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Identification of Multiple Levels of Trauma Induced Coagulopathy

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Identification of Multiple Levels of Trauma Induced Coagulopathy

A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy at Virginia Commonwealth University.

by

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List of Abbreviations

12-HETE	12-hydroxyeicosatetraenoic acid
12-LOX	12-lipoxygenase
5-HT	serotonin
A-V	annexin-V
α2-AP	α2-antiplasmin
α2β1	α2β2 integrin
α5β1	α5β2 integrin
α6β1	α6β2 integrin
α8β1	α8β2 integrin
αVβ3	αVβ3 integrin
αIIβ3	αIIβ3 integrin
AA	arachadonic acid
AA	arachadonic acid
ABP	actin binding protein
AC	adenylyl cyclase
ACoT	acute coagulopathy of trauma
ADP	adenosine diphosphate
AGG	aggregates
ALB	albumin
ALP	alkaline phosphatase

ALT	alanine aminotransferase
AMY	amylase
ANOVA	analysis of variance
APC	activated protein C
APS	activated protein S
Asc A	ascorbic acid
AT	anti-thrombin
ATP	adenosine triphosphate
B	blunt
B/P	blunt and penetrating
BD	base deficit
BE	base excess
BPD	diastolic blood pressure
BPS	systolic blood pressure
BUN	blood urea nitrogen
Ca2+	Calcium ion
cAMP	cyclic adenosine monophosphate
CAT	calibrated automated thrombogram
CCO	continuous cardiac output
CEM	clot elastic modulus
CFT	clot formation time
cGC	cytosolic guanylate cyclase

cGMP	cyclic guanosine monophosphate
cGMP	cyclic guanosine monophosphate
cMP	circulating micro-particle
COHb	carboxyhemoglobin
Coll	collagen
COT	coagulopathy of trauma
COX-1	cyclooxygenase 1
$cPLA_2$	cytosolic phospholipase A_2
Cre	creatinine
CRP	collagen reactive peptide
CT	closure time (PFA-100)
CT	Clotting Time (ROTEM)
ctO_2	total blood O_2
CVP	central venous pressure
CVX	convulxin
D80	oxygen debt = 80 mL/kg
DAG	1,2-diacylglycerol
DIC	disseminated intravascular coagulopathy
ECG	electrocardiograph
ECoT	early coagulopathy of trauma
EPCR	endothelial cell protein C receptor
EPI	epinephrine

$etCO_2$	end-tidal CO_2
FAK	focal adhesion kinase
FDP	fibrin degradation product
FDP	freeze dried plasma
FFP	fresh frozen plasma
FIB	fibrinogen
FII	Factor II
FIV	Factor IV
FIX	Factor IX
FOT	force onset time
FpA	fibrinopeptide A
FpB	fibrinopeptide B
FSC	forward scatter
FV	Factor V
FVII	Factor VII
FVIII	Factor VIII
FWB	fresh whole blood
FX	Factor X
FXI	Factor XI
FXII	Factor XII
FXIII	Factor XIII
Gα12	G protein alpha subunit 12

Gα13	G protein alpha subunit 13
Gαi	G protein alpha subunit i
GAP	GTPase-activating protein
Gαq	G protein alpha subunit q
Gαz	G protein alpha subunit z
Gβγ	G protein beta-gamma subunit
GC	guanylate cyclase
GCS	Glasgow Coma Scale
GDP	guanosine diphosphate
GLOB	globulin
GP	glycoprotein
GPCR	G protein coupled receptor
GRA	granulocytes
GTP	guanosine triphosphate
H_2O_2	hydrogen peroxide
HAS	hemostasis analyzer system
Hb	hemoglobin
HC	hypercoagulability
HCT	hematocrit
HGB	hemoglobin
HMWK	high molecular weight kininogen
HSP27	heat shock protein 27

INR	International Normalized Ratio
IP3	inositol 1,4,5-trisphosphate
IP3R	IP3 receptor
IQR	interquartile range
IRB	Institutional Review Board
ISS	Injury Severity Score
ISTH	International Society on Thrombosis and Haemostasis
ITAM	immunoreceptor tyrosine-based activation motif
KD	kinase domain
LAT	linker of activated T-cells
LTA	light transmission aggregometry
LYM	lymphocytes
MA	maximum amplitude
MAP	mean arterial pressure
MAPK	mitogen-activated protein kinase
MAPKK	mitogen-activated protein kinase kinase
MAPKKK	mitogen-activated protein kinase kinase kinase
MCF	maximum clot firmness
MethHb	methemoglobin
MFI	mean fluorescent intensity
MKK	MAP kinase kinase
MLC	myosin light chain

MLCK	myosin light chain kinase
MLCK	myosin light chain kinase
MOD	multiple organ dysfunction
MON	monocytes
MPase	myosin phosphatase
NO	nitric oxide
NOS	nitric oxide synthase
NS	normal saline
NW	non-white
O_2^-	superoxide
OD	oxygen debt
P	penetrating
PA	plasminogen activator
PAI-1	plasminogen activator inhibitor 1
PAP	pulmonary artery pressure
PAR	protease activated receptor
PBS	phosphate buffered saline
PCF	platelet contractile force
pCO_2	partial pressure CO_2
PDE	phosphodiesterase
PDE3	phosphodiesterase 3
PDE5	phosphodiesterase 5

PE	phosphatidylethanolamine
PECAM-1	platelet/endothelial cell adhesion molecule 1
PGE_1	prostaglandin E_1
PGE_3	prostaglandin E_3
PGG_2	prostaglandin G_2
PGH_2	prostaglandin H_2
PGI_2	prostacyclin
PH	pleckstrin homology domain
PH	pre-hospital
PI3K	phospoinositide 3-kinase
PIP_2	phosphatidylinositol 4,5-bisphosphate
PIP_3	phosphatidylinositol-3,4,5-trisphosphate
PIP_5K	phosphatidylinositol 4-phosphate 5-kinase
PKA	protein kinase A
PKA	protein kinase A
PKB	protein kinase B
PKC	protein kinase C
PKD	protein kinase D
PKG	protein kinase G
PL	phospholipid
PL	phospholipid
PLCβ	phospholipase C β

PLCγ_2	phospholipase C γ_2
PLT	platelet
pMP	platelet microparticle
pO_2	partial pressure O_2
PPP	platelet poor plasma
PRP	platelet rich plasma
PS	phosphatidylserine
PT	prothrombin time
PT	prothrombin time
PTT	activated partial thromboplastin time
RAIM	Rap1 interacting adapter molecule
RBC	red blood cells
RESP RATE	respiratory rate
rFVIIa	recombinant FVIIa
RGD	arginine-glycine-aspartic acid
RGS	regulator of GTPase-activating signaling proteins
Rho	Ras homology
Rho-GEF	Rho-guanine nucleotide exchange factor
ROCK	Rho-associated protein kinase
$ScvO_2$	mixed venous hemoglobin saturation
SDP	spray dried plasma
SFK	serine family kinase

sGC	soluble guanylate cyclase
SHIP1	SH2-containing inositol phosphatase 1
sO_2	O_2 saturation
SSC	side scatter
t-PA	tissue-type plasminogen activator
TAFI	thrombin activatable fibrinolytic inhibitor
TAT	thrombin-anti-thrombin complex
TBI	traumatic brain injury
TBIL	total bilirubin
TEG	thromboelastography
TF	tissue factor
TFPI	tissue factor pathway inhibitor
TIC	trauma induce coagulopathy
TM	thrombomodulin
TP	total protein
TSP	thrombospondin
TxA_2	thromboxane A_2
u-PA	urokinase type plasminogen activator
u-PAR	urokinase type plasminogen activator receptor
VASP	vasodilator-stimulated phosphoprotein
VCUMC	VCU Medical Center
Vit C	Vitamin C

VO_2	volume of oxygen consumed
vWF	von Willebrand factor
W	White
WB	whole blood
WBA	whole blood aggregometry
WBC	white blood cell
WP	washed platelets
X	xanthine
XO	xanthine oxidase

Abstract

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Major Directors: Directors: Donald F. Brophy, Pharm.D.
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Trauma continues to be a major cause of death across the globe. While the exact causes of trauma differ greatly between the military and civilian lifestyles, the ability to stop bleeding after trauma is paramount for survival. Over the past decade coagulation research has transitioned from a classical understanding of plasma based protein coagulation to the current cell focused research. As part of this shift, platelets have become a central player in hemostasis. Unfortunately little is currently understood about how platelet function is affected by trauma.

In an effort to better define platelet function during trauma and the resulting shock from exsanguination, a multipronged approach was developed. The hypothesis that the introduction of a state of clinical shock in a controlled environment would allow for an in-depth assessment of trauma-induced coagulopathy led to the development of a swine based model of hemorrhagic shock.

In this model a composite injury consisting of soft tissue damage, long bone fracture, and controlled hemorrhage was used to induce a moderate state of

hypovolemic shock. As a result of this injury the animals showed both the beginning of a plasma protein consumption coagulopathy as well as kinetic quickening in the clotting process. These surprising results show competing up-regulation and down-regulation of the coagulation system in response to trauma induced shock.

To better define the effect of polytrauma on platelet function in a human population a clinical study was conducted. The hypothesis behind the development of this study was that the examination of platelet function during polytrauma would lead to a more complete understanding of the effects of trauma on hemostasis.

This study resulted in the identification of two separate but not mutually exclusive coagulopathies in response to trauma. The first was the traditional consumption based coagulopathies recently suggested to be varying degrees of disseminated intravascular coagulopathy. The second was a development a hypercoagulable state that may be attributed to increased platelet function.

The identification of these two competing coagulopathies in separate models highlights the inadequacies of the current plasma based clinical testing, and the need for increased whole blood testing in the trauma treatment environment.

Chapter 1:

Introduction and Background

1.1 Significance of Hemostasis in Traumatic Injury

Trauma is a major cause of death across the world, both in the civilian world as well as during military conflicts.[1,2] Uncontrolled hemorrhage accounts for 40% of civilian trauma deaths, and in conjunction with the resulting shock represents the major causes of preventable deaths on the battlefield.[2–4] These facts highlight that hemostasis is a critical physiological response to trauma. The ability for blood to clot is paramount to avoid exsanguination once the vasculature is disturbed. Our understanding of the biological coagulation response has evolved over the years, with initial theories revolving around a cascading series of enzymatic reactions of plasma based proteins and current models integrating the cellular aspect of clot propagation.

Currently there is little agreement on the actual specifics related to the coagulation changes that occur early after trauma. Recent work by Gando performed on behalf of the Scientific and Standardization Committee on DIC of the International Society on Thrombosis and Hemostasis (ISTH) highlights the current gaps in understanding around trauma related coagulopathies. While this work focused mainly on the distinction between coagulopathy of trauma (COT), acute coagulopathy of trauma-shock (ACoT), and disseminated intravascular coagulation (DIC), it highlights the need for better understanding of the mechanisms behind the pathologies.

To further add to the complexity, the observation of hypercoagulopathy (HC) in recent studies performed on whole blood show that the current methods of defining and treating pathologies based on plasma tests may not be sufficient to truly discriminate between the subtle changes that occur to hemostasis in response to trauma.[5] An increased understanding of whole blood coagulation in response to trauma may lead to both improved diagnosis of coagulopathies and more effective treatment strategies to treat or even prevent trauma related coagulation disorders.

1.2 Literature Review

Plasma Proteins and the Coagulation Cascade

The first published mention of the coagulation cascade came in 1964, as a theory to describe the newly standardized terminology of blood clotting factors. The International Committee on Blood Clotting Factors adopted a roman numeral terminology scheme clearing up the confusion caused by synonyms generated through separate lines of research.[6] This initial system (Figure 1.1) is essentially

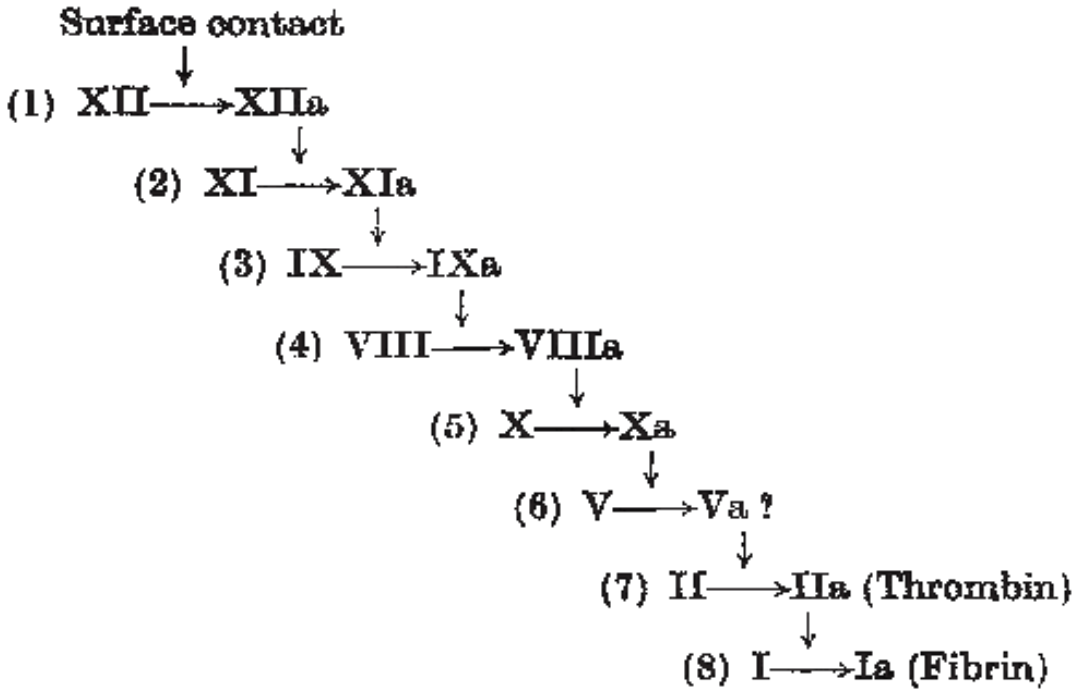

Figure 1.1 Original Coagulation Cascade. Adapted from MacFarlane 1964.[6]

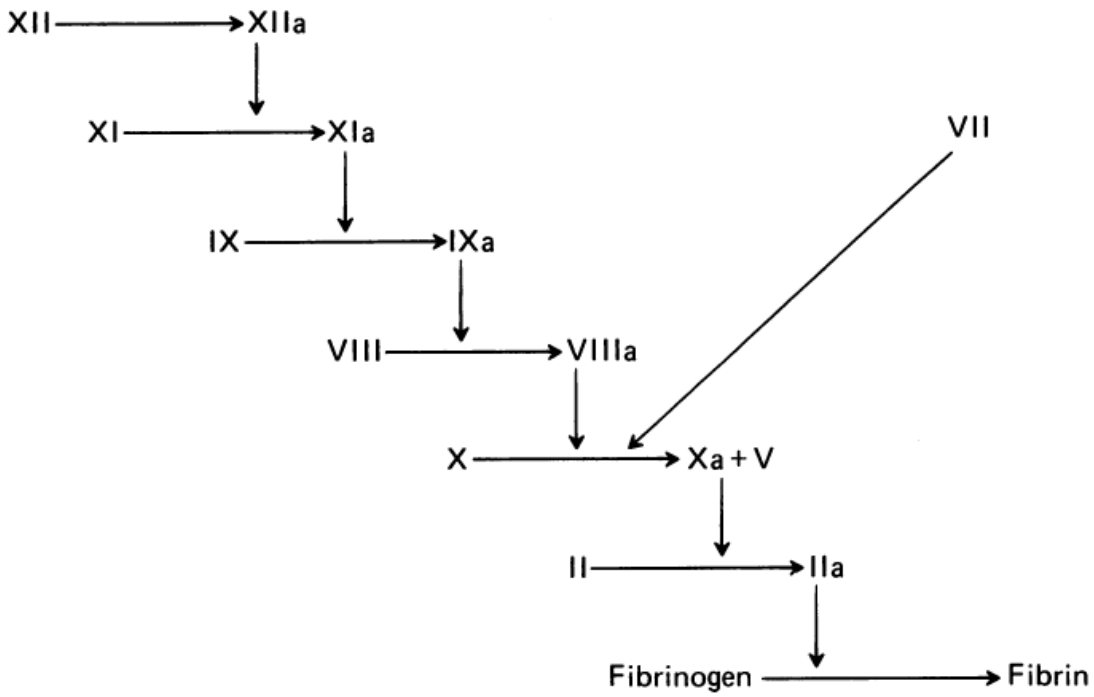

Figure 1.2: Currently Accepted Coagulation Cascade. Adapted from Hayne 1976.[7]

correct in the stepwise activation of coagulation due to surface contact, but completely ignores Factor VII (FVII) as well as Factor XIII (FXIII). Interestingly enough the requirement of phospholipid was also discussed even in these early stages, almost foreshadowing the development of the cell based model of coagulation. The identification of the extrinsic pathway and the interaction of FVII with membrane bound tissue factor (TF) was soon to follow.[8] While not always shown as part of the coagulation cascade it has been know since the mid 1960s that Factor XIII (FXIII) was required to stabilize the fibrin network generated by the cascade, as well as the fact that thrombin activates FXIII activity.[9]

By the 1970s the coagulation cascade included FVII in most printed articles (Figure 1.2).[7] During the development of the understanding of the coagulation proteins, various tests were developed to test the effectiveness coagulation and some even resulted in diagnostically relevant tests. Publications all the way back to 1916 refer to the re-calcification of plasma as testing the prothrombin time (PT). In 1935 Dr. Armand Quick published the version of the PT test that's is still used today, albeit with some minor modifications.[10]

The activated partial thromboplastin time (PTT) test developed in the late 1950s, but the use of cephalin didn't appear in literature until 1962.[11] Each test was developed to test what was thought as independent methods of coagulation cascade activation.[7]

PT is designed to test the extrinsic pathway, also known as the tissue factor (TF) pathway. The pathway moves stepwise with TF activating FVII, FVIIa ("a" was adopted to denote activated factors when the roman numeral system was adopted)

then moves to activate Factor X (FX). FXa begins what is called the common pathway in the cascade. FXa combines with activated Factor V (FVa) to activate Factor II (FII). Factor II is also called prothrombin, and FIIa thrombin. Thrombin then cleaves fibrinogen to fibrin, ending what is typically considered the coagulation cascade (Figure 1.3).[7] While still used diagnostically today, the fact that PT measures the activity of FVII, FX, FV, thrombin, and fibrinogen as a system.[7] This limits the usefullness of the test to determine the actual cause of a perturbance of clotting. The same can be said of PTT testing.

