient plasmas.

A

	Control	5µg/mL	10µg/mL	20µg/mL	40µg/mL	60µg/mL
Lag Time (min)	26.4 ± 6.1	28.8 ± 1.9	24.8 ± 3.0	25.5 ± 3.2	17.5 ± 1.9*	12.7 ± 1.6*
Peak Thrombin (nM)	75.0 ± 9.1	67.1 ± 3.3*	70.3 ± 3.9	69.4 ± 2.9	87.1 ± 5.1*	113.3 ± 7.3*
Time to Peak Thrombin (min)	42.3 ± 6.6	46.8 ± 2.6*	42.3 ± 2.7	43.0 ± 5.1	29.5 ± 2.7*	20.0 ± 2.5*
Velocity Index (nM/min)	5.1 ± 1.9	3.8 ± 0.5*	4.1 ± 0.6	4.1 ± 0.7	7.4 ± 1.3*	16.0 ± 3.6*
ETP (nM•min)	2932.7 ± 87.0	2921.8 ± 48.6	2974.5 ± 40.8	2999.2 ± 35.8	3103.2 ± 45.0*	3159.8 ± 93.0*

B

	Control	5µg/mL	10µg/mL	20µg/mL	40µg/mL	60µg/mL
Lag Time (min)	26.4 ± 6.1	23.8 ± 5.2	25.3 ± 4.2	20.3 ± 2.7	14.0 ± 2.0*	11.7 ± 1.4*
Peak Thrombin (nM)	75.0 ± 9.1	77.6 ± 4.1	81.2 ± 8.2	92.2 ± 13.2*	120.8 ± 4.7*	124.3 ± 7.7*
Time to Peak Thrombin (min)	42.3 ± 6.6	39.7 ± 5.6	39.3 ± 6.1	30.2 ± 3.2*	20.2 ± 2.9*	17.8 ± 1.9*
Velocity Index (nM/min)	5.1 ± 1.9	5.0 ± 0.9	6.0 ± 1.6	9.6 ± 2.2*	20.2 ± 4.2*	20.7 ± 3.9*
ETP (nM•min)	2932.7 ± 87.0	3035.0 ± 55.9*	3008.3 ± 92.2	3023.7 ± 215.7*	3194.2 ± 33.4*	3198.5 ± 66.8*

Table 1: Effects of Increasing Concentrations of Nucleic Acids on Thrombin Generation.

Normal plasma was incubated with 0 to 60µg/mL of (**A**) A549 DNA or (**B**) RNA before thrombin generation was initiated with the addition of 40mM $CaCl_2$ and 2mM Z-Gly-Gly-Arg-AMC. Results are summarized by the various parameters of the thrombin generation assay: Lag time, peak thrombin, time to peak thrombin, velocity index, and endogenous thrombin potential (ETP). Data are presented as the mean ± standard deviation of n = 3 in duplicate. *$p<0.05$ for nucleic acid-stimulated conditions compared with control as tested by the Mann-Whitney rank sum test.

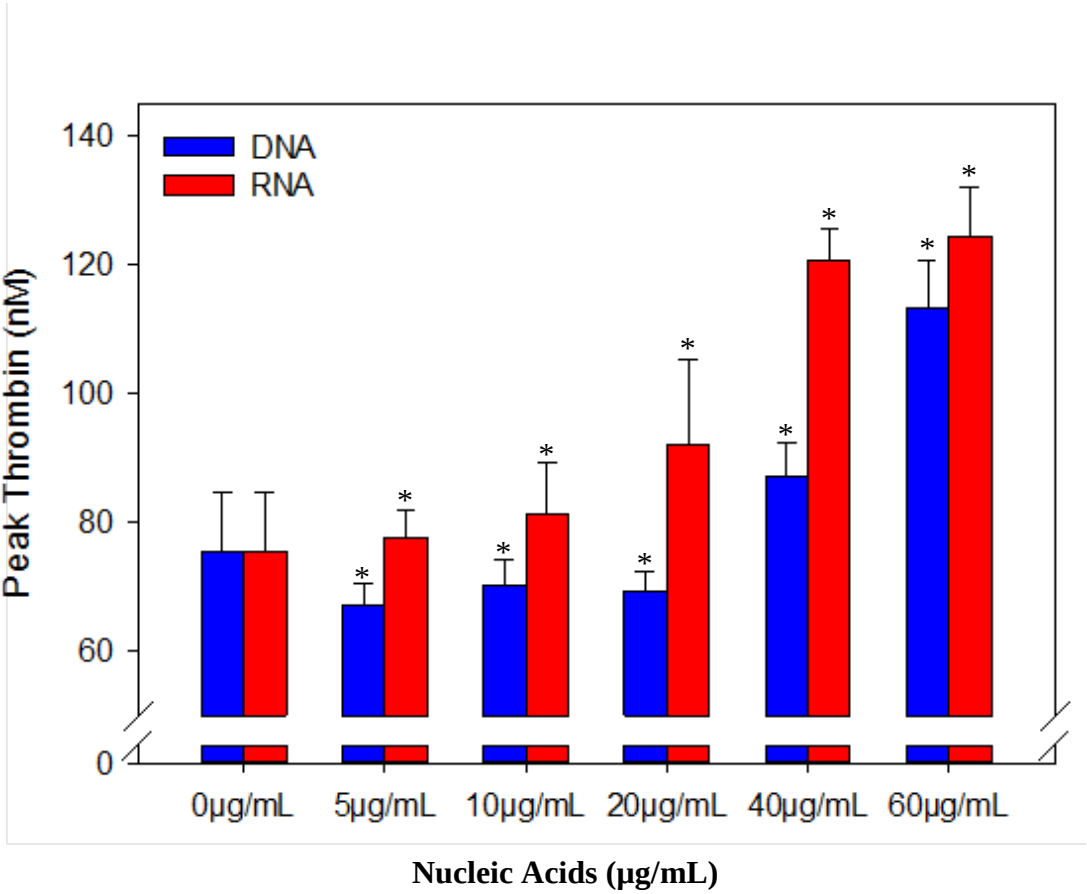


Figure 7: Comparing DNA vs RNA's Effect on Peak Thrombin Concentration. Normal plasma was incubated with 0 to 60µg/mL of A549 DNA or RNA before thrombin generation was initiated with the addition of 40mM $CaCl_2$ and 2mM Z-Gly-Gly-Arg-AMC. Peak thrombin concentration is presented as mean ± standard deviation of n = 3 in duplicate. *$p<0.05$ between DNA and RNA at equal concentrations from 5 to 60µg/mL as tested by the Mann-Whitney rank sum test.

3.1.2 Deficient Plasmas Stimulated By Nucleic Acids

It has been previously reported that DNA and RNA promote thrombin generation through the intrinsic pathway of coagulation (Kannemeier *et al.*, 2007, Vu *et al.*, 2016). We set out to confirm this using a thrombin generation assay. We tested the addition of 40µg/mL of A549 DNA or RNA in platelet poor normal, FVII-deficient (FVII-def), FXII-deficient (FXII-def), FXI-deficient (FXI-def), or PK-depleted (PK-dep) plasma for their effect on thrombin generation.

As expected, the thrombin generation profile of normal plasma revealed enhanced thrombin generation upon addition of 40µg/ml DNA or RNA (Figure 8A). We next incubated nucleic acids in FVII-def plasma to study the procoagulant effect of nucleic acids that operates exclusively through the intrinsic pathway of coagulation. The addition of DNA or RNA to FVII-def plasma resulted in similar shifts of the curve indicating enhanced thrombin generation (Figure 8B). There was a 46% reduction in lag time from 33.4 ± 10.1 min to 18.0 ± 3.7 min upon addition of DNA and a 37% reduction to 21.0 ± 3.5 min upon addition of RNA (Table 2). This is comparable to the reduction in lag time in normal plasma upon addition of nucleic acids.

The addition of nucleic acids enhanced thrombin generation in a statistically significant manner in both normal and FVII-def plasma (Table 2). Comparison of thrombin generation between FVII-def plasma and normal plasma in the absence of nucleic acids revealed that differences in the lag times and peak thrombin concentrations were not statistically significant (Table 3). This indicates that normal plasma is

generating thrombin through the intrinsic pathway similar to FVII-def plasma. Enhancement of thrombin generation by nucleic acids in FVII-def plasma confirms that this assay operates through the intrinsic pathway. Additional studies targeting the components of intrinsic pathway can confirm that nucleic acids promote thrombin generation through the intrinsic pathway of coagulation.

Without the addition of nucleic acids, plasma devoid of FXII or FXI has thrombin generation profiles that are shifted to the right with reduced peaks and ETP compared with FVII-def or normal plasma (Figure 8F). This indicates that FXII-def or FXI-def plasma's capacity to generate thrombin is compromised compared with FVII-def or normal plasma. This highlights the importance of FXII and FXI and consequentially confirms that thrombin is being generated through the intrinsic pathway of coagulation upon stimulation by nucleic acids.

Thrombin generation in FXII-def or FXI-def plasma indicated enhanced thrombin generation upon addition of DNA or RNA. The leftward shift of the curve in the presence of DNA or RNA indicated a reduction in lag time and the increased peaks of the curve indicated enhanced peak thrombin concentration (Figure 8C,D).

Quantitative analysis revealed that the addition of DNA to FXII-def plasma resulted in a 37% reduction in lag time from 48.6 ± 12.4 min to 30.5 ± 4.7 min, and a 15% reduction to 41.5 ± 3.8 min upon addition of RNA. In FXI-def plasma, addition of DNA resulted in a 25% reduction in lag time from 38.9 ± 2.9 min to 29.3 ± 3.5 min upon addition of DNA and 21% to 30.8 ± 1.8 min upon addition of RNA (Table 2).

