PHYSIOLOGICAL IMPLICATIONS FOR MURINE DEFICIENCIES OF KEY PROTEINS OF THE COAGULATION CASCADE

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PHYSIOLOGICAL IMPLICATIONS FOR MURINE DEFICIENCIES OF KEY PROTEINS OF THE COAGULATION CASCADE

Abstract

by

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Deficiencies of key proteins of the coagulation cascade have a measurable impact in haemostasis when murine models are used. In our studies, these models allow us to study the implications of factor XI (FXI)\(^-\), factor XII (FXII)\(^-\), FXI/\(^-\)/FXII/\(^-\), very low-expressing factor VII (FVII\(^{TA/TA}\)), fibrinogen (Fg)\(^-\), and protein C (PC)\(^+\) mice in biological systems other than coagulation. These systems include inflammation and the resulting tissue modifications from such process. All of the murine models used in these studies exhibit extended life spans and do not develop the life threatening conditions seen in other murine deficiencies. Most of these deficient mice slowly develop pathologies and abnormalities caused by the partial or total lack of the coagulation protein. Occasionally a phenotype with no obvious compromises is seen under resting conditions, however, the deficiencies become very obvious after inflammatory or vascular challenge when
compared to the responses observed in wild type (WT) mice. Evaluation of spontaneous and induced phenotypes of mice deficient in proteins from the contact system, reveal evidence implicating FXI and potentially FXII in the regulation of the normal inflammatory response. This response was evident in unchallenged FXI⁻/⁻ mice and becomes more conspicuous after arterial oxidative challenge when a silicon-copper cuff is used (copper-based oxidative challenge). Potential implications of the contact system in the regulation of inflammation are observed by exacerbated counts of granulocytes in the blood of FXI and FXII double deficient mice. Similarly, a low expression of FVII in mice of the same genetic background produces an inflammatory response that results in spontaneous left ventricular fibrosis. This phenomenon seems to be caused by an angiogenesis-driven cardiomyocyte degeneration. This abnormality in endothelial cell behavior was only seen in mice with compromised tissue factor (TF)/FVIIa complex levels. The direct impact of the deficiency of Fg in haemostasis is frequently observed by clot instability if examined by transluminescent video microscopy and extension of coagulation times in Fg-specific, PT, and aPTT tests. However, cellular and structural differences are apparent when clots are studied at an ultrastructural level. Detailed differences that impact the size and stability of the clot, as well as the degree of platelet activation, become evident in electron micrographs of platelet clots in the absence of Fg. The impact of the anticoagulant PC on the regulation of thrombin-dependant angiogenesis and inflammation in mice exposed to copper-based oxidative arterial challenge was evaluated. A partial deficiency of normal PC levels (≈60% of WT) supports a normal development and viability in PC⁺/⁻ mice. Nevertheless, these levels in PC⁺/⁻ mice are enough to cause significant phenotypical differences upon copper-based
oxidative challenge. Characterization of each one of these deficiencies under resting or challenging conditions reveal the extent of their effects on coagulation and, when also studied, in inflammation. Such effects often involve implications beyond the thrombin and/or fibrin-dependent induced cell behavior or inflammatory changes. These models for murine deficiencies disclose new or understudied potential interactions of each one of these proteins with other biological systems.
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<td>ALB</td>
<td>Albumin</td>
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<tr>
<td>ALKP</td>
<td>Alkaline Phosphatase</td>
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<tr>
<td>APTT</td>
<td>Activated Partial Thromboplastin Time</td>
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<tr>
<td>BK</td>
<td>Bradykinin</td>
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<tr>
<td>CBC</td>
<td>Complete blood count</td>
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<tr>
<td>CDA</td>
<td>Cytosine Deaminase</td>
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<td>CHOL</td>
<td>Cholesterol</td>
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<tr>
<td>CK1</td>
<td>Cytokeratin 1</td>
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<tr>
<td>ECs</td>
<td>Endothelial Cell(s)</td>
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<tr>
<td>ECM</td>
<td>Extra Cellular Matrix</td>
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<tr>
<td>EPCR</td>
<td>Endothelial Cell Protein C Receptor</td>
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<td>ELISA</td>
<td>Enzyme Linked Immunosorbent Assay</td>
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<tr>
<td>ES</td>
<td>Embryonic Stem Cells</td>
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<td>FVII</td>
<td>Coagulation Factor VII</td>
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FXIII ................................................................. Coagulation Factor XIII
Fg ............................................................................. Fibrinogen
FRET .......................................................... Förster resonance energy transfer
gC1qR .......................................................... Glycoprotein C1q Receptor
GPIIb/IIIa .......................................................... Glycoproteins IIb/IIIa
H&E ................................................................. Hematoxylin and Eosin
Hb ................................................................. Hemoglobin
Hct ................................................................. Hematocrit
HK ................................................................. High Molecular Weight Kininogen
KK ................................................................. Kallikrein
LDH ................................................................. Lactate Dehydrogenase
LK ................................................................. Low Molecular Weight Kininogen
Mac-1 .......................................................... Macrophage-1 Antigen
MCV ............................................................. Mean Corpuscular Volume
MMP .......................................................... Matrix Metalloproteinases
NEO ............................................................. Neomycin Cassette
NF-κB .................................................. Nuclear Factor Kappa Light-Chain-Enhancer of Activated B Cells
PAI-1 .................................................. Plasminogen Activator Inhibitor-1
PAR .......................................................... Protease Activated Receptor
PAS .......................................................... Periodic Acid-Schiff
PC .......................................................... Protein C
PK .......................................................... Prekallikrein
PT .......................................................... Prothrombin Time
RBC..........................................................................................Red Blood Cell
RDW.......................................................................................Red Blood Cell Distribution Width
SEM...................................................................................Scanning Electron Microscopy
SMC.......................................................................................Smooth Muscle Cell
TAFI..............................................................Thrombin Activatable Fibrinolysis Inhibitor
TAT..............................................................Thrombin Antithrombin III Complex
TEM......................................................................................Transmission Electron Microscopy
TF..............................................................................................Tissue Factor
TFPI..............................................................Tissue Factor Pathway Inhibitor
TGFβ..............................................................Transforming Growth Factor-Beta
TMB..............................................................3,3’,5,5’-Tetramethylbenzidine
TNFα..............................................................Tumor Necrosis Factor-alpha
TRIG......................................................................................Triglycerides
tTA..............................................................................................Tetracyclin Transactivator
uPAR..........................................................Urokinase Plasminogen Activator Receptor
WBC......................................................................................White Blood Cell
WT..............................................................................................Wild Type
vWF...........................................................................................von Willebrand Factor
INTRODUCTION

Hemostasis is the process responsible for avoiding excessive blood loss after vascular injury and is comprised of two states of vascular stability. The first state takes place under normal resting conditions, when blood circulates through the vessels and remains fluid. Under such conditions the endothelial wall is protected from coagulation by a mutual repulsion between the endothelium and blood cells. Both the endothelium and blood cells are covered with a protective glycocalix, or gelatinous layer of glycoproteins. This layer of glycoprotein cushions the blood from the continuous shear forces present in circulation while also providing an anionic surface for charge repulsion between blood cells and endothelium. The second state occurs when vessels are injured, resulting in coagulation. Blood coagulation begins when endothelial cells covering the lumen of blood vessels are damaged and subendothelial components, such as collagen and glycosaminoglycans, FVII high affinity receptors, and tissue factor (TF) become exposed to blood. The result is a rapid response involving vessel constriction and the formation of blood clots at the site of injury, a phenomenon known as thrombosis.

The coagulation cascade is the mechanism that leads to the formation of blood clots (Fig.1). It is composed of a sequence of proteolytic enzymes present in the blood plasma that mainly function to produce fibrin aggregates. Together with platelets, these fibrin aggregates form a clot to prevent blood loss after injury. The number of proteolytic enzymes that constitute this cascade, their protein structures, and their gene sequences are
not fully conserved among vertebrates. However, the capacity of these proteins for preventing blood loss through fibrin aggregation after their activation is a highly conserved feature, present from fish to birds and mammals (1).

Associated with coagulation, a third process, fibrinolysis, takes place after fibrin clots are formed. Fibrinolysis promotes the lysis and degradation of the blood clot, as well as the healing of the vessels, in order to restore normal circulating conditions. Fibrinolysis converts fibrin into fibrin-degradation products through the activity of plasmin, the enzyme that results from the release of an activation peptide (Arg560-Val561) from the protein plasminogen. The main function of plasmin is to turn fibrin into degradable fragments, such as D-dimers and X-oligomers. Those fragments are the result of plasmin activity on deposited fibrin fibrils generating smaller soluble fragments of variable molecular weight named X-oligomers. Further processing of X-oligomers generates D-dimers, generally containing two covalently bound D domains and fragments from the E domain (Fig.1.2). Both X-oligomers and D-dimers can then be released into circulation and further degraded by other enzymes.

Coagulation is a delicate balance between thrombosis and fibrinolysis. Protection from excessive fibrinolysis and proteolytic enzymes occurs through the action of inhibitors such as Plasminogen Activator Inhibitor 1 (PAI-1), alpha-2 antiplasmin, Thrombin Activatable Fibrinolysis Inhibitor (TAFI), and alpha-2 macroglobulin. Similarly, protection from excessive coagulation occurs through the action of anticoagulant proteins, such as protein C (PC), as well as inhibitors like antithrombin III and Tissue Factor Pathway Inhibitor (TFPI). Of particular importance is the anticoagulant
PC. Activation of PC into its protease form, aPC, is a key event in the regulation of thrombin formation. Activation of PC, and therefore downregulation of thrombin generation, requires the formation of the thrombin/thrombomodulin complex and binding of PC to the endothelial cell PC receptor (EPCR). Downregulation of thrombin by aPC, as well as PC profibrinolytic activity, occurs through secondary interactions with other proteins. Thrombin generation is regulated by aPC through inactivation of FVα and FVIIIa (Fig.1.1) (8). These two factors are crucial in the amplification of thrombin generation after its initial burst from prothrombin. Additionally, aPC can act as a profibrinolytic agent by inhibiting plasminogen activator inhibitor-1 (PAI-1) and favoring the generation of plasmin from plasminogen. These aPC effects, together with the regulation of thrombin generation, promote fibrinolysis (9,10).

The presence of aPC is essential for normal embryonic development and haemostasis during adulthood in both humans and mice (2,3,4,5,6,7). Defects in the ability of aPC to downregulate thrombin generation, inhibiting PAI-1 or consumption of the proteins involved in these mechanisms, can create procoagulant conditions. If thrombosis does occur and fibrin deposition is not resolved, inflammation typically follows. These events can also occur in the reverse order, with inflammation deregulating anticoagulant mechanisms, triggering coagulation, and diminishing fibrinolysis. Once coagulation is triggered by thrombosis or infection (i.e., sepsis), activation of protein C can impact the outcome of these events by downregulating inflammation through its anticoagulant activity on thrombin generation (68,69). Additionally, aPC appears to
affect inflammation through inhibiting activation of NF-κB and activator protein-1 independently of its anticoagulants properties (70).

The link between coagulation and inflammation goes beyond the aPC-thrombin interaction because other coagulation proteins, such as the complex formed by activated factor VII (FVIIa) and Tissue Factor (TF), are also involved in inflammation (13). The formation of the TF/FVIIa complex is the first event in blood coagulation after vessel injury. This complex contributes to thrombin-induced inflammation through the conversion of Factor X (FX) into its activated form, FXa, as well as factor IX (FIX) into FIXa. Activation of FIXa leads to the formation of a complex with FVIIIa, which was previously activated by thrombin. FVIIIa serves as a cofactor with FIXa for the further generation of FXa, favoring additional conversion of prothrombin into thrombin. Moreover, the TF/FVIIa complex also affects inflammation owing to its capacity to promote cell adhesion and inflammatory cell migration through endothelial layers (11,12). The impact of low levels of FVII in plasma of elderly humans has been related to higher risk for heart failure (71). The connections between low levels of FVII, inflammation, and heart physiology are one of the subjects of our research in this work. We address the impact of FVII on heart physiology by studying the behavior of ECs in murine models with low level of FVII.

The final product of the cascade initiated by the TF/FVIIa complex is the formation of fibrin fibrils from the precursor fibrinogen. Fibrinogen is a 340 KDa protein composed of three polypeptides that form a triplet of dimeric chains. The fibrinogen molecule is arranged into two equally long arms, each containing one Aα, one Bβ and
one γ chain in a coiled coil conformation of α-helices linked by disulfide bonds. The α-helices are joined at a central point by the amino terminals through two smaller chains designated fibrinopeptides A and B (FPA and FPB). The three polypeptides named Aα, Bβ, and γ are 610, 461 and 411 a.a. in length, respectively. The Aα chains extend at the carboxy terminal end to form the Aα polar appendages present in each arm (Fig.1.2). Variations of the Aα carboxy terminal ends can be a product of gene alternative splicing.

Formation of fibrin occurs after the removal of the fibrinopeptides A and B domains from fibrinogen by thrombin. This cleavage exposes the GlyProArg sequences of the Aα chain and the GlyHisArg sequences of the Bβ chain at the E domains, allowing these sites to interact with the amino terminal ends of the γ and Bβ chains at the D domains (Fig.1.3). The result is the formation of a fibrin polymer built of laterally joined fibrinogen units or D:D sites. Formation of fibrin fibrils is also promoted by interactions between the αc domains exposed after thrombin-catalyzed FPA and FPB removal (66). These polymers can be further modified by the transglutaminase coagulation Factor XIII and the addition of the structural protein fibronectin, resulting in the production of a high affinity cross-linked fibrin polymer (67,68).

Fibrin and fibrinogen are the most abundant components of the blood clot and are essential for the stability of platelet thrombi. The binding of fibrin to its platelet receptor complex glycoproteins IIb/IIIa (GPIIb/IIIa) after platelet activation forms the blood clot (14). Furthermore, both von Willebrand factor, a multimeric glycoprotein present in blood plasma, and fibronectin can bind to the GPIIb/IIIa receptors on the platelet membrane, facilitating platelet aggregation (15,16). A tight and stable thrombus is
capable of attaching itself to the site of injury and resisting the high shear forces present in the main arteries of the body. Defective fibrin binding or the absence of its platelet receptor complex GP IIb/IIIa translates into unstable clots with poorly aggregated platelets, as occurs in the human disorder Glanzmann thrombasthenia (17,18). We dedicate part of this work to the study of the impact that the total absence of fibrinogen has in the aggregation of platelets and the stability of arterial clots.

Activation of thrombin from its precursor prothrombin occurs through the action of another serine protease, activated coagulation factor X (FXa). This is the protein at which the extrinsic and the intrinsic pathways of the coagulation cascade converge, making FX the first serine protease of the common pathway. The relevance of this factor is reflected by the low frequency of its deficiency in the human population (1 case/50,000 newborns), suggesting that most conceptions with inherited FX deficiency do not result in a viable pregnancy. The acquired deficiency of FX can be caused by insufficient dietary vitamin K, a vitamin required to form functional FX and other serine proteases of the coagulation cascade. Thrombin activation, and consequently fibrin generation, depends directly on FXa activity. The role of FX, as well as many other coagulation proteins, is not limited to clotting. Studies have shown FXa participates in inflammation through its interactions with PAR1 and PAR2 receptors on endothelial cells (ECs) (13). Cleavage of PAR1 and PAR2 may occur upon FXa’s association with EC membranes. This cleavage translates into activation of NF-κB and initiates other intracellular events, resulting in the potential modification of ECs and connective tissue behavior (13).
The intrinsic pathway, the second branch of the coagulation cascade, appeared later in evolution, presumably from gene duplication (1). Four plasma proteins: Prekalikrein (PK), Factor XI (FXI), Factor XII (FXII), and High Molecular Weight Kininogen (HK) constitute the initial part of the intrinsic pathway (19). Also known as the contact system, these proteins participate in the maintenance of thrombin generation after an initial burst of thrombin is produced by the extrinsic pathway (Fig.1.1 and Fig.1.4). This augmentation of thrombin generation allows for the further catalysis of fibrinogen and the formation of the fibrin clot. The additional thrombin and fibrin produced also favor clot stability and resistance to fibrinolysis (20,21,22,23). The availability in plasma of each one of the contact system proteins varies considerably (Table.1.1). The levels of these proteins in plasma may correlate with their interactions and particular function in haemostasis.

Without activation of the intrinsic pathway, coagulation is limited to thrombin generated by the activation of the TF/FVIIa complex, and stability of the fibrin-platelet clot is compromised. The total absence of FXI or FXII in mice translates in vitro into a delayed coagulation time, as determined by aPTT tests (24,25). Both FXII and FXI appear to be required for the formation of stable clots in mice (26,27). Deficiencies of factor VIII or IX, both members of the intrinsic pathway, most likely produce the well known conditions haemophilia A and B in humans (28). Though deficiency in any of the contact system proteins does not produce life threatening bleeding in human patients, FXI deficiencies can exhibit serious bleeding diathesis (29,30,31). This phenomenon is particularly noted after surgery, major injuries, and menorrhagia and during delivery or
TABLE 1.1

MOLECULAR MASS AND PLASMA CONCENTRATION

OF CONTACT SYSTEM PROTEINS

<table>
<thead>
<tr>
<th>Contact system protein</th>
<th>Molecular mass (Da) (^a)</th>
<th>[Plasma] (µg/mL) (^b)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Prekallikrein (PK)</td>
<td>80,000</td>
<td>50</td>
</tr>
<tr>
<td>High molecular weight kininogen (HK)</td>
<td>120,000</td>
<td>70-80</td>
</tr>
<tr>
<td>Coagulation factor XI</td>
<td>160,000</td>
<td>4-8</td>
</tr>
<tr>
<td>Coagulation factor XII</td>
<td>80,000</td>
<td>24-40</td>
</tr>
</tbody>
</table>


post partum (32,33,34,22). On the other hand, elevated levels of FXI or FXII have been associated with venous thromboembolism and increased risk for cardiovascular disease in humans (35,36,37). Paradoxically, low levels of FXII have also been associated with an increased risk for cardiovascular disease (38). Due to the low frequency of deficiencies of proteins from the contact system in the human population and the limited data available, little is known about them and their symptomatic implications.
Murine models for most contact system protein deficiencies have been generated (e.g. HK, FXI and FXII (24,39,40,41). Despite the clear physiological differences between mice and humans, murine models are extremely valuable due to their capacity for mimicking the symptoms and physiological conditions of their human counterparts. Most murine models are fully deficient in contact system proteins, whereas cases of human deficiencies generally exhibit some degree of protein functionality. Exceptions in human cases are genetic mutations that compromise the transcription of the normal version of the gene, rendering either no protein or a completely dysfunctional protein post-translation. Nevertheless, a total lack of these proteins in animals leads to models with exacerbated symptoms and defects that are not fully manifest in a partially deficient human.

Activation of FXII takes place upon contact with a negatively charged surface. These surfaces include phospholipids from the inner face of the cellular membrane, proteins and proteoglycans from the ECM of the subendothelial tissue, negatively charged misfolded proteins, artificial surfaces, and bacterial cell walls. Such contact induces a series of conformational changes in FXII that facilitates its self-activation, resulting in the further catalysis of PK into kallikrein (KK) and/or activation of FXI into FXIa (42,43). KK catalyzes the cleavage of additional FXII, rendering even more FXIIa, while FXIa catalyzes the cleavage of FIX into FIXa, allowing the cascade to continue. A second cleavage of FXIIa renders the catalytic domain in the light chain soluble, and therefore unable to bind to surfaces. This form of FXIIa, named βFXIIa, can further catalyze the activation of PK for the generation of KK, but is unable to contribute to FXI
activation in the absence of a surface (42). In addition, the contact system has implications beyond coagulation, including participation in the regulation of vascular tone and contributions to inflammation, immunity, and angiogenesis.