PTT is designed to test the activity of the intrinsic pathway as a whole. The intrinsic pathway depends on the exposure of Factor XII (FXII) to a charged surface. Once activated FXIIa activates Factor XI (FXI), FXIa activates Factor IX (FIX), FIXa activates Factor VIII (FVIII), and FVIII then activates FX, then following the same common patway mentioned above (Figure 1.4). PTT evaluates the activity of all of the proteins in the coagulation cascade except for FVII.[7] While both of these tests can identify problems in the respective pathways, additional testing must be performed to determine individual factor concentrations and activities to determine the actual underlying problem. Also there are multiple amplification loops, requirements for Calcium ions ($Ca^{2}+$) once called Factor IV, and phospholipid associations that makes the pathway far more complicated (Figure 1.5).[12]

Cell Based Model of Coagulation

While the coagulation cascade provided an easily followed waterfall model of blood clotting, it was unable to account for the phospholipid membrane requirements or the emerging idea of platelets being the main mediators of the

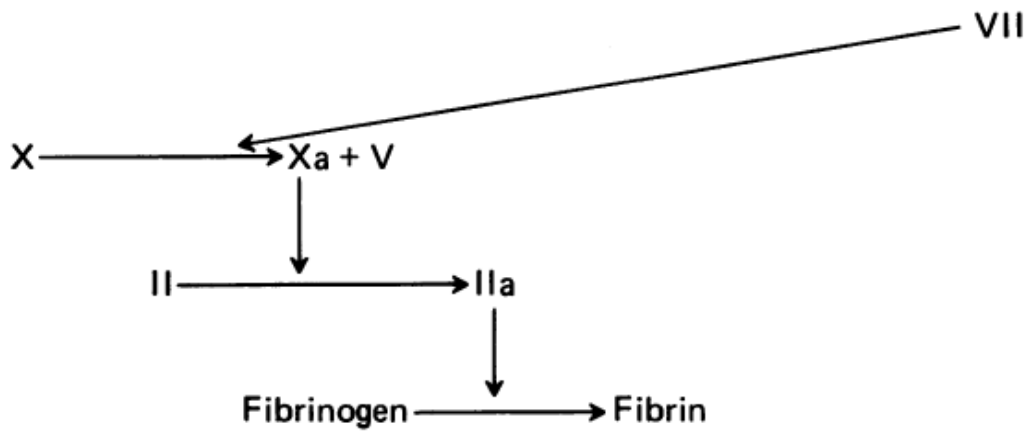

Figure 1.3: Extrinsic Pathway of the Coagulation Cascade. Adapted from Hayne 1976.[7]

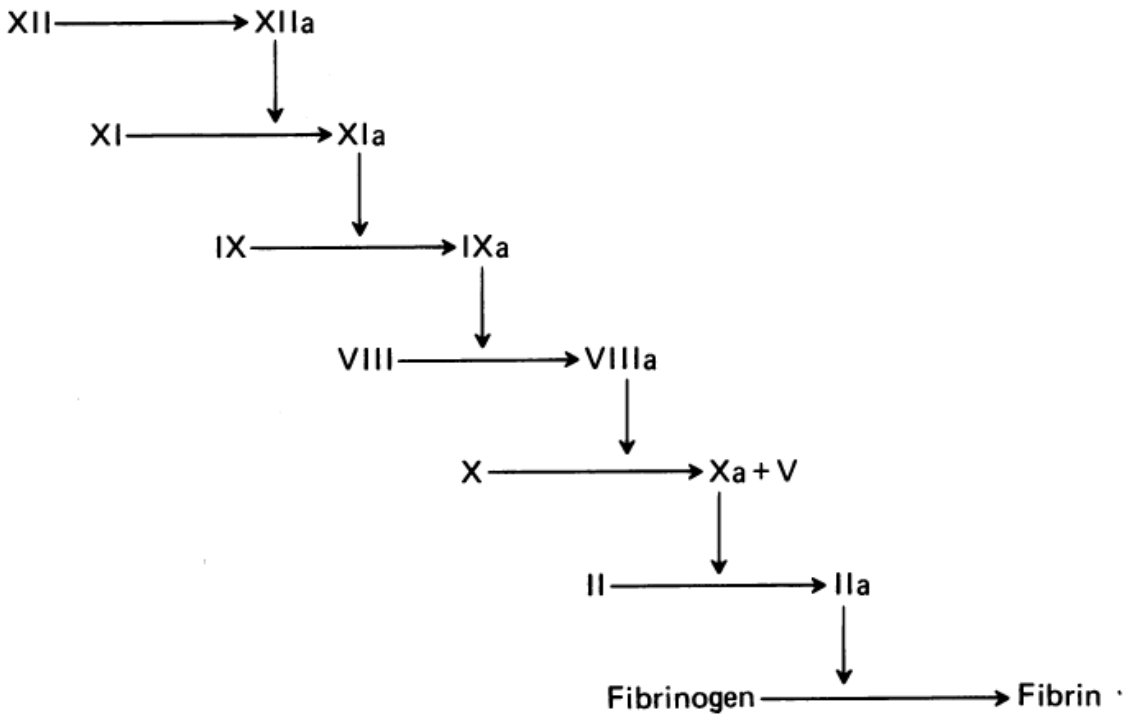

Figure 1.4: Intrinsic Pathway of the Coagulation Cascade. Adapted from Hayne 1976.[7]

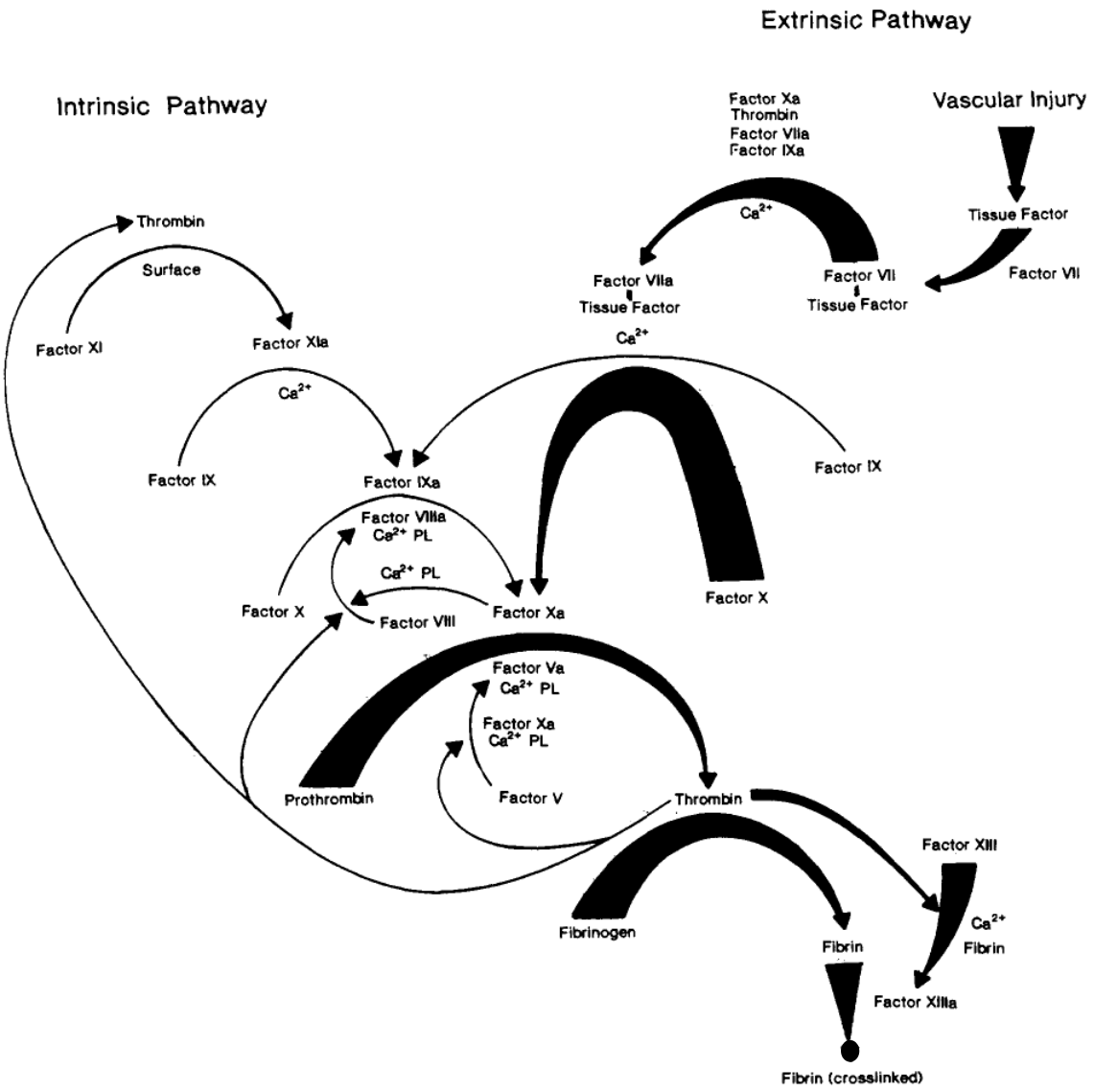

Figure 1.5: Coagulation Cascade with Amplification Loops and Cofactors. PL; phospholipid. Adapted from Davie 1991.[12]

physiological coagulation response. The cell based model of coagulation was introduced to address these issues in 2003 by Maureane Hoffman.[13] This model highlighted the need for cell membrane surfaces to provide the required phospholipid requirement to optimize enzymatic reactions (Figure 1.6). The model was based on the theory that coagulation occurs in three overlapping steps (initiation, amplification, propagation) that take place on different cell surfaces (Figure 1.7). The model emphasizes the assembly of membrane phosphatidylserine (PS) bound factor complexes in activation of the coagulation proteins. PS is an integral component of the platelet membrane inner leaflet.

The tenase complex (sometimes referred to as the intrinsic tenase complex) is composed of FVIIIa and FIXa and is responsible for the generation of FXa.[14] The prothrombinase complex consists of FXa and FVa bound together through a linkage in FVa to PS. The prothrombinase complex is responsible for generation of large amounts of thrombin from prothrombin.[14] Assembly of both these complexes is dependent on the expression of a PS rich pro-coagulant surface on activated platelets (Figure 1.8).

Initiation

Initiation occurs when circulating FVIIa encounters a TF bearing cell in the presence of Ca2+ and activates factor X. TF is normally not in contact with blood until injury or inflammation.[13] There is circulating tissue factor in the blood but it is unable to activate FX. It is thought that either the circulating TF requires cleavage to become activated, or if its lack of PS binding prevents proper tenase assembly.[15]

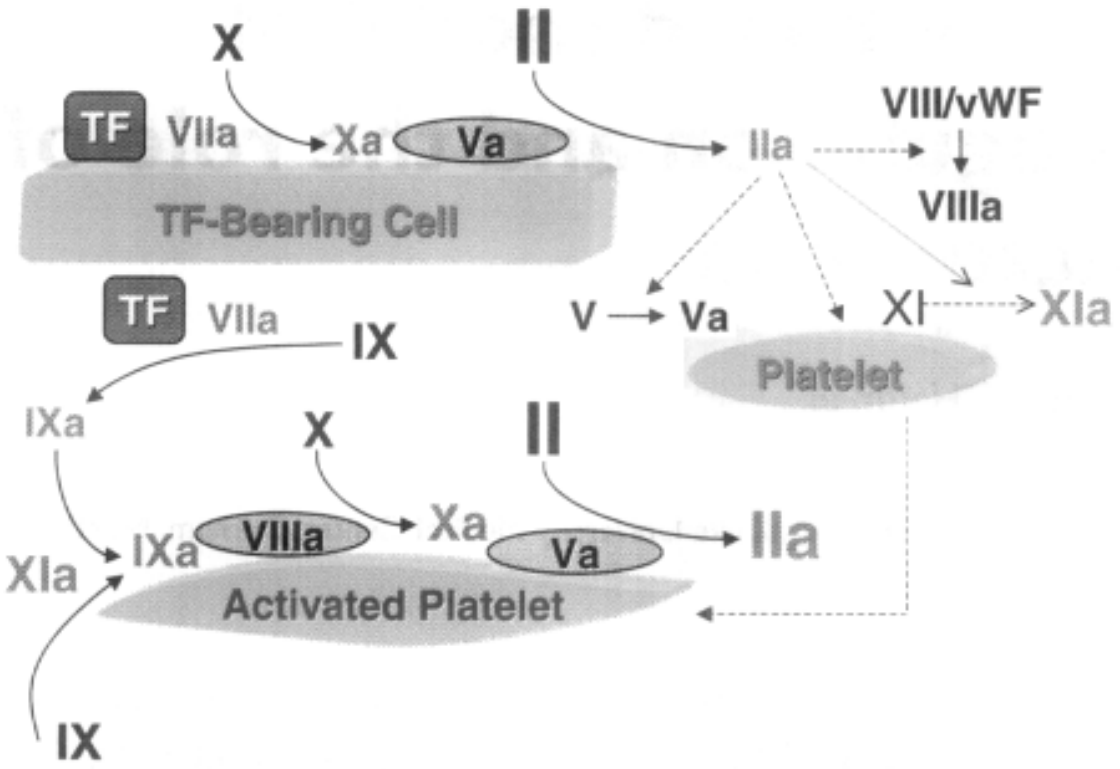

Figure 1.6: Cell Surface Assembly of Coagulation Factors. TF; tissue factor, vWF; Von Willebrand factor. Adapted from Hoffman 2003.[13]

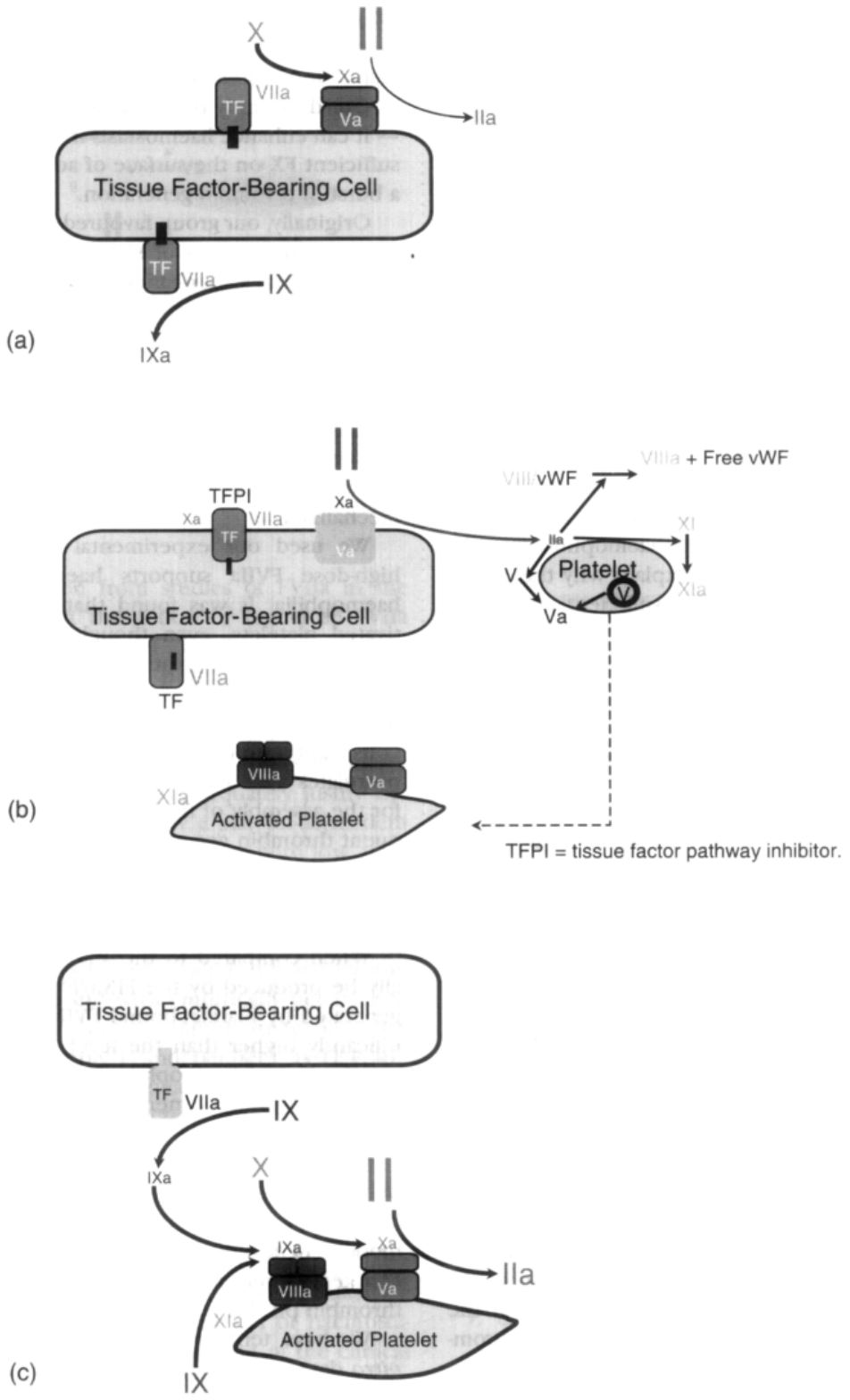

Figure 1.7: Cell Based Model of Coagulation. A) Initiation. **B)** Amplification. **C)** Propagation. TF; tissue factor, vWF; Von Willebrand factor. Adapted from Hoffman 2003.[12]