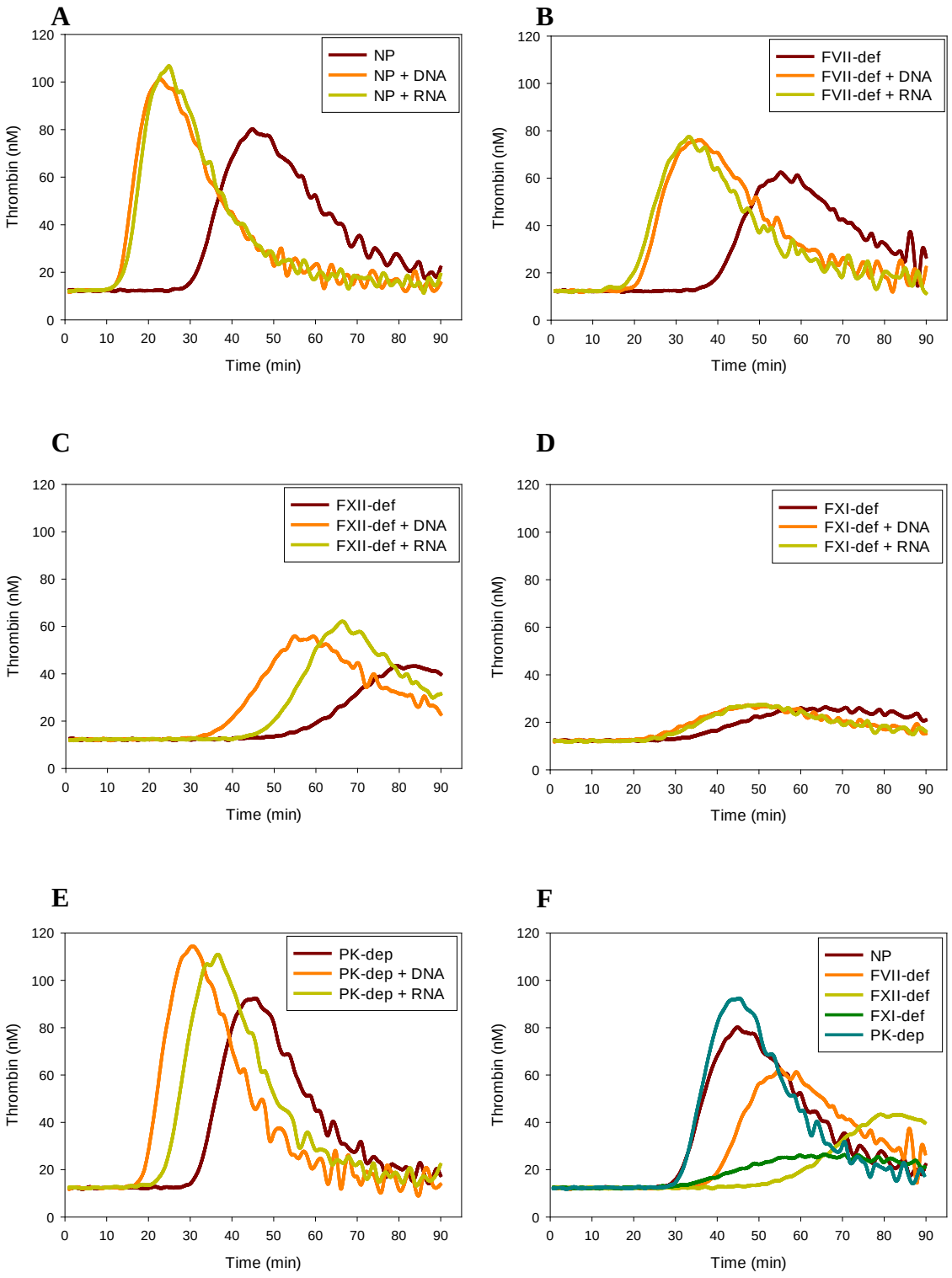

A
NP
NP + DNA
NP + RNA
B
FVII-def
FVII-def + DNA
FVII-def + RNA
C
FXII-def
FXII-def + DNA
FXII-def + RNA
D
FXI-def
FXI-def + DNA
FXI-def + RNA
E
PK-dep
PK-dep + DNA
PK-dep + RNA
F
NP
FVII-def
FXII-def
FXI-def
PK-dep
Thrombin (nM)
Time (min)

Figure 8: The Effect of DNA or RNA on Thrombin Generation in Factor-Deficient Plasmas.

Plasmas were incubated with 40µg/mL of A549 DNA or RNA before thrombin generation was initiated with the addition of 40mM $CaCl_2$ and 2mM Z-Gly-Gly-Arg-AMC. Data are displayed by representative thrombin generation profiles of (**A**) NP, (**B**) FVII-def, (**C**) FXII-def, (**D**) FXI-def, and (**E**) PK-dep plasma. These plasmas were also compared head to head without the addition of nucleic acids (**F**).

	Lag Time (min)	Peak Thrombin (nM)	Time to Peak Thrombin (min)	Velocity Index (nM/min)	ETP (nM•min)
NP	28.3 ± 5.9	72.2 ± 15.6	44.0 ± 8.9	5.3 ± 2.6	2902.4 ± 218.0
NP + DNA	13.3 ± 3.3*	107.6 ± 13.6*	21.3 ± 5.2*	14.6 ± 5.2*	3253.6 ± 64.7*
NP + RNA	16.1 ± 1.1*	108.7 ± 9.0*	25.0 ± 2.4*	12.9 ± 3.6*	3249.0 ± 56.9*
FVII-def	33.4 ± 10.1	66.1 ± 3.6	48.1 ± 9.9	4.5 ± 0.6	2753.8 ± 156.0
FVII-def + DNA	18.0 ± 3.7*	85.1 ± 6.3*	28.3 ± 5.3*	8.7 ± 2.3*	3178.3 ± 110.1*
FVII-def + RNA	21.0 ± 3.5*	78.3 ± 7.7*	33.3 ± 3.5*	6.4 ± 0.9*	2891.1 ± 220.7*
FXII-def	48.6 ± 12.4	49.0 ± 3.8	79.5 ± 11.8	1.7 ± 0.3	1977.4 ± 337.8
FXII-def + DNA	30.5 ± 4.7*	56.1 ± 4.7*	53.6 ± 5.2*	2.5 ± 0.5*	2535.4 ± 171.6*
FXII-def + RNA	41.5 ± 3.8	62.4 ± 3.5*	60.1 ± 5.4*	3.4 ± 0.2*	2448.5 ± 100.8*
FXI-def	38.9 ± 2.9	25.9 ± 1.1	68.3 ± 4.5	0.9 ± 0.1	1647.9 ± 66.0
FXI-def + DNA	29.3 ± 3.5*	29.5 ± 4.7*	49.0 ± 5.4*	1.5 ± 0.3*	1789.3 ± 164.7*
FXI-def + RNA	30.8 ± 1.8*	27.1 ± 0.7*	48.0 ± 2.5*	1.6 ± 0.1*	1659.1 ± 29.5
PK-dep	28.6 ± 2.8	96.5 ± 3.5	41.8 ± 3.2	7.4 ± 0.5	3162.5 ± 63.9
PK-dep + DNA	18.8 ± 3.4*	118.1 ± 12.3*	29.0 ± 4.9*	11.9 ± 2.8*	3375.0 ± 58.4*
PK-dep + RNA	20.9 ± 2.0*	112.8 ± 3.6*	32.6 ± 2.7*	9.7 ± 1.0*	3319.4 ± 33.0*

Table 2: Effects of Nucleic Acids on Thrombin Generation Parameters in Normal and Factor-Deficient Plasmas.

Plasma was incubated with 40µg/mL of A549 DNA or RNA before thrombin generation was initiated with the addition of 40mM $CaCl_2$ and 2mM Z-Gly-Gly-Arg-AMC as shown in Figure 8. Results of normal plasma (NP), FVII-def, FXII-def, FXI-def, and PK-dep plasma are summarized by the various parameters of the thrombin generation assay: Lag time, peak thrombin, time to peak thrombin, velocity index, and endogenous thrombin potential (ETP). Data are presented as the mean ± standard deviation of n = 4 in duplicate. *$p<0.05$ for nucleic acid-stimulated conditions compared with plasma without nucleic acids, as tested by the Mann-Whitney rank sum test.

	Lag Time (min)	Peak Thrombin (nM)	Time to Peak Thrombin (min)	Velocity Index (nM/min)	ETP (nM•min)
NP	28.3 ± 5.9	72.2 ± 15.6	44.0 ± 8.9	5.3 ± 2.6	2902.4 ± 218.0
FVII-def	33.4 ± 10.1	66.1 ± 3.6	48.1 ± 9.9	4.5 ± 0.6	2753.8 ± 156.0*
FXII-def	48.6 ± 12.4*#	49.0 ± 3.8*#	79.5 ± 11.8*#	1.7 ± 0.3*#	1977.4 ± 337.8*#
FXI-def	38.9 ± 2.9*	25.9 ± 1.1*#	68.3 ± 4.5*#	0.9 ± 0.1*#	1647.9 ± 66.0*#
PK-dep	28.6 ± 2.8	96.5 ± 3.5*#	41.8 ± 3.2	7.4 ± 0.5#	3162.5 ± 63.9*#

Table 3: Thrombin Generation in Factor-Deficient Plasmas in the Absence of Nucleic Acids.

Thrombin generation of platelet poor plasma was initiated with the addition of 40mM $CaCl_2$ and 2mM Z-Gly-Gly-Arg-AMC. Results of normal plasma (NP), FVII-def, FXII-def, FXI-def, and PK-dep plasma are summarized by the various parameters of the thrombin generation assay: Lag time, peak thrombin concentration, time to peak thrombin, velocity index, and endogenous thrombin potential (ETP). Data are presented as the mean ± standard deviation of n = 4 in duplicate. *$p<0.05$ for deficient plasmas compared with NP and #$p<0.05$ for deficient plasmas compared with FVII-def platelet poor plasma as tested by the Mann-Whitney rank sum test.

This is interesting because it is believed that nucleic acids generate thrombin through the intrinsic pathway yet nucleic acids are still found to be procoagulant in FXII-def or FXI-def plasma. However, it should be noted that compared with normal plasma, the lag time was much higher and the peak thrombin concentration was much lower in FXII-def or FXI-def plasma upon addition of DNA or RNA (Table 2). This suggests that although nucleic acids promote thrombin generation in FXII-def or FXI-def plasma, the procoagulant effect is not as pronounced compared to normal platelet poor plasma. The limited procoagulant effect in FXII-def plasma upon addition of nucleic acids could be due to the presence of minute amounts of FXII and/or the possible ability of nucleic acids to activate proteins downstream of FXII such as FXI. In FXI-def plasma it is harder to explain the limited procoagulant effect of nucleic acids as FXI serves as a key protein that activates downstream proteins and can be activated by thrombin (Gailani and Broze, Jr., 1991). Minute amounts of available FXI and the ability of nucleic acids to enhance thrombin activation of FXI can be thought to be responsible for the limited procoagulant effect of nucleic acids in FXI-def plasma (Vu *et al.*, 2016). This once again confirms that nucleic acids promote thrombin generation primarily through the intrinsic pathway.

In contrast to the role of FXII or FXI, studies conducted with PK-dep plasma provided vastly different results regarding the role of PK in mediating thrombin generation through the intrinsic pathway. The thrombin generation profile of PK-dep plasma overlapped the profile of normal plasma but had higher peak (Figure 8F). This indicates a similar lag time and an increased peak thrombin concentration compared with normal plasma. Plasmas devoid of FXII or FXI had greatly reduced thrombin generation,

whereas depletion of PK did not reduce thrombin generation compared with normal plasma. This suggests that depletion of PK alone does not diminish the plasma's capacity to generate thrombin through the intrinsic pathway.

Thrombin generation profile of PK-dep plasma indicated enhanced thrombin generation upon addition of DNA or RNA. The leftward shift of the curve upon addition of DNA or RNA indicated a reduction in lag time and the increased peaks of the curve indicated enhanced peak thrombin concentration (Figure 8E).

Quantitative analysis revealed that the addition of DNA to PK-dep plasma resulted in a 34% reduction in lag time from 28.6 ± 2.8 min to 18.8 ± 3.4 min upon addition of DNA, and a 27% reduction to 20.9 ± 2.0 min upon addition of RNA (Table 2). The reductions in lag time in PK-dep plasma are comparable to normal plasma as there was a 53% reduction in lag time from 28.3 ± 5.9 min to 13.3 ± 5.9 min upon addition of DNA, and a 43% reduction to 16.1 ± 1.1 min upon addition of RNA. The increase in thrombin generation upon addition of nucleic acids in PK-dep plasma suggests that PK-dep plasma still contains components necessary to generate thrombin comparable with normal plasma.