HK circulates in complex with either PK or dimeric FXI in plasma. Preferential binding of one complex to EC seems to depend highly on plasma Zn$^{2+}$ concentration (44). The existence of HK receptors on both ECs and neutrophils has implications for HK and its ligands, PK and FXI, in haemostasis and inflammation. Cytokeratin 1 (CK1), urokinase plasminogen activator receptor (uPAR), and glycoprotein C1q receptor (gC1qR) have been described as the receptors for HK on the surface of ECs, while Mac1 (leukocyte integrins CD11b/18, αMβ2), uPAR and gC1qR were reported as HK receptors on neutrophils and monocytes (45,46,47,48). Although it is unclear what types of interactions might exist among them, it is possible that these proteins form a complex that serves as a receptor for HK on EC and neutrophils. Its formation may also have an effect on cell activation. Additionally, blockage with specific antibodies against gC1qR, uPAR and CK1 in cell culture assays, together with colocalization and flow cytometry experiments, suggests the interaction of FXII with all of these EC membrane receptors (49). This interaction occurs in a Zn$^{2+}$- and HK-dependent manner (49). Binding of FXII to its receptor(s) on ECs could enhance the generation of KK, which otherwise would rely on a less specific activation mechanism for the catalysis of PK. The catalytic action of KK also generates HKa and bradykinin from HK, as well as LKa and bradykinin from tissue low molecular weight kininogen (LK). LK is located inside the cell however, and is not always available in the plasma.
Bradykinin (BK), a nine amino acid long peptide derived from domain 4 of HK, has potent vasodilatory and inflammatory properties (50). This nonapeptide induces vasodilatation and increases vascular permeability by its interaction with B2 receptors present on ECs. It also affects vascular neutrophil recruitment and glutamate signaling in the nervous system upon binding to B1 receptors (51, 52, 53). Additionally, the interaction of BK with its B1 receptor appears to induce angiogenesis, although the expression of this receptor on the vasculature seems to be inflammation and injury-dependent (54, 55). The generation of BK is accompanied by the activation of HK into HKa, a form of HK with antiangiogenic and proapoptotic effects on ECs (56). It is not clear how those two molecules, BK and HKa, originating from the same precursor protein, can regulate the angiogenic process on ECs.

Both HK and LK are products of the same gene, Kng1 (57). Alternative splicing of this gene produces different C-terminal light chains for each kininogen (HK consists of domains 5 and 6) but identical heavy chains (domains 1 through 4) (57). HKH19, a His and Lys rich region of domain 5 in HK, has been mapped as the LPS and cell binding domain of HK (58). This binding interaction was inhibited in vitro by heparin in a dose-dependent manner (IC50= 0.3 U heparin/mL) in the presence of physiological amounts of HK (600nM) (58). The use of a monoclonal antibody against HK in a rat model of reactive arthritis ameliorated the KK-dependent inflammatory response observed in these animals (59). The contribution of KK to the inflammatory response seen with this model was linked to the capacity of the HK/PK complex to recruit neutrophils upon KK generation (59).
KK favors neutrophil chemotaxis, aggregation, and degranulation (60,61,63). Furthermore, the generation of antimicrobial peptides (AMPs) has implicated kininogens in the innate immune response. Upon contact with bacterial surfaces, the cleavage by neutrophil elastase of domain 3 of either HK or LK generates peptides with antimicrobial properties similar to those of the human antibacterial peptide LL-37 (64,65). All evidence regarding the participation of different members of the contact system in diverse biological phenomena points towards potential implications of these proteins in such diverse events as inflammation and immune reactions, independent of their roles in coagulation. A portion of the first chapter in this manuscript is dedicated to the further characterization of a murine deficiency in FXI, a key enzyme of this system and the only deficiency of the contact protein system that produces a bleeding diathesis in humans. Such studies focus on the presence of inflammatory signs in FXI deficient mice. Later on we study the impact that such inflammatory signs have when mice are subjected to an arterial oxidative challenge. Events that may trigger inflammation in our oxidative model include platelet degranulation, activation of endothelial and SMCs in the vessel wall, and the recruitment of inflammatory cells through induction of cell-cell interaction on the endothelial surface.
Fig.1.1 Coagulation cascade. Physiological events and key enzymatic reactions (black), regulators (red capped lines), and the participation of thrombin in additional enzymatic reactions (green capped lines). Roman numbers correspond to the different coagulation factors and their activated forms (a).
Fig. 1.2) Structural domains and binding regions of the fibrinogen molecule. The E domain connects two equilateral arms of the molecule where the amino terminals of each chain locate. This domain also contains the fibrinopeptides A and B. The D domains contain the γ and β globular domains, and the carboxy terminal ends of each chain. The γ globular domains display the D:D and Da sites as well as the γxL appendages, all key sites for fibrin polymerization. The cleavage site for plasminogen (sphere), factor XIII transglutamination of γxL appendages, αC sites, and the platelet binding domain are also shown. Adapted from Mosesson MW et al. The structure and biological features of fibrinogen and fibrin. In: Nieuwenhuizen W, Mosesson MW, De Maat MPM, eds. Fibrinogen, Ann. New York Acad. Sci. Vol. 936, New York; 2001: 11 (66).
Fig.1.3) Basic fibrin fibril diagram. Fibrils are formed after the thrombin-catalyzed removal of FPA and FPB. Notice the antiparallel arrangement of fibrin molecules and D:D and Da contact sites between the fibrin monomers. Crosslinking of $\gamma xL$ appendages (C terminal of $\gamma$ chains) has taken place in cis (end to end) and trans (transverse) fashion between monomers. Adapted from Mosseson MW et al, PNAS 1998 (67).
Fig.1.4) The contact system of coagulation. 1 and 2 occur in the luminal side of the vessels and correspond to the initial part of the intrinsic pathway. (1) Initial damage of the cell membrane exposes phospholipids to the circulation. Contact of FXII with negatively charged phospholipids lead to a conformational change and its self-activation. The newly generated FXIIa can further catalyze the generation of Kallikrein (K) from prekallikrein (PK), Factor XIa (FXIa) from Factor XI (FXI) or can be further processed for the creation of βFXIIa, a form of FXII that only participates in the generation of K. Production of FXIa continues the activation of the intrinsic pathway to form thrombin, whereas K can either convert more FXII into FXIIa in a feedback reaction or further process the catalysis of high molecular weight kininogen (HK). (2) HK catalysis by K leads to the generation of bradykinin (BK) and activated HK (HKa). Both products, BK and HKa, have important effects in the vasculature such as, induction of vascular permeability and modification of EC’s behavior.
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49. 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.


CHAPTER 1:

SPONTANEOUS PHENOTYPES IN FVII\textsuperscript{tTA/tTA}, FXI\textsuperscript{−/−} AND FXI\textsuperscript{−/−}/FXII\textsuperscript{−/−} DOUBLE DEFICIENT MICE

1.1 Introduction

This chapter deals with the study of spontaneous phenotypes found in mice with individual or combined deficiencies of coagulation proteins. These phenotypes are the result of physiological changes resulting from the lack of one or more proteins that are present in WT mice under normal conditions. Such physiological changes can develop into unregulated and pathological conditions that, in the most severe cases, impede normal physiological functions and may negatively impact life expectancy in the long run. The murine genetic deficiencies examined in the present study comprise coagulation FVII, FXI, FXII and the combination of FXI and FXII. The aforementioned deficiencies are part of the initiation of the extrinsic pathway (FVII) or the intrinsic pathway (FXII and FXI) of the coagulation cascade. Activation of both pathways in normally functioning mice allow for the balance of haemostasis. For such studies we used total murine deficiencies of FXI and FXII as models as well as, mice expressing very low levels of FVII (FVII\textsuperscript{tTA/tTA} mice).
Exposure of TF from the intravascular tissue followed by formation of the TF/FVIIa complex begins the process for thrombin generation through the extrinsic pathway of coagulation. In contrast with FVII, which is mainly synthesized in the liver, TF is expressed by fibroblast, vascular SMC, cardiomyocytes and some blood cells such as leukocytes and platelets (1). This expression pattern makes TF availability almost ubiquitous in all tissues. Although the formation of the TF/FVIIa complex is required for the activation of the extrinsic pathway of coagulation, their individual deficiencies manifest different symptoms. TF deficiency impairs normal cardiac functions in mice (2). Its importance in humans can be inferred because no congenital deficiency has been described (2) and deficient humans may not survive into postnatal stage. The relevance of the TF/FVIIa complex is reflected in the impaired haemostasis and defective circulation observed in utero and in neonates of individual murine total deficiencies of TF and FVII respectively (3, 4, 5). The murine TF total deficiency leads to vessel fragility and rupture around the vitello of the embryos leading to a bleeding lethality around day 9.5 of embryonic development (3). In contrast, a murine FVII total deficiency produces normal embryos that survive delivery; however, either intraperitoneal bleeding at 24 h or later intracranial bleeding limits their survival to zero past 24 days post birth (5). In murine models expressing very low levels of either TF or FVII (≤1% of wild types), survival is highly improved. Occurrence of prolonged hemorrhage in the heart, demonstrated by hemosiderosis, inflammatory infiltration, and tissue fibrosis, compromises normal physiologic functions (2,6). In the first part of this chapter, a detailed description of this hemorrhagic phenomenon is described for low FVII expressing mice. The spontaneous pathology observed in these mice was marked by consumption of coagulation factors
from the intrinsic pathway available in plasma, low levels of TAT, and cardiac abnormalities (6). As a consequence of the unregulated coagulation in these mice, prolonged PT and aPTT values were observed (6). While we describe the pathological findings surrounding this cardiac fibrosis, special focus was placed on the initial stages of this cardiac myopathology. Studying the initial stages allows us to find the root cause of the malfunction of this organ seen at later time points.

Spontaneous phenotypes in mice with total deficiencies of FXI, FXII and its combination displayed different patterns of abnormalities. Such abnormalities ranged from irregular complete blood counts (CBCs) to tissue abnormalities that compromised their response to inflammation. Particular focus was put on FXI murine deficiency due to the lack of detailed information about this deficiency in mice and the discrepancies between bleeding manifestations and active protein plasma levels seen in humans. Currently there is no factual explanation for the lack of relationships between the degree of FXI protein activity in plasma and coagulation times seen with humans deficient in this factor. Although the FXI human and murine deficiency share common characteristics such as prolonged aPTTs and no obvious bleeding diathesis, the total lack of the coagulation factor in the murine model probably exacerbates any of the symptoms that may be found in humans. Therefore, the murine model of the disease provides an excellent tool for the study of this deficiency.

The pathological findings and physiological discrepancies that we found in the FXI- mice when compared to WT pairs consisted of blood and vascular abnormalities, as
well as, hepatic inflammation. A description of these findings, together with their implications for aging, and predisposition to inflammation is detailed here.

1.2 Materials and Methods

1.2.1 Animal housing, generation and breeding

All mice used in these studies were housed in microisolation cages with a maximum capacity of 5 mice per cage, a 12 hour daily light cycle, and with permanent access to food and water ad libitum. Animal breeding, housing and manipulation were performed following the regulations established by the IACUC and the AALAC, including the certification of all the personnel on Human care and use of laboratory animals by the Freimann Life Science Center of Notre Dame.

1.2.2 Generation of FVII\textsuperscript{tTA/tTA}, FXI\textsuperscript{-/-} and FXI\textsuperscript{-/-}/FXII\textsuperscript{-/-} double deficient mice

The generation of FVII\textsuperscript{tTA/tTA} mice expressing very low levels of coagulation factor FVII was performed in our laboratory as described previously with a targeted replacement of the FVII gene with the FVII cDNA (7). FXI fully deficient mice (FXI\textsuperscript{-/-}) were generated by Dr. David Gailani’s group (8). Here, exon V of the region coding for FXI was replaced with NEO in a targeting vector, disrupting the gene sequence after homologous recombination. For the targeted generation of FXII deficient mice in our laboratory (9), exons 3 through 8 were disrupted. FXI/FXII double deficient mice were
generated through multiple crossing of FXI<sup>-/-</sup> mice with FXII<sup>-/-</sup> mice until full double deficient mice were obtained. All of the crosses survived and mice appeared fully viable and survived into adulthood with no obvious bleeding events after full body necropsy.

1.2.3 Mouse genotyping

Tissue from ear punches was used as the source of DNA for genotyping the deficient mice in these studies. Isolation of the DNA from the tissue requires alkaline hydrolysis in 20μL of 0.2M sodium hydroxide (NaOH) at 75°C for 10 minutes, followed by neutralization in 180μL of 0.04M Tris-Cl buffer. Detection of WT and deficient genes was performed by PCR DNA amplification using a conventional thermal cycler (Peltier Thermal Cycler-200, BIORAD, USA) or a LightCycler real-time PCR machine (Roche, Mannheim, Germany). The LightCyler uses FRET for the detection of the amplified DNA with primers specific for the desired gene as well as sequence complementary fluorescent probes attached to fluorophores. In contrast, the conventional thermal cycler uses the reverse and forward primers for the generation of the DNA amplicon. DNA amplification is followed by gel electrophoresis for verification of the amplicon size. When the probes hybridize to the sequence during the annealing cycle, FRET occurs and fluorescence is detected. Additionally, Taqman real-time PCR (RT-PCR) was also used for the detection of some of the alleles present in the DNA samples. Although Taqman technology also uses fluorescence, this method is based on a different principle involving the release of a fluorescent reporter that is cleaved by the polymerase during extension. The release of the reporter to the reaction medium separates it from the quencher placed
in close proximity in the probe and allows the fluorophore to emit fluorescence previously repressed by the quencher. The list of primers used for the detection of the different genotypes and the methodology used are shown in Table 1.2.

1.2.4 Necropsy

Mice were anesthetized using a rodent cocktail (0.075 mg ketamine/0.015 mg xylazine/0.0025 mg aceprozamine/g weight of mice) before any intervention. Blood was collected from the vena cava in 0.17 M EDTA at a volume ratio of 9:1 to check for standard CBC values in a Hemavet cell counter (DREW Scientific, Oxford, Connecticut, USA) or citrate buffer for coagulation tests, enzymatic activity assays, ELISA and blood chemistry analysis from plasma samples. Plasma samples were obtained by centrifugation of whole blood preparations at 2,500xg for 10 min. Blood chemistry was performed by colorimetric readings of test-specific slides incubated with plasma samples in a Vet Test 8008 chemical analyzer (IDEXX laboratories, Inc, Westbrook, Maine, USA). Collection of arteries from WT and FXI<sup>−/−</sup> and hearts from WT and FVII<sup>TAI/TA</sup> was followed by fixation in Karnovsky solution and processing for SEM or TEM; livers, spleens, hearts, lungs, kidneys, large intestines and testis of WT and FXI<sup>−/−</sup> mice were fixed in NBF (neutral buffered formalin) or PLP (acetone/periodate-lysine-paraformaldehyde). Following fixation, samples were dehydrated in 70% ethanol and paraffin infiltrated for later sectioning, H and E staining, and histological analysis. Blood smears from WT, FXI<sup>−/−</sup>, FXII<sup>−/−</sup>, and FXI<sup>−/−</sup>/FXII<sup>−/−</sup> mice were developed on histological slides, stained with the Wright-Giemsa method and subjected to morphological analysis.
TABLE 1.2

PRIMERS AND METHODOLOGY FOR DETECTION OF GENOTYPES

<table>
<thead>
<tr>
<th>Gene detected</th>
<th>Primers</th>
</tr>
</thead>
<tbody>
<tr>
<td>FXI</td>
<td>5’ common primer  TTGCAGCAAAGATGAGTGACGTGAAC</td>
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<td></td>
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<tr>
<td>WT</td>
<td>3’ WT  ATGGTGCACTGGGGAAAATACCC</td>
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<tr>
<td></td>
<td></td>
</tr>
<tr>
<td>FXI Neo</td>
<td>FXI Neo&lt;sup&gt;5&lt;/sup&gt;  ATTCGCAGCGCATCGCCTTATC</td>
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<tr>
<td></td>
<td></td>
</tr>
<tr>
<td>WT</td>
<td>5’ FITC&lt;sup&gt;6&lt;/sup&gt;  CGAGAATGCCCAGGAGAGATGCAC Fluorescence&lt;sup&gt;6&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td>WT</td>
<td>3’ 640TexasRed&lt;sup&gt;7&lt;/sup&gt;  ACGATGCCACTGCGCATCGCCTTTCACATACG Phosphate&lt;sup&gt;8&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td>FXI&lt;sup&gt;7&lt;/sup&gt;</td>
<td>5’ FITC  GTCAAGAAGGCAAGGGAAGGCAAC Fluorescence&lt;sup&gt;8&lt;/sup&gt;</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>FXI&lt;sup&gt;7&lt;/sup&gt;</td>
<td>3’ 705 Cy5.5&lt;sup&gt;8&lt;/sup&gt;  CAGATGCTGGCAACTAGAAGGCACAGTC Phosphate&lt;sup&gt;8&lt;/sup&gt;</td>
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<tr>
<td></td>
<td></td>
</tr>
<tr>
<td>WT</td>
<td>5’  GACTTGAGGGTCGGAAC'TG</td>
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</tr>
<tr>
<td>WT</td>
<td>3’  CAGAGCAGTTTGGTGGTCTC</td>
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<tr>
<td>WT</td>
<td>5’ FITC  GACTCGCTGCTGTCCCTTTTGACT Fluorescence&lt;sup&gt;8&lt;/sup&gt;</td>
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<td>WT</td>
<td>5’  640TexasRed  TTGCCCTGAGAATTCTGCTGAAC'TG Phosphate&lt;sup&gt;8&lt;/sup&gt;</td>
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<tr>
<td>FVII-tTA</td>
<td>5’  GACTTTGAGGGTCGGAAC'TG</td>
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<tr>
<td>FVII-tTA</td>
<td>3’  GGCAATGTCGTGGTGGTAT</td>
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<tr>
<td>FVII</td>
<td>5’ FITC  CACAGTGTTGATGGGTAACATGAC Fluorescence&lt;sup&gt;8&lt;/sup&gt;</td>
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<tr>
<td></td>
<td></td>
</tr>
<tr>
<td>FVII</td>
<td>3’ 705 Cy5.5&lt;sup&gt;8&lt;/sup&gt;  CAGTGAGAAGGATGGGGATGAGCAA Phosphate&lt;sup&gt;8&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a,b,c,d,e</sup> Fluorophores emitting at different wavelengths when in FRET distance.  
<sup>f</sup> Detection with Light cycler RT-PCR/ conventional PCR.  
<sup>g</sup> Detection with Light cycler RT-PCR.
Total body weight measurements of at least 20 WT and 20 FXI<sup>+/−</sup> mice per time point were made at the ages of 3, 6, 8, and 11 months in a Mettler type BD202 scale (Mettler-Toledo Inc., Columbus, Ohio, USA). Similarly, average amount of food weight consumed daily was estimated for mice of 3, 6 and 9-12 months of age as well.

1.2.5 Hepatic histology

Histological analysis and quantification of hepatic inflammatory foci and number of inflammatory cells were assessed in 4μm paraffin sections after H&E staining. Results were expressed by the number of cells, foci and fields for each genotype. A minimum of 30 fields per liver sample at 20X were examined.

1.2.6 Coagulation assays

Fibrinogen (Fg) levels, factor X (FX) and factor XII (FXII) relative percentage, activated partial thromboplastin time (aPTT) and thrombin time in the plasma of WT and FXI<sup>+/−</sup> mice were determined by clotting times in a Start 4 coagulometer (Diagnostica Stago, Asnières, France) with reagents from Diagnostica Stago. Plasma samples and reagents were both prewarmed at 37°C and mixed in plastic cuvettes provided with standardized magnetic balls.

Fg levels were determined by incubating 50 μL of diluted (1:20) plasma with 100 μL of Fibri-Prest Automate reagent (Diagnostica Stago). Standards were made before
each series of measurements with dilutions of Unicalibrator in Owen-Koller buffer from the same manufacturer. Results were expressed in mg/dL.

aPTTs were determined by incubating 50 μL of undiluted plasma with 50 μL of PTT Automate reagent (Diagnostica Stago) and 50 μL of 0.025 M CaCl₂. Results for coagulation time were expressed in seconds.

FXII plasma percentage was determined by incubating 50 μL of STA-deficient XII with 50 μL of plasma sample, 50 μL of PTT Automate reagent and 50 μL of 0.025 M CaCl₂; conversely, FX plasma percentage used STA deficient X reagent, incubated with 50 μL of plasma sample and 100 μL of Néoplastine reagent. Results for each genotype were expressed as % of pooled WT control plasma.