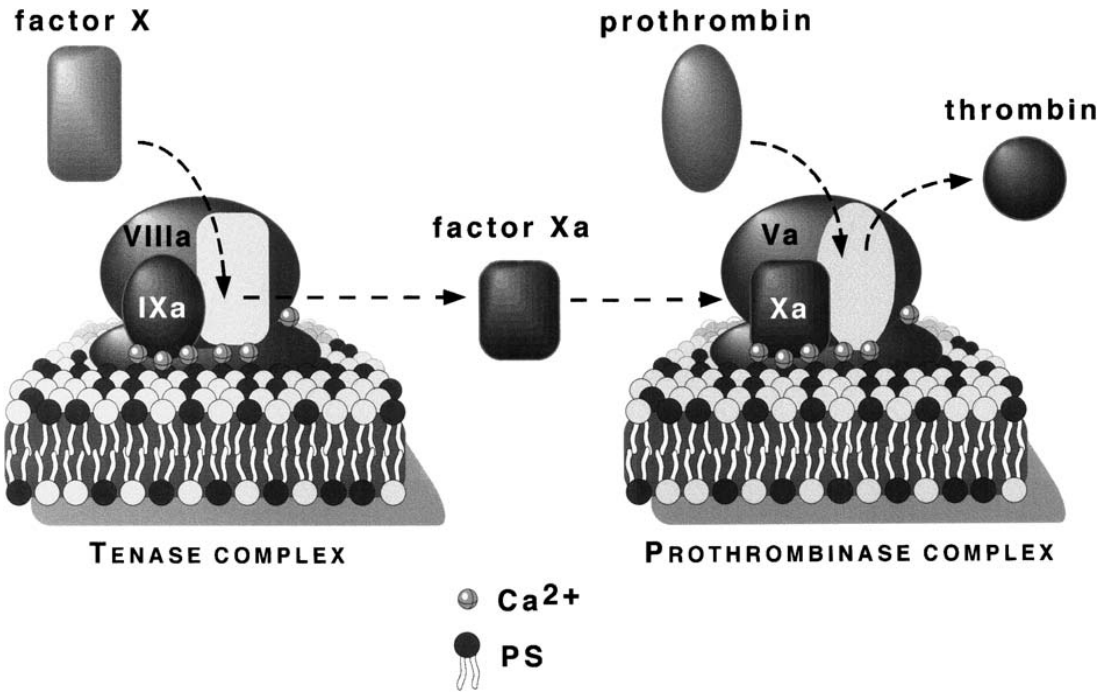

Figure 1.8: Coagulation Enzyme Complexes. PS; phosphatidylserine. Adapted from Zwaal 2004.[16]

FVII is the only factor that circulates in an active form, with basal levels of FVIIa around 1%.[15] The fact that TF, FVII, and FX all are expressed in tissues outside the vasculature suggests that there is initiation occurring in an idling fashion, increasing the ability for the body to respond rapidly at the site of injury.[13]

There are a couple of different regulatory pathways that regulate the initiation phase. First the TF-FVII complex is rapidly shut off by both TFPI and AT. This rapid shut off only generates trace amounts of thrombin.[17] This fits well with the second following amplification step. The other method can be looked at like a tenase / prothrombinase switch. FX and FXa have the same affinity for binding with PS. As FXa is generated it can displace FX from the platelet membrane by competing for the PS binding sites. This allows for the assembly of the prothrombinase complex and the progression into the later steps in the model (Figure 1.7).[18]

Amplification

During the amplification step the platelets begin to ramp up their pro-thrombotic response. Small levels of thrombin generated through initiation diffuse away from the initiation site. This diffused thrombin has multiple effects to the surrounding platelets. It activates platelets through the protease-activated receptors 1 and 4 (PAR1, PAR4) in humans causing the secretion of α and dense granules that contain many autocrine and paracrine factors.[13] It also causes the exposure of PS on the surface of the platelet creating a pro-coagulant surface for complex assembly.[15] Thrombin also activates FXI, FV, and cleaves vWF from FVIII.[17] At the end of the amplification phase platelets activated by the limited release of

thrombin from the amplification phase are clad in activated factors on their surface (Figure 1.7).[13]

Propagation

Propagation is the step of clotting progression where the thrombin burst occurs.[17] During this phase the assembly of the tenase complex continues to occur on adjacent platelets, as well the assembly of the prothrombinase complex. The tenase complex continues to generate FXa, which allows for large-scale assembly of the prothrombinase complex, generating large amounts of thrombin. Along with platelet stimulation by thrombin, this large local concentration of thrombin promotes the cleavage of fibrinogen to fibrin.[13]

Thrombin is also responsible for the activation of FXIII, crosslinking and stabilizing the thrombin clot. It is during this step that hemophilia patients begin to show dysfunction. Both hemophilia A (FVIII deficiency) and hemophilia B (FIX deficiency) cause issues with complex assembly and propagation of thrombin signaling through complex assembly.[17] Phospholipid scrambling due to Scott syndrome also causes inhibition of complex formation to lack of PS exposure. [16]

Coagulation Factors

Although the cell based model stresses the importance of cellular response in the coagulation process, an understanding of the coagulation factors is required to understand the final fibrin clot formation that is required for hemostasis. There are 14 proteins involved in the pro-coagulant response (Table 1.1). When examining the individual proteins in the context of coagulation they can be divided into three

Table 1.1: Plasma Coagulation Proteins

Factor Name	Common Name	MW (kDa)	Description
Factor I	Fibrinogen	340	Adhesive glycoprotein that forms the basis of a fibrin clot
Factor II	Prothrombin	72	Vitamin K-dependant serine protease, main coagulation enzyme
Factor III	Tissue Factor, thromboplastin	37	Lipoprotein, initiator of the extrinsic pathway with Factor VII
Factor IV	Ca++ ion	40.078*	Divalent cation required for multiple coagulation reactions
Factor V	Labile Factor	330	Cofactor for activation of thrombin from prothrombin
Factor VII**	Proconvertin	50	Vitamin K-dependant serine protease, initiator of the extrinsic pathway with Factor III
Factor VIII	Antihemophilic Factor	330	Cofactor for activation of Factor X
Factor IX	Christmas Factor	55	Vitamin K-dependant serine protease, enzyme responsible for the activation of Factor X
Factor X	Stuart-Prower Factor	59	Vitamin K-dependant serine protease, enzyme responsible for activation of thrombin
Factor XI	Plasma thrombospondin antecedent	160	serine protease, enzyme responsible for activation of Factor IX, circulates in complex with high molecular weight kininogen
Factor XII	Hageman Factor	80	serine protease, initiator of the intrinsic pathway with prekallikrein
Factor XIII	Fibrin Stabilizing Factor	320	Transamidase, cross-links fibrin clot
High-Molecular Weight Kininogen***	Fitzgerald, Flaujeac, or William Factor	110	Cofactor, circulates in complex with inactive Factor XI
Prekallikrein***	Fletcher Factor	85	Serine protease, initiator of the intrinsic pathway with Factor XII

* atomic mass units, ** there is no Factor VI, *** no factor number assigned, MW; molecular weight. Adapted from Lefkowitz 2008.[9]

separate pathways. Traditionally these pathways are referred to as the intrinsic, extrinsic, and common pathways.

Extrinsic Pathway

Tissue Factor (Factor III)

Tissue factor (TF) is typically referred to by its common name rather than by its factor number. TF is a 37,000 Da lipoprotein that is the main initiator of the extrinsic pathway.[10] TF is expressed constitutively on the cell membrane of subendothelial fibroblasts, epithelial cells of the skin and mucosa, stroma cells in the endometrium, and astrocytes in the brain.[19–21] TF combines with Factor VII (FVII) to activate the initiation phase of the cell-based model of coagulation. TF serves as the cell based receptor of circulating F VII.[22] Once bound to circulating FVII TF activates FVII (FVIIa) and accelerates the activation of circulating FVII.[22] Tissue factor is also referred to as CD142 and thromboplastin.

Proconvertin (Factor VII)

Proconvertin is typically referred to as Factor VII (FVII). FVII is a 50,000 Da vitamin K dependent serine protease. It combines with TF on the membrane of TF bearing cells and becomes activated (FVIIa).[23,24] FVIIa activates two separate clotting factors (Factor X and Factor IX) via proteolytic mechanisms that cleave an activation peptide.[22] As discussed above it should also be noted that small amounts of FVII circulate in the active form. It is the only clotting factor to do so.[20]

Intrinsic Pathway

Prekallikrein (Fletcher Factor)

Prekallikrein is an 80,000 Da serine protease that is involved in the initiation of the intrinsic pathway in conjunction with Factor XII.[10] Deficiency in Prekallikrein causes increased PTT measurements, but has no thrombotic or bleeding phenotype.[10] Once activated, kallikrein is able to activate plasminogen through the urokinase type plasminogen activator (u-PA).[25]

Hageman Factor (Factor XII)

Hageman factor is commonly referred to as Factor XII. It is an 80,000 Da serine protease that is commonly considered the start of the intrinsic pathway.[10] Deficiency in FXII does not result in either a bleeding or thrombotic phenotype.[9] Factor XII can be activated by either the serine protease kallikrein or spontaneously when the blood is exposed to a negatively charged surface. In a positive feedback loop FXIIa can act on Prekallikrein cleaving it to kallikrein, which in turn can activate more factor XII. FXIIa is also responsible for the activation of Factor XI as well as its disassociation from high molecular weight kininogen (HK).[9]

Plasma Thromboplastin Antecedent (Factor XI)

Plasma thromboplastin antecedent is commonly referred to as Factor XI (FXI). It is a 160,000 Da serine protease that is involved in the intrinsic activation of Factor IX.[9] FXI binds both specifically and reversibly to GP Ibα in the presence of either high molecular weight kininogen and Zn2+ or prothrombin and Ca2+.[26] This binding appears to occur only on activated platelets and is localized to lipid rafts on

the membrane surface.[27] This binding then serves to promote activation of FXI by thrombin.[26,27]

Christmas Factor (Factor IX)

Christmas factor is traditionally referred to as Factor IX (FIX). FIX is a 55,000 Da vitamin K dependent serine protease and a component of the intrinsic tenase complex.[9] Factor IX can be activated by both the intrinsic and extrinsic pathways via FXIa and FVIIa respectively.[28] FIX binding to the phospholipid surface of the platelet is Ca2+ dependent. [22,29] FIXa assembles on the lipid membrane along with FVIIIa and FX to form the tenase complex, which also requires Ca2+ for full activity.[30]

Antihemophillic Factor (Factor VIII)

Antihemophillic factor is commonly referred to as Factor VIII (FVIII). It is a 330,000 Da protein that is a cofactor for the intrinsic activation of Factor X.[10] FVIII binds to and circulates with Von Willebrand Factor (vWF) as an inactive precursor.[31] FVIII can be activated either by thrombin or activated factor X (FXa) via a cleavage event, and following disassociation with vWF is concentrated via phospholipid binding to cellular membranes.[32,33] Once bound to the membrane surface the FIXa/FVIIIa/FX complex (intrinsic tenase complex) enhances the catalysis of FXa 100,000 times the normal rate.[28,34] Thrombin mediated cleavage of FVIII does not require the presence of Ca2+, but once activated Ca2+ is required for proper functioning of the tenase complex.[35] Hemophilia is the result of either qualitative or quantitative deficiencies of FVIII.[31]

Common Pathway

Stuart-Prower Factor (Factor X)

Stuart-Prower factor is commonly referred to as Factor X (FX). FX is a 58,900 Da vitamin K dependent serine protease.[10] Activated FX (FXa) is the initiator of the final common pathway and is the enzymatic force behind the activation of thrombin.[9] Due to the importance of FX's role in the coagulation protein pathway, it is often considered central to the cascade.[36] FX is the only coagulation factor to be involved in the formation and function of both the tenase as well as the prothrombinase complexes, and its activity is highly dependent on its association with the plasma membrane.[34]

FX can be activated by either the intrinsic or extrinsic pathway as a result of activation of the coagulation pathway, and has been shown to be activated in unrelated pathways as well. MAC-1 (CD11b/ CD18) present on the surface of monocytic cells is capable of binding FX and be activated by cathepsin G secreted during periods of inflammation.[37] Various pathological agents have been shown to activate FX as well, which creates a link between infection, inflammation, and coagulation. Cytomegalovirus and herpes simplex virus (types 1 and 2) express phospholipids on their surface that allow for the imitation of the FVII-TF complex and subsequent activation of FX.[38] Bacterial proteinases have also been shown to activate FX, with the most commonly studied being gingipain-Rs produced by *Porphyromonas gingivalis*.[39]

Regulation of FX is of upmost importance when maintaining homeostasis in the coagulation pathway. Because of the central role it plays, FX inhibition is the focus of multiple physiological coagulation inhibitors. TFPI binds strongly to the TF-FVII complex in the presence of FX, creating a quaternary complex that prevents the

formation of FXa.[40,41] TFPI is the strongest endogenous inhibitor of the coagulation pathway due to it's ability to stop the extrinsic pathway at the initiation phase, essentially stopping the TF response directly at the site of injury.[36] Antithrombin is also a direct inhibitor of FXa activity, binding to FXa and creating an inactive complex in the presence of heparin.[42] Both inhibitory FX complexes are quickly cleared from the blood and transported to the liver where they are degraded.[36,43,44]

FXa also plays a significant role in cell signaling, as it is able to activate protease-activated receptors (PARs) in various cell types. FXa has been shown to be able to activate protease activated receptors (PARs) 1 and 2 via proteolytic cleavage.[45,46] PARs are G protein coupled receptors, and will be discussed later in this work in detail. For current purposes however it should be mentioned that the main result of FX activation in the blood is the subsequent generation of thrombin from prothrombin. Thrombin is the most effective singular mediator of PAR activation, and widely accepted to be the receptor's physiological agonist of choice. The fact that FXa is also able to activate the receptor provides a fall back mechanism that allows for the activation of the receptor in the absence of thrombin generation.[36] While the activation of PAR signaling by FXa alone is inefficient (requiring nM concentrations), when complexed with TF and FVII the ability to activate PAR-1 increases to that of thrombin.[47,48]

As mentioned above, the main physiological role of FXa is the conversion of prothrombin to thrombin. FXa is relatively efficient alone in the conversion and activation of thrombin, requiring less than nM amounts in vitro to generate thrombin.[48] Thrombin generated at this stage activates factor V (FV), which then

complexes on a lipid membrane surface with FXa creating what is known as the prothrombinase complex. The prothrombinase complex generates massive thrombin production, often termed the thrombin burst.[22]

Labile Factor (Factor V)

Labile factor is commonly referred to as factor V (FV). FV is a 330,000 Da protein that acts as a cofactor alongside FXa to generate thrombin.[10] FV's main mechanism of action is through the assembly of the prothrombinase complex. FV is tightly regulated by protein C, providing an inhibitory feedback loop in the regulation of coagulation.[49] Factor V Leiden is caused by a mutation that inhibits the ability of protein C to inactivate FVa, and shows both a thrombotic phenotype as well as protective qualities in hemophilia.[49]

Prothrombin (Factor II)

Prothrombin is the precursor for thrombin, the central enzyme in the coagulation cascade. It is a 72,000 Da vitamin K-dependent serine protease, and is normally referred to as prothrombin.[9] Thrombin conversion happens in two separate steps in the cell-based model. Small amounts of thrombin are generated during the initiation phase, which diffuses away from the immediate site of injury.[15]

Binding of small amounts of thrombin to PARs on the platelet surface induce the rearrangement of phospholipids on the membrane surface.[16,50,51] PAR signaling is capable of complete activation of the platelet response through G proteins as well, and both these responses will be discussed later in this work. Thrombin also activates positive feedback loops generating more FXIa and FVa (demonstrated in figure 1.5). In addition, thrombin is able to cleave the FVIII-vWF complex liberating

FVIII. This FVIII is then also converted to FVIIIa by thrombin as well. Thrombin is central to the structure of a clot, as it cleaves soluble fibrinogen to a loosely associated fibrin network. To stabilize the clot thrombin is also responsible for the activation of the cross-linking transamidase FXIIIa.[15]

Fibrinogen (Factor I)

Fibrinogen is the final substrate in the protein clotting system. Fibrinogen is produced in the liver and circulates as a dimer composed of a pair of three individual protein chains.[9] The individual protein components of the monomers are called Aα, Bβ, and γ. These individual units are linked together at their N terminal ends by a disulfide linkage (Figure 1.9). [9] Once activated thrombin cleaves two small peptides (Fibrinopeptides A and B) forming a fibrin monomer. The resulting fibrin monomers align by non-covalent interactions into an unstable fibrin polymer, which requires FXIII for stabilization.[9] A schematic of this process can be found in Figure 1.9.