These thrombin generation studies revealed that lack of FVII, a key extrinsic pathway factor, does not supress nucleic acid-mediated thrombin generation compared to normal plasma and that nucleic acids mediate thrombin generation through the intrinsic pathway. Studies conducted with FXII-def or FXI-def plasma revealed reduced thrombin generation compared to normal plasma (Figure 8F), thus highlighting the importance of

FXII and FXI in the intrinsic pathway for nucleic acid-mediated thrombin generation. Activation of specific reactions of the contact system in a purified system will reveal more information regarding the capacity of nucleic acids to activate the contact system.

3.2 FXIIa Generation Assay

3.2.1 Activation of FXII Stimulated by Nucleic Acids

Nucleic acids enhanced thrombin generation in platelet poor plasma. To dissect the mechanisms responsible for this activity, studies were performed in a purified system using various components of the contact system. First, we examined whether nucleic acids activate FXII, the initiator of the contact system. Studies were performed in the presence of 15nM PK to permit reciprocal activation. The experiment was conducted by incubating 150nM FXII with increasing concentrations of nucleic acids from 0 to 60µg/mL for 60 minutes at 37°C in the absence or presence of 15nM HK. After addition of STI to inhibit generated kallikrein, FXIIa-directed substrate S2302 was then added and its hydrolysis was monitored as an index of FXIIa generation.

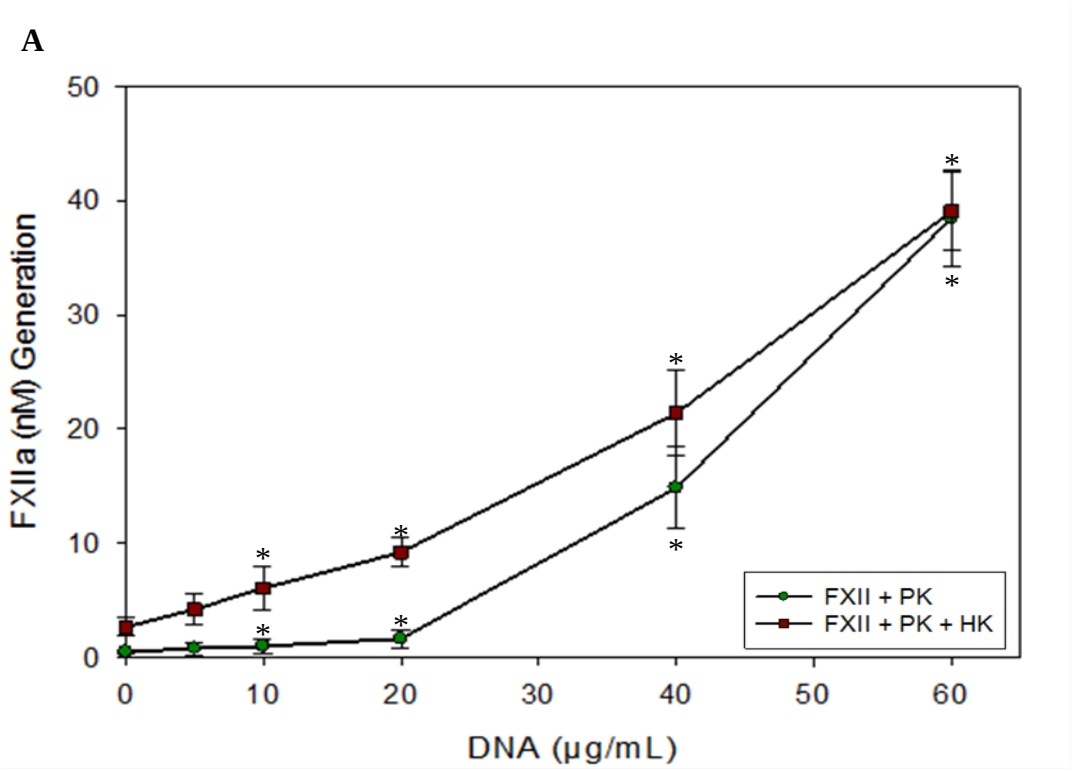
Nucleic acid dose response for FXII activation was conducted to assess if it is similar to the dose response found in normal plasma. PK-mediated activation of FXII was augmented with increasing concentrations of nucleic acids. PK-mediated activation of FXII was augmented by DNA with statistical significance ($p<0.01$) starting at 10µg/mL (Figure 9A). This resulted in 1.0 ± 0.6 nM FXIIa generation, a 2.1-fold increase over background. RNA augmented PK-mediated activation of FXII with statistical significance ($p<0.01$) starting at 40µg/mL (Figure 9B). This resulted in 24.7 ± 4.3 nM

FXIIa generation, a 14.5-fold increase over background. Linear regression analysis demonstrated a positive correlation between PK-mediated FXII activation and increasing concentration of DNA ($r^2 = 0.87$) or RNA ($r^2 = 0.78$). These results suggest an important role of PK in mediating FXII activation stimulated by nucleic acids and that at least 10µg/mL of DNA or 40µg/mL of RNA are required to achieve efficient activation of FXII in the presence of PK.

An important cofactor that plays the role of an assembly platform for the contact system is HK. Its ability to assemble the contact system and bind anionic surfaces for contact activation makes it an important cofactor to include in these studies (Wu, 2015). Since HK alone doesn't result in the activation of FXII, HK was added in addition to PK in the activation of FXII. Addition of HK to PK-mediated activation of FXII in the absence of polyanions resulted in 3.2-fold increase in FXIIa generation. Activation of FXII in the presence of PK and HK was augmented with increasing concentrations of nucleic acids.

DNA augmented PK/HK-mediated activation of FXII with statistical significance ($p<0.01$) starting at 10µg/mL (Figure 9A). This resulted in 6.1 ± 1.9 nM FXIIa generation, a 2.3-fold increase over background. RNA augmented PK/HK-mediated activation of FXII with statistical significance ($p<0.01$) starting at 5µg/mL (Figure 9B). This resulted in 8.6 ± 2.6 nM FXIIa generation, a 2.0-fold increase over background. Linear regression analysis demonstrated a positive correlation between PK/HK-mediated FXII activation and increasing concentration of DNA ($r^2 = 0.94$) or RNA ($r^2 = 0.97$).

A


FXIIa (nM) Generation
DNA (µg/mL)
FXII + PK
FXII + PK + HK

B

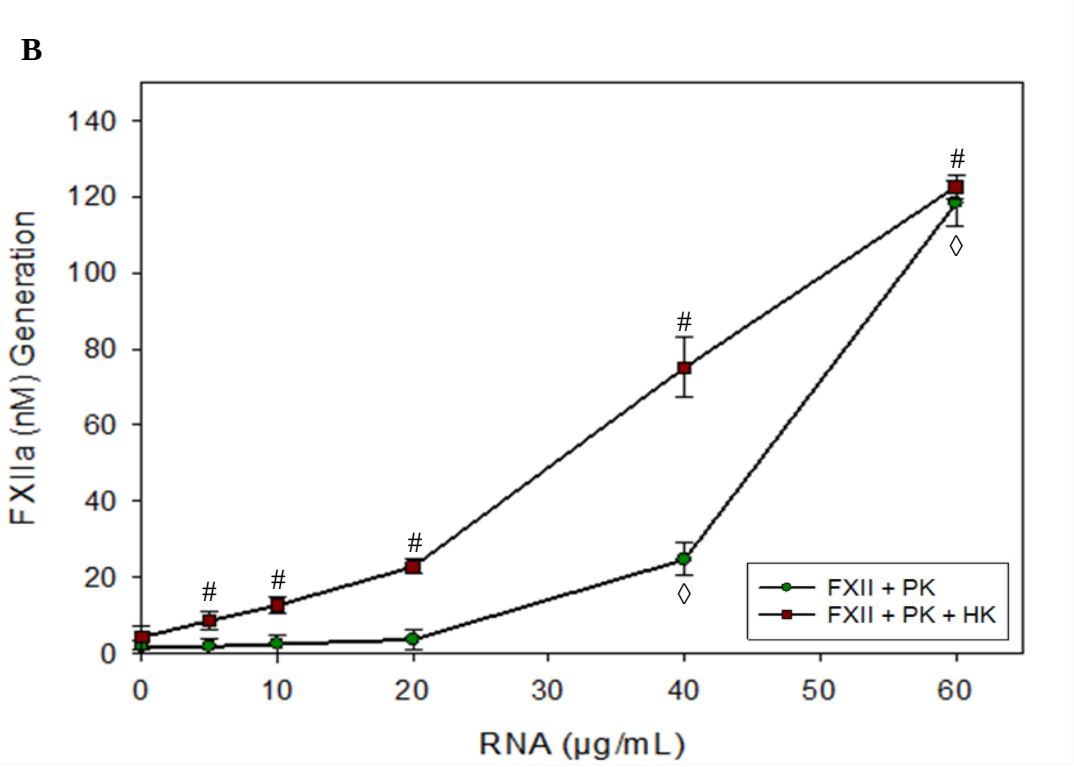

FXIIa (nM) Generation
RNA (µg/mL)
FXII + PK
FXII + PK + HK

Figure 9: Effect of Nucleic Acids on FXIIa Generation.

FXII activation was assessed by evaluating FXIIa generation in the presence of PK or both PK and HK. Solutions containing150nM FXII and 15nM PK, without or with 15nM HK were incubated with increasing concentrations of (**A**) A549 DNA or (**B**) RNA up to 60µg/mL. Kallikrein activity was inhibited with STI before FXIIa generation was measured by the hydrolysis of FXIIa-directed S2302 chromogenic substrate and determined using the FXIIa standard curve. Data are presented as mean ± standard deviation of n = 3 in duplicate. *$p<0.01$, compared with control for FXII activation stimulated by DNA and mediated by PK or both PK and HK. ◊$p<0.01$, compared with control for PK-mediated activation of FXII that is stimulated by RNA. #$p<0.01$ compared with control for PK- and HK-mediated activation of FXII that is stimulated by RNA. p-values determined by the Mann-Whitney rank sum test.

The required concentration for RNA to augment PK-mediated activation of FXII with statistical significance is 5µg/mL in the presence of HK compared with 40µg/mL in the absence of HK. The lower concentration of RNA highlights the importance of HK in PK-mediated activation of FXII. Linear regression analysis also revealed that PK/HK-mediated activation of FXII stimulated by nucleic acids was statistically better than the activation without HK based on the higher y-intercept ($p<0.001$) confirming the importance of HK. This experiment showed that increasing concentrations of nucleic acids increased FXII activation in agreement with the procoagulant effect seen with increasing concentrations of nucleic acids in plasma. This also showed that nucleic acid concentration of 40µg/mL is efficient for FXII activation in the two reactions described above and it will be used in further analyses of FXII activation.

Activation of FXII was further analyzed with 40µg/mL of DNA or RNA. Experiments were conducted with various combinations of PK and HK to determine the contents of the reaction that was most efficient for FXII activation. FXII activation was conducted in the absence or presence of PK and/or HK with DNA or RNA in a similar manner as described above. Dextran sulfate, a well-established non-physiological activator of FXII, was used as a positive control (Wu, 2015).