Thrombin times were determined by adding 100 μL of STA Thrombin reagent to 100 μL of plasma sample. Results were expressed as the number of seconds that the samples took to coagulate.

Enzymatic activity assays for FXa were developed in 96 well plates using 100 μL of plasma sample diluted with 50 μL of 50 mM Tris-Cl buffer containing the chromogenic substrate S2772 (Chromogenix, Lexington, MA, USA) at a 2.2 mM concentration. Incubations were run for 3 min., after which the reactions were stopped by adding equal volumes of 1N HCl. Readings were then taken at 405 nm OD against the blank in a SPECTRA max plus 384 spectrometer (Molecular Devices, Sunnyvale, California, USA). Standards were made with dilutions of hFXa (Enzyme Research
Laboratories, Ltd, South Bend, IN, USA) ranging from 0.0004 μg/mL to 2 μg/mL. Similar activity assays were performed for KK using S2302 substrate at a 0.05 mM concentration and measured at 405 nm OD against the blank. Activity was measured with and without FXIIa activation. Blank wells were set up for subtracting the catalytic activity generated by the addition of FXIIa to the mix. Standards for KK assays were based on dilutions of hKK (Enzyme Research Labs, South Bend, IN, USA) ranging from 0.00125 μg/mL to 12.5 μg/mL.

Peroxidase enzymatic activity was measured in plasma, before and after incubation, of whole blood from WT and FXI-/- mice in 0.2 μg/mL of complement C5a protein (R&D Biosystems, Minneapolis, MN, USA) for 10 min, using a 3,3',5,5'-tetramethylbenzidine (TMB) chromogenic substrate kit solution (R&D Biosystems, Minneapolis, MN, USA). The reaction was stopped by adding equal volumes of 1 N HCL followed by readings at 450 nm OD. Standards were based on dilutions of horseradish peroxidase (PIERCE, Rockford, IL, USA) incubated for 3 min with the same substrate. All the enzymatic tests were performed within 4 hours after the collection of blood from the mice. Blood collection was immediately followed by preparation of plasma for the enzymatic tests. Measurements were performed within the linear part of the initial velocity curve.
1.2.7 ELISA

Levels of thrombin in the plasma were assessed by measuring the thrombin-antithrombin III (TAT) complex concentration by ELISA. Standards were made based on a 1 μM of TAT complex solution in 0.05 U/mL of heparin and ranged from 20 pM to 600 pM of TAT complex (Enzyme Research Laboratories, South Bend, IN, USA). Both the capture and the detection antibodies were raised in Sheep. The capture antibody (1:100 dilution) reacts against human α-thrombin and the detection antibody (1:100 dilution), peroxidase conjugated, reacts against antithrombin III. A TMB chromogenic substrate kit solution (R&D Biosystems, Minneapolis, MN, USA) was used (100 μL/well in a 96 well plate) as substrate for detection after 3 min. incubation. The reactions were runned in the linear part of the velocity curve and then stopped by adding 50 μL/well of 2.5 M H₂SO₄. Results were read at 450 nm wavelength and values were obtained after subtraction of the blank absorbance at 490 nm and expressed in ng/mL of TAT.

1.2.8 Transmission Electron Microscopy (TEM) and morphometric analyses

Ultrastructural analysis on carotid arteries from 10 WT and 10 FXI⁻/⁻ 3 month old mice and left cardiac ventricles from 8 WT and 8 FVII⁻¹TA⁻¹TA mice, ages 1.5 and 4 months old, were fixed in Karnovsky solution, rinsed in 1X PBS, pH7.4, postfixed in OsO₄, dehydrated in ethanol and embedding in plastic resins (Polysciences, Warrington, PA) as previously described (10). Plastic thick (0.5 μm) and ultrathin (90-80 nm) sections were obtained from each sample for morphological analysis in a Nikon Eclipse E600 optical
microscope (Nikon, Yokohama, Japan) and a Hitachi H600 transmission electron microscope (Hitachi, Tokyo, Japan) at 75 kV accelerating voltage. Thick sections were stained in 2% Toluidine blue, while ultrathin sections were stained in 2% uranyl acetate and Reynold’s lead stain. Additionally, morphometric measurements of thick cross-sections from the same arteries were made in triplicate in sections separated by a 500 µm distance. Using Metamorph 4.01 software, variables such as wall thickness and the number and height of luminal undulations were measured for WT and FXI⁺⁻ carotid arteries.

1.2.9 Scanning Electron Microscopy (SEM) analysis

Arteries from at least 3 perfused mice of each genotype were harvested, fixed for 4 hours at 4° C in Karnovsky's solution, and post-fixed in OsO₄. Post-fixation was followed by dehydration up to 100% ethanol in solution. Complete sample dehydration was achieved in a CO₂ critical point dryer containing absolute ethanol. Next the arteries were cut longitudinally and sputter-coated on an aluminum block with a gold/platinum mix. SEM analysis was performed with a JEOL JSM-T300 scanning electron microscope (JEOL USA, Peabody, MA) at 25 kV.

1.2.10 Vascular permeability assays and fecal blood detection

Tail vein injections of Evans blue (20mg/kg) diluted in 100 µL PBS were performed in at least 10 mice (3 months old) of each genotype. After 15 min post-dye
injections, paw and facial areas were exposed to 5% mustard oil (allyl isothiocyanate, diluted in mineral oil) via skin application. Detection of blood in mice stool samples was developed early in the day (8:00-9:00 am) using a Beckman Coulter Hemoccult *Sensa* kit for detection of low but abnormal levels of Hemoglobin (Hb) (Fisher Scientific, Pittsburgh, PA, USA).

1.2.11 Statistical measurements

Morphometric analysis data were processed using the NCSS-2001 software package, Microsoft Excel 2002 for t-test comparisons and manual computation performed for \( \chi^2 \). A \( p \) value \( \leq 0.05 \) was considered statistically significant. Results for averages in all the tables and plots were expressed accompanied with standard errors of the mean (\( \pm \)SEM).

1.3 Results

1.3.1 Spontaneous phenotype in FVII\(^{TA/TA}\) mice

The presence of a spontaneous left ventricular fibrosis in FVII\(^{TA/TA}\) mice was observed as early as 6 weeks of age. Previous necrotic examination and echocardiographic measurements of these FVII\(^{TA/TA}\) mice hearts at four months of age suggested the existence of an abnormal physiology evidenced by increased heart size,
reduced left ventricular volume and irregular cardiac blood output (6). Later histological analyses of these hearts demonstrated the occurrence of internal bleeding and hemosiderin deposition. Those histological analyses were followed by more detailed histological and ultrastructural examination in plastic sections (6). Detailed examination of these left ventricles by optical and transmission electron microscopy revealed the occurrence of intramuscular bleeding, angiogenesis, and fibrosis (Fig.1.5 and Fig.1.6). After studying heart samples at six weeks and four months of age, a mechanistic sequence of events was constructed. We hypothesize that an angiogenic phenomenon takes place in the left ventricles of the low FVII expressing mice. Angiogenesis is by nature a blood leaking process due to a lack of a restrictive barrier that keeps the blood from the existent vessel from leaking into the forming neovessels. Extended examination of the affected areas confirmed that the cardio-muscular bleeding was associated with newly forming vessels. This angiogenesis often occurred with ECs migrating through the cardiomyocytes (Fig.1.6 and Fig.1.7). As a consequence of this angiogenic phenomenon, the integrity of cardiomyocytes is highly compromised, and consequently degeneration occurs. Internal bleeding and cell degeneration trigger local inflammation, evidenced by the presence of macrophages that engage in hemorrhage and cell debris clearance (Fig.1.8). Cell debris clearance is the initial stage of a tissue repair process that ends in the replacement of degenerated cardiac tissue with fibroblast-rich tissue. We also found that fibroblasts produced substantial amounts of collagen and other extracellular matrix proteins, filling their surrounding areas with connective tissue.
1.3.2 Spontaneous phenotype in FXI⁻/⁻ mice and accumulative effects of the combined deficiency of FXI/FXII in mice.

The complete absence of FXI or FXII from mice plasma results in a delayed coagulation time when assayed in vitro, as determined by aPTT tests (8,9,11). Although the activation of FXI can occur independently of FXII (12,13,14), both FXI and FXII seem to be required for the formation of stable clots (15,16). Furthermore, the effects of the combined absence of FXI and FXII, in the formation of clots in vivo, remain unknown. The initial phenotypical characterization of young adult mice with a total deficiency in FXI failed to show any obvious physical, reproductive or behavioral abnormalities (8). Nevertheless, some differences became noticeable as the mice aged, and blood and morphological analyses of the liver and main arteries were performed. We also report some of the effects derived from combining both murine deficiencies for FXI and FXII.

1.3.2.1 Blood chemistry, cell counts, and histology

Morphological examination of blood smears from FXI⁻/⁻ mice showed signs of microcytic anemia and iron deficiency (Fig.1.9). This morphological analysis was complemented with CBCs from samples of WT and FXI⁻/⁻ mice (Table 1.3). Differences on the number of RBCs, Hb concentration, and RDW were statistically significant and confirmed the observations from the blood smears. The young adults revealed higher concentration of Hb (13.31±0.13 g/dL for WT vs. 12.16±0.20 g/dL for FXI⁻/⁻, p=0.007),
higher number of RBCs (8.74 ± 0.21 M/mm$^3$ for WT vs. 7.10 ± 0.31 M/mm$^3$ for FXI$^{-/-}$, 
$p=0.0003$) and higher hematocrit (46.25% for WT vs. 35.07% for FXI$^{-/-}$) in the WT when compared to the FXI$^{-/-}$ mice. Also, RDW values were higher for the WT (9.63 ± 0.15 for WT vs. 8.14 ± 0.34 for FXI$^{-/-}$, $p=0.0005$). In the FXI$^{-/-}$ mice WBCs tended to be diminished (3.37 ± 0.33 m/mm$^3$ for WT vs. 2.42 ± 0.36 m/mm$^3$ for FXI$^{-/-}$, approaching significance), though granulocytes were increased in number (20.94% for WT vs. 29.08% for FXI$^{-/-}$). Some of these differences, such as WBC number (3.92 ± 0.51 m/mm$^3$ for WT vs. 3.20 ± 0.39 m/mm$^3$ for FXI$^{-/-}$, $p=0.29$) and hemoglobin concentration (11.40 ± 0.38 g/dL for WT vs. 10.92 ± 0.44 g/dL for FXI$^{-/-}$, $p=0.36$), are mitigated at late ages (9-12 months of age) (Table 1.3). However, RBC, hematocrit, MCV, RDW and granulocyte values maintain their initial tendencies. No significant changes were seen on MCV values, although they were always slightly diminished in FXI$^{-/-}$ mice. A second CBC analysis performed specifically for producing differential counts of WBCs was performed with another group of mice, revealing a similar increase in granulocytes but different WBC proportion for three month old mice (Table 1.4). Of particular interest was the increase in number of neutrophils (0.46 ± 0.03 K/μL for WT vs. 1.16 ± 0.16 K/μL for FXI$^{-/-}$, $p=6.7 \times 10^{-5}$). Blood chemistry analysis also exposed some of the differences between the two genotypes, showing increased lactate dehydrogenase (LDH) and decreased alkaline phosphatase (ALKP) values that reached significance in FXI$^{-/-}$ mice (Table 1.5). Values such as globulins (GLOB) showed a slight tendency for higher numbers in the plasma of FXI$^{-/-}$ mice ($p=0.5$), whereas albumin (ALB) tended to diminish almost reaching significance ($p=0.078$). Cholesterol (CHOL) and triglyceride (TRIG) values were no different between the two genotypes.
1.3.2.2 Vascular abnormalities

A series of abnormalities were found during the histological and ultrastructural examination of the main vessels of FXI<sup>−/−</sup> mice, including the carotid and aorta arteries. The first defects were observed in histological plastic sections (0.25µm thick) which showed a clear difference in the arterial wall thickness, and number and deepness of the typical undulations after fixation. Quantification of the wall thickness and the depth of the undulations in the intima of carotid arteries showed a clear reduction of these variables in FXI<sup>−/−</sup>mice (Table 1.6). These observations were confirmed by SEM analysis of longitudinal halves of aorta arteries (Fig.1.10). A generalized lack of the typical waves is seen throughout the aorta arteries. The SEM analysis also provided detailed information of the state of the endothelium. The main features seen on the intimal layer were marked by flattened ECs with atypical cell shape and loss of cellular folding edges and included the appearance of irregularities and openings (Fig.1.11). Cross-sections of carotid arteries were obtained for TEM analysis, revealing additional differences between the WT and FXI<sup>−/−</sup>mice (Fig.1.12). A partial detachment of ECs from their basal lamina was observed in numerous areas of the endothelium. These findings were not observed in WT controls that displayed a tight attachment of ECs to their basal membrane in all of the examined areas of the endothelium.
1.3.2.3 Vascular permeability

The evidence of vascular abnormalities in the arteries of the FXI−/− mice and low values of ALKP and ALB determined by the blood chemistry tests were strong indicators that the integrity of the endothelium was compromised. Those analyses were followed by assessment of vascular permeability through injections of Evans blue into the mice systemic circulation. These assays provide an indication of the extent of impact of these vascular irregularities in the vascular physiology of FXI−/− mice. After repeated external applications of mustard oil onto the facial and paw areas post Evans blue injections, a noticeably greater intra-dermal infiltration of the dye was observed for most of FXI−/− mice (Fig. 1.13). Although mice from both genotypes experienced the penetration of the dye into their skin, the greater intensity of the Evans blue in the challenged areas in FXI−/− mice was more obvious. This difference suggested a tendency for greater vascular permeability. Additionally, a test for detecting the presence of blood in the stool of these mice was developed in order to detect any potential gastrointestinal (GI) bleeding. The Hemoccult test revealed that for three month old mice, 38 out of 44 FXI−/− mice tested positive. Conversely, only 5 out of 30 of the WT mice tested positive, suggesting a higher occurrence of GI bleeding in the FXI−/− (χ² (1,N=74) = 35.57, p<0.01).

1.3.2.4 Total body weight measurements

Body weight measurements were collected at different ages according to mice genotype. FXI−/− mice were significantly heavier than WT mice after the third month of
age (t-test, $p \leq 0.05$), when mice reached adulthood (Fig.1.14). Mice necropsy revealed an accumulation of fat surrounding the peritoneal vascular system at six months of age and the entire peritoneal area at later time points (9-12 months). Even though fat accumulation was observed in the mentioned areas for both genotypes, it was more conspicuous in FXI$^{-/-}$ mice. Weight augmentation during aging also suggested the persistence of a compromised vascular permeability in the FXI$^{-/-}$ mice as a function of time. Monitoring of average daily food consumption for mice of each genotype did not show differences in food ingestion habits during their life span (Table 1.7). Even though a continuous decrease of food intake was seen as mice aged, the average amount of food consumed per mouse of both genotypes was equivalent. This suggests the increase in total body weight is attributable to other physiological causes.

### 1.3.2.5 Coagulation assays and coagulation factor levels

The lack of FXI in the deficient mice compromises the capacity of the endothelium to selectively permeate molecules in and out of circulation. A series of assays for key coagulation factors was performed to assess possible changes in normal levels of these proteins in plasma. Levels of thrombin were measured by two means: ELISAs of the thrombin/anti-thrombin III (TAT) complex, and a thrombin time assay (Table 1.8). Both tests suggested a tendency for lower thrombin values in the FXI$^{-/-}$ mice plasma. Although the differences between genotypes were not statistically significant, a tendency for lower thrombin levels opens the possibility for an elevated consumption of thrombin in the FXI$^{-/-}$ mice.
These assays were followed by determination of fibrinogen levels in plasma (Table 1.8). The measurements for plasma Fg levels showed the opposite tendency. Increased values in the FXI−/− mice (Table 1.8) were seen for every Fg test performed with two out of four tests reaching statistical significance. Because this test is based on coagulation times, those findings suggest a greater generation of fibrin in the FXI−/− mice plasma samples. A greater availability of fibrinogen or a greater concentration of FX/FXa, would explain the reduction of coagulation times in the plasma of FXI deficient mice. Both FX and FXa were found to be increased in the FXI−/− mice plasma samples, although statistical significance was only seen in the FXa activity assays (Table1.8 and Fig.1.15). FX is the serine protease responsible for thrombin activation and indirectly affects fibrin generation through activation of prothrombin. The FXa activity assays were also performed in the presence of a specific inhibitor, fondaparinux (IC50≈ 10nM for human FXa), at the concentrations of 4 and 20nM. The use of fondaparinux at these concentrations suggested that the activity seen in the WT and FXI−/− mice was FXa dependent and not due to the catalytic activity of other proteases present in the plasma samples.

Additional assays involved the determination of levels of FXII (Table 1.8) and Kallikrein (KK) activity (Fig.1.16), two contact system proteins. Also, total blood peroxidase activity was determined in resting conditions and after stimulation of whole blood with complement C5a protein (Fig.1.17). FXII and KK are proteins directly involved in the activation of the intrinsic pathway and are likely to be affected by the absence of FXI in the deficient mice. FXII relative plasma levels reveal a slight increase
of FXII in the plasma of FXI⁻/⁻ mice although the \( p \) value only approached significance (\( p=0.08 \)). On the other hand, measuring the peroxidase activity in whole blood before and after C5a stimulation can be used as an indicator of neutrophil myeloperoxidase activity in the blood. Neutrophil degranulation, the process that releases the enzyme content from the cytoplasmic granules into the extracellular space, is a factor that can contribute to the degradation of the basal lamina that supports the ECs. Even though both the peroxidase and KK levels in whole blood seem reduced for FXI⁻/⁻ mice, the differential increase in activity tends to be greater between the resting and treated states for the whole blood in FXI⁻/⁻ mice. These results suggest an overall greater degranulation of neutrophils upon C5a stimulation and a greater generation of KK upon activation by FXIIa. Such overall increase in degranulation occurs as a result of the greater number of neutrophils preexisting in FXI⁻/⁻ mice.

1.3.2.6 Hepatic inflammation and fatty lesions

Small clusters of inflammatory cells were commonly found in the liver of both WT and FXI⁻/⁻ mice during examination of H&E stained paraffin sections (Fig.1.18). However, a noticeable increase in size and number of these inflammatory foci was observed in FXI⁻/⁻ mice (Table 1.9). Characterization of these foci revealed the occurrence throughout the liver at random locations, in irregular shapes, and without a surrounding capsule. The foci predominantly consisted of lymphocytes and neutrophils, although occasional macrophages were also found (Fig.1.19 Left). In order to discard a fungal or bacterial origin of these foci, PAS and Brown and Hopps histochemical staining for the
cell wall of these microorganisms were separately performed. In both cases the results of these histological stains were negative when compared to positive controls of reference slides. A small proportion of FXI⁻/⁻ mice (1/10 of the offspring) developed a fatty liver like lesion that can affect extended areas of this organ (Fig.1.19 Right). Histological characterization of these fatty lesions exhibited advanced necrosis and cell degeneration.

1.3.3 Combined murine deficiency of FXI and FXII

We have described the phenotype of mice deficient in FXI, which is the zymogen protein responsible for the sequential activation of the intrinsic pathway of coagulation. Such activation has been reported to occur independently of FXII activation, the classical activator for this intrinsic pathway. By knocking out both of the proteins responsible for initiation and propagation of the pathway, we produced a model for studying the role of the initial part of this pathway in coagulation and other systems. An initial phenotypical description of these double deficient mice revealed irregular CBCs, exacerbated hemosiderin deposition in the spleen, extended aPTTs, and weight differences when compared to the individual deficiencies. As mentioned before, FXII⁻/⁻ mice do not display any observable abnormality when compared to WT controls other than a smaller body size and weight. FXII⁻/⁻ mice have the tendency to be smaller in size, a characteristic not seen in the double deficient mice and most likely compensated by the combination of both deficiencies (Fig.1.23). However, necropsy of 3 month-old double deficient mice suggested that a significant part of that weight compensation may be a consequence of abdominal fat accumulation.
A noticeable increase in numbers of WBCs, particularly of granulocytes, were seen in the FXI\(^{-/-}\)/FXII\(^{-/-}\) mice when compared to FXII\(^{-/-}\) and WT controls (Table 1.10). These discrepancies suggest the involvement of these factors in the regulation of cell genesis from the bone marrow or perhaps clearance of aged cells from the circulation by organs like the spleen. Although there was an increased number of RBCs for the double deficient mice (8.66 ± 0.11 M/μL for FXI\(^{-/-}\)/FXII\(^{-/-}\) vs. 8.39 ± 0.08 M/μL for WT, \(p=0.27\)), the differences did not reach statistical significance. In contrast with FXI\(^{-/-}\) mice, the FXI\(^{-/-}\)/FXII\(^{-/-}\) mice did not display any observable abnormalities in their RBCs (Fig.1.20). An irregular deposition of hemosiderin in the spleens of FXI\(^{-/-}\)/FXII\(^{-/-}\) mice was found after histological examination of paraffin sections when compared to WT, FXI\(^{-/-}\) or FXII\(^{-/-}\) controls (Fig.1.21).