Fibrinogen is unique as it is active in the clotting process both in the fluid and the solid phase. Soluble fibrinogen is the mediator of platelet-platelet aggregation. Once activated the platelet integrin GP IIb/IIIa binds to fibrinogen.[52] Platelet aggregation occurs when multiple platelets are linked through the fibrinogen molecule.[53] In comparison, the main structural component of the solid phase is fibrin generated from the thrombin mediated cleavage of fibrinogen.[52,54]

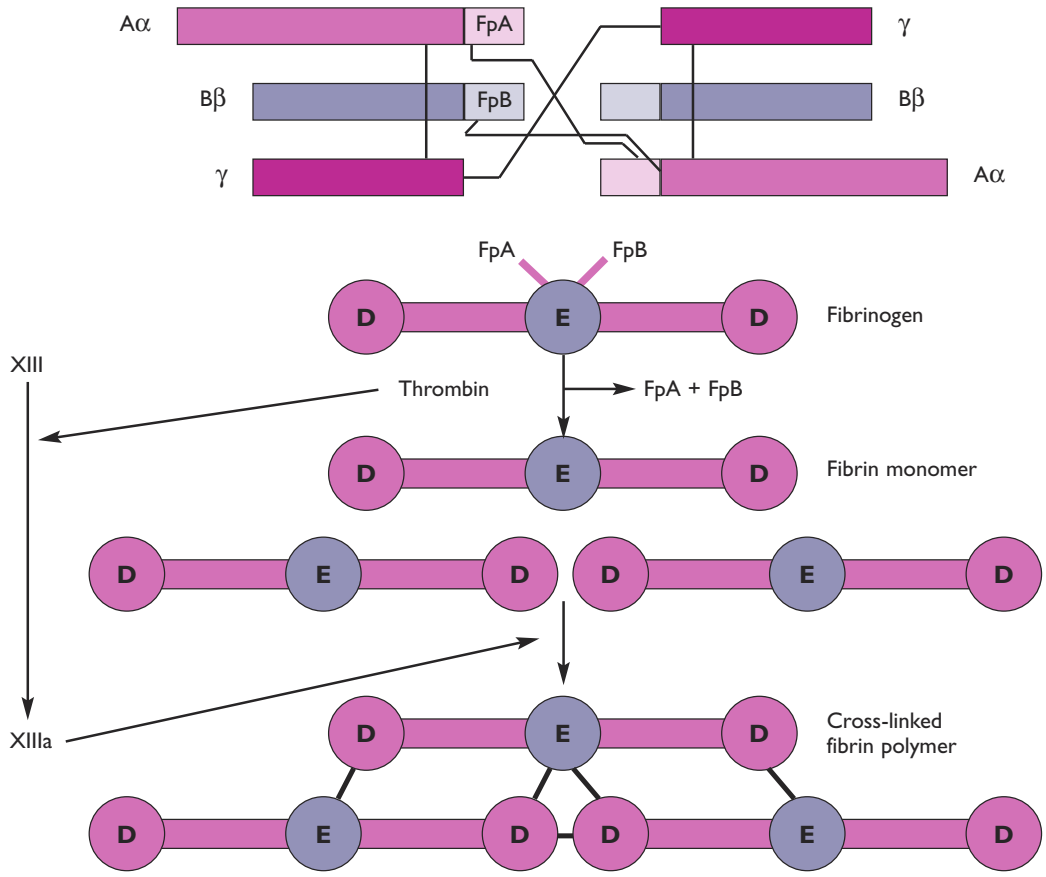

Figure 1.9: Schematic of Fibrinogen Structure and Fibrin Polymer Generation. FpA; fibrinopeptide A, FpB; fibrinopeptide B. Adapted from Lefkowitz 2008.[9]

Fibrin Stabilizing Factor (Factor XIII)

Fibrin stabilizing factor is more commonly referred to as Factor XIII (FXIII). It is a 320,000 Da transamidase that is activated by thrombin.[9] FXIII covalently cross-links the fibrin network generated by thrombin by connecting the γ monomers by attacking glutamic acid and lysine in side chains.[36]

FXIII also covalently attaches plasminogen, antiplasmin, and other materials to the growing clot. This action serves to alter the composition of the clot, and it is thought to increase wound healing and tissue repair activity inside the clot.[10]

High Molecular Weight Kininogen

High molecular weight kininogen (HMWK) is an 110,000 protein that has no enzyme activity. It acts as a cofactor for the conversion of kallikrein and FXII in the initiation of the intrinsic pathway.[9] It is also required for the activation of FXI by FXIIa. HMWK also serves a purpose in the binding and regulation of endothelial cells, and is a strong inhibitor of cysteine proteases.[55]

Anticoagulant Plasma Proteins

The coagulation cascade is kept under tight regulation to avoid unwanted activation. The main mechanisms of action are by enzyme inhibition and modulation of the activity of cofactors.[22] There is significant cross talk between the anticoagulant pathway and the fibrinolytic pathway, which will be discussed later in this work.

Tissue Factor Pathway Inhibitor (TFPI)

TFPI is a 33,000 Da protein that is the main inhibitor in the extrinsic TF pathway. TFPI is secreted from the endothelium, and rapidly inactivates the TF/FVII complex.[10]

Antithrombin (AT)

Antithrombin is a 58,000 Da serpin that directly inhibits several serine proteases (FXa, FIXa, and FXIa) in the coagulation cascade. AT requires heparin as a cofactor for proper activity.[9] Heparin is often given as an anticoagulant during pharmacological therapy, but physiologically its role is somewhat less defined. It is known to be present on endothelial cells and to be released by mast cells at the site of injury.[56] Once bound to heparin AT undergoes a conformational change that increases the efficiency of the inhibitor 1000 fold.[10]

Protein C

Protein C is a 62,000 Da vitamin K dependent serine protease that is able to cleave and inactivate both FVa and FVIIIa.[9] Protein C is responsible for controlling thrombin formation in the physical area around the clot.[57] In the inactive state protein C is localized to the surface of the endothelium by the endothelial cell protein C receptor (EPCR).[58] Thrombin generated during the clotting process binds to thrombomodulin (TM) on the endothelial surface as well. This binding itself inhibits thrombin's ability to interact with fibrinogen, but also brings it into proximity to protein C.[59] Thrombin cleaves protein C from the endothelium generating activated protein C (APC). APC then moves to act on its main targets, FVIIIa and FVa reducing generation of thrombin away from the site of endothelial

injury.[40] Protein C's inhibitory activity is greatly increased in the presence of the cofactor protein S.[60]

Protein S

Protein S is a 75,000 Da vitamin K-dependent serine protease that acts as a cofactor for protein C.[9] It has been shown that activated protein S (APS) greatly increases the ability of activated protein C (APC) to inactivate FVa.[60] It has also been shown that APS increases the binding of APC to a negatively charged phospholipid surface as well as increase the interaction of APC with activated platelets.[60–62] APS also increases the inherent inactivation of FVIIIa by APC in a dose dependent manner. Interestingly, APS is also able to further cleave the breakdown products generated by the cleavage of FVIIIa by APC.[33]

1.3 Fibrinolysis

In comparison to the coagulation cascade, the fibrinolytic pathway is comprised of far less active participants. Fibrinolysis is accomplished through an inactive proenzyme (plasminogen) that is converted to the active serine protease plasmin by two separate activators (tissue-type plasminogen activator, urokinase-type plasminogen activator). Inhibition of the pathway is accomplished at two levels. α2-antiplasmin inactivates plasmin, and plasminogen activator inhibitor specifically and thrombin-activatable fibrinolytic inhibitor inhibit the activity of the plasminogen activators.[63] A schematic of the fibrinolytic pathway can be found in Figure 1.10.[64] Each component is discussed further below.

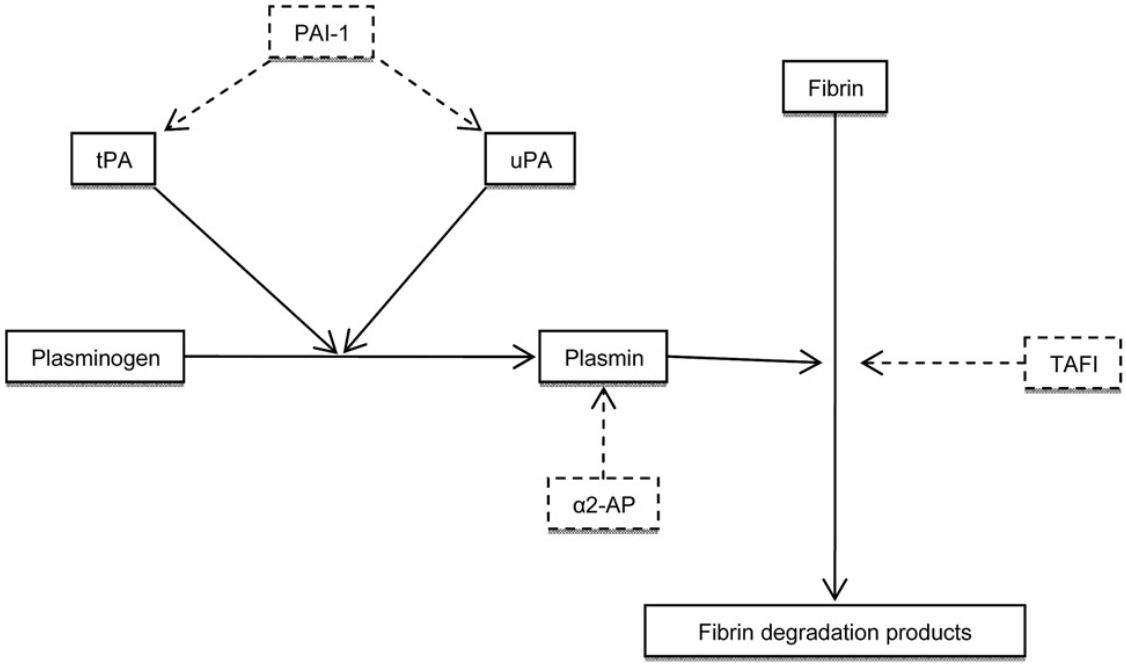

Figure 1.10: Schematic Representation of Fibrinolytic Pathway. PAI-1; plasminogen activator inhibitor 1, t-PA; tissue-type plasminogen activator, u-PA; urokinase-type plasminogen activator, α2-AP; α2-antiplasmin, TAFI; thrombin-activatable fibrinolysis inhibitor. Dashed lines indicate inhibition. Adapted from Thelwell 2010.[64]

Plasminogen

Plasminogen is a 92,000 Da glycoprotein proenzyme. It is converted by plasminogen activators by a cleavage mechanism that creates a two-chain trypsin-like serine protease named plasmin.[65] Plasmin digests and cleaves fibrin indiscriminately at different sites, resulting in the generation of various sized fibrin degradation products (FDPs). The half life of free plasminogen is extremely short (0.1 seconds) due to inactivation by α2-antiplasmin, but this reaction is much slower (10 to 100 seconds) when plasmin is bound to fibrin.[66] This fact leads to the understanding that fibrinolysis occurs almost exclusively at the fibrin surface.

Plasminogen Activators (PAs)

There are two types of plasminogen activators. Tissue type plasminogen activator (t-PA) is produced by endothelial cells, and is a 70,000 Da serine protease.[63] t-PA is a very inefficient enzyme on its own, but upon binding to fibrin its efficiency increases by at least two orders of magnitude. The association with fibrin does not change the catalytic ability of the enzyme, but instead increases its rate of association with plasminogen.[67] In a similar fashion, the activation rate of t-PA on cell bound plasminogen is approximately 10 times that of the activation rate in solution.[63] Plasmin generated by the reaction in turn inactivates t-PA through a hydrolysis bond, providing feedback inhibition of this pathway.[68]

Urokinase-type plasminogen activator (u-PA) is a 54,000 Da protease that circulates in a single chain (prourokinase). u-PA can be activated by plasmin or kallikrein generating an active enzyme. Active u-PA does not bind to fibrin, but does bind to a specific cell surface receptor (u-PAR).[63] Inactive u-PA does bind to the

surface of fibrin however, where it is brought into close proximity to plasmin and subsequently activated.[65] u-PA is inactivated by thrombin at a slow rate, but this reaction is strongly accelerated in the presence of thrombomodulin.[25]

α2-Antiplasmin

α2-antiplasmin is a 70,000 Da serpin that has a high affinity for plasmin/plasminogen. This involves the formation of a 1:1 complex between the two molecules, and a fast initial reversible step, followed by a slower irreversible permanent inactivation of both molecules.[63,65]

Thrombin Activatable Fibrinolytic Inhibitor (TAFI)

TAFI is also referred to as plasma procarboxypeptidase B, and is a 60,000 Da proenzyme. TAFI can be activated by thrombin, but this reaction rate is increased 1250 fold in the presence of thrombomodulin.[63] TAFI is unique as it can be both activated as well as inactivated by plasmin.[69] TAFI acts by removing C-terminal lysine and arginine residues on the surface of partially degraded fibrin, which decreases the ability of plasminogen to bind and become activated.[70]

Plasminogen Activator Inhibitor 1 (PAI-1)

PAI-1 is a 52,000 Da member of the serpin superfamily. PAI-1 is the main inhibitor of both PAs, and is present in the circulation in low concentrations normally.[63] Platelets have been shown to contain relatively large amounts of inactive PAI-1, and recent work also shows that they can convert this PAI-1 to its active form and release it.[71] PAI-1 can act on t-PA and active u-PA, but cannot bind to inactive u-PA. It follows a similar mechanism of inhibition as TAFI, with the quick

formation of a reversible 1:1 complex, followed by a slow covalent linkage that permanently inactivates the PA.[63]

Additional Plasma Proteins Involved In Hemostasis

Von Willebrand Factor (vWF)

Von Willebrand Factor (vWF) is present in a soluble form in the plasma, but is also present as a component of the subendothelial matrix. In the plasma it circulates in complex with FVIII, which protects it from catalysis by APC. Once thrombin activates FVIII the two molecules separate and vWF and FVIIIa move on to promote coagulation.[72] Matrix vWF is recognized by the platelet integrin GP Ib, and this interaction is the only adhesion reaction that works under high shear situations found in the arteries and arterioles.[73]

Additionally vWF is able to fully support platelet aggregation through GPIIb/IIIa in the absence of fibrinogen. vWF shares a binding domain with fibrinogen (RGD domain) so they compete for binding. The concentration of fibrinogen is two orders of magnitude higher than vWF however, which causes most activated platelets to bind to fibrinogen and eventually end up incorporated to the fibrin clot.[74,75] Soluble vWF in the plasma also recognizes and binds to exposed components of the extracellular matrix, most notably collagen.[76,77] This allows for proper platelet recognition and attachment to any break or irregularity in the vasculature, regardless of the presence of subendothelial vWF. The platelet vWF interaction will be discussed more in depth later in this work.

Thrombospondin

Thrombospondin (TSP) is an 150,000 Da adhesive glycoprotein that comprises a large portion of the platelet α-granule.[78] TSP has been shown to bind to multiple platelet integrin receptors, vWF, collagen, plasminogen, fibrinogen, thrombin, fibrinectin, and multiple other molecules.[72] Both adhesive and anti-adhesive properties have been reported, and it is also able to mediate cell apoptosis, angiogenesis, cell migration and tumor progression.[79] TSPs diverse array of biological functions may be attributed to its ability to bind multiple receptors simultaneously.[78] TSP also directly activates the platelet scavenger receptor CD36 (GPIV).[72]

Vitronectin

Vitronectin is a 75,000 Da adhesive glycoprotein that participates in cell differentiation, complement assembly, and cell proliferation in addition to its role in platelet aggregation and adhesion.[80,81] Perhaps the most important role it plays in thrombosis is its ability to prevent fibrinolysis by binding to PAI-1 and allowing it to become incorporated into the platelet plug at the site of injury.[72]

Collagen

Collagen is not technically a plasma protein, but its effects on platelet activity and interactions with vWF warrant visitation of this protein further. There are 25 different types of collagen, representing up to 40% of the total protein in the vessel wall. Collagen provides an attachment surface for the adhesion of vascular cells and attachment of matrix proteins.[76] Platelets are directly able to adhere to collagen types I, III, IV, V, and VI which comprise the majority of collagen in the vessel walls.[82]

This adhesion is accomplished through a variety of integrin moieties, but is mainly accomplished through GPVI and the GPIb-vWF complex.[82,83]

Every form of collagen tested has been able to induce aggregation of stirred platelets *in-vitro*, suggesting that all forms of collagen can induce platelet activation under appropriate conditions.[82] This ability to serve as both an adhesion molecule as well as potent activator makes collagen unique among the plasma proteins.