Without polyanion, 0.06 ± 0.01 nM FXIIa was generated in the presence of HK, 0.4 ± 0.2 nM FXIIa was generated in the presence of PK, and 1.9 ± 1.4 nM was generated in presence of both PK and HK (Figure 10). This represents a 4.5- and 20.9-fold increase

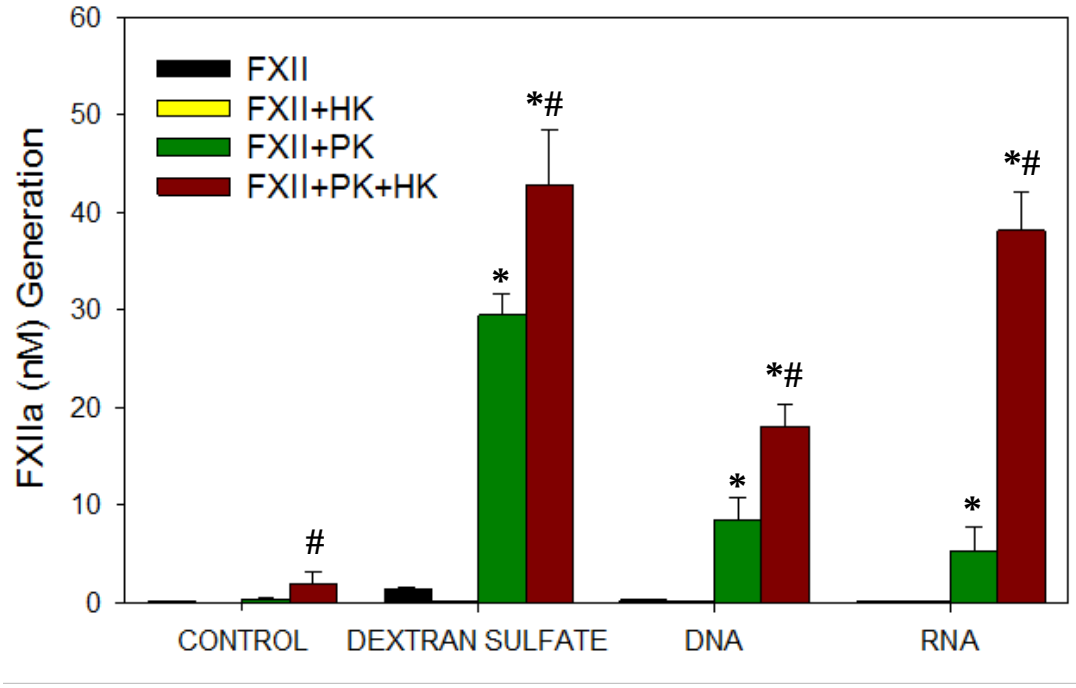


Figure 10: FXII Activation Stimulated By Nucleic Acids.

FXII activation was assessed by evaluating FXIIa generation in the absence and presence of PK, HK, and polyanion. FXII (150nM), 15nM PK, and 15nM HK were incubated in buffer containing 0.05µg/mL dextran sulfate, 40µg/mL A549 DNA, or 40µg/mL A549 RNA. FXIIa generation was measured by the hydrolysis of FXIIa-directed S2302 chromogenic substrate and determined using the FXIIa standard curve. Data are presented as mean ± standard deviation of n = 3 in duplicate. #$p<0.01$ between FXII+PK and FXII+PK+HK within each polyanion as tested by the Mann-Whitney rank sum test. *$p<0.01$ between buffer and polyanion for each respective condition as tested by the Mann-Whitney rank sum test.

in FXIIa generation over background in the presence of PK and both HK and PK, respectively. Although addition of HK alone to FXII does not result in increased FXIIa generation, addition of HK to PK-mediated activation of FXII augments FXIIa generation by 4.6-fold (Figure 10).

With addition of 40µg/mL DNA, 0.3 ± 0.1 nM FXIIa was generated in the absence of HK and PK. In the presence of HK or PK, 0.2 ± 0.1 nM and 8.6 ± 2.2 nM FXIIa were generated, respectively, while in the presence of both HK and PK, 18.1 ± 2.2 nM FXIIa was generated. Thus, HK on its own has no effect whereas PK increased FXIIa generation 28.8-fold over background. In contrast, with both PK and HK, FXIIa generation is increased 61.1-fold; a 2.1-fold increase over that with PK alone (Figure 10).

With addition of 40µg/mL RNA, 0.1 ± 0.03 nM FXIIa was generated. Addition of HK resulted in 0.1 ± 0.04 nM FXIIa generation, whereas PK addition resulted in 5.2 ± 2.5 nM FXIIa generation and addition of both HK and PK resulted in 38.1 ± 4.0 nM FXIIa generation. Therefore, HK alone has no effect on FXIIa generation, whereas PK alone enhances FXIIa generation by 50.7-fold. With both HK and PK, FXIIa generation is increased 369.0-fold over background; a 7.3-fold increase over that with PK alone (Figure 10).

Dextran sulfate was used as a positive control in this experiment. Based on a dose response of dextran sulfate for FXII activation, 0.05µg/mL was chosen as an efficient concentration to serve as a control. Addition of dextran sulfate resulted in the generation of 1.4 ± 0.2 nM FXIIa in the absence of HK and PK. Addition of HK had no effect on

FXIIa generation, whereas addition of PK resulted in 29.5 ± 2.2 nM FXIIa generation. With addition of both HK and PK, 42.9 ± 5.7 nM FXIIa was generated. This represents a 20.9-fold increase in FXIIa generation over background with PK addition and a 30.5-fold increase in FXIIa generation with addition of PK and HK; a 1.5-fold increase over that with PK alone (Figure 10).

The capacity of nucleic acids to augment FXII activation was demonstrated by analyzing the results of PK-mediated activation of FXII in the absence or presence of HK. The addition of DNA resulted in a 21.0-fold increase in PK-mediated activation of FXII and a 9.7-fold increase in the presence of both PK and HK. The addition of RNA enhanced PK-mediated activation of FXII by 12.9-fold and by 20.3-fold in the presence of both PK and HK. Dextran sulfate, which was used as a positive control, increased PK-mediated activation of FXII by 72.4-fold and by 22.8-fold in the presence of HK. Therefore, in the presence of PK and HK, DNA or RNA increased FXII activation over background by 201.4- and 423.8-fold, respectively. Based on the comparison of fold-increases, it was found that RNA stimulated FXII activation better than DNA ($p<0.01$) when in the presence of both PK and HK.

Taking these results together, PK augments FXII activation by 4.5-fold in the absence of polyanions and by 28.8-, 50.7-, and 20.9-fold in the presence of DNA, RNA, and dextran sulfate, respectively. These findings identify PK as an important stimulator of FXII activation. Although HK has little effect on its own, when added with PK, it further increases FXII activation by 4.6-fold in the absence of polyanions and by 2.1-,

7.3-, and 1.5-fold in the presence of DNA, RNA, and dextran sulfate, respectively. Moreover, the addition of both PK and HK augmented the activation of FXII by 20.9-fold in the absence of polyanions and by 61.1-, 369.0-, and 30.5-fold in the presence of DNA, RNA, and dextran sulfate, respectively. The significant increase in FXII activation upon addition of PK and HK, across the various treatments, demonstrated their importance in FXII activation.

These results showed that nucleic acids did not promote efficient autoactivation of FXII even in the presence of HK. Since the addition of PK allowed FXII activation to occur, it is thought that minimal autoactivation due to the presence of nucleic acids activated PK to kallikrein which reciprocally activated FXII to generate FXIIa. The role of HK, as previously established in literature, is thought to augment activation of FXII by providing an assembly platform for the nucleic acids and the contact system proteins (Wu, 2015). However it should be noted that, although PK/HK-mediated activation of FXII resulted in the highest amount of FXIIa generation upon stimulation by polyanions, polyanions were able to stimulate activation of FXII in the absence of HK as well. This may suggest that although HK increases PK-mediated activation of FXII, it is not essential for this reaction.

3.2.2 Effect of Zinc on FXII Activation Stimulated by Nucleic Acids

It has been established that zinc serves as a cofactor for the activation of the contact system on non-physiological surfaces (Vu et al., 2013). This raises interesting questions about the role of zinc in FXII activation stimulated by nucleic acids, PK, and

HK. It has been previously established in literature that Zn^{2+} binds to FXII and results in a conformational change which results in enhanced FXII activation (Bernardo *et al.*, 1993b, Bernardo *et al.*, 1993a, Rojkjaer and Schousboe, 1997, Shore *et al.*, 1987, Vu *et al.*, 2013). The effect of Zn^{2+} on nucleic acid-stimulated contact system can be assessed by evaluating FXIIa generation.

In this experiment, 150nM FXII was incubated with 15nM PK, 15nM HK, in presence or absence of 5µg/mL A549 DNA or RNA, and in presence or absence of 10µM Zn^{2+} for 5 minutes at 37°C. Zinc concentration was chosen based on ou previously conducted Zn^{2+} dose response with FXII activation and physiological levels published in literature (Vu *et al.*, 2013). This was followed by an additional incubation period of 5 minutes with STI at 37°C to inhibit kallikrein activity. Finally, S2302 was added to measure FXIIa generation as determined by the FXIIa standard curve.

Without polyanions, 0.2 ± 0.03 nM FXIIa was generated in the presence of PK and HK. However, the addition of Zn^{2+} generated 1.0 ± 0.2 nM FXIIa, a 4.5-fold statistically significant increase ($p<0.01$) (Figure 11). It should be noted that zinc-mediated effects on FXII activation were only observed in the presence of PK and HK.

In the presence of DNA or RNA, PK/HK-mediated activation resulted in 0.3 ± 0.03 nM or 0.3 ± 0.04 nM FXIIa generation, respectively. The addition of Zn^{2+} augmented FXII activation in a statistically significant ($p<0.01$) manner to generate 15.0 ± 3.5 nM FXIIa in the presence of DNA or 18.0 ± 4.0 nM FXIIa in the presence of RNA,

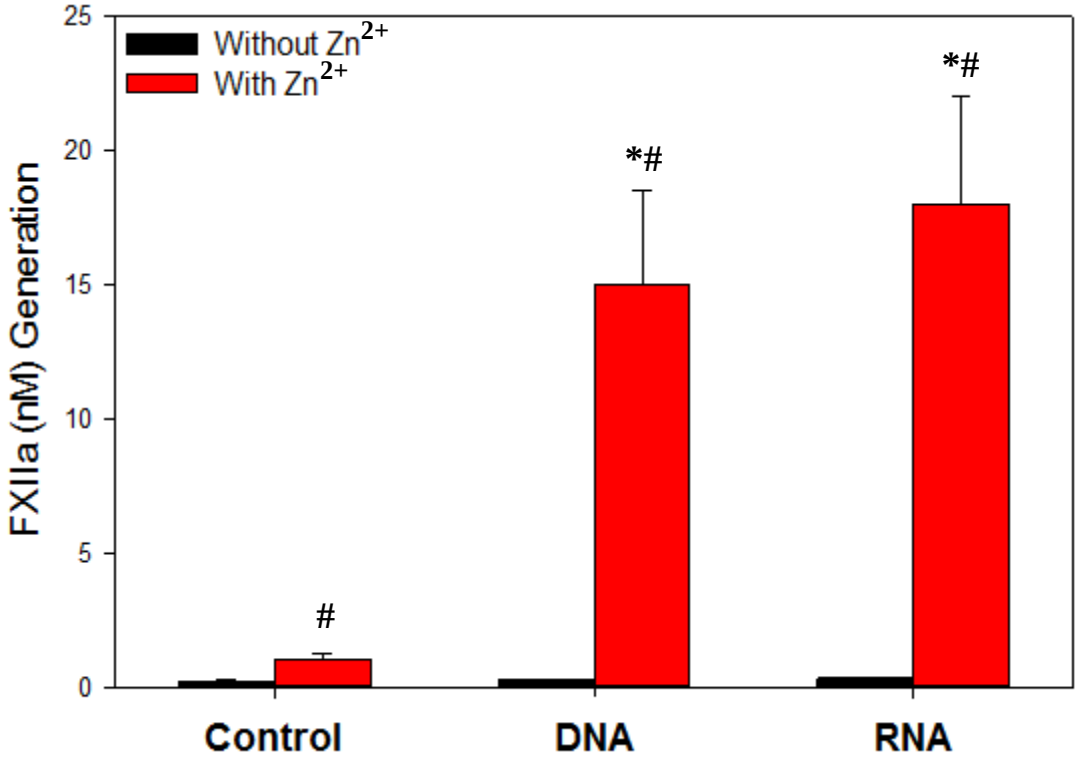


Figure 11: The Effect of Zn^{2+} on Nucleic Acid-Mediated FXII Activation.

