Characterization of a Feline Model of Factor XII Deficiency: The In Vivo Role of FXII in Vascular Injury and Inflammatory Responses

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ABSTRACT

Diane Elizabether Bender: *Characterization of a Feline Model of Factor XII Deficiency: The In Vivo Role of FXII in Vascular Injury and Inflammatory Responses*

(Under the direction of Dwight A. Bellinger, D.V.M, Ph.D)

For nearly 6 decades scientists have been identifying and defining the many roles of the blood serine protease Factor XII (FXII) *in vitro* and *in vivo*. Each year we gain new information about this elusive protein, however conflicts still exist over the importance of FXII in human disease states. Factor XII participates in coagulation, fibrinolysis, renin-angiotensin, and immune system responses making it an integral part of mammalian life. Animal models offer the best way to study the importance of a factor *in vivo*, however only two readily available FXII deficient models exist, i.e. the gene-manipulated mouse and our cats. This work characterizes the origin of the genetic defect causing FXII deficiency in a colony of domestic cats, and investigates *in vivo* acute responses to intravascular and intradermal (extravascular) injury. Ultimately we aim to provide a well defined animal model for therapeutic targeting

strategies, and gain new insights into the importance of FXII in cellular and extracellular interactions.

Chapter 1 provides a comprehensive overview of the biological features of FXII: its importance as a central player in coagulation, fibrinolysis, complement activation, the innate immune system, and vascular repair; its molecular and genetic structure and cellular interactions; and the attributed pathophysiology derived from human and mouse studies. Chapter 2 details the feline FXII gene and the mutation discovered in our colony along with protein studies to confirm our findings.

Vascular injury and associated fibrinolytic and inflammatory response studies comprise Chapter 3. We found that FXII procoagulant activity is not necessary to initiate clotting and to form stable thrombi upon an initial venous or arterial injury. However, FXII appears to aid in clot stability following a second intravascular injury 48 hours later on the contralateral veins. Inflammatory mediators are also significantly reduced in FXII deficient animals. Chapter 4 addresses the role of FXII in recruiting inflammatory cells to a site of heme-induced skin injury. Our studies explore a new method of nitric-oxide driven innate immune response, finding significantly fewer inflammatory cell infiltrates in FXII deficient cats. Chapter 5 offers future studies to pursue with this model based on results of these dissertation findings.

DEDICATION

The work presented here could not have been done without the intense efforts and compassion of many people. Working with a feline model can be physically and emotionally straining at times. Every attempt was made to release any qualified animal to a good home where he or she could live out his/her days in comfort and peace. I dedicate the findings of this dissertation work to all the cats who could not qualify and the people whose lives are enhanced by them. I pray the knowledge gained for the benefit of human kind is worth their sacrifice.

ACKNOWLEDGEMENTS

For the last 6 years and longer I have worked with a remarkable group of individuals whom I deeply admire and consider my 'family'. I wish I could name all of them, but I will resign my comments to a few who dedicated large blocks of their time to helping me and have emotionally supported me through life and graduate school. To all others, I am also indebted to you and hope you feel your efforts were appreciated by me and the animals you've helped.

When I first began working as a technician for Dwight Bellinger, D.V.M, Ph.D, he offered me salvation from an allergy inducing research environment—he offered me minimal animal contact and lots of bench assays to keep me busy. As fate would guide me, I soon became intrigued with one of his small side projects which later became the impetus for my applying to graduate school. During my undergraduate studies, I developed a love for learning about hemostasis and the immune system—two complex systems that interact in the cardiovasculature and are responsible for the leading causes of death in the United States each year. Dr. Bellinger simply wanted to know if FXII was important in thrombosis and fibrinolysis, in addition to identifying the gene defect (for genotyping purposes). He

graciously gave me the project and the freedom to pursue other FXII-related avenues. He did not considercould devise an experiment that would push his surgical skills and bore him to death simultaneously. But his patience with me paid off and I learned valuable lessons in experimental design and eliciting the help of others. The countless hours spent together in the surgical suite, discussing meaningless and sometimes controversial issues (with his usual cynicism), missing lunches/chugging down Coca Cola, and dealing with a demanding graduate student will hopefully be fond memories for everyone. I wish to thank him for all he has done to shape me as a scientist and a person, and for supporting me from start to finish as a good advisor does. He does not stand alone in his support.

I recognize Virginia Godfrey, D.V.M, Ph.D., as a cohort advisor and outstanding committee member, an inspiration, a friend, a boss, and a long-time supporter of my achievements. Virginia suggested I switch to a mouse model, but I wouldn't take her advice (the only instance I'm happy I did not take her advice). I am inspired by her passion for teaching pathology and patience while working with students, especially those of us who seem hopeless and clueless. Her phenomenal knowledge of veterinary pathology is admired by all who meet her. And her ability to identify common-sense solutions to most any problem is not only astonishing but comforting to those who can appreciate simplicity in life. I thank you Virginia for the pep talks regarding your own challenges in finishing graduate school(s) and standing up for me when needed.

As for my other committee members, I wouldn't have been able to complete these studies without your help. Herb Whinna, M.D., Ph.D. not only provided the

crucial Doppler equipment that allowed sensitive measurements of *in vivo* thrombus formation, but his mentoring and straightforward comments saved me much time and effort. I am forever grateful for your help. Tom Fischer, Ph.D, has been a delight to work with over the years. Tom has an amazing talent to trick you into thinking he is clueless about your field so you feel comfortable with your knowledge, then he slams you with a concept or question that shows great knowledge and understanding while you're left looking like an ignoramus. I admire him not only for his ability to ask the right questions, but also for his achievements in science and business. I hope to accomplish a similar success in my own future. You're a genius in my eyes dude! My last committee member (but not the least of whom I am indebted) Dougald Monroe, Ph.D, otherwise known as 'Mac', is a gift from heaven. I did not personally know Mac before approaching him to be on my committee, but I was well aware of his outstanding achievements in the field of thrombosis and hemostasis. Mac graciously offered me critical assistance to complete this project and offered explanations for some confusing data that sparked new interests in the roles of FXII. His mentorship and availability to talk or review my writing was first rate and probably the most helpful in building my confidence about the work I had done. Thank you.

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She offered the best suggestion for studying skin inflammation: “Why can’t we find a way to inject some irritant into the skin?” Ah ha! Such a simple solution would not have crossed our ‘higher educated’ minds. Pam saved me countless weeks in trying other strategies to get a blister to form or a bottle cap to stick—thank you Pam! I don’t know what I would’ve have done without you by my side.

Achievements in science rarely happen without a team of people helping to carry out the research design. I was blessed with a wonderful team of Laboratory Animal Medicine staff who gladly offered their assistance and went beyond their required duty to provide the highest quality of animal care. I thank all the husbandry staff, veterinary technician staff, veterinarians, and clinical diagnostic staff (Yumiko Hayes) for their compassion and ongoing support of the animals and myself. I wish to make a special mention of my dear friend and co-worker, Mrs. Yumiko Hayes, for the many years of optimism, compliments, and emotional support she provided me. She always greeted me with a warm hello, and forgave me for the many age-related comments I made. Yumi is one of the most dedicated workers I have ever met—I am truly honored to have worked with her and shared my life experiences with her (and family).

Certain people were responsible for teaching me about molecular biology and protein chemistry. First I would like to recognize the great patience and mind of Mark Kloos, Ph.D. who taught me all I know about gene sequence analysis and proper primer design. Mark reviewed my sequences and discovered the gene defect in our cat colony. To him I am forever grateful, but I am still trying to figure out what he was talking about behind all that jargon! Secondly, I would like to recognize

Dave Klapper, Ph.D. who runs the UNC Peptide Facility and patiently walked me through protein purification, SDS-PAGE and Western blotting techniques. I hope my poor quality gels do not reflect on his teaching abilities—it's solely my fault for not taking the time to repeat the procedure until it is perfect. I promise to do better in the future.

Finally, I would like to recognize and thank my friends and family who have supported me over the years. My friends have always provided the encouragement to get through tough classes and exams, then later through finishing the experiments and writing this dissertation. They've listened to my complaining, cared for my children, cared for me, and offered me unconditional love and a place to crash when needed. I've ignored many of them in the past year and hope they will forgive me for this.

To my family: You have shaped me into the person I am today because you love and support me in every decision I make. My mother, Florence Bender, endured many hardships to provide me with the financial support and education that carried me this far. Her recent stroke helped funnel my energy to completing these studies in order to offer the medical community more insight into thrombus development. And her assistance in watching over her toddler grandson while I wrote part of this dissertation was a physical hardship she wasn't expecting, but never voiced a complaint. I pray she will continue to be proud of me and look fondly on the times we shared. My brother, Robert, has always offered sound advice and caring support over the years. I admire him for his strength and stubbornness in not giving up on life when his health failed. My sufferings seem small compared to his. He serves as a constant reminder that we should never give up on our dreams and passions until the

casket is 6 feet in the ground. My oldest son, Nolan, was starting preschool when I began my graduate education. Today he is a budding scientist and my number one fan, however I suspect his motives for supporting me to finish are driven by his desire to spend more quality time with me. Regardless of his reason, I appreciate his patience and unconditional love—may he aspire to being the best at whatever he chooses. My youngest son, Ayyub, will have grown up in a lab and not understand what challenges we have endured as a family. May he continue to think that scientists are funny and cool, and research labs are a secondary residence. And finally I would like to thank my husband, Radwan Abu-Issa, Ph.D. for his love and support in all realms. He is a companion, husband, father, mentor, reviewer, advisor, spiritual guide and more. He has supported me every step of the way and received little recognition in return--to him I am most grateful, second only to God.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

a.a. (amino acids)

Ala (Alanine)

Asn (Asparagine)

Asp (Aspartate)

Arg (Arginine)

APS (Anti-phospholipid Syndrome)

aPE (Phosphatidylethanolamine)

ATIII (Antithrombin III)

APTT (Activated Partial Thromboplastin Time)

ΔA (delta absorbance or change in absorbance)

ANOVA (Analysis of Variance)

BSA (Bovine Serum Albumin)

BCIP (5-bromo-4-chloro-3-indolyl phosphate)

bp (base pairs)

C1-INH (Complement C1 inhibitor)

CAM (Cell Adhesion Molecule)

CBC (Complete Blood Cell Count)

CD11b/CD18c (Cluster of Differentiation 11b/Cluster of Differentiation 18c)

cDNA (complementary DNA)

CHD (Coronary Heart Disease)

CK1 (Cytokeratin 1)

CTAB (hexadecyltrimethylammonium bromide)

C-Terminus (Carboxy terminus end)

CTI (Corn Trypsin Inhibitor)

CU (Clottable Unit)

Cys (Cysteine)

CXCL (Cys-X-Cys Chemokine Ligand)

DEF (Dextran Sulfate Euglobulin Fraction)

DLL4 (Delta-like ligand 4)

DNA (Deoxyribonucleic Acid)

dNTP (deoxyribonucleotide triphosphates)

2-D DIGE (two-dimensional difference in gel electrophoresis)

ECL (enhanced chemiluminescence)

ECM (Extracellular Matrix)

EDTA (ethylenediaminetetraacetic acid)

EGF (Epidermal Growth Factor)

FIIa (Activated FII or thrombin)

FV (Factor V)

FVIIa (Activated Factor VII)

FVIII (Factor VIII)

FIX (Factor IX)

FX (Factor X)

FXa (Activated Factor X)

FXI (Factor XI)

FXII (Factor XII)

FXIIa (Activated Factor XII)

FXII:C (Factor XII Coagulant activity)

FXIIf or β-FXIIa (Factor XII fragment derived from cleavage of FXIIa)

FcγRI (Fc Fragment Gamma Receptor I)

FDP (Fibrinogen Degradation Product)

Fe^{3+} (Ferric iron)

FGF-1 (Fibroblast Growth Factor-1)

GPIbα (Glycoprotein Ibα)

GPIIb/IIIa (Glycoprotein IIb/IIIa receptor)

GPVI (Glycoprotein VI)

Glu (Glutamate)

gC1qR (globular Complement C1q Receptor)

g (gram)

HCT (Hematocrit)

HET (Heterozygote)

H&E (Hematoxylin and Eosin)

His (Histidine)

HK (High Molecular Weight Kininogen)

HKa (Activated High Molecular Weight Kininogen)

$HOCl^-$ (Hypochlorous)

hpf (high power field)

HRP (Horse Radish Peroxidase)

HZY (Homozygote)

H_2O_2 (Hydrogen Peroxide)

H_2O (water)

IEF (isoelectric focusing)

Ig (Immunoglobulin)

IHCS (Immunohistochemical Staining)

IL-1β (Interleukin-1 beta)

IL-6, IL-8 (Interleukin-6, Interleukin-8)

Ile (Isoleucine)

IU (International Unit)

kg (kilogram)

80 kDa (80 kilo Daltons)

KK (Kallikrein)

KKS (Kallikrein-Kinin System)

Leu (Leucine)

L (liter)

Lys (Lysine)

MCP-1 (Monocyte Chemoattractant Protein 1)

MI (Myocardial Infarction)

µg (microgram)

µl (microliter)

µM (micromolar)

mg (milligram)

ml (milliliter)

min (minutes)

M (Molar)

MMP (Matrix Metalloproteinase)

MP (Microparticles)

MPO (Myeloperoxidase)

MW (Molecular Weight)

NBT (p-nitroblue tetrazolium chloride)

ng (nanogram)

NIH (National Institute of Health)

NL (Normal)

nm (nanometer)

NO (Nitric Oxide)

N-terminus (Amino-terminus)

ΔOD (Change in Optical Density)

PA (Plasminogen Activator)

PAI-1 (Plasminogen Activator Inhibitor-1)

PAI-2 (Plasminogen Activator Inhibitor-2)

pAb (polyclonal antibody)

PBS (Phosphate Buffered Saline)

PCF (Permanent Cessation of Flow)

PCI (Protein C Inhibitory)

PDGF (Platelet Derived Growth Factor)

PEG (polyethylene glycol)

Phe (Phenylalanine)

pI (isoelectric point)

P1-P1’ (Peptidyl bond 1-peptidyl bond 1’)

PK (Prekallikrein)

PKC (Protein Kinase C)

PKd (Prekallikrein deficient)

PLG (Plasminogen)

PMN (Polymorphonuclear leukocyte or Neutrophil)

1˚ (Primary)

PRCP (Prolylycarboxypeptidase)

Pro (Proline)

PS (phosphatidylserine)

PVDF (polyvinylidene fluoride)

rcf (relative centrifugal force)

RNA (Ribonucleic acid)

RT-PCR (Reverse Transcription-Polymerase Chain Reaction)

SBTI (Soy Bean Trypsin Inhibitor)

SDS-PAGE (Sodium dodecyl sulfate-polyacrylamide gel electrophoresis)

Ser (Serine)

TAFI (Thrombin-activatable fibrinolysis inhibitor)

TBS (Tris Buffered Saline)

TF (Tissue Factor)

TGF-b (Transforming Growth Factor beta)

TIMPs (Tissue Inhibitor of Metalloproteinases)

Tm (melting temperature)

TNF-a (Tissue Necrosis Factor alpha)

tPA (Tissue-type Plasminogen Activator)

Trp (Tryptophan)

Tyr (Tyrosine)

UNC-CH (University of North Carolina at Chapel Hill)

uPA (Urokinase Plasminogen Activator)

uPAR (Urokinase Plasminogen Activator Receptor)

Val (Valine)

VEGF (Vascular Endothelial Growth Factor)

WBC (White Blood Cell)

BACKGROUND AND SIGNIFICANCE

Vascular Thrombosis: Incidence and Impact

Each year in the United States of America nearly 700,000 people suffer a fatal thrombotic event.[1] An additional 200-600,000 people are diagnosed and undergo treatment for an intravascular thrombus, making vascular thrombosis the leading cause of mortality and morbidity.[1,2] Today's therapeutic strategies for thrombolysis and thrombus removal can be effective but they carry risks of their own. We need greater knowledge of the mechanisms and risk factors associated with thrombophilia so we can develop safer therapies and earlier interventions. We need better public awareness of thrombosis and it's symptoms in order to combat this major disease.

FXII in Coagulation: History and Controversy

Concepts in hemostasis and thrombosis are still emerging. Lipids, lipoproteins, coagulation proteins, cell membrane receptor proteins, and other soluble factors interact in a perfectly orchestrated manner under specific conditions to form a blood clot. Unfortunately scientists today have limited understanding of how these elements specifically interact. One factor still shrouded in mystery is the blood serine

protease, factor XII or Hageman factor named after the first patient diagnosed with its deficiency. In 1955, Drs. Ratnoff and Colopy described a patient, Mr. John Hageman, who presented with a prolonged test tube clotting time yet suffered no bleeding tendencies.[3] They concluded Mr. Hageman lacked a blood protein that initiated clotting in a glass test tube. Ratnoff and colleagues further established that coagulation factors circulate as inactive precursors until becoming activated in the blood. Factor XII's activation upon contact with a negatively charged surface was termed 'autoactivation' and further initiated a sequence of proteolysis in a proposed cascading manner thereby giving it the name 'contact activator' of the intrinsic pathway of coagulation.[4] Since Mr. Hageman was able to form intravascular blood clots (and eventually died of a pulmonary embolism), FXII was thought to be of minor importance *in vivo* because some other factor(s) could act in its absence. After the index patient was reported, a few additional FXII deficient (or Hageman trait) people and animals were identified. Many of these deficient humans appeared to have a thrombotic tendency i.e. thrombophilia, however it was unclear if FXII alone or in combination with other risk factors was the cause of their disease. Various clinical investigations have looked at the impact of FXII on cardiovascular disease, venous thrombosis and pregnancy/fetal loss, however the results have been mixed and controversy still exists. From the 1960s through today, researchers defined many of the *in vitro* characteristics of FXII from humans and other species. The *in vivo* importance of FXII in hemostasis, pathological thrombus formation and other biological pathways has been difficult to study due to low numbers of Hageman trait persons and different findings in FXII deficient animal models of mice and cats.

The incidence of congenital FXII deficiency in human and domestic feline populations appears to be low. Factor XII deficiency is generally an inherited autosomal-recessive trait in humans and cats with an incidence of 1-5%.[5-7] Small studies in Asian populations report an incidence of mild and severe FXII deficiency as high as 10%.[8] This higher rate could be attributed to assay reagent differences and to a FXII gene mutation frequency.[8,9] Most of the population incidence and thrombotic risk studies have focused on families and individuals of German, Spanish, Italian, and Swiss ethnicity who carry a congenital abnormality. Acquired FXII inhibitors have also been identified in patients with anti-phospholipid syndrome (APS), metastatic cancer, and liver disease.[10-12] Acquired FXII inhibitors are generally antibodies directed against FXII.

Controversy about the role of FXII in thrombotic events and pregnancy loss continues to be a topic of research and speculation. Early reports of severe FXII deficient individuals (having less than 1% procoagulant activity determined by an activated partial thromboplastin time or APTT) indicated a possible association with the risk of developing a venous thrombus. Seven out of eleven cases reported from 1968 through 1990 had some other risk factor known to predispose someone to venous thrombosis, such as pregnancy, Factor V Leiden or antithrombin mutation, traumatic injury, or sepsis.[13] The four other cases had no information about associated risk factors or family history of the individuals reported. Girolami et al. argue that concluding an association of prothrombitic risk and low levels of FXII is unjustified even in arterial thromboses such as myocardial infarctions since they have followed

21 severely deficient patients and 58 patients moderately deficient in FXII for 16 years and only 1 patient developed a heart attack.[14] Other large studies of coronary heart disease (CHD) and myocardial infarction (MI) have reported conflicting results concerning the impact of FXII levels on disease outcome and risk. A study of 2464 middle aged men who had at least one previous MI found a statistically significant, independent, and positive association of FXIIa with other CHD risk factors of serum cholesterol and triglyceride concentrations, blood pressure, body mass index, factor VII activity, plasma fibrinogen concentration, and tobacco smoking.[15] Another study examining survivors of myocardial infarction found FXIIa levels positively correlated with factor VII coagulant activity, body mass index, cholesterol, insulin, plasminogen activator inhibitor-1 (PAI-1) antigen, tissue plasminogen activator (tPA) antigen and triglycerides. Factor XIIa levels were associated strongly with the extent of coronary stenosis. The data suggests that the high levels of FXIIa could be a marker of increased coagulability in these patients and it could represent a persistent hypercoagulable state for further coronary thrombosis.[16] In contrast, a coronary prevention study of Scottish men with high cholesterol showed an association of a common FXII gene mutation (resulting in lower levels of circulating FXII) with a higher risk of developing CHD.[17] Factor XIIa levels may reflect contact activation induced by higher levels of lipids (see next section on Serine Proteases Activation) or inactivated FXIIa bound to an inhibitor yet still detectable with an antibody. Lower FXIIa levels could indicate a loss of fibrinolytic activity (see section on FXII In Fibrinolysis) resulting in enhanced thrombus retention in the vessel. Other studies agree with one outcome or the other, leaving it unclear if FXII should be considered a

marker of atherosclerotic vascular damage[18], an independent risk factor for CHD[19], or just a consequence of a thrombotic disease state.[20]

The question of FXII's importance in pregnancy retention or fetal loss is also under debate. In the early 1990s FXII levels were evaluated during pregnancy and found to increase during the last trimester.[21] Fertility researchers and clinicians began to report cases in which patients with low levels of FXII had multiple spontaneous abortions before the third trimester.[22,23] Some of these cases may have underlying thrombophilic risk factors such as Factor V Leiden mutation, autoantibodies to phosphatidylethanolamine (aPE), or anti-phospholipid syndrome (APS) that are associated with pregnancy loss.[24,25] Again, the associations of low FXII levels with pregnancy complications were contradicted by findings of no increased pregnancy losses or thrombotic events in severely FXII deficient women.[26] Animal models of FXII deficiency have addressed these questions of thrombosis and pregnancy outcomes. In 2004, Pauer et al. generated a mouse model by targeted deletion of the FXII gene (referred to as FXII -/- for homozygous gene deletion) that has no FXII transcript nor protein production.[27] When male FXII -/- and female FXII -/- mice were mated, litter sizes were the same as related normal (referred to as wild-type or FXII +/+) mice from the same genetic backgrounds. They also found no thrombophilia in the FXII deficient mice.[27] The mouse model of FXII deficiency indicates there is no association of FXII with fetal loss or increased thrombotic tendencies. The feline model of congenital FXII deficiency is currently being studied for fetal loss. Preliminary observations suggest there might be an association of low

levels of FXII with fewer kittens born (unpublished data) however issues of genetic background and breeding environment need to be addressed before any conclusions can be drawn. More direct assessment of FXII's *in vivo* role during pregnancy is needed to settle this controversy.

The mouse FXII deficient model was created to clarify the discrepancies between studies of humans with Hageman trait, however new conflicts have arisen. Vascular injury studies on aortas and carotid arteries indicate FXII-/- mice form reduced size thrombi that are less stable at sites of attachment to the vasculature. Further studies with FXII-/- mice suggested that thrombin-induced activated platelets may promote thrombus formation in a FXII-dependent way.[28] These animal studies and others confirmed that FXII is not necessary for hemostasis, but opened new discussions about the role of FXII in pathological thrombus formation (see next section for more details) and mouse coagulation.[29] Factor XII deficiency may have a protective effect by forming smaller, looser thrombi, however these thrombi could break away and form pathological thrombi elsewhere. The vascular injury studies presented in this dissertation continue the discussions and add new conflicting data about the *in vivo* role of FXII.

Serine Proteases Structure, Function, and Activation in Contact Activation

Nearly all the proteins involved in blood coagulation and complement activation are serine proteases. Serine proteases are enzymes that cleave specific

peptides and have at least one serine amino acid residue in their active site. Serine proteases are grouped into families and clans according to structural similarities or homologies and sequence similarities based on their catalytic domain characteristics.[30] Most serine proteases are secreted as inactive precursors or zymogens which require proteolysis to become active and capable of hydrolyzing peptide bonds of their target protein. Cardinal features of serine proteases are the formation of an oxyanion hole after zymogen activation, the conserved geometrical location of a nucleophilic Serine (Ser) adjacent to a histidine (His) in the catalytic site, and a buried 'proton accepting' aspartate (Asp) in the catalytic apparatus.[30] The Ser-His-Asp formation is referred to as the 'catalytic triad' and provides for strong hydrogen bonding between His-Asp and proton transferring between the non-covalently bound substrate and active site residues. Coagulation and fibrinolytic proteases have inhibitors (serine protease inhibitors or serpins) that prevent their activation and limit the clotting or fibrinolytic process—a way to maintain homeostasis. Factor XII is a serine protease that has multiple inhibitors: Complement 1 inhibitor (C1-INH), antithrombin III (ATIII), alpha-2-antiplasmin, and the non-serpin inhibitor alpha-2-macroglobulin.[31,32] Complement 1 inhibitor is the major plasma inhibitor of FXII and its substrate kallikrein.[32] Factor XII can be 'autoactivated' via a conformational change when it contacts certain surfaces, hence it is called a contact activation factor.[4,33] Previous thinking was that FXII zymogen became activated to FXIIa when it contacted anionic 'negatively charged' hydrophilic surfaces. New evidence shows spontaneous activation of FXII occurs upon contact with anionic surfaces, however FXIIa production can occur when the zymogen is

putatively adsorbed to a hydrophobic (water repelling) surface then displaced by some other protein.[33] Furthermore, the manner of zymogen adsorbing to a surface (transiently vs. directly) can influence whether the kallikrein-kinin or coagulation pathway is activated by FXIIa.[34] Over the years, various FXII-activating substances have been identified: glass, polyethylene, silicone rubber, metals, kaolin, ellagic acid, dextran sulfate, sulphatides, endothelial cell-associated glycosaminoglycans, polyphosphates, phospholipids, sodium urate crystals, free nucleic acids, aggregated amyloid peptides, articular cartilage, heparins, bacterial endotoxins, misfolded proteins, type I collagen and many other agents. [4,33,34] Recently it was reported that aggregates of misfolded proteins (often found in inflammatory diseases) could activate FXII without initiating the coagulation cascade protease target factor XI (FXI)[34], while type I collagen also activates FXII[35]. This new information about physiologically relevant activators has sparked new interest in the role of FXII in inflammation (to be discussed later).

Coagulation is the process in which blood serine proteases activate each other to form a clot (thrombus). The thrombus is composed of a network of polymerized fibrin, platelets, and blood cells that associate in a way that allows immediate reduction or stoppage of fluid flow yet can be lysed at a later time. A delicate balance between hemostasis and thrombosis is provided by complex protein interactions, feedback activation mechanisms, and specific proteolytic inhibitors. When a vessel is injured, the blood cells, platelets, endothelial cells, pericytes and surrounding matrix provide substances that are thrombogenic i.e. they promote clot

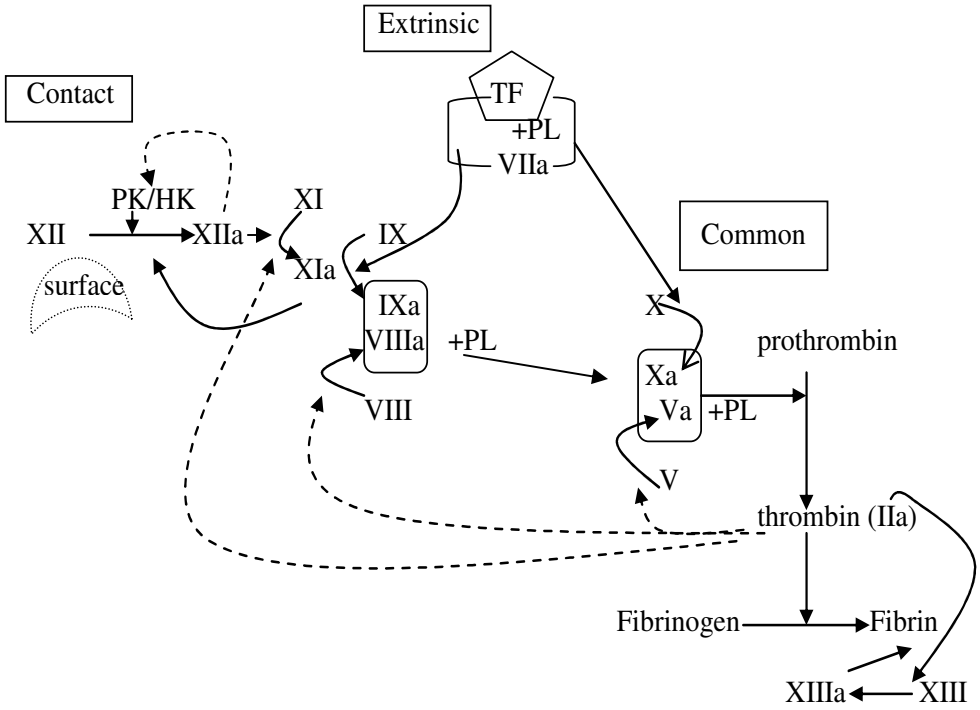
formation by the intrinsic (contact activation factors plus factor IX and VIII) and extrinsic (tissue factor required) coagulation pathways.[36-38] The two pathways are not exclusive of each other: Tissue factor expressed on perturbed pericytes/adventitial cells and monocytes or released from platelets, leukocytes, and endothelial cells in the form of microparticles (MP) binds with factor VIIa to activate the intrinsic pathway at factor IX (FIX) and the extrinsic pathway at factor X (FX). The pathways converge into a "common" pathway (at factor Xa) which continues the proteolytic cascade until the final target, fibrinogen, is activated to fibrin by thrombin.[29,36-38] Figure 1.1 illustrates the interactions of the pathways. Factor IXa requires a cofactor, factor VIII (FVIII), and phospholipids in order to convert FX to FXa. Absence of FVIII or FIX results in the bleeding disorders hemophilia A or B, respectively.[36] In hemophiliac patients, clotting can be induced by giving them supraphysiological doses of factor VIIa (FVIIa), a serine protease that complexes with TF to initiate the extrinsic pathway.[36] Factor X and its ability to complex with factor V (FV) and phospholipids to form the "prothrombinase" complex is critical for activating small amounts of thrombin. Thrombin (FIIa) is a serine protease that acts on multiple substrates including fibrinogen, factor XIII (fibrin stabilizing factor), FV, FVIII, and FXI, and glycoprotein V on platelet surfaces. The positive feedback action induced by trace amounts of thrombin generated during extrinsic pathway activation helps to accelerate thrombin production and create a "burst" of thrombin at the injury site.[36] Thrombin is a crucial element of clotting, therefore most anticoagulant therapies target or involve thrombin inhibition. The main plasma inhibitors of thrombin are

antithrombin III, heparin (in complex with antithrombin III), protein C inhibitor (PCI), and plasminogen activator inhibitor-1.

In vivo coagulation begins with the adherence and spreading of platelets to the damaged endothelial cells, followed by a secondary phase mostly initiated by the extrinsic pathway via tissue factor (TF) either bound to a membrane or blood-borne.[35-38] *In vitro* coagulation is initiated by FXII directly adsorbing to a surface of glass or polystyrene.[33,36-38] In humans and animals, the loss of contact activation factors FXII, prekallikrein (PK), and high molecular weight kininogen (HK) does not result in a bleeding tendency, thus the intrinsic coagulation pathway provides an alternate way to generate more thrombin.[35,36,38] *Ex vivo* studies have shown that the amount and rate of thrombin activation may determine the strength and stability of the clot formed.[39-41] When the large hexameric glycoprotein fibrinogen is cleaved by thrombin, the Aα and Bβ chains of fibrinogen lose their N-termini and "half-staggered, double-stranded protofibrils [form] followed by thickening of protofibril chains".[39] The double-stranded protofibrils interact laterally to form long thin strands/fibers and eventually polymer chains then networks.[36] Factor XIII (with calcium) is activated by thrombin, induces cross-linking of the fibrin polymers, and confers stability i.e. resistance to plasmin degradation.[36,39] Clots formed *in vitro* by contact activation/FXIa[40] or under higher concentrations of thrombin[41] are composed of thinner fibers and are more stable and recalcitrant to lysis. Since fibrin is a substrate and a cofactor for the main fibrinolytic protease plasmin, its availability for interacting with plasmin can influence the rate of clot lysis.[39] Thin fibrin fibers

generate plasmin at a reduced rate[42] by possibly limiting the access of plasminogen activators or by increasing an inhibitor of fibrinolysis. Clots containing loosely assembled, thicker fibrin fibers are easier to lyse and can be generated by TF-FVIIa/Xa initiated coagulation.[40] New evidence that collagen can activate FXII and activate platelets at their immunoglobulin receptor glycoprotein VI (GPVI) suggests an "initial" appearance of the contact activation pathway is seen *in vivo* during the primary phase of hemostasis.[35] The early activation of FXII can provide a way to generate coagulation factors (i.e. thrombin) at the attached platelet surface and help strengthen and stabilize the clot. These intriguing findings suggest that driving a coagulation pathway toward an extrinsic activation method could affect clot structure and stability which may be important in pathological outcomes. The contact activation pathway may be more important than previously thought.