The impact of this double deficiency on coagulation was tested by aPTT assays (Fig.1.22). The combination of both FXI and FXII deficiencies seem to lengthen the amount of time necessary for fibrin generation in comparison to either of the individual deficiencies. These results suggest that the double deficient mice exhibit a more severe, but not necessarily additive, coagulation phenotype than the individual deficiencies. Furthermore, the results suggest that FXII deficiency may play a more important role in the regulation of the coagulation cascade than what originally thought and it is only revealed when FXI is totally absent. A characterization of the levels of proteins from the extrinsic pathway may be necessary to complement the certainty of such statements.
1.4 Discussion

1.4.1 Spontaneous phenotype in FVII\textsuperscript{TA/TA} mice

Obvious effects of low levels of FVII on the behavior of ECs were seen through cardiac angiogenesis. This phenomenon caused uncontrolled internal bleeding that resulted in inflammation and later ventricular fibrosis. Although the intracellular mechanism involved in the cardiopathology described in FVII\textsuperscript{TA/TA} mice was not elucidated, the events involved in such mechanism may involve a loss of ECs attachment to the ECM and cell-to-cell contact in order for the ECs to migrate from their residing sites. We demonstrated that the ventricular bleeding in FVII\textsuperscript{TA/TA} mice is the result of a prominent angiogenic process. This angiogenesis-derived bleeding results from the formation of neovessels rather than an unbalanced haemostasis. However, the limited capacity for thrombin/fibrin production may be an important contributing factor in facilitating the pass of migrating ECs through cardiac tissue. Fibrin is an important component of the ECM, particularly during inflammatory processes, and when deposited in the tissue can difficult the migration of EC by forming a barrier or slowing cell movement.

Measurements of the expression levels of TGFβ, TNFα and several MMPs in hearts samples at three weeks and four months of age were used as indicators of inflammation and tissue remodeling. These analyses suggested that bleeding precedes the fibrotic events, as shown by a significant increase of these indicators at four months but
not at three weeks of age (6). Inflammatory mediators serve as stimuli for ECs and inflammatory cells to migrate towards target areas. The angiogenesis-driven bleeding described here differs from the bleeding described in embryonic and neonatal fatalities of TF and FVII total deficient mice. Nonetheless, there is a clear dependence on TF/FVIIa complex formation in both total deficient and low FVII expressing mice for endothelial integrity and EC normal behavior. The limited availability of FVII triggers the transformation of ECs from a resting membrane-attached state to a migratory and angiogenic state.

We speculate that the mechanism for this transformation of ECs might involve the activation of the cytoplasmic domain of TF and/or the participation of limited amounts of TF/FVIIa/FXa complex with PAR receptors on the surface of EC. Such mechanisms could facilitate the maintenance of cell-to-cell and/or cell to ECM attachments more stable for ECs facilitating residence and contraresting migration under normal conditions. However, under low availability of FVII, conditions seen to be different. In tumors, activation of TF/FVIIa/PAR-2 signal promotes proliferation and metastasis (17). Whereas, in primary cell cultures of SMCs, FVIIa promotes expression of cytokines such as, IL-8 and IL-6 in a TF and PAR-2 expression-dependent manner (18). Such reports reveal the importance of the level of expression of the TF/FVIIa complex on tumor and vascular cells behavior, and even though none of those studies were performed under low expression levels of FVII, we can not dismiss the hypothesis that a similar mechanism may be acting to exert drastic changes in ECs phenotype. Additionally, detailed research on the intracellular events involved with the ECs of these low FVII expressing mice is
necessary to evaluate the observed phenomena. Together with studies directed to the effects of different levels of FVII in the development of cardiac fibrosis, elucidation of the critical signals that inducethose changes in EC behavior may be possible.

1.4.2 Spontaneous phenotype in FXI<sup>-/-</sup> mice and accumulative effects of the combined deficiency of FXI/FXII in mice.

The phenotypical findings seen in FXI<sup>-/-</sup> mice when compared to the WT controls are indicative of several features. Some features are signs of a compensatory mechanism generated by the coagulation system, and other features indicate hepatic inflammation and abnormal vascular structure. Compensation through coagulation comes from increased FX/FXa levels and shorten ed Fg coagulation times. The coagulation system of FXI<sup>-/-</sup> mice appears to use the common pathway to regulate, to a certain extent, the lack of the thrombin-generating boost derived from the activation of the intrinsic pathway. Evidence of this compensation is the increased levels of FX/FXa and Fg seen in *in vitro* tests.

The increased inflammation found in the liver may well be a consequence of vascular abnormalities accentuated by predisposition to inflammation in these deficient mice. Inflammatory foci in the liver is a sign of greater tissue damage via inflammation in FXI<sup>-/-</sup> mice as indicated by the leukocyte cell counts and higher LDH levels in their plasma. We believe that the fatty liver lesions observed in 1/10 of the FXI<sup>-/-</sup> mice could
be a consequence of cellular debris accumulated over time through cell damage and ultimately tissue necrosis, as seen in histological sections of the affected livers.

We also believe that the abnormal vascular structure observed in FXI<sup>−/−</sup> mice leads to several consequences involving diverse blood components. The lower RBC count, lower Hb and htc and ALKP values, as well as the presence of microcytic and hypochromatic RBCs in the blood smears of FXI<sup>−/−</sup> mice are strong signs for microcytic anemia. Moreover, the vascular abnormalities, the tendency for lower albumin levels, greater numbers of GI bleeding positive mice, and greater Evans blue permeability suggest an increased vascular permeability in the FXI<sup>−/−</sup> mice. The occurrence of all the signs for microcytic anemia and vascular permeability were examined by two independent animal pathologists, Dr. Mark A. Suckow and Dr. Harm HugenEsch, who corroborated these observations. Quantification of Evans blue permeability remains to be done in order to generate comparative data. A method for generating data based in a fluorescence scale after subjecting the mice to Evans blue injections has been tested with a promising outcome. The method filters the wavelength transpassing the mice so the desired light from the spectrum excites the Evans blue molecules. Examination of experimental subjects under these conditions will validate the differences observed in the previous assays.

The irregular endothelium exhibited by FXI<sup>−/−</sup> mice clearly compromises the capacity for this arterial layer to act as a semipermeable barrier to the blood content. This results in loss of RBCs and plasma proteins such as serum albumin. Serum albumin is a regulator of oncotic pressure, a carrier for minerals and trace elements, and it is important
in the balance of capillary pressure and fluid exchange. A decrement of 0.15 g/dL of ALB was observed in FXI−/− compared to WT mice, which approached statistical significance (p=0.078). The consequences of the loss of proper endothelial permeability in FXI−/− mice lead to the occurrence of microcytic anemia, the average weight increase after the third month of age and the lower ALKP levels found in plasma. Although the source of ALKP varies depending on the pathological state of the animal, low level of this enzyme in plasma is a well known sign of malnutrition. An increased vascular permeability and continued loss of cells and essential nutrients may cause low ALKP levels. The weight increase observed in the FXI−/− mice did not seem to be dependent on differences in fat metabolism, as indicated by normal levels of CHOL and TRIG (Table 1.5), but rather a long term response to unregulated fat molecule traffic through the vascular endothelium. The additional differences found in the levels of GLOB and Ca^{2+} in plasma were not significant enough to be considered important indicators of any pathology.

Capillaries in the liver may be particularly susceptible to vascular defects due to the high permeability rate they experience. Fenestrations are common along small hepatic vessels making the hepatic vasculature overexposed to any inflammatory agents present in the local or systemic circulation. Other areas of high permeability, such as the GI tract, are also susceptible to vasculature disruption and increased permeability. FXI−/− mice are also prone to greater GI bleeding than control mice as corroborated within their stool samples. This bleeding is indicative of a major irregular permeability in the GI tract considering that blood cells are lost through this tissue. An alternative way to test for
vascular permeability in the GI tract could involve the ingestion of small concentrations of Evans blue in the water supply for these mice for small periods of time (e.i. 24 h). A whole body evaluation of the fluorescence that is emitted using the previously described technique could elucidate differences without the need of promoting local inflammation in these mice. Such a technique can be extended to particular organs. Lastly, an additional way to test directly differences in the permeability of these mice, vascular endothelium can be performed by adapting the Ussing chamber for the evaluation of molecular trafficking from the luminal side to the interior of the arteries. An adaptation remains to be used for this kind of tissue.

FXII activation is required for the generation of KK from its precursor prekallikrein (PK). Once KK is generated, a catalytic positive feedback between FXIIa and KK takes place due to their capacity for mutual activation. The increased level of FXII seen in FXI<sup>-/-</sup> mice may translate into a greater activation of PK via FXIIa, and therefore, more KK can be generated. Additionally, the absence of FXI from plasma will disturb the 7:1 proportion existing between PK and FXI in plasma. This allows for HK to complex more PK favoring additional availability of KK in plasma upon PK activation. We suspect that such conditions may be part of the key factors responsible for the phenotypical changes seen in FXI<sup>-/-</sup> mice. Generation of BK due to processing of HK will allow for the interaction of this peptide with their constitutive β2 and, if present, also inducible β1 receptors on ECs. Both β1 and β2 are G protein-coupled receptors and will affect ECs behavior after activation. The β2 receptor couples to both G<sub>q</sub> and G<sub>i</sub> therefore activating phosphorylation through PKC increasing [Ca<sup>2+</sup>] in the cell and inhibiting
adenylate cyclase. Additionally, this receptor can stimulate the MAPK pathways, inducing release of arachidonic acid and activating eNOS (19,20).

The activation of the β1 receptor follows the same pathways mentioned for the β2 receptor, however, they differ in stimulation patterns. The β1 receptor is highly dependent on extracellular Ca\(^{2+}\) influx than intracellular free Ca\(^{2+}\) like in the β2 case (20). This difference translates into different regulation times for activation, desensitization and/or receptor internalization (20). The vascular effects of these receptors’ activation, by agonists like BK include SMCs relaxation through the effects of NO that diffuses from the endothelium to the underlying muscle layers, and prostacyclin that potentiates vasorelaxation in SMCs through cAMP production (21). This may explain the overall reduction of arterial undulations and greater lumenal area seen in plastic sections of carotid and aorta arteries of FXI\(^{-/-}\) mice.

The effect of BK and any other synergistic molecules coming from inflammatory cells such as neutrophils, seen in the liver, will induce vascular permeability. Assesment of the expression levels of β1 and β2 receptors on the endothelium of WT and FXI\(^{-/-}\) mice will elucidate the connection between the observed phenotype and the participation of BK and other stimulatory molecules affecting vasodilatation and vascular permeability. Measuring mRNA expression levels for such proteins in the vascular tree together with immunohistochemistry labeling of arteries with specific antibodies for such receptors are two ways to assay that task. Injection of mice with specific fluorescent antibodies directed to endothelial proteins prior to dissection, fixation and observation under the confocal microscope has been proven to be an effective method for detecting the
presence of endothelial proteins. Directed blockage of β2 receptors with specific inhibitors such as HOE140 will minimize the activation of intracellular event that lead to increased vascular permeability. This synthetic peptide has a long term effect and a half life of more than two weeks in the mice systemic circulation. Serial injections of such a peptide could translate into a long term inhibition of such receptors, perhaps enough to evaluate any changes in vascular permeability. This approach provides an opportunity to test the effects of a molecule more stable than FXI in the circulation.

Substances like reactive oxygen species (ROS), heparin-binding protein (HBP) and leukotrienes produced by neutrophils will induce an increase of intracellular Ca\(^{2+}\) through G protein-couple receptors on the EC membrane. These interactions will activate myosin light chain kinases and myosin phosphorylation, inducing EC contraction and junctional disruption (22,23). Similar to the activation of β2 receptors, Ca\(^{2+}\)-dependent PKC activation also activates RhoA and its effector Rho kinase, inducing stress fiber formation through actin polymerization (24). The accumulated effects are EC contraction and intercellular junction disruption, that leads to an increase in vascular permeability. A first approach to evaluation of the state of cell junctions was assayed in WT and FXI\(^{-/-}\) mice. Quantification of ZO-1, an essential protein in tight cell junctions, by immunohistochemistry was performed in intestinal samples. The GI tract of FXI\(^{-/-}\) mice exhibit exacerbated vascular permeability, so assessing the state of intestinal and vascular tight junctions is a valid approach to evaluate their integrity. The preliminary data showed a great variability in the staining with zones of high intensity intermixed with low or negative stained areas. This approach has been proven to be very inconsistent to be
considered reliable. Perhaps a similar approach using arterial vessels would be more reliable considering that ECs from this tissue were already examined and found to be affected both morphologically and functionally. The inclusion of the staining and measurements of mRNA expression level of other essential proteins participating in the structure and physiology of tight junctions, such as, occluding and E-cadherin, will complement greatly such assessments.

Endothelial abnormalities in FXI/− mice were marked by the appearance of irregularities, gaps and even disruption of ECs as well as basal lamina disintegration in some areas as evidenced by electron microscopy. Such abnormalities are signs of a modified vascular structure and are most likely a consequence of not only the effects of BK and synergistic molecules affecting the ECs behavior, but also the action of erosive external factors that can cause cell disruption and sub-endothelial damage. Because of the involvement of neutrophils in hepatic inflammatory foci, and the greater number of these leukocytes seen in FXI/− mice we believe that this type of inflammatory cell are direct actors in causing the abnormal vasculature. The link between the contact system and neutrophils occurs through binding of HK to its receptors on the neutrophil membrane. Such binding occurs only complex to PK (since FXI is absent) which favors the generation of KK. Consequently this unbalance of contact system proteins could lead to more neutrophil degranulation. Degranulation releases oxidative enzymes, proteases and other ROS that induces extracellular matrix degradation of the basal lamina as well as permeability of the endothelium.
The tendency to exhibit more inflammation in the liver of FXIₜ⁻ mice is an indicator of a greater susceptibility to such reactions. There are no indications in these inflammatory foci that signal a limitation of such inflammation only to the hepatic tissue (i.e. encapsulation of the foci). Furthermore, the appearance of numerous lymphocytes in these foci is an indicator that chronic or prolonged inflammatory reactions are taking place in such areas of the liver. An increase in peroxidase activity was also seen in whole blood preparations after C5a incubation in these mice. This tendency favors the assumption that inflammation in these mice is not restricted to the liver. Complement C5a is a known agent for induction of neutrophils, chemotaxis and activation (25,26). Since myeloperoxidase is one of the lysosomal enzymes from the azurophilic granules of neutrophils, its activity can be used as a direct indicator of neutrophil degranulation. Together with the tendency for greater plasma KK activity, augmentation of peroxidase activity was a sign of increased neutrophil activity in FXIₜ⁻ mice. Such increased peroxidase activity may be due to either a greater sensitivity to complement C5a in neutrophils from FXIₜ⁻ mice or the greater number of neutrophils found in these mice. However, further experimentation is required to confirm those assumptions.

The overall phenotypical findings on the FXIₜ⁻ mice point at a predisposition to inflammation accentuated in the vasculature and hepatic tissues. Such predisposition most likely occurs through neutrophil activation and may be originated from unbalanced concentrations contact system proteins. A critical step of these reactions is the activation of FXII and the loop reaction FXII-PK that greatly accelerates the generation of both KK and FXIIa. In our whole blood model, activation of PK through addition of exogenous
FXIIa is a determining factor in the increased KK generation seen in FXI<sup>+</sup> mice. A greater availability of KK in the plasma can be directly related to the vascular phenotype observed in the FXI<sup>−/−</sup> mice through the effects of activated High Molecular Weight Kininogen (HKa) and bradykinin (BK) on EC physiology and behavior. Both HKa and BK are KK generation-dependent molecules that result from the processing of HK.

In other experimental settings, neutrophil aggregation has been proven in a KK dependent fashion in the presence of Ca<sup>2+</sup> and a concomitant respiratory burst (27). KK also promotes secretion of azurophilic granules during coagulation (28). Moreover, such neutrophil degranulation is highly reduced (1/2 of normal levels) if neutrophils are resuspended in PK or FXII-deficient plasma (28). These results show the relevance of PK and FXII on neutrophil degranulation. Evaluation of neutrophil degranulation from isolated neutrophils resuspended in WT or FXI<sup>−/−</sup> mice plasma would corroborate the higher tendency for degranulation upon stimulation seen in whole blood experiments. Moreover, comparative measurements of the levels of C1INH in plasma, an important KK and FXIIa plasma inhibitor, could reveal if an increased generation of KK and/or FXIIa is being antagonized in FXI<sup>−/−</sup> mice mice. C1INH is a serpin synthetized in the liver, a tissue subjected to increased inflammation in FXI<sup>−/−</sup> mice, usually in response to inflammatory cytokines. Additionally, KK-specific inhibitors such as DX-88 could be used over time to evaluate the reversion of any symptom such as increased vascular permeability or abnormal CBC in FXI<sup>−/−</sup> mice. DX-88 or Ecallantide is a very specific KK inhibitor (Ki= 44pM) based on the first Kunitz domain of TFPI. The use of DX-88 will discern between potential effects of KK from other factors that could be contributing to
the phenotype of FXI<sup>−/−</sup> mice. In the next chapter, a description of a copper-induced arterial challenge performed in WT and FXI<sup>−/−</sup> mice is presented. The results from those experiments helped support the statements regarding predisposition to inflammation in a murine FXI deficiency and a greater involvement of neutrophils, PK and FXII in such reactions.

1.4.3 Combined murine deficiency of FXI and FXII

The extension of aPTT times seen in the double deficient mice is more evident than the delays seen for either of the individual deficiencies. This observation suggests a greater impact of FXII in the generation of thrombin than suspected. An incremental effect of the individual deficiencies is seen with the combination of these deficiencies. Most of the evidence suggesting that FXII is available for activation of the intrinsic pathway comes from measurements of both human and murine deficiencies of this factor. The evidence for these results rests on the fact that both PK and FXI can be activated in the absence of FXII (29). The interpretation of these results depicts FXII as a non essential factor for the maintenance of normal haemostasis, however in most of those experimental settings, FXI has also been present (30,31,32). The contribution of FXI to the generation of thrombin has always been accounted for in those types of experiments.

By removing both of the genes responsible for triggering the initiation of the intrinsic pathway, we eliminate the capacity for activating FIX through this pathway and generating KK though FXIIa. These new results show that the elimination of any
functional FXI and FXII from the circulation impacts not only the time required for thrombin to be generated through the intrinsic pathway, but also spleen physiology and blood cell numbers. Further characterization of these mice under resting and challenging conditions remains to be done.

Additional roles for these factors may involve the regulation of blood cell numbers, in particular granulocytes, in the circulation. Potential implications on granulocyte genesis by regulation of the precursor cell lineage of these cells in the bone marrow may be some of the unknown functions for these two contact system proteins. Such leukocytosis was very obvious and the neutrophilia observed surpassed the WT controls in numbers by 2.8 fold. A change of this magnitude will most likely impact the way these double deficient mice will react to any inflammatory challenge.

1.5 General discussion

We have learned that non-lethal genetic deficiencies of coagulation factors in mice are useful for studying how these proteins help maintain haemostasis and other functions. These murine models are great tools for observing gradual phenotypic changes that would not be evident in the absence of challenging conditions. Evidence continues to indicate that coagulation factors play roles in processes beyond coagulation. In our study, murine FVII, FXI and combined FXI/FXII deficiencies had several effects ranging from compromised haemostasis and vascular physiology to cardiac fibrosis.
A partial deficiency on FVII revealed a regulatory role for the factor and/or the TF/VIIa complex on EC behavior and physiology. The low availability of FVII in FVII$^{TA/TA}$ mice drives the occurrence of an unexpected angiogenesis in the left cardiac ventricle. This observation shows once more the importance of FVII not only for mice survival and haemostasis but for normal cardiac function.