Thrombomodulin

Like collagen, thrombomodulin is not typically considered a plasma protein. Thrombomodulin is an endothelial cell protein that binds to thrombin and acts like a molecular switch, changing the enzyme's role from prothrombotic to antithrombotic.[63,65,84] The ability of thrombin to activate protein C and other anticoagulant and fibrinolytic proteins when bound to thrombomodulin is approximately equal to that of unbound thrombin's ability to cleave coagulant proteins.[84]

1.4 Platelet Literature

Introduction

The cell based model stresses the importance of platelet function in the clot formation. Platelets do more than just provide a surface for the assembly of enzymatic complexes. Activation of the coagulation system causes distinct responses including aggregation, secretion, shape change, and membrane rearrangement. To understand the complex response of platelets to the surrounding environment a detailed look at platelet signaling is required. Platelet response is based on both traditional receptor interactions as well as integrin

interaction with extracellular matrix components (Figure 1.11). These pathways normally function together in the physiological platelet response, with crosstalk and autocrine amplification pathways providing redundancy ensuring a robust platelet response. The pathways will be examined individually first to allow for identification of each individual component, and then the crosstalk will be examined allowing for a better understanding of the concerted platelet response.

Platelet Response

Platelets are the main functional cells in the hemostasis system. Unlike the protein coagulation system mentioned above, platelets are able to adapt to their environment and thus differentiate their response based on the signals they receive. Platelets can be induced to produce a variety of responses including shape change, secretion of their granule contents, aggregation with other cell types, adhesion to extracellular matrix components, membrane rearrangement, and microvesiculation. These responses are required for proper hemostasis and will be discussed further below.

Shape Change

In a step wise analysis of platelet activation the first response of the platelet is shape change. The normally disc shaped anucleate cells undergo a cytoskeletal rearrangement that causes them to become spheres and extend filopodia.[85,86]

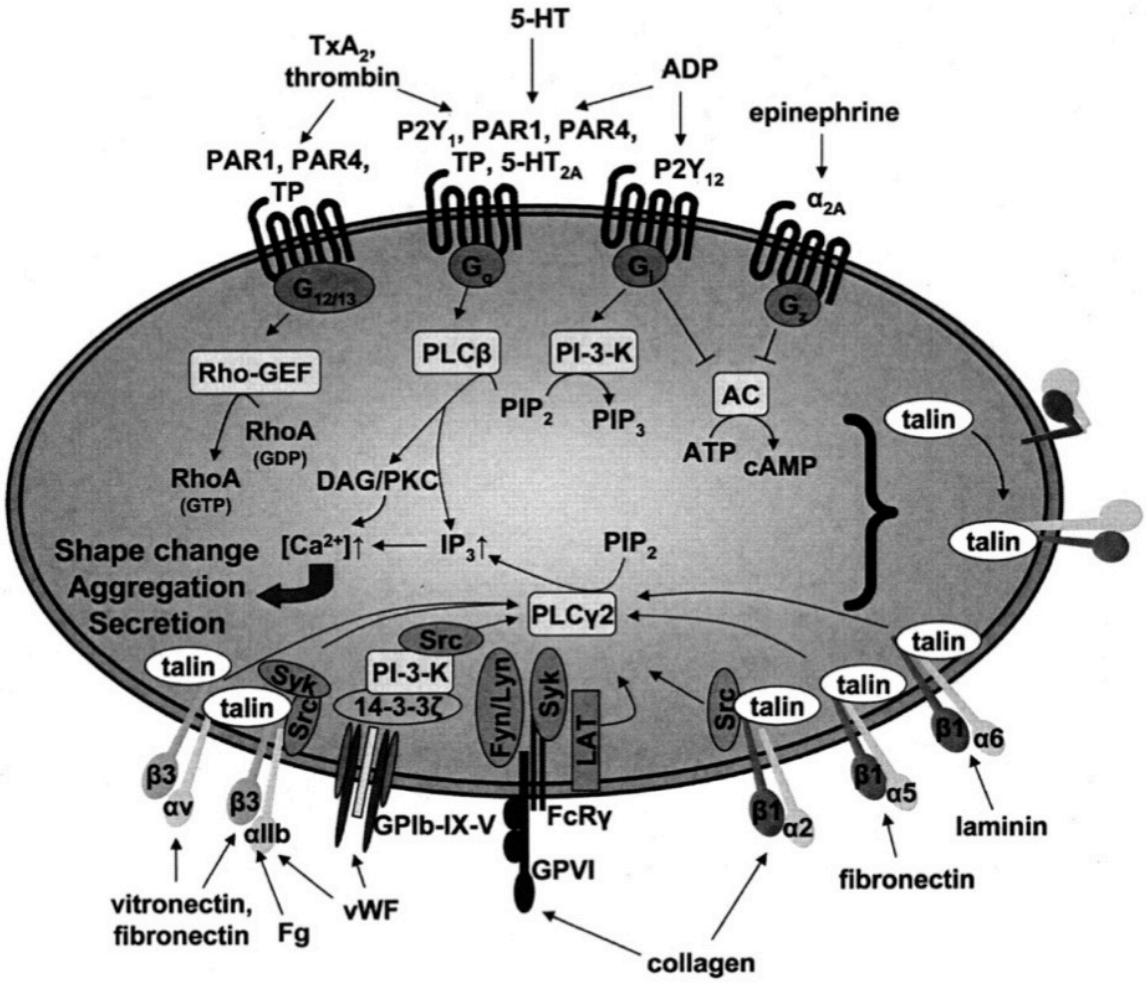

Figure 1.11: Common Platelet Signaling Pathways. Figure depicts agonist stimulation of both G protein coupled receptors (top) and integrin receptors (bottom) and their respective main effectors. 5-HT; serotonin, TxA_2; thromboxane A_2, AC; adenylyl cyclase, ATP; adenosine triphosphate, ADP; adenosine diphosphate, AMP; adenosine monophosphate, cAMP; cyclic AMP, PI-3-K; phospoinositide 3-kinase, PLCγ2, phospholipase Cγ2, PLCβ; phospholipase Cβ. LAT; linker of activated T-cells, Rho-GEF; Rho guanine nucleotide exchange factor, PKC; protein kinase C, DAG; diacylglycerol, PIP_2; phosphatidylinositol 4,5-bisphosphate, PIP_3; phosphatidylinositol (3,4,5)-trisphosphate, vWF; Von Willebrand factor, GPVI; glycoprotein VI. Adapted from Jennings 2009.[110]

Studies have shown that phosphorylation of the regulatory myosin light chain is required to initiate shape change.[87] The small GTP-binding protein families Rho, Rac, and Cdc42 have been implicated in platelet shape change.[88] These reactions have been shown to occur with or without Ca++ mobilization, which is recognized as the final common response to all strong platelet agonists.[89] The individual small GTP binding protein involved in the shape change is dependent on the agonist used to elicit the response, and will be discussed in detail when examining the individual signaling pathways.

Secretion

Platelets have been shown to contain three separate types of granules (α, dense, and lysosomal), which upon stimulation they can secrete into their environment. The exact mechanism of platelet secretion has not been identified as of yet, but it is understood that the cytoskeletal remodeling involved in shape change causes centralization of the granules. A fusion event then allows the contents of the granules to exocytose.[90] α-granules are thought to release their contents into the open canicular system on the surface of the platelets, where as dense granules fuse directly with the plasma membrane and allow for exocytosis of their contents.[91] Lysosomes are membrane bound vesicles and their role in coagulation is still poorly understood.[90] Similar lysosomes in other cell types, they are believed to have a cellular degradation purpose, as well as possible an immune function inside platelets.[92,93] The contents in the individual granules can be found in Table 1.2.[94] Secretion allows for autocrine and paracrine signaling by the platelets, as well as expression of new surface markers and adhesion molecules.

Table 1.2: Platelet Granule Contents

Alpha granules[a]	Dense granules	Lysosomal granules[b]
Albumin	Serotonin	Cathepsin D
Fibrinogen	ATP	Cathepsin E
Fibronectin	ADP	Carboxypeptidase A
Vitronectin	Calcium	Carboxypeptidase B
Osteonectin	Pyrophosphate	Proline carboxypeptidase
von Willebrand factor		β-*N*-acetyl-D-hexosaminidase
von Willebrand antigen II		β-D-glucuronidase
Thrombospondin		β-D-galactosidase
Platelet factor 4		α-D-mannosidase
IgG, IgA, IgM		α-L-arabinofuranosidase
C1 inhibitor		α-D-galactosidase
Plasminogen		α-L-fucosidase
Plasminogen activator inhibitor-1		β-D-fucosidase
Platelet-derived collagenase inhibitor		β-D-glucosidase
High molecular weight kininogen		α-D-glucosidase
Protein S		Acid phosphatase
α_2-antitrypsin		Arylsulphatase
α_2-macroglobulin		
α_2-antiplasmin		
Multimerin		
Platelet basic protein		
β-thromboglobulin		
Histidine-rich glycoprotein		
Connective tissue-activating protein III		
Neutrophil-activating protein II		
Platelet-derived growth factor		
Transforming growth factor β-		
Endothelial cell growth factor		
Coagulation factor V		
Coagulation factor VIII		

Adapted from McNicole and Israels 1999.[94]

Aggregation/ Adhesion

Platelet aggregation and adhesion can be viewed together as they are similar processes. Both are mediated by integrin binding, but are spatially dependent. Aggregation occurs when platelets attach themselves to other circulating platelets or cell types. While platelet-white blood cell aggregates have been identified, for the purpose of hemostasis the platelet-platelet aggregation response is more physiologically involved. Adhesion is the process by which platelets become attached to an immobile surface, such as exposed collagen of vWF at the site of injury. As discussed previously platelet attachment to collagen and vWF is mediated mainly through the GP Ib-IX-V complex, and this complex will be reviewed in depth in the integrin section.[82,83] Platelet aggregation occurs through the activated GP IIb/IIIa integrin, and this interaction will be discussed in more detail during the integrin review as well.[95,96]

Exposure of Pro-Coagulant Membrane Surfaces

The availability of a phospholipid surface to allow for the assembly of the tenase and prothrombinase complexes is a vital for proper hemostatic function. While this assembly can happen at the site of injury in TF bearing cells, this local extrinsic response is unable to provide an adequate response to allow for the propagation of a clot. The ability of platelets to rearrange their membranes to expose PS in response to activation allows them to act like a template for assembly of the coagulation complexes further away from the actual immediate site of injury.[97] The identification of a platelet population that exposes high amounts of PS after dual stimulation with thrombin and collagen have been termed COAT or

coated platelets, and appear as a subpopulation of the entire platelet pool.[97,98] They show not only increased binding of Annexin V, but also binding of coagulation factors, serotonin, and fibrinogen. Interestingly these coated platelets will not bind to the PAC-1 antibody used to detect GP IIb/IIIa activation, although normally the binding affinity for PAC-1 is orders of magnitude higher for the integrin, allowing it to displace fibrinogen.[98,99]

In platelets the rearrangement of the lipid membrane is accomplished in three separate systems (Figure 1.12). Two of the systems are considered to be responsible for the steady state maintenance of the asymmetric phospholipid composure of resting platelets. A third system is responsible for the rapid breakdown of this symmetry and exposure of PS and PE on the platelet membrane in response to activation.[14]

Steady state membrane maintenance is accomplished through two separate enzymes. Aminophospholipid translocase is a PS and PE specific translocase that transports these lipids against a gradient from the outer membrane to the inner membrane leaflet. This enzyme is ATP dependent and inhibited by Ca2+.[51] To balance the action of the translocase a slow acting and yet unidentified floppase is postulated to be responsible for the outward non-specific transport of phospholipids to the outer membrane from the inner leaflet. [51] Due to the slow acting nature of both these processes it is unlikely they are able to respond to platelet activation, and elevated intracellular Ca2+ levels associated with the activation response likely would inhibit their activity.

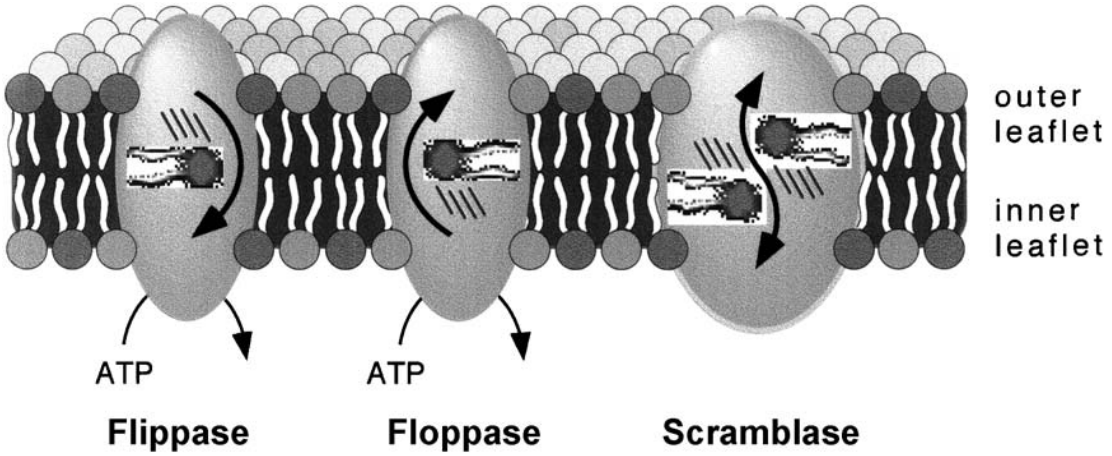

Figure 1.12: Enzymes Responsible for Platelet Membrane Rearrangement. Floppase is also commonly referred to as translocase. ATP; adenosine triphosphate. Adapted from Zwaal 2004.[16]

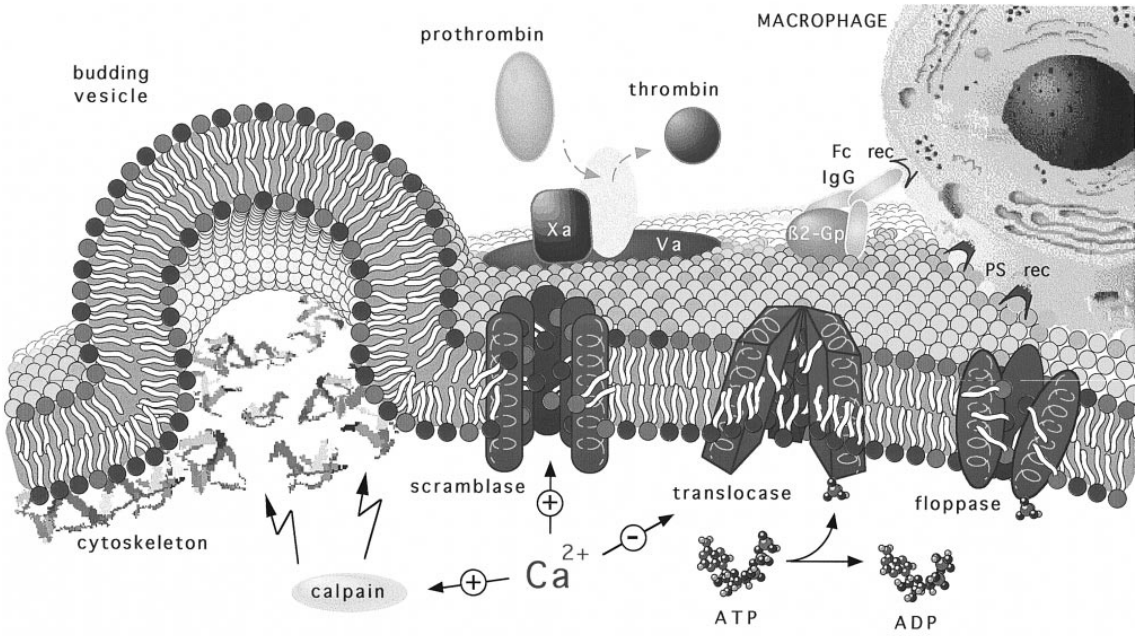

Figure 1.13: Microvesicle Generation in Platelets. Translocase is often referred to as floppase. ATP; adenosine triphosphate, ADP; adenosine diphosphate, PS; phosphotidylserine. Adapted from Zwaal and Schroit 1997.[51]

The third system is fast acting, bi-directional, and activated by Ca2+.[50] This system is regulated by the enzyme "scramblase" although the random nature of the term does not actually reflect a random system.[100] It is this system that is believed by many groups o be responsible for the rapid translocation of PS to the platelet outer membrane, allowing for assembly of the factor complexes.

Microvesiculation

The formation of microparticles (MP) is also associated with the platelet activation response. These resulting small phospholipid microvesicles have a membrane skeleton and are capable of expressing cell surface receptors and antigens.[101] MPs are generally defined as having a general size range of 0.1-1.0 μm in diameter, however can be found as large as 1.5 μm and as small as 40-80 nm.[102] Many other cell types are also capable of producing MPs, however 70-90% of all circulating MPs (cMPs) originate from platelets (pMPs).[103] These pMPs have been shown to enhance platelet deposition and thrombus formation both in healthy individuals as well as those with cardiovascular atherothrombotic disease.[104] A diagram of this process can be found in Figure 1.13.[16]

Platelet Signaling

Integrin signaling

Adhesive proteins expressed on the platelet surface not only act as adhesion molecules for the physiological adhesion and aggregation response but can also participate in both outside-in and inside-out signaling. All integrins contain an α subunit and a β subunit. In platelets there are two β subunits expressed, β2 and β3. These subunits are associated with various a subunits.[105]

Each subunit protein has a large extracellular domain, single transmembrane domain, and intracellular domain. The ligand recognition and binding pocket is formed by the extracellular domains of both subunits.[106] In most cases a divalent cation presence (either Mg2+ or Ca2+) is required to maintain the ligand binding site.[107]

The arginine-glycine-aspartic acid motif (RGD) is the recognition site for ligand binding to integrins, and they bind a variety of soluble molecules as well as extracellular matrix proteins.[74,108] Integrins present on the platelet surface and their ligands can be found in Table 1.3.