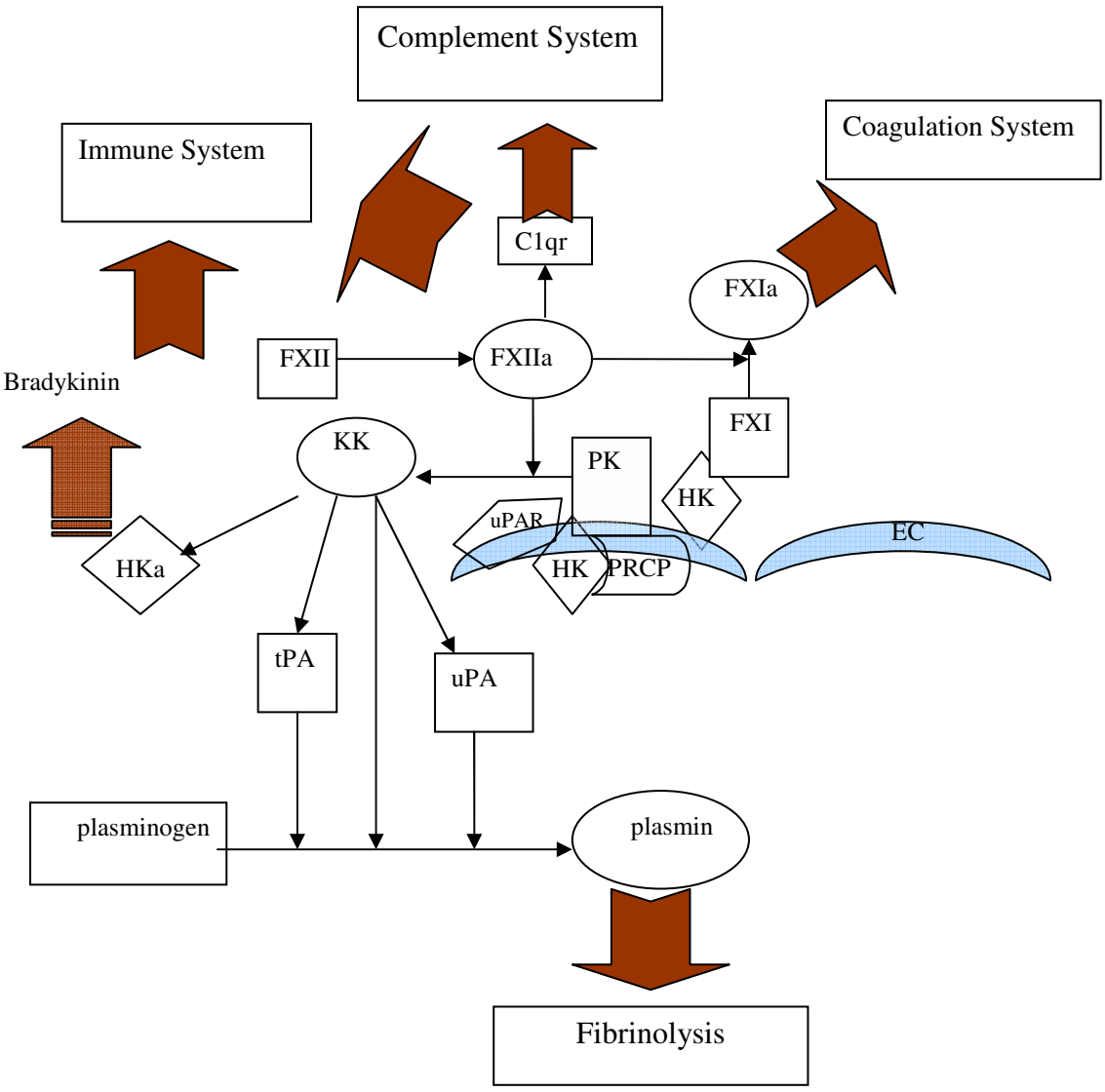
Figure 1.1: *Coagulation Pathways*



Contact activation factors connect hemostasis with other biologically important pathways such as kallikrein-kinin, complement activation, fibrinolysis, and extracellular remodeling. Activated FXII or prolylycarboxypeptidase cleave the zymogen prekallikrein to kallikrein thereby initiating the plasma kallikrein-kinin system (KKS).[32] Plasma and tissue kallikreins are serine proteases that cleave prourokinase, prorenin, FXII, and HK (further cleaves into bradykinin).[36] Kallikrein's activation of FXII generates a positive feedback regulatory mechanism to amplify the contact activation pathway in plasma and on the surface of endothelial cells. Prekallikrein reversibly binds to the surface of neutrophils, platelets, endothelial cells

and matrix and can activate HK in its membrane-bound receptor complex. Kallikrein activates a second plasminogen activator, prourokinase (single chain uPA) to urokinase (uPA) on the endothelial cell—initiating fibrinolysis at the cell/vessel surface.[32,36] High molecular weight kininogen is a cofactor/substrate that forms a complex with globular complement C1q receptor (gC1qR), urokinase plasminogen activator receptor (uPAR), cytokeratin 1 (CK1), and prolylycarboxypeptidase (PRCP).[32,36] This complex of HK-gC1qR-uPAR-CK1 can serve as a template on endothelial cell surfaces to bind and activate FXII, or in the absence of FXII it can activate PK (if PRCP is present).[32] High molecular weight kininogen also serves as a bridge for neutrophil binding to platelets at the glycoprotein Ibα-V-IX complex.[43] Activated HK (HKa) has anti-adhesive and apoptotic properties attributed to its interactions with extracellular matrix proteins (ECM).[44] Activated HK is also important in liberating bradykinin (a nonpeptide hormone) which is a potent activator of tissue-type plasminogen activator (tPA), and releasing agent of nitric oxide (NO) and prostacyclin from endothelial cells.[32,45] Bradykinin reduces/regulates blood pressure (as an endogenous vasodilator), causes capillary leakage and edema (and pain), inhibits platelet responses, and stimulates intimal hypertrophy and smooth muscle cell proliferation after vascular damage.[46] Over generation of bradykinin impacts inflammatory diseases such as hereditary angio-edema, vasculitis, ulcerative colitis, arthritis, and encephalomyelitis.[46-48] Since the KKS is important in inflammation, fibrinolysis, and vascular remodeling, its modulation is important in many diseases. The multiple pathway interactions of the contact activation system are shown in Figure 1.2.

Factor XII has a more pivotal role *in vivo* because it can directly activate complement, plasminogen (fibrinolysis target), leukocytes, and extracellular matrix metalloproteinases (remodeling enzymes) in addition to its indirect actions via the KKS. Each of these interactions will be discussed in depth in the subsequent sections. **Figure 1.2**: *FXII/Contact Activation In Multiple Pathways*



FXII Domain Structures: Analyses and Implications They Carry

Human FXII protein exists as a ~80,000 Daltons (~80 kDa) zymogen in the blood until being activated by surfaces, kallikrein, plasminogen (PLG), or itself. Activation of FXII at the arginine (Arg)^{353}-valine (Val)^{354} (a newer numbering system lists it as Arg^{373}-Val^{374} however I will use the traditional nomenclature) bond results in two main products FXIIa and FXII fragment (FXIIf or β-FXIIa, ~30 kDa).[49] Factor XIIa is a two-chain molecule (~80 kDa) having a heavy chain (353 residues, ~52 kDa) and a catalytically active light chain (243 residues, ~28 kDa) held together by a disulfide bond. Factor XIIa is further hydrolyzed by kallikrein at the Arg^{334}-Asn^{335} and Arg^{343}-leucine (Leu)^{344} residues into products lacking the heavy chain, therefore it cannot bind surfaces and activate FXI. The two β-FXIIa products retain the catalytic triad active region allowing them to cleave some zymogens such as PK, but they differ in structure by being disulfide linked to either a 10- or 19-amino acid residue carboxyl-terminal end.[49] Computer-modeling suggests β-FXIIa shares a 3-dimensional structural homology to pancreatic serine proteases, but little is known about its function(s).[50]

The light chain of FXIIa is the serine protease portion of the molecule and homologous to tPA, with the highest homology occurring at the catalytic triad region (His^{393}, Asp^{442}, Ser^{544}).[49,50] The light chain comprises Val^{354}-Ser^{596} amino acid residues and contains one amino-linked carbohydrate chain on Asn^{414}.[49] Plasma

activity of the light chain is fully expressed when binding of the heavy chain to a surface, HK, and kallikrein or FXI are all contiguous.[49] Bound two-chain FXIIa can hydrolyze the substrates FXI or PK which are noncovalently associated with HK in the plasma. Zinc accelerates the autoactivation and hydrolysis by kallikrein.[51] The main inhibitor of FXIIa, C1-INH, reversibly binds in the exposed reactive site by presenting a peptidyl bond (P1-P1') that matches the P1(Arg) –P1'(Threonine) of its substrate (FXIIa).[52] Specificity of the inhibitor binding is determined by the P1 residue of the substrate—Arg^{353} for FXIIa.

The heavy chain of FXIIa has multiple structural domains of which little is known about their specific function. The human FXII heavy chain amino acid structure was first reported[53] and confirmed[54] for the predicted primary structure in 1985 however no three-dimensional structure has been reported to date. The heavy chain comprises isoleucine (Ile)[1] through Arg^{353}. Amino acids Ile^{1} through Cys^{28} on the amino-terminus (N-terminus) bind platelets at the glycoprotein Ibα-IX-V complex and are part of the ~60 residue region (Lys^{13}-Cys^{69}) that shares homology with the type II region of fibronectin.[49,55] This type II fibronectin-like domain is predicted to be a collagen binding region where FXII putatively binds anionic surfaces[56] and the HK-gC1qR-uPAR-cytokeratin 1 complex on endothelial cells[57] (at FXII residues Tyr^{39}-Arg^{47}). The next secondary structural domains are two epidermal growth factor-like domains (residues Cys^{79}-Cys^{111} and Cys^{159}-Cys^{190}) and a type I fibronectin-like domain. Epidermal growth factor-like domains are found in many proteins including transforming growth factor type 1, tPA, factor IX, and several

coagulation proteins.[49] The epidermal growth factor domains in FXII may be regions of neutrophil and monocyte binding.[49,58] Factor XII residues Cys^{116}-Cys^{144} comprise the "finger shaped" type I fibronectin-like domain that are thought to be important in binding fibrinogen, heparin, amyloid, and ECM proteins fibronectin and vitronectin.[49,54,59,60] The remaining amino acid residues of the FXIIa heavy chain form a kringle domain (Cys^{198}-Cys^{276}) and a proline-rich region (proline $(Pro)^{313}$-Arg^{334} and Leu^{344}-Arg^{353}). The kringle domain function in FXII is unclear but suspected to participate in binding fibrinogen and fibrin. Multiple kringle domains have been found in prothrombin, plasminogen, urokinase (uPA), and tPA.[49] The proline-rich region participates in artificial surface binding[61] and perhaps other unknown functions.

FXII In Fibrinolysis

Fibrinolysis (also called thrombolysis) is the break-down or degradation of a fibrin clot into fibrin degradation products (FDPs) by a variety of enzymes. The fibrin clot is actually a mixture of plasminogen, thrombin, and other hemostasis proteins bound to fibrinogen and fibrin strands. Fibrin can regulate its own degradation by binding plasminogen and its activator tPA to its surface.[62] In the presence of fibrin, tPA's catalytic activity is increased by more than 2 orders of magnitude. The main enzyme that initiates the fibrinolytic process is the serine protease plasmin, derived from the zymogen plasminogen. Plasminogen binds to fibrin through kringle domains and can be activated to plasmin by tPA, uPA, FXIIa, and possibly unknown proteases.[62] As plasmin cleaves fibrin, soluble degradation

products are formed and carboxy-terminal lysine residues are exposed. The kringle domains of tPA and plasminogen contain lysine-binding sites to aid in fibrin binding and enhance degradation. The thrombin-activatable fibrinolysis inhibitor (TAFI) can remove carboxy-terminal lysine residues, thereby inhibiting plasmin generation and fibrinolysis.[62] Plasmin has two main endogenous inhibitors: alpha-2-antiplasmin and alpha-2-macroglobulin. When plasmin is bound to fibrin, alpha-2-antiplasmin cannot bind to plasmin. However, TAFI can remove the lysine residues that would bind plasminogen thereby attenuating fibrinolysis. The activation of plasminogen is controlled by the fast-acting plasma protease inhibitors plasminogen activator inhibitor-1 and -2 (PAI-1 and PAI-2), C1-INH, and the broadly specific alpha-2-macroglobulin. Plasminogen activator inhibitor-2 is found in high levels only during pregnancy, thus not normally a significant serpin. Almost immediately upon secretion, tPA and uPA bind to PAI-1 which is secreted by endothelial cells, macrophages, monocytes, adipocytes, and platelets. The tPA-PAI-1 complex is stabilized by matrix proteins (primarily vitronectin).[63] Tissue plasminogen activator and uPA are secreted as "less active" single chain molecules by endothelial cells, but uPA is also expressed on macrophages and renal epithelial cells. The single chain-uPA is cleaved to a high and a low molecular weight fragment, but only the high molecular weight can bind to uPA-receptor on endothelial cells, monocytes, macrophages, fibroblasts.[62] The function of the uPAR is still unclear, but it is thought to be important in regulating cell adhesion and cell signaling.[64] Most of the fibrinolytic activity of plasma is attributed to tPA, whereas uPA appears to be more active in tissues.

Currently very little is known about FXII's participation in fibrinolysis, with most of our knowledge being gained through *in vitro* assays. Braat et al. published the most extensive evaluation and quantification of FXII as a plasminogen activator.[65] Their studies used a purified system and dextran sulfate-activated human plasma euglobulin fractions that were evaluated in a fibrin clot microtiter plate assay. The euglobulin fraction is formed by precipitation of plasma proteins and collection of the precipitate. Kluft determined that a large amount of C1-INH and other inhibitors were also precipitated in the euglobulin fraction, and altering the ionic strength or pH could influence their amounts.[66] Activating the plasma with dextran sulfate (an activator of FXII), altering the pH, and adding flufenamate (inhibits C1-INH and alpha-2-antiplasmin) yields the highest level of fibrinolytic activity that is contact activation factor dependent.[65,67,68] Braat et al. tested purified FXII activated with dextran sulfate in buffer and found low levels of plasminogen activator (PA) activity. However, dextran sulfate added to plasma (dextran sulfate euglobulin fraction or DEF) and blocking of tPA and uPA activity resulted in a 6 fold increase in PA activity. The direct contribution of FXIIa as a PA in normal plasma was determined to be about 12% (or 20% of contact activation-dependent PA activity).[65] Earlier studies by Kluft et al. and Levi et al. determined contact activation factor contribution was 50%[69], however FXII may only contribute half of that or ~25% (tPA contributes 50% overall, uPA contributes 25% overall).[70] It appears FXII can provide 12-25% of the fibrinolytic activity in plasma.

Persons with severe FXII deficiency have been reported to have low plasma (euglobulin fraction) fibrinolytic activity related to FXII plasminogen activation.[70-72] Speculation has been made that FXII deficient individuals may be prone to pathological thrombus formation (attributed to reduced fibrinolytic ability), yet no clear evidences have been found. *In vivo* animal studies using FXII deficient subjects have not fully addressed this question.

FXII In Inflammation

Inflammation is a multi-step process that occurs as a response to injury or infection in vascularized tissues. Serine proteases, such as FXII and kallikrein, play a pivotal role in initiating primary response elements necessary for host defense and homeostasis after an injury. Factor XII is particularly interesting because it has the power to activate four systems involved in the inflammatory process: kallikrein-kinin, clotting, fibrinolytic, and complement. Each of these systems have a cascading effect on other systems through the production of chemical mediators acting to recruit cellular responders (e.g. leukocytes, fibroblasts, smooth muscle cells, endothelial cells) while maintaining primary host defenses (e.g. bacterial removal) and hemostasis.

For over 50 years the contact activation system's involvement in inflammation has been studied, however mostly in a venue of *in vitro*, manipulated environments using Boyden chemotaxis chambers, microcapillary flow chambers, and cell or tissue culture methods. Factor XIIa can directly bind to immune cells and

endothelial cells[73]. Neutrophils (PMNs) have been shown to directly bind fibrin and FXIIa through the Mac-1 or CD11b/CD18 receptor via the protease-mediated pathways of cathepsin G and elastase stored in their azurophil granules.[74,75] Upon binding FXIIa, the PMNs can release the contents of the granules to aid in degrading bacteria, collagen, elastin, and other proteoglycans. Degradation products of collagen are chemotactic for PMNs, monocytes, and fibroblasts. Macrophages (or tissue monocytes) are important in host defense against bacteria and clearing immunoglobulin (Ig)-coated cells or Ig-complexes that bind to the macrophage Fc gamma receptor I (FcγRI). Factor XIIa can reduce the number of FcγRI ligand binding sites on macrophages by 80%.[58] Reduced efficiency of FcγRI-IgG interactions can compromise the clearance of Ig complexes and exacerbate an inflammatory response.[76] Factor XII has been shown to bind platelets at their glycoprotein Ibα (GPIbα) receptor, the receptor where HK can act as a bridge for PMN-surface-adherent platelet interactions.[77] The binding of FXII to the GPIbα receptor can inhibit thrombin binding and platelet aggregation[78], suggesting FXII may be important in homeostatic regulation of leukocyte-platelet interactions on the endothelium.

Factor XII can indirectly effect inflammatory reactions by inducing chemokines, activating complement, and liberating bradykinin. Early studies in FXII-deficient humans[79,80] and cats[81] found skin reactions to FXII activators or complement activators resulted in significantly less local inflammation. The results of these experiments suggested the complement system and/or the KKS were not

effectively activated in the absence of FXII. Recently Zabel et al. found FXIIa is a potent activator of the serum chemoattractant chemerin which is known to activate dendritic cells and macrophages.[82]

Factor XIIa has been shown to activate complement protein $C1qr_2s_2$ to C1s and C1r[83], the first components of a "classical" complement protease cascade in which products C3a, C4a, and C5a serve as chemoattractants, platelet activators, and lysis-inducing agents of pathogens and cells.[84] Complement activation generates a proinflammatory response by the body that is primarily controlled by C1-INH—giving this serpin multiple biological properties also.

Very little research has addressed the importance of FXII in leukocyte chemotaxis as a result of vascular injury. Thrombus development is an inflammatory process involving chemokine and cytokine driven cellular responses to the site of vessel injury. Various groups have looked at monocyte[85], neutrophil[86-88], and mast cell[89] recruitment to the thrombus and have defined certain inflammatory mediators are important in thrombus development and resolution. Most of this research has focused on chemokine, cytokine, and matrix metalloproteases roles in vascular inflammation without addressing contact activation factors participation in this process.

FXII and Matrix Metalloproteinases In The Wound Repair and Remodeling Process

Besides a role for FXII in the initial inflammatory responses to injury, FXII may also play a role in proliferation of tissue by activating matrix metalloproteinases.

Matrix metalloproteinases (MMPs) are zinc-dependent endopeptidases capable of degrading all types of matrix proteins (such as basement membrane, collagens, proteoglycans, fibronectin, laminin) and plasma proteins such as fibrinogen and FXII.[90] The MMPs are synthesized as zymogens then secreted or membrane-bound until catalyzed to their active form. Fibroblasts, smooth muscle cells[91], monocytes/macrophages[92], NK cells[93], and neutrophils[94] are reported to produce MMP-1. Fibroblasts, macrophages and neutrophils can produce MMP-9.[91,92,94] Factor XII can be degraded by MMP-8, MMP-12, MMP-13, or MMP-14 by proteolysis down-stream of the catalytic region at Gly^{376}-Leu^{377}.[90,91] Activation of MMPs by coagulation serine proteases has recently become a topic of interest for regulating vascular remodeling and local inflammatory processes in damaged tissues. Factor XII's role in vascular regression/matrix degradation was identified through *in vitro* studies with endothelial cell capillary tubes, finding plasma PK induced MMP-1 activation and contraction of the tubes only in the presence of FXII or HK.[92] Currently there are no reports regarding the interactions of MMPs and FXII *in vivo*.

Vascular remodeling and wound repair normally begin immediately after injury. After damage occurs, hemostasis begins and initiates/coincides with an inflammatory response. Damaged cells release tissue factor, heme proteins, superoxide particles and other chemotactic factors that activate platelets and signal the immune system, surrounding fibroblasts, endothelial cells, and smooth muscle cells to respond. Platelets adhere to the ECM and release growth factors while providing a procoagulant surface for thrombin activation and eventually clot formation (as described earlier). Thrombin, plasmin, FXII, complement, and other

blood proteins can induce leukocyte chemotaxis and provide growth factors for propagating the inflammatory and proliferative responses just beginning. Proinflammatory cytokines interleukin-1β (IL-1β), tissue necrosis factor-alpha (TNF-α), monocyte chemoattractant protein-1 (MCP-1), IL-6 and IL-8 are secreted by infiltrating lymphocytes, macrophages and smooth muscle cells to recruit other cells and begin matrix degradation via MMP-1,-2 and-9.[91-93,95] Degradation of the matrix serves to make migration easier, recruit cells (collagen fragments are chemoattractants and MMPs can process chemokines) and remodel infrastructure so new vessels and cell connections can develop. The inflammatory phase takes place from 1 hour to 4 days in large mammals.[97] Matrix metalloproteinase-2 gelatinase/collagenase activity is increased in mice at 4 days post intimal injury[98], whereas MMP-9 gelatinolytic activity markedly increases at 4 hours.[99] Matrix metalloproteinase-1 is a collagenase, therefore it would be expected to be increased around the same time as MMP-2. Proliferation involves mesenchymal and epithelial cell migration, fibroblast proliferation (to lay down new matrix), and angiogenesis.[100] During the proliferative phase chemokine suppression is increased by IL-10 and transforming growth factor β (TGFβ) (it also promotes growth), while angiogenesis is promoted by vascular endothelial growth factor (VEGF), fibroblast growth factor-1 (FGF-1), angiopoeitins, Delta-like ligand 4 (DLL4), plasminogen activators, platelet derived growth factor (PDGF), tissue factor, and other factors.[101] The proliferative phase is considered to take place from four days to two weeks (in mice it is 48 hours to 5 days), overlapping with inflammation and the next phase of remodeling.[97] Remodeling involves laying down new collagen matrix thereby suppressing MMP

activity with tissue inhibitor of metalloproteinases (TIMPs) and scavenging of debris by macrophages. This final process takes place between 14 days and 2 months in large mammals (5 days to 28 days in rodents).[97] Not surprising, wound healing processes are delayed in hemophiliacs because a multitude of issues must be overcome from the lack of proper coagulation pathway(s) interactions.[102,103] Precise timing of cellular/protein interactions along with proper quantities of factors are required in these complex biological processes to avoid a pathological state. We need more *in vivo* research in areas of hemostasis, fibrinolysis, inflammation, and wound repair—especially in the case of FXII.

Experimental Vascular Injury: Methods and Locations

As anyone may assume, there are many ways to injure a vessel. The goal of this section is to delineate why one general method may be better than the other, and why we chose our methods and locations.

This brief background on vessel differences and thrombi differences is mostly derived from the Bodary and Eitzman[104] and Whinna[105]reviews. Not all thrombi are the same in composition and how they develop. Thrombus formation is complex and depends on factors such as blood flow rate, shear stresses, cell types, coagulation or fibrinolytic factors present, the amount of collagen/matrix exposure, and length of time of stasis to name a few. The size of the vessel and the thickness of its wall matters. Microvessels (capillaries, arterioles and venules) have smaller diameters that can accommodate one or two cell widths, and they have thin (one to four layered) walls to contain a slow flow of blood for cellular respiration. Their thin

walls and slow flow allow for easier real-time monitoring of labeled cellular or protein interactions at the site of injury. Circulating tissue factor is more important for thrombus formation in microvessels[104, 106] whereas vessel wall TF expression is more relevant to macrovessel (carotid and inferior vena cava) [107, 108] thrombus formation. Microvessel venules are less sensitive to collagen and thrombin inhibition compared to arterioles, however they both respond to inhibition of platelet phosphatidylserine (PS).[109] Platelet aggregation and the length of time needed to interact with the arteriole wall have also been found important in microvessels—this may be true in larger arteries but real-time evaluation of this effect is more difficult.[110]

In fast flowing vessels having high pressure and high stress forces (i.e. thick walled arteries) thrombus formation is primarily platelet dependent. Platelet tethering to the vessel wall via the GPIbα receptors (with von Willebrand factor binding)[111], activation by the GPIIb/IIa receptor[112], and thrombin[113] are identified factors needed during high shear flow conditions and can be induced by the forces[114,115]. In mice it appears FXII is needed during arterial thrombosis.[28] The collagen-rich arterial walls provide an activating agent for platelets and FXII after the protective endothelium is removed (denuded). Of course the quantity and quality of platelets are important for arterial thrombosis too. Thrombi formed in the high flow rate arteries are platelet-rich and have tails that are fibrin rich (where flow is reduced).[116] The rapid conversion of fibrinogen by platelets and thrombin in these thrombi lead to more cross-linking of fibrin strands, thereby incorporating more plasminogen. These

thrombi tend to be more readily lysed by tPA (in their fibrin-rich "red" regions) but only during a 3 hour window after formation, for reasons unknown.[104,117]

Venous thrombosis development is viewed in the historical context given by Virchow in 1855. Virchow ascribed "a triad" of conditions involving reduced hemodynamics of flow (stasis), dysfunction of the endothelium (or injury), and a thrombogenic (hypercoaguable) environment are needed for venous thrombus development.[118,119] Thrombi formed under stasis conditions are thought to be more dependent on coagulation factors and fibrinolysis inhibition than rapid platelet and thrombin activation.[116] The pooling blood may contribute to contact activation and endothelial dysfunction, however stasis alone does not appear to induce thrombosis.[120] Little is known about the exact mechanisms involved. White blood cells (WBC) have been found important in the development[88] and resolution of venous thrombi.[87,89] The availability of cell adhesion molecules (CAMs) and selectins (P- and E-selectin) is important for leukocyte transmigration through the vascular walls.[120] Thrombi formed during low flow conditions tend to have more red blood cells and less platelets in their composition[121], however platelets are important for the formation of a clot regardless of the vessel type.[122]

Various mechanical methods of vessel injury can be used experimentally to study thrombus formation or resolution. Arterial studies emulating coronary, carotid, or femoral artery damage relevant to human disease conditions generally employ chemical materials (ferric chloride, rose Bengal photochemical) or physical trauma (forceps, clamps, catheters, brushes, lasers) that are externally or internally applied.[105] The method chosen mostly depends on the animal used, the equipment available, the

question being asked, and the molecular/cellular mechanism wanting to be studied. We chose a brush injury model because it offered a way to denude endothelial cells and cause "mild" intimal injury without generating a large amount of collagen/matrix exposure as seen in Folt's[123,124] and ferric chloride methods[125]. The mild localized damage causes moderate flow alterations (detectable with a Doppler probe) that may reflect injury seen in humans patients undergoing endarterectomy and catheterization procedures.[124] The milder brush damage may reflect or promote a contact activation pathway manner of thrombus development.

Objectives Of This Dissertation Research

Many questions concerning FXII and its *in vivo* roles have developed over the years. The unexpected observations seen in the knock-out mice bring more questions about the role of FXII in humans and other species. The main objective of this dissertation work is to define whether FXII deficiency results in altered thrombotic and fibrinolytic responses in large, deep vessels after injury. Additionally, we asked if inflammation was reduced in FXII deficients at the site of injury and tried to provide insight to the reason (assuming there might be reduced inflammation). We also explored the role of FXII in a skin inflammation model to determine the extent of its participation in local inflammation using an endogenously present inflammatory agent, the blood cell degradation product heme (or hemin). Finally, we aimed to define the FXII gene defect and protein associated alterations in our cat colony.

CHAPTER 2

FELINE FXII GENE AND MUTATION

ABSTRACT

Coagulation factor XII (FXII) assists in thrombus formation and may be important in cardiovascular and inflammatory mediated diseases. We have identified a naturally occurring mutation in the feline FXII gene that encodes a catalytically inactive mutant protein. We sequenced the genomic loci encoding factor XII in wild type cats and analyzed mRNA sequences encoding FXII in wild type and FXII deficient mutants. The feline Factor XII gene comprises fourteen exons ranging in size from 57 to 222 base pairs and spanning 11 kilobases on chromosome A1. The wild type feline FXII gene encodes a transcript that is 1833 base pair long and contains an open reading frame encoding a protein of 610 amino acids. Analysis of RNA from factor XII wild type and mutant cats revealed one single base deletion in exon 11 of the FXII coding gene in the FXII deficient cats. The deletion caused a frame-shift at L^{442} ($Leu^{442}Cys$) resulting in a nonsense mutation, a premature stop codon, and a truncated factor XII protein of 561 amino acids. The mutant FXII protein lacks the catalytic triad domain located in the C-terminus between His^{405} and

Asp[454] explaining its loss of enzymatic activity. This study is the first to identify the genomic sequence and structure of the feline FXII gene. We identified a FXII mutation in the domestic cat, providing insights into the origin and nature of feline FXII deficiency.

INTRODUCTION

Deficiency of factor XII (or Hageman factor) was first described in 1955 in a human patient who lacked plasma enzymatic activity.[3] Although FXII deficiency does not present as a bleeding tendency, recent evidences indicate FXII may be important in maintaining blood clot stability and pregnancy.[28,126] The gene encoding FXII in multiple species has been sequenced and various mutations have been reported in humans. The prevalence of moderate to severe FXII deficiency in humans is between 1-5% of a given Caucasian population.[5,9] Feline FXII deficiency, first reported in 1977 by Green and White[127], presents with very similar features to the human disease, thus FXII deficient cats represent an invaluable resource for studying the role of factor XII in health and disease. As in humans, the prevalence of FXII deficiency in domestic cats of the United States is 2%.[8] We took advantage of a colony of factor XII deficient cats established first by Kier et al.[6], to study the molecular basis of FXII deficiency.

Factor XII is a serine protease that is primarily produced in the liver and circulates in the plasma as an inactive precursor enzyme or zymogen. The FXII protein is a two-chain molecule consisting of a heavy chain (50,000 Daltons) and a

light chain (30,000 Daltons) connected by a disulfide bond.[54] The enzymatic activity of FXII is contained in a typical serine protease active site located in its light chain (C-terminus). A serine protease forms an oxyanion hole after zymogen activation that has a nucleophilic serine (Ser) adjacent to a histidine (His), and a buried 'proton accepting' aspartate (Asp) in the catalytic apparatus.[30] The Ser-His-Asp formation is referred to as the 'catalytic triad'. When the FXII heavy chain contacts negatively charged surfaces or misfolded proteins, the FXII zymogen changes conformation to expose its serine protease active site in a process termed 'autoactivation'.[31-33] This conformational change allows other coagulation factor zymogens, prekallikrein and factor XI, to bind at the newly exposed catalytic triad site and become cleaved to kallikrein and factor XIa respectively. Because autoactivation of FXII is the initial step for *in vitro* coagulation induction, it has been labeled a 'contact activation factor' and initiator of the intrinsic coagulation pathway.[32] Activated FXII (FXIIa) has also been shown to bind to cell surfaces via its heavy chain and participate in other biological processes such as inflammation and fibrinolysis, however little is known about the extent of its participation and its importance in pathological outcomes.[32,34] The heavy chain has amino acid regions that are homologous to fibronectin type I and type II, epidermal growth factor, and kringle structural domains. Fibronectin type I and II domains can bind extracellular matrix proteins and collagen, and are thought to be involved in the cell-mediated binding of FXIIa.[54,60]

In humans, congenital FXII deficiency is caused by a variety of genetic defects that result mostly in null defects or loss of function mutants. The most common defect is a single 46C to T nucleotide substitution in the promoter region of

exon 1 on one allele (heterozygous) or both alleles (homozygous).[9,128] Caucasians have a 20% incidence of a 46T (2-6% incidence for a 46T substitution on both alleles) resulting in a decreased translation of FXII due to disruption of the translation initiation site. Individuals having this mutation produce less functional protein and can have FXII activity and antigen levels at or slightly below standard normal ranges, hence they may not be identified with FXII deficiency during coagulation screening tests.[9,128,129] Very few mutations result in detectable FXII antigen (referred to as cross-reacting material positive) however these tend to occur in exons 9 through 14 and involve amino acid substitutions.[9,128-133] Only 3 cases have been reported to carry single base deletions and subsequent frame shifts via mutations in either exon 12 (10590DelC and 10586DelG) or exon 14 (splice site mutation 11397G→A), and all 3 transcribed mutant proteins are considered unstable.[131]

In this manuscript we sequenced the genes encoding the wild type FXII and encoding a mutant allele causing FXII deficiency in the cat. Our findings explain the molecular origin of FXII deficiency in cats.

MATERIALS AND METHODS

Animals

Four normal cats from outside the colony were tested for FXII enzymatic activity and considered normal (>85% of a normal cat plasma pool) for FXII levels. Three normal (NL) cats were partially sequenced for genomic DNA and RNA, while

one cat was fully sequenced. One severely FXII deficient (homozygote or HZY) having <2% activity was completely sequenced for all exons and RNA, and 4 others partially sequenced. Three moderately deficient for FXII (heterozygote or HET), as determined by an enzymatic activities between 30-85% of a normal cat plasma pool, were partially sequenced for FXII genomic DNA and RNA products.

Factor XII Activity Assays

A modified one-stage activated partial thromboplastin time (APTT) was used to measure FXII coagulant activity (FXII:C) while a chromogenic peptide (S-2302) was used to measure amidolytic activity.[81] A normal platelet-poor citrated (3.8%) plasma pool was created using 5 normal cats (outside and colony sources). A homozygote cat plasma pool created from 4 living colony cats identified through breeding and APTT test was used as a substrate to dilute plasmas to 1:10, 1:20, and 1:40 for the FXII coagulant assay. One hundred micro liters (µl) of prewarmed phospholipid reagent (Dade APTT-FSL, Dade Behring Inc., Newark, DE) containing kaolin was added to 100 µl of diluted plasma then warmed to 37˚C for 3 minutes before 100 µl of prewarmed 25mM $CaCl_2$ was added. A Stago ST4 coagulation analyzer (Diagnostica Stago Inc., Parsippany, NJ) was used to monitor clotting time in seconds. A standard curve was generated using serial dilutions (1:10, 1:20, 1:40) of normal cat plasma pool which had been given an assigned value of 100% activity. Individual samples (run in duplicate) were compared to this curve and activity expressed as percent of a normal cat plasma pool. Chromogenic substrate S-2302

(Diapharma Group Inc, OH) H-D-Pro-Phe-Arg-pNA was used to test kallikrein activation as an indicator of FXIIa. Briefly, 25 µl of plasma diluted 1:5 with 0.15M Barbital/0.125M NaCl/0.25% bovine serum albumin pH 7.5 is placed in a 96 well microtiter plate with 25 µl of APTT reagent (Dade) to achieve a final plasma dilution of 1:10. The plate is incubated at 37˚C for 45 minutes then 50 µl of 0.0005 M S-2302 in 0.05M Tris/0.05M Imidazole/0.15M NaCl pH 8.2 is added to the activated plasma and wells are monitored every 10 minutes at 405nm for 1 hour. A standard curve of serially diluted normal cat pooled plasma is included on the plate to determine a % of normal pool activity for each test sample (run in duplicate). The normal cat FXII:C range for our colony is 85-110%. Normal human plasma was run as an internal control to assess reagent and machine functions. The addition of the kallikrein inhibitors, soy bean trypsin inhibitor (SBTI) and Kallistop (American Diagnostica, CT), resulted in no activity seen in normal cat pool plasma therefore it was left out.

Molecular Techniques

Genomic DNA was extracted from peripheral blood leukocytes or liver samples using the Qiagen DNeasy Blood and Tissue kit (Qiagen Inc., Valencia, CA) or Promega's Wizard Genomic DNA Isolation kit (Promega Corp., Madison, WI) according to manufacturer's instructions. The DNA concentration was adjusted to 100 ng/ml for use in polymerase chain reactions (PCR). The region of the 1.9x cat genome (Pontius et al.)[134] identified as being a putative ortholog for F12 (cat chromosome A1:206664989..206674943) was retrieved using GARFIELD

(http://lgd.abcc.ncifcrf.gov/cgi-bin/gbrowse/cat/, Pontius and O'Brien 2007)[135]. The cat sequence was aligned to GenBank sequences representing transcripts for this gene from human (NM_000505), mouse (NM_021489), rat (XM_225172, currently NM_001014006), cow (XM_883314, currently NM_001075119), and dog (XM_546206). Candidate primers were selected in immediately flanking intronic sequences located 50 bases upstream/downstream of acceptor/donor splice sites such to extend through each identified exon pair (7 exon pairs in total). Primer sequences and PCR conditions for each pair are defined in Table 2.1. A Perkin Elmer GeneAmp PCR Systems 9700 thermocycler was used along with Qiagen's HotStar HiFidelity Taq polymerase kit to amplify the specified cDNA. Cycles varied according to primer set melting temperature (Tm); 95˚C for 15mins initial denaturing and enzyme activation, then generally step down amplifications of 0.3˚C to 1.0˚C decreases per amplification step at 10 cycles per temperature were performed (see Table 2.1). For example, primer set genomic DNA Exon 7-8 had: 95˚C for 15min; 94˚C for 45sec, 60.3˚C for 45sec, 72.0˚C for 1min x10 cycles; 94˚C for 45sec, 60.0˚C for 45sec, 72.0˚C for 1min x 10 cycles; 94˚C for 45sec, 59.7˚C for 45sec, 72.0˚C for 1min x 10 cycles; 72.0˚C for 10min, and 4˚C infinity hold.