Characterization of the spontaneous phenotype seen in FXI$^{-/-}$ mice uncovered the impact of this deficiency on vascular physiology and permeability and its implications in inflammation. This characterization also revealed a potential regulatory role for this protein in maintaining normal levels of granulocytes in blood. Generation of mice with a double deficiency in FXI and FXII also supported its implications in the regulation of granulocyte numbers. Additionally, we also provide a model for studying coagulation without the main proteins responsible for the activation of the intrinsic pathway.
Fig. 1.5) Toidine blue stain of plastic sections showing cardiomyocytes from left cardiac ventricule at six weeks of age. A.- Cross-section of a normal cardiomyocytes in WT mice. Two small vessels (arrows). B.- Cross-section of a fibrotic area in FVII<sup>TA/TA</sup> mice cardiomyocytes. Notice the presence of numerous microvessels throughout the tissue (arrows) and a prominent collagenous matrix in the tissue parenchyma (*). Mag. bar 25µm. Mag. in A and B, 40X.
Fig1.6) Toluidine blue stained plastic sections of FVII$^{TA/TA}$ mice cardiac tissue. A.- Panoramic view of a fibrotic area in left cardiac ventricule at six weeks of age. Observe the cardiomyocytes undergoing degeneration (arrows). Also a former muscle-rich contractile area appears replaced by fibrotic tissue (*). Mag. bar 100µm. Mag.10X. B.- Extravascular bleeding in left cardiac ventricule at four months of age. Observe the abundant extravascular RBCs (re) seen in between cardiomyocytes (Cm). Also, infiltrated macrophages (m) appear displaying numerous fat vacuoles in their cytoplasm product of cell debris clearance (arrows). Mag. bar 10µm. Mag.100X.
Fig.1.7) Electron micrographs of left ventricle cardiomyocytes at four months of age. A.- Some extravascular RBCs (re) appear inside and outside a cardiomyocyte (Cm) in FVII_{TA/TA} mice left cardiac ventricle. A recently formed vessel (rv) is seen at the left of the cardiomyocytes as well as migrant ECs with long cellular processes (arrow). Mag. bar 10 μm. Mag. 3000X. B.- A functional blood vessel (bv) containing an RBC is seen in the interstitial space (I) between cardiomyocytes of the ventricular parenchyma of WT mice. Mag. bar 10 μm. Mag. 35000X.
Fig. 1.8) Low and high magnification images of two regions of a fibrotic area found in the left ventricule of a FVII\textsuperscript{TtA/TtA} four months old mouse. A.- Toluidine blue stain of plastic section showing local fibrosis with hemorrhage (re) in close proximity to macrophages containing numerous fatty vacuoles (m). The entire area is enriched in a collagen matrix (cg). Mag. bar 10 µm. Mag. 100X. B.- Electron micrographic detail of a macrophage from the same area. Notice the engulfed RBCs (e) containing iron deposits (dark black material) and a nascent siderosome made of erythrocyte membranes (arrow). Also observe the abundance of collagen in the matrix surrounding the macrophage (cg). Mag. bar 4 µm. Mag. 3500X. C.- Electron micrograph of advanced fibrosis. Observe the common presence of fibroblast (f) and macrophages with cytoplasmic vacuoles (m) in this fibrotic area. Also, observe the abundant collagenous matrix (cg) delimitated by cardiomyocytes (Cm). Mag. bar 10 µm. Mag. 2500X
Fig. 1.9) Wright-Giemsa stained blood smears from WT and FXI⁻/⁻ six-month-old males. A.- Normal blood from a WT mouse. B.- Blood from a FXI⁻/⁻ mouse. Notice the appearance of small, and hypochromatic RBCs, the elliptic shape of some of the RBCs and the greater central pallor. Mag. bar 10 µm. Mag. 100X for A and B.
Fig. 1.10) SEM micrographs showing a lumenal view of half sectioned murine aorta. A.- Aorta of WT mice. Observe the normal emergence of the numerous undulations that follow the blood flow. Few flat areas can be seen. B.- Aorta of FXI−/− mice. Notice the overall flat appearance of the lumen surface and the occurrence of few undulated areas. Mag. in A and B, 50X.
Fig. 1.11) SEM of aorta lumen showing smooth and undulated areas. A.- Aorta of WT mice. ECs appear with well defined oval shapes and typical dotted and protruding folding ends (arrows) indicating their cell borders. B.- Aorta of FXI\(^/-\) mice. Note the abnormally enlarged shape of ECs and the poor prominence of their folding ends. Also, observe the abundant stress fibers aligned in the direction of the blood flow (arrows). A prominent number of irregularities can be seen as well (*). Mag. 500X. Detail: irregularities of the endothelium. Observe the prominent opening of the endothelial surface (arrowhead) and the thin folding end of most EC (arrow). Mag. 5000X.
Fig. 1.12) TEM micrographs of FXI−/− and WT carotid arteries. A.- Spontaneous damage found in the endothelium of FXI−/− mice carotid arteries. Observe the detachment of the endothelial cell from its basal lamina (arrows). B.- WT carotid arteries. Notice the integrity of the EC and the close contact between the basal lamina and the elastic lamina (EL). Mag. bar 2µm. Mag. for A and B 5000X.
Fig. 1.13) Vascular permeability assay. A. Facial area and B. Fore paws of FXI<sup>−/−</sup> (left) and WT (right) mice after Evans blue injection and repeated local mustard oil application. Notice the more intense penetration of the dye into the skin of the FXI<sup>−/−</sup>mice. Mag. 6X.
Fig.1.14) Genotype independent weight measurements of mice at 3, 6, 8 and 11 month of age. Note the increments in weight for FXI−/− mice after the 3 month time point (p≤0.05 * for statistical significance).
Fig. 1.15) Measurements of FXa activity in WT and FXI<sup>−/−</sup> mice plasma. Observe the differences in activity between genotypes in the presence of 4nM and 20nM concentration of the FXa specific inhibitor fondaparinux. (n=8 per group of mice or treatment, <i>p</i>≤0.05 * for statistical significance).
Fig. 1.16) Measurements of KK activity in WT and FXI<sup>−/−</sup> mice whole blood after activation with FXIIa. Notice the greater differential increase in KK activity for the FXI<sup>−/−</sup> mice group when compared to the WT control. Statistical differences were found between the WT and FXI<sup>−/−</sup> mice groups before and after activation (*p = 0.05).
Fig.1.17) Measurements of total peroxidase activity in WT and FXI<sup>−/−</sup> mice whole blood in the presence of 0.2 μg/mL of C5a. Notice the differential in relative activity between the FXI<sup>−/−</sup> and the WT group before and after incubation of whole blood with complement C5a. (n=11 per group of mice or treatment, *p<0.05 for statistical significance).
Fig. 1.18) Inflammatory foci found in hepatic tissue of WT and FXI<sup>−/−</sup> mice. A.- Liver section of 3 month-old WT mouse. Notice the occurrence of a small inflammatory focus and the hyperplastic state of the hepatocytes surrounding it (arrow). B.- Liver section of 3 month-old FXI<sup>−/−</sup> mouse. Note the relative abundance of inflammatory clustered cells when compared to the WT mice. Mag. bar 100µm. Mag. 20X.
Fig.1.19) Left. H & E stain of 3 month-old FXI⁻/⁻ mouse liver. Detail of an inflammatory focus. The majority of cells forming these foci are lymphocytes (arrows), though neutrophils are also frequently seen (arrowhead). Mag. bar 20 µm. Mag. 100 X. Right. Panoramic view of a fatty liver lesion in a FXI⁻/⁻ mouse (arrow). Mag. 10 X.
Fig.1.20) Wright-Giemsa stained blood smears from 3 month old FXII$^{-/-}$ and FXI$^{-/-}$/FXII$^{-/-}$ mice. Observe the similar shape and central parlor of the RBCs. Mag.bar 10 µm. Mag. 100 X.
Fig.1.21) H&E stain sections of spleen. A.- WT mice spleen. Observe the well defined red (top right area) and white pulp (left bottom) areas. B.- FXI<sup>−/−</sup> mice spleen. Notice the lack of definition of the white pulp area as well as the localization of RBCs (intense red cells) in the red pulp. C.- FXII<sup>−/−</sup> mice spleen. Observe the clear separation between the white and the red pulp. D.- FXI<sup>−/−</sup>/FXII<sup>−/−</sup> mice spleen. Notice the abnormal enrichment in RBCs in the red pulp and the hemosiderin (brown-yellow precipitates) frequently seen in the white pulp. Mag. bar 100 µm. Mag. 20 X.
Fig. 1.22) Coagulation aPTT assay for plasma samples of WT, FXI⁻/⁻, FXII⁻/⁻, and FXI⁻/⁻/FXII⁻/⁻ mice. Observe the slight increment of time registered for the double deficient mice plasma samples. Significant differences were found between the FXI⁻/⁻ and the FXI⁻/⁻/FXII⁻/⁻ mice ($p=0.01$), and the WT and all the groups ($p \leq 2.5 \times 10^{-6}$). (n≥12 for all groups).
Fig. 1.23) Total weight measurements for WT, FXI<sup>−/−</sup>, FXII<sup>−/−</sup>, and FXI<sup>−/−</sup>/FXII<sup>−/−</sup> mice. Notice the weight compensation seen when the two deficiencies of FXI and FXII combine compared with the weight exhibited by FXII<sup>−/−</sup> mice which exhibited statistical differences (*p<0.05). (n≥19 for all the groups).
**TABLE 1.3**

GENOTYPE SPECIFIC COMPLETE BLOOD COUNTS OF WT AND FXI<sup>–/–</sup> MICE AT 3 AND 9-12 MONTHS OF AGE.

<table>
<thead>
<tr>
<th>Variable</th>
<th>3 Months</th>
<th></th>
<th>p value</th>
<th>9-12 Months</th>
<th></th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>RBC (M/mm³)</td>
<td>WT</td>
<td>8.74 ± 0.21</td>
<td>7.1 ± 0.31</td>
<td>0.0003*</td>
<td>FXI&lt;sup&gt;–/–&lt;/sup&gt;</td>
<td>7.84 ± 0.89</td>
</tr>
<tr>
<td>[Hb]g/dL</td>
<td>WT</td>
<td>13.31 ± 0.13</td>
<td>12.16 ± 0.2</td>
<td>0.007*</td>
<td>FXI&lt;sup&gt;–/–&lt;/sup&gt;</td>
<td>11.4 ± 0.38</td>
</tr>
<tr>
<td>Htc</td>
<td>WT</td>
<td>46.25%</td>
<td>35.97%</td>
<td>0.332</td>
<td>FXI&lt;sup&gt;–/–&lt;/sup&gt;</td>
<td>41.56%</td>
</tr>
<tr>
<td>MCV fl</td>
<td>WT</td>
<td>52.62 ± 0.88</td>
<td>51.66 ± 0.49</td>
<td>0.332</td>
<td>FXI&lt;sup&gt;–/–&lt;/sup&gt;</td>
<td>52.02 ± 0.65</td>
</tr>
<tr>
<td>RDW</td>
<td>WT</td>
<td>9.63 ± 0.15</td>
<td>8.14 ± 0.34</td>
<td>0.0005*</td>
<td>FXI&lt;sup&gt;–/–&lt;/sup&gt;</td>
<td>9.44 ± 0.24</td>
</tr>
<tr>
<td>WBC m/mm³</td>
<td>WT</td>
<td>3.37 ± 0.33</td>
<td>2.42 ± 0.36</td>
<td>0.06</td>
<td>FXI&lt;sup&gt;–/–&lt;/sup&gt;</td>
<td>3.92 ± 0.51</td>
</tr>
<tr>
<td>Granulocytes</td>
<td>WT</td>
<td>20.94%</td>
<td>29.08%</td>
<td>32.93%</td>
<td>FXI&lt;sup&gt;–/–&lt;/sup&gt;</td>
<td>45.80%</td>
</tr>
<tr>
<td>Platelets(K/mm³)</td>
<td>WT</td>
<td>913 ± 39</td>
<td>908 ± 51</td>
<td>0.942</td>
<td>FXI&lt;sup&gt;–/–&lt;/sup&gt;</td>
<td>NA</td>
</tr>
</tbody>
</table>

NOTE: NA = no data is available for these ages.

*p<0.05 (*)&.
### Table 1.4

**DIFFERENTIAL CBC OF 3 MONTHS OLD WT AND FXI⁻/⁻ MALE MICE**

<table>
<thead>
<tr>
<th>Genotype</th>
<th>wbc (K/μL)</th>
<th>ne (K/μL)</th>
<th>ly (K/μL)</th>
<th>mo (K/μL)</th>
<th>eo (K/μL)</th>
<th>ba (K/μL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>3.88 ± 0.20</td>
<td>0.46 ± 0.03</td>
<td>3.18 ± 0.18</td>
<td>0.23 ± 0.01</td>
<td>0.01 ± 0.0</td>
<td>0.01 ± 0.0</td>
</tr>
<tr>
<td>FXI⁻/⁻</td>
<td>4.22 ± 0.21</td>
<td>1.16 ± 0.16</td>
<td>2.75 ± 0.21</td>
<td>0.29 ± 0.03</td>
<td>0.01 ± 0.0</td>
<td>0.01 ± 0.0</td>
</tr>
<tr>
<td>t-test (p value)</td>
<td>0.246</td>
<td>6.7 x 10⁻⁵ *</td>
<td>0.128</td>
<td>0.07</td>
<td>0.5</td>
<td>0.5</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Genotype</th>
<th>ne (%)</th>
<th>ly (%)</th>
<th>mo (%)</th>
<th>eo (%)</th>
<th>ba (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>12.08</td>
<td>81.58</td>
<td>6.01</td>
<td>0.26</td>
<td>0.06</td>
</tr>
<tr>
<td>FXI⁻/⁻</td>
<td>27.87</td>
<td>64.90</td>
<td>6.82</td>
<td>0.33</td>
<td>0.06</td>
</tr>
</tbody>
</table>

**NOTE:** n= 21 for each group.

wbc: white bloodcells; ne: neutrophils; ly:lymphocytes; mo: monocytes; eo: eosinophils; ba: basophils; Plt: platelets.
# TABLE 1.5

## BLOOD CHEMISTRY ANALYSIS OF WT AND FXI<sup>−/−</sup> MICE

### AT 3 MONTHS OF AGE

<table>
<thead>
<tr>
<th>Values</th>
<th>WT</th>
<th>FXI</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>ALKP (U/L)</td>
<td>65.33± 2.08</td>
<td>59.27± 2.36</td>
<td>0.044*</td>
</tr>
<tr>
<td>GLOB (g/dL)</td>
<td>3.15 ± 0.09</td>
<td>3.23± 0.08</td>
<td>0.506</td>
</tr>
<tr>
<td>LDH (U/L)</td>
<td>451.92± 50.55</td>
<td>641.80± 72.68</td>
<td>0.032*</td>
</tr>
<tr>
<td>ALB (g/dL)</td>
<td>1.50± 0.06</td>
<td>1.35± 0.05</td>
<td>0.078</td>
</tr>
<tr>
<td>Ca&lt;sup&gt;2+&lt;/sup&gt; (mg/dL)</td>
<td>8.86± 0.56</td>
<td>8.48± 0.17</td>
<td>0.090</td>
</tr>
<tr>
<td>CHOL mg/dL</td>
<td>93.19± 6.48</td>
<td>96.57± 7.59</td>
<td>0.784</td>
</tr>
<tr>
<td>TRIG mg/dL</td>
<td>150.98± 15.23</td>
<td>181.03± 16.22</td>
<td>0.293</td>
</tr>
</tbody>
</table>

**NOTE:** n=10 for each group.

Statistical differences were considered at p≤ 0.05.

ALKP: alkaline phosphatase, GLOB: globulins, LDH: lactate dehydrogenase, ALB: albumin, Ca<sup>2+</sup>: free calcium, CHOL: cholesterol, TRIG: triglycerides. (n≥10 for each group).
MORPHOMETRIC MEASUREMENT OF CAROTID ARTERIES CROSS-SECTIONS

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Wall thickness (µm²)</th>
<th>Deepness of undulations (µm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>78.52 ± 5.00</td>
<td>22.36 ± 1.83</td>
</tr>
<tr>
<td>FXI⁻/⁻</td>
<td>51.33 ± 2.96</td>
<td>15.67 ± 2.45</td>
</tr>
</tbody>
</table>

*p value* 0.002 * 0.053

NOTE: n=30 for each group, N=10 per group.
### TABLE 1.7

AVERAGE FOOD INTAKE PER MOUSE AT THE AGES OF 3, 6, AND 9-12 MONTHS OLD

<table>
<thead>
<tr>
<th>Genotype</th>
<th>average daily food consumed (g)</th>
<th>age (months)</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>5.84 ± 0.23</td>
<td>3</td>
</tr>
<tr>
<td>FXI&lt;sup&gt;−/−&lt;/sup&gt;</td>
<td>6.15 ± 0.35</td>
<td>3</td>
</tr>
<tr>
<td>WT</td>
<td>4.87 ± 0.15</td>
<td>6</td>
</tr>
<tr>
<td>FXI&lt;sup&gt;−/−&lt;/sup&gt;</td>
<td>4.86 ± 0.15</td>
<td>6</td>
</tr>
<tr>
<td>WT</td>
<td>4.50 ± 0.21</td>
<td>9 to 12</td>
</tr>
<tr>
<td>FXI&lt;sup&gt;−/−&lt;/sup&gt;</td>
<td>4.46 ± 0.28</td>
<td>9 to 12</td>
</tr>
</tbody>
</table>
### TABLE 1.8

TAT COMPLEX ELISA AND PLASMA ASSAYS FOR THROMBIN, FIBRINOGEN, FX AND FXII.

<table>
<thead>
<tr>
<th>Plasma assay</th>
<th>WT</th>
<th>FXI&lt;sup&gt;+&lt;/sup&gt;</th>
<th>t-test ( p value)</th>
</tr>
</thead>
<tbody>
<tr>
<td>[TAT] ng/mL</td>
<td>14.92 ± 1.12</td>
<td>12.12 ± 1.6</td>
<td>0.182</td>
</tr>
<tr>
<td>Thrombin time (sec)</td>
<td>48.95 ± 1.54</td>
<td>52.77 ± 1.82</td>
<td>0.126</td>
</tr>
<tr>
<td>[Fibrinogen] mg/dL</td>
<td>365.67 ± 44.7</td>
<td>490.17 ± 30.72</td>
<td>0.045&lt;sup&gt; * &lt;/sup&gt;</td>
</tr>
<tr>
<td>FX % plasma</td>
<td>102.07 ± 1.58</td>
<td>111.52 ± 6.02</td>
<td>0.203</td>
</tr>
<tr>
<td>FXII % plasma</td>
<td>103.34 ± 5.15</td>
<td>116.0 ± 5.09</td>
<td>0.08</td>
</tr>
</tbody>
</table>

NOTE: n≥12 for TAT, n=11 for thrombin time, n≥6 for Fg, n≥10 for FX%, n≥8 for FXII%.
TABLE 1.9

COMPARISON BY GENOTYPE OF THE NUMBER OF INFLAMMATORY CELLS
AND INFLAMMATORY FOCI OF 3 MONTH-OLD MICE.

<table>
<thead>
<tr>
<th>Genotype</th>
<th># inf. cells/total fields</th>
<th># foci/total fields</th>
<th># inf.cells/total foci</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>1.185</td>
<td>0.078</td>
<td>15.44</td>
</tr>
<tr>
<td>FXI&lt;sup&gt;−/−&lt;/sup&gt;</td>
<td>5.085*</td>
<td>0.131</td>
<td>38.82</td>
</tr>
</tbody>
</table>

A significant difference in the proportion of inflammatory cells per field studied in histological sections is seen between the genotypes, *p*<0.01. (n≥5 per group).
# TABLE 1.10

CBC VALUES FOR 3 MONTHS-OLD FXII<sup>−/−</sup> AND FXI<sup>−/−</sup>/FXII<sup>−/−</sup> MICE.