The αIIbβ3 Integrin (GP IIb/IIIa)

The αIIbβ3 integrin (GP IIb/IIIa) is the most abundant protein expressed on the platelet surface, and accounts for roughly 17% of the total protein composition of the platelet membrane.[96] This integrin is commonly referred to as the fibrinogen receptor, and is responsible for the platelet-platelet aggregation response. This integrin can participate in both inside-out as well as outside in signaling.[109] While fibrinogen is the main ligand for GP IIb/IIIa, the integrin has also been shown to recognize vWF, fibrinectin, vitronectin, thrombospondin, collagen, PECAM-1, prothrombin, serum amyloid A, and CD40L. It should be noted that these ligands may be involved with the adhesion response, but are not able to support platelet-

Table 1.3: Common Platelet Integrins, Their Ligands, and Function.

GP Receptor	Ligand	Biologic Function
GP Ia/IIa ($\alpha_2\beta_1$, VL2-A)	Collagen	Adhesion
GP Ib/IX/V	von Willebrand factor	Adhesion
GP Ic/IIa ($\alpha_5\beta_1$)	Fibronectin	Adhesion, stabilizing GP Ia/IIa
GP IIb/IIIa ($\alpha_{IIb}\beta_3$)	Collagen, fibrinogen, fibronectin, vitronectin, von Willebrand factor	Aggregation, but with a secondary role in adhesion under conditions of high shear stress
GP IV	Thrombospondin	Adhesion
GP VI	Collagen	Signal transduction, activation
Vitronectin ($\alpha_v\beta_3$)	Thrombospondin, vitronectin	Adhesion
VLA-6 ($\alpha_6\beta_1$)	Laminin	Adhesion

GP; glycoprotein. Adapted from Jennings 2009.[110]

platelet aggregation.[105] In fact it has been shown that these alternate ligands may have an inhibitory effect on the platelet aggregation response.[105]

Once bound to ligand GP IIb/IIIa undergoes a conformational change initiating outside-in signaling. This conformational change allows the β3 subunit to bind to the G protein α13 subunit inside the cytoplasm.[111] This interaction activates Src family kinases (SFKs), with the main effector being c-Src.[112] c-Src moves forward to activate PLCγ resulting in the increase of IP3 and resultant increase in intracellular calcium mobilization.[110]

The GP Ib-IX-V Complex

The complex of GP Ib-IX-V complex serves as a major adhesive receptor on the platelet surface. It is composed of four separate glycoproteins, GP Ibα, GP Ibβ, GP IX, and GP V. [113] This receptor complex is vital to proper platelet function due to the fact it is able to mediate adhesion under high sheer via interactions with vWF. Sheer rates vary greatly between veins, large arteries, arterioles, and stenotic vessels (Table 1.4).[114]

The GP Ib-IX-V complex is constitutively expressed on the surface of the platelet at around 25,000 copies per cell.[115] Structurally it is composed of four distinct glycoprotein subunits. GP Ibα and GP Ibβ subunits are linked via a disulfide bond, and then non-covalently associated with GP IX and GP V. These subunits maintain a stoichiometric ratio of 2:2:2:1.[116] It has been shown that the GP Ib-IX-V complex is found to be enriched in lipid rafts on the platelet membrane, and that after stimulation more of the complex is recruited to the rafts to increase signaling potential.[117] Improper expression of the subunits GP Ibα, GP Ibβ, and GP IX have

Table1.4: Typical Range of Wall Shear Rates.

Blood Vessel	Wall Shear Rate (/s)	Wall Shear Stress (dynes/cm^2)
Large arteries	300-800	11.4-30.4
Arterioles	500-1,600	19.0-60.8
Veins	20-200	0.76-7.6
Stenotic vessels	800-10,000	30.4-380

Assuming a viscosity of 0.038 Poise. Adapted from Kroll 1996. [60]

been found to cause the hereditary Bernard-Soulier syndrome.[118]

The process by which GP Ib-IX-V mediates adhesion under high shear rates involves engagement of GP Ibα to the A1 binding sites on vWF. vWF is bound to collagen at the site of vascular injury through its A3 binding sites. The binding of vWF to collagen at high shear causes a stretching of the vWF protein, this exposing the A1 binding sites.[119] The interaction with vWF has a high disassociation rate but causes a significant slowing of platelet movement allowing for the engagement of the GP IIb/IIIa receptor to the RGD domain of vWF.[120]

After receptor engagement with vWF a number of intracellular events mediate the outside-in signaling (Figure 1.14). The cytoplasmic tails of the complex interact with a number of intracellular proteins in a resting state including actin binding protein (ABP), filamin A, 14-3-3ζ, and calmodulin.[113,121–123] ABP has been shown to be required for maintaining the binding of the receptor complex under high shear as well as platelet slowing and rolling along vWF.[124–126] Filamin A interactions also help link the adhesion receptor complex to the cytoskeleton.[127] Loss of any of these cytoskeletal-binding proteins negatively impacts the platelet's ability to bind to vWF under high shear and loss of intracellular signaling.

14-3-3ζ protein is associated with the cytoplasmic region of the receptor, but its activation causes different effects depending on its spatial location on the intracellular domain (Figure 1.14). 14-3-3ζ can associate with GP Ibα, GP Ibβ, and GP V.[113] Binding of 14-3-3ζ to the GP Ib integrins is cooperative, with association of

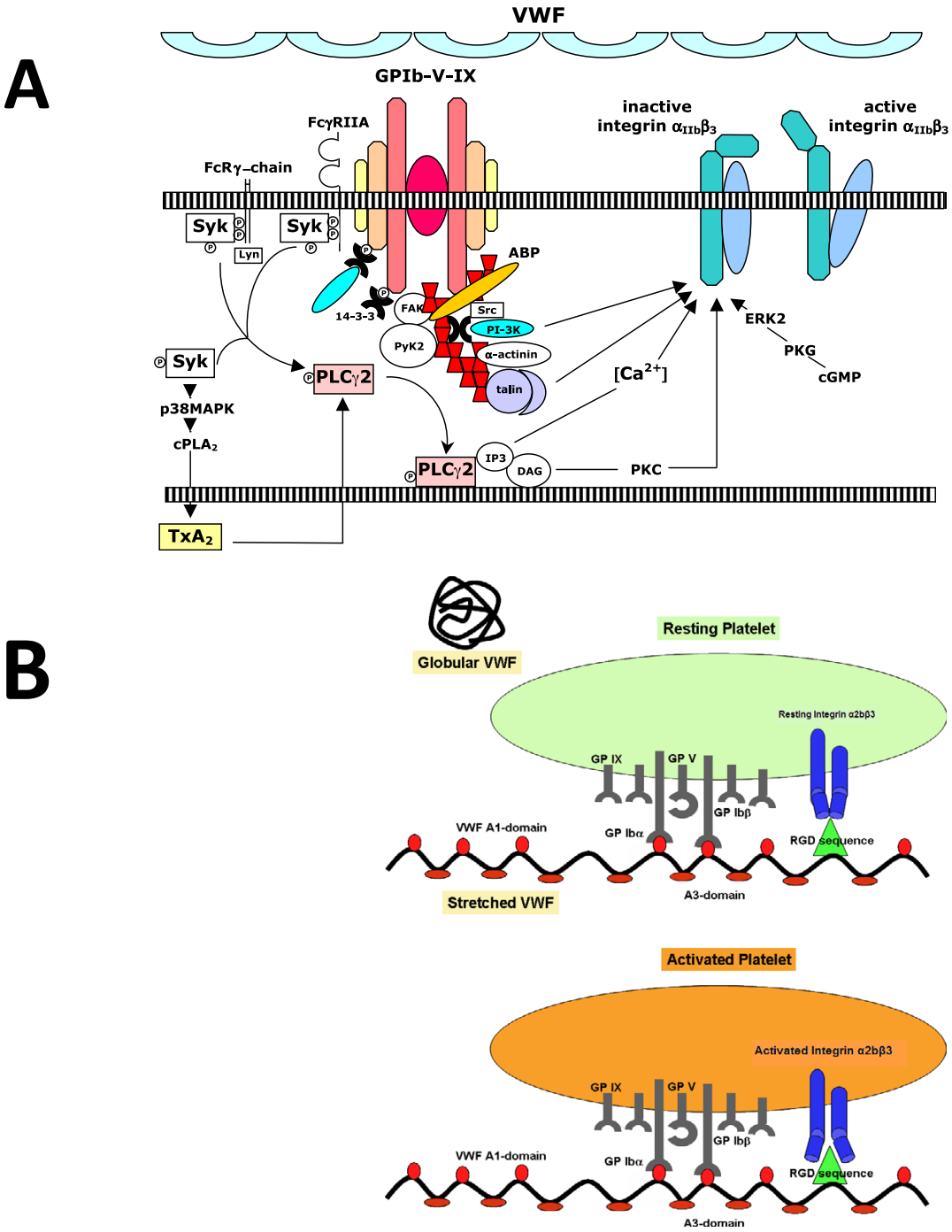

Figure 1.14: The Platelet GP Ib-IX-V complex A) Model of GP Ib-IX Complex. B) Model of GP Ib-IX-V Complex Facilitating GP IIb/IIIa engagement of vWF. vWF; von Willebrand factor, TXA2; thromboxane A_2, cPLA2; cytosolic phospholipase A_2, ABP; actin binding protein, PKC; protein kinase C, PKG; protein kinase G, PLCγ2; phospholipase Cγ2, cGMP; cyclic guanosine monophosphate, DAG; diacylglycerol, IP3; inositol trisphosphate, FAK; focal adhesion kinase. Adapted from Canobbio 2004.[128]

the protein with one subunit increasing the association of the protein with the other subunit.[129] This association has been shown to be a result of phosphorylation of 14-3-3ζ by protein kinase A (PKA) and negatively regulates the ability of the receptor to bind to vWF.[121] Alternately 14-3-3ζ can also associate with the cytoplasmic domain of GP V in resting platelets.[113] This association level increases when the platelets are stimulated with vWF. Signal transduction through 14-3-3ζ is accomplished via activation of tyrosine kinases Syk, FAK, and Pyk2 as well as activation of PI3K and subsequently PLCγ.[130–133]

It has also been found that vWF interaction with GP Ib-IX-V induces phosphorylation of the immunoreceptor tyrosine-based activation motif (ITAM) containing receptors FcγRIIA and FcR γ-chain.[128,134,135] ITAM signaling proceeds through Syk to initiate an intracellular tyrosine kinase cascade that will be addressed when the receptors are looked at individually. These ITAM receptors are most commonly known for their requirement for competent GP VI – collagen signaling, but it has been demonstrated that vWF alone is able to initiate this signaling pathway in platelets.[128] The final outcome from ITAM mediated signaling is TXA_2 generation and GP IIb/IIIa activation (Figure 1.14).[113]

While vWF is the main physiological ligand for the GP Ib-IX-V complex, it also interacts with multiple other ligands. Thrombin, thrombospondin-1, factors XI and XII, high molecular weight kininogen, MAC-1, and P-selectin all have been shown to interact with the extracellular N-terminal domain of GP Ibα.[113] Thrombospondin-1, and P-selectin binding are implicated in the platelet adhesion response.[29] MAC-1 binding is responsible for platelet clearance seen in chilled platelet administration

by leukocytes.[136,137] Factors XI and XII as well as kininogen bind to the complex when localized to lipid rafts after activation of the platelets by thrombin.[26,27]

Perhaps the most interesting role of the GP Ib-IX-V complex involves its interaction with thrombin. It has been shown that thrombin binding to the complex induces adhesion, aggregation, and secretion.[138] While it was originally assumed that thrombin binding was responsible for the platelet response, it is now believed that GP Ib binds thrombin and presents it to the PAR1 receptor to allow activation at low concentrations of thrombin.[139] This model implicates GP Ib as a catalytic cofactor, forming a ternary complex with PAR-1 and thrombin.[140] It has been shown that the interaction between GP Ib and thrombin occurs after cleavage of GP V from the receptor complex by the protease activity of activated thrombin.[140] This interaction mimics the action of the PAR3 and PAR4 interaction seen in murine platelets at low thrombin concentrations.

GP VI

GP VI is the main collagen receptor found on platelets. It is a 60,000 Da glycoprotein that belongs to the immunoglobulin superfamily.[141] It has two extracellular immunoglobulin like domains, a mucin like core, a transmembrane domain, and short cytoplasmic tail that associates with FcRγ-chain through a salt bridge (reviewed by Surin).[142] Once bound to collagen the initial step in activation involves the recruitment of Fyn and Lyn allows for the activation of the immunoreceptor tyrosine based motif on the FcRγ-chain.[143] As depicted in Figure 1.15 this activation allows for the recruitment SLP76 [144], multiple Syk and Rho kinases (Gab2, Vav, Grb2, and Rho)[145], and activation of the membrane bound linker

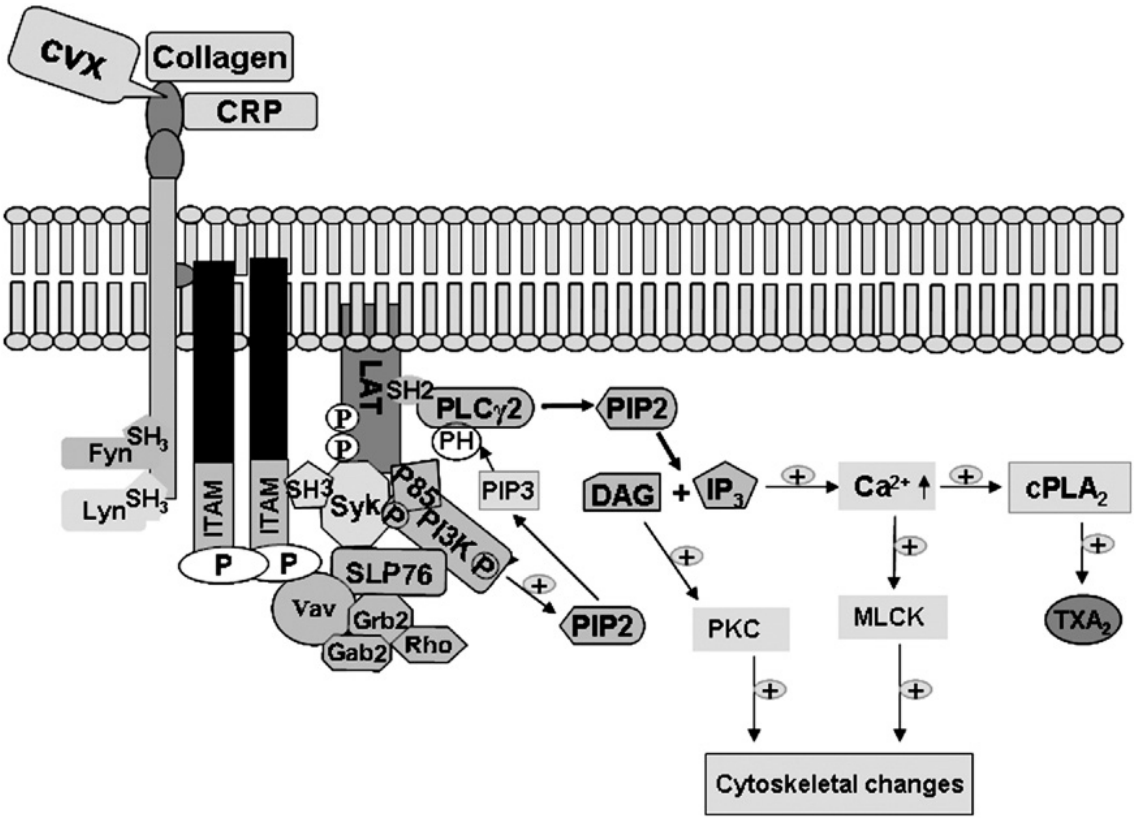

Figure 1.15: Signaling Events Following GP VI Ligand Activation. CVX; convulxin, CRP; collagen reactive protein, TXA_2; thromboxane A_2, $cPLA_2$; cytosolic phospholipase A_2, ABP; actin binding protein, PKC; protein kinase C, MLCK; myosin light chain kinase PLCγ2; phospholipase Cγ2, cGMP; cyclic guanosine monophosphate, DAG; diacylglycerol, IP3; inositol trisphosphate, PI3K; phospoinositide 3-kinase, ITAM; immunoreceptor tyrosine based motif LAT; linker of activated T-cells, PKC; protein kinase C, DAG; diacylglycerol, PIP2; phosphatidylinositol 4,5-bisphosphate, PIP3; phosphatidylinositol (3,4,5)-trisphosphate Adapted from Surin 2008.

for activation of T-cells (LAT). This results in activation of PI3 kinase, PLCγ, and generation of IP3 and DAG.[146] This results in the final pathway of intracellular Ca++ release, $cPLA_2$ activation[147], and thromboxane A_2 generation.