Total RNA was extracted from liver tissue samples collected and immediately frozen in liquid nitrogen then stored at -70˚C until being processed with the Stratagene Absolutely RNA RT-PCR miniprep kit (Stratagene, La Jolla, CA, USA) or Invitrogen's Purelink Total RNA Purification System (Invitrogen Corp./Life Technologies Corp., Carlsbad, CA) according to the manufacturer's instructions. One normal and one HZY cat total RNA were further purified for messenger RNA

products using Sigma's GenElute mRNA Miniprep kit (Sigma-Aldrich, St. Louis, MO). All RNA samples were treated with 1 unit DNase I (Stratagene, La Jolla, CA, USA) per microgram RNA for 15-30 minutes at room temperature, then heated to 65˚C for 10 minutes prior to reverse transcription. Two-step reverse transcription polymerase chain reaction (RT-PCR) of liver RNA was performed in a 20 micro liter volume using Promega's ImProm-II Reverse Transcription System (Promega Corp., Madison, WI) under manufacturer's suggested conditions. Briefly, each reaction included 4µl of 25mM MgCl2, 4µl of 5X buffer, 1µl of reverse transcriptase, 2µl of dNTP mix (10mM each), 0.5µl of 2µg/µl random hexamers or 0.8 µg/µl oligo d(T)15, 0.3µl of ABI-Perkin Elmer RNase Inhibitor (20U), 4.2 µl of nuclease-free water, and 4µl of sample RNA (100-300 ng/µl). The PCR was then performed using GoTaq® DNA Polymerase or Qiagen's HotStar Hifidelity DNA Polymerase Kit with the following conditions: each 25µl reaction contained 5µl cDNA, 5µl GoTaq® Reaction or HotStar 5x Buffer, 0.3µl dNTP (10mM) only with GoTaq® DNA Polymerase, 0.3µl GoTaq® DNA Polymerase or HotStar Hifidelity DNA Polymerase, 0.4µl each primer (50 µM), 0.5µl of 25mM MgCl2 or Qiagen's 5x Q Solution, and 13.1µl nuclease-free water in GoTaq® mix or 13.4µl nuclease-free water in HotStar mix. PCR was performed in a Touch-down procedure similar to the following: 95˚C for 15 minutes (HotStar polymerase) or 2 minutes (GoTaq® polymerase); 94˚C for 30sec, touch-down 62.5˚C for 30 sec then decrease 0.5˚C per cycle x 5 cycles, 72˚C for 30 sec; then 30 cycles of 94˚C for 15 sec, 60˚C for 45 sec, 72˚C for 45 sec, with final holds of 72˚C for 7mins then 4˚C for infinity. (see Table 2.2 for each primer set's conditions). PCR products were visualized by

electrophoresis on 1.5% Low EEO agarose gel stained with ethidium bromide. Bands were excised and cleaned using Qiagen's QIAEXII kit. Automated sequencing was done by the UNC-CH Automated DNA Sequencing Facility and the University of Michigan DNA Sequencing Core. Analysis was performed using 'Sequencher 4.8' software (Gene Codes Corp., Ann Arbor, MI).

Table 2.1: *Primers used for genomic DNA amplification of exons and exon/intron boundaries of feline F12*

Coverage of	Forward primer 5'→ 3'	Reverse primer 5'→ 3'	Size bp	PCR conditions (annealing temperature
Exon 1→2	ATTCCCAGGACTCAAGGACC	ACCTGACCATCTGCACACTCC	637	56˚C x10 cycles, 55.8˚C x10 cycles, 55.5˚C x 10 cycles
Exon 3→4	CTGTTTTCCAATCAGGCCC	GGGTAAGAACTGATGATACCCAG	648	56˚C x10 cycles, 55.5˚C x10 cycles, 55.0˚C x 10 cycles
Exon 5→6	AAGCAGGTTTGGGTAGGGAC	GGTTGCCTAACAGCTTTTCC	548	59˚C x10 cycles, 58.5˚C x10 cycles, 58.0˚C x 10 cycles
Exon 7→8	AGCTGTTAGGCAACCAGTCG	GGCGCTGTTAAAGGGTAAGG	589	60.3˚C x10 cycles, 60.0˚C x10 cycles, 59.7˚C x 10 cycles
Exon 9→10	CATCTCCCTCTGCCCCAG	CCAGACTCTGCGGCACTC	573	56˚C x10 cycles, 55.8˚C x10 cycles, 55.5˚C x 10 cycles
Exon 11→12	GAAACCCAGCAAGGGACTC	CCCTAGTGGTACCAAAGCTGAG	556	60˚C x10 cycles, 59.5˚C x10 cycles, 59.0˚C x 10 cycles
Exon 13→14	CTGCAACAGAGGCCAGTTAG	AGAGGAATCAAACCCAACCC	577	59.0˚C x10 cycles, 58.0˚C x10 cycles, 57.0˚C x 10 cycles

Table 2.2: *Primers used for cDNA amplification and sequencing of feline FXII RNA*

Coverage of	Forward primer 5'→ 3'	Reverse primer 5'→ 3'	Size bp	PCR conditions (annealing temperatures)
Exons 1-4	ATGAGGGCTCTCCTGTTCCT	GCTCGAAGTTGGGACTGGTA	241	62.5˚C*-0.5˚C/cycle x 5 cycles 60˚C 45sec x 30 cycles
Exons 3-5	TTCTCACTGTCACTGGGGAGC	TGAAGTGTTCTGGACAGATGCA	261	62.5˚C*-0.5˚C/cycle x 5 cycles 60˚C 45sec x 30 cycles
Exons 4-6	GCTACCAGTCCCAACTTCGAGCA	AGTGGGCATCAGGACCCTTACAC	288	62.5˚C*-0.5˚C/cycle x 5 cycles 60˚C 45sec x 30 cycles
Exons 6-8	GAGAAATGCTTTGAGCCTCAGC	GCTCTGCAGTCACGTTCCGGTA	364	61.5˚C*-0.5˚C/cycle x 7 cycles 58.2˚C 45sec x 30 cycles
Exons 7-9	GGATACGCAGGACGCTTCT	CTTGCTCTGCAGTCACGTTC	157	60.5˚C*-0.5˚C/cycle x 7 cycles 59.0˚C 30sec x 30 cycle
Exons 8-9	ACACTGAGGCGCGATGCTACGA	TGCAGTGCTGAAAGCGAGGGCA	346	62.5˚C*-0.5˚C/cycle x 5 cycles 60.2˚C 45sec x 30 cycles
Exons 10-12	GTACTGGCGCCACAATTTCT	AGGTCGTGCTGGTAGGTGAT	183	61.5˚C*-0.5˚C/cycle x 7 cycles 58.3˚C 45sec x 30 cycles
Exons 11-14	TTCTCGCCCATCACCTACC	GGTCAGGAATTGGTGTGCTC	500	60.5˚C*-0.5˚C/cycle x 7 cycles 58.3˚C 45sec x 10 cycles, then 58.5˚C x10 cycles, followed by 58.7˚C x10 cycles
Exons 13-14	ACGTGCACGGAGTCTCTTTT	CTGGATGGTCAGGAATTGGT	50	60.5˚C*-0.5˚C/cycle x 7 cycles 59.0˚C 30sec x 30 cycles
*Cat GAPDH	GCCGTGGAATTTGCCGT	GCCATCAATGACCCCTTCAT	82	Works between 55C-56C for 30 sec x 30 cycles

*primer sequences and conditions are from C.M. Leutenegger et al. Vet. Immunology and Immunopathology 71 (1999) p. 291-305

Protein Isolation and Identification Techniques

a) Inhibition of Feline FXII by Corn Trypsin Inhibitor (CTI) and mixing studies

Mixing studies involving human prekallikrein-deficient (PKd) plasma (Sigma Diagnostics) and FXII-deficient plasma (George King Biomedicals, Overland Park, KS), corn trypsin inhibitor (Enzyme Research Laboratories, South Bend, IN), and specific FXIIa antibodies were performed on normal cat plasmas using the Stago ST4 and the modified APTT procedure. Human PKd plasma was used as a diluent

instead of HZY cat plasma pool to rule out a prekallikrein deficiency in our colony. Antibodies or corn trypsin inhibitor (CTI) were used to check their ability to inhibit feline FXII amidolytic activity. Plasma duplicates were incubated 15-60 minutes at 37˚C with each inhibitor prior to performing the APTT. One normal cat, 3 HZY cats, and the HZY cat plasma pool were evaluated in these studies. An overall 1:2 dilution of plasma was used with HZY cat plasmas while a >1:10 dilution of normal cat plasma was used because of the extremely short clotting time observed with normal cat plasma (<18 seconds at a 1:2 dilution) would not be detected on the ST4. A standard curve was not used, only the ability to correct the prolonged clotting time was observed. Results are given in Table 2.3.

Table 2.3: *Mixing and inhibition studies of colony plasmas*

Sample Identification	Sample dilution, diluent or inhibitor added (if any)	Averaged APTT (seconds)	Determination
NL 0827 cat	1:40 with human PKd plasma	31.5	PK sufficient
HZY 6034 cat	1:2 with human PKd plasma	46.5	PK sufficient
HZY 6B1E cat	1:2 with human PKd plasma	46.3	PK sufficient
HZY 171A cat	1:2 with human PKd plasma	47.7	PK sufficient
HZY cat pool	1:2 with human FXII-deficient plasma	>200	FXII deficient
Human PKd	1:2 with barbital-saline buffer*	144.7	PKd;dilutionary control
NL 0827 cat	1:10, 300 ug/ml CTI (10ul volume)	64.7	inhibitory
NL 0827 cat	1:10, 400 ug/ml CTI (10ul volume)	66.5	inhibitory
NL 0827 cat	1:10, 500 ug/ml CTI (10ul volume)	92.0	inhibitory
NL 0827 cat	1:10, 10 ul of saline (vehicle) or nonspecific IgG	<20	control
NL 0827 cat	1:10, 10 ul of 0.4uM anti-FXII B7C9	20.2	inhibitory
NL 0827 cat	1:10, 10 ul of 1.0uM anti-FXII B7C9	21.6	inhibitory
Normal Human	10 ul of 0.4uM anti-FXII B7C9	56	Control; inhibitory

b) Protein electrophoresis and Western blotting (WB)

Plasma samples from a normal cat and homozygote FXII-deficient cat were subjected to reducing conditions and 12% SDS-PAGE prior to electroblotting onto nitrocellulose or polyvinylidene fluoride (PVDF). The blotted antigens were detected with two polyclonal (pAb) anti-human FXII antibodies (purchased from Cedarlane [CL20055A] and Affinity Biologicals) and a monoclonal 'B7C9' heavy chain specific anti-human FXII antibody (a gift from Dr. Robin Pixley) according to conditions described by Pixley et al..[49] Prestained (Sigma P8748, Bio-Rad Kaleidoscope C3312) and unstained (Bio-Rad #80715) molecular weight standards (MW) were used to determine the size and migration of detected antigens. Secondary antibodies of either rabbit anti-goat IgG or rabbit anti-mouse IgG conjugated to peroxidase (Sigma Aldrich A5420 and A9044) or rabbit anti-mouse alkaline phosphatase (Jackson Laboratories) were used and detected with Vector Labs NovaRed or Sigma BCIP/NBT B6404 substrates. An example of plasma blotting is given in Figure 2.1. Additional studies of feline plasmas involved two-dimensional difference in gel electrophoresis (2-D DIGE) with subsequent WB analysis.[132] Normal feline plasma stained with CyDye3 (green) and HZY feline plasma stained with CyDye 5 (red) were run on a pH 3-10 non-linear isoelectric focusing (IEF) followed by 10.5% SDS-PAGE for the 2nd dimension prior to imaging on ImageQuant software (GE Healthcare). Electroblotting and immunodetection were performed as described[137], using a peroxidase conjugated rabbit anti-goat secondary antibody (Sigma A5420) at 1:20,000 in Tris buffered saline (TBS)/1% bovine serum albumin/0.1% Tween-20 and enhanced chemiluminescence (ECL) detection kit (Amersham™ ECL). The membrane was exposed to Kodak Scientific Processing film and developed with a

table top film processor. Results of the 2-D DIGE and WB are shown in Figures 2.2 and 2.3 respectively.

c) Protein isolation methods

Normal feline FXII protein was isolated on CTI-CNBr Sepharose 4B and pAb (Cedarlane) goat anti-human FXII-CNBr Sepharose affinity columns prepared as described by Ratnoff et al.[138] and Kier et al.[81], respectively. Briefly, 20-50 milliliters of fresh frozen citrated plasma (pH 6.0) was thawed at 37˚C and treated with 100 ug/ml soybean trypsin inhibitor (SBTI) and 50 ug/ml polybrene (hexadimethrine bromide, Sigma Aldrich, St. Louis, MO). In order to remove contaminating proteins including FXIIa, plasmas were run over a benzamidine-HCl bound to agarose column and fractions collected. Those fractions with detectable FXII activity (using the APTT assay with HZY cat pool plasma) were dialyzed in appropriate loading buffers and further purified over either CTI- or anti-FXII-CNBr Sepharose columns. Bound feline FXII was eluted with 0.05M glycine/50% ethylene glycol buffer pH 3.2 on the immunoaffinity column or eluted with a barbital saline buffer (0.15M sodium barbital, 0.125M sodium chloride, 100 ug/ml SBTI, 50 ug/ml polybrene) with a linear gradient of 0.15-1.5M sodium chloride from the CTI column. Two to 5 ml fractions were collected with a LKB optical fractionator, dialyzed/desalted, concentrated and tested for FXII antigen (WB) and activity (APTT). Flow through material was collected and re-applied to the column(s) in order to maximize FXII recovery. The CTI column was carefully stripped with 0.1M sodium acetate/1.0M sodium chloride pH 4.0 then re-equilibrated with barbital saline buffer prior to isolating HZY feline plasma FXII as described above. The anti-FXII-CNBr column was stripped with 10

ml of 2M guanidine hydrochloride and re-equilibrated with 0.01M Tris/0.5M sodium chloride/100ug/ml SBTI/50ug/ml polybrene pH 7.5 buffer prior to use. All fractions of HZY plasma with 280 nm absorbance readings above 0.1 were tested for antigen and activity. Results of WB are shown in Figure 2.4 (normal cat) and Figure 2.5 (HZY cat).

Figure 2.1: Western Blot of Feline and Human Plasmas With Monoclonal Heavy Chain Antibody

Normal (lane 3) and homozygote (lane 4) for feline FXII citrated plasma was reduced, electrophoresed, then blotted onto PVDF. Human plasma (lane 5) and purified human FXIIa (lane 6) are positive controls. Lane 1 contains purified normal cat FXII and lane 2 contains a molecular weight standard (Bio-Rad Kaleidoscope). Primary immunodetection was done with a mouse anti-human FXII heavy chain (B7C9) monoclonal antibody then detected with a rabbit anti-mouse IgG whole molecule alkaline phosphatase conjugated antibody followed by BCIP/NBT development. Arrows indicate expected bands (longer arrow=80 kDa, shorter=50 kDa)

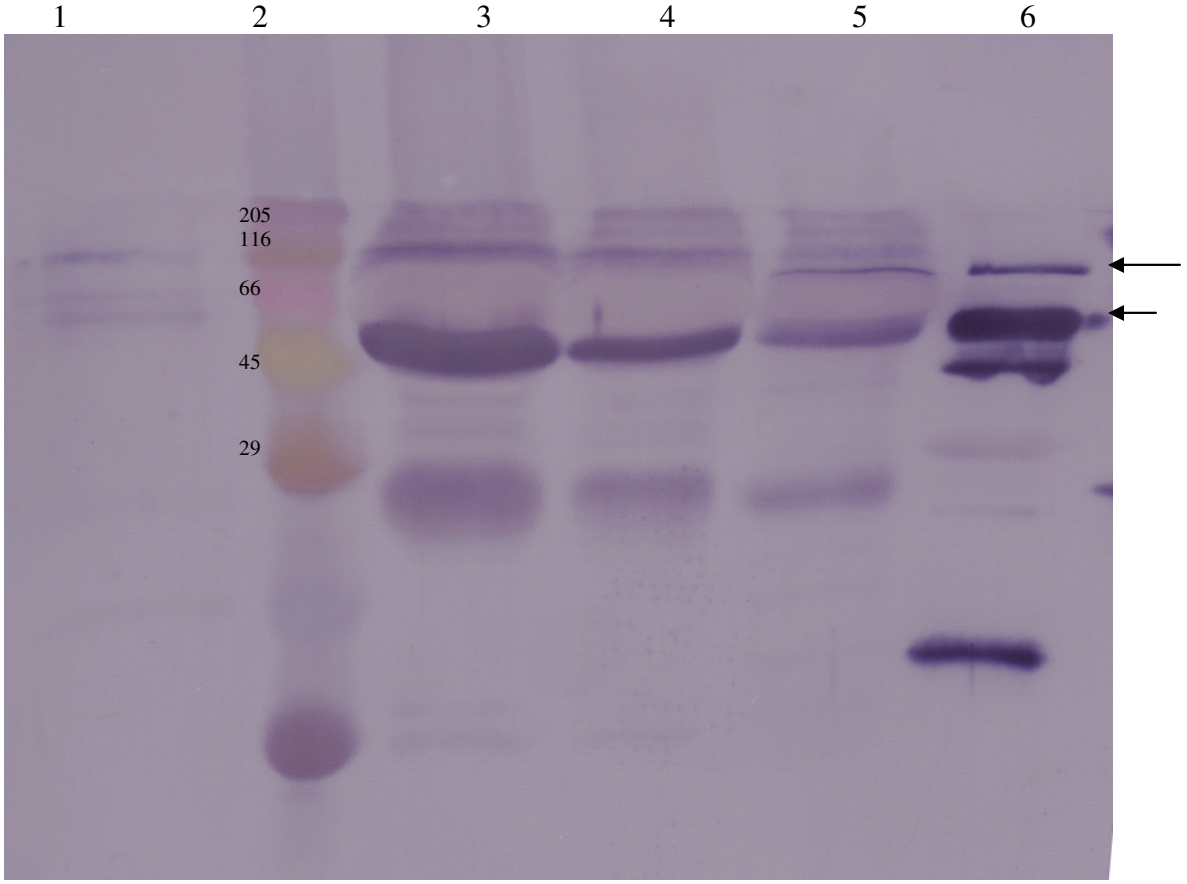


Figure 2.2: *2-D DIGE of One Normal Cat (green) and One Homozygote Cat (red) Citrated Plasma*
The circled spots correspond to suspected FXII proteins detected by WB techniques.

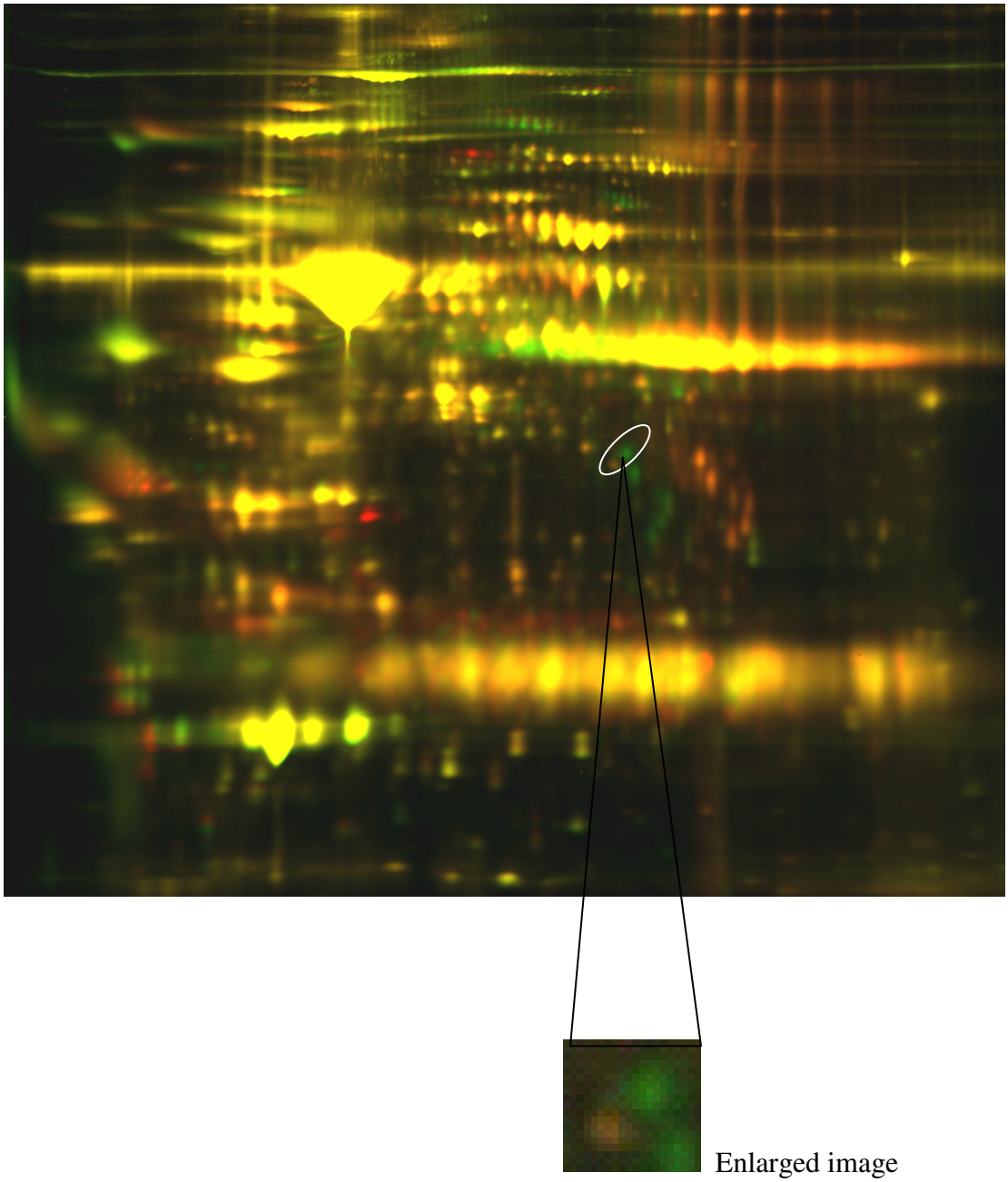


Figure 2.3: *Western Blot of 2-D DIGE*
Primary antibody used for probing was a polyclonal goat anti-human FXII (Affinity Biologicals). Secondary antibody was a peroxidase conjugated rabbit anti-goat IgG (Sigma) detected with ECL kit. The circled spots are suspected feline FXII (upper right spot=normal cat, lower left spot=HZY cat).

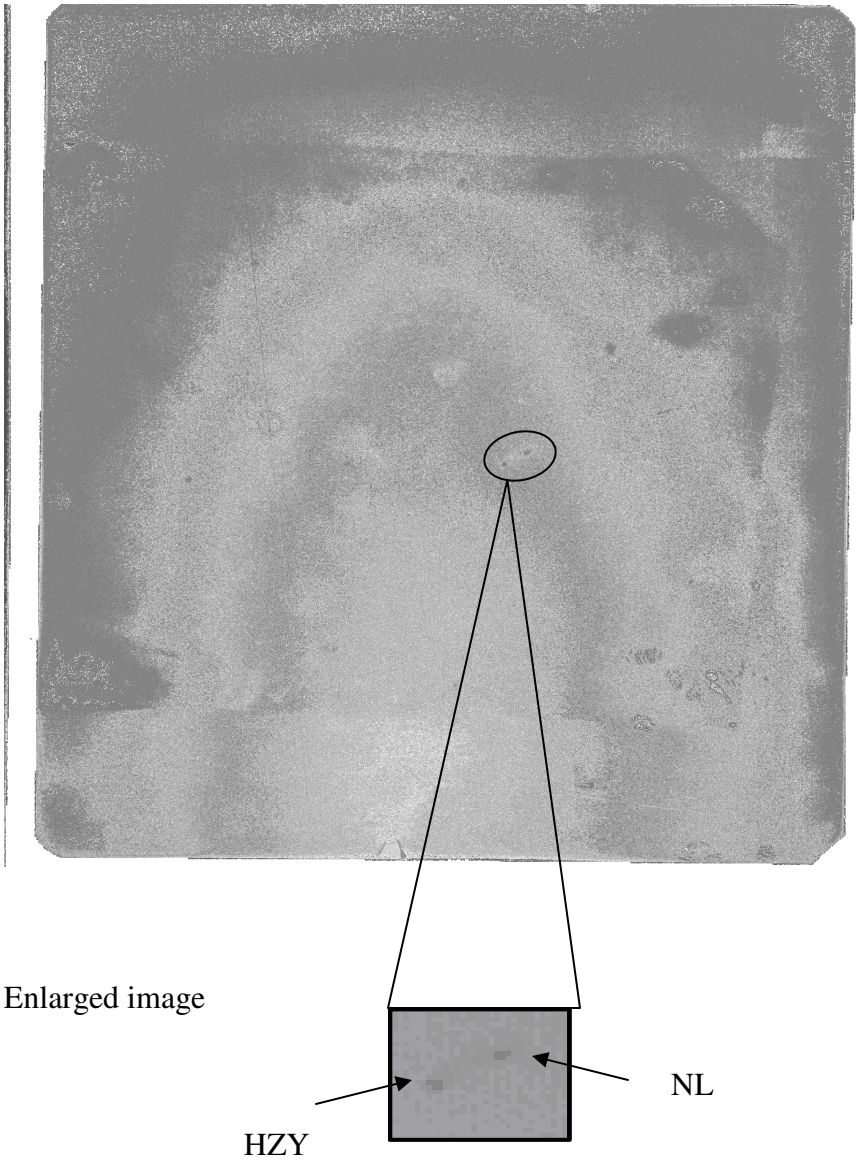


Figure 2.4: *Western Blot of Fractions From Normal Feline Plasma FXII Purification*
Lanes are as follows: 1=unstained MW, 2=human FXII deficient plasma, 3=purified human FXIIa, 4=normal human plasma, 5=eluted cat fraction 36, 6=cat fraction 35, 7=cat fraction 34, 8=empty, 9=cat fraction 33, 10=cat fraction 32, 11=flow through from FXII immunoaffinity column, 12=benzamidine column eluate. Primary antibody used to probe antigens was mouse anti-human heavy chain (B7C9). Secondary antibody was rabbit anti-mouse IgG alkaline phosphatase conjugated that was detected with BCIP/NBT. Arrows indicate expected band size (long arrow=80 kDa, short arrow=50 kDa)

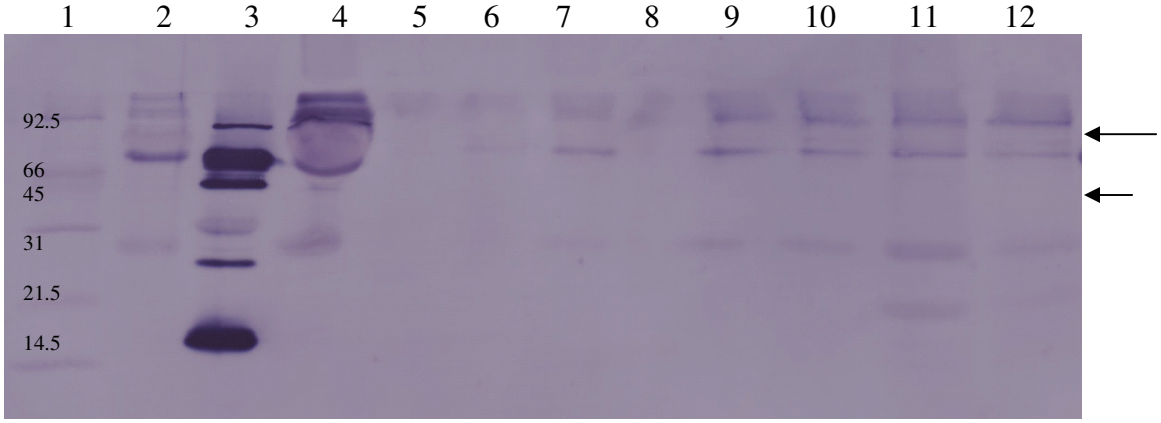
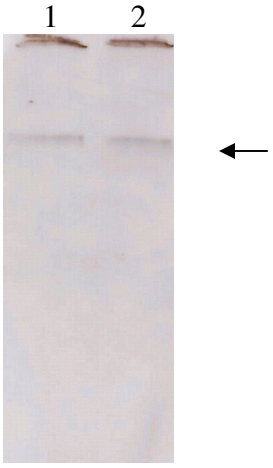


Figure 2.5: *Western Blot of Purified Mutant Feline Plasma FXII Fractions Using An Anti-FXII CNBr Column*
Primary antibody was a polyclonal goat anti-human FXIIa (Affinity Biologicals). Lanes are: 1= HZY cat fractions 1&2, 2= HZY cat fractions 1&2 2nd loading of flow through material. Arrow indicates an expected band of identity (~66 kDa).



RESULTS

The feline FXII gene consists of 14 exons and 13 introns which results in a protein of 610 amino acids (a.a.) having high sequence similarity to human (80% nucleotide, 73% a.a.), and mouse (79% nucleotide, 68% a.a.) FXII genes as shown in Figure 2.6. The coding sequence for one normal cat FXII (accession number GQ_981174) and the mutant cat FXII (accession number GQ_981175) were determined and submitted to GenBank.. Several polymorphisms were found and reported while using the 1.9x cat genomic sequence as an alignment reference for our cat samples. Most of the polymorphisms occurred in exons (7, 8, 10, 11 and 14) yet are predicted to yield synonymous substitutions. The exceptions being a Val→Ala change in exon 7, and our colony's mutation found in exon 11.

Figure 2.6. *Nucleotide sequence comparison of mRNA transcripts encoding for mature FXII protein*

Shaded areas indicate identical sequence. Dashed lines indicate no nucleotide. The beginning of each exon and the termination codon are indicated by an underlined letter with the exon identified above it. The location of the FXII-deficiency mutation in our cats is in exon 11 and indicated by a bold, underlined, boxed red letter.