FXIIa generation was evaluated by incubating 150nM FXII with 15nM PK and 15nM HK in buffer, 5µg/mL A549 DNA, or 5µg/mL A549 RNA in the absence or presence of 10 µM Zn^{2+}. FXIIa generation was measured by the hydrolysis of FXIIa-directed S2302 substrate and determined using the FXIIa standard curve. Data are presented as mean ± standard deviation of n = 3 in duplicate. #$p<0.01$ compared with reaction in the absence of Zn^{2+} for each condition as tested by the Mann-Whitney rank sum test. *$p<0.01$ compared with control in the presence of Zn^{2+} as tested by the Mann-Whitney rank sum test.

representing 52.5- and 53.8-fold increases, respectively (Figure 11). Addition of DNA or RNA to PK/HK-mediated activation of FXII in the presence of Zn^{2+} resulted in 14.3- and 17.1-fold increases, respectively. These results showed that addition of Zn^{2+} significantly increased PK/HK-mediated activation of FXII in the absence of polyanions, but at the same time the presence of polyanions is also important as it leads to significantly ($p<0.01$) increased FXII activation. The presence of both nucleic acids and Zn^{2+} resulted in increased activation of FXII and this effect should be further analyzed in other reactions of the contact system.

3.3 Kallikrein Generation Assay

3.3.1 Prekallikrein Activation Stimulated By Nucleic Acids

Another important reaction of the contact system is the activation of PK to kallikrein so that it can activate FXII which subsequently stimulates the intrinsic pathway (Wu, 2015). First, we studied the activation of PK by FXIIa in the absence or presence of HK with a dose response of nucleic acids from 0 to 60µg/mL to establish suitable conditions. The experiment was conducted by incubating 5nM PK with increasing concentrations of nucleic acids in the absence or presence of 5nM HK for 5 minutes at 37°C before incubation with 0.2nM FXIIa for 1 minute at 37°C. After addition of CTI to inhibit FXIIa, kallikrein-directed substrate S2302 was added to measure kallikrein generation as determined by the standard curve of kallikrein.

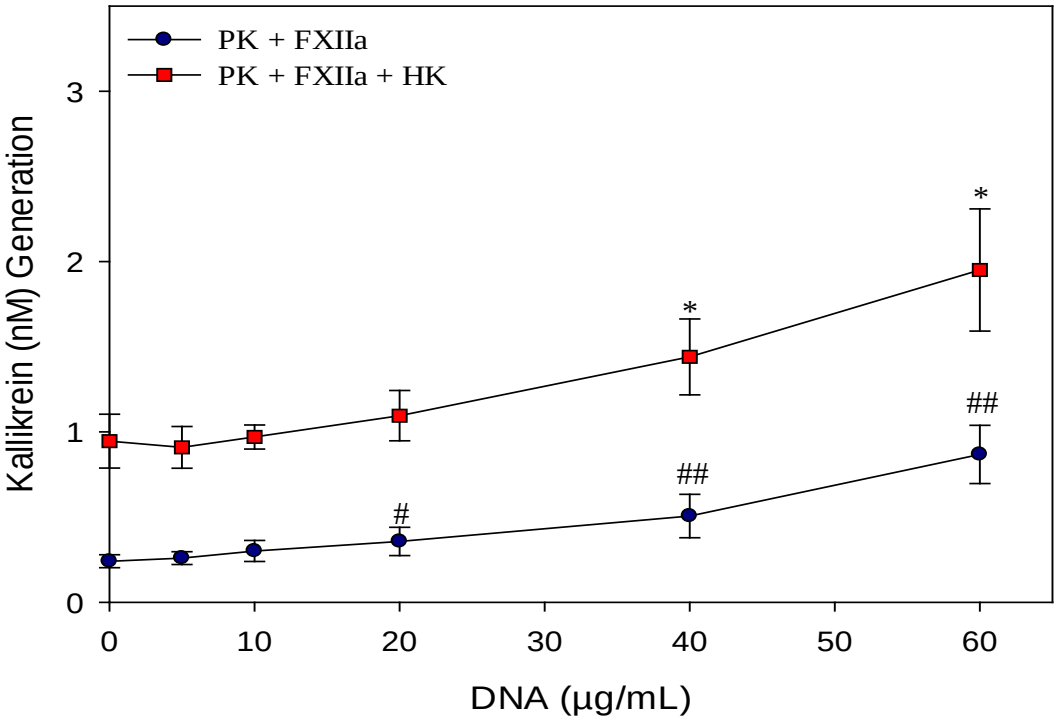
FXIIa-mediated activation of PK is augmented by nucleic acids in a concentration dependent manner from 0 to 60µg/mL. FXIIa-mediated activation of PK was augmented

by DNA with statistical significance ($p<0.05$) starting at 20µg/mL DNA (Figure 12A). This resulted in 0.36 ± 0.08 nM kallikrein generation, a 1.5-fold increase over control. FXIIa-mediated activation of PK was augmented by RNA with statistical significance ($p<0.05$) starting at a concentration of 10µg/mL (Figure 12B). This resulted in 0.30 ± 0.04 nM kallikrein generation, a 1.2-fold increase over control. Linear regression analysis demonstrated a positive correlation between FXIIa-mediated PK activation and increasing concentration of DNA ($r^2 = 0.80$) or RNA ($r^2 = 0.76$). These results suggest an important role of FXIIa in mediating PK activation stimulated by nucleic acids and that at least 20µg/mL of DNA or 10µg/mL of RNA are required to achieve efficient activation of PK in the presence of FXIIa.

HK is an important part of the contact system due to its role in assembling the contact system and binding anionic surfaces, was also tested in this experiment (Wu, 2015). Since HK alone doesn't mediate PK activation, it was added in addition to FXIIa to assess PK activation. Addition of HK to FXIIa-mediated activation of PK in the absence of polyanions resulted in 3.8-fold increase in kallikrein generation.

FXIIa/HK-mediated activation of PK was augmented by nucleic acids in a concentration dependent manner from 0 to 60µg/mL. DNA augmented FXIIa/HK-mediated activation of PK with statistical significance ($p<0.01$) starting at 40µg/mL (Figure 12A). This resulted in 1.44 ± 0.22 nM kallikrein, a 1.5-fold increase compared with activation of PK by FXIIa/HK without DNA. FXIIa/HK-mediated activation of PK was found to be augmented by RNA with statistical significance ($p<0.01$) starting at

A


PK + FXIIa
PK + FXIIa + HK
Kallikrein (nM) Generation
DNA (µg/mL)
0
1
2
3
0
10
20
30
40
50
60
*
*
#
##
##

B

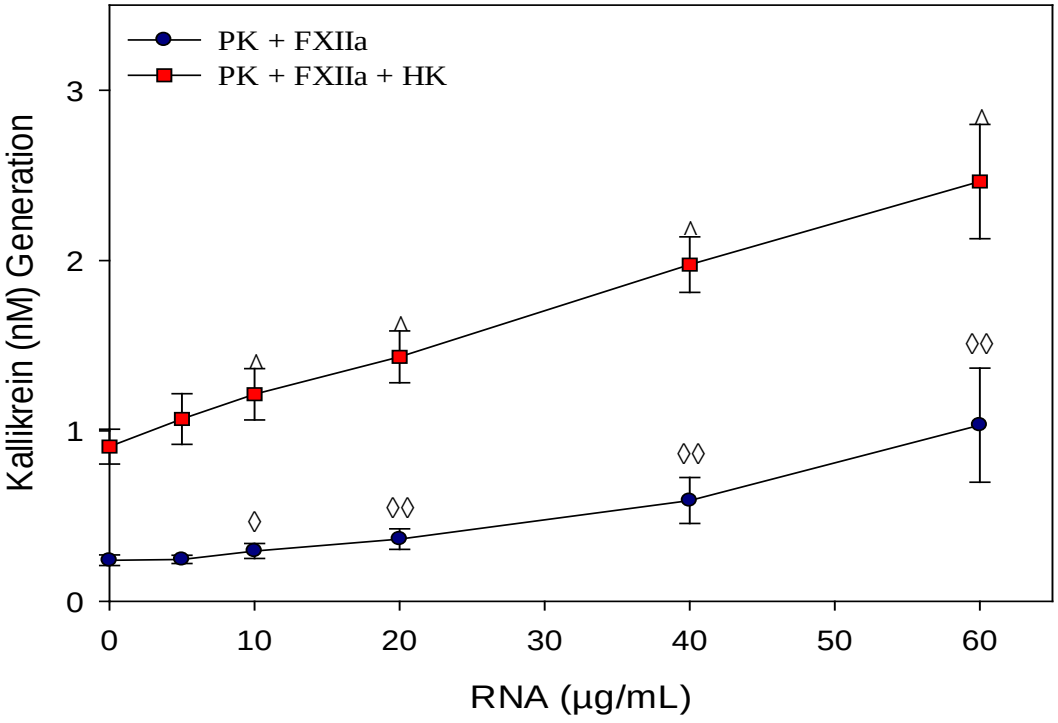

PK + FXIIa
PK + FXIIa + HK
Kallikrein (nM) Generation
RNA (µg/mL)
0
1
2
3
0
10
20
30
40
50
60
△
△
△
△
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Figure 12: The Effect of Nucleic Acids on Prekallikrein Activation by FXIIa.

PK activation was assessed by evaluating kallikrein generation in the presence of FXIIa without and with HK. PK (5nM), 0.2nM FXIIa, and 5nM HK were incubated with increasing concentrations of (**A**) A549 DNA or (**B**) RNA up to 60µg/mL. Kallikrein generation was measured by the hydrolysis of kallikrein-directed S2302 substrate and determined using the kallikrein standard curve. Data are presented as mean ± standard deviation of n = 3 in duplicate. #$p<0.05$, ##$p<0.01$ compared with control for PK+FXIIa stimulated by DNA. *$p=0.01$ compared with control for PK+FXIIa+HK stimulated by DNA. ◊$p<0.05$, ◊◊$p<0.01$ compared with control for PK+FXIIa stimulated by RNA. ^$p<0.01$ compared with control for PK+FXIIa+HK stimulated by RNA. p-values determined by the Mann-Whitney rank sum test.