Combined signaling through the TxA_2 receptor and increased intracellular Ca++ concentration allow for the activation of GP IIb/IIIa and exposure of pro-coagulant membrane surfaces (Figure 1.16), elucidating a complete activation of the platelet (Reviewed by Surin).[142]

G Protein Coupled Receptor Signaling Pathways

A large portion of the platelet's response to its environment is through G protein coupled receptors (GPCRs.) GPCRs are the largest family of proteins in the human genome. Activation of GPCRs can be accomplished through a variety of ligands including amines, lipids, peptides, ions, nucleotides, or proteases.[148] This agonist diversity allows GPCR signaling in platelets to respond to a variety of soluble ligands, and Figure 1.17 depicts the currently identified GCPRs in platelets as well as their physiological ligands. [149] GCPRs transduce their signals through heterotrimeric guanine nucleotide-binding proteins (G proteins.) G proteins are composed of α, β, and γ subunits, and act in a molecular switching fashion. α subunits can be classified into four subfamilies: Gs, Gi, Gq, and G12/13.[150–152] as part of the activation process the G protein cycles through an inactive GDP bound state, and an active GTP bound state. [151] Upon ligand engagement a conformational change induced in the GPCR allows it to

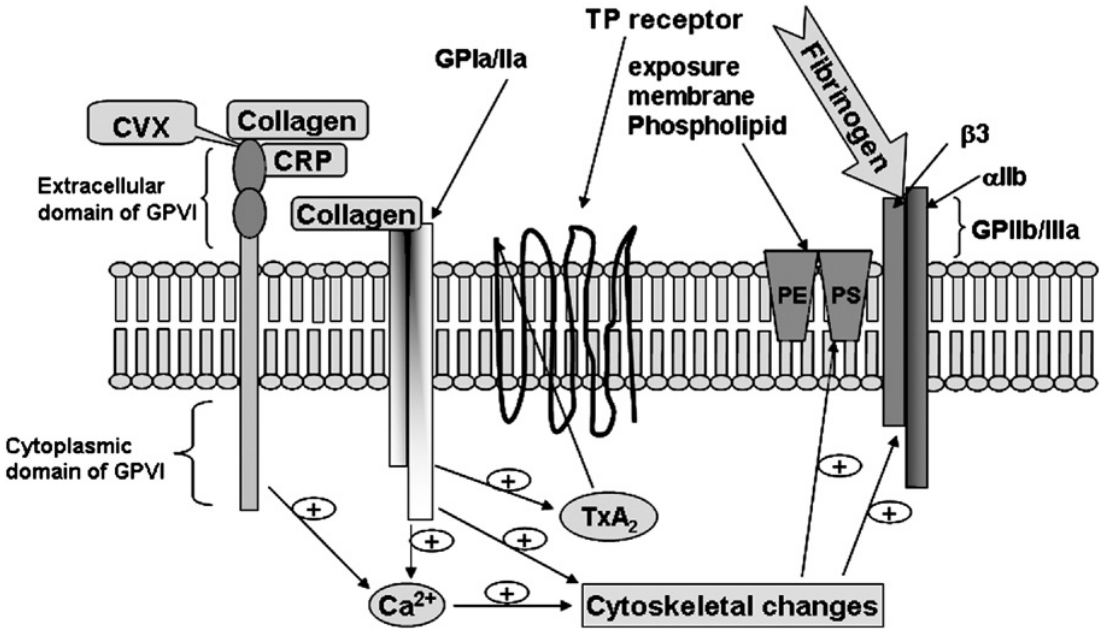

Figure 1.16: Final Results of Collagen GP VI signaling. CVX; convulxin, CRP; collagen reactive protein, TxA2; thromboxane A_2, PE; phosphatidylethanolamine, PS; phosphatidylserine. Adapted from Surin 2008.[142]

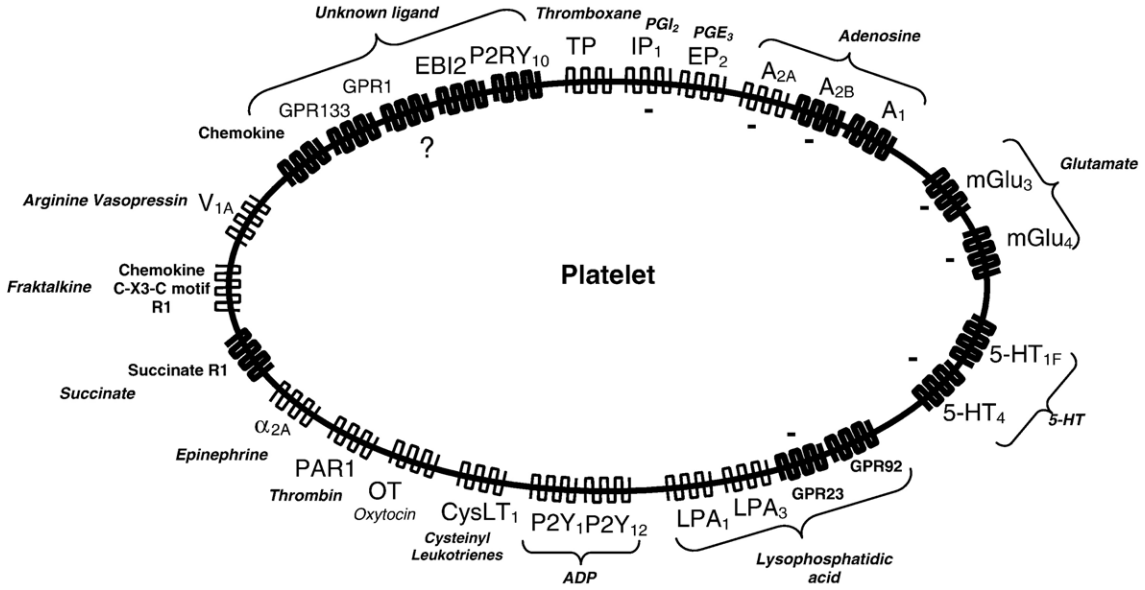

Figure 1.17: Currently Identified G Protein Coupled Receptors and Their Ligands. ADP; adenosine di-phosphate, 5-HT; serotonin, LPA; lysophosphatidic acid, PAR; protease activated receptor, PGI_2; prostacyclin, PGE_3; prostaglandin E_3. Adapted from Amisten 2007.[153]

facilitate a GDP-GTP exchange reaction on the α subunit. Once bound to GTP the α subunit disassociates from the βγ dimer, and then both continue to transmit their signals downstream. [149–151,154]

The α subunit contains an intrinsic hydrolysis activity, but this activity is greatly increased by the binding of GTPase-activating proteins (GAPs) such as regulator of GTPase-activating signaling (RGS) proteins. GAPs bind to the switching regions of the α subunit, greatly increasing its hydrolysis activity. After hydrolysis the GDP bound α subunit re-associates to the βγ subunit and can recouple to a receptor, allowing for another round of signaling.[151] Due to the fact that each receptor activation event results in activation of a βγ subunit, there is a level of commonality to the signaling response. Receptor activation also results in activation of one of four α subunit species, which vary between receptors. [149,151,154,155] These individual species regulate a variety of platelet activation and inhibition responses through both distinct and cooperative pathways.

G Protein βγ Subunits

G Protein βγ Subunits (Gβγ) once activated move across the membrane to activate Phosphoinositide 3-kinase (PI3K.)[155] PI3K phosphorylates the membrane component phosphatidylinositol 4,5-bisphosphate (PIP_2), generating phosphatidylinositol 3,4,5-triphosphate (PIP_3). PIP_3 acts as a lipid second messenger that activates exchange factors for Rho family GTPases as well as protein kinase B (PKB/Akt). [156] Rho GTPases are highly involved in multiple pathways of platelet shape change, integrin inside out signaling, and secretion. [157] PKB has been the subject of some controversy. One downstream effector of activated PKB is Rap1.

[158] It has been shown however that PKB can be activated independently of PKB *in vitro* in platelets, suggesting that more than one pathway for PKB activation may be present. [159] It has been shown however that PKB activation in platelets is dependent on Gαi coupled signaling.[160] The role of PI3K in the activation of PKB as well as the observation that PKB activation is dependent on Gαi coupled signaling lends credit to the emerging idea that the main source of Gβγ signaling in platelets occurs through Gαi coupled receptors. [154]

G Protein αs Subunits

G Protein αs Subunits (Gs) are the only inhibitory GCPR signaling molecules in platelets. They act by stimulating membrane bound adenylate cyclase (AC).[149] Stimulation of AC increases the intracellular level of cyclic AMP (cAMP), increasing the activity of protein kinase A (PKA).[149,155]

PKA is involved in multiple inhibition pathways in platelets. PKA phosphorylates myosin light chain kinase (MLCK), impairing its ability to bind to the Ca2+-Calmodulin complex. This phosphorylation inhibits cytoskeleton rearrangement needed to induce secretion and shape change responses.[155] PKA also phosphorylates the IP_3 receptor on the dense tubule system, lowering the intracellular Ca2+ release response to the second messenger. [149,155] PKA is also responsible for VASP phosphorylation, which modulates the activity of GPIIb/IIIa.[155]

G Protein αi Subunits

G Protein αi Subunits (Gi) show the most diversity in reference to the individual receptors they are associated with.[149] The main physiological role of Gi signaling is inhibition of AC.[151] Inhibition of AC reduces the intracellular

concentration of cAMP, lowering the activity of protein kinase A (PKA). [155,161] This pathway acts in direct inhibition of Gs signaling, and vice versa. As noted above, Gi signaling is also believed to be responsible for most of the Gβγ seen upon platelet stimulation, making it the main pathway that PKB and Rap1b signaling occurs. [154]

There are two different isoforms of Gi found in platelets, Gi2 and Gi3.[154] Mouse knockout studies have revealed a larger role of Gi2 than Gi3 in agonist induced activation. Gi2 has been shown to preferentially interact with the ADP receptor $P2Y_{12}$. Gi2 knock out platelets showed reduced aggregation and loss of cAMP inhibition *in vitro* in response to both Thrombin and ADP.[162,163]

Another Gi family member, G protein αz (Gz), is also expressed in platelets. It is coupled to the α2a receptor, and is responsible for the cells response to epinephrine. Gz follows the same activation pathway seen in Gi stimulation. Gz knockout studies produced platelets with no observable defects.[158] This is to be expected, as ADP autocrine and paracrine signaling occurs on a large scale after initial activation.

G Protein αq Subunits

G Protein αq Subunits (Gq) have been extensively shown to target phospholipase Cβ (PLCβ) their main effector. Once the molecule is activated the membrane bound PLCβ moves to the membrane component PIP_2 generating IP_3 and DAG. [164]

IP_3 activates the IP_3R on the dense tubule system causing an intracellular Ca2+ flux. This Ca2+ flux facilitates the translocation of PKC to the membrane where it is tethered to phosphatidylserine (PS).[165,166] PKC then undergoes a conformational

change induced by DAG causing the expulsion of a pseudosubstrate domain from the substrate binding pocket.[167] Activated PKC can then move on to cause granule secretion through protein kinase D (PKD) and the exposure of the RGD binding domain of GPIIb/IIIa.[155,168,169]

An additional effect of the elevated Ca2+ and DAG allows for the formation of the guanine nucleotide exchange factor CalDAG-GEF. CalDAG-GEF has been implicated in activation of GPIIb/IIIa.[168] As expected, CalDAG-GEF -/- platelets show a reduced aggregation response to all agonists.[170]

Intracellular release of Ca2+ also stimulates cytosolic phospholipase A_2 ($cPLA_2$), allowing for the liberation of Arachadonic Acid (AA) from the platelet inner membrane.[171] AA moves through two separate enzyme pathways in the cytosol. AA is converted by cyclooxygenase-1 (COX-1) to prostaglandin H_2 (PGH_2). PGH_2 is then converted by thromboxane synthase to thromboxane A_2. TxA_2 then diffuses out of the platelet and acts as an autocrine and paracrine stimulator of platelet activity. cPLA2 -/- platelets show that 95% or more of the TxA_2 generation is due to $cPLA_2$ activation.[172]

AA is also converted to 12-hydroxyeicosatetraenoic acid (12-HETE) in an oxidation reaction by 12-lipoxygenase (12-LOX). 12-HETE has been demonstrated to be an autocrine moderator of platelet function, and interestingly knockout studies have shown that more than 95% of 12-HETE is generated from $cPLA_2$ activation as well.[172] These two competing pathways do seem to have some pathway specificity, with evidence showing that thrombin activation of $cPLA_2$ to COX-1 is coupled to a separate signaling pathway than $cPLA_2$ to 12-LOX.[173]

G Protein α12/13 Subunits

G Protein α12 subunits (G12) consist of two separate α subunits, α12 (G12) and α13 (G13). Both subunits are expressed ubiquitously, and binding specificity to specific receptors is not strict among the two family members.[151] It should also be noted that most receptors that bind Gq also Bind G12 and G13. In addition to receptor agonist related activation, there is also proof that PKC can phosphorylate G12 family members.[174] This phosphorylation modification causes G12 to lose its affinity to the βγ subunits, which provides feedback modulation of the G12 and G13 pathways after thrombin of TxA_2 stimulation.[151] G13 has been shown to stimulate the RhoGEF activity p115RhoGEF, but G12 is unable to activate this pathway. [175,176] Both subunits show an similar affinity to p115RhoGEF however.[151]

This data taken together suggests a model where G12 and G13 compete for binding with p115RhoGEF with activation stemming from only G13 signaling. In this context activation of receptors linked to heterogeneous mix of G12 and G13 lead to a balanced response of p115RhoGEF activity. Activation of PKC, through either integrin engagement or Gq activation, would then result in the phosphorylation of G12 and its disassociation with the βγ subunit in its inactive form. Suppression of G12 regulation of the G13 signal would allow the preferential binding of G13 to p115RhoGEF, promoting signal transduction.

P115RhoGEF is a Ras homology (Rho) guanine nucleotide exchange factor that binds to G12 and G13 increasing their GAP activity, terminating their signaling potential.[175] Rho signaling is the main pathway initiated with G13 signaling, leading to platelet shape change through ROCK. ROCK activation causes a decrease in

MPase. This decrease in MPase coupled with the increase in MLCK activity associated with increased Ca2+ levels allow for the modification of MLC and subsequent shape change associated with platelet activation.[154] In contrast G13 -/- platelets show reduced aggregation and secretion with TxA_2 and thrombin, as well as inhibited shape change and RhoA activation *in vitro*. *In vivo* the mice show an increased bleeding time and they are protected against arterial thrombosis.[177]

Signaling Discussion

To draw general conclusions from GPCR signaling a more generalized picture of receptors and effectors is presented in Figure 1.18. As witnessed in a combined depiction of integrated platelet signaling (Figure 1.19) the platelet response is complicated and diverse. The cross talk that occurs between the pathways complicates any individual agonist responses when examining *in-vitro* platelet function. His becomes even more complicated due to the interplay with inflammation and the endothelium. Care needs to be taken to ensure the proper conclusions are drawn from any isolated experiments, and distinction needs to be drawn when examining platelet function outside the vasculature compared to function inside the circulation.