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CLUSTAL 2.0.10 multiple sequence alignment
                                                        exon 1
feline      ------------------------------------------------------ATGAGGGCTCT 11
human       CTATTGATCTGGACTCCTGGATAGGCAGCTGGACCAACGGACGGATGCCATGAGGGCTCT 60
mouse       --------------TCCTGGGCAGGCAGCGGGGCCATCGG-CAGACGCCATGACGGCTCT 45

                                                    exon 2
feline      CCTGTTCCTGGGGTCCCTGCTGGGAAGCCTGGAGTCAGCGTTTTTGACTCCACCTTGGAA 71
human       GCTGCTCCTGGGGTTCCTGCTGGTGAGCTTGGAGTCAACACTTTCGATTCCACCTTGGGA 120
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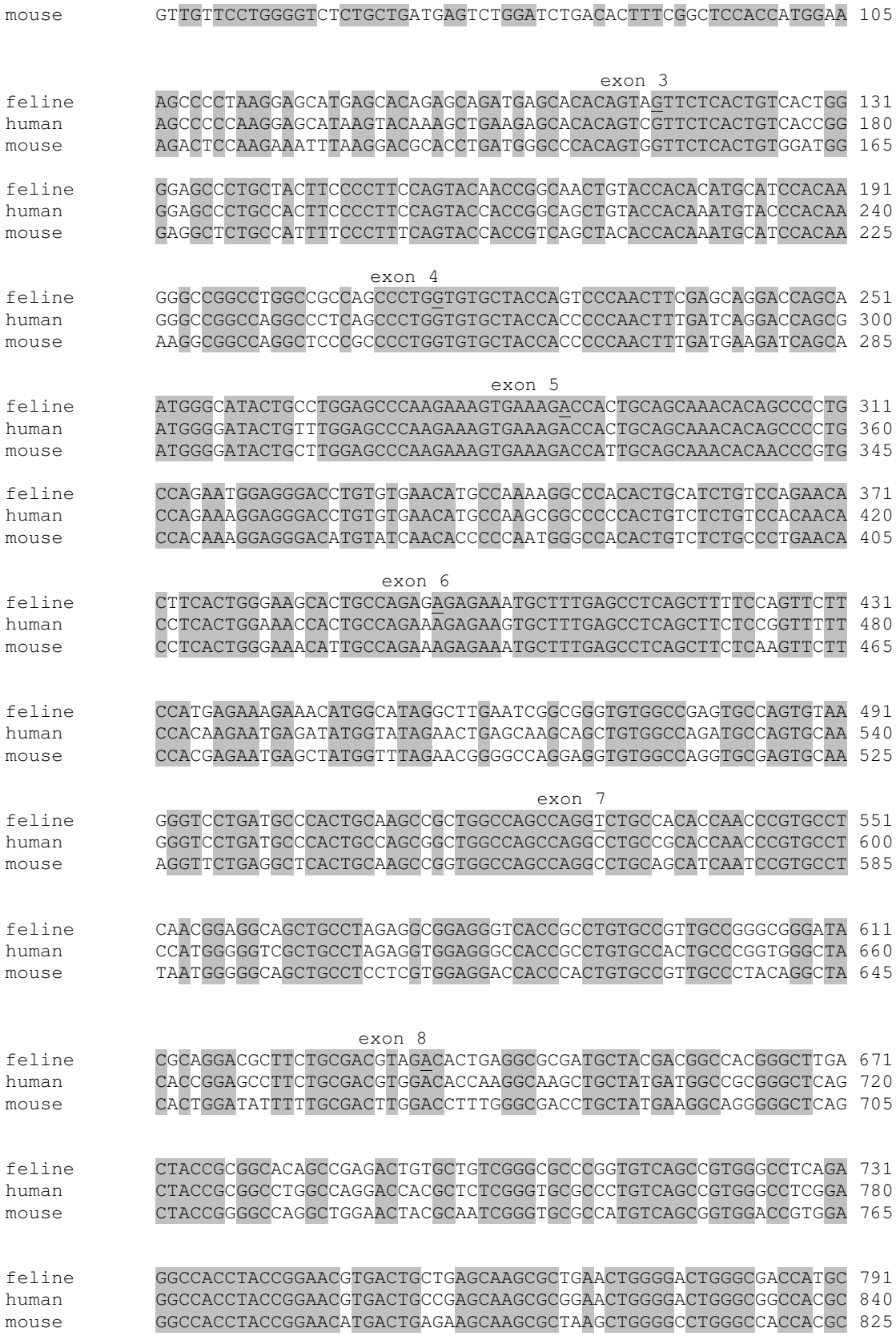
mouse GTTGTTCCTGGGGTCTCTGCTGATGAGTCTGGATCTGACACTTTCGGCTCCACCATGGAA 105
exon 3
feline AGCCCCTAAGGAGCATGAGCACAGAGCAGATGAGCACACAGTAGTTCTCACTGTCACTGG 131
human AGCCCCCAAGGAGCATAAGTACAAAGCTGAAGAGCACACAGTCGTTCTCACTGTCACCGG 180
mouse AGACTCCAAGAAATTTAAGGACGCACCTGATGGGCCCACAGTGGTTCTCACTGTGGATGG 165
feline GGAGCCCTGCTACTTCCCCTTCCAGTACAACCGGCAACTGTACCACACATGCATCCACAA 191
human GGAGCCCTGCCACTTCCCCTTCCAGTACCACCGGCAGCTGTACCACAAATGTACCCACAA 240
mouse GAGGCTCTGCCATTTTCCCTTTCAGTACCACCGTCAGCTACACCACAAATGCATCCACAA 225
exon 4
feline GGGCCGGCCTGGCCGCCAGCCCTGGTGTGCTACCAGTCCCAACTTCGAGCAGGACCAGCA 251
human GGGCCGGCCAGGCCCTCAGCCCTGGTGTGCTACCACCCCCAACTTTGATCAGGACCAGCG 300
mouse AAGGCGGCCAGGCTCCCGCCCCTGGTGTGCTACCACCCCCAACTTTGATGAAGATCAGCA 285
exon 5
feline ATGGGCATACTGCCTGGAGCCCAAGAAAGTGAAAGACCACTGCAGCAAACACAGCCCCTG 311
human ATGGGGATACTGTTTGGAGCCCAAGAAAGTGAAAGACCACTGCAGCAAACACAGCCCCTG 360
mouse ATGGGGATACTGCTTGGAGCCCAAGAAAGTGAAAGACCATTGCAGCAAACACAACCCGTG 345
feline CCAGAATGGAGGGACCTGTGTGAACATGCCAAAAGGCCCACACTGCATCTGTCCAGAACA 371
human CCAGAAAGGAGGGACCTGTGTGAACATGCCAAGCGGCCCCCACTGTCTCTGTCCACAACA 420
mouse CCACAAAGGAGGGACATGTATCAACACCCCCAATGGGCCACACTGTCTCTGCCCTGAACA 405
exon 6
feline CTTCACTGGGAAGCACTGCCAGAGAGAGAAATGCTTTGAGCCTCAGCTTTTCCAGTTCTT 431
human CCTCACTGGAAACCACTGCCAGAAAGAGAAGTGCTTTGAGCCTCAGCTTCTCCGGTTTTT 480
mouse CCTCACTGGGAAACATTGCCAGAAAGAGAAATGCTTTGAGCCTCAGCTTCTCAAGTTCTT 465
feline CCATGAGAAAGAAACATGGCATAGGCTTGAATCGGCGGGTGTGGCCGAGTGCCAGTGTAA 491
human CCACAAGAATGAGATATGGTATAGAACTGAGCAAGCAGCTGTGGCCAGATGCCAGTGCAA 540
mouse CCACGAGAATGAGCTATGGTTTAGAACGGGGCCAGGAGGTGTGGCCAGGTGCGAGTGCAA 525
exon 7
feline GGGTCCTGATGCCCACTGCAAGCCGCTGGCCAGCCAGGTCTGCCACACCAACCCGTGCCT 551
human GGGTCCTGATGCCCACTGCCAGCGGCTGGCCAGCCAGGCCTGCCGCACCAACCCGTGCCT 600
mouse AGGTTCTGAGGCTCACTGCAAGCCGGTGGCCAGCCAGGCCTGCAGCATCAATCCGTGCCT 585
feline CAACGGAGGCAGCTGCCTAGAGGCGGAGGGTCACCGCCTGTGCCGTTGCCGGGCGGGATA 611
human CCATGGGGGTCGCTGCCTAGAGGTGGAGGGCCACCGCCTGTGCCACTGCCCGGTGGGCTA 660
mouse TAATGGGGGCAGCTGCCTCCTCGTGGAGGACCACCCACTGTGCCGTTGCCCTACAGGCTA 645
exon 8
feline CGCAGGACGCTTCTGCGACGTAGACACTGAGGCGCGATGCTACGACGGCCACGGGCTTGA 671
human CACCGGAGCCTTCTGCGACGTGGACACCAAGGCAAGCTGCTATGATGGCCGCGGGCTCAG 720
mouse CACTGGATATTTTTGCGACTTGGACCTTTGGGCGACCTGCTATGAAGGCAGGGGGCTCAG 705
feline CTACCGCGGCACAGCCGAGACTGTGCTGTCGGGCGCCCGGTGTCAGCCGTGGGCCTCAGA 731
human CTACCGCGGCCTGGCCAGGACCACGCTCTCGGGTGCGCCCTGTCAGCCGTGGGCCTCGGA 780
mouse CTACCGGGGCCAGGCTGGAACTACGCAATCGGGTGCGCCATGTCAGCGGTGGACCGTGGA 765
feline GGCCACCTACCGGAACGTGACTGCTGAGCAAGCGCTGAACTGGGGACTGGGCGACCATGC 791
human GGCCACCTACCGGAACGTGACTGCCGAGCAAGCGCGGAACTGGGGACTGGGCGGCCACGC 840
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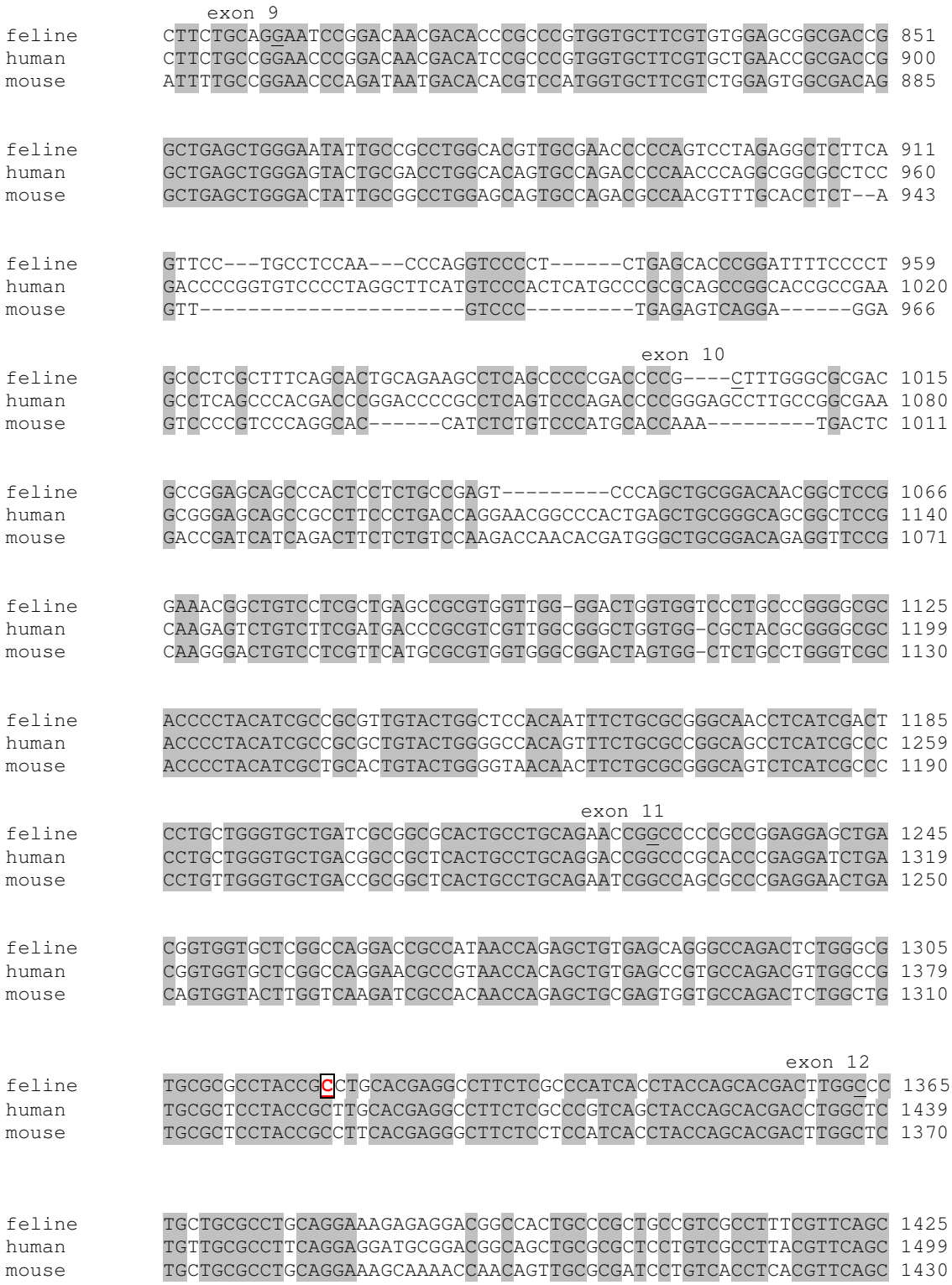

exon 9
feline CTTCTGCAGGAATCCGGACAACGACACCCGCCCGTGGTGCTTCGTGTGGAGCGGCGACCG 851
human CTTCTGCCGGAACCCGGACAACGACATCCGCCCGTGGTGCTTCGTGCTGAACCGCGACCG 900
mouse ATTTTGCCGGAACCCAGATAATGACACACGTCCATGGTGCTTCGTCTGGAGTGGCGACAG 885
feline GCTGAGCTGGGAATATTGCCGCCTGGCACGTTGCGAACCCCCAGTCCTAGAGGCTCTTCA 911
human GCTGAGCTGGGAGTACTGCGACCTGGCACAGTGCCAGACCCCAACCCAGGCGGCGCCTCC 960
mouse GCTGAGCTGGGACTATTGCGGCCTGGAGCAGTGCCAGACGCCAACGTTTGCACCTCT--A 943
feline GTTCC---TGCCTCCAA---CCCAGGTCCCCT------CTGAGCACCCGGATTTTCCCCT 959
human GACCCCGGTGTCCCCTAGGCTTCATGTCCCACTCATGCCCGCGCAGCCGGCACCGCCGAA 1020
mouse GTT----------GTCCC---------TGAGAGTCAGGA------GGA 966
exon 10
feline GCCCTCGCTTTCAGCACTGCAGAAGCCTCAGCCCCCGACCCCG----CTTTGGGCGCGAC 1015
human GCCTCAGCCCACGACCCGGACCCCGCCTCAGTCCCAGACCCCGGGAGCCTTGCCGGCGAA 1080
mouse GTCCCCGTCCCAGGCAC------CATCTCTGTCCCATGCACCAAA---------TGACTC 1011
feline GCCGGAGCAGCCCACTCCTCTGCCGAGT---------CCCAGCTGCGGACAACGGCTCCG 1066
human GCGGGAGCAGCCGCCTTCCCTGACCAGGAACGGCCCACTGAGCTGCGGGCAGCGGCTCCG 1140
mouse GACCGATCATCAGACTTCTCTGTCCAAGACCAACACGATGGGCTGCGGACAGAGGTTCCG 1071
feline GAAACGGCTGTCCTCGCTGAGCCGCGTGGTTGG-GGACTGGTGGTCCCTGCCCGGGGCGC 1125
human CAAGAGTCTGTCTTCGATGACCCGCGTCGTTGGCGGGCTGGTGG-CGCTACGCGGGGCGC 1199
mouse CAAGGGACTGTCCTCGTTCATGCGCGTGGTGGGCGGACTAGTGG-CTCTGCCTGGGTCGC 1130
feline ACCCCTACATCGCCGCGTTGTACTGGCTCCACAATTTCTGCGCGGGCAACCTCATCGACT 1185
human ACCCCTACATCGCCGCGCTGTACTGGGGCCACAGTTTCTGCGCCGGCAGCCTCATCGCCC 1259
mouse ACCCCTACATCGCTGCACTGTACTGGGGTAACAACTTCTGCGCGGGCAGTCTCATCGCCC 1190
exon 11
feline CCTGCTGGGTGCTGATCGCGGCGCACTGCCTGCAGAACCGGCCCCCGCCGGAGGAGCTGA 1245
human CCTGCTGGGTGCTGACGGCCGCTCACTGCCTGCAGGACCGGCCCGCACCCGAGGATCTGA 1319
mouse CCTGTTGGGTGCTGACCGCGGCTCACTGCCTGCAGAATCGGCCAGCGCCCGAGGAACTGA 1250
feline CGGTGGTGCTCGGCCAGGACCGCCATAACCAGAGCTGTGAGCAGGGCCAGACTCTGGGCG 1305
human CGGTGGTGCTCGGCCAGGAACGCCGTAACCACAGCTGTGAGCCGTGCCAGACGTTGGCCG 1379
mouse CAGTGGTACTTGGTCAAGATCGCCACAACCAGAGCTGCGAGTGGTGCCAGACTCTGGCTG 1310
exon 12
feline TGCGCGCCTACCGCCTGCACGAGGCCTTCTCGCCCATCACCTACCAGCACGACTTGGCCC 1365
human TGCGCTCCTACCGCTTGCACGAGGCCTTCTCGCCCGTCAGCTACCAGCACGACCTGGCTC 1439
mouse TGCGCTCCTACCGCCTTCACGAGGGCTTCTCCTCCATCACCTACCAGCACGACTTGGCTC 1370
feline TGCTGCGCCTGCAGGAAAGAGAGGACGGCCACTGCCCGCTGCCGTCGCCTTTCGTTCAGC 1425
human TGTTGCGCCTTCAGGAGGATGCGGACGGCAGCTGCGCGCTCCTGTCGCCTTACGTTCAGC 1499
mouse TGCTGCGCCTGCAGGAAAGCAAAACCAACAGTTGCGCGATCCTGTCACCTCACGTTCAGC 1430

```
feline      CGGTGTGCCTGCCAAGCAGCGCTGCCCGCCAGCTGAGGCCGAGGCCGCCCTCTGTGAGG 1485
human       CGGTGTGCCTGCCAAGCGGCGCCGCGCGACC--CT----CCGAGACCACGCTCTGCCAGG 1553
mouse       CTGTGTGTCTACCCAGCGGCGCGGCCCCACC--CT----CTGAGACAGTGCTCTGCGAGG 1484

                                  exon 13
feline      TGGCAGGCTGGGGCCACCAGTTTGAGGGGGCCGGGGAATATTCCAGCTTCCTGCAGGAAG 1545
human       TGGCCGGCTGGGGCCACCAGTTCGAGGGGGCGGAGGAATATGCCAGCTTCCTGCAGGAGG 1613
mouse       TGGCCGGCTGGGGTCACCAGTTCGAGGGGGCTGAAGAATACTCCACCTTCCTGCAGGAGG 1544

feline      CACAGGTGCCGCTCATCCCTTCTGAGCGCTGCTCCGCCCAGGACGTGCACGGAGTCTCTT 1605
human       CGCAGGTACCGTTCCTCTCCCTGGAGCGCTGCTCAGCCCCGGACGTGCACGGATCCTCCA 1673
mouse       CACAGGTTCCCTTTATCGCCCTGGATCGCTGCTCCAACTCTAACGTGCACGGAGACGCCA 1604

                                                                exon 14
feline      TTACTTCGGGCATGCTCTGCGCTGGCTTCCTTGAGGGTGGCACCGACGCCTGCCAGGGTG 1665
human       TCCTCCCCGGCATGCTCTGCGCAGGGTTCCTCGAGGGCGGCACCGATGCGTGCCAGGGTG 1733
mouse       TTCTCCCTGGGATGCTTTGCGCTGGCTTCTTGGAGGGAGGCACCGATGCCTGCCAGGGTG 1664

feline      ACTCCGGGGGCCCGCTAGTGTGTGAGGAGGAGGCCGCAGAGCACCAGCTCGTCCTGCGAG 1725
human       ATTCCGGAGGCCCGCTGGTGTGTGAGGACCAAGCTGCAGAGCGCCGGCTCACCCTGCAAG 1793
mouse       ACTCCGGGGGCCCTCTGGTGTGTGAGGAAGGAACTGCAGAACATCAGCTCACCCTGCGCG 1724

feline      GCATCGTCAGCTGGGGTTCCGGTTGTGGCGACCGCTACAAACCAGGTGTGTACACCGACG 1785
human       GCATCATCAGCTGGGGATCGGGCTGTGGTGACCGCAACAAGCCAGGCGTCTACACCGATG 1853
mouse       GAGTCATCAGCTGGGGCTCCGGCTGTGGTGACCGCAACAAGCCCGGAGTCTACACAGACG 1784

                                                stop
feline      TGGCCAGCTACCTGGCCTGGATCCAGGAGCACACCAATTCCTGACCATCCAGAGCGANAT 1845
human       TGGCCTACTACCTGGCCTGGATCCGGGAGCACACCGTTTCCTGATTGCTCAGGGACTCAT 1913
mouse       TGGCCAACTACCTGGCTTGGATCCAGAAGCATATTGCTTCATAACTAACCAG-GCTTTAT 1843

feline      TTTTNNNTT-------------------------------------------------- 1854
human       CTTTCCCTCCTTGGTGATTCCGCAGTGAGAGAGTGGCTGGGGCATGGAAGGCAAGATTGT 1973
mouse       CCTTCCCTCCTTGTGTGCTCCTTGG----------GATGGGACGATGAATGTGGCATGCT 1893

feline      ------------------------------------------------------------
human       GTCCCATTCCCCCAGTGCGGCCAGCTCCGCGCCAG-GATGGCGCAGGAACTCAATAAAGT 2032
mouse       GGGTCA------CAGTGAAGCTAGTGCCCCGACACTGGGGGCACAGAAACTCAATAAAGT 1947

feline      ----------------------------
human       GCTTTGAAAATGCTGAGAAAAAAAAAAA 2060
mouse       GCTTTGAAAACGTT-------------- 1961
```

Analysis of the sequenced feline RNAs indicates the FXII gene encodes for a mature protein of 610 amino acids and is predicted to contain all the characteristic structural domains reported in human FXII, i.e. a Type I and II Fibronectin-like domain, two epidermal growth factor (EGF)-like domains, a kringle domain, a

proline-rich domain, and the typical trypsin-like serine protease domain of the light chain. The feline light chain's predicted catalytic triad of His^{405}, Asp^{454}, Ser^{558} (it is His^{393}, Asp^{442}, Ser^{544} in human FXII) has all 3 amino acids aligning with the human translated sequence. However, only 2 of the 3 kallikrein cleavage sites in human (Arg^{334}-N^{335} and Arg^{343}-Leu^{344}) FXII share homology with the cat sequence as illustrated in Figure 2.7. Using the

Figure 2.7: *Amino Acid Sequence Homology of Normal Cat, Human, and Mutant Cat FXII*

Regional alignment of predicted normal and mutant feline FXII protein sequences compared with human. Teal highlighted amino acids indicate potential kallikrein cleavage sites, yellow highlighted a.a. are members of the well conserved serine active site or catalytic triad (Ser-His-Asp) which is linked to the heavy chain by a disulfide bond (indicated by highlighted green). The light grey colored a.a. sequence is a portion cleaved off at the signal peptidase site after secretion of the protein. The red colored a.a. indicate the nonsense sequence caused by the mutation in the colony.

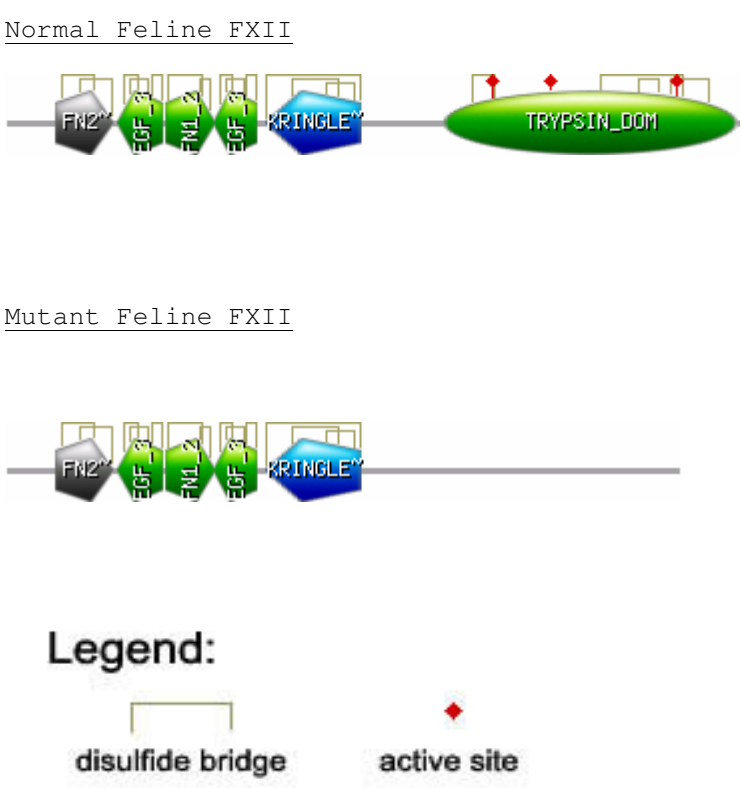
```
Normal cat     MRALLFLGSLLGSLESAFLTPPWKAPKEHEHRADEHTVVLTVTGEPCYFPFQYNRQLYHT 60
human          MRALLLLGFLLVSLESTLSIPPWEAPKEHKYKAEEHTVVLTVTGEPCHFPFQYHRQLYHK 60
mutant cat     MRALLFLGSLLGSLESAFLTPPWKAPKEHEHRADEHTVVLTVTGEPCYFPFQYNRQLYHT 60
               *****:** ** ****::  ***:*****:::*:*************:*****:*****:

normal cat     CIHKGRPGRQPWCATSPNFEQDQQWAYCLEPKKVKDHCSKHSPCQNGGTCVNMPKGPHCI 120
human          CTHKGRPGPQPWCATTPNFDQDQRWGYCLEPKKVKDHCSKHSPCQKGGTCVNMPSGPHCL 120
mutant cat     CIHKGRPGRQPWCATSPNFEQDQQWAYCLEPKKVKDHCSKHSPCQNGGTCVNMPKGPHCI 120
               *:******:******:***:**:**********************:********.****:

normal cat     CPEHFTGKHCQREKCFEPQLFQFFHEKETWHRLESAGVAECQCKGPDAHCKPLASQVCHT 180
human          CPQHLTGNHCQKEKCFEPQLLRFFHKNEIWYRTEQAAVARCQCKGPDAHCQRLASQACRT 180
mutant cat     CPEHFTGKHCQREKCFEPQLFQFFHEKETWHRLESAGVAECQCKGPDAHCKPLASQVCHT 180
               **:*:**:***:********::***::* *:* *.*.**.**********: ****.*:*

normal cat     NPCLNGGSCLEAEGHRLCRCRAGYAGRFCDVDTEARCYDGHGLDYRGTAETVLSGARCQP 240
human          NPCLHGGRCLEVEGHRLCHCPVGYTGPFCDVDTKASCYDGRGLSYRGLARTTLSGAPCQP 240
mutant cat     NPCLNGGSCLEAEGHRLCRCRAGYAGRFCDVDTEARCYDGHGLDYRGTAETALSGARCQP 240
               ****:** ***.******:*::**:*:******:* ****:**.*** *.*.**** ***

normal cat     WASEATYRNVTAEQALNWGLGDHAFCRNPDNDTRPWCFVWSGDRLSWEYCRLARCEPPVL 300
human          WASEATYRNVTAEQARNWGLGGHAFCRNPDNDIRPWCFVLNRDRLSWEYCDLAQCQTPTQ 300
mutant cat     WASEATYRNVTAEQALNWGLGDHAFCRSPDNDTRPWCFVWSGDRLSWEYCRLARCEPPVL 300
               *************** *****.*****.**** ****** . ******** **:*:.*.
```

prediction program SignalP3.0, a signal peptidase cleavage site has been suggested between a. a. position 16 and 17 (LysGluSer-AlaPhe) in the feline FXII protein, indicating the secreted protein would be 594 a. a. in length.[139] A mutation consisting of a deletion of one 'c' nucleotide in exon 11 was found in all FXII-deficient cats tested (see Figure 2.6 and Figure 2.6 Supplemental for identified base). As expected, the homozygote cats had the deletion on both alleles whereas the heterozygotes had only one allele affected. We discovered a single nucleotide polymorphism of a c>t nucleotide change at this site in one normal cat that helped to determine which 'c' had been deleted. The deletion mutation appears to result in a reading frame-shift during translation which would yield a nonsense amino acid sequence with a premature stop codon. Exons 11 through 14 encode most of the trypsin-like serine protease domain

(active site). This nonsense mutation is predicted to be expressed as a truncated, catalytically inactive protein that retains normal fibronectin Type I and Type II, EGF-like, and kringle domains as shown in Figure 2.8.

Figure 2.8: *ExPASy ScanProsite Domain View of Predicted Normal vs. Mutant feline FXII Protein*

The normal feline FXII shares complete domain homology with human FXII by having a Fibronectin Type II, a Fibronectin Type I, a kringle, a trypsin, and two EGF-like, domains. The mutant feline protein is predicted to lack the trypsin domain, thus having no serine protease catalytic activity.



Analysis of the predicted feline FXII proteins revealed many interesting characteristics. The theoretical isoelectric point (pI) and molecular weight (MW) for normal feline FXII are 6.02 and 67.4 kDa, respectively. The predicted/theoretical pI

and MW for the mutant protein are 9.01 and 62.5 kDa, respectively.[140] Predictions of arginine cleavage sites (autoactivation or kallikrein cleavage sites in human FXII) suggest that normal and mutant feline FXII have one site at amino acid Arg^{364}-Val^{365} (site a.a. sequence RLSSLSR|VV) similar to human, however the human FXII has an additional site at Arg^{345}-Glu^{346} (R^{345}-E^{346}) based on the ProP v.1.0b ProPeptide Cleavage Site Prediction program (see Figure 2.6 for locations).[141] Mouse FXII also has one predicted site at Arg^{354} in the same region of the C-terminus, suggesting activation differences may exist between human and other mammals.

Protein studies of plasma and purified feline FXII confirm some of the predicted characteristics. According to WB analyses with various antibodies directed to human FXII, the feline reduced protein migrates at about 67 kDa as seen in Figures 2.1, 2.4 and 2.5. Table 2.3 illustrates that normal feline plasma has expected prekallikrein cleavage ability and can be inhibited with CTI and a monoclonal heavy chain anti-human FXII antibody (directed against Thr^{134} through Arg^{153}). Human studies show similar enzymatic inhibition properties using both agents.[39,142] When the purified and concentrated fractions of FXII protein from normal cats were added to a homozygote cat plasma pool, the activated partial thromboplastin time (APTT) was corrected to near normal values (from >200 seconds corrected to 33.6-43.1 seconds) indicating the purified FXII protein was intact and active and that there was not a potential inhibitor of the FXII activity in the mutant plasma. When the abnormal FXII protein was isolated from deficient cat plasma, no correction of the APTT was given, further indicating the primary defect in FXII is present in the mutants. Mutant feline FXII was detected in plasma samples and purified fractions

using various antibodies (see Figures 2.1, 2.3, 2.5). The apparent size of the mutant FXII protein is close to 66 kDa (as seen in Figure 2.5) with its heavy chain of appropriate size (50 kDa) recognizing the monoclonal antibody B7C9 (Figure 2.1). Using isoelectric focusing and reduced SDS-PAGE separation (Figure 3b, circled, red dot) followed by western blot analysis (Figure 3c, circled), the mutant FXII protein has a slightly different migration pattern than the wild-type protein. These results demonstrate that feline FXII is enzymatically similar to the human protein, that the mutant feline factor lacks activity, and that its truncation is due to the single nucleotide cytosine deletion. Under isoelectric focusing and reduced SDS-PAGE (2-D DIGE) conditions it appears the mutant FXII protein expressed in the plasma has a slightly different migration pattern (Figure 2.2 circled, red dot). Western blot analysis with a polyclonal anti-human FXII antibody suggests the two identified spots (circled) in Figure 2.3 should be FXII proteins which correspond with the identified gel proteins (Figure 2.2 circled).

DISCUSSION

This study has confirmed sections of the LGD 1.9x WGS cat genome and successfully identified normal and abnormal feline mRNA transcripts coding for FXII. The mutation identified in exon 11 of our FXII-deficient cat colony is the first molecular characterization of this defect in domestic short haired cats. Although no identical nonsense mutation has been described in humans, other single base deletions in exons 12 and 14 result in similar phenotypes.[131] Studies of our HZY cat plasmas

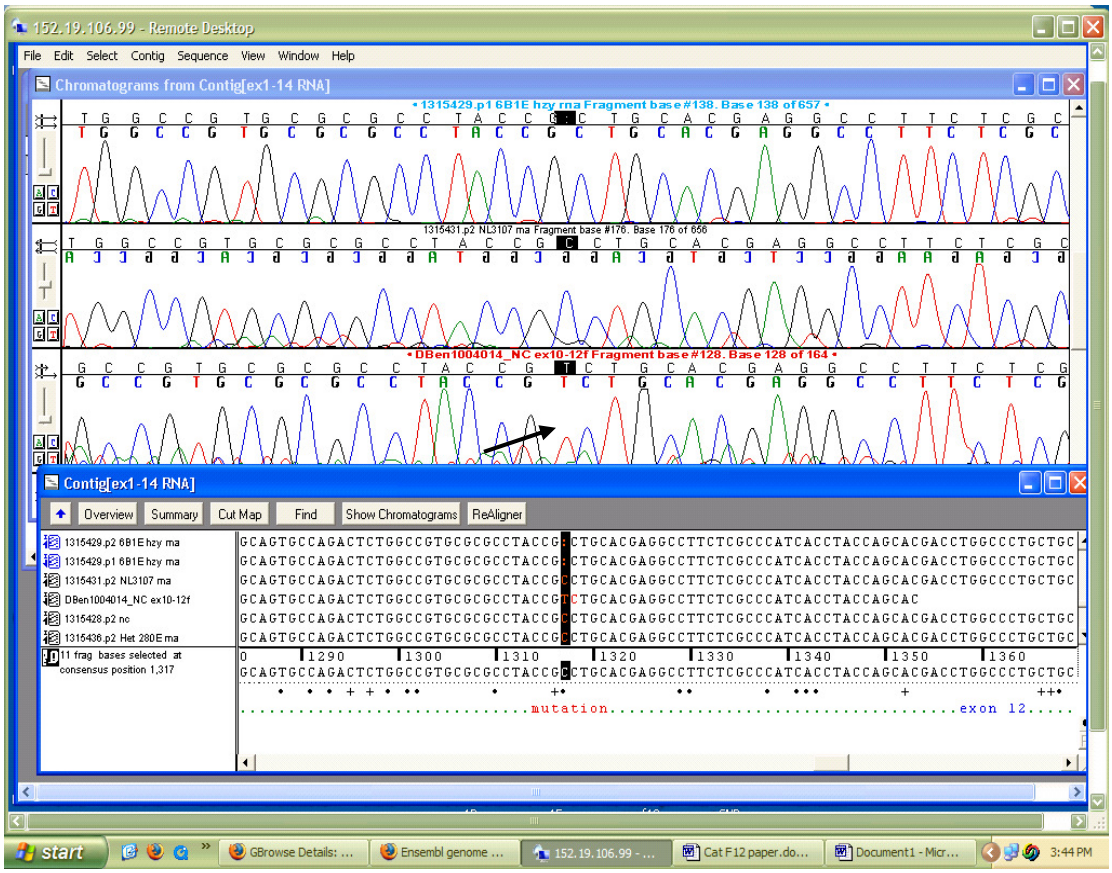
indicate a catalytically inactive cross reacting material-positive protein, as detected by various commercially available polyclonal and monoclonal anti-human FXII antibodies, may be expressed. The active site and heavy chain binding site appear to be similar in feline and human FXII according to their amino acid sequences and protein studies. In humans, the heavy chain has high homology with urokinase and tissue plasminogen activator, along with type II fibronectin regions which may contribute to FXII's binding to cellular surfaces and collagen, respectively.[49,54,60] Corn trypsin inhibitor is thought to bind in the carboxy-terminal region of the light chain in an area distinct from the active site[142], possibly explaining why the mutant cat FXII protein is able to bind the CTI-CNBr affinity column yet not have enzymatic activity. Our studies suggest the expressed mutant cat FXII protein retains a portion of its C-terminal light chain region yet lacks the proper active site--in agreement with the predicted protein characteristics. However we cannot eliminate the possibility that our antibodies are detecting some other cross-reacting protein able to activate FXII (thus inhibited by antibody and CTI) when tested in an APTT assay as shown in Table 2.3. Studies in plasmas from certain FXII-deficient humans indicate that amino acid alterations occurring in the C-terminal region of the protein can yield an expressed protein lacking enzymatic function. The human variant FXII Washington has a G to A nucleotide change in the 3'splice acceptor site of exon 14 that causes the exon 13-exon 14 splicing product to lack one nucleotide. This splice site mutation results in a Cys^{571} to Ser replacement that translates to a detectable truncated transcript with a disrupted active site.[143] Other cross reacting material-positive mutations in FXII deficient patients were identified as an Arg^{353} to Pro (FXII

Locarno) substitution in exon 10 resulting in a loss of the kallikrein cleavage site[144], and a substitution (Trp^{486} to Cys) in exon 12 resulting in a reduced translation and a reduced secretion of FXII due to incorrect protein folding.[132] Most likely the severity of the predicted changes to our colony's mutant FXII protein would result in a misfolded protein that is subject to rapid proteasome degradation.[145] Further studies of the mutant cat FXII protein are needed in order to determine the expression status and sequence of their predicted protein.

In conclusion, new insights into the molecular characterization of feline FXII have been gained. Identification of the FXII genetic defect causing a loss of coagulant and amidolytic activity in a research colony of cats will aid in future analyses of studies investigating the in vivo role of FXII.

Figure 2.6 Supplemental: *Chromatogram of Feline Nucleotide Sequence Analysis Showing Mutation Detection*

Arrow indicates the SNP c>t nucleotide change that identified which 'c' is deleted.



CHAPTER 3

VASCULAR INJURY RESPONSES IN A FELINE MODEL OF FXII DEFICIENCY

ABSTRACT

Vascular injury of the endothelium and exposure of the intima can lead to thrombosis and subsequent activation of the fibrinolytic and immune systems *in vivo*. Factor XII (Hageman factor) can contribute to all of them simultaneously. Humans with FXII deficiency have no bleeding problems after surgery or intravascular procedures.[4] Mice deficient in FXII, however, do not form stable occlusive thrombi after arterial injury which appears to be related to collagen-induced FXII activation and platelet interactions.[35] We used a feline model of congenital FXII deficiency to evaluate thrombotic, fibrinolytic, and inflammatory changes after venous and arterial injuries. Cats deficient in FXII formed stable occlusive thrombi in femoral veins and arteries at the same rate as normal animals. However, contralateral vein injuries 48 hours later showed significantly delayed thrombus formation in FXII deficient cats. Fibrinolysis, thrombin generation, and fibrinogen plasma levels were reduced but not significantly in deficient animals. Fibrinogen degradation products were present in

only one deficient and 2 normal cats. Leukocyte numbers in veins, MMP-1 and MMP-9 expression in veins were significantly lower in FXII deficient cats in 48 hour old vein segments. Leukocyte and/or platelet interactions with FXII in the developing thrombus may play a significant role *in vivo*. Factor XII may have a greater impact on inflammatory responses and the ability to resolve a formed thrombus more so than hemostasis.

INTRODUCTION

For many years the importance of the role of FXII *in vivo* has been disputed. Humans and animals lacking FXII activity do not have bleeding problems and seem to form pathological thrombi, thus it was thought to be unimportant in coagulation. Researchers and clinicians argue over whether FXII deficiency is related to thrombophilia because studies in humans have yielded controversial results, possibly due to unknown risk factors individuals may carry and the inability to control other factors. In recent years targeted gene deletion mouse models have been created to study *in vivo* properties of FXII. The mice studies have yielded many new unexpected findings of its role in thrombosis, however new questions arose as to whether these findings are species specific and possibly not reflective of thrombus formation in humans.