<table>
<thead>
<tr>
<th>Genotype</th>
<th>wbc (K/μL)</th>
<th>ne (K/μL)</th>
<th>ly (K/μL)</th>
<th>mo (K/μL)</th>
<th>eo (K/μL)</th>
<th>ba (K/μL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>FXII&lt;sup&gt;−/−&lt;/sup&gt;</td>
<td>3.49 ± 0.46</td>
<td>0.54 ± 0.14</td>
<td>2.71 ± 0.34</td>
<td>0.22 ± 0.02</td>
<td>0.01 ± 0.004</td>
<td>0.01 ± 0.0</td>
</tr>
<tr>
<td>DKO</td>
<td>6.14 ± 0.35</td>
<td>1.29 ± 0.17</td>
<td>4.08 ± 0.29</td>
<td>0.40 ± 0.05</td>
<td>0.27 ± 0.07</td>
<td>0.11 ± 0.03</td>
</tr>
<tr>
<td>t-test (p value)</td>
<td>0.0002 *</td>
<td>0.01 *</td>
<td>0.01 *</td>
<td>0.03 *</td>
<td>0.02 *</td>
<td>0.03 *</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Plt (K/μL)</th>
<th>ne (%)</th>
<th>ly (%)</th>
<th>mo (%)</th>
<th>eo (%)</th>
<th>ba (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>FXII&lt;sup&gt;−/−&lt;/sup&gt;</td>
<td>1007.40 ± 42.04</td>
<td>14.74 ± 2.07</td>
<td>78.19 ± 1.83</td>
<td>6.72 ± 0.92</td>
<td>0.31 ± 0.08</td>
<td>0.04 ± 0.02</td>
</tr>
<tr>
<td>DKO</td>
<td>1212.31 ± 47.47</td>
<td>20.59 ± 2.36</td>
<td>67.10 ± 4.07</td>
<td>6.49 ± 0.65</td>
<td>4.19 ± 1.06</td>
<td>1.63 ± 0.46</td>
</tr>
<tr>
<td>t-test (p value)</td>
<td>0.01 *</td>
<td></td>
<td></td>
<td></td>
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<td></td>
</tr>
</tbody>
</table>

NOTE: Observe the statistically significant difference for all of the WBC values and the noticeable increase in the number of granulocytes (n=11 for the DKO and 6 for FXII<sup>−/−</sup> mice).

wbc: white blood cells; ne: neutrophils; ly:lymphocytes; mo: monocytes; eo: eosinophils; ba: basophils; Plt: platelets.
1.6. References


CHAPTER 2

VASCULAR RESPONSE TO CAROTID ARTERY OXIDATIVE INJURY
IN FG\(^{-}\), PC\(^{+/}\) AND FXI\(^{-}\) MICE

2.1 Introduction

In the vascular endothelium, oval-shaped ECs are arranged in a cobblestone pattern set in the direction of blood flow. The ECs tightly cover the entire surface forming a layer between the blood and the vessel wall. This layer, or endothelium, provides the vessel with an interface that has selective properties that regulates most molecule traffic in a semi-permeable fashion. The tight junctions and cell adhesion molecules (CAMs) among the ECs in conjunction with anchorage of the ECs to its basal membrane are responsible for the attachment, stability, and maintenance of the selective permeability of the endothelium.

Under pathological or stress conditions, ECs undergo a series of physiological and morphological changes indicative of its activation. Physiological changes are marked by production of NO, cytokines and chemokines, among others. The release of those molecules into circulation favors the activation of mediators from the complement system, that can further activate the endothelium and trigger immune and inflammatory
responses (1, 2). Activation of the endothelium induces permeability between the ECs, resulting in the exposure of subendothelial surfaces and leakage of plasma components. The activation of ECs is also accompanied by the production of adhesive molecules such as E-selectin, VE-cadherin and, ICAM-1, among others. These molecules induce leukocyte adhesion, attachment and trafficking into the vascular wall, and production of P-selectin, that induces platelet activation and attachment (1, 3, 4).

Deficiency of a key gene that encodes a protein of the coagulation cascade modifies the conditions by which the organism maintains haemostasis. If the genetic deficiency affects the function or production of a coagulation protein, it is most likely to have an effect on fibrin or the levels of thrombin. These two proteins are closely tied to inflammation via a myriad of interactions with PAR receptors; induction of cytokines such as TNFα, and both IL-6 and 8; and induction of inflammatory cells chemotaxis. Thrombin is also associated with the immune response through the activation of C5a of the complement system (2, 5). The most severe cases involving either partial or total protein deficiencies include the development of a generalized coagulopathy, derived from excessive fibrin deposition, or the production of unstable clots in the absence of fibrin. Diverse pathologies and interconnections with other biological pathways have been observed from studies of different coagulation protein deficiencies (6, 7, 8, 9, 10).

The close relationship between thrombin and fibrin makes the separation of thrombin’s functions and effects difficult to evaluate in vivo. However, the availability of specific inhibitors, such as hirudin, which forms a tight complex with thrombin (Kd≈6 x 10^-11 M) (11), makes possible the prolonged inhibition of thrombin at relatively low
dosages. Hirudin is a 65 a.a. peptide, rich in carboxylic side chains, which react stochiometrically via the formation of salt bridges with residues on thrombin. The binding residues for hirudin on thrombin correspond to the Fg binding site residues and the acylated serine from the active center (12, 13). Although the binding of hirudin to thrombin is reversible, the fact that it occurs with such a high affinity, and with the occupancy of sites involved in fibrinogen binding renders the enzyme functionally incapable of catalyzing fibrin generation (11).

Experimental models that challenge the normal physiological response of animals help test the implications of a gene/protein deficiency in multiple physiological events. The use of oxidative injury models in the mice vascular system is one method of reproducing, in an accelerated fashion, pathological conditions that occur over long time periods. The oxidative injury approach when taken together with genetically modified mice allows a good approximation of the implications a specific deficiency may have for vascular processes.

Chemical injuries may result in different degrees of damage to the vascular wall. These injuries may severely damage the endothelial layer, exposing the subendothelial and inner vascular tissue to the circulation. This exposure triggers both the activation of coagulation factors and the release of agonists for platelet activation, such as ADP and serotonin. Platelets rely on mechanisms, such as binding of fibrinogen/fibrin to its receptor glycoprotein IIb/IIIa (αIIbβ3) on the platelet surface, for platelet adhesion to other platelets, leukocytes or ECs. Platelet-collagen binding occurs through a different mechanism. It may include the direct binding of α2β1 (VLA-2,GPIIaIIa) integrin onto
collagen or may be the result of vWF-glycoprotein 1b-mediated collagen binding (14,15). Additional mechanisms include proteins such as JAM A and C, also present on platelet membranes. These proteins belong to the CTX immunoglobulin family, a type I transmembrane group of proteins involved in cell-cell adhesion that localizes at cellular junctions (16). JAM-A can bind with integrins across several regions of the Ig domain, from the membrane proximal to the N-terminal region (17,18). JAM-C, on the other hand, can bind to the leukocyte integrin Mac-1(α₂β₁) favoring platelet-leukocyte interactions and supporting the close contact seen between these two cell types in blood clots (19). The absence of any of these mechanisms results in the formation of an unstable clot with limited platelet adhesive properties. The binding of fibrin to GP IIb/IIIa activates the Src and Syk mechanisms that translate into platelet aggregation and clot retraction (20). Fibrin GP receptor on the surface of platelets, in connection with myosin-actin complexes, seem to be the molecules primarily responsible for the clot retraction event (20).

Ferric trichloride (FeCl₃) is a chemical oxidant that has been repeatedly used as a thrombus-inducing agent in mice veins and arteries, such as the femoral, carotid and aorta. The platelet-rich thrombus generated by this injury is suitable for short term studies, and its evolution can be easily monitored by visual examination and Doppler probes (21). The oxidative injury induces not only endothelial denudation, but also collagen exposure and erythrocyte lysis. The lysis of RBCs results in the release of heme groups that interact with FeCl₃ to form oxHb, an additional injury agent to the vascular wall (22).
A modified silicon-copper cuff model, redesigned by our research group, consisting of a cylindrical silicon hollow mold filled with copper powder, and opened longitudinally, was used to induce arterial oxidative injury. The central hollow and longitudinal opening of this design facilitates the cuff implantation around the artery (Fig.2.1).

Fig.2.1 Adapted from Völker W. et al. Artherosclerosis. 1997;130:29-36.

Copper induced injury is based on the effects of copper ions on neutrophil chemotaxis and the correlation between cardiovascular disease prevalence and elevated copper plasma levels (23, 24, 25, 26). Particularly in our model, the oxidation of copper ions results in a release of ions from the cuff into the arterial wall, causing a progressive lesion similar to restenosis/arteriosclerosis-like neointima hyperplasia (27, 28). The initial stage of the resulting arterial injury is marked by the activation of the ECs from the intima arterial layer and the adhesion and transmigration of leukocytes into the intima. These events take place during the first week post cuff implantation (28, 29). The leukocyte invasion is accompanied by gradual but incremental infiltration of macrophages and transformation of the SMC of the medial layer from a contractile to a synthetic phenotype (28, 29). After several weeks, we see proliferation of the SMCs, fibrin deposition, appearance of foam cells (cells storing oxidized LDL), and rupture of
the endothelium as the injury persists (28, 29). This model allows for the observation of gradual inflammatory injury, with clear implications for inflammatory cells and vascular wall behavior.

Our interest is focused on three proteins with particular roles in the coagulation cascade: Factor XI (FXI), protein C (PC), and fibrinogen /fibrin (Fg). FXI is a key protein in the intrinsic pathway of the coagulation cascade. Its total deficiency disrupts the activation of factor IX and, consequentially, the generation of additional thrombin via FIX-dependent FX activation.

A total deficiency of FXI translates into augmented coagulation times in aPTT tests and a survival advantage after induced peritoneal sepsis (30,31). Activated PC (aPC) is known to have anti-coagulant properties through its interactions with protein S, which can inactivate factors Va and VIIIa. Also, aPC is capable of forming a complex with its receptor on ECs (EPCR) that can cleave at least one of the protease-activated receptors (PAR-1) on the surface of ECs. This activation ultimately triggers inhibition of apoptosis via reduction of NF-κB expression and surface adhesion molecules (32). Binding of aPC to EPCR can also induce increased phosphorylation of myosin light chain, peripheral actin reorganization and central stress fiber reduction facilitating the maintenance of endothelial permeability (60).

Viability of a murine total protein C deficiency is highly compromised by a perinatal consumptive coagulopathy of the brain and liver during mice development, leading to embryonic or neonatal lethality (33). This murine lethal condition is partially
attenuated when a total protein C deficiency is combined with a total deficiency in factor XI, resulting in double deficient mice with lifespans extending to early adulthood (34). The partial deficiency of PC heterozygote mice provided an excellent model for developing our study due to its significant protein C expression level (≈60% of WT level) (35). This PC expression level translates to a normal viability and lack of spontaneous phenotype but a differential response after arterial inflammatory challenge when compared to WT mice (35,28).

Fibrinogen, the precursor of Fg, is an essential protein of the platelet clot, which after cleavage and polymerization confers stability to platelets and allows the clot to grow. Additionally, Fg/Fb deposition is a major inflammatory mediator (36). Here we challenge mice using the silicon-cooper cuff model in both murine deficiencies: FXI (FXI−/− mice), and PC (PC+/− mice) to evaluate the total lack of a key protein of the intrinsic pathway of the coagulation cascade, and the partial lack of a protein with anticoagulant properties in arterial physiology. In this chapter, we evaluate the impact of the lack of Fg/Fb (Fg−/− mice) on the formation and structure of an induced arterial blood clot. We describe the implications of these individual murine deficiencies in prothrombotic, anticoagulant and inflammatory events.
2.2 Materials and Methods

2.2.1 Animal housing and breeding

Mice were housed in microisolation cages with a maximum capacity of 5 mice per cage, kept in a 12 hour daily light cycle, and allowed access to food and water \textit{ad libitum}. Animal breeding, housing, and manipulation strictly conformed to regulations established by the IACUC and AALAC, including the certification by the Freimann Life Science Center of Notre Dame of all personnel on Human care and the use of laboratory animals. All mice used in these studies were between 7 and 8.5 weeks of age.

2.2.2 Generation of $Fg^{-/-}$, $PC^{+/+}$ and $FXI^{-/-}$ mice

The generation of $Fg^{-/-}$, $PC^{+/+}$ and $FXI^{-/-}$ mice used in the study has been described previously (30, 33, 37). These mice were generated by targeted deletion through introduction of a neomycin gene (NEO-cassette) in the genomic sequence of the gene, resulting in the interruption of the particular gene after homologous recombination. Briefly, for the production of a Fg deficient allele, the NEO cassette was directed to the region that encodes the $\gamma$ chain of murine Fg. For the generation of a FXI deficient allele, a NEO cassette was directed to the exon V of the FXI encoding gene (more detailed description in section 1.2.2 of Chapter 1). For the PC deficient allele, the NEO cassette was directed to the whole encoding region between the 5’ and 3’ flanking points. In the PC deficient allele, a cda cassette was also cloned upstream of the 5’ point for further
negative selection. Targeting vectors containing the PC or the Fg modified genes were inserted into R1 embryonic stem cells (ES). Positive heterozygous ES cells were confirmed by Southern blot with specific probes for each vector, followed by insertion into mouse blastocysts for the generation of heterozygous mice. Further crossing of heterozygote mice was performed for the production of Fg or FXI fully deficient mice. Detection of the PC deficient alleles was performed by Southern and Northern blots, and aPC activity levels were measured by plasma activity assays (33). For Fg deficient mice, Western blots and thrombin clotting time assays were utilized to detect any Fg activity in the plasma (37).

2.2.3 Mouse genotyping

Tissue from ear punches was used as the source of DNA for genotyping in these studies. Isolation of the DNA from the tissue requires alkaline hydrolysis in 20μL of 0.2M sodium hydroxide (NaOH) at 75°C for 10 minutes. Neutralization in 180μL of 0.04M Tris-Cl buffer, pH 7.4 follows the lysis. Detection of WT and deficient genes was performed by PCR DNA amplification using a conventional thermal cycler (Peltier Thermal Cycler-200, BIORAD, USA) or a LightCycler real-time PCR machine (Roche, Mannheim, Germany). The LightCycler uses FRET for the detection of the amplified DNA with primers specific for the desired gene as well as sequence complementary fluorescent probes attached to fluorophores. In contrast, the conventional thermal cycler uses the reverse and forward primers for the generation of the DNA amplicon, followed by gel electrophoresis for verification of the amplicon size. When the probes hybridize to
the sequence during the annealing cycle, FRET occurs and fluorescence is detected. Additionally, Taqman real-time PCR (RT-PCR) was also used for the detection of some of the alleles present in the DNA samples. Although Taqman technology also uses fluorescence signaling for the detection of DNA amplicons, this method is based on a different principle involving the release of a fluorescent reporter which is cleaved by the polymerase during extension. The release of the reporter to the reaction medium separates it from the quencher placed in close proximity in the probe allowing the fluorophore to emit fluorescence. The list of primers used for the detection of the different genotypes and the methodology used are shown in Table 2.1.

2.2.4 Silicon-Copper cuff and osmotic pump implantation

Construction and implantation of the Silicon-Copper cuff has been described previously (29). Briefly, a mixture of prosthetic silicone and 325 mesh powder copper is degassed and embedded into cylindrical molds with steel rods of a similar diameter of the mouse carotid arteries placed along the center of each cylinder. This mixture is then polymerized at 80°C for 8 hours after which the rods are removed and the cylinders cut every 1.5 mm. The cuffs are placed through a longitudinal opening around the previously exposed left carotid artery of each anesthetized mouse. The opening is sealed surgically and mice are left to recover on a 37°C plate. The right carotid arteries are used as controls. The cuffs were left in the mice during periods of 7 or 21 days, after which the arteries were collected and analyzed by histochemistry and TEM. In addition to the Silicon-Copper cuff implants in PC+/− and FXI−/− mice, a group of PC+/+ mice was
simultaneously treated with lepirudin to assess the influence of fibrin in this model (Wayne, NJ, USA). The lepirudin was delivered at a dosage/rate of 7.5μg/mL/hr through ALZET osmotic pumps (2001 model from Durect Corporation, Cupertino, CA, USA) implanted subcutaneously in the peritoneal cavity.

2.2.5 Necropsy

Mice were anesthetized using rodent cocktail (0.075 mg ketamine/0.015 mg xylazine/0.0025 mg aceprozamine/g weight of mice) before any intervention. Blood was collected from the vena cava in EDTA at a volume ratio of 9:1 to check for standard CBC values in a Hemavet cell counter (DREW Scientific, Oxford, Connecticut, USA) or citrate buffer for lepirudin-treated mice. Injured carotid arteries from Fg<sup>−/−</sup> mice were harvested after the chemical injury with FeCl<sub>3</sub> was performed, whereas carotid arteries from PC<sup>−/−</sup> and FXI<sup>−/−</sup> mice were harvested after full perfusion of the mice with saline solution. Collection of the arteries was followed by fixation in Karnovsky solution and processing for SEM or TEM.

2.2.6 Ferric chloride chemical injury

The procedure for this chemical injury was described previously (38, 39). Platelet clots were induced in three different groups: WT, Fg<sup>−/−</sup> and Fg<sup>+</sup> mice injected intravenously with Fg (Fg<sup>−/−</sup> i.v.). Briefly, each group of n=3 mice was anesthetized and exposed to chemical injury with the oxidizing agent FeCl<sub>3</sub> on the left carotid artery.
FeCl$_3$ was applied in 1mm wide x 0.2 mm thick strips of Whatman No.4 filter paper. Paper strips, previously immersed in a 200mg/mL solution of FeCl$_3$ in 10% glycerol, were put in contact with the carotid arteries during 3 min after which the strips were removed and the arteries were left to produce a stable thrombus for periods of time that ranged from 15 to 30 min. Injection of 4 mg/mL/weight ($\approx$9μM) of human fibrinogen in the Fg$^{+/-}$ i.v. group was delivered via tail vein in Fg$^{-/-}$ mice before inducing formation of the platelet clot.

2.2.7 Coagulation assays

Prothrombin times from lepirudin treated WT and PC$^{+/-}$ mice were performed using 50μL of pre-warmed and undiluted mouse plasma added to 100 μL of pre-warmed Neoplastine-CL (Diagnostica Stago, Asnières, France) and mixed in plastic cuvettes provided with standardized magnetic balls to be used in a ST art 4 coagulometer (Diagnostica Stago, Asnières, France). The obtained values were compared to saline treated WT and PC$^{+/-}$ mice controls.

2.2.8 ELISA

Enzyme linked Immuno-Sorbent Assays were performed with plasma obtained from WT and PC$^{+/-}$ mice for the determination of Thrombin-Anti-Thrombin III (TAT) complex levels in the mice blood before subjecting the groups to lepirudin treatment. Procedures were described in section 1.2.6 of Chapter 1.
### TABLE 2.1

PRIMERS AND METHODOLOGY FOR DETECTION OF GENOTYPES

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primers</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>FXI</strong></td>
<td>Reported in Chapter 2.</td>
</tr>
<tr>
<td><strong>Detected with Taqman-RT-PCR</strong></td>
<td></td>
</tr>
<tr>
<td><strong>PC null</strong></td>
<td>5’ common primer: CGTGATGAGTTTCAGGCAGTGAGAG</td>
</tr>
<tr>
<td></td>
<td>3’ WT: GCACACGTGTGGACCAGGGATAAT</td>
</tr>
<tr>
<td></td>
<td>PC Neo*: ACAAGCAAAAACCAAATTAAGGGCCA</td>
</tr>
<tr>
<td></td>
<td>PC FAM*: CCTTCATCCTTCCCCAGGCATTGTC- BHQ1*</td>
</tr>
<tr>
<td></td>
<td>PC Null TAMRA*: CATAGCCTGAAGACGAGATCAGCAGCC- BHQ2*</td>
</tr>
<tr>
<td><strong>Detected with RT-PCR Light cycler</strong></td>
<td></td>
</tr>
<tr>
<td><strong>Fg</strong></td>
<td>Map3 common 5’: CAGCGGCTTGTCATTAG</td>
</tr>
<tr>
<td></td>
<td>TV1-WT*: 3’: CTGAAAGACCTGTCTTTGC</td>
</tr>
<tr>
<td></td>
<td>Fg Neo*: ACAAGCAAAAACCAAATTAAGGGCCA</td>
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TABLE 2.1 (Continued)

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<th>Gene</th>
<th>Primers</th>
<th>Fluorophore</th>
<th>Phosphate</th>
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<tr>
<td></td>
<td>5’ FITC&lt;sup&gt;f&lt;/sup&gt; TGCTGATCTCGTTCTTCAGGCTAT-</td>
<td>Fluoresceine</td>
<td></td>
</tr>
<tr>
<td></td>
<td>3’ Cy5.5&lt;sup&gt;g&lt;/sup&gt; AACTGACACATTTGGAAACCACAGTACTTAGAACCAC</td>
<td>Phosphate</td>
<td></td>
</tr>
</tbody>
</table>

NOTE: <sup>a</sup>PC Neo and <sup>e</sup>Fg Neo are primers in the neomycin cassette region; <sup>b</sup>PC FAM, <sup>f</sup>FITC and <sup>g</sup>Cy5.5 are fluorophores; <sup>d</sup>TV1-WT is a primer in the targeting vector region; <sup>c</sup>PC Null TAMRA, BHQ1<sup>h</sup> and BHQ2<sup>i</sup> are fluorescence quenchers.