10µg/mL (Figure 12B). This resulted in 1.22 ± 0.15 nM kallikrein, representing a 1.3-fold increase compared to activation of PK by FXIIa/HK without RNA. Linear regression analysis demonstrated a positive correlation between FXIIa/HK-mediated PK activation and increasing concentrations of DNA ($r^2 = 0.77$) or RNA ($r^2 = 0.91$). HK enhanced FXIIa-mediated activation of PK as shown by higher kallikrein generation. Linear regression analysis revealed that FXIIa/HK-mediated activation of PK stimulated by nucleic acids was statistically better ($p<0.001$) than the activation without HK based on its steeper slope confirming the cofactor effect of HK. This study showed that kallikrein generation increased with increasing concentration of nucleic acids similar to the effect seen in plasma. This work also revealed that nucleic acid concentration of 40µg/mL is efficient for PK activation in the two reactions described above and this concentration will be carried over for further analyses of PK activation.

Activation of PK was further analyzed with 40µg/mL of DNA or RNA. Experiments were conducted with various combinations of FXIIa and HK to determine the contents of the reaction that was most efficient for PK activation. PK activation was conducted in the absence or presence of FXIIa and/or HK with DNA or RNA in a similar manner as described above. Dextran sulfate, a known non-physiological activator of prekallikrein, was used as a positive control (Tans *et al.*, 1987).

In buffer alone, significant amounts of kallikrein were only generated in the presence of FXIIa or FXIIa/HK. Incubation of PK with FXIIa resulted in 0.24 ± 0.04 nM kallikrein generation, and in the presence of HK, 0.72 ± 0.19 nM kallikrein was generated

(Figure 13). This suggests that although HK-mediated activation of PK did not result in kallikrein generation, HK does however enhance FXIIa-mediated activation of PK by 3.0-fold in the absence of polyanions.

Incubation of PK with 40µg/mL DNA did not promote autoactivation even in the presence of HK. DNA-treated activation of PK by FXIIa generated 0.46 ± 0.10 nM kallikrein, while in the presence of FXIIa and HK, 1.40 ± 0.33 nM kallikrein was generated (Figure 13). Addition of HK enhanced FXIIa-mediated activation of PK by 3.0-fold.

Similar to DNA, incubation with 40µg/mL RNA did not promote autoactivation of PK even in the presence of HK. RNA-treated activation of PK by FXIIa resulted in 0.65 ± 0.08 nM kallikrein generation, while in the presence of FXIIa and HK, 2.43 ± 0.23 nM kallikrein was generated (Figure 13). Addition of HK enhanced FXIIa-mediated activation of PK by 3.7-fold.

Incubation with 0.1µg/mL dextran sulfate did not promote autoactivation of PK even in the presence of HK. Dextran sulfate-treated activation of PK by FXIIa resulted in 0.54 ± 0.09 nM kallikrein generation and the addition of HK to FXIIa-mediated activation of PK resulted in 3.64 ± 0.39 nM kallikrein generation (Figure 13). Addition of HK increased FXIIa-mediated activation of PK by 6.8-fold.

In summation, the addition of FXIIa is necessary to activate PK. The addition of FXIIa augmented kallikrein generation by 140-fold in the absence of polyanions and by 144-, 99-, and 123-fold in the presence of DNA, RNA, and dextran sulfate, respectively.

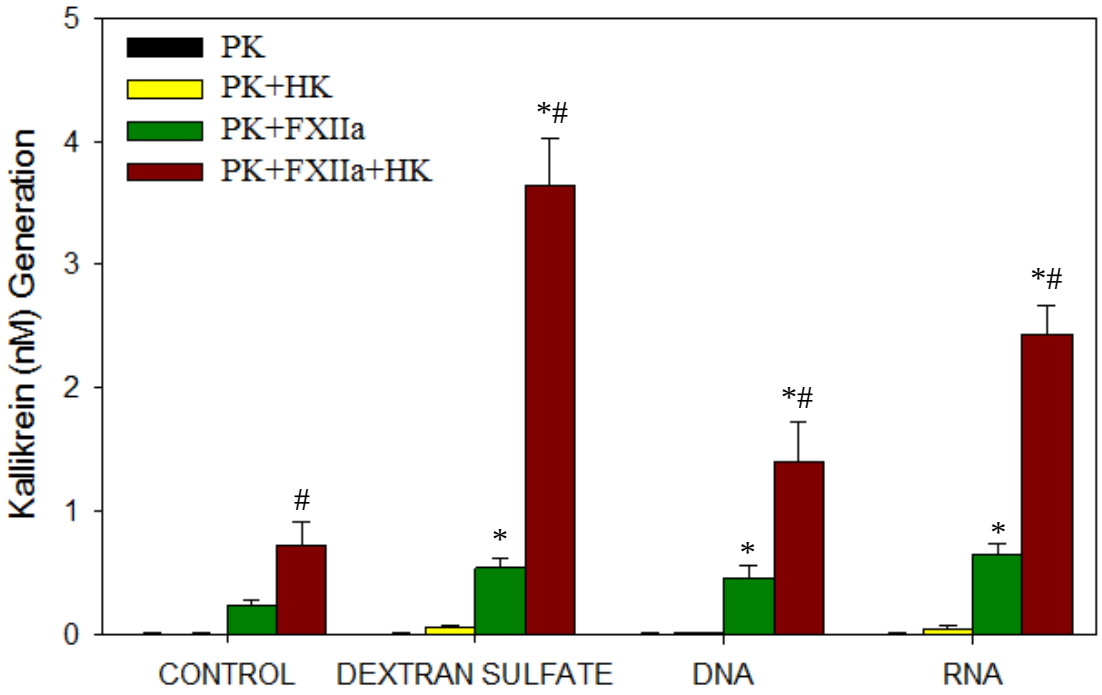

Figure 13: PK Activation Stimulated By Nucleic Acids.

PK activation was assessed by evaluating kallikrein generation in the absence and presence of FXIIa, HK, and polyanion. PK (5nM), 0.2nM FXIIa, and 5nM HK were incubated in buffer containing 0.1µg/mL dextran sulfate, 40µg/mL A549 DNA, or 40µg/mL A549 RNA. Kallikrein generation was measured by the hydrolysis of kallikrein-directed S2302 substrate. Data are presented as mean ± standard deviation of n = 3 in duplicate. #$p<0.01$ between PK+HK and PK+FXIIa+HK within each polyanion as tested by the Mann-Whitney rank sum test. *$p<0.01$ between buffer and polyanion for each respective condition as tested by the Mann-Whitney rank sum test.

The similarity of the fold-increases between the absence and presence of polyanions may indicate that FXIIa plays a much larger role in the activation of PK than nucleic acids. It suggests that nucleic acids play a much larger role in the activation of FXII compared to PK.

This study revealed that the addition of DNA, RNA, or dextran sulfate augmented FXIIa-mediated activation of PK by 2-, 3-, or 2-fold, respectively. This suggests that addition of FXIIa and nucleic acids augment kallikrein generation. Addition of HK to FXIIa-mediated activation of PK was shown to further augment kallikrein generation. The addition of HK to FXIIa-mediated activation of PK resulted in a 3-fold increase in the absence of polyanions and by 3-, 4, and 7-fold in the presence of DNA, RNA, or dextran sulfate, respectively. The capacity of nucleic acids to augment FXIIa/HK-mediated activation of PK is also shown by this study. It was found that FXIIa/HK-mediated activation of PK was augmented by 2-, 3-, and 5-fold upon addition of DNA, RNA, or dextran sulfate, respectively. These results suggest that polyanion-triggered activation of PK by FXIIa is enhanced by HK. This highlights the importance of both FXIIa and HK as well as the capacity of nucleic acids to augment activation of PK.

3.3.2 Effect of Zinc on PK Activation Stimulated by Nucleic Acids

The role of zinc in the activation of prekallikrein should also be assessed to attain a better understanding of the role of zinc in the activation of the contact system. It has been previously established that Zn^{2+} accelerates surface-mediated activation of PK by FXIIa in the presence of HK (Shimada *et al.*, 1987). In this experiment, 5nM PK was

incubated with 5nM HK, in presence or absence of 5µg/mL A549 DNA or RNA, and in the presence or absence of 10µM Zn^{2+} for 5 minutes at 37°C. A lower concentration of nucleic acids was used in this experiment to focus on the effect of zinc over nucleic acids. This was followed by the addition of 0.2nM FXIIa and incubation for 2 minutes at 37°C with a subsequent incubation with CTI for 5 minutes at 37°C to inhibit the remaining FXIIa from the system. Kallikrein generation was then evaluated upon addition of S2302 kallikrein-directed substrate and determined by the kallikrein standard curve.

In buffer, kallikrein was only generated in the presence of HK and FXIIa. FXIIa/HK-mediated activation of PK resulted in 0.66 ± 0.14 nM kallikrein generation (Figure 14). Upon addition of Zn^{2+}, 1.38 ± 0.71 nM kallikrein was generated, representing a 2.1-fold statistically significant ($p<0.01$) increase.

In the presence of DNA or RNA, FXIIa/HK-mediated activation of PK generated 0.71 ± 0.07 nM or 0.97 ± 0.08 nM kallikrein, respectively. Upon addition of Zn^{2+}, PK activation was augmented in a statistically significant manner ($p<0.01$) to generate 1.89 ± 0.10 nM kallikrein in the presence of DNA or 1.81 ± 0.12 nM kallikrein in the presence of RNA, representing 2.6- and 1.9-fold increases, respectively (Figure 14).

Zinc enhanced FXIIa/HK-mediated activation of PK in the absence and presence of DNA or RNA. Comparison of the effect induced by Zn^{2+}in the absence and presence of nucleic acids revealed that the effect of Zn^{2+} is similar in both conditions. This suggests that although Zn^{2+} enhances PK activation, its effect is much more pronounced in FXII activation.

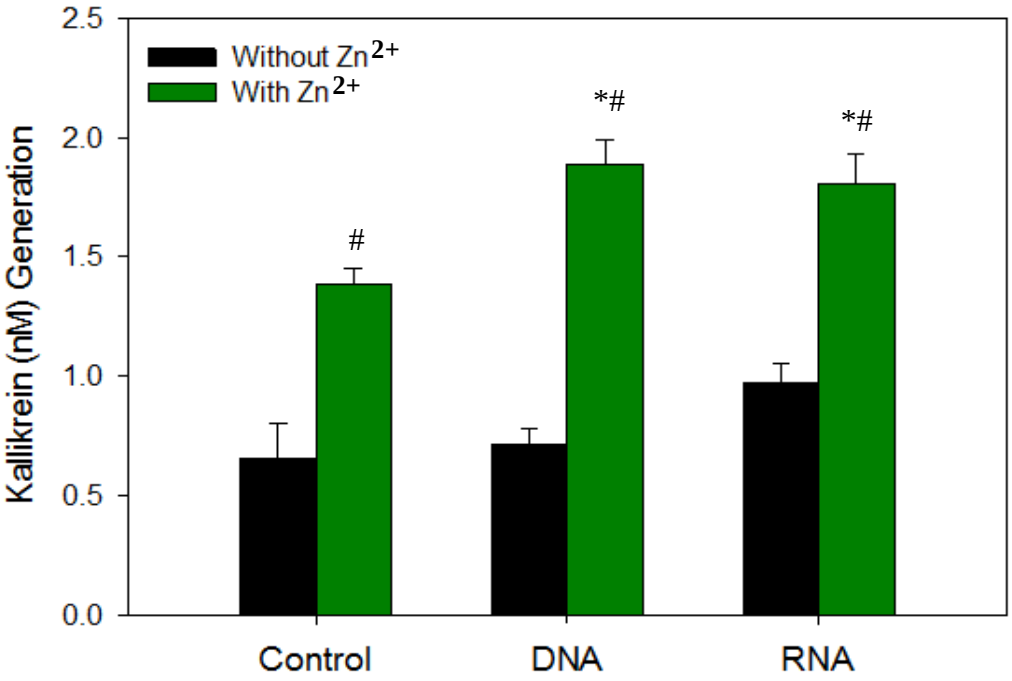

Figure 14: The Effect of Zn^{2+} on Nucleic Acid-mediated Prekallikrein Activation.