The current thinking of the hemostatic and thrombotic processes may change based on new discoveries about FXII. When a vessel is injured, collagen and matrix underneath the protective endothelial cells is exposed. The pericytes and

adventitial cells surrounding the endothelial cells express tissue factor that combines with blood factor VIIa and activates other coagulation proteases involved with clotting.[36-38] Tissue factor initiated clotting is termed extrinsic (not from the blood) and is thought to be the first pathway of coagulation triggered after traumatic vessel injury. Factor XII was first identified through *in vitro* studies as an initiator of coagulation (intrinsic pathway) when blood deficient in FXII did not clot in a glass test tube.[3] Activation of FXII was found to require a negatively charged surface, such as glass or metals, however a normally present physiological i.e. *in vivo* substance was not identified until recently. Exposure of Type I collagen fibers[34] as seen in traumatic vascular damage and misfolded proteins[35] present in certain inflammatory disease states can activate FXII *in vivo*. The activation of the intrinsic coagulation pathway provides a way to amplify thrombin generation during thrombosis. Thrombin is critical in thrombosis because it cleaves fibrinogen into fibrin strands which then provide a network to trap cells and proteins needed for a stable thrombus formation. Thrombin also activates other coagulation factors, in particular FXI, which can activate FXII thereby accelerating the clotting process. In the absence of FXII, a thrombus can still form because of the TF/VIIa pathway. The FXII deficient mice experiments open new questions as to whether the unstable clots could embolize and become pathologic thrombi somewhere else.[35] Those who argue no firm evidence of a thrombophilia risk exists for severely FXII deficient humans point to many familial and large population studies showing no associations.[13,14,26] The FXII controversy raises serious concerns about species differences and mechanisms of hemostasis, thrombosis and fibrinolysis. The feline model of FXII deficiency may

help answer this question. The congenital FXII deficiency in cats was first described by Green and White.[127] We took advantage of a colony of factor XII deficient cats established first by Kier et al.[6], to study thrombosis, fibrinolysis, and inflammatory differences associated with FXII deficiency.

MATERIALS AND METHODS

Animals

Animal care was provided in accordance with the procedures outlined in the "Guide for Care and Use of Laboratory Animals" (National Research Council; 1996; National Academy Press; Washington, D.C.) and in accordance with a protocol approved by the Institutional Animal Care and Use Committee of the University of North Carolina at Chapel Hill. Domestic breeder cats (Felis catus) were received from P. Bresnahan and limited in-breeding performed to maintain the line. Animals were phenotyped for FXII activity (FXII:C) based on a modified APTT clotting assay as described by Kier et al.[81]and in Chapter 2. Factor XII:C levels of <2% of a normal cat plasma pool defined cats as homozygote (HZY), <85% but >2% of a normal cat plasma pool as heterozygote (HET), and >85% as normal (NL). No spontaneous bleeding tendencies or thrombotic events have been observed in the colony. No cardiomyopathies, immunodeficiencies or autoimmune problems have been observed.

One female and 8 male HZY cats were compared to 4 female and 3 male NL cats (6 derived from outside sources). The ages of the animals varied from 1 year to 11 years, all are considered adult animals.

Surgical procedures

Animals were fasted 8 hours prior to surgery. All cats were anesthetized before venepuncture and surgical procedures with a mixture of ketamine (20mg/ml/kg body weight) and acepromazine maleate (0.11mg/kg body weight). Atropine sulphate (0.25 mg) was given at the time of intubation. Isoflurane gas inhalation (1-3% with oxygen) was used to maintain anesthesia through out the procedure. Sterile surgical technique was used. Each animal received 250 ml of saline over the course of the surgery. No heparins or anticoagulants were used. Blood pressure, pulse, heart rate, body temperature were monitored with Vetspecs VSM6 (Canton, GA) and respirations were charted from the time of intubation until the animal was extubated. All exposed vessels were moistened with sterile saline-soaked gauze to prevent drying. Animals were revived, placed on antibiotics and analgesics, and monitored until they could return to the colony. Two separate surgeries were performed on each animal as follows.

Day 1: The right femoral artery and femoral vein were isolated and baseline blood flow monitored with 20-MHz Doppler flow probe (Indus Instruments). Data from the Doppler probe were collected on an Apple iMac using a PowerLab 4/20 and Chart software (ADInstruments, Colorado Springs, CO). Blood flow was primarily monitored on the femoral vein after injury since the artery required ligation upon catheter removal. A one millimeter disposable cytology brush purchased from Wilson-Cook Medical (Winston-Salem, NC) was introduced into a branching

saphenous vein (distal to the site of injury) and fed cranially to the main portion of the common/external femoral artery or vein approximately 10 cm caudal to the aortic bifurcation. Figure 3.1 illustrates the spatial relationships of the injury site. The brush was chosen based on the apparent diameter of the vessel with the intention to have a close but not too tight fit in order to introduce a moderate amount of intimal damage. Arterial saphenous branches were too narrow in general for our smallest brush, therefore we introduced the catheter-sheathed brush directly into the common femoral artery. This required ligation of the artery at the catheter introduction site afterward. Visual monitoring was used for thrombosis formation proximal to the ligature. The brush was released from its sheath five times before removing the catheter from the animal. Pressure was applied at the introduction site until bleeding was stopped. The probe was placed on the site of injury and flow rates monitored until flow stopped (permanent cessation of flow or PCF) or until 240 minutes was reached. Blood samples were taken immediately distal to the injury site (considered 'local' samples) at timed intervals (0, 5, 15, 30, 60, 90, 120, 180, and 240 minutes) after injury or up to the moment of PCF. Additional 'systemic' blood samples were taken from the cephalic or jugular veins at timed intervals of presurgery, 30, 90, 180, and 240 minutes. After the venous injury was made and initial blood samples collected, the femoral artery injury procedure began (usually around 70 minutes after venous injury). The Doppler probe was removed for the 15 minute procedure. Arterial injury was made similar to the venous injury, however only a pre-injury blood sample was taken, i.e. no further blood samples were taken. No Doppler flow measurements were taken on the artery. Blood flow and visual changes were noted at the site of the

injury. Surgery was ended by closing the wound site with absorbal (polyglycolic acid) and non-absorbal (ethilon) exterior suture material. Antibiotics (ampicillin and amoxicillin) were administered for 10 days.

Day 3: The above anesthesia and prepatory procedures were made on the same cat at 48 hours after the first surgery. The left femoral vein and artery were isolated and injured as described above. The same blood sampling procedures were followed. At the end of blood flow monitoring or 240 minutes, all injured vessels were retrieved. Vessels were placed in 10% neutral buffered formalin for 48 hours then 70% ethyl alcohol prior to standard histological processing (at the UNC Lineberger Cancer Center Histopathology Lab). Tissue orientation was done by silk suture attached to the end of the vessel located cranial to the injury site (referred to as the proximal end). Animals were then euthanized or revived for later terminal studies.

Figure 3.1: Injury Site Diagram For Femoral Vessels

The catheter-sheathed brush (grey line) was introduced in the branch of the saphenous vein (thin arrow) then fed caudally to ~10 cm before the aorta. Five repeated brushings (red area, thick arrow) were made in the femoral vein or artery.

Blood Sampling

Blood was collected into plastic syringes either having 3.8% sodium citrate pH 6.0 preloaded to achieve a 1:9 ratio of sodium citrate to blood respectively, or directly placed into BD Vacutainer tubes with ethylenediaminetetraacetic acid (EDTA). Samples were immediately placed on ice (except EDTA samples). Citrated blood was spun at 10,000 relative centrifugal force (rcf) for 6 minutes at 4˚C, then the platelet poor plasma was immediately separated and stored at -70˚C until used. Samples for blood indices (complete blood count or CBC) were mixed well at room temperature and analyzed on a Beckman Coulter ACTdiff (Miami, FL) automated blood analyzer that was set for feline blood cell parameters. Frozen plasma samples

were thawed at 37˚C then stored on ice when assayed. All samples were run in duplicate for all assays except for fibrinogen degradation products in which 2 different concentrations of plasma were tested. A normal cat plasma pool was made from cats that had not been used for experimental procedures.

Hemostasis Assays

Thrombin Assay: The thrombin specific chromogenic substrate BIOPHEN CS-01 (38) was purchased from Aniara (Mason, OH) and used at a concentration of 1.25 mg/ml. Plasma samples were first removed of their fibrinogen by incubating equal volumes of plasma with 10% polyethylene glycol (PEG)-8000 for 6 minutes at room temperature.[146] They were centrifuged at 10,000 rcf for 3 minutes and the supernatant removed for further assay steps.[147] One hundred microliters (µl) of defibrinated plasma was diluted with 27 µl of 0.05M Tris-HCl/0.3M sodium chloride buffer pH 8.4 and 10 µl of activated partial thromboplastin time (APTT) reagent (Dade) then incubated at 37˚C for 4 minutes in a 96 well microtiter plate (Costar EIA/RIA plate). Eight microliters of chromogenic substrate containing 100 µg/µl of soy bean trypsin inhibitor (SBTI) was added to each well using a multichannel pipetor. One reading was for absorbance at 405nm was immediatelyl taken with a Bio-TEK EL600 (Bio-TEK Instruments, Winooski, VT., U.S.A.) spectrophotometeric plate reader. Ten microliters of 180 mM calcium chloride was added. Readings at 405nm were taken every 30 seconds for 30 minutes. A normal cat plasma pool was used to generate a standard curve using serial dilutions

(undiluted, 1:2, 1:4, 1:8) of defibrinated plasma. The undiluted sample was assigned a value of 100% activity with serial dilutions representing 50%, 25%, and 12.5% respectively. Normal human plasma was run on every plate as an internal control to assess reagent and machine functions. Percentage of thrombin generated was determined by plotting the optical density values against time and calculating the time to one half of the maximum velocity reached (½Vmax) for each time point, then extrapolating their value from a curve generated from the normal cat pool's time to ½ Vmax. Samples that were clotted upon thawing were not used.

Factor XIIa: As desribed in Chapter 2, chromogenic substrate S-2302 (Diapharma Group Inc, OH) H-D-Pro-Phe-Arg-pNA was used to test kallikrein activation as an indicator of FXIIa. Briefly, 25 µl of plasma diluted 1:5 with 0.15M Barbital/0.125M NaCl/0.25% bovine serum albumin pH 7.5 is placed in a 96 well microtiter plate with 25 µl of APTT reagent (Dade) to achieve a final plasma dilution of 1:10. The plate is incubated at 37˚C for 45 minutes then 50 µl of 0.0005 M S-2302 in 0.05M Tris/0.05M Imidazole/0.15M NaCl pH 8.2 is added to the activated plasma and wells are monitored every 10 minutes at 405nm for 1 hour. A standard curve of serially diluted normal cat pooled plasma is included on the plate to determine a % of normal pool activity for each test sample (run in duplicate). The addition of the kallikrein inhibitors, SBTI and Kallistop (American Diagnostica, CT), resulted in no activity seen in normal cat pool plasma therefore it was left out.

Fibrinogen (% of normal pool): A turbidimetric fibrinogen method[148] was modified for use with small sample volumes. The lower detection limits of this

method are reported as 0.2 grams/L. Standard curves of normal cat pool plasma and purified clottable bovine fibrinogen (Sigma-Aldrich) were generated on every plate by running serial dilutions (undiluted, 1:2, 1:4, 1:8) and assigning a value of 200% to the undiluted pooled plasma sample. All samples were run in duplicate. The method is as follows: Citrated test plasmas (25 µl) were diluted 1:2 with 6% bovine serum albumin (BSA)/ 10 mM Na_2HPO_4/138 mM NaCl/2.7 mM KCl /400 µg/ml polybrene pH 7.4 in a 96 well microtiter plate. One reading was taken at 405 nm room temperature to determine a time zero (pre-clotting) absorbance value. One hundred microliters of 6% BSA/ 10 mM Na_2HPO_4/138 mM NaCl/2.7 mM KCl /400 µg/ml polybrene/0.3IU/ml bovine thrombin pH 7.4 were added to each well, being careful to avoid bubbles. Bovine thrombin was aliquoted and stored at -20˚C to preserve its quality and avoid interassay variations. Absorbance increase (ΔA) was measured every 30 seconds for 20 minutes at 405nm. Change in absorbance versus time was plotted for the standard curve and the area of the curve showing the greatest linearity was chosen for comparing ΔA/minute. Usually this time of greatest linearity was between 3 minutes and 5 minutes. The test sample values were extrapolated from the standard curve and reported as percentage of normal cat pool plasma. Four homozygotes and one normal experimental cat were not assayed due to compromised samples.

Fibrin Degradation Products (FDP): A commercially available latex agglutination kit assay for FDP was purchased from Diagnostica Stago catalog # 00540 and used according to manufacturer's instructions. Briefly, test plasmas were diluted 1:2 and 1:8 with Reagent 2 (a glycine buffer). Twenty microliters of diluted

plasma are mixed with 20 µl of Reagent 1 (latex beads coated with a mouse monoclonal anti-human FDP antibody). The reagents are mixed on a special card and rocked for exactly 3 minutes. A determination of "positive" or "negative" is made by comparing agglutination patterns to manufacturer supplied controls. The detection limit for this assay is 2.5 µg/ml. A positive result in a 1:2 diluted sample reflects a level of >5 µg/ml FDPs. This kit is used at the North Carolina School of Veteriniary Medicine (Raleigh, NC) clinical diagnostic lab for feline patients and proven to work in our cat plasmas by tPA treating a clot and testing it after 4 hours. Only presurgery and 240 minute citrated plasma samples were tested for FDPs.

Fibrinolytic Ability Assay: This is a modified[65] fibrin plate lysis assay in which a plasma dextran sulfate euglobulin fraction (DEF) is mixed with a fibrinogen-plasminogen reagent in a microtiter plate. Thrombin and calcium are added and a fibrin clot forms. Plasminogen activating factors present in the DEF will lyse the clot and the optical density (Δ % Transmittance at 405nm) is monitored over time. The details of this procedure are as follows.

DEF preparation (on ice): One hundred microliters of citrated plasma was mixed with 800 µl of cold sterile distilled water then 100 µl of cold sterile dextran sulphate (100 mg/L) then 0.25% acetic acid in this order. Samples were vortexed briefly then spun at 12,000 rcf at 4˚C for 10 minutes. Supernatants were removed by pipetting and 100 µl of sterile 0.05 M Sodium Barbital/ 0.1M NaCL/0.25% gelatin/2.7mM EDTA pH 7.75 was added to the pellet for resuspending the precipitate. Next 1.6 µl of flufenamic acid (final concentration of 4mM) in 0.25M

sodium hydroxide was added to the precipitate and mixed well until all elements appeared dissolved. For samples receiving plasminogen activator inhibitors, 0.5 µl of mouse anti-human uPA (200µg/ml from NeoMarkers Ab-2) and 1 ul of goat anti-human tPA (50µg/ml from American Diagnostica #387) were added to 23.5 µl of the DEF and incubated 30 minutes at 37˚C. A normal cat pool plasma DEF (100%) and a "no DEF" (buffers only) were used as controls that received 1.5 µl of non-immune mouse sera instead of specific antibodies.

For Fibrin Clot Formation: Purified, clottable (>95%) bovine fibrinogen (Sigma-Aldrich) was prepared fresh daily using the same buffer (sterile and refrigerated). An equal volume of 0.2% (weight/volume) fibrinogen in 50mM Sodium Barbital/HCl pH 7.75 was mixed with an equal volume of a 1 Clottable Unit (CU)/ml human Lys-plasminogen (Hematologic Technologies Inc, Essex Junction, VT) in 150mM sodium chloride to achieve a final concentration of 0.1% fibrinogen/0.5 CU/ml plasminogen. One hundred microliters of 0.1% plasminogen-rich fibrinogen (final concentration) was added to a polypropylene 300 µl capacity 96 well microtiter plate well. Samples were run in duplicate. Twenty-five microliters of DEF were added to the fibrinogen mixture and gently mixed. Twenty-five microliters of 36 Unit (NIH)/ml of bovine thrombin diluted in 20mM CaCl/0.1% Tween-80 were added and immediately mixed by gentle tapping of the plate. The microtiter plate was monitored at room temperature on the Bio-TEK EL600 (Bio-TEK Instruments, Winooski, VT, U.S.A.) spectrophotometeric plate reader at 405nm for 24 hours with readings 1 hour apart. A standard curve of normal cat plasma pool DEF (undiluted,

1:2, 1:4, 1:8 dilutions with 150mM sodium chloride) was generated for calculations in which undiluted DEF was given a value of 100%. The percent transmittance will increase over time as the clot is lysed by plasminogen activators in the DEF. The time to reach ½ Vmax was determined for each test sample and compared against the standard curve to calculate the percentage of normal cat plasma fibrinolytic ability. Antibodies for uPA and tPA were tested for their cross reactivity to cat tPA by immunodetection on a Western Blot assay as described in Chapter 2 and by fibrinolytic plate assay described above. Normal cat plasma was diluted and run under reducing conditions in a 12% SDS-PAGE. The gel was transferred to a membrane, blocked with 5% milk, then the primary goat anti-human tPA was incubated overnight at 4˚C at a 1:200 dilution in Tris Buffered Saline (TBS)/1% Bovine Serum Albumin (BSA). A rabbit anti-goat IgG peroxidase conjugated antibody (Cappel Worthington) used at 1:2000 in TBS/1% BSA for 2 hours. Human plasma was run as a control and molecular weight determinations of antigens detected were done by comparison to a molecular weight marker (Sigma P8748). Results are shown in Figure 3.2. Anti-uPA was not detectable with Western Blotting however its ability to inhibit plasminogen activity in the fibrin plate assay was evaluated in normal cat pool plasma. Serial increases of antibody placed in normal cat plasma DEF caused a reduction of clot lysis as expected for inhibition. Normal human plasma DEF was run as a control. Results of the uPA inhibition study are shown in Figure 3.3. The amount of lysis reflects the overall fibrinolytic ability (plasminogen activators and their inhibitors) of the test subject. Reduced lysis reflects either few

plasminogen activators are present, or an increased level of plasminogen activator inhibitors is present.

Figure 3.2: *Western Blot of Goat Anti-Human tPA On Cat Plasmas*

Lane1=kaleidoscope MW marker, Lane 2=empty, Lane 3=Normal human plasma 1:20, Lane 4=Normal cat plasma 1:20, Lane 5=HZY cat plasma 1:20. Thick arrow indicates expected band for two-chain tPA at 70 kDa, thin arrow indicates expected band for sc-tPA at 40 kDa (very faint bands for cat plasmas).

1 2 3 4 5

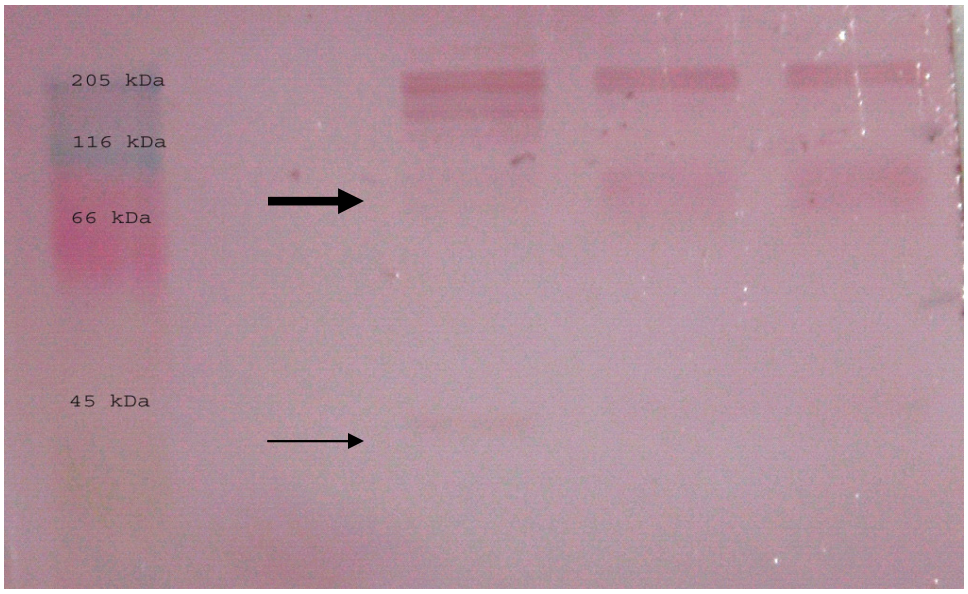
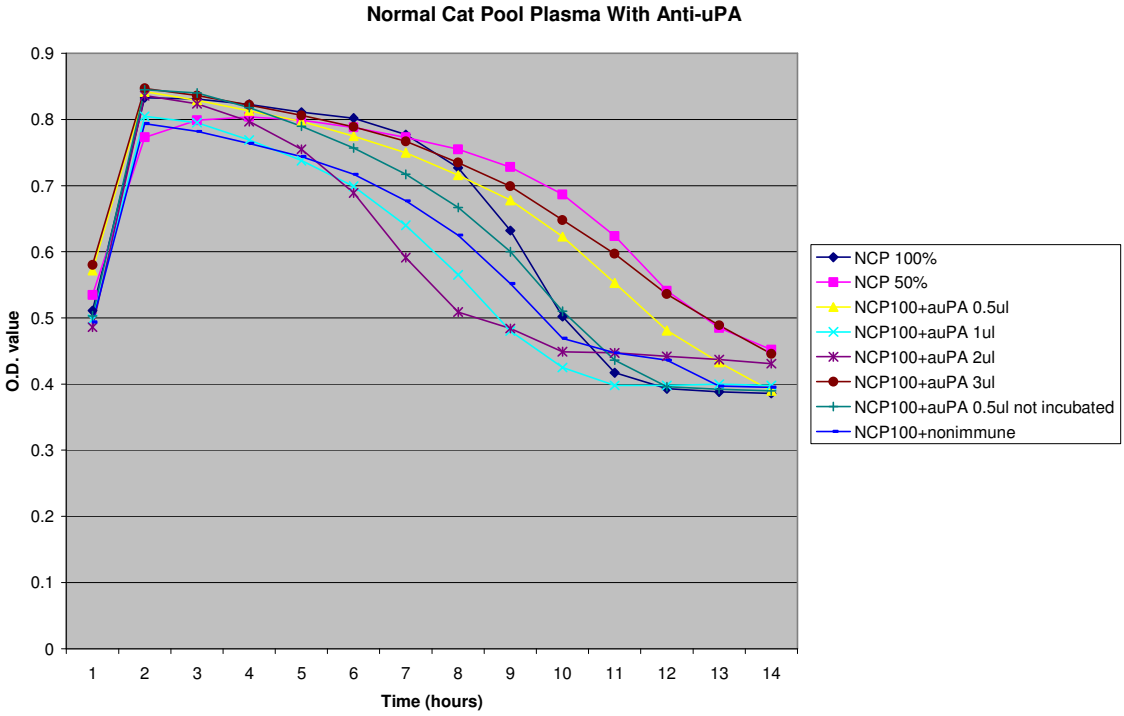


Figure 3.3: *uPA Antibody: Inhibition Studies on Cat Fibrinolytic Activity*



Histopathology Evaluation and Staining

Standard Hematoxylin and Eosin Evaluation: Tissue samples were cut in half cross-sectionally, oriented with the cut edges (middle of the injured area) down for sectioning, were dehydrated in alcohols, embedded in paraffin, and serially cut at 5 micrometers (µm). Two pieces or three pieces of vessel were included on

each slide. The first section of the tissue block was stained with Mayer's Hematoxylin and eosin (H&E) by the UNC LCCC Histopathology Lab, with the remaining serial unstained sections for immunohistochemical staining (IHCS). A single trained evaluator (D.E.B) performed damage score assessments and leukocyte counts (cell#/hpf) of H&E stained slides by the following methods.

Damage Score Assessment: An arbitrary system was created to rank the damage of the vessel wall and the presence of attached and/or occlusive thrombi. The assumption was made that damage into the deeper layers would provide more thrombogenic material thus a thrombus would be present and attached. Scores range from minimal damage (1) to severe damage (5). Since 2-3 separate regions of the injured area were evaluated, scores were averaged for each animal then means were compared using a Mann-Whitney U test. A p value <0.05 was considered significant. The rating system is as follows:

1=endothelium disrupted minimally; thrombus may or may not be present.

2=endothelial and intimal damage of 50% or more of the surface area; thrombus may or may not be present.

3=25% of the media is damaged along with endothelium and most of the intima is removed; attached luminal thrombus may be present. .

4= ~50% of the media remains (no other layers present), much hemorrhage to the media, luminal thrombus is attached and >80% occluding if present.

5=almost no media present, the adventitia has much hemorrhaging, a luminal thrombus is attached and >80% occluded.

Inflammatory Cell Number Evaluation: Each vessel was assessed for an average number of leukocytes present per high power field (20x). A grid system was used to maintain consistency and prevent biasing by the evaluator. A vessel was divided into 5 regions as compared to a clock face: 12-O'clock, 3-O'clock, 6-O'clock, 9-O'clock and the center point. Each region was counted at 20x for all leukocytes present for a total of five hpf counts. The five regions were then averaged and the means were compared using the Mann-Whitney U test in order to measure inter-group differences having large variances. A p value of <0.05 was considered significant.

Immunohistochemistry Methods: All tissue sections were deparaffinized and rehydrated in xylenes (5 minutes x 2), 100% ethanol (3 minutes x 2), 95% ethanol (3 minutes x 2), 75% ethanol (3 minutes x 2), 50% ethanol (3 minutes x 2), then phosphate buffered saline (PBS) for 3 minutes x2 washings. Endogenous peroxidases were blocked with 0.3% (v/v) aqueous hydrogen peroxide in methanol for 19 minutes. Tissues were then subjected to heat-induced antigen retrieval using a microwave method[149] and citrate buffer. Briefly, slides were submerged in 0.1M citrate buffer pH 6.0 and heated to 100˚C and maintained for 18 minutes by

intermittent starting and stopping of the microwave. Slides were allowed to gradually cool to room temperature before washing with PBS. Tissues were block of non-specific proteins at room temperature for 30-60 minutes with the appropriate normal sera or milk (5%) according to the secondary antibody species used. The primary antibody was applied at a defined dilution for overnight incubation at 4˚C. Table 3.1 shows the specific details of each antibody used. The MMP-1 and MMP-9 were previously reported to work in cat tissue.[150]

Table 3.1: *Antibodies Used For Immunohistochemistry of Vessels*

	1' antibody	clonality	species	blocking agent	1' dilution	2' antibody	2' dilution
MMP-1	Lab Visions Corp, Ab-6	poly	rabbit	goat	1:50	goat-HRP	1;200
MMP-9	Lab Visions Corp, purified, 9234-p1	epitope specific	rabbit	goat	1;100	goat-HRP	1;200
tPA	Innovative Research, purified on protein G	poly	sheep	horse	1:50	donkey-HRP	1;100
uPA	Innovative Research, antiserum	poly	rabbit anti-mouse	goat	1:50	goat-HRP	1;300

The secondary antibodies, horse radish peroxidase (HRP) labeled goat anti-rabbit or donkey anti-sheep (Sigma-Aldrich), were diluted in PBS/1% BSA, and applied at room temperature for 1 hour. Slides were washed three times in PBS/0.1% Tween-20 then developed with Vector Labs NovaRed and counterstained with 0.01% (w/v) Methylene blue stain. Tissues were washed in water then dehydrated in the same series of alcohols and xylenes before permanently coverslipping. A positive control tissue slide of cat placental tissue was run with each batch of staining. Positive staining is indicated by a red precipitate against a light blue background. The primary

antibody was not added for negative control specimens (no 1˚). Each tissue stained had a negative control along side of it (the next serial section).

Staining Evaluation: The intensities of immunohistochemical staining for uPA, tPA, MMP-1, and MMP-9 were ranked in a semi-quantitative blinded manner by 1 histologist (DEB), using a scale from 0 to 3 as follows:

(0) indicating no staining (background);

(1) indicating weak staining in a few cells and/ or matrix;

(2) indicating weak staining in the majority of cells or strong staining in a few cells and/or matrix;

(3) indicating strong positive staining in the majority of cells and/or matrix.

The observations were made in the media-intima area of cross-sections (1-3 tissue pieces per cat) of the injured region of the vessel. Two uninjured vessels collected from an area about 10 centimeters distal to the injury site were stained for MMPs only. Differentiation was made between the cellular staining intensities versus matrix component staining intensities. The mean score for staining intensity of cells in each animal was compared within groups and between groups using an analysis of variance (ANOVA) and Mann-Whitney U test with a p value <0.05 indicating significance. The mean score for staining intensity of matrix components in each animal was compared within groups and between groups using an analysis of variance (ANOVA) and using a Mann-Whitney U test with a p value <0.05 indicating significance. A total staining intensity was made by averaging the cell staining mean

value with the matrix staining mean value of each animal, then comparing the total staining intensity means of each group using a Mann-Whitney U test.

Statistical Analysis:

The statistical software Statmost 32 (Dataxiom) was used for analyses. The arithmetic means were used for most analyses except the time to 50% flow reduction which had large positive outliers therefore the median and nonparametric comparisons were used. For statistical purposes, a cat that did not have a 50% flow reduction during the experiment was given the value "240". One standard deviation (SD) value was used as a measure of variance around the mean. Comparisons of between group means were made using a one-way ANOVA and Mann-Whitney U test. When the sample distribution was found to be normally distributed, an unpaired Student's t-test was employed to compare means. Linear correlations of damage with 50% flow reduction, cell numbers in veins, and IHCS staining intensities were done using the Spearman's rank correlation. Groups are identified as severely deficient in FXII (HZY), normal FXII levels (NL), first day of surgery (Day 1), second surgery 48 hours later (Day 3), vessel retrieved after 4 hours (4 hr), and vessel retrieved after 48 hours (48 hr).

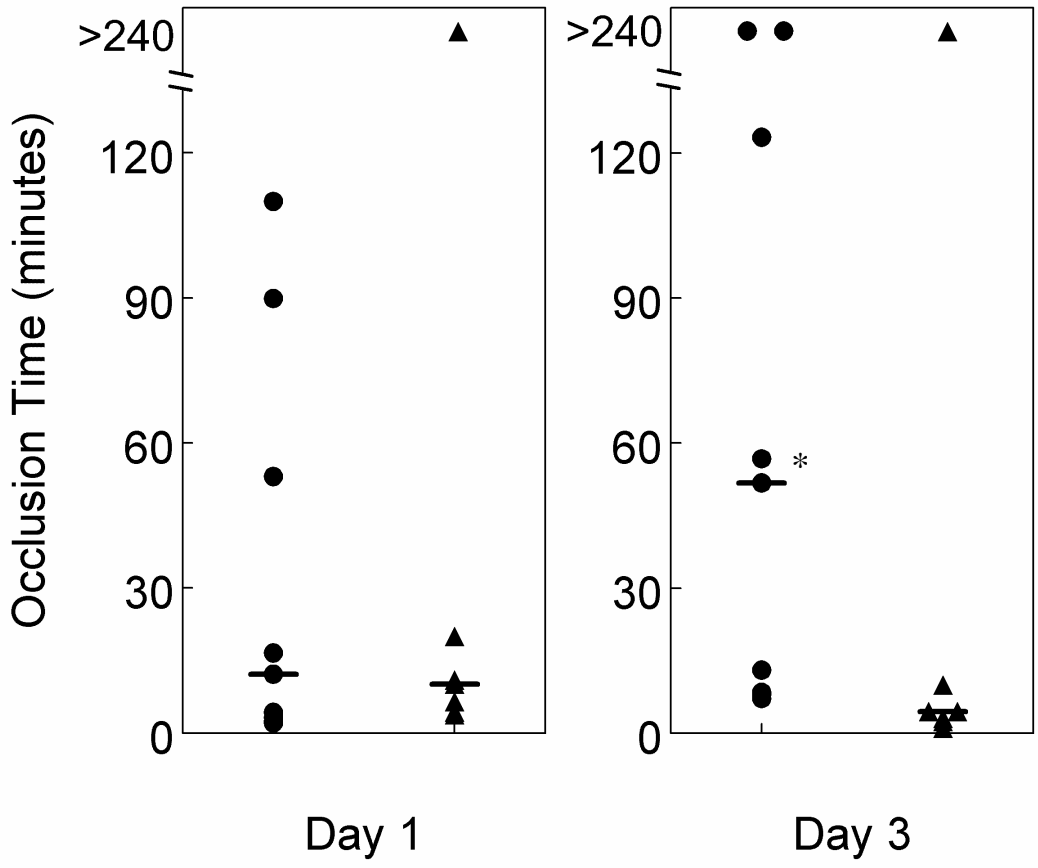
RESULTS

A. Venous Blood Flow Changes

There was no difference between groups in the time taken to achieve a 50 percent reduction in blood flow velocity upon first injury of the right femoral vein (Day 1). However, after injury on the contralateral (left) leg femoral vein (Day 3), the HZY were significantly impaired in forming thrombi compared to NL cats of Day 3. There were no significant differences between Day 1 and Day 3 values. The results are given in Figure 3.4.

Figure 3.4: *Time Taken To Achieve A 50% Reduction In Blood Flow*

Homozygote cats (circles) had a median time to occlusion of 12.2 minutes on first vein injury (Day 1), while normal cats (triangles) median time was 10.1 minutes. Upon contralateral leg injury (Day 3), homozygote cats median time (51.8 minutes) was significantly longer (asterisk indicates p=0.03 at 95% confidence interval) than normals (4.5 minutes) using a Mann-Whitney U test for Day 3 only.



Analysis of the number of cats having a permanent cessation of blood flow (PCF) after the brush injury is given in Table 3.2. A PCF is indicated on Doppler by a flow velocity measurement of less than 15 Hz/ms with no spontaneous return to flow. The percentage of animals having a PCF on Day 1 was nearly the same for both groups (67% HZY vs. 57% NL). However, a dramatic difference is seen on Day 3. None of the HZY cats formed occlusive thrombi in their femoral vein, whereas 43% of the normals did.

B. Hematological Parameters:

Complete blood cell counts were measured pre-surgery for each cat and values are listed in Table 3.2. Platelet counts were evaluated on the blood smears made for each cat and indicated normal numbers present. Machine platelet counts were unreliable due to platelet clumping and size differences. A significant elevation of WBCs was seen at Day 3 for both groups, however between groups (HZY vs. NL) WBC counts were not significantly different as shown in Figure 3.5 There was a significant decrease ($p=0.04$) in HCT between Day 1 and Day 3 for HZY, but again no intra-group differences. No excess bleeding was observed to explain this difference in hematocrit.