2.2.9 Histochemistry

Plastic cross-sections of 200nm thickness obtained from the arterial area covered by the Silicon-copper cuff were stained with toluidine blue and analyzed in a Nikon Eclipse E600 optical microscope (Melville, NY). Vessel count was carried out by counting the microvessels present in the adventitia layer of 10 serial cross sections separated by a distance of 200μm.

2.2.10 Electron microscopy (TEM and SEM)

Arteries subjected to chemical injury with FeCl₃ or cooper based injury were harvested from the mice, fixed and processed for TEM or SEM following the procedures previously detailed (29). Electron micrograph of cross-sections 80 to 90nm in thickness
of the areas previously examined on Toluidine blue stained sections were taken for ultrastructural examination in a Hitachi H 600 transmission electron microscope (Hitachi, Tokio, Japan) at 75kV accelerating voltage. Harvesting and processing of carotid arteries for SEM was performed as previously described (28). Briefly, mice carotid arteries covered with the Silicon-copper cuff were collected from WT and PC+/- mice, sectioned longitudinally in two halves, fixed in Karnovsky solution, post-fixed in OsO4 and dehydrated in a critical-point drier before being coated on carbon-platinum for SEM examination.

2.2.11 Platelet clot characterization

Two types of measurements were performed on images from electron-micrographs obtained of platelet clots after FeCl3-induced chemical injury to the carotid arteries. The first measurement included the average area among the platelets obtained by a grayscale threshold contrast of the platelets vs. the area among them. The data obtained from each replica was normalized against the correspondent total area of the electron micrograph. The second measurement involved the average distance between cell membranes of neighboring platelets. Both distance and area measurements were performed using the distance bar and a combination of threshold/area functions provided in the free source software ImageJ from the N.I.H. (40,41). Distances measurements were based on the exact length of a pixel registered in digital versions of electron micrographs magnified 20000X, scanned at 600 ppi and provided with a magnification bar, whereas, area measurements were obtained in pixel/μm 2.
2.2.12 Statistics

Student’s t-test was used for the comparison of numbers of vessels observed in the adventitia of WT and PC+/- mice carotid arteries after 7 days of cooper induced injury. One-way ANOVA was performed to examine the differences among WT, Fg+/- and Fg i.v mice for measurements of both the area and the distance between platelets that had formed clots. The NCSS-2001 software package was used for ANOVA analyses (42). Results for averages and plot bars were accompanied with standard errors of the mean (±SEM). For the measurement of distance between platelets, random sampling of at least one quadrant out of six that constituted every picture was used for quantification. The average number of pixels counted per quadrant was later subjected to power analysis, with α=0.01 and an effect size=0.35, for the estimation of a good sample size (see appendix A for complete power analysis values). Statistical significance was considered when \( p \leq 0.05 \) for all tests.

2.3 Results

2.3.1 Protein C partial deficiency

Protein C deficiency, in both mice and humans, is generally manifested with intravascular coagulation. The thrombosis derived from that coagulation is directly dependent on the levels of aPC in plasma (33,34). In PC+/- mice the occurrence of such thrombosis is mitigated by a relatively high availability of aPC in plasma originated from
more than 50% of normal PC levels. However, under arterial damaging conditions, where EC activation and thrombosis are induced, these levels of PC can be consumed favoring the occurrence of thrombosis. We compared the development of arterial inflammation in PC+/− mice and their WT controls after oxidized copper induced damage.

2.3.1.1 Copper induced injury after 7 days

The reaction to copper-based oxidative injury in the carotid arteries of WT mice was marked by the activation of EC, attachment and transvasation of circulating leukocytes into the subendothelial space, and a gradual transformation of SMC from the medial arterial layer. Activation of EC is caused by the effect of released oxidized Cu²⁺ ions from the cuff on the endothelium and it is obvious through an increase of biosynthetic organelles, vascular permeability and EC volume. Such activation was confirmed by rolling and attachment of circulating leukocytes on ECs, followed by transcellular or pericellular penetration of these leukocytes into the vessel wall. This reaction was exacerbated in the PC+/− carotid arteries (Fig.2.2). The full diapedesis of leukocytes into the subendothelial space generates the formation of a neointima. This modified arterial layer is constituted mainly of neutrophils located between the ECs and the elastic lamina (28). The Cu²⁺ ions together with the transvasation of leukocytes induce the transformation of SMCs from the medial compartment from contractile to synthetic cells. These cells react to the Cu²⁺ ions, inflammatory mediators released from invading leukocytes, and ECs by synthesizing collagen and other components of the ECM. This results in an enlargement of the medial compartment, also noticeably enlarged
in the PC\textsuperscript{+/-} case (Fig.2.3). The increased attachment and infiltration of inflammatory cells into the intima of PC\textsuperscript{+/-} carotid arteries extends similarly to the medial compartment, a phenomenon observed in the WT arteries only after weeks of copper induced injury. This extended infiltration into the media generates inflammation, fat accumulation and transformation of SMCs phenotype (Fig.2.4 A and B). Compared to the WT, SMCs from the PC\textsuperscript{+/-} carotid arteries displayed fewer contractile elements and were surrounded by a more disorganized collagenous matrix. Those findings were confirmed by histological H&E stains and immunohistochemistry for smooth muscle actin (SMA), and CD45\textsuperscript{+} cells performed in our lab (28). Additional stains also revealed an increased fibrin(ogen) deposition and loss of organized collagen fibrils in the matrix of the medial compartment (28). A similar reaction is observed in the adventitia layer where abundant collagen and fibrin deposition seem to generate an enlargement of this arterial layer. Intermixed throughout strings of fibrin and collagenous matrix, abundant inflammatory cells are seen in arteries from both genotypes (Fig.2.3). Interestingly, when compared to the PC\textsuperscript{+/-} carotid arteries, the WT arteries display fewer microvessels in the adventitia compartment per section (63 ± 4/mm\textsuperscript{2} for WT and 353 ± 26/mm\textsuperscript{2} for PC\textsuperscript{+/-}, n=3; p=0.0043) (Fig.2.3). However, with the exception of the abundant newly formed microvessels and the presence of macrophages in the adventitia of PC\textsuperscript{+/-} arteries, the amount of neutrophils and collagen in the adventitia was comparable between the two genotypes (Fig.2.4 C and D). Thrombin plasma levels may have a direct impact on fibrin tissue deposition and then affect the occurrence of angiogenesis. To assess the potential impact of thrombin in the neovascularization observed in the adventitia thrombin plasma levels were assessed for mice from both genotypes. TAT levels in the plasma of non-injured mice were
determined to discard any predisposition to form more fibrin(ogen) as seen in animals with a more severe deficiency in PC (PC levels lower than 50%). However, results from these immunoassays did not show any significant difference between genotypes (Fig.2.5). To further investigate the combined effects of thrombin/fibrin and the partial deficiency of PC on the angiogenic response seen in the adventitia, mice exposed to the cooper-induced injury were treated with lepirudin for 7 days. A dosage/rate of 7.5 μg/mL per hour of the specific thrombin inhibitor was enough to extend the aPTT times above 5 min (≈ 7 fold the normal time) in the plasma of mice after 7 days of treatment. Although PC+/− mice exhibited a greater tendency for medial compartment enlargement, after parallel treatment of copper induced injury and lepirudin, no neovessels were observed for both genotypes after 7 days of treatment (Fig.2.6).

2.3.1.2 Copper induced injury after 21 days

A generalized increment on the size of every arterial layer was noticed for both genotypes after 21 days. When compared to their WT counterparts, injured PC+/− arteries displayed a thinner endothelial cell layer due to overexpansion of the neointima into the luminal area (Fig.2.7 A and B). The ECs that cover the arterial lumen tend to extend their cytoplasms in order to cover the augmented area occupied by the enlarged neointima as seen in the PC+− arteries. This extension compromises the integrity of the endothelium, making it fragile and susceptible to ruptures, as seen in some areas (Fig.2.7 A). The neointima from both genotypes exhibited highly synthetic SMCs with abundant ECM surrounding them, however, the presence of eosinophils together with migratory ECs
seem to be unique to the PC<sup>+/−</sup> injured arteries at this stage (Fig.2.7 C,D and E). Synthetic SMCs displaying accumulated fat were often found in the arterial media from both genotypes, nevertheless ECs forming RBC-containing neovessels were only seen in the PC<sup>+/−</sup> arteries (Fig.2.7 F and G). In contrast to the WT neointima and media layers, a greater number of neutrophils were found in these compartments in the PC<sup>+/−</sup> mice arteries, contributing to the enlargement of the arterial wall, as confirmed by morphometric measurements performed in histological sections (28). Additionally, a remarkable clearance of cell debris was noticed in the adventitia layer of PC<sup>+/−</sup> mice arteries when contrasted with WT arteries, which exhibited numerous neutrophil remnants (Fig.2.6 H and I).

2.3.2 FXI total deficiency

Murine total FXI deficiency is characterized by vascular and hepatic abnormalities, irregular blood cell and coagulation factors levels. All of these altered characteristics suggest that this protein can act as an inflammatory regulator and its absence from circulation predispose deficient mice to inflammation. Moreover, this protein may be acting by affecting the interaction of the endothelium with leukocytes, such as neutrophils, via the contact system of coagulation. We believe that in a total deficiency for murine FXI unbalanced generation of KK and coagulation factors combined with neutrophilia transform the vascular endothelium making it more susceptible to inflammatory reactions. Here, we support some of those findings through the use of a copper-based arterial challenge for the induction of vascular inflammation.
2.3.2.1 Copper induced injury after 7 days

As mentioned previously, the reaction of WT carotid arteries to the copper-induced injury at this stage is characterized by the formation of a thin neointima marked by diapedesis of leukocytes, mainly neutrophils, into the subendothelial space. Although this process involves the disintegration of cell junction complexes that keep ECs together and connected to their basal membranes, the integrity of the endothelium was maintained in places where inflammatory cells are absent. In contrast to the WT intima, the endothelium of FXI$^{−/−}$ arteries at this stage is mostly separated from the basal lamina due to a massive neutrophil invasion into the subendothelial space (Fig.2.8 A and B). This extravasation of neutrophils is noticeably extended to the medial layer, generating fat accumulations and transformation of SMCs, whereas, in WTs the SMCs appear normal (Fig.2.8 C and D). Additionally, the presence of eosinophils seen in the FXI$^{−/−}$ arterial wall, at this early time point, suggested the expression of specific attachment molecules on the EC surface, or the secretion of chemotaxins for these granulocytes in the arterial wall.

2.3.2.2 Copper induced injury after 21 days

Upon comparison between genotypes, most features in the injured arteries at this stage appear remarkably different. The degree of injury found in WT arteries shares certain similarities to damage found in FXI$^{−/−}$ arteries after 7 days post cuff implantation. An obvious detachment of the endothelial cell layer from its basal lamina due to a
massive invasion of neutrophils into the subendothelial space was observed (Fig. 2.9 A and B), that contrasted enormously with the amorphous and at some points disrupted endothelial cell layer seen in FXI−/− arteries. Multilayered neointima were found in both arterial genotypes, although the presence of neutrophils and an increased number of SMCs in FXI−/− arteries was obvious (Fig. 2.9 C and D). More differences were found in the medial compartment. In WT arteries, the SMCs appeared elongated with a mild synthetic phenotype and are surrounded by a well defined collagenous matrix. In contrast, rounded and highly synthetic SMCs intermixed with neutrophils in an irregular collagenous matrix were found in the media of FXI−/− arteries. The shape and sheer number of SMCs present in FXI−/− arteries suggested a high proliferation rate of this cell type in the medial layer (Fig. 2.9 E and F). Observation of the adventitial compartments revealed the presence of abundant neutrophils in the FXI−/− arteries when compared to WT counterparts. Furthermore, an increased size of the entire adventitia with scarce presence of repair cells such as fibroblasts was seen in the FXI−/− arteries (Fig. 2.9 G and H). No differences in the amount of microvessels in this compartment were found.

2.3.3 Fg total deficiency

Murine total deficiency of Fg is associated with abnormal embryonic development, susceptibility to infections and platelet clot instability (48,49,50). Fg presence between platelets affects the stability of the clot by keeping platelets together and decreasing the distance between these cells, perhaps also affecting the capacity for
clots to contract. Here, we use a chemical injury model to show in fine detail some of the structural aspects involved in clot instability in the presence and absence of Fg.

2.3.3.1 FeCl₃ induced arterial clot

Ultrastructural analysis of the chemically induced clots from carotid arteries shows major differences between WT and Fg⁻/⁻ mice. Panoramic view of the WT mice showed an aggregation of platelets that fully covered the arterial lumen. These clots displayed abundant plasma proteins and fibrin deposited among the platelets (Fig. 2.10A). A heterogeneous degree of platelet activation was also seen, with medium degranulation as the predominant stage of platelet activation. Contrary to the WT case, the Fg⁻/⁻ clots were not occlusive; they covered only part of the injured area and consisted of platelets displaying a more advanced degree of degranulation (Fig. 2.10B). This observation suggested instability and/or limitations for clot growth due to the lack of Fg. Nevertheless, it reflects the capacity for platelets to form small clots through alternative adhesion mechanisms under arterial blood flow conditions. A closer view of the platelet clots at a magnification of 20,000X revealed a clear difference in the degree of compactness of platelets between genotypes (Fig. 2.11 A and B). Measurements of both the average area among the platelets, and the average distance between platelets show a significant separation for clots of Fg⁻/⁻ mice. Additionally, intravenous injection of physiological amounts of Fg into Fg⁻/⁻ mice (Fg⁻/⁻ i.v.) restores the capacity to produce occlusive clots. The clots in the injected mice display similar platelet spacing to the WT clots (Fig. 2.11C). Statistical analysis of the area and distance between the platelets
forming the different clots shows a significant difference between Fg\(^{-}\) and both WT and Fg\(^{-}\) i.v. groups. No statistical difference was found between WT and Fg\(^{-}\) i.v. mice (Table 2.2 and Table 2.3). Measurement of the distance between platelets was performed by random sampling of equivalent areas on the electron micrographs, with average values being subjected to one-way ANOVA power analysis for the estimation of sample size. On average, the clots formed in the Fg\(^{-}\) animals had approximately 1.7 times more space between the platelets and 3.8 times more distance than both Fg\(^{-}\) i.v. and WT. These differences were significant even though the variability of the data for Fg\(^{-}\) clots was considerably greater.

2.4 Discussion

2.4.1 Protein C partial deficiency

The increased inflammatory response seen in PC\(^{+/}\) mice arteries at 7 days is observed by the greater attachment and infiltration of leukocytes into the vascular wall and by transformation of the medial compartment. These observations correspond to the enlarged neointima and phenotypical transformation of SMCs in the medial compartment. Such findings are in agreement with histochemical and immune staining for SMA and CD45\(^{+}\) cells performed in these arteries. The partial deficiency of aPC displayed by PC\(^{+/}\) mice accounts for a greater fibrin(ogen) deposition in the medial and adventitial compartments, as confirmed by immunohistochemistry (28). Increased fibrin(ogen) deposition also favors inflammatory cell infiltration into these areas. This
infiltration facilitates the development of neovessels in the adventitia by both clearing the path for migratory ECs and releasing chemoattractive molecules for these cells to migrate towards those target areas. Perhaps the increase on cell numbers of the arterial wall is a key factor in the observed neovessels formation. An increase in cellularity would demand a greater oxygen and nutrient supply to those areas.

Although the direct effects of aPC on proliferative and migratory behavior of ECs has not been fully studied; the differences on angiogenesis observed in the formation of neovessels seem to be highly dependent on fibrin(ogen). This dependence was confirmed by the disappearance of vessels after lepirudin treatment for 7 days in both WT and PC+/− arteries. Even though the differences between the numbers of vessels/genotype were not statistically significant after the lepirudin treatment, there was a clear tendency of an increased inflammation in PC+/− mice. This tendency is demonstrated by the larger neointima and medial layers seen post lepirudin treatment.

The mentioned observations support the role of aPC as a key anti-inflammatory molecule even in the presence of very low levels of thrombin. Equivalent TAT levels in the plasma of resting mice indicate the absence of pro-thrombotic conditions for both WT and PC+/− murine genotypes, an aspect present in resting mice with a more severe PC deficiency (43). Experiments using another challenging model that allows for the measurement of a quicker inflammatory response could give us a finer detail on how aPC influences such response under very low thrombin levels.
Extension of the copper induced injury for 21 days resulted in an exacerbation of the size of the multilayered neointima and media of PC\(^{+/−}\) arteries when compared to the WT. PC\(^{+/−}\) arteries displayed neovessels in the media and the neointima, as well as a regular presence of eosinophils not often found in WT arteries. Comparatively, the greater number of neutrophils in PC\(^{+/−}\) arteries was indicative of an increased inflammatory response to the injury, an aspect also observed at the 7 day time point. The extension of the injury to three weeks revealed the importance of aPC levels even after prolonged exposure to this oxidative arterial injury in PC\(^{+/−}\) mice.

The exacerbated inflammatory response accounted by PC\(^{+/−}\) mice during the copper induced injury supports the multiple effects that aPC can have regulating thrombin levels, fibrinolysis, apoptosis, and PAR receptors activation. Generation of aPC occurs in the presence of the thrombin-thrombomodulin complex. The later binding of aPC to its cofactor Protein S can act on FVIIIa and FVa, rendering these two pro-coagulant enzymes inactive. This inactivation limits the generation of thrombin from its precursor prothrombin. Protein S, although a cofactor for aPC, is not always required for thrombin inactivation. It has been suggested that aPC can affect PAI-1 activity favoring the action of uPA and tPA in the activation of plasminogen, the key protein of fibrinolysis (43). Activities of uPA and tPA lead to the generation of plasmin followed by degradation of fibrin and eventual clot dissolution.

It is clear that lower levels of aPC can affect the levels of fibrin generated by the coagulation cascade (33). Additionally, aPC has the capacity to regulate inflammation through its interaction with EPCR and PARs on EC. If we add to these inflammatory
effects the interaction of aPC on such EC receptors, the augmented response seen in the arteries of PC partially deficient mice can be explained. The partial lack of PC generally involves greater production of inflammatory mediators, such as IL-6, IL-1B, KC and TNF-α. Such molecules affect not only the endothelium but also ECs from the microvasculature of the adventitia layer. The expression of other molecules from the CAM group such as, V-CAM, I-CAM and E and P-selectins may occur in response to those inflammatory mediators as well. The exacerbated infiltration of leukocytes seen in PC+/- arteries may well be dependent on the greater presence of such attachment molecules on the endothelial surfaces. Experiments directed to assess the differential expression of such attachment molecules on the injured endothelium at early times of injury would extend the knowledge on how aPC influences the leukocyte-ECs interaction.