Kallikrein generation was evaluated by incubating 5nM PK with 0.2nM FXIIa and 5nM HK in buffer, 5µg/mL A549 DNA, or 5µg/mL A549 RNA in the absence or presence of 10µM Zn^{2+}. Kallikrein generation was measured by the hydrolysis of kallikrein-directed S2302 substrate and determined using the kallikrein standard curve. Data are presented as mean ± standard deviation of n = 3 in duplicate. #$p<0.01$ compared with reaction in the absence of Zn^{2+} for each condition as tested by the Mann-Whitney rank sum test. *$p<0.01$ compared with control in the presence of Zn^{2+} as tested by the Mann-Whitney rank sum test.

Chapter 4: Discussion

4.1 Overview

The contact system has received renewed interest over the past several years due to the identification of its role in thrombosis. In the past the contact system received little importance because it was not considered to play a role in hemostasis. But the importance of the contact system has been highlighted upon findings of reduced thrombus formation in FXII- or FXI-deficient mice in arterial and venous models (van Montfoort and Meijers, 2014).

The contact system is the preliminary component of the intrinsic pathway and consists of FXII, PK, and HK. Activation of FXII can occur through interaction with negatively charged surfaces and it subsequently results in the activation of the intrinsic pathway to generate thrombin. Many artificial surfaces in the past had been identified as activators of the contact system including glass, silica, kaolin, ellagic acid, and dextran sulfate (Wu, 2015).

Although all of the above surfaces activate the contact system, they are non-physiological substances. Recent work has identified physiological activators of the contact system. Physiological surfaces include polyphosphates released from platelets, DNA and RNA released from apoptotic and necrotic cells. The discovery of physiological activators of the contact system and role of the contact system in thrombosis, though absent in hemostasis, highlights the importance of contact system (Morrissey *et al.*, 2012, Smith *et al.*, 2010, Wisler and Becker, 2014).

The ability to stimulate the contact system has been displayed by the aforementioned physiological anions (Muller *et al.*, 2009). Platelet-derived polyP are relatively short, 60 to 100 phosphate units long, compared with the long polyP found in microorganisms (Morrissey *et al.*, 2012). Although long polyP have been shown to be more potent in activating the contact system, platelet-derived polyP have also been shown to promote the activation of the contact system (Morrissey *et al.*, 2012, Muller *et al.*, 2009). Platelet-derived polyP have been shown to promote the activation of FXII in the presence of PK but to a lesser extent in the autoactivation of FXII (Muller *et al.*, 2009). *In vivo* murine studies demonstrated that the release of polyP upon stimulation of platelets by Trap6 resulted in lethality while $FXII^{-/-}$ mice were largely protected in the lethal pulmonary embolism model. This suggests that platelet-derived polyP activate the contact system through the activation of FXII (Muller *et al.*, 2009).

Similarly, nucleic acids are also thought to promote the activation of the contact system. Nucleic acids were first thought to be involved in the activation of coagulation when extracellular RNA was found to serve as a cofactor for the autoactivation of FVII-activating protease (Nakazawa *et al.*, 2005, Wisler and Becker, 2014). Subsequent work had identified that both DNA and RNA stimulated that activation of the contact system in the purified system. It demonstrated the ability of nucleic acids to stimulate FXII activation in the presence of PK and HK (Kannemeier *et al.*, 2007, Vu *et al.*, 2016). Nucleic acids also demonstrated the capacity to augment thrombin generation in normal plasma but this was attenuated in FXII- or FXI-deficient plasma. This established that

nucleic acids augment thrombin generation through the contact system (Swystun *et al.*, 2011, Vu *et al.*, 2016).

We studied the role of nucleic acids and zinc in the contact system by testing the capacity of nucleic acids to activate specific reactions of the contact system. This allowed us to outline the necessary components required for the activation of FXII and PK. In order to achieve efficient activation of FXII stimulated by nucleic acids, we hypothesized that the presence of PK and HK are necessary and zinc enhances this reaction. Similarly, we hypothesized that efficient activation of PK stimulated by nucleic acids requires addition of FXIIa and HK and zinc enhances this reaction.

4.2 Thrombin Generation Stimulated By Nucleic Acids

In order to evaluate the role of nucleic acids on the activation of the contact system, we started by assessing the effect of nucleic acids in plasma by measuring thrombin generation. Thrombin generation assay conducted using plasmas deficient in certain coagulation proteins allowed us to evaluate the effect of nucleic acids in intrinsic- or extrinsic-exclusive pathways of coagulation. Thrombin generation assay using FVII-def plasma represents a system that generates thrombin primarily through the intrinsic pathway due to the lack of FVII, an essential component of the extrinsic pathway. Addition of DNA or RNA to FVII-def plasma enhanced thrombin generation as suggested by various parameters of the thrombin generation assay such as reduced lag time and increased peak thrombin concentration. Thrombin generation is similarly enhanced upon addition of nucleic acids in normal pooled plasma, indicating that

thrombin generation stimulated by nucleic acids occurs through the intrinsic pathway. This corroborates previously published work on the ability of nucleic acids to augment thrombin generation through the intrinsic pathway (Swystun *et al.*, 2011, Vu *et al.*, 2016).

Compared with FVII-def plasma and normal plasma, FXII-def or FXI-def plasma had reduced thrombin generation as indicated by reduced peak thrombin concentrations and ETP. Addition of nucleic acids to FXII-def or FXI-def plasma enhanced thrombin generation but the effect was not as pronounced compared with the effect found in FVII-def or normal plasma. These results corroborate previously published work on the effect of nucleic acids in FXII- or FXI-def plasma compared with FVII-def or normal plasma (Swystun *et al.*, 2011, Vu *et al.*, 2016). These findings also confirm that thrombin generation in these plasmas stimulated by nucleic acids is occurring primarily through the intrinsic pathway.

Interestingly, PK-dep plasma's ability to generate thrombin was not compromised compared with normal plasma. Similar to normal plasma, addition of nucleic acids to PK-dep plasma enhanced thrombin generation as indicated by the reduced lag time among other parameters of the thrombin generation assay. This could be attributed to the fact that FXII is still available in PK-dep plasma to be stimulated by nucleic acids and activate downstream proteins to generate thrombin through the intrinsic pathway.

In normal plasma the effect of increasing concentrations of nucleic acids also revealed a dose dependent effect on thrombin generation. Increasing concentrations of nucleic acids showed changes in the individual parameters of the thrombin generation

assay, such as prolonged lag times and increased peak thrombin concentrations, indicative of enhanced thrombin generation. These results were expected as previously published work demonstrated increasing DNA concentrations augmented thrombin generation in a dose-dependent manner (Gould *et al.*, 2014, Swystun *et al.*, 2011). Our work revealed that nucleic acid concentration of 40µg/mL was effective to enhance thrombin generation and show a statistically significant change across all parameters of the thrombin generation assay. The procoagulant nature of nucleic acids in normal plasma was also supported by previously published work that have indicated similar findings to our work (Gould *et al.*, 2014, Swystun *et al.*, 2011, Vu *et al.*, 2016).

Overall, thrombin generation studies revealed that nucleic acids stimulated the intrinsic pathway to enhance thrombin generation and also provided a concentration of nucleic acids that is sufficient to promote thrombin generation in the plasma system. The effective concentrations of nucleic acids derived from this plasma system were used in the purified buffer system to evaluate its effect on FXII and PK in an isolated setting.

4.3 Contact System Activation Stimulated by Nucleic Acids

Nucleic acids did not promote autoactivation of FXII effectively. However, PK- and PK/HK-mediated activation of FXII was significantly increased by 10- to 20-fold upon addition of 40µg/mL nucleic acids. It should be noted that although the folds are similar for both reactions, in the absence of nucleic acids, FXII activation was increased by 5-fold upon addition of PK but in the presence of nucleic acids, FXII activation was augmented by 30- to 50-fold upon addition of PK. This demonstrated the importance of

nucleic acids and PK for the activation of FXII. Addition of HK augmented PK-mediated activation of FXII stimulated by nucleic acids by another 2- to 7-fold. It is thought that nucleic acids autoactivate a very small amount of FXII which activates PK and reciprocally activates FXII. HK is thought to provide an assembly platform this activation as previously established in literature (Wu, 2015). Increasing concentrations of nucleic acids stimulated PK-mediated activation of FXII in a dose dependent manner. Similarly, increasing concentrations of nucleic acids also stimulated PK/HK-mediated activation of FXII in a dose dependent manner. The importance of PK and HK to the activation of FXII stimulated by nucleic acids was also highlighted by previous work and these results corroborate those findings that nucleic acids stimulate PK-mediated activation of FXII which is enhanced in the presence of HK (Kannemeier *et al.*, 2007, Vu *et al.*, 2016).

Although Kannemeier and colleagues were the first to analyze this, it should be noted that they did not specifically measure the activation of a single coagulation protein but rather a combination of PK and FXII cleaving a non-specific chromogenic substrate (Kannemeier *et al.*, 2007). They did not use an inhibitor such as STI to inhibit the kallikrein generated in the process so that the activity of only one coagulation protein was being measured. This led to non-specific proteolysis of the chromogenic substrate and a premature conclusion about the role of nucleic acids in activation of the contact system.

Similar to the activation of FXII, nucleic acids did not autoactivate PK effectively. However, FXIIa- and FXIIa/HK-mediated activation of PK was increased by 2- to 3-fold upon addition of 40µg/mL nucleic acids. Addition of HK augmented FXIIa-

mediated activation of PK stimulated by nucleic acids by another 3- to 4-fold. Nucleic acid dose response studies conducted to test FXIIa-mediated activation of PK in the presence or absence of HK revealed increased activation of PK with increasing concentrations of nucleic acids. This novel work showed that nucleic acids enhanced FXIIa-mediated activation of PK and that the activation is augmented to generate more kallikrein in the presence of HK.

4.4 The Role of Zinc in the Contact System

Zinc is an important ion to the contact system due to its ability to bind to FXII and subsequently induce conformational changes that make it better suitable for activation (Vu *et al.*, 2013). This study was conducted with 10μM Zn^{2+}. This may raise concern as literature has established that free, unbound concentration of Zn^{2+} in plasma is less than 1μM. However, it has also been shown that upon accumulation of activated platelets, the concentration of Zn^{2+} can rise to 10μM (Mahdi *et al.*, 2002, Vu *et al.*, 2013).

In the absence of polyanions, PK/HK-mediated activation of FXII was increased by 5-fold upon addition of Zn^{2+}. However, the addition of Zn^{2+} to PK/HK-mediated activation of FXII stimulated by nucleic acids augments FXII activation by 50-fold compared with that in the absence of Zn^{2+}. This novel finding demonstrates that Zn^{2+} augments PK/HK-mediated activation of FXII stimulated by nucleic acids. This work is developed from previous findings in literature indicated zinc's ability to accelerate sulfatide-mediated activation of FXII by kallikrein in the presence of HK (Shimada *et al.*, 1987).