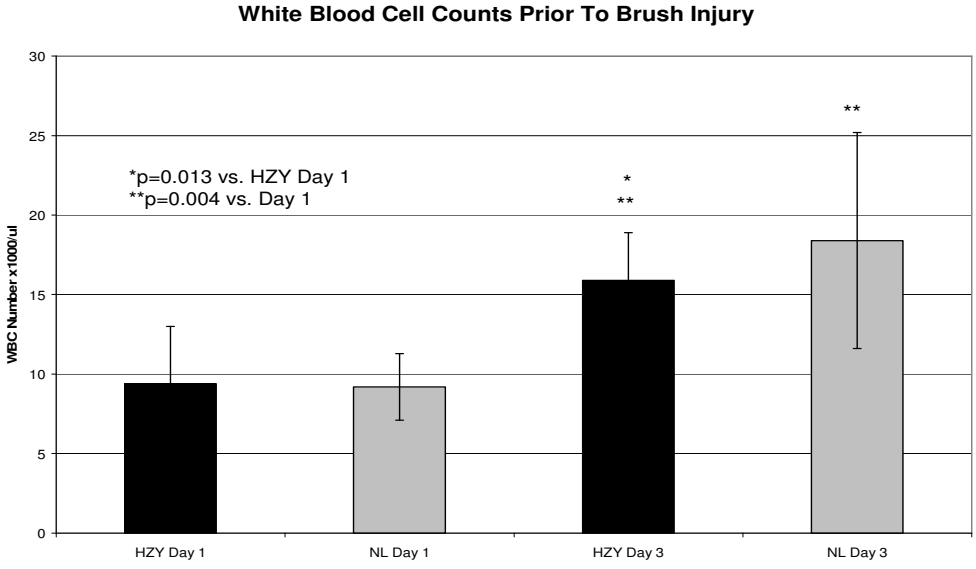
Table 3.2: *Individual Animal Data: Blood Flow Reductions, WBC, and HCT Values*

FXII:C (FXII clotting activity), if PCF (permanent cessation of flow) occurred is a yes (Y) or did not occur is a no (N) and the percentage of animals that had PCF,

WBC (white blood cell count) values are multiplied by 10^3 to indicate the number of cells per cm^2, and HCT (hematocrit) values are listed as percentage of packed red blood cells. Averages listed are means +/- SD except for 50% flow reductions are given in medians.

			Phenotyping	50% flow reduction	50% flow reduction			WBC	HCT%	WBC	HCT%
	sex	age	FXII:C	Day 1 (mins)	Day 3 (mins)	Day 1 PCF	Day 3 PCF	Day 1	Day 1	Day 3	Day 3
HZY 1	M	10 Y	<1%	12.2	8.5	Y	N	nd	nd	nd	nd
HZY 2	M	4 Y	<2%	3.2	56.8	Y	N	10.1	27.4	17	21.8
HZY 3	M	4 Y	<2%	16.6	13.1	Y	N	9.8	35.1	16.8	24.6
HZY 4	M	4 Y	<2%	4.3	>240	Y	N	8	31	nd	nd
HZY 5	M	3 Y	<2%	89.9	123.3	N	N	17.4	29.5	19.7	27.5
HZY 6	M	3 Y	<2%	2.1	7.2	Y	N	7.3	25.6	11.1	24
HZY 7	M	3 Y	<2%	110	8	N	N	5.6	29.6	13.7	28.9
HZY 8	M	3 Y	<2%	53.1	51.8	N	N	10.3	26	nd	nd
HZY 9	F	8 Y	<1%	2.5	>240	Y	N	6.9	25.5	17.1	23.8
				median= 12.2	median= 51.8	67%	0%	9.4+/-3.6	28.7+/-3.3	15.9+/-3.0	25.1+/-2.6
NL 1	M	2 Y	99%	6.5	3.1	Y	N	12.7	27.6	26.1	34
NL 2	M	1 Y	101%	>240	10	N	Y	9.37	27.6	21.4	23.8
NL 3	M	1 Y	96%	11	1.1	N	N	8.9	28.1	12.9	25.2
NL 4	F	2 Y	105%	3.8	240	Y	N	9.5	29.6	20.8	25.6
NL 5	F	1 Y	93%	4.4	4.5	Y	Y	8.6	31.5	10.4	21.1
NL 6	F	11 Y	86%	10.1	4.5	Y	Y	9.9	21.6	26.1	19.9
NL 7	F	1 Y	100%	20	2.5	N	N	5.72	26.5	11.1	19.7
				median= 10.1	median= 4.8	57%	43%	9.2+/-2.1	27.5+/-3.1	18.4+/-6.8	24.2+/-5.0

Figure 3.5: *Differences in Systemic WBC Counts*

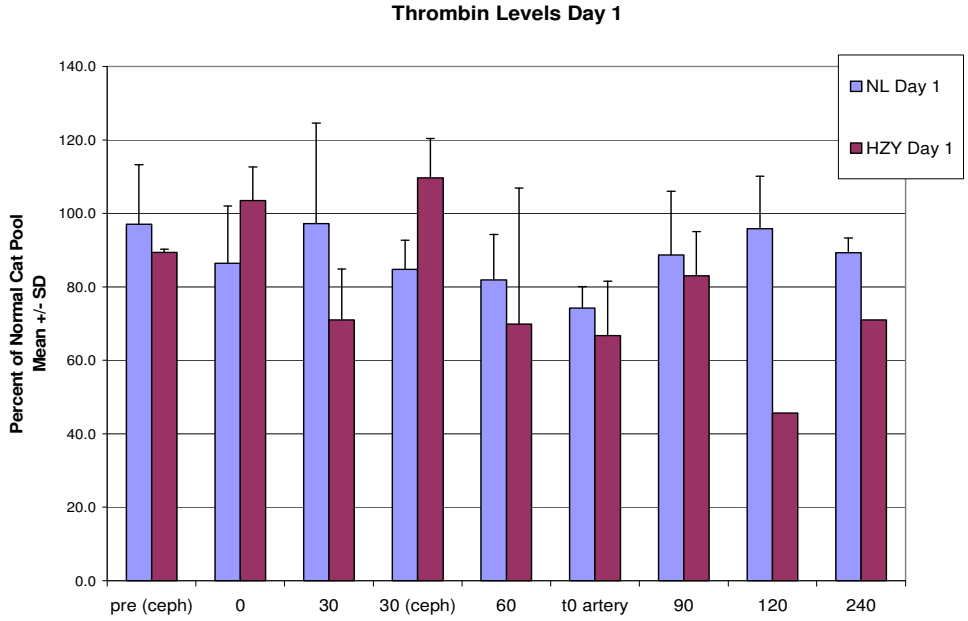


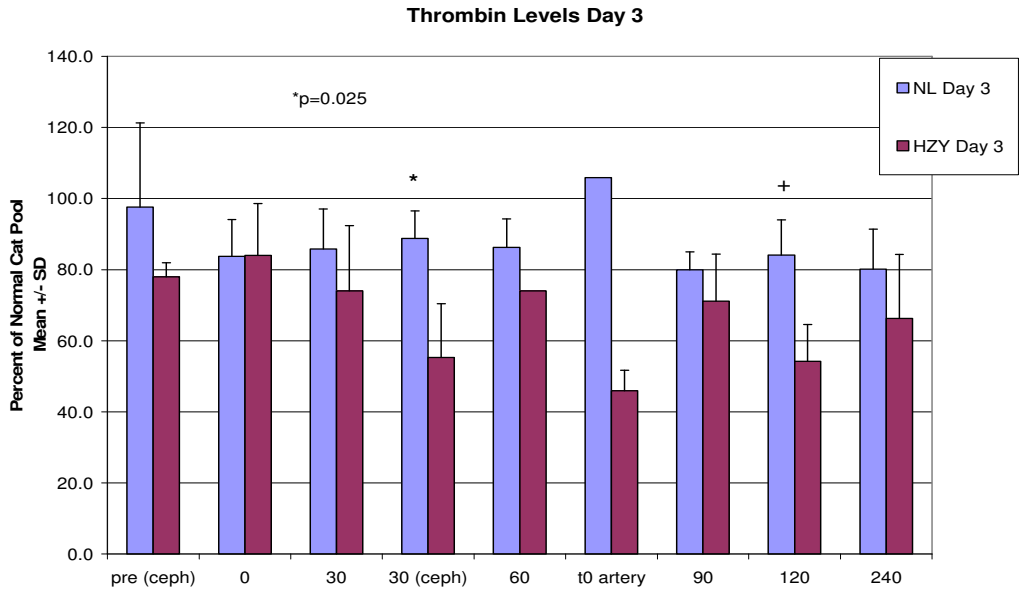
C. Hemostasis Parameters

Thrombin Levels*:* Although HZY cats had lower levels of thrombin generation compared to NL animals, only one time point showed a significant difference yet another was nearly significant. The 30 minute cephalic "systemic" was significantly different with a p=0.025 and the 120 minute (p=0.052) was nearly significant on Day 3. Not all animals and time points were tested therefore statistical analysis were only done on presurgery, 0, 30, 30 cephalic, 90, 180, and 240 minutes after injury samples. Numbers of animals analyzed were: HZY (n=3 to 5) and NL (n=4-6). Figure 3.6 compares means of both groups at each sampling time point (in minutes).

Figure 3.6: *Thrombin Levels Before And After Injury For Each Day*

The "t0 artery" refers to a sample taken from the femoral artery prior to injuring. This time point varied but generally it was collected between 60 and 90 minutes. Pre (ceph) refers to presurgery sample taken from the cephalic vein. The "30 ceph" refers to a 30 minute sample taken from the cephalic vein.

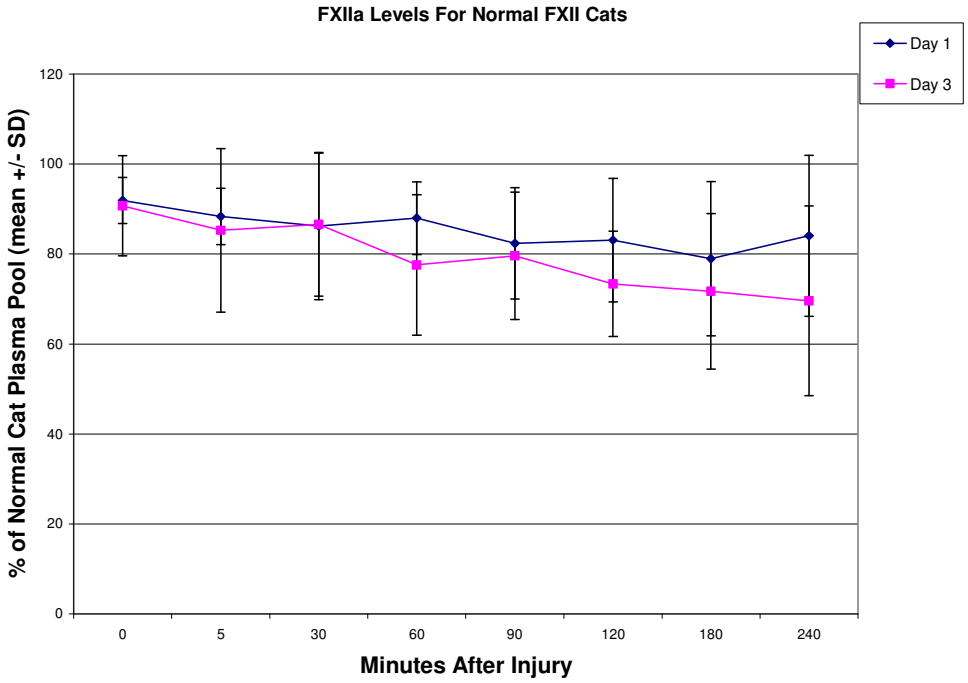




FXIIa levels: Only the NL cats were analyzed for FXIIa. No significant differences were seen between days or across time points. A general decrease in FXIIa level was seen on both days from the initial presurgery time point (see Figure 3.7)

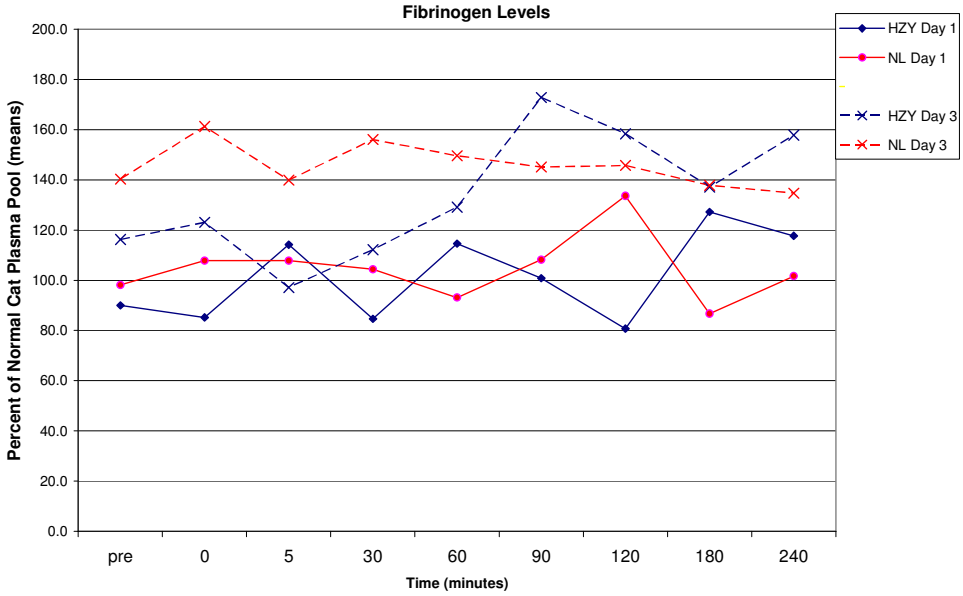
Figure 3.7: *Factor XIIa Levels In Normal Cats Before And After Injury*

The line graph depicts the change in activated FXII through the duration of the surgeries on Day 1 and Day 3. Homozygote cats have no FXIIa therefore they were not assayed. Data is shown as means +/- SD.



Fibrinogen levels: No significant differences were seen in any parameters. Standard deviations were large and numbers of HZY samples were low (n=3). A general increase in fibrinogen was seen on Day 3 in all animals, possibly reflecting an acute phase physiological inflammatory response by the animals. Data means are shown in Figure 3.8.

Figure 3.8: *Fibrinogen Levels Before and After Injury*



FDPs: Only three animals in total (HZY #5, NL #5, and NL #6) had a positive agglutination at 1:2 dilution in their 240 minute sample on Day 1, indicating a level of FDPs being >5 ug/ml. No other positive FDPs were seen.

Fibrinolytic Ability: There were significant differences seen in early time points (presurgery, 0, 5, 30 minutes) between the HZY and NL groups. Comparisons of means were performed on DEF samples and not the anti-uPA and anti-tPA treated clots. Large variations of individual samples of each group made the anti-uPA and anti-tPA plasminogen activator blocking data unreliable so only the no antibody

treated wells were used for comparisons. Figure 3.9 shows a plot of fibrinolytic activity (plasminogen activating activity) over the time course of the surgery(s). Normal cats started with 36% more fibrinolytic activity than HZY (p value is 0.067) on Day 1. The group means +/- SD are as follows:

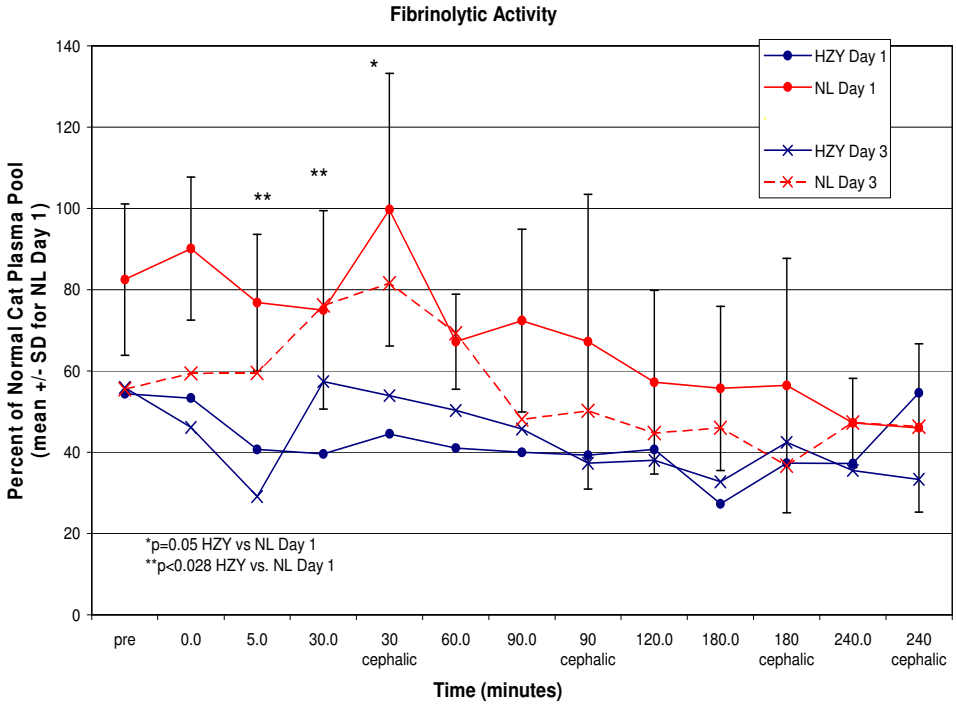
NL Day 1 presurgery:	82.5 % +/- 18.6
HZY Day 1 presurgery:	54.4 % +/- 30.4

NL Day 3 presurgery:	55.5% +/- 32.6
HZY Day 3 presurgery:	55.9 % +/- 20.4

After 5 minutes, a significant reduction of fibrinolytic activity (p=0.028) developed on Day 1 in the HZY compared to the NL plasma as illustrated in Figure 3.9. The 30 minute time points from Day 1 also showed significantly reduced levels of FXIIa (p=0.018) as well as the 30 minute cephalic source (systemic) p=0.05 between groups (Mann-Whitney U) as shown in Figure 3.7. No other time points were significant for fibrinolytic activity. Additional comparisons of data revealed no significant correlations with FXIIa or thrombin levels. We normally see a 25-40% reduction in fibrinolytic ability between non-experimental normal and homozygote cat plasma DEFs when run in this assay.

Figure 3.9: *Fibrinolysis Activity of Plasma DEF Before and After Injury*

The change in fibrinolytic ability is shown below for both normal and homozygote groups. Data is depicted in means. The standard deviation (SD) is shown only for the normal cats on Day 1 since the variances are large in all groups.



D. Histological Findings

Damage Scores: Table 3.3 contains the means and a one standard deviation variance for the amount of damage assessed due to brush injury. A nomenclature of "4 hours" and "48 hours" is given to vessels from the groups of Day 3 and Day 1 of injury respectively. This time refers to the length of time the injured vessels remained in the body. No significant differences were found between damage scores. The

higher amount of damage seen in arteries is probably related to the small size of the artery compared to the brush diameter. Unfortunately no smaller size brushes were available to try.

Table 3.3: *Damage Score and Leukocyte Counts of H&E Stained Vessels*
Data is given in means +/- SD. One asterisk (*) indicates significance between 4 hour vein groups i.e. HZY vs. NL. Two asterisk (**) indicates significance between HZY 4 hour vein vs. NL 48 hour vein.

		Damage Score		
	4hr vein	**48hr vein**	**4hr artery**	**48hr artery**
	mean+/-SD	mean+/-SD	mean+/-SD	mean+/-SD
HZY	**1.7+/-0.8**	**2.9+/-1.2**	**3.1+/-1.9**	**3.0+/-1.6**
n=	7	7	7	7
NL	**2.6+/-1.6**	**2.6+/-2.6**	**3.1+/-1.1**	**3.6+/-0.9**
n=	7	7	7	7
		Vessel Leukocyte Counts		
	4hr vein	**48hr vein**	**4hr artery**	**48hr artery**
	WBC #/hpf	WBC #/hpf	WBC #/hpf	WBC #/hpf
	mean+/-SD	mean+/-SD	mean+/-SD	mean+/-SD
HZY	**84+/- 42**	**113+/-60**	**99+/-95**	**155+/-109**
n=	9	7	9	7
NL	**153 +/- 74**	**164+/-52**	**112+/-73**	**155+/-93**
n=	7	7	7	7
	*	**		

Leukocyte Counts of Injured Vessels: The average number of white blood cells counted per 20x high power field (hpf) of vessel is given in Table 3.3. A significant difference in WBC/hpf was found between HZY 4hour veins and NL 4 hour veins (p=0.039 Mann-Whitney U) and between HZY 4 hour veins and NL 48 hour veins (p=0.009 Mann-Whitney U). No differences were found in arteries. The

amount of damage only correlated with leukocyte numbers for the HZY 48 hour vein samples, having a correlation value of 0.8046 and a p value of 0.029. It is uncertain why there is a correlation only in this group.

Immunohistochemical Staining Results:

MMP-1 Staining: The staining pattern was separately scored for cells and matrix components (vessel wall and advential regions) since MMPs are present in both and may function differently. Upon analysis of each parameter (cells staining positive versus matrix components staining positive) in HZY versus NL, significant differences were seen. Normal cats had higher intensities in cells ($p=0.0165$ ANOVA) and in matrix components ($p=0.0086$ ANOVA) staining positive. There was significantly more staining in the vein matrix than in artery matrix for NL cats in their 48 hour vessels ($p=0.012$ Mann-Whitney U). The HZY cats *did not* show this vein>artery matrix intensity difference. At 48 hours the HZY cats had more *cellular* staining in their vein segments compared to their vein matrix staining ($p=0.021$). The staining pattern (cell>matrix) was nearly significant ($p=0.052$) in 4 hour vein segments. Arterial segments had much larger significant differences in cell versus matrix staining positive within groups (i.e. HZY 4 hour versus 48 hour) for both HZY and NL cats ($p<0.001$). Comparisons between groups (NL vs. HZY) showed a significant increase in total staining of the NL veins (derived by averaging cell intensity and matrix intensity values in each grouping) at 48 hours using the Mann-Whiney U test. Table 3.4 outlines these differences. Seven normal and 9 homozygote cats were evaluated for MMP-1 staining intensity.

Table 3.4: *Intensities of Staining Scores for MMP-1 and MMP-9*

MMP-1	n = 9	n = 7	
	HZY mean+/- SD	NL mean +/- SD	
4hr Vein cells	1.9 +/- 0.4	2.4 +/- 0.5	
4hr Vein matrix	1.1 +/- 1.1	1.3 +/- 0.7	
48hr Vein cells	2.0 +/- 0.8	*2.6 +/- 0.4	
48h Vein matrix	1.0 +/- 0.5	**2.2 +/- 0.8	
4hr Artery cells	2.8 +/- 0.4	2.6 +/- 0.9	+p=0.012
4hr Artery matrix	0.9 +/- 0.5	0.7 +/- 0.7	
48hr Artery cells	2.7 +/- 0.4	2.6 +/- 0.5	
48hr Artery matrix	0.9 +/- 0.6	0.8 +/- 0.7	

MMP-9	HZY	NL	
	mean+/- SD	mean +/- SD	
4hr Vein cells	1.9 +/- 0.4	2.4 +/- 0.5	
4hr Vein matrix	1.1 +/- 1.1	1.3 +/- 0.7	
48hr Vein cells	2.0 +/- 0.8	*2.6 +/- 0.4	
48h Vein matrix	1.0 +/- 0.5	**2.2 +/- 0.8	
4hr Artery cells	2.8 +/- 0.4	2.6 +/- 0.9	+p=0.023
4hr Artery matrix	0.9 +/- 0.5	0.7 +/- 0.7	
48hr Artery cells	2.7 +/- 0.4	2.6 +/- 0.5	
48hr Artery matrix	0.9 +/- 0.6	0.8 +/- 0.7	

*p=0.049 vs HZY 48hr
**p=0.009 vs. HZY 48hr

MMP-9 Staining: The same approach as in MMP-1 was used with MMP-9. Nearly identical results were seen (see Table 3.4). Normal cats had higher

intensities in cells (p=0.0081 ANOVA) and in matrix components (p=0.0004 ANOVA) compared to the HZY. The only other difference was the p value for the 48 hour NL vein matrix versus 48 hour NL artery matrix was slightly higher (p=0.023 using a Student's t-Test since the distributions were normal). Seven normal and 9 homozygote cats were evaluated for MMP-9 staining intensity.

In summary of the MMP staining data, NL cats had significantly more MMP-1 and MMP-9 expressed in their veins at 48 hours than the HZY cats. Normal cats also had more MMP expression in their vein matrix compared to their arterial matrix. The HZY cats did not have this vein>artery matrix staining difference. When all parameters (cells and matrix) were averaged together, the NL cats showed a significant increase in total MMP-1 staining of their *arteries* compared to their veins at 48 hours (p=0.005 Student's t-Test). This increased artery MMP-1 staining correlated positively with the number of WBCs in their (NL) arteries (p=0.039, correlation coefficient of 0.8332) at 48 hours. In contrast, combining all parameters for MMP-9 staining in the NL cats at 48 hours showed an increased staining intensity in their *veins* compared to their arteries. No other significant correlations were seen with MMP staining and WBC per high power field. No significant correlations were seen between the damage score and MMP staining, or between MMP-1 and MMP-9 in all combinations tested.

Urokinase plasminogen activator and tPA staining: Only 3 animals in each group were stained with these antibodies. No specific positive control was available so the pattern of staining fibrin strands and endothelial cells was the indicator for specificity and quality of staining. There were no differences found in uPA or tPA

staining intensities between animal groups or vessel types. The limited number of animals stained did not allow for correlation analysis. Microphotograph examples of all stainings are given in Figure 4.0.

The staining intensities for each group are as follows (mean +/-SD):

uPA:	HZY 48hr Vein	2.7 +/- 0.6
	NL 48hr Vein	2.7 +/- 0.3
	HZY 4hr Artery	2.25 +/- 0.4
	NL 4hr Artery	2.25 +/- 0.4
tPA:	HZY 48hr Vein	1.7 +/- 0.6
	NL 48hr Vein	1.6 +/- 1.2
	HZY 48hr Artery	2.1 +/- 0.9
	NL 48hr Artery	2.2 +/- 0.7

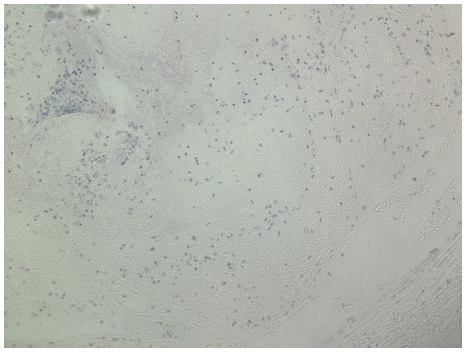
Figure 4.0: *Examples of Immunohistochemitry Staining*

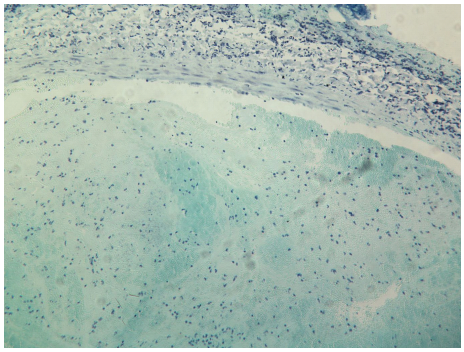
All photos are at 10x magnification unless otherwise stated. Positive staining is indicated by a purplish-red precipitate. Bright blue coloring is the background stain.

IMMUNOHISTOCHEMISTRY STAININGS FOR CATS

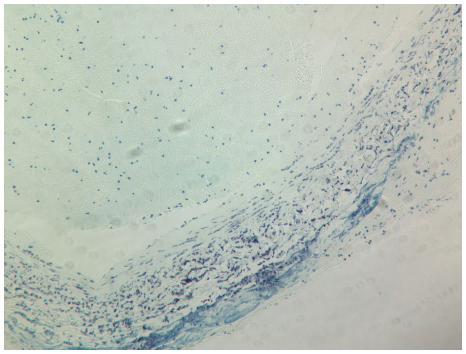
HZY 48 HOUR VEIN NL 48 HOUR VEIN

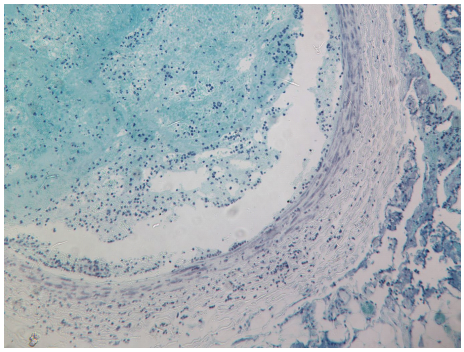
MMP-1



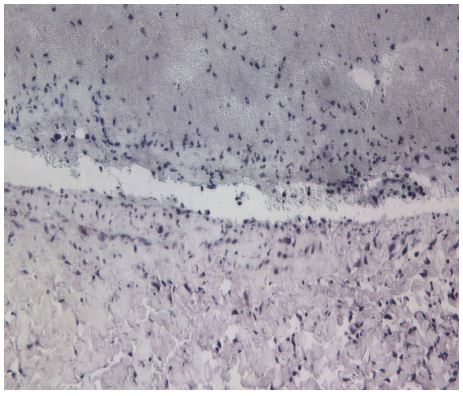


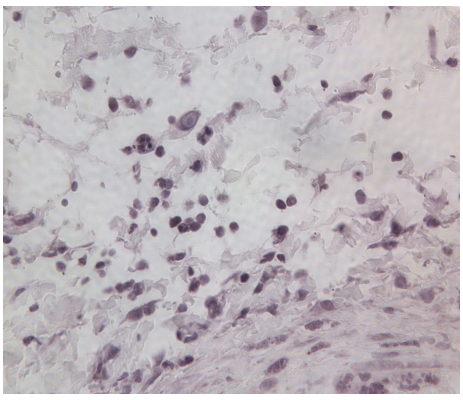
MMP-9





uPA



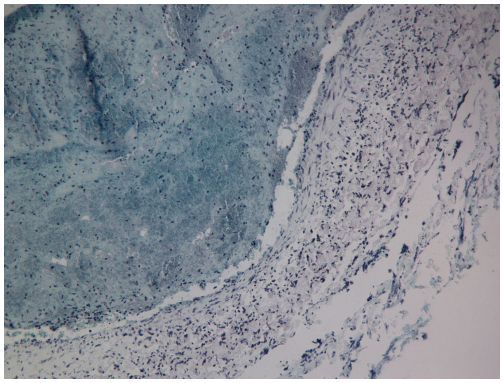


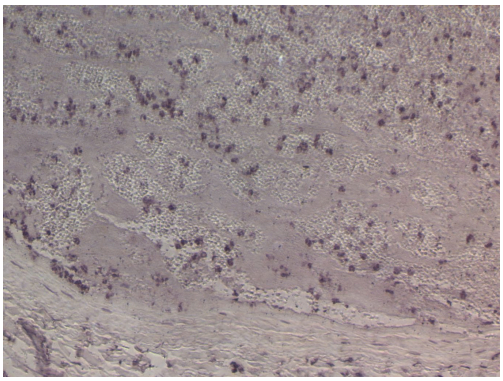
20x 40x

HZY 48hr VEIN

NL 48hr VEIN

tPA





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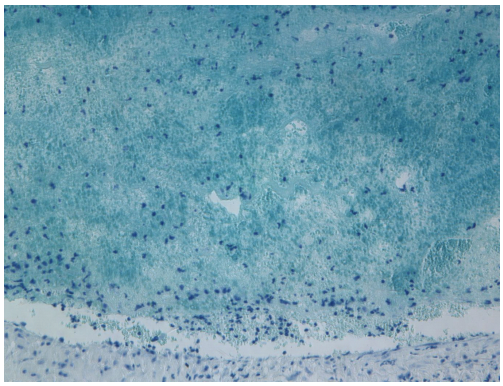
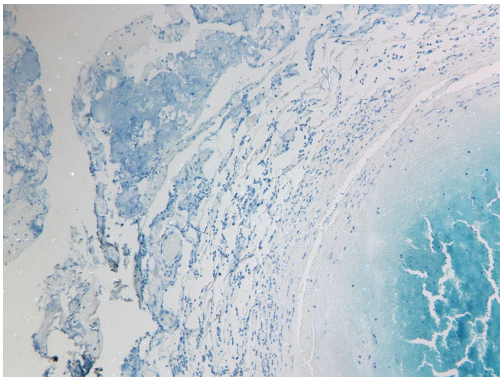
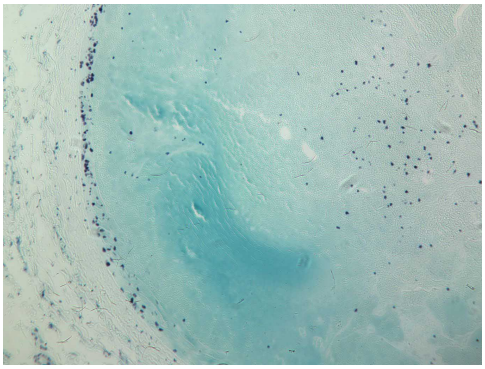


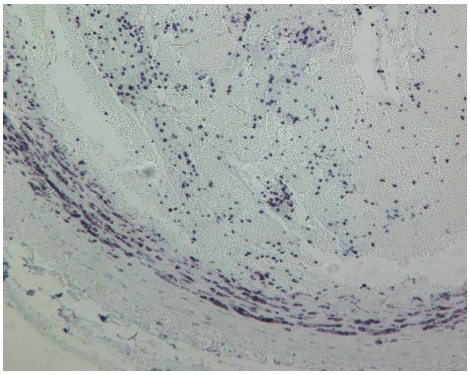


HZY

NL

MMP-9
Arteries
48 hours





DISCUSSION

The recent unexpected findings in the FXII deficient mouse model have created a growing interest for more *in vivo* studies concerning the role of FXII in thrombosis. Our experiments also have shown unexpected findings in a different species, adding more intrigue to this "elusive" protease. The FXII deficient cats are able to form stable occlusive thrombi in veins at the same rate as normals on first injury. Since we were not able to measure blood flow in the adjacent artery, little can be concluded about the role of FXII in arterial thrombosis in the cat. However personal observations and unpublished data using the Folt's model of carotid artery injury and measuring blood flow hemodynamics indicate that FXII deficient cats are able to form occlusive thrombi at nearly the same rate as normal cats. The exciting new observation we report is that FXII deficient animals are significantly impaired in being able to form stable occlusive thrombi upon a second venous injury 48 hours later. The significance of this finding may have impact on therapeutic strategies and monitoring of patients after repeated intravenous catheterization procedures or other vascular surgery procedures. We attempted to find the reason(s) for the hemostatic difference by evaluating different factors important to thrombosis and fibrinolysis. Is the delay in thrombus formation and inability to fully occlude on Day 3 due to a lack of thrombin generation or the presence of more profibrinolytic agents? Our findings indicate that thrombin levels on Day 3 are not significantly different, albeit they are

reduced compared to normal cats. It is possible that the slightly lower thrombin levels in FXII deficient cats contribute to their thrombotic change. The significant finding (reduction) in the 30 minute cephalic samples may be due to sampling techniques since the cephalic vein can be difficult to access during surgery. To answer the question concerning a profibrinolytic agent we looked at the fibrinolytic ability of plasma in euglobulin fractions and the presence of FDPs. Only a few animals had detectable fibrin degradation taking place, therefore rapid clot dissolution is not likely the explanation. Further confirmation of this idea is given by the fibrinolytic ability assay. The HZY cats had no significant changes in their ability to lyse a clot on Day 3 compared to Day 1. In fact the NL cats had a steady decrease in fibrinolytic ability after injury on Day 3, however not a significant reduction. This could explain why the normal cats formed occlusions rather rapidly on Day 3 (Figure 3.4). Unfortunately we were not able to directly measure uPA and tPA activity in cats, but this data probably would not explain the lack of stable thrombus formation based on the other fibrinolytic indicators.