2.4.2 FXI total deficiency

Since the early stage of 7 days post cuff implantation, a great difference in neutrophil attachment and infiltration was seen in FXI−/− carotid arteries compared to WT. The noticeable infiltration of neutrophils into the subendothelial space of FXI−/− arteries led to endothelial detachment and degradation of the basal lamina. This was a sign of greater affinity for these inflammatory cells on the endothelium. This infiltration extended to the media compartment inducing phenotypical transformation of SMCs from contractile to synthetic cells. Rounded SMCs shape and reduced cytoplasmic myofibrils were some of the signs for this transformation into a synthetic phenotype. Additional
complementary assays included morphometric measurements of the arterial compartments and immune staining for CD45$^+$ cells, SMA, and vWF and fibrin(ogen). These assays also reveal greater inflammation for FXI$^{-/-}$ injured arteries, thicker arterial walls and early loss of contractile phenotype of SMCs.

Although there was a tendency for higher levels of granulocytes in resting FXI$^{-/-}$ mice, as mentioned in Chapter 1 (Section 1.3.2.1), the greater number of neutrophils and eosinophils seen in FXI$^{-/-}$ injured arteries is thought to be a consequence of a greater chemotaxis of these cells on the endothelium and not a direct effect of the number of granulocytes in the blood. It is unknown however, exactly how the total lack of FXI modifies the behavior of ECs and/or granulocytes. The effect appears to be a predisposition of the endothelium to more severe inflammatory reactions. This results in an increased chemotaxis and ultimately greater infiltration of neutrophils into the artery. Both types of cells, neutrophils and ECs, express several membrane receptors for HK, the protein that forms complexes with FXI and PK when circulating in blood. Absence of FXI could have an impact on the proportion of PK that binds HK in the circulation. Consequently, increased amounts of BK generated after HK cleavage as well as FXII activation may result from excessive KK. More KK in the circulation may translate into increased neutrophil and EC activation through induction of degranulation and increased vascular permeability as described in Chapter 1. It is also completely unknown how an imbalance of contact system proteins could affect the expression of adhesion molecules such as selectins, chemokines and/or integrins on the EC surface. The binding of monoclonal antibodies against VE-cadherin results in both an increase in vascular
permeability and neutrophil diapedesis in thioglycollate-challenged mice and VE-cadherin transfected CHO cells (44). It is highly probable that during our arterial injury challenge, the occurrence of several of these events were promoted. Moreover, generation of BK can induce separation of ECs through its effects on β2 receptors, facilitating vascular permeability, activating ECs and promoting FXII activation. As described in Chapter 1 (Section 1.3.2.3), vascular permeability was augmented when FXI<sup>−/−</sup> mice were subjected to localized inflammatory challenge after injection of Evans blue. Differences in the first events of inflammation, such as neutrophil attachment and diapedesis, seem to dictate the evolution of the arterial injury in this model. Augmented inflammatory responses in FXI<sup>−/−</sup> mice were also seen in older mice subjected to a peritoneal septic challenge performed in our lab. Increased infiltration of neutrophils into the peritoneum and lower survival of FXI<sup>−/−</sup> mice, when compared to WT, were some of the outcomes of that study (manuscript in preparation).

2.4.3  Fg total deficiency

Analysis of the size and structure of the platelet clots in Fg<sup>−/−</sup> mice support the essential role of Fg in the formation and stability of the clot. After chemical injury the sparse distribution of the clot formed in Fg<sup>−/−</sup> arteries was in contrast to the occlusive WT clots. The impact of the lack of fibrinogen in the expansion and stability of clots was also reflected in the degree of platelet activation and the separation between them. The more advanced state of platelet activation, generally observed in Fg<sup>−/−</sup> clots, could be a consequence of a higher molecular traffic among the platelets. This greater molecular
traffic could be provided by the lack of fibrin(ogen) molecules as well as the increased space among platelets. Platelets and EC secrete stimulatory molecules, including ADP, vWF, prostaglandins/thromboxane A2, NO and others, which can diffuse more freely under more spacious conditions, inducing platelet aggregation and activation. Studies aimed at characterization of spontaneous development of arterosclerosis in Fg−/− mice, in combination with a double deficiency that predisposes the mice for arterial LDL accumulation, revealed an increment on the expression of soluble P-selectin in plasma (45). These findings suggest the occurrence of an adaptative response to the total lack of Fg, a condition not found in the combined singly deficient mice with the intact Fg gene.

The increment in the distance and area between platelets in Fg−/− clots with respect to WT or Fg−/− i.v. clots correlate with the size of clot formed. In the absence of Fg, the platelets rely on other mechanisms, such as integrin-integrin interaction and membrane receptor binding of adhesive proteins, for the adhesion of platelets to the endothelium and to each other. Additional results from blood flow experiments performed in our lab show that the treatment of WT mice with the specific inhibitor G4120 completely limits the stability of the clot after FeCl₃-induced injury. G4120 is a RGD-containing cyclic peptide that blocks the binding of ligands to the GPIIb/IIIa integrins on the surface of platelets. The specific blockage of GPIIb/IIIa, the receptor for Fg on the surface of platelets, abolished the formation of permanent and stable platelet clots. An intermediate degree of occlusion was seen in injured Fg−/− mice arteries. These findings suggest that GPIIb/IIIa could be interacting with other adhesive proteins such as fibronectin and vWF. We speculate that these secondary interactions between GPIIb/IIIa and adhesive proteins,
other than Fg, support platelet adhesion to some degree. This will allow for the clot to form however, the limited strength of these interactions restrict the stability of the clot. As expected, the injured carotid arteries on WT mice display a steady clot growth with relatively permanent lumenal occlusion (manuscript in preparation). Other studies performed in Fg\(^{-/-}\) mice subjected to FeCl\(_3\)-induced chemical injury of mesenteric arterioles result in the formation of non-occlusive clots (46). Those observations were confirmed in our blood flow measurements of injured Fg\(^{-/-}\) mice. They also reveal an increased fibronectin platelet content, perhaps indicating the existence of compensatory mechanisms in the lack of fibrinogen in these mice (46). Nevertheless, these studies indicate that the absence of Fg translates into an incapacity for platelets to adhere in a stable fashion, not allowing the clot to grow normally when arterial shear forces are present.

2.5 General discussion

Two different deficient genotypes, FXI\(^{-/-}\) and PC\(^{+/+}\) mice, with potential opposite effects on thrombosis, exhibited greater inflammation after prolonged arterial oxidative injury. Those results reflect the degree of complexity and the delicate regulatory balance that exists among the proteins in the coagulation cascade. Of particular interest is the influence on inflammation that the partial lack of PC has on these arteries in the presence of low levels of thrombin. Such observations extend the anti-inflammatory properties of aPC beyond the control over thrombin, and in consequence fibrin, generation. This aspect may involve effects of aPC on ECs behavior independent of its effects on thrombin
generation. Perhaps such effects are related to its interactions with PAR and EPCR on ECS and deserves further studies.

Overall, WT carotid arteries display a delayed response to copper-induced injury when contrasted with FXI$^+$ and PC$^{-/-}$ arteries. In the FXI$^+$ case, at both early and late time points, the arteries had a greater inflammatory response to the extent of developing thicker arterial walls. It is not known how this increased inflammatory response in FXI$^+$ arteries would comparatively evolve after 21 days of oxidative injury. The initial response in this model demonstrated some major differences ranging from endothelial behavior to generalized enlargement of the arterial wall. Considering the phenotypical findings in the arteries and the liver of resting FXI$^-/$ mice, predisposition for augmented endothelial damage was expected after copper-oxidative injury. The fact that the damage affected the entire carotid artery due to a generalized increased inflammation was a novel observation. These findings revealed the importance of FXI in regulating leukocyte diapedesis in arteries and a link between this coagulation factor and inflammation.

A synthesis of initial sequential events in this oxidative model would include the following stages:

1- Secretion of inflammatory mediators such as IL-6, IL-1B, KC and TNF$\alpha$ released by SMCs, ECs and even fixed macrophages from the reticuloendothelial system upon exposure to Cu$^{2+}$ ions.

2- Neutrophil recruitment/activation with attachment and rolling on the endothelium through ICAM-1, VCAM-1 and E and P selectin (51).

3- Docking of neutrophils and adherence to ICAM-1 via LFA-1 and Mac-1. Followed by, increased intracellular Ca$^{2+}$ in ECs and the consequential cytoskeletal changes (52,53).
4- Migration of neutrophils through a paracellular or transcellular route. A transcellular route is mostly favored under ICAM-1 overexpression otherwise the classical paracellular traffic is the preferred route (54,55).

5- Formation of endothelial domes that encapsulate the migrating neutrophils or other leukocytes during diapedesis. Endothelial domes are structures enriched with ICAM-1 and VCAM-1 that surround the leukocyte during migration, limiting the transference of extra and intracellular content during this process (56,57).

6- Rearrangement of LFA-1 and ICAM-1 around the cell junctions so changes in permeability are minimized during leukocyte transit through the endothelium (58,59).

In the case of FXI−/− mice arteries, the increased endothelial permeability, seen in resting animals, facilitates the transit of leukocytes through the first vascular layer of the carotid arteries. The result is a greater neointima and further invasion of subsequent arterial layers like the media. Whereas in the PC+/− mice arteries the reduced aPC activity could increase endothelial permeability through a diminished control on actin fibers reorganization and activation of PAR-1 receptor induced by increased levels of thrombin or other alternative mechanism. Higher levels of thrombin derived from the non-regulated activation of FVIII and FV expected in a limited presence of aPC. The final result is an increased generation of fibrin and therefore induction of more inflammation.

Several attempts were made to extract mRNA from the injured arterial area to generate quantitative gene expression data, however only picograms of starting material were obtained. This technical limitation makes it difficult to continue an extended mechanistic study of the injured arteries with this model.
Lastly, the effects in mice and humans of a fibrinogen deficiency are well known. Compromised pregnancy and excessive bleeding due to certain Fg molecule defects are part of the generalized observations seen in mice and humans. We showed the impact that this protein deficiency has in the structure and size of the platelet clot. Additional experiments using the G4120 inhibitor supports the interaction of the fibrinogen receptor with other adhesive proteins that constitute the platelet clot, also suggested by others (47). The presence of such interactions was not fully compensatory for the lack of Fg in the platelet clot. Although it is not known if the higher degree of platelet activation observed in the Fg⁻/⁻ deficient clots is a direct effect of P-selectin expression levels or increased molecular traffic between platelets, this observation merits further research.

The role of fibrinogen and inflammation in atherosclerosis in humans is well known; however, it is not yet clear if the role of fibrinogen in its progression is highly specific, or just a contributor to the overall processes (36). The enhanced angiogenic response seen in the carotid arteries of PC⁺⁻ mice showed the impact of fibrinogen deposition in our model. This indicator could be used for prognosis and as a monitoring tool for the progression of carotid atherosclerosis.

By using a FeCl₃-induced injury, we were able to evaluate the immediate impact of the lack of Fibrin(ogen) in the clot structure, the degree of platelet activation their separation, and clot stability. These observations may have applications for evaluating the impact of Fg in the development of prothrombotic and proinflammatory processes of human diseases. Similar measurements, performed on clots induced in Fg⁻/⁻ mice, and consisting of different Fg concentrations (i.e. from physiological levels to very low
concentrations) would allow for the generation of a model that correlates the distance between platelets and area between them with the levels of fibrin in plasma. Such correlation could be used as an indicator for clot stability and prothrombotic conditions. Evaluation at a fine level of the clot structure may be use in the future as an indicator for effectiveness of antithrombotic therapies.
Fig. 2.2) SEM of carotid arteries at day 7 post-copper cuff implantation. Noticeable differences are seen in the number of leukocytes adherent to the endothelia of WT (A) and PC$^{+/-}$ (B) mice. A detail of the top left corner area seen in micrograph B, shows the attachment of several leukocytes to the endothelium, one of them undergoing diapedesis. (n=4 for each group).
Fig. 2.3) Toluidine blue stained plastic cross sections of WT and PC<sup>+</sup>_− carotid arteries 7 days post-silicon-copper-cuff implantation. Observe the enlargement of the media layer of the arterial wall (m) and the prominent vascularization of the adventitia (arrows) in the PC<sup>+</sup>_− arteries (right) when compared to the WT control (left). Inflammatory cells are abundantly seen as dark blue-round dots in both sections. (n=3 per group). Mag. bar 100 µm. Mag. 10X.
Fig. 2.4) TEM of WT and PC<sup>+</sup>/<sup>-</sup> carotid arteries at day 7 post-copper cuff implantation. (A) Intima and media of PC<sup>+</sup>/<sup>-</sup> mice. Observe the endothelium with partially detached (arrows) and deformed EC. Some invasive neutrophils (*) are seen in diapedesis towards or in the medial compartment. Also, round-shaped SMC with reduced cellular prolongations (S) and cytoplasmic fat (f) accumulations are evident. (B) Intima and media of WT mice. A continuous and slightly changed endothelium is observed. Invasive neutrophils (n) are often found attached to the endothelium, migrating through it or in the media. The SMCs and ECM in the media conserve their typical appearance. (C) WT mouse adventitia. A large accumulation of apoptotic neutrophils (n), cellular debris (-D-), fat droplets and fibrin(ogen) (*) are found in this layer. (D) Adventitia of PC<sup>+</sup>/<sup>-</sup> mice. An abundant presence of scavenger macrophages (m) containing cytoplasmic fat accumulations and invasive neutrophils (n) are seen intermixed with fibrin(ogen) deposits (*). Some of the neutrophils are undergoing apoptosis (-A-). Functional neovessels are often seen in this layer, containing RBCs and platelets (inset). (n=3 for each group). All magnification bars = 5 µm.
Fig. 2.5) ELISA results of TAT plasma levels for WT and PC<sup>+-</sup> mice. No significant differences were found between the genotypes. (n=6 mice per group).
Fig.2.6) Toluidine blue stained plastic cross sections of WT and PC<sup>+/−</sup> carotid arteries 7 days post-silicon-copper-cuff implantation and lepirudin treatment. Observe the enlargement of the medial layer (m) in the PC<sup>+/−</sup> arteries (right) when compared to the WT controls (left). Also, notice the absence of vessels in the adventitia layer (a) for both genotypes. (n=4 mice per group). Mag. bar 100 µm. Mag. 10X.
Fig. 2.7) TEM of carotid arteries at day 21 after copper cuff implantation. A.- Intima of PC<sup>+/−</sup> mice. A thin endothelium of highly invaginated and vacuolated EC is seen (EC), covering an engrossed neointima composed of SMCs with synthetic phenotype (*) surrounded by a collagenous matrix. Some detachment of the EC from the basal lamina can be observed (arrowhead). B.- Intima of WT mice. An endothelium of rounded and very active EC is seen, followed by a neointima of SMC embedded in a collagenous and proteoglycan rich matrix. EC present numerous mitochondria (*) and an unusually engrossed basal lamina (arrows). C and D neointima of PC<sup>+/−</sup> mice; C.- An EC with abundant cellular prolongations incursions into the neointima to form microvessels are observed in this layer (arrow); elastic lamina (E). D.- Elongated SMC (S), neutrophils (n) and eosinophils (e) are often found in the neointima of these mice. E.- WT neointima. Abundant mitochondria and RER of the SMCs that compose this layer (arrowheads) can be observed; elastic lamina (EL). The arrow indicates the direction of the arterial lumen. F.- Media of PC<sup>+/−</sup> mice. SMCs appear with cellular processes (S). Elongated EC (EC) with multiple invaginations and neutrophils (n) are often present throughout this layer. RBCs (r) are also present in areas of neovessel formation. Clear spaces (c) in the ECM and surrounding the EC are seen. Clusters of neutrophils and foam cells are occasionally found in the media (inset). G.- WT media. Rounded and highly active SMC with few myofibrils (S). Cytoplasmic fat accumulations in the SMC (arrow) are noted. H.- Adventitia of PC<sup>+/−</sup> mice. Cellular debris (D) and deposits of fibrinogen (arrows) are seen in abundance. Invasive neutrophils (n) are commonly found throughout the area. I.- WT adventitia with deposits of fibrinogen (arrow). Invasive neutrophils (n) and neutrophil remnants (r) are seen in the same area. Fat droplets, abundant cell debris, and foam cells (fc) can also be observed. (n=4 mice per group). All magnification bars = 5 µm.
Fig. 2.8) TEM of carotid arteries at day 7 post-copper cuff implantation. A. FXI$^+$ intima. Notice the overall detachment of the EC layer (EC) from the elastic lamina (EL) and the invasion of neutrophils (n) to the subendothelial space. B. WT carotid. Observe the maintenance of the attachment of the EC layer (EC) to the elastic lamina (EL) in the presence of migrating neutrophils (n). A and B, mag. bar 5μm. C. FXI$^+$ media. Observe the presence of fat accumulations (*), transformed SMCs (arrow) and neutrophils (n) in this arterial layer. Detail: a focal group of neutrophils found in the media. D. WT media. SMCs conserve their typical elongated shape with contractile cell prolongations (arrows). Notice as well the lack of enlargement of the medial layer and the smooth elastic lamina (EL). (n=4 mice per group). C, D and detail mag. bar 2μm.
Fig. 2.9) TEM of carotid arteries at day 21 post-copper cuff implantation. A. FXI<sup>f</sup> intima. A detached and amorphous ECs (EC) layer appears covering numerous neutrophils (n) in process of diapedesis. Highly amorphous SMCs from the neointima are also seen containing cytoplasmic vacuoles (arrows). B. WT intima. An irregular but continuous EC layer (EC) is seen with attached and subendothelial neutrophils. C. FXI<sup>f</sup> neointima. Multilayered neointima rich in SMC (S) and neutrophils (n). Arrow indicates the direction of the arterial lumen. D. WT neointima. Multilayered neointima with semi-synthetic SMC (S) and abundant collagenous ECM (cg). Neutrophils are also frequently found (n). E. FXI<sup>f</sup> media. Notice the rounded shaped and fully synthetic SMC (S) and the presence of numerous neutrophils (n). F. WT media. Normal, elongated and contractile SMCs (S) surrounded by a typical and regular collagenous matrix (cg). G. FXI<sup>f</sup> adventicia. Notice the abundant number of neutrophils in this layer. H. Fibroblasts (f) and occasional neutrophils (n) are seen in the collagen rich adventitial parenchyma. (n=4 mice per group).
Fig. 2.10) Overall view of FeCl₃-induced platelet arterial clots. A.- WT clot. An extensive platelet clot appears covering a large area of the arterial lumen. Platelets appear compacted among each other conserving noticeable amounts of granular content (p). Fibrin fibrils are seen intermixed with platelets (arrows). Deformed RBCs trapped from the circulation during clot formation are also seen (r). The elastic lamina appear in direct contact with the platelets after endothelium denudation by FeCl₃-induced damage (E). B.- Fg⁺ clot. A small size platelet clot is observed attached to the damaged endothelium (E). Notice the full activation of the platelets mostly lacking any granular content and the increased space among them (p). RBCs are seen normal in shape as part of the arterial circulation (r). Observe the absence of electron-dense plasma proteins and fibrin in the clot interstitial spaces (i). (n=3 mice per group). Mag bar 4 µm. Mag. in A and B, 2500X.
Fig. 2.11) Detailed view of platelet clots by genotype and treatment. A) WT clot. B) Fg\(^{-/-}\) clot. Notice the greater interstitial space among platelets when compared to the WT counterpart. C) Fg\(^{-/-}\) i.v. clots. Observe the restoration of the degree of compactness seen in the WT controls. (n=3 mice per group). Mag. bar 0.5μm, all images: 20,000X.
Fig. 2.12) Average surface area/total area ratio among platelets. Observe the significantly greater area existing among platelet in the total absence of Fg. (n=7 for WT or Fg\(^{-/-}\) i.v, n=22 for Fg\(^{-/-}\) group, N=3 per treated group).
Fig. 2.13) Average distance between platelets. Notice the difference in distance and variability of distance from platelet to platelet in the absence of Fg. (n=573 for WT, 900 for Fg−/− and 504 for Fg−/− i.v, N=3 mice per group).
2.6. Appendix A:

Power analysis: P=power, K=number of groups, n=average group sample size, 
$\alpha$=probability of rejecting a true null hypothesis, effect size=ratio of SEM to Sd.
2.7. References


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