Aside from its role in the activation of FXII, zinc was also shown to modestly accelerate FXIIa-mediated activation of PK in the presence of HK. Without polyanions, FXIIa/HK-mediated activation of PK is increased by 2-fold upon addition of zinc. Similarly, the addition of Zn^{2+} to FXIIa/HK-mediated activation of PK stimulated by nucleic acids augments PK activation by 1.9- to 2.6-fold compared with that in the absence of Zn^{2+}. These results indicate a milder effect of Zn^{2+} on the activation of PK compared with FXII. This work is also developed from previously published work indicating zinc's ability to accelerate sulfatide-mediated activation of PK by FXIIa and HK. Similar to our findings, it was also found that accelerating effect of zinc is less pronounced in the activation of PK than FXII (Shimada *et al.*, 1987).

4.5 Structural Properties of Nucleic Acids

The physical properties of nucleic acids have also been explored in literature in order to determine structural factors that may contribute to its procoagulant nature. These properties include charge, size, and secondary structure.

Artificial surfaces such as dextran sulfate and kaolin had been previously identified as activators of the contact system. The primary structural characteristic of these artificial surfaces has been that they are anionic (Wu, 2015). Similarly, nucleic acids are also negatively charged molecules that promote contact activation. Amongst physiological surfaces, inorganic polyphosphates have also been identified as activators of the contact system. Inorganic polyphosphates share an anionic polyphosphate backbone with nucleic acids. The negative charge in these physiological structures is

derived from their polyphosphate backbone (Geddings and Mackman, 2014, Vu *et al.*, 2015). Thus, these findings confirm that a negative charge is an important attribute for nucleic acids to promote activation of the contact system.

Another structural property of nucleic acids that could contribute to its procoagulant nature is strand length. Inorganic polyphosphates vary in phosphate-unit length depending on its origin. Inorganic polyphosphates secreted by human platelets are 60-100 phosphate units long while polyphosphates derived from microorganisms are over 1000 phosphate units long. The difference in the length of the polyphosphates affects its function in coagulation (Geddings and Mackman, 2014, Smith *et al.*, 2010).

Studies with different size variants of polyP revealed that shorter polyP were better able to accelerate FV activation and shorten the FXa-initiated clotting time compared with longer polyP. Conversely, longer polyP enhanced fibrin clot turbidity compared with shorter polyP. One of the most interesting effects observed with the different sizes of polyP was that short polyP are poor activators of FXII compared with long polyP (Geddings and Mackman, 2014, Smith *et al.*, 2010). The size-varying effects with polyP raise interesting questions about the relationship between the size of a nucleic acid and its ability to activate the contact system.

Previously published studies conducted with varying lengths of single-stranded DNA revealed that increasing lengths of oligonucleotides from 9 to 35 nucleotides resulted in increased FXII activation in the presence of zinc (Pavlov *et al.*, 2006). However, longer single-stranded DNA, ranging from 587 to 831 nucleotides, were found

to be not as effective in promoting FXII activation compared with DNA 35 nucleotides long. This particular study proposes that nucleic acids of longer length are relatively ineffective for promoting FXII activation (Pavlov *et al.*, 2006). In regards to studies performed with RNA, RNA longer than 50 nucleotides were able to activate the contact system (Kannemeier *et al.*, 2007).

Secondary structures of nucleic acids have also been studied to determine its effect on activation of the contact system. In addition to single-stranded DNA, double-stranded DNA has also been explored to determine its ability to activate the contact system. Different results have been reported in regards to double-stranded DNA's ability to activate the contact system. Pavlov et al. reported that unlike single-stranded DNA, double-stranded DNA is not able to activate FXII. The authors suggest that this may be due to the lack of flexibility in the double-helix conformation compared with single-stranded DNA which hinders its capacity to activate FXII (Pavlov *et al.*, 2006). Conversely, Gansler et al. reported that single-stranded DNA is a poor activator in the prekallikrein activation assay and has low procoagulant activity in turbidity clot lysis. They suggest that nucleic acid's ability to form hairpin structures is a more important factor in determining their procoagulant nature (Gansler *et al.*, 2012).

The ability of nucleic acids to form hairpin structures has also been explored by different groups. Pavlov et al. reported that although hairpin-forming DNA-oligonucleotides are able to activate FXII, linear oligonucleotides are better activators.

The authors suggest that this may be due to FXII's inability to open the hairpin structure (Pavlov *et al.*, 2006).

Gansler et al. has reported opposing findings on this matter. They found that hairpin-forming DNA- and RNA-oligonucleotides were able to effectively reduce clotting time in a plasma recalcification clotting assay while linear oligonucleotides exhibited poor procoagulant activity (Gansler *et al.*, 2012). Functional studies involving PK activation in the presence of HK also revealed similar findings. Hairpin-forming DNA- and RNA-oligonucleotides were both able to better autoactivate PK compared with linear oligonucleotides. Specifically, hairpin-forming DNA-oligonucleotides were found to activate PK remarkably (Gansler *et al.*, 2012).

An important aspect to consider about the secondary structure of nucleic acids is their stability in plasma. It has been found that both hairpin-forming and linear DNA-oligonucleotides remain stable in plasma. However, linear RNA-oligonucleotides degraded immediately while hairpin-forming RNA-oligonucleotides were only partially degraded. This suggests that forming a hairpin secondary structure, especially in the case of RNA-oligonucleotides is important for remaining stable in plasma (Gansler *et al.*, 2012).

Further work is required in studying the structural properties of nucleic acids to better understand what makes nucleic acids procoagulant. Thrombin generation studies should be performed to assess the effect of chain length with single- and double-stranded nucleic acids. Also, inconsistencies between different groups regarding the secondary

structure of nucleic acids and their capacity to elicit a procoagulant response need to solved. This will also allow us to better study nucleic acids that are found in the physiologically setting as messenger (mRNA), ribosomal (rRNA), and transfer (tRNA) RNA can exist as hairpin-forming structures (Gansler *et al.*, 2012).

4.6 Nucleic Acids in Physiology

4.6.1 Neutrophil Extracellular Traps

As previously mentioned, nucleic acids are released from apoptotic and necrotic cells in the physiological environment (Jahr *et al.*, 2001, Schwarzenbach *et al.*, 2014, Ziegler *et al.*, 2002). DNA is also released from neutrophils as a part of neutrophil extracellular traps (NETs) in a process called NETosis. In addition to DNA, NETs also consist of histones and other proteins. NETs primarily act as a defense mechanism against infections from bacteria and fungi by entrapping and killing them. In addition to their role in the immune system, NETs have also been reported to enhance thrombosis in arterial and venous models. As component of NETs, DNA is the negatively-charged surface that activates FXII and histones activate platelets (Geddings and Mackman, 2014, Martinod and Wagner, 2014). Histones have been reported to enhance thrombin generation by interacting with toll-like receptor 2 and 4 on platelets (Wisler and Becker, 2014). Certain histone proteins have also been reported to activate FXII *in vitro*. The existence of NETs provides a physiological source for the release of DNA into the circulation which can activate the contact system (Geddings and Mackman, 2014).

4.6.2 Cell-free DNA in Cancer and Sepsis

Physiologically, cell-free DNA has been found to be a relevant species in various diseases. DNA has been found in the circulation of cancer patients and septic patients (Hisada *et al.*, 2015, Mai *et al.*, 2015). Tumor cells are known to release nucleic acids such as DNA, mRNA, and microRNA. It is also known that cancer patients exhibit elevated levels of cell-free DNA in their circulation. It has also been reported that the concentration of cell-free DNA increases depending on the severity of the tumour (Hisada *et al.*, 2015). The increase of cell-free DNA in the circulation provides stimulus for the activation of the contact system. Studies have also revealed that administration of chemotherapy in breast cancer patients further increased the cell-free DNA concentration in the plasma and promoted contact system activation through FXII (Hisada *et al.*, 2015).

Similar to cancer patients, septic patients also have increased levels of cell-free DNA in their circulation (Mai *et al.*, 2015). A study by Dwivedi et al. has reported that in survivors the mean cell-free DNA levels were 1.16 ± 0.13 ng/µL, similar to the healthy control group, while the non-survivors had cell-free DNA concentrations of 4.65 ± 0.48 ng/µL (Dwivedi *et al.*, 2012). The study by Dwivedi et al. allows cell-free DNA to act as a potential indicator for severe sepsis (Dwivedi *et al.*, 2012). This study can be expanded to other diseases as well because it has been previously established in literature that level of circulating cell-free nucleic acids increase in a variety of disorders such as cancer, stroke, and myocardial infarction (Swarup and Rajeswari, 2007).

It has also been reported that some severely septic patients had "two prominent DNA bands of ~150 bp and ~300 bp" (Dwivedi *et al.*, 2012). This was quite different from the 10 kbp genomic DNA that we used in this study. However, it was also found that chemotherapy-treated whole blood yielded DNA ranging in size from 3kbp to 10kbp in a cancer study (Swystun *et al.*, 2011). This suggests that we should focus on these sizes of DNA that are found in the circulation and perform functional assays with more physiological accuracy.

4.7 Future Directions

Although the procoagulant effects of both DNA and RNA have been established on the contact system *in vitro,* it is interesting to note that there are discrepancies in literature that suggest that only one may be responsible *in vivo* (Kannemeier *et al.,* 2007, Vu *et al.,* 2015). *In vivo* studies conducted with the murine arterial thrombosis model revealed that RNA, and not DNA, was detected in the thrombi of $FeCl_3$-treated mice. These studies also demonstrated that the administration of RNase had a protective, antithrombotic effect on the mice while the administration of DNase did not and resulted in occlusive thrombus formation (Kannemeier *et al.*, 2007, Vu *et al.*, 2015). This work suggests that RNA is solely responsible for arterial thrombosis yet studies conducted with NETs suggest that DNA is important for thrombus formation in DVT. This study revealed that mice treated with DNase1 reduced NETs formation and thrombus weight in the IVC (von Bruhl *et al.*, 2012). This raises interesting questions about the absence of DNA and its prothrombotic role in the arterial thrombosis model. Further work is

required to understand this discrepancy as it has been established that at least *in vitro* DNA and RNA both promote the activation of the contact system (Kannemeier *et al.*, 2007, Vu *et al.*, 2016).

Future work could explore the role of nucleic acids holistically down the intrinsic pathway, analyzing the generation of FXIa upon stimulation of the contact system proteins by nucleic acids. Work can also be conducted to assess if there is any nucleic acid-length dependency on its ability to generate thrombin in plasma. This would be tested due to published work indicating the polyphosphates have a length-dependent effect on its ability to activate the contact system (Morrissey *et al.*, 2012). Expanding our understanding on the structural properties of nucleic acids and *in vivo* findings on the prothrombotic nature of nucleic acids could help us understand the relevance of nucleic acids in clinical disorders.

4.8 Summary

To summarize, we showed that DNA and RNA are procoagulant species in normal plasma and they generate thrombin through the intrinsic pathway of coagulation. We were able to translate that work to a purified buffer setting to confirm previous findings that PK/HK-mediated activation of FXII is enhanced by nucleic acids. We also proposed that FXIIa/HK-mediated activation of PK is enhanced by nucleic acids. Novel work showed zinc's ability to accelerate PK/ HK-mediated activation of FXII stimulated by nucleic acids. Zinc was also shown to accelerate FXIIa/HK-mediated activation of PK

stimulated by nucleic acids. The present study confirmed the procoagulant effects of DNA and RNA on the contact system and the role of zinc in accelerating its activation.

Chapter 5: References

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