Findings in the FXII-/- mice indicate platelet activation or some other FXII-dependent interactions may be responsible for impaired thrombus formation in carotid arteries.[35] Thrombus formation in arteries is more dependent on platelet activation by high shear force mechanisms than in veins. However femoral veins are large vessels having higher flow velocities therefore platelets may be a factor in explaining our model differences. One interaction that may be important and involves FXII is the binding of platelets and fibrin to neutrophils. Factor XII can bind fibrin strands on neutrophils and dramatically enhance fibrin deposition in a developing thrombus.[74]

Neutrophils can provide a source of tissue factor in the growing thrombus. Upregulation of P-selectin by endothelial cells and neutrophils aids in leukocyte tethering to the endothelial cells and promotes thrombus formation by attracting microparticles (a source of TF) to the area of stasis.[37] Platelets are also critical to thrombus initiation and helping to generate the "burst" of thrombin at the site of injury. The interaction of FXII and platelets at the endothelial surface appears to be important in thrombin generation and subsequent thrombus formation *in vivo*. Factor XII may co-localize with HK at the platelet glycoprotein Ibα receptor and regulate thrombin's ability to activate platelets.[78]

In our study we looked at the role of FXII in inflammatory responses at the injury site. We found FXII may promote leukocyte recruitment to the injured vein and effect MMP activation or expression. Although systemic WBC counts did not indicate a difference between groups at 4 hours (Figure 3.5), a significantly higher number of leukocytes were present in the veins of NL animals (Table 3.3). The inflammatory vascular response begins with a large influx of neutrophils/leukocytes from the vasa vasorum into the thrombus from about 2 to 48 hours after experimental injury in veins.[118, 120, 151] Our data indicate that significantly fewer WBCs are present in FXII deficient animals at that high recruitment period (4 hours) in the veins. Previous studies agree with our findings and have confirmed that leukocytes in these FXII deficient cats can respond to chemotaxins normally.[81] Since FXII can activate neutrophils and activate chemotactic agents, the lack of FXII most likely contributes to these mechanistic processes.

Factor XII can also activate proteolytic enzymes involved in matrix degradation. Matrix metalloproteinase-1 can be activated by kallikrein (substrate of FXII) in a FXII-dependent manner. The main role of MMP-1 is to cause the degradation of collagen, presumably to make leukocyte trafficking into the thrombus easier and to aid in capillary tube regression during healing. Significantly increased levels of MMP-1 (has collagenase activity) and MMP-9 (has gelatinolytic activity) were seen in normal cats primarily in the veins after 48 hours. The antibodies used in this study do not distinguish between activated and zymogen proteinase MMP-1 or -9, therefore we cannot address the question of activity of enzymes present. In normal cats there was a significant increase in the total amount (intensity) of MMP-1 in their arteries at 48 hours which correlated with an increased WBC in the arteries. Neutrophils can activate MMPs by dumping proteolytic enzymes (elastase, cathepsin G, tryptase, chymase) from their primary granules into the matrix bed.[96] Our findings agree with what is expected, i.e. increased numbers of leukocytes are associated with increased MMP activity.[88,90-93] The loss of FXII activity would result in a loss of PMN influx, degranulation, and activation of MMP-1 and MMP-9. The role of FXII appears to be significant at 48 hours. Although no significant correlation was seen between FXIIa levels and MMP expression, an indirect relation through inflammatory modulation may be indicated. More studies are needed to determine if there is any direct cause and effect of FXII on MMP activation *in vivo*, and outcomes of such mechanisms. Neutrophils, monocytes, mast cells, eosinophils, and MMPs are extremely important in venous resolution. The finding of increased MMP-9 in our normal cat veins at 48 hours is in agreement with other studies.[88] Kowalewski et al.

found MMP-1,-2 and -9 to be significantly increased in inflamed veins taken from human patients suffering from thrombophlebitis.[151] This supports our finding that the NL cat veins have more inflammation (WBC/hpf), and higher expression of MMP-1 and -9. It is an interesting finding that vein matrix had more staining than arterial vessel matrix in NL cats. This finding may indicate an increased level of matrix degradation in the smaller walled veins at that time point. A difference in MMP-1 and -9 activity in the vein versus artery (vein>artery) has been reported in humans.[152] Our study was not designed to address issues of MMP activation or the role of FXII in thrombus resolution, however future studies are indicated by findings presented here.

The findings presented in this study indicate that FXII has a very important role in vascular thrombosis and its associated inflammatory responses *in vivo*. Our data show a different thrombotic response in large veins and arteries than what the mouse model investigators report upon initial injury, however there is a similarity in a reduced thrombotic response at a proinflammatory phase (indicated by the significant increase in systemic WBCs and increased fibrinogen levels in all cats) at 48 hours. The differences we show may reflect species differences or experimental design differences. Activation of FXII is important *in vivo* and warrants further studies concerning pathological thrombi and platelet-leukocyte-endothelial cell interactions.

CHAPTER 4

IN VIVO FXII RESPONSES INTRADERMAL INJURY TO HEME-INDUCED

ABSTRACT

Very little is known about the *in vivo* role of coagulation Factor XII or Hageman Factor in inflammation. An inflammatory response is initiated during vascular injury in which neutrophils, monocytes, and platelets have been shown to play pivotal roles in thrombus attachment to the injured endothelium and recruiting new cells to the site to aid in the repair process. Factor XII can activate neutrophils (PMNs) directly or indirectly via chemoattractants, however the magnitude of Factor XII's cellular recruiting role is not known. Our recent findings of reduced numbers of inflammatory cells at the site of vascular damage in Factor XII deficient cats sparked our interest in understanding FXII's role in inflammatory responses related to blood cell degradation. We used free heme, a break-down product of hemoglobin and potent proinflammatory agent, to evaluate responses in FXII deficient cats. Heme is plentiful in hemorrhagic tissues and induces neutrophil chemotaxis via G protein-

coupled receptor activation.[154] Factor XII has been shown to enhance leukocyte infiltrations into areas of immune-complex mediated (complement activation) inflammation.[81] By injecting heme intradermally then retrieving tissues at 24 hours, we found a significant reduction in dermal leukocyte recruitment (peroxidase activity) in animals having less than 2% Factor XII activity compared to animals with normal (NL) factor XII. Absolute leukocyte numbers were also reduced in FXII deficient animals. Vascular and dermal inflammatory responses after injury appear to be affected by Factor XII levels, suggesting Factor XII may be more important in inflammatory responses than coagulation.

INTRODUCTION

A complex process of cell communication, migration and proliferation occurs when tissues or cells are injured. The damaged cells signal for help from leukocytes, smooth muscle cells, and fibroblasts recruiting them to the injured area to aid in the containment of the "disaster" and repair of the damage. If this injury disrupts the integrity of the vascular system, bleeding will need to be controlled and repair to the endothelium and underlying media and matrix components will need to be initiated. One particular initiator of this healing process is heme or heme proteins. Heme proteins are found in all cells, particularly in erythrocytes which carry large amounts of hemoglobin. When freed from its protein, free heme has been shown to be a potent pro-inflammatory agent that can induce adhesion molecule expression,

increase vascular permeability, and act as a chemoattractant for leukocyte recruitment.[154,155] Leakage of the vasculature results in blood entering the tissues where it becomes lysed, and allows the pro-oxidative free heme and heme proteins to be released. Once released, the free heme signals inflammatory cells and induces oxidative stress that must be neutralized to prevent further damage. Heme scavengers in the plasma such as albumin and hemopexin are normally present but they can be overwhelmed in large hemorrhages, leaving heme to accumulate in extravascular tissues.[155] Thus large amounts of free heme can act as a beacon to alert the body to damage. Degradation of free heme occurs by heme oxygenase (HO) enzymes which break down heme into biliverdin/bilirubin, iron, and carbon monoxide.[154] The key to successfully repairing the damaged tissue lies in the body's ability to mount an appropriate immune response involving many other blood and tissue components.

Once injury and/or vascular permeability occur, the inflammatory process begins. Endothelial cells retract, leaving small gaps between them, and they begin to express leukocyte adhesion markers.[154] Leukocytes interact with the adhesion molecules (E-selectin, ICAM-1, VCAM-1) and become activated—further resulting in reactive oxygen species (ROS) and proteases. The intracellular gaps are large enough to allow plasma proteins to enter the extracellular tissue while retaining blood cells in the vessel.[154] Certain plasma proteins have been shown to have chemoattractant properties. Zabel et al. showed serine proteases, coagulation factor twelve (FXII) and plasmin in particular, elastase and cathepsin G from neutrophils, along with mast cell tryptase had high chemerin activating abilities. Chemerin is a

potent chemoattractant that triggers a rapid immune response via G-protein coupled receptor mediated chemotaxis.[82] Others have shown FXII can induce chemotaxis of neutrophils (PMNs) directly through binding to the CD11b/18c receptor.[74,75] Factor XII is critical in activating the kallikrein-kinin system (pro-inflammatory) and can do so without initiating coagulation in patients with an inflammatory disease, suggesting it may be important in inflammation regulation.[34]

The question remains as to the significance of plasma coagulation proteins in the maintenance of inflammatory responses, whereas the importance of leukocytes in thrombus resolution has been known for years. Neutrophils and their associated cytokines such as CXCL chemokines, IL-8, and IL-10 are important in venous thrombolysis[86], and mast cells/monocytoid perivascular cells[87,88] are important in vascular thrombosis and fibrinolysis. In addition to thrombolysis, PMNs and mast cells can dump their proteolytic granules into the extra cellular tissue and activate matrix metalloproteinases (MMPs)—acting to digest collagen and elastin to aid in vessel remodeling. It remains to be shown that FXII and kallikrein may be significant contributors to the vascular and tissue repair processes.

We hypothesize that the continual breakdown of heme proteins occurring with thrombolysis of a clot or with hemorrhage provides an important initiating agent for localized inflammation. However if FXII is not available to amplify this response, there may be a delay in resolving the thrombus and activating MMPs to aid in tissue repair and neointimal formation. This could lead to a pathological thrombotic state and possibly delay wound healing. By studying the effects of heme-induced

inflammation in the skin, we hope to clarify the extent of the *in vivo* role of FXII in this complex process.

Materials and Methods:

Animals

Animal care was provided in accordance with the procedures outlined in the "Guide for Care and Use of Laboratory Animals" (National Research Council; 1996; National Academy Press; Washington, D.C.) and in accordance with a protocol approved by the Institutional Animal Care and Use Committee of the University of North Carolina at Chapel Hill. Domestic breeder cats (Felis catus) were received from P. Bresnahan and limited in-breeding performed to maintain the line. Animals were phenotyped for FXII activity (FXII:C) based on a modified APTT clotting assay as described by Kier et al.[81] and in Chapter 2. Factor XII:C levels of <2% of a normal cat plasma pool defined cats as homozygote (HZY), <85% but >2% of a normal cat plasma pool as heterozygote (HET), and >85% as normal (NL). The number of animals used for each group was 15 HZY, 6 HET, and 4 NL cats. Two cats in each group under went the procedure twice in order to increase the sampling numbers. These cats were rested at least 2 months between procedures and received the inflammatory agent on the opposite flank at a location far from the first site.

Clinical Pathology: All animals were screened for physical abnormalities and signs of disease. White blood cell counts and skin evaluations were performed by the clinical veterinary staff and all cats were found to be within the normal range. Vaccinations were administered at a period greater than 6 months prior to the animal being used. Animals were examined after the experimental procedure for any signs of abnormal wound healing (no abnormalities were detected in any of the groups).

Inflammatory Agent (Heme) Preparation:

Heme or hemin was purchased from Frontier Scientific (Logan, UT) as a dry powder. A stock solution was prepared with limited light exposure as 5 mg/ml in 0.1M sodium hydroxide, vortexed for 5 minutes, pH adjusted to 7.50 with HCl, then filter sterilized into a sterile tube wrapped in foil. Immediately before administering, the stock solution was diluted to a 300 microMolar concentration of heme in pyrogen-free saline. A vehicle control, pyrogen-free saline, was used on the opposite flank.

Injection and Biopsy Procedure:

Animals were sedated and gas anesthesia (1-3% isoflurane) was administered by mask for the short 15 minute procedures. The skin was shaved of its hair and 70% alcohol was applied in a rubbing motion to remove surface contaminants. On the first day (Day 1) the animals received 0.2 ml intradermally of 300 μM heme on one flank and 0.2 ml of pyrogen-free saline on the opposite flank. Injection sites were circled with permanent marker for ease of retrieval 24 hours later. Twenty-four hours later (Day 2) animals were anesthetized and two 5 mm punch

biopsies were taken from each injection site (heme and saline): one punch biopsy sample was placed in a tube and immediately frozen in liquid nitrogen, the other sample was fixed in 10% neutral buffered saline for 48 hours before performing routine histology preparations. The incision sites were closed with surgical glue and animals returned to their quarters. Frozen biopsies were stored at -70˚C until further processing.

Peroxidase Assay:

This assay is a modification of an enzyme-substrate assay for the detection of peroxidases in skin samples as described by Ipaktchi et al.[157]. The basic principle of the reaction is as follows: Myeloperoxidase (MPO) and other hemoprotein peroxidases catalyze the hydrogen peroxide (H_2O_2)-dependent oxidation of halide ions and other organic compounds. In its native form, MPO is in the ferric state (Fe3+) and can react with H_2O_2 to form a compound that can accept 2 electrons. This reactive compound then oxidizes a halide such as Cl^- or Br^- which can combine with water (H_2O) to form a hypohalous compound such as hypochlorous ($HOCl^-$) that is a powerful anti-microbial. The final step of this reaction is that the aromatic amine (ortho-dianisidine) is oxidized by the hypohalous acid and a color is produced.[158] Frozen tissue samples were thawed at room temperature, weighed, and homogenized with a Polytron (PT 1200 Kinematica AG) electric homogenizer in 2 ml of cold homogenization buffer consisting of 0.044 M monobasic potassium phosphate, 0.0062 M dibasic potassium phosphate, 5 g/L hexadecyltrimethylammonium bromide (CTAB), and 0.001 M EDTA. Skin is difficult to dismember therefore an additional

step of sonication was added using a Fisher Sonic Dismembranator Model 300 (Fisher Scientific, USA). Three cycles of homogenization then sonication for 10-15 seconds, then rapid freeze-thawing were performed to each biopsy with care to add only 0.5 ml of homogenization buffer (to rinse the probe) per step. A total volume of homogenization buffer at the end of tissue processing was 3.5 milliliters or adjusted to this volume before the peroxidase detection assay was performed. Homogenates were centrifuged at 12,000 rcf for 30 minutes at 4˚C. The supernatant was removed and stored at -70˚C until all samples could be analyzed as one batch.

The supernatants were thawed and 20 µl added to a 96-well microtiter plate (run in duplicate). Two hundred microliters of substrate buffer (made immediately before the experiment) was added to the well. The substrate buffer consisted of 2.19 ml of 1M monobasic potassium phosphate, 0.307 ml of 1M dibasic potassium phosphate, 4.15mg of ortho-dianisidine HCl, and 40.6 µl of 0.3% H_2O_2 adjusted with distilled water to a total volume of 25 milliliters. A second substrate buffer was made containing the above reagents with 0.5 ml removed (to decrease the volume) and 0.5 ml of 0.001M sodium azide in distilled water added. This 10µM azide containing buffer was added to a separate well (run in duplicate also) on the same plate with another 20 µl of test subject's supernatant (or homogenization buffer alone) in order to block non-myeloperoxidase activity (eosinophil peroxidase, catalase, heme, and lactoperoxidase mainly) that can react to the chromogenic substrate (ortho-dianisidine).[159] Azide inhibits heme proteins (myeloperoxidase included) but at a 10 µM final concentration, it primarily blocks eosinophil peroxidase and lactoperoxidase

activities with only causing a 10% reduction in myeloperoxidase activity.[159] The reaction was monitored every 30 seconds for optical density changes at 450 nm for 30 minutes. A lactoperoxidase (Calbiomed, CA) sample was used as a positive control for both ortho-dianisidine HCl and for checking the blocking ability of azide. Sample values were subtracted from the corresponding azide-treated well then averaged. Peroxidase activity was expressed as ΔOD per minute per milligram tissue (ΔOD/min/mg tissue). This assay measures all peroxidases present. The majority of peroxidases are released from neutrophils and eosinophils found in the skin tissues. Samples that had large amounts of activity not blocked by azide were repeated. If they continued to show high activity, those samples were discarded since large amounts of heme interfering proteins were suspected. Only two saline-injected homozygote cat samples were discarded, of note both cats had been previously injected with heme on that same side.

Histological Processing and Evaluations:

Skin biopsies were cut in half and oriented on their edge for sectioning in order to visualize all dermal layers with light microscopy. Standard tissue processing, embedding, and sectioning were performed by a professional histopathology laboratory. Serial sections were cut at 5μm each for 30 sections such that 2 sections per glass slide in serial order were collected (15 slides all together). Four test animals were used to determine the number of sections necessary to be reviewed in order to find a depth of tissue that will show any inflammatory changes created and the consistency of section to section differences. These test animal biopsies were serially

sectioned in full and stained with Mayer's H&E. Histological evaluations revealed that the first 6 slides (12 serial sections) provided a good estimation of the degree of inflammation therefore we chose to have experimental animals sectioned and stained for H&E (for leukocyte counting) in this manner. Additional serial paraffin sections were taken for immunohistochemical analysis after the first 12 sections were taken.

Inflammation scoring:

Two independent, trained reviewers (DAB and DEB) blinded to the animal treatment group counted and differentiated inflammatory cell types in 6 of the 12 serial sections (ever other section) according to the following procedure. (1) Each section was evaluated for the most inflamed region (indicated by the presence of leukocytes and tissue disruption) and the reviewer focused counting here. This biases the reviewer to an inflamed area. (2) The most inflamed region was then subdivided into 4 fields (F):

F1=epithelial/upper epidermis (papillary dermis)

F2=layer of collagen fibers with hair shafts present

F3=fatty hypodermis region of the dermis

F4=muscle layer/lower hypodermis region of the dermis

(3) At a 40x magnification, each reviewer counted/differentiated the number of eosinophils, neutrophils, and mononuclear cells found in one high power field (hpf) of each region. A total of 24 counts (6 sections x 4 fields) were made for each cell

type. (4) Reviewer evaluations were compared for inter-rater reliability. The highest number of inflammatory cells was found to be in F3 and F4. Neutrophil, macrophage, and eosinophil numbers in F3 and F4 in two animal's left side biopsy sample were compared using a Spearman's correlation coefficient. The differential analyses (PMN, macrophage, eosinophils) did not correlate between reviewers. Correlation was defined as having a >0.5 correlation coefficient. However the total number of leukocytes counted in field 3 (F3) did correlate (coefficients of 0.5649 and 0.7201 for HZY and NL respectively) therefore only this field and total numbers were used for statistical purposes. The reviewer data was averaged and the group means were compared using the Mann-Whitney U test since the samples were not normally distributed. Only the HZY and NL groups were reviewed, the HET group was not reviewed. The data is reported as means +/- 1 standard deviation (SD).

Immunohistochemistry Staining:

Immunohistochemical staining for matrix metalloproteinase-1 (MMP-1) and -9 (MMP-9), cathepsin G, and mast cell tryptase were performed as described in Chapter 3 with exceptions of antibody changes for cathepsin G and mast cell tryptase. Cathepsin G: sheep anti-human polyclonal purified antibody to cathepsin G protease was purchased from Abcam (Cambridge, MA) and reported to cross react to cat neutrophils[160]. Blocking was done with normal horse serum and the primary antibody dilution was at 1:50. Overnight incubation at 4˚C was performed before washing and adding the secondary antibody (Sigma Donkey anti-sheep HRP conjugated) as describe in Chapter 3. The mouse anti-human monoclonal mast cell tryptase AA1

antibody was purchased from Thermo Fisher Scientific (Fremont, CA) and reported to cross react with feline protein according to the datasheet provided at a different supplier.[161] The dilution of primary antibody used was 1:25 and incubation carried out at 4˚C overnight. All other steps were the same as described in Chapter 3.

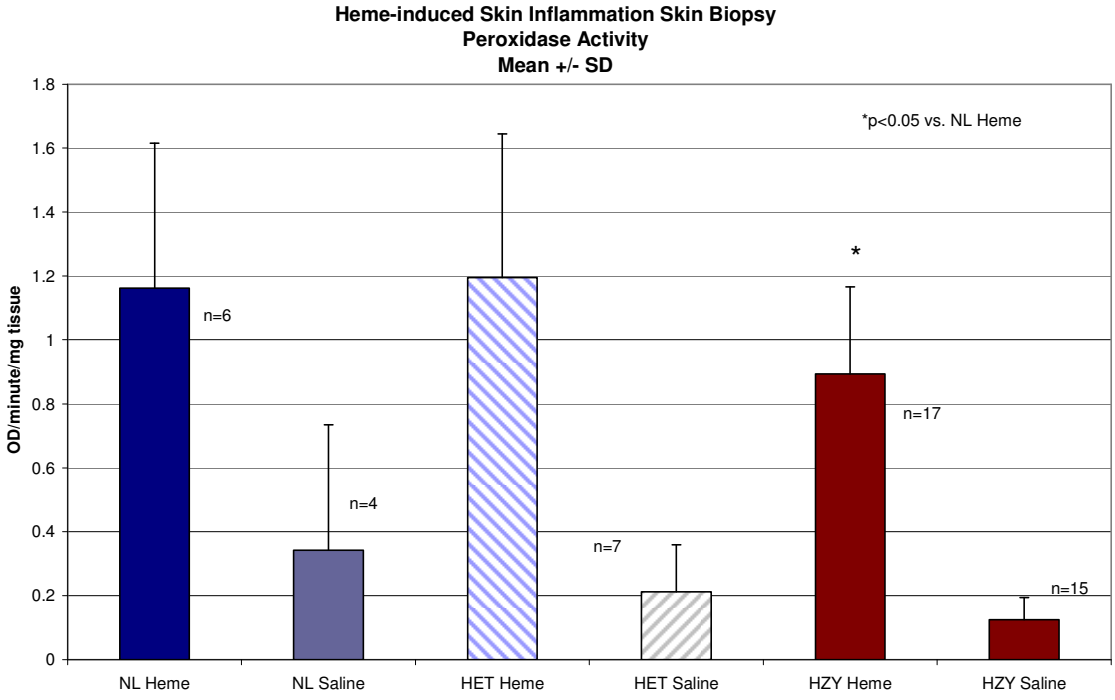
Due to harsh conditions of antigen retrieval, the tissue specimens were lost or damaged to a point that would not allow reliable analysis. The immuno-histochemistry stained slides were not graded nor included in this study.

RESULTS

Heme induces a granulocytic response--The peroxidase assay measures the amount of peroxidases present in the skin biopsy tissue, generally a reflection of the number of granulocytic white blood cells (PMNs and eosinophils) present. Our results revealed a significant reduction of ($p=0.0499$) in peroxidase activity in HZY animals compared to NL, but not with HET cats. Reduced peroxidase activity could mean fewer granulocytes are present but inflammation is the same (a monocytic/macrophagic response could be taking place in HZY cats instead). Therefore white blood cell differentials of the injury site are warranted in order to interpret this result as meaning "less inflammation". As expected the level of peroxidase activity in the saline injected tissues was significantly less ($p=0.019$) than heme-injected tissues and reflective of the nearly absent inflammation seen on H&E evaluation. Figure 4.1 illustrates these differences in activity.

Figure 4.1: *Peroxidase Activity of Cat Skin Biopsies After Injury*

"NL, HET, HZY Heme" indicates normal, heterozygote FXII deficient, and homozygote FXII deficient cats injected with heme, respectively. "NL, HET, HZY Saline" refers to tissues injected with saline.



Factor XII deficient cats have fewer leukocytes at the site of injury--Normal cats had a larger inflammatory response (in all cell types) as indicated by the increased number of leukocytes per high power field present in one region of the skin. The difference between numbers of leukocytes in NL and HZY heme-injected tissues was approaching significance ($p=0.073$ with Mann-Whitney). Unfortunately only 3 NL cats had histological evaluations in the heme receiving tissues, making statistical analysis difficult. The means and their variances in the total inflammatory cells

counted per high power field for region (F3) in NL and HZY groups receiving heme- and saline-injections are shown in Table 4.1. Increasing the numbers of NL animals being evaluated may bring significance to this finding. There was a significant difference between the heme-injected and saline-injected tissues of the normal cats ($p=0.025$) which indicates the heme did act as a proinflammatory agent. There was no difference in the numbers of WBC per high power field in heme-injected and saline-injected sites of the FXII deficient cats, indicating either they did not mount a response to heme, or this method of enumerating has a high error rate. No correlations were found with the peroxidase data, suggesting this method can help explain the peroxidase activity, however it should not be the sole means of measuring inflammatory responses. An example of histological differences is given in Figure 4.2, showing H&E sections of heme-injected skin for one normal and one homozygote cat.

Table 4.1: *Average Number of Inflammatory Cells (WBC) Per High Power Microscopic Field In Hypodermis*

"NL Heme" indicates a normal cat having received heme in that skin site. "NL Saline" indicates a normal cat received saline in the injection site. "HZY Heme" indicates a homozygote FXII deficient cat received heme in the injection site. "HZY Saline" indicates a homozygote FXII deficient cat received saline in the injection site.

WBC/hpf in
F3 (Hypodermis)

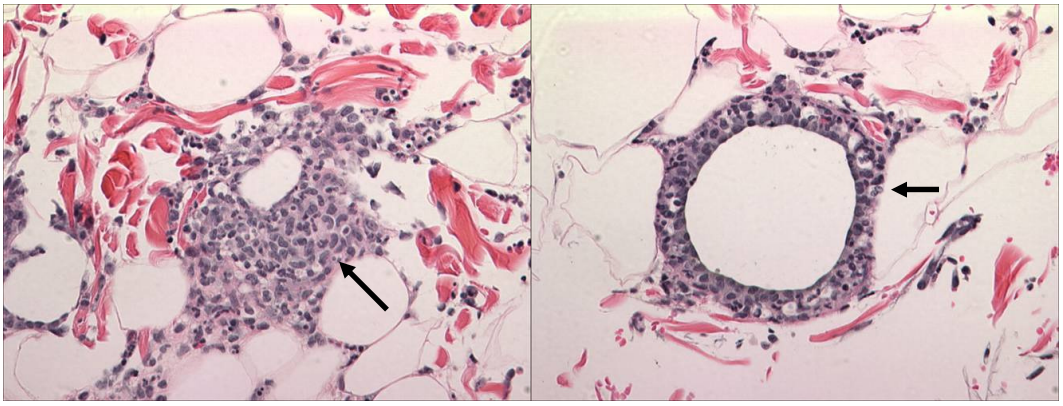
	Mean +/- SD	n	
NL Heme	94.5 +/- 96.1	3	p=0.025
NL Saline	6.5 +/- 5.2	5	
HZY Heme	15.2 +/- 14.7	11	
HZY Saline	14.5 +/- 24.2	4	

p=0.073 NL heme vs HZY heme

Figure 4.2: *Histology (H&E) Examples of Heme-Injected Cat Skin*

One normal (NL) and one homozygote (HZY) representative photomicrograph illustrates the general inflammatory response differences between the two groups after receiving a single intradermal heme injection. The area of focus is a small venule or adipocyte surrounded by inflammatory cells (arrow indicates inflamed area) located in the hypodermis. Magnifications are at 20x.

NL cat skin HZY cat skin



DISCUSSION

The results of this study offer new insights into the role of FXII in acute inflammatory responses to a naturally occurring chemotactic agent—heme derived from lysed blood cells. Previous findings by others agree with our findings that severely FXII deficient individuals have reduced responses in pro-inflammatory situations.[80,81] After injecting purified heme into the dermis of skin, an inflammatory response was seen in all animals as indicated by the peroxidase activity (Figure 4.1). The severely FXII deficient animals (HZY) have a significantly reduced granulocytic response compared to animals with approximately 50% or more of normal levels of FXII. This finding was confirmed by semi-quantitative histological analyses indicating a pan-cellular response (with predominately granulocytic lineage) was diminished in HZY animals.

Careful evaluation of the HZY peroxidase activity reveals these animals did mount a significant ($p<0.0001$) response because their average heme-injected peroxidase values (0.9 +/- 0.2) are higher than their saline-injected values (0.2 +/- 0.1). This particular assay is based on the utilizing a redox reaction in which other contaminating heme proteins or peroxidases could be a source of falsely elevated oxidizing agents. We took precautions in limiting the effects of other peroxidases[159], however there could be differences related to these. Most likely the increase in peroxidase activity reflects the influx of leukocytes to the injury site and their subsequent dumping of myeloperoxidase into the tissues. This is supported by our findings (Table 4.1) of increased numbers of WBCs per high power field in cats

having high peroxidase activity (this is an observation and not based on statistical analysis which showed no correlation).

Unfortunately we were not able to identify any mechanism(s) that could help explain the reduced response. Studies of neutrophil chemotactic responses to purified heme using human and mouse neutrophils show heme induces the protein kinase C (PKC) signalling pathway and other pathways by stimulating seven transmembrane receptors coupled to $G_{\alpha i}$ protein.[154] Since there appeared to be a general reduction in all cell numbers in FXII deficients, not just the PMNs, the mechanism may be more broad. The major WBC type found acutely after ischemia-reperfusion and vascular injury is the PMN.[88,153,154,162] Neutrophils are more prevalent in the circulation (40-60% of the WBC population) and can rapidly increase in numbers (neutrophilia) upon a sudden stress to the body, lending to their prevalence at injury sites. Free heme enhances the inflammatory response[156] and increases cell adhesion molecules on endothelial cells and vascular permeability[154] thereby making it easier for PMNs to respond. We know FXIIa can bind PMN at their Mac-1 receptor[74,75,77] and perhaps this mechanism is involved in heme-induced inflammatory responses.

The information gained through our experiments with heme adds new knowledge to the role of FXII in acute inflammation driven by oxidative stress. More investitgations are needed to identify the specific mechanisms associated with FXII and how it enhances leukocyte recruitment, especially in response to heme proteins.

CHAPTER 5

FUTURE DIRECTIONS AND CLINICAL IMPLICATIONS

The work presented in this dissertation has brought support and new questions to the importance of FXII *in vivo*. We have shown that FXII in cats can significantly impact their ability to form stable thrombi during periods of acute inflammation. But we don't know why. We have found that FXII enhances leukocyte recruitment to the site of injury, but is this related to free heme proteins or some other chemotactic factor(s) involved in mounting an inflammatory response? Does FXII impact the body's ability to resolve venous thrombi? Certainly it is expected to have some sort of an effect, perhaps a significant one, based on our findings. It would be helpful to know if cytokines and MMPs are regulated differently in FXII deficient individuals after injury, and if a specific cell type, such as neutrophils, are responsible for any differences. We also don't know how the FXII protein interacts with its heavy chain structural domains during *in vivo* processes.

The clinical implications of our findings are significant if the cat model translates to what happens in humans. If patients are undergoing multiple surgeries or

intravascular procedures, FXII interactions may be a factor in how they are managed with anticoagulants or antithrombolytic agents. Perhaps regulation of a local inflammatory response can be done through targeting FXII? Of course this is tricky because FXII is involved in so many beneficial pathways that we may not want to alter. A word of caution: drug targeting may not be effective since it appears FXII levels <2% of normal are needed to significantly impact inflammation at sites of injury. The heterozygote cats (moderate FXII deficient animals) appear to act like normals in their thrombotic and inflammatory responses. This brings even more questions for *in vivo* studies to pursue because we don't know what minimal level is needed for FXII to impact the normal function of biological systems. All these new possibilities and questions can be pursued with this animal model that is now better defined through our efforts presented here.

REFERENCES

1. Rosamond W, Flegal K, Friday G, Furie K, Go A, Greenlund K, Haase N, Ho M, Howard V, Kissela B, Kittner S, Lloyd-Jones D, McDermott M, Meigs J, Moy C, Nichol G, O'Donnell CJ, Roger V, Rumsfeld J, Sorlie P, Steinberger J, Thom T, Wasserthiel-Smoller S, Hong Y, for the American Heart Association Statistics Committee and Stroke Statistics S. Heart Disease and Stroke Statistics–2007 Update: A Report From the American Heart Association Statistics Committee and Stroke Statistics Subcommittee. Circulation. 2007; 115:e69–e171.

2. American Public Health Association, White Paper, Public Health Leadership Conference on Deep Vein Thrombosis. Deep-Vein Thrombosis: Advancing Awareness To Protect Patients Lives. Washington, D. C., February 26, 2003.

3. Ratnoff OD, Colopy JE. A familial hemorrhagic trait associated with a deficiency of a clot-promoting fraction of plasma. J Clin Invest. 1955; 34: 602.

4. Schmaier AH. The elusive physiological role of FXII. J Clin Invest. 2008; 118: 3006-3009.

5. Halbmayer WM, Haushofer A, Schön R, Mannhalter C, Strohmer E, Baumgarten K, Fischer M. The prevalence of moderate and severe FXII (Hageman factor) deficiency among the normal population: evaluation of the incidence of FXII deficiency among 300 healthy blood donors. *Thromb Haemost*. 1994; 71(1):68-72.

6. Kier AB, Bresnahan JF, White FJ, Wagner JE: The inheritance pattern of factor XII (Hageman) deficiency in domestic cats. *Can J Comp Med*. 1980; 44(3): 309-14.

7. Brooks M, DeWilde L: Feline FXII deficiency. Compendium on Continuing Education For The Practicing Veterinarian. 2006 February; 28 (2): 148-156.

8. Song KS. High incidence of plasma factor XII deficiency in normal Korean subjects. *Thromb Res*. 2006;117(6):732-3.

9. Kanaji T, Okamura T, Osaki K, Kuroiwa M, Shimoda K, Hamasaki N, Niho Y: A common genetic polymorphism (46C to T substitution) in the 50-untranslated region of the coagulation factor XII gene is associated with low translation efficiency and decrease in plasma factor XII level. *Blood*. 1998; 91: 2010–2014.

10. Halbmayer WM, Haushofer A, Schön R, Mannhalter C, Strohmer E, Baumgarten K, Fischer M. The prevalence of moderate and severe FXII (Hageman factor) deficiency among the normal population: evaluation of the incidence of FXII deficiency among 300 healthy blood donors. *Thromb Haemos*t. 1994; 71(1):68-72.

11. Gallimore MJ, Jones DW, Winter M. Factor XII determinations in the presence and absence of phospholipid antibodies. *Thromb Haemost*. 1998; 79(1):87-90.

12. Chalkiadakis G, Kyriakou D, Oekonomaki E, Tsiaoussis J, Alexandrakis M, Vasilakis S, Kouroumalis E. Acquired inhibitors to the coagulation factor XII associated with liver disease. A*m J Gastroentero*l. 1999; 94(9): 2551-3.

13. Girolami A, Randi ML, Gavasso S, Lombardi AM, Spiezia F. The occasional venous thromboses seen in patients with severe (homozygous) FXII deficiency are probably due to associated risk factors: a study of prevalence in 21 patients and review of the literature. *J Thromb Thrombolysis*. 2004; 17(2):139-43.

14. Girolami A, Gavasso S, Pacquola E, Cabrio L, Lombardi AM, Girolami B. Comparable levels of activity and antigen in factor XII deficiency: a study of 21 homozygotes and 58 heterozygotes. *Clin Appl Thromb Hemost*. 2005; 11(3):335-8.

15. Miller GJ, Esnouf MP, Burgess AI, Cooper JA, Mitchell JP. Risk of coronary heart disease and activation of factor XII in middle-aged men. *Arterioscler Thromb Vasc Biol.* 1997; 17(10):2103-6.

16. Kohler HP, Carter AM, Stickland MH, Grant PJ. Levels of activated FXII in survivors of myocardial infarction--association with circulating risk factors and extent of coronary artery disease. *Thromb Haemost*. 1998; 79(1):14-18.

17. Zito F, Lowe GD, Rumley A, McMahon AD, Humphries SE; WOSCOPS Study Group West of Scotland Coronary Prevention Study. Association of the factor XII 46C>T polymorphism with risk of coronary heart disease (CHD) in the WOSCOPS study. *Atherosclerosis*. 2002; 165(1):153-8.

18. Kanaji T. Lower factor XII activity is a risk marker rather than a risk factor for cardiovascular disease: a rebuttal. *J Thromb Haemost*. 2008; 6: 1053–4.

19. Colhoun HM, Zito F, Norman Chan N, Rubens MB, Fuller JH, Humphries SE. Activated factor XII levels and factor XII 46C>T genotype in relation to coronary artery calcification in patients with type 1 diabetes and healthy subjects. *Atherosclerosis*. 2002; 163: 363–9.

20. Bach J, Endler G, Winkelmann BR, Boehm BO, Maerz W, Mannhalter C, Hellstern P. Coagulation factor XII (FXII) activity, activated FXII, distribution of FXII C46T gene polymorphism and coronary risk. *J Thromb Haemost*. 2008; 6(2):291-6.

21. Briseid K, Hoem NO, Johannesen S, Fossum S. Contact activation factors in plasma from pregnant women--increased level of an association between factor XII and kallikrein. *Thromb Res.* 1991; 61(2):123-33.

22. Braulke I, Pruggmayer M, Melloh P, Hinney B, Köstering H, Günther E. Factor XII (Hageman) deficiency in women with habitual abortion: new subpopulation of recurrent aborters? *Fertil Steril*. 1993; 59(1):98-101.

23. Yamada H, Kato EH, Ebina Y, Kishida T, Hoshi N, Kobashi G, Sakuragi N, Fujimoto S. Factor XII deficiency in women with recurrent miscarriage. *Gynecol Obstet Invest*. 2000; 49(2):80-3.

24. Jones DW, Gallimore MJ, Winter M. Antibodies to factor XII: a possible predictive marker for recurrent foetal loss. Immunobiology. 2003; 207(1):43-6.

25. Sugi T, McIntyre JA. Certain autoantibodies to phosphatidylethanolamine (aPE) recognize factor XI and prekallikrein independently or in addition to the kininogens. *J Autoimmun*. 2001; 17(3):207-14.

26. Girolami A, Zocca N, Girolami B, Lombardi AM, Fabris F. Pregnancies and oral contraceptive therapy in severe (homozygons) FXII deficiency: a study in 12 patients and review of the literature. *J Thromb Thrombolysis*. 2004; 18(3):209-12.

27. Pauer HU, Renné T, Hemmerlein B, Legler T, Fritzlar S, Adham I, Müller-Esterl W, Emons G, Sancken U, Engel W, Burfeind P. Targeted deletion of murine coagulation factor XII gene-a model for contact phase activation in vivo. *Thromb Haemost*. 2004; 92(3):503-8.

28. Renné T, Pozgajová M, Grüner S, Schuh K, Pauer HU, Burfeind P, Gailani D, Nieswandt B. Defective thrombus formation in mice lacking coagulation factor XII. J Exp Med. 2005; 202(2):271-81.

29. Gailani D, Renne´ T. The intrinsic pathway of coagulation: a target for treating thromboembolic disease? *J Thromb Haemost*. 2007; 5: 1106–12.

30. Perona J, Craik C. Structural basis of substrate specificity in the serine proteases. *Protein Sci*. 1995; 4: 337–60.

31. Pixley RA, Schapira M, Colman RW. The regulation of human factor XIIa by plasma proteinase inhibitors. *J Biol Chem.* 1985;260 (3):1723-9.

32. Schmaier AH, McCrae KR. The plasma kallikrein–kinin system: its evolution from contact activation. *J Thromb Haemost.* 2007; 5: 2323–9.

33. Chatterjee K, Thornton JL, Bauer JW, Vogler EA, Siedlecki CA. Moderation of prekallkrein-factor XII interactions in surface activation of coagulation by protein-adsorption competition. Biomaterials. 2009; 30(28):4915-20.

34. Maas C, Govers-Riemslag JW, Bouma B, Schiks B, Hazenberg BP, Lokhorst HM, Hammarström P, ten Cate H, de Groot PG, Bouma BN, Gebbink MF. Misfolded proteins activate factor XII in humans, leading to kallikrein formation without initiating coagulation. J Clin Invest. 2008; 118(9):3208-18.

35. van der Meijden PE, Munnix IC, Auger JM, Govers-Riemslag JW, Cosemans JM, Kuijpers MJ, Spronk HM, Watson SP, Renné T, Heemskerk JW. Dual role of collagen in factor XII-dependent thrombus formation. *Blood*. 2009 Jul 23;114(4):881-90.

36. Colman RW. 2006. Contact Activation (Kallikrein-Kinin) Pathway: Multiple Physiologic and Pathophysiologic Activities. In *Hemostasis and Thrombosis: Basic Principles and Clinical Practice* 5th edn. Colman RW, Marder VJ, Clowes AW, George JN, Goldhaber SZ, editors. Lippincott Williams & Wilkins, Philadelphia, PA. 107-130.

37. Ramacciotti E, Hawley AE, Farris DM, Ballard NE, Wrobleski SK, Myers DD Jr, Henke PK, Wakefield TW. Leukocyte- and platelet-derived microparticles correlate with thrombus weight and tissue factor activity in an experimental mouse model of venous thrombosis. *Thromb Haemost*. 2009 Apr;101(4):748-54.

38. Mackman, N. Role of tissue factor in hemostasis, thrombosis, and vascular development. *Arterioscler Thromb Vasc Biol*. 2004; 24: 1015-1022.

39. Wolberg AS, Campbell RA. Thrombin Generation, Fibrin Clot Formation and Hemostasis. *Transfus Apher Sci*. 2008; 38(1): 15–23.

40. Torbet J. The thrombin activation pathway modulates the assembly, structure and lysis of human plasma clots in vitro. *Thromb Haemost*. 1995; 3(5):785-92.

41. Ryan EA, Mockros LF, Weisel JW, Lorand L. Structural origins of fibrin clot rheology. *Biophys J*. 1999; 77(5):2813-26.

42. Gabriel DA, Muga K, Boothroyd EM. The effect of fibrin structure on fibrinolysis. *J Biol Chem.* 1992; 267:24259–24263.

43. Chavakis T, Santoso S, Clemetson KJ, Sachs UJH, Isordia-Salas I, Pixley RA, Nawroth PP, Colman RW, and Preissner KT. High molecular weight kininogen regulates platelet-leukocyte interactions by bridging Mac-1 and glycoprotein Ib. *J of Biol Chem*. 2003; 278(46): 45375-45381.

44. Guo YL, Wang S, Cao DJ, Colman RW. Apoptotic effect of cleaved high molecular weight kininogen is regulated by extracellular matrix proteins. *J Cell Biochem*. 2003; 89(3):622-32.

45. Madeddu P, Emanueli C, El-Dahr S. Mechanisms of Disease: the tissue kallikrein–kinin system in hypertension and vascular remodeling. *Nature Clinical Practice Nephrology*. 2007; 3: 208-221.

46. Karpman D, Kahn R. The contact/kinin and complement systems in vasculitis. *APMIS Suppl.* 2009 Jun;(127):48-54.

47. Colman RW, Schmaier AH. Contact system: a vascular biology modulator with anticoagulant, profibrinolytic, antiadhesive, and proinflammatory attributes. *Blood*.1997; 90:3819–43.

48. Schulze-Topphoff U, Prat A, Prozorovski T, Siffrin V, Paterka M, Herz J, Bendix I, Ifergan I, Schadock I, Mori MA, Van Horssen J, Schröter F, Smorodchenko A, Han MH, Bader M, Steinman L, Aktas O, Zipp F. Activation of kinin receptor B1 limits encephalitogenic T lymphocyte recruitment to the central nervous system. *Nat Med*. 2009 Jul; 15(7):788-93.

49. Pixley RA, Colman RW. Factor XII: Hageman factor. *Methods Enzymol*. 1993; 222:51-65.

50. Shan J, Baguinon M, Zheng L, Krishnamoorthi R. Expression, refolding, and activation of the catalytic domain of human blood coagulation factor XII. *Protein Expr Purif*. 2003; 27(1):143-9.

51. Bernardo MM, Day DE, Olson ST, Shore JD. Surface-independent acceleration of factor XII activation by zinc ions. I. Kinetic characterization of the metal ion rate enhancement. *J Biol Chem.* 1993; 268(17):12468-76.

52. Eldering E, Huijbregts CC, Lubbers YT, Longstaff C, Hack CE. Characterization of recombinant C1 inhibitor P1 variants. *J Biol Chem*. 1992; 267(10):7013-20.

53. McMullen BA, Fujikawa K. Amino acid sequence of the heavy chain of human alpha-factor XIIa (activated Hageman factor). *J Biol Chem*. 1985 May 10; 260(9):5328-41.

54. Cool DE, Edgell CJ, Louie GV, Zoller MJ, Brayer GD, MacGillivray RT. Characterization of human blood coagulation factor XII cDNA. Prediction of the primary structure of factor XII and the tertiary structure of beta-factor XIIa. *J Biol Chem*. 1985 Nov 5; 260(25):13666-76.

55. Bradford HN, Pixley RA, Colman RW. Human factor XII binding to the glycoprotein Ib-IX-V complex inhibits thrombin-induced platelet aggregation. *J Biol Chem*. 2000; 275: 22756–22763.

56. Citarella F, te Velthuis H, Helmer-Citterich M, Hack CE. Identification of a putative binding site for negatively charged surfaces in the fibronectin type II domain of human factor XII--an immunochemical and homology modeling approach. *Thromb Haemost*. 2000; 84(6):1057-65.

57. Mahdi F, Madar ZS, Figueroa CD, Schmaier AH. Factor XII interacts with the multiprotein assembly of urokinase plasminogen activator receptor, gC1qR, and cytokeratin 1 on endothelial cell membranes. *Blood.* 2002; 99(10):3585-96.

58. Chien P, Pixley RA, Stumpo LG, Colman RW, Schreiber AD. Modulation of the Human Monocyte Binding Site for Monomeric Immunoglobulin G by Activated Hageman Factor. *J Clin Invest.* 1988; 82:1554-1559.

59. Maas C, Schiks B, Strangi RD, Hackeng TM, Bouma BN, Gebbink MF, Bouma B. Identification of fibronectin type I domains as amyloid-binding modules on tissue-type plasminogen activator and three homologs. *Amyloid*. 2008;15(3):166-80.

60. Schousboe I, Nystrøm BT, Hansen GH. Differential binding of factor XII and activated factor XII to soluble and immobilized fibronectin--localization of the Hep-1/Fib-1 binding site for activated factor XII. *FEBS J*. 2008; 275(20):5161-72.

61. Citarella F, Ravon DM, Pascucci B, Felici A, Fantoni A, Hack CE. Structure/function analysis of human factor XII using recombinant deletion mutants. Evidence for an additional region involved in the binding to negatively charged surfaces. *Eur J Biochem*. 1996; 238(1):240-9.

62. Cesarman-Maus G, Hajjar KA. Molecular mechanisms of fibrinolysis. *British Journal of Haematology*. 2005; 129 (3): 307–21.

63. Minor KH, Peterson CB. Plasminogen Activator Inhibitor Type 1 Promotes the Self-association of Vitronectin into Complexes Exhibiting Altered Incorporation into the Extracellular Matrix. J Biol Chem. 2002; 277(12): 10337-10345.

64. Chapman HA. Plasminogen activators, integrins, and the coordinated regulation of cell adhesion and migration. *Current Opinion in Cell Biology*. 1997; 9: 714–724.

65. Braat EA, Dooijewaard G, Rijken DC. Fibrinolytic properties of activated FXII. *Eur J Biochem*. 1999;263(3):904-11.

66. Kluft C. Occurrence of Cl Inactivator and Other Proteinase Inhibitors in Euglobulin Fractions and Their Influence on Fibrinolytic Activity. *Haemostasis*. 1976; 5:136-146.

67. Hauert J, Nicoloso G, Schleuning WD, Bachmann F, Schapira M. Plasminogen activators in dextran sulfate-activated euglobulin fractions: a molecular analysis of factor XII- and prekallikrein-dependent fibrinolysis. *Blood.* 1989;73(4):994-9.

68. Miles LA, Burnier JP, Verlander MS, Goodman M, Kleiss AJ, Griffin JH. Inactivation of purified human alpha 2-antiplasmin and purified human C1 inhibitor by synthetic fibrinolytic agents. *Blood.* 1981; 57(6):1015-24.

69. Kluft C, Dooijewaard G, Emeis JJ. Role of the contact system in fibrinolysis. Semin Thromb Hemost. 1987; 13:50-68.

70. Levi M, Hack CE, de Boer JP, Brandjes DP, Büller HR, ten Cate JW. Reduction of contact activation related fibrinolytic activity in factor XII deficient patients. Further evidence for the role of the contact system in fibrinolysis in vivo. *J Clin Invest.* 1991;88(4):1155-60.

71. Rodeghiero F, Castaman G, Ruggeri M, Cazzavillan M, Ferracin G, Dini E. Fibrinolytic studies in 13 unrelated families with factor XII deficiency. *Haematologica*. 1991; 76:28-32.

72. Lodi S, Isa L, Pollini E, Bravo AF, Scalvini A. Defective intrinsic fibrinolytic activity in a patient with severe factor XII-deficiency and myocardial infarction. *Scand J Haematol*. 1984;33(1):80-2.

73. Schousboe I. Rapid and cooperative binding of factor XII to human umbilical vein endothelial cells. *Eur J Biochem*. 200; 268(14):3958-63.

74. Goel MS, Diamond S. Neutrophil enhancement of fibrin deposition under flow through platelet-dependent and -independent mechanisms. *Arterioscler Thromb Vasc Biol*. 2001; 21(12): 2093-8.

75. Wachtfogel YT, Pixley RA, Kucich U, Abrams W, Weinbaum G, Schapira M, Colman RW. Purified plasma factor XIIa aggregates human neutrophils and causes degranulation. *Blood*. 1986; 67(6): 1731-7.

76. Bournazos S, Woof JM, Hart SP, Dransfield I. Functional and clinical consequences of Fc receptor polymorphic and copy number variants. *Clin Exp Immunol*. 2009;157(2):244-54.

77. Chavakis T, Santoso S, Clemetson KJ, Sachs UJ, Isordia-Salas I, Pixley RA, Nawroth PP, Colman RW, Preissner KT. High molecular weight kininogen regulates platelet-leukocyte interactions by bridging Mac-1 and glycoprotein Ib. *J Biol Chem*. 2003;278(46):45375-81.

78. Bradford HN, Pixley RA, Colman RW. Human factor XII binding to the glycoprotein Ib-IX-V complex inhibits thrombin-induced platelet aggregation. *J of Biol Chem*. 2000;275 (30): 22756-22763.

79. Margolis J. Hageman factor and capillary permeability. *Aust J Exp Biol Med Sci.* 1959 Jun; 37:239-44.

80. Poon MC, Moore MR, Castleberry RP, Lurie A, Huang ST, Lehmeyer J. Severe Fletcher factor (plasma prekallikrein) deficiency with partial deficiency of Hageman factor (factor XII): report of a case with observation on in vivo and in vitro leukocyte chemotaxis. *Am J Hematol*. 1982;12(3):261-70.

81. Kier AB, McDonnell JJ, Stern A, Ratnoff OD. The Arthus reaction in cats deficient in Hageman factor (factor XII). *J Comp Pathol*. 1990;102(1):33-47.

82. Zabel BA, Allen SJ, Kulig P, Allen JA, Cichy J, Handel TM, Butcher EC. Chemerin activation by serine proteases of the coagulation, fibrinolytic, and inflammatory cascades. *J Biol Chem*. 2005; 280(41):34661-6.

83. Ghebrehiwet B, Randazzo BP, Dunn JT, Silverberg M, Kaplan AP. Mechanisms of activation of the classical pathway of complement by Hageman factor fragment. *J Clin Invest.* 1983;71(5):1450-6.

84. Caliezi C, Wuillemin WA, Zeerleder S, Redondo M, Eisele B, Hack CEC1-Esterase Inhibitor: An Anti-Inflammatory Agent and Its Potential Use in the Treatment of Diseases Other Than Hereditary Angioedema. *Pharmacol Rev*. 2000; 52(1):91-112.

85. Carter AM. Inflammation, thrombosis and acute coronary syndromes. *Diab Vasc Dis Res*. 2005; 2(3): 113-21.

86. Henke, P.K., Wakefield, T.W., Kadell, A.M., Linn, M.J., Varma, M.R., Sarkar, M., Hawley, A., Fowlkes, J.B., Strieter, R.M.: "Interleukin-8 administration enhances venous thrombosis resolution in a rat model". *J Surgical Research*, VOL 99, No. 1, July 2001, pp. 84-91.

87. Henke, P.K., DeBrunye, L.A., Strieter, R.M., Brornber, J.S., Prince, M., Kadell, A.M., Sarkar, M., Londy, F., Wakefield, T.W.: "Viral IL-10 gene transfer decreases inflammation and cell adhesion molecule expression in a rat model of venous thrombosis". *J Immunology*, VOL 164, No. 4, February 15, 2000, pp. 2131-41.

88. Varma, M.R., Varga, A.J., Knipp, B.S., Sukheepod, P., Upchurch, G.R., Kunkel, S.L., Wakefield, T.W., Henke, P.K.: "Neutropenia impairs venous thrombosis resolution in the rat". *J Vasc Surg*, VOL 38, No. 5, November 2003, pp. 1090-8.

89. Bankl HC, Valent P. Mast cells, thrombosis, and fibrinolysis: The emerging concept. *Thrombosis Research*. 2002;105: 359-65.

90. Hiller O, Lichte A, Oberpichler A, Kocourek A, Tschesche H. Matrix metalloproteinases collagenase-2, macrophage elastase, collagenase-3, and membrane type 1-matrix metalloproteinase impair clotting by degradation of fibrinogen and factor XII. *J Biol Chem*. 2000; 275(42):33008-13.

91. Verschuren L, Lindeman JH, van Bockel JH, Abdul-Hussien H, Kooistra T, Kleemann R. Up-regulation and coexpression of MIF and matrix metalloproteinases in human abdominal aortic aneurysms. *Antioxid Redox Signal*. 2005 Sep-Oct;7(9-10):1195-202.

92. Zhang Y, Zhou ZH, Bugge TH, Wahl LM. Urokinase-type plasminogen activator stimulation of monocyte matrix metalloproteinase-1 production is mediated by plasmin-dependent signaling through annexin A2 and inhibited by inactive plasmin. *J Immunol.* 2007 Sep 1;179(5):3297-304.

93. Goda S, Inoue H, Umehara H, Miyaji M, Nagano Y, Harakawa N, Imai H, Lee P, Macarthy JB, Ikeo T, Domae N, Shimizu Y, Iida J. Matrix metalloproteinase-1 produced by human CXCL12-stimulated natural killer cells. *Am J Pathol.* 2006 Aug;169(2):445-58.

94. Gebbia JA, Coleman JL, Benach JL. Borrelia spirochetes upregulate release and activation of matrix metalloproteinase gelatinase B (MMP-9) and collagenase 1 (MMP-1) in human cells. *Infect Immun.* 2001 Jan; 69(1):456-62.

95. Tschesche H, Lichte A, Hiller O, Oberpichler A, Büttner FH, Bartnik E. Matrix metalloproteinases (MMP-8, -13, and -14) interact with the clotting system and degrade fibrinogen and factor XII (Hageman factor). *Adv Exp Med Biol*. 2000;477:217-28.

96. Saunders WB, Bayless KJ, Davis GE. MMP-1 activation by serine proteases and MMP-10 induces human capillary tubular network collapse and regression in 3D collagen matrices. *J Cell Sci.* 2005; 118(10): 2325-40.

97. Dobaczewski M, Gonzalez-Quesada C, Frangogiannis NG. The extracellular matrix as a modulator of the inflammatory and reparative response following myocardial infarction. *J Mol Cell Cardiol*. 2009 Jul 23.

98. Dahi S, Lee JG, Lovett DH, Sarkar R. Differential transcriptional activation of matrix metalloproteinase-2 and membrane type-1 matrix metalloproteinase by experimental deep vein thrombosis and thrombin. *J Vasc Surgery*. 2005; 42(3): 539-45.

99. Cho A, Reidy MA. Matrix metalloproteinase-9 is necessary for the regulation of smooth muscle cell replication and migration after arterial injury. *Circ Research*. 2002; 91:845-851.

100. Hoffman M. Some things I thought I knew about tissue factor that turn out to be wrong. *Thromb Res*. 2008;122 Suppl 1:S73-7.

101. Adams RH, Alitalo K. Molecular regulation of angiogenesis and lymphangiogenesis. *Nature Reviews Molecular Cell Biology 8.6*. 2007;464-479.

102. Hoffman M, Harger A, Lenkowski A, Hedner U, Roberts HR, Monroe DM. Cutaneous wound healing is impaired in hemophilia B. *Blood*. 2006;108:3053-60.

103. Sutherland MA. Wound healing problems in haemophilia. *J Wound Care*. 1995;4(4):166-8.

104. Bodary PF, Eitzman DT. Animal models of thrombosis. *Current Opinion in Hematology*. 2009 June 22,16:000–000 (5 pages)

105. Whinna HC. Overview of murine thrombosis models. *Thromb Res*. 2008;122 Suppl 1:S64-9.

106. Chou J, Mackman N, Merrill-Skoloff G, et al. Hematopoietic cell-derived microparticle tissue factor contributes to fibrin formation during thrombus propagation. *Blood*. 2004; 104:3190–3197.

107. Day SM, Reeve JL, Pedersen B, Farris DM, Myers DD, Im M, Wakefield TW, Mackman N, Fay WP. Macrovascular thrombosis is driven by tissue factor derived primarily from the blood vessel wall. *Blood.* 2005;105:192–198.

108. Wang L, Miller C, Swarthout RF, et al. Vascular smooth muscle-derived tissue factor is critical for arterial thrombosis after ferric chloride-induced injury. *Blood* .2009; 113:705–713.

109. Kuijpers MJ, Munnix IC, Cosemans JM, Vlijmen BV, Reutelingsperger CP, Egbrink MO, Heemskerk JW. Key role of platelet procoagulant activity in tissue factor and collagen-dependent thrombus formation in arterioles and venules in vivo differential sensitivity to thrombin inhibition. *Microcirculation*. 2008; 15:269–282.

110. Sim DS, Merrill-Skoloff G, Furie BC, Furie B, Flaumenhaft R. Initial accumulation of platelets during arterial thrombus formation in vivo is inhibited by elevation of basal cAMP levels. *Blood*. 2004;103:2127-2134.

111. Stoll G, Kleinschnitz C, Nieswandt B. Molecular mechanisms of thrombus formation in ischemic stroke: novel insights and targets for treatment. *Blood*. 2008; 112(9):3555-62.

112. Massberg S, Schurzinger K, Lorenz M, et al. Platelet adhesion via glycoprotein IIb integrin is critical for atheroprogression and focal cerebral ischemia: an in vivo study in mice lacking glycoprotein IIb. *Circulation*. 2005;112: 1180-1188.

113. Jang IK,Gold HK, Ziskind AA, Leinbach RC, Fallon JT, Collen D. Prevention of platelet-rich arterial thrombosis by selective thrombin inhibition. *Circulation*. 1990; 81: 219-225.

114. Coller BS. Blockade of platelet glycoprotein GIIb/IIIa receptors as an antithrombotic strategy. *Circulation.* 1995; 92:2373-80.

115. Jørgensen L, Straume B, Mustard JF. Platelet deposition in rabbit common carotid arteries promoted by arterial stenosis and spasm. A quantitative and morphological study. *APMIS*. 2008;116(9):801-10.

116. Kovanen PT, Carpen O, Lassila R, Kaste M. Carotid plaques and stenosis: molecular mechanisms affecting the development of symptomatic lesions. 2001. In *Prevention of Ischemic Stroke*. Cesare Fieschi and Marc Fisher, editors. Martin Dunitz, London. 193-216.

117. Kirchhof K, Welzel T, Mecke C, Zoubaa S, Sartor K. Differentiation of white, mixed, and red thrombi: value of CT in estimation of the prognosis of thrombolysis phantom study. *Radiology*. 2003 Jul;228(1):126-30.

118. Mammen, E.F. Pathogenesis of venous thrombosis. *Chest.* 1992; 102:640S-644S.

119. Bagot CN, Arya R. Virchow and his triad: a question of attribution. *Br J Haematol*. 2008;143(2):180-90.

120. Wakefield TW, Myers DD, Henke PK. Mechanisms of venous thrombosis and resolution. *Arterioscler Thromb Vasc Biol*. 2008;28:387-391.

121. Sakai T, Inoue S, Matsuyama TA, Takei M, Ota H, Katagiri T, Koboyashi Y. Eosinophils may be involved in thrombus growth in acute coronary syndrome. *Int Heart J*. 2009 May;50(3):267-77.

122. Furie B, Furie BC. Mechanisms of thrombus formation. *N Engl J Med*. 2008;359:938-49.

123. Folts JD, Gallagher K, Rowe GG. Blood flow reductions in stenosed canine coronary arteries: vasospasm or platelet aggregation? *Circulation*. 1982;65:248–255.

124. Colman RW, White JV, Scovell S, Stadnicki A, Sartor RB. Kininogens are antithrombotic proteins in vivo. *Arterioscler Thromb Vasc Biol*. 1999;19:2245-2250.

125. Woollard KJ, Sturgeon S, Chin-Dusting JP, Salem HH, Jackson SP. Erythrocyte hemolysis and hemoglobin oxidation promote ferric chloride-induced vascular injury. *J Biol Chem*. 2009 May 8;284(19):13110-8.

126. Ogasawara MS, Aoki K, Katano K, Ozaki Y, Suzumori K: Factor XII but not protein C, protein S, antithrombin III, or factor XIII is a predictor of recurrent miscarriage. *Fertil Steril*. 2001; 75(5):916-9.

127. Green RA, White F. Feline factor XII (Hageman) deficiency. *Am J Vet Res*. 1977; 38(6): 893-5.

128. Endler G, Exner M, Mannhalter C, Meier S, Ruzicka K, Handler S, Panzer S, Wagner O, Quehenberger P: A common C-->T polymorphism at nt 46 in the promoter region of coagulation factor XII is associated with decreased factor XII activity. *Thromb Res*. 2001;101(4): 255-260.

129. Tirado I, Soria JM, Mateo J, Oliver A, Souto JC, Santamaria A, Felices R, Borrell M, Fontcuberta J: Association after linkage analysis indicates that

homozygosity for the 46C-->T polymorphism in the F12 gene is a genetic risk factor for venous thrombosis. *Thromb Haemost*. 2004;91(5):899-904.

130. Wuillemin WA, Huber I, Furlan M, Lammle B: Functional characterization of an abnormal factor XI1 molecule (FXII Bern). *Blood.* 1991;78: 997-1004.

131. Schloesser M, Zeerleder S, Lutze G, Halbmayer WM, Hofferbert S, Hinney B, et al.: Mutations in the human factor XII gene. *Blood*. 1997;90: 3967–3977.

132. Wada H, Nishioka J, Kasai Y, Kato-Nakazawa K, Abe Y, Morishita Y, Nakatani K, Nobori T: Molecular characterization of coagulation factor XII deficiency in a Japanese family. *Thromb Haemost*. 2003;90(1): 59-63.

133. Lombardi AM, Bortoletto E, Scarparo P, Scapin M, Santarossa L, Girolami A: Genetic study in patients with factor XII deficiency: a report of three new mutations exon 13 (Q501STOP), exon 14 (P547L) and -13C>T promoter region in three compound heterozygotes. *Blood Coagulation and Fibrinolysis*. 2008;19: 639–643.

134. Pontius JU, Mullikin JC, Smith DR; Agencourt Sequencing Team, Lindblad-Toh K, Gnerre S, Clamp M, Chang J, Stephens R, Neelam B, Volfovsky N, Schäffer AA, Agarwala R, Narfström K, Murphy WJ, Giger U, Roca AL, Antunes A, Menotti-Raymond M, Yuhki N, Pecon-Slattery J, Johnson WE, Bourque G, Tesler G; NISC Comparative Sequencing Program, O'Brien SJ: Initial sequence and comparative analysis of the cat genome. *Genome Res*. 2007 Nov; 17(11):1675-89.

135. Pontius JU, O'Brien SJ: Genome Annotation Resource Fields--GARFIELD: a genome browser for Felis catus. *J Hered.* 2007; 98(5): 386-9.

136. Unlu M, Morgan ME, Minden JS: Difference gel electrophoresis: a single gel method for detecting changes in protein extracts. *Electrophoresis*. 1997; 18: 2071–2077.

137. Pixley RA and Colman RW: Purification of human factor XII from plasma using zinc chelate affinity chromatography. *Thromb Res*. 1986; 41(1):89-98.

138. Ratnoff OD, Everson B, Donaldson VH, Mitchell BH: Purification of Hageman factor (factor XII) on columns of popcorn-agarose. *Blood* 1986 June, 67(6):1550-3.

139. Bendtsen DJ, Nielsen H, von Heijne G, Brunak S: Improved prediction of signal peptides: SignalP 3.0. *J Mol Biol*. 2004; 340: 783-795.

140. Gasteiger E, Hoogland C, Gattiker A, Duvaud S, Wilkins MR, Appel RD, Bairoch A. 2005. *Protein Identification and Analysis Tools on the ExPASy Serv*er. The Proteomics Protocols Handbook. John M. Walker, editor. New York, Humana Press. 571-607.

141. Peter Duckert, Søren Brunak and Nikolaj Blom. Prediction of proprotein convertase cleavage sites. *Protein Engineering, Design and Selection*. 2004; 17: 107-112.

142. Kambhu SA, Ratnoff OD, Everson B: Inhibition of Hageman factor (factor XII) by popcorn inhibitor. *J Lab Clin Med*. 1985; 105 (5): 625-8.

143. Miyata T, Kawabata S-I, Iwanga S, Takahashi I, Alving B, and Saito H: Coagulation factor XII (Hageman factor) Washington D.C.: Inactive factor XIIa results from Cys-571-Ser substitution. *Proc Natl Acad Sci USA*. 1989; 86: 8319-8322.

144. Hovinga JK, Schaller J, Stricker H, Wuillemin WA, Furlan M, Lämmle B: Coagulation factor XII Locarno: the functional defect is caused by the amino acid substitution Arg 353-->Pro leading to loss of a kallikrein cleavage site. *Blood*. 1994 Aug 15, 84(4): 1173-81.

145. Kopita RR. ER quality control: The cytoplasmic connection. *Cell*. 1997;88: 427-430.

146. Li A, Taylor FB Jr, Chang AK, Peer GT, Dong F, Hinshaw LB. Functional assay of plasma antithrombin using polyethylene glycol (PEG) defibrinated plasma. *Thromb Res*. 1995 Aug 15;79(4):395-403.

147. Hemker HC, Willems GM, Béguin S. A computer assisted method to obtain the prothrombin activation velocity in whole plasma independent of thrombin decay processes. *Thromb Haemost*. 1986 Aug 20;56(1):9-17.

148. Stief TW. The Fibrinogen Functional Turbidimetric Assay. *Clinical and Applied Thrombosis/Hemostasis*. 2008;14 (1): 84-96.

149. Suurmeijer AJ, Boon ME. Notes on the application of microwaves for antigen retrieval in paraffin and plastic tissue sections. *Eur J Morphology*. 1993; 31: 144-150.

150. Walter I, Schönkypl S. Extracellular matrix components and matrix degrading enzymes in the feline placenta during gestation. *Placenta*. 2006 Feb-Mar; 27(2-3):291-306.

151. Kowalewski R, Sobolewski K, Wolanska M, Gacko M. Matrix metalloproteinases in the vein wall. *Int Angiol.* 2004 Jun;23(2):164-9.

152. Anstadt MP, Franga DL, Portik-Dobos V, Pennathur A, Bannan M, Mawulawde K, Ergul A. Native matrix metalloproteinase characteristics may influence early stenosis of venous versus arterial coronary artery bypass grafting conduits. *Chest*. 2004 May;125(5):1853-8.

153. McGuinness CL, Humphries J, Waltham M, Burnand KG, Collins M, Smith A. Recruitment of labelled monocytes by experimental venous thrombi. *Thromb Haemost*. 2001 Jun;85(6):1018-24.

154. Porto BN, Alves LS, Fernandez PL, Dutra TP, Figueiredo RT, Graca-Souza AV, Bozza, MT. Heme induces neutrophil migration and reactive oxygen species generation through signaling pathways characteristic of chemoatactic receptors. *J Biol Chem*. 2007;282(33):24430-6.

155. Wagener FADTG, van Beurden HE, von den Hoff JW, Adema GJ, and Figdor CG. The heme-heme oxygenase system: a molecular switch in wound healing. *Blood.* 2003; 102:521-528.

156. Wagener FA, Eggert A, Boerman OC, Oyen WJ, Verhofstad A, Abraham NG, Adema G, van Kooyk Y, de Witte T, Figdor CG. Heme is a potent inducer of inflammation in mice and is counteracted by heme oxygenase. *Blood*. 2001;98(6):1802-11.

157. Ipaktchi K, Mattar A, Niederbichler AD, Hoesel LM, Hemmila MR, Su GL, Remick DG, Wang SC, Arbabi S. Topical p38MAPK inhibition reduces dermal inflammation and epithelial apoptosis in burn wounds. *Shock*. 2006;26(2):201-9.

158. Ohno Y, Gallin JI. Diffusion of extracellular hydrogen peroxide into intracellular compartments of human neutrophils. Studies utilizing the inactivation of myeloperoxidase by hydrogen peroxide and azide. *J Biol Chem*. 1985;260(14):8438-46.

159. Thomas EL, Bozeman PM, Learn DB. 1991. Lactoperoxidase: structure and catalytic activity. In *Peroxidases in Chemistry and Biology, vol. I*. Everse J, Everse KE, Grisham, MB, Eds. CRC Press, Boca Raton. 127-129.

160. Lundgren JD, Rieves RD, Mullol J, Logun C, Shelhamer JH. The effect of neutrophil protenase enzymes on the release of mucus from feline and human airway cultures. *Respir Med*. 1994 Aug;88(7):511-8.

161. Abcam website datasheet: http://www.abcam.com/Mast-Cell-Tryptase-antibody-AA1-ab2378.html. August 16, 2009.

162. Hernandez LA, Grisham MB, Twohig B, Arfors KE, Harlan JM, Granger DN. Role of neutrophils in ischemia-reperfusion-induced microvascular injury. *Am J Physiol*. 1987 Sep; 253(3 Pt 2):H699-703.