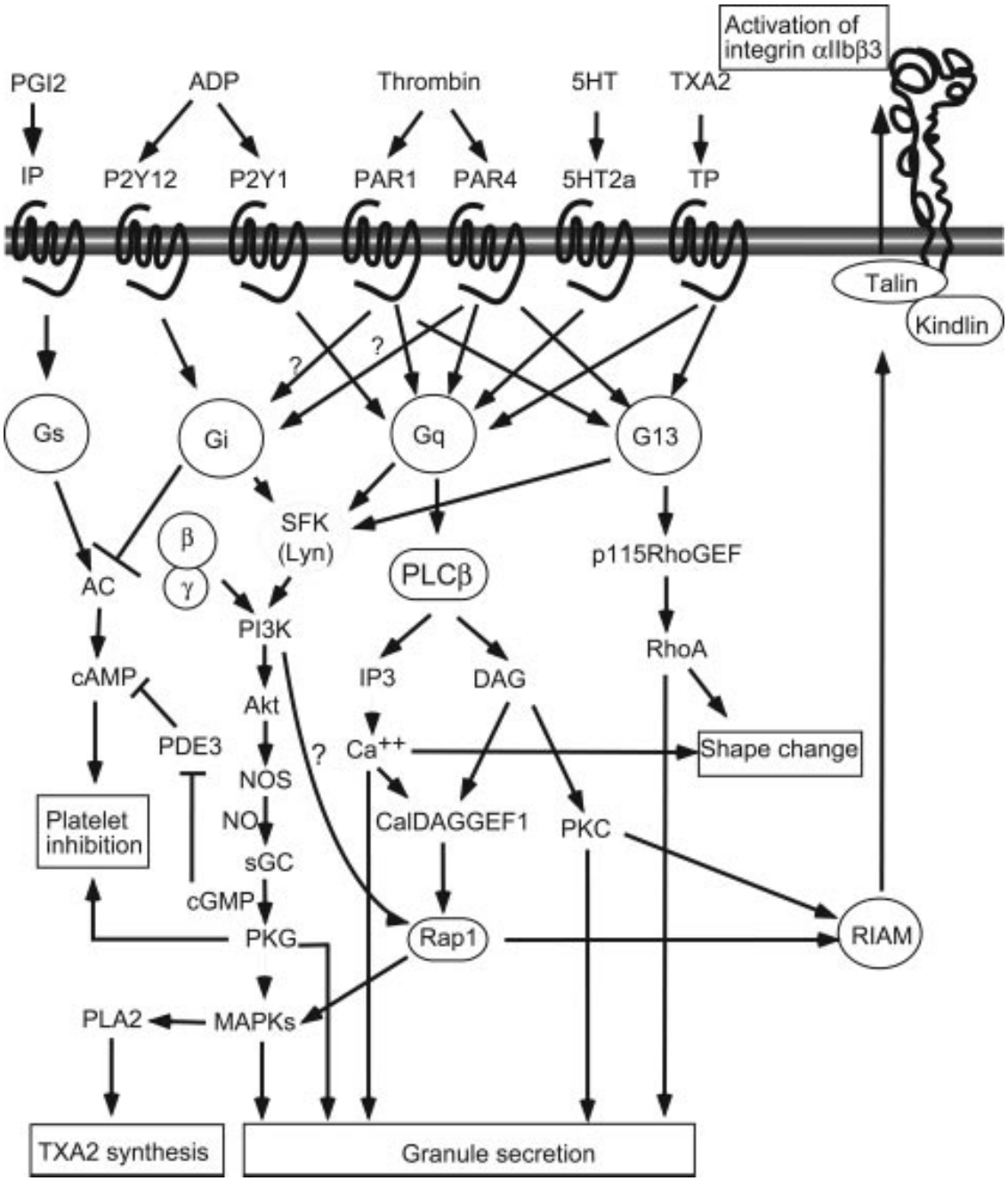

Figure 1.18: Generalized Schematic of Platelet GCPR Signaling. PGI_2; prostacyclin, TXA2; thromboxane A_2, ADP; adenosine di-phosphate, 5-HT; serotonin, AC; adenylyl cyclase, cAMP; cyclic adenosine monophosphate, PDE3; phosphodiesterase E_3 PAR; protease activated receptor, $cPLA_2$; cytosolic phospholipase A_2, PKC; protein kinase C, PKG; protein kinase G, NO; nitric oxide, NOS; nitric oxide synthase, PLCβ; phospholipase Cβ, cGMP; cyclic guanosine monophosphate, sGC; guanylate cyclase, DAG; diacylglycerol, IP3; inositol trisphosphate, PI3K; phospoinositide 3-kinase, RIAM; Rap1 interacting adapter molecule. Adapted from Li 2010.[178]

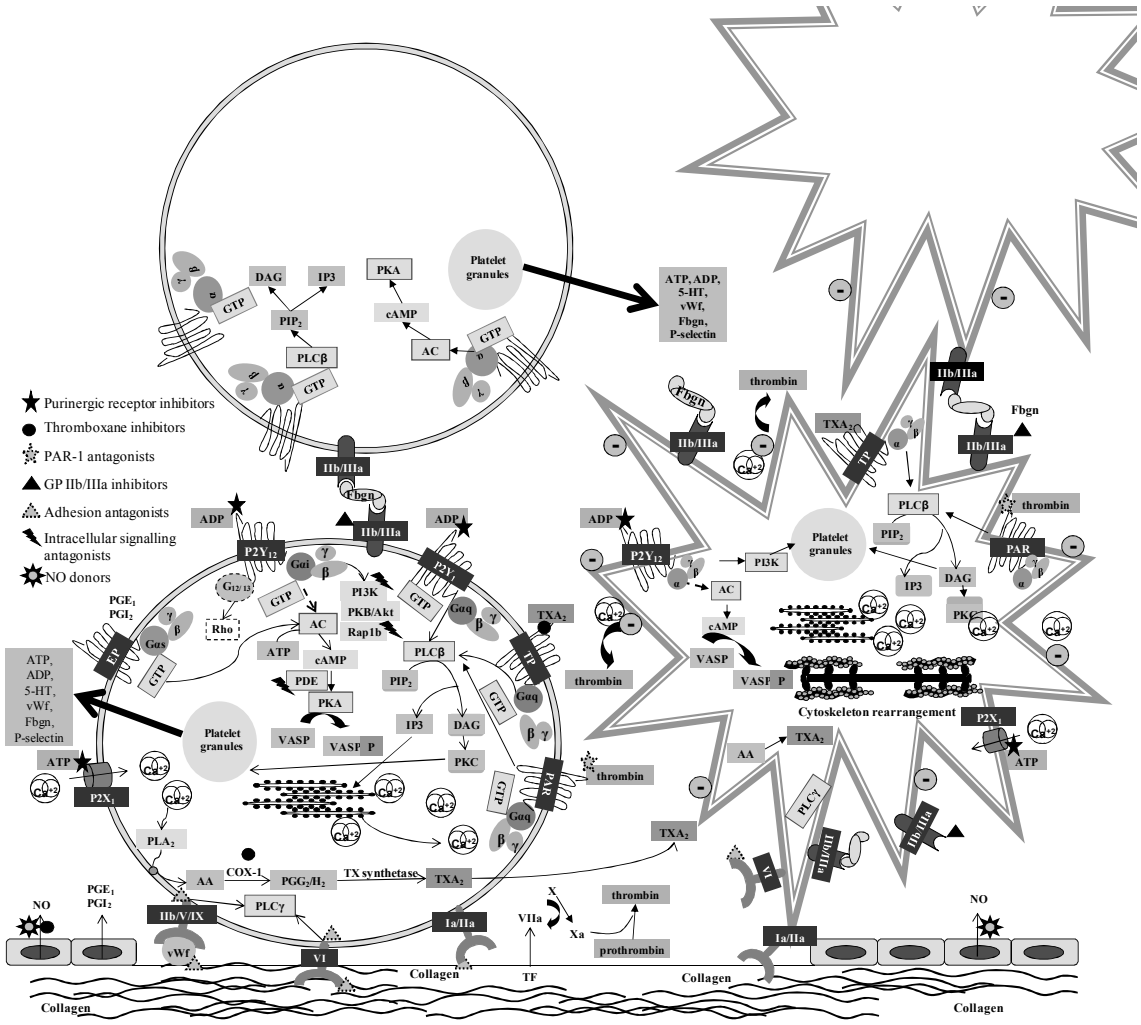

Figure 1.19: Integrated Platelet Signaling. PGI_2; prostacyclin, TXA_2; thromboxane A_2, ATP; adenosine triphosphate, ADP; adenosine di-phosphate, 5-HT; serotonin, AC; adenylyl cyclase, cAMP; cyclic adenosine monophosphate, PDE; phosphodiesterase E, PAR; protease activated receptor, PLA_2; cytosolic phospholipase A_2, PKA; protein kinase A, PKC; protein kinase C, PKG; protein kinase G, NO; nitric oxide, NOS; nitric oxide synthase, PLCβ; phospholipase Cβ, PLCγ; phospholipase Cγ, cGMP; cyclic guanosine monophosphate, sGC; guanylate cyclase, DAG; diacylglycerol, IP3; inositol trisphosphate, PI3K; phospoinositide 3-kinase, PIP2; phosphatidylinositol 4,5-bisphosphate, PIP3; phosphatidylinositol (3,4,5)-trisphosphate, AA; arachadonic acid, VASP; vasodilator-stimulated phosphoprotein, vWF; von Willebrand factor, Fbgn; fibrinogen, TF; tissue factor, PGE_1, prostaglandin E_1, COX-1; cyclooxygenase-1, PGG_2; prostaglandin G_2, PGH_2, prostaglandin H_2, TX synthase; thromboxane synthase. Adapted from Tello-Montoliu 2012.[155]

Pathophysiology of Trauma Literature

Introduction

Currently there are a variety of conditions associated with coagulopathy following trauma. Understanding these conditions better may allow for new treatment strategies increasing survival in the trauma population. Recently The Scientific and Standardization Committee on DIC of the International Society on Thrombosis and Haemostasis (ISTH) has released a review indicating that a vast majority of these conditions could be considered one.[179] This idea would suggest that coagulopathy of trauma (COT), acute coagulopathy of trauma (ACoT), and early coagulopathy of trauma (ECoT) are all a component of disseminated intravascular coagulopathy (DIC). There is also emerging evidence of a separate disorder termed simply as hypercoagulability (HC).[5] For the purpose of this review DIC, ACoT, and ECoT will be considered generally the same condition of a consumption disorder at decreasing levels of severity. HC will also be discussed, and the traditional categorization of trauma-induced coagulopathy (TIC) will encompass any condition induced by trauma that effects coagulation (hyper or hypo).

Disseminated Intravascular Coagulopathy (DIC)

Disseminated Intravascular Coagulation (DIC) can be associated with trauma as well as septic infections. It is most commonly observed in severe sepsis, but can be observed in response to severe trauma as well. Septic induced DIC has been shown to involve activation of the innate immune response, which in turn is capable of initiating coagulation as well as inhibiting fibrinolysis.[180–182] Pathologically, sepsis related DIC results in systematic elevated thrombin generation combined with

degradation and dysfunction in the anticoagulant and fibrinolytic pathways. The combined effects of this dysregulation can been associated with intravascular fibrin deposition and multiple organ dysfunction (MOD).[182] While initial effects of DIC are thromboembolic in nature, the consumption of protein coagulation factors and increased fibrinolytic activity often present as a hypocoagulability in testing.

Coagulopathy of Trauma (ACoT/ECoT)

Controversy surrounds the identification and characterization of ACoT and ECoT. Many different groups have used different diagnostic and qualification criteria leading to confusion. Current ISTH opinion is that these conditions are related closely to, if not part of the development of DIC.[179] These conditions are commonly characterized as an extension of plasma protein function testing (PT/PTT) due to consumption of factors.[179,183,184] Currently it is assumed that clotting factors are consumed at the site of injury during the body's natural clotting response. Unfortunately it has yet to be proven if there is a true loss of factors, or if the functionality of the pathways are effected as well.

Lending to the mechanistic mystery, these conditions are diagnosed in platelet poor plasma (PPP) removing a main component of the physiological response. A main difference between DIC and ACoT and ECoT is the lack of a fibrinolytic phenotype in the latter two.[183,185]

Hypercoagulability (HC)

Whole blood testing by thromboelastography (TEG) has been used recently in the treatment of trauma patients to better understand the complete coagulation profile of the blood. It has been able to identify hypercoagulable patients that

traditional plasma based testing has not.[5,186] These patients present with TEG R times < 3.7 minutes, and other kinetic parameters may be quickened as well.[185]

Coagulation Monitoring

Over the years there have been numerous tests developed to monitor coagulation status. The original testing included simple observation tests such as bleeding time and plasma protein functionality tests. While these tests are imperfect, they are still used to this day in one form or another to assess bleeding status both in the research environment as well as clinically. To better understand the current state of coagulation testing the following currently accepted coagulation tests are examined in detail below.

Classical Coagulation Protein Testing

Coagulation testing is a critical part of monitoring hemostasis. The established clinical plasma based clotting assays are used to determine both the functionality of the enzymatic pathways involved in clotting as well as the concentration and activity of the individual plasma proteins themselves. While extremely useful in identifying factor deficiencies such as Hemophilia, they offer little insight into actual cellular processes involved in clotting. Monitoring platelet aggregation allows for the examination of the platelet-platelet interaction mediated by fibrinogen in both PRP as well as WB, but does so in a non-clotting system. Adhesion studies allow for the monitoring of the ability platelets to adhere to an immobile surface through integrin interactions. Flow Cytometry allows for a thorough investigation into the signaling involved in the platelet hemostatic response on a cellular level. Thromboelastography allows for the examination of

the entire whole blood system, integrating plasma, platelets, and the rest of the blood components to proceed down normal coagulation pathways. Taken together, these tools provide.

Plasma Based Clotting Assays

Prothrombin Time (PT) is a test that was developed in 1935 by Dr. Armand Quick by using a rabbit brain extract to mimic tissue factor and recalcification of a citrated blood sample.[9] While the terminology and understanding of the extrinsic pathway has changed over the years, the method for assessing PT is relatively unchanged. PT tests the functionality of the extrinsic and common pathways together as a whole group. Both PT and PTT were covered in depth at the beginning of this chapter.

Partial Thromboplastin Time (PTT) was developed in 1953 by a group of researchers, and was named as such due to the use of only a partial extract.[9] This assay was also modernized and optimized over the years, but also remains theoretically unchanged over the years. PTT is able to test the functionality of the intrinsic and common pathways as a group. Because of the inclusion of the common pathway in both tests, more testing would be needed if both tests produce an abnormal result.

Fibrinogen concentration can be measured in two manners. The first method is mechanically by the method of Clauss. This method is most commonly used for clinical evaluation, as well as for research uses. It is a clot based quantitative assay that is able to determine functional fibrinogen levels. It involves the addition of purified thrombin to a diluted plasma sample.[187] Second, fibrinogen ELISA kits are

commercially available, and can be used to determine if reduced functional results are due to actual consumption of fibrinogen, or if a state of dysfibrinogenemia exists.

Coagulation factor and protein assays can be performed in a variety of manners. With small manipulations the same tests developed to monitor PT and PTT can be used to determine the functional concentration of the individual components of each system. Another technique involves the identification of the total molecule concentration by commercially available ELISA kits. The advantage to using both options is the ability to generate a functional to total concentration ratio of the components being tested.

Aggregation

Aggregometry

Aggregometry is commonly performed in two ways, by light transmission in PRP, or by impedance in whole blood (WB). Light transmission aggregometry (LTA) is the oldest method of measuring platelet aggregation, and relies on the formation of platelet-platelet aggregates to deflect and absorb light passing through the plasma sample. LTA has considerable downfalls, including a high labor intensity factor, extended time requirements (20-30 minutes) to prepare plasma samples, and the lack of WBCs and RBCs that the platelets typically encounter in the circulation.[188]

Whole blood aggregometry (WBA) is measured by impedance generated by platelets between two electrodes in a WB sample. Typically the sample is diluted 1:1 in saline, and spun at a low rate to ensure mixing and mimic a low shear environment.[188] The disadvantages of LTA are effectively nullified with WBA, as it

requires no centrifugation, little labor intensity, and has all the normal components of the circulating blood. As a bonus it also requires less sample than LTA. Recent advances have automated this process even further with microplate aggregation and multiple probe aggregation further lowering the required sample volume and increasing throughput.[189–191]

Platelet Function Analyzer (PFA-100®)

The PFA-100 uses high shear conditions to measure the formation of a platelet plug, which in turn generates data on platelet adhesion and aggregation. The system uses disposable cartridges with internal membranes coated with collagen and either ADP (Coll/ADP) or epinephrine (Coll/EPI) to cause a contact activation of the blood. Blood is then passed through an aperture at high shear, and the amount of time required for the complete occlusion of this aperture is reported as the closure time (CT).[192] Normal human range for Coll/ADP and Coll/EPI are 70-125 seconds and 90-200 respectively.

Thromboelastography

The TEG 5000 Thromboelastograph® Hemostasis Analyzer (Haemoscope Corp., Niles, IN, USA) is used to measure viscoelastic properties of clotting in a whole blood (WB) system under low shear. The technology is well known and used in both the clinical and research setting. The assay is typically performed using recalcification with or without kaolin activation as per manufacturers instruction.[193] An explanation of the most commonly reported parameters can be found in Table 1.5.

Platelet Mapping® (Haemoscope Corp., Niles, IN, USA) is a modified form of TEG, designed to isolate and determine platelet contributions to whole blood clotting. All required reagents were purchased in kit form directly from Haemoscope, and assays were performed according to manufacturer's specifications. Briefly, platelet mapping involves comparing the kaolin-activated MA ($MA_{thrombin}$) obtained in citrated WB samples to testing performed on heparinized blood. The use of heparinized blood eliminates the thrombin contribution to the clotting process. In order to then determine the fibrin-based contribution to the clot activator F (reptilase and FXIII) is added and MA is obtained (MA_{fibrin}). In the final set of samples activator F and a platelet activator is added, which currently includes ADP and Arachadonic Acid (AA) when purchased as a kit. Literature searches also reveal published work using collagen by separate groups using standardized methods.[194,195] The resulting MA from the activated sample ($MA_{activator}$) is used to determine the activator inhibition activator related %aggregation (TEG-MAactivator) is measured according to the following equation:[196]

$$\textbf{TEG-MA}_{\textbf{activator}}\ \textbf{(\%)} = [(\textbf{MA}_{\textbf{activator}} - \textbf{MA}_{\textbf{fibrin}})/\ (\textbf{MA}_{\textbf{thrombin}} - \textbf{MA}_{\textbf{fibrin}})] \times 100$$

The resulting aggregation level is compared to the thrombin-activated sample and inhibition is calculated. Normal human range provided by the company is <70% for any individual activator. This assay was originally designed to monitor anticoagulant therapies, but recent work has begun to highlight other applications.[196,198,199]

Table 1.5: Description of HAS, TEG, and ROTEM Parameters.

Abbreviation	Name	Explanation	Reference range
TEG/ROTEM			
r/CT	Clotting time	Initial clot formation time. It is the time interval between the addition of sample to the cup and the production of a signal of at least 2 mm amplitude. It is interpreted as a measure of the speed at which fibrin is produced and cross-linked	TEG: 3.0–8.0 min ROTEM: 4–10.5 min
k/CFT	Clot formation time	The time from initial clot formation until an amplitude of 20 mm is reached. It represents the clot formation dynamics	TEG: 1.0–3.0 min ROTEM: 1.5–4.5 min
MA/MCF	Maximum amplitude/maximum clot firmness	This is a reflection of the maximum structural integrity obtained by the clot. It is dependent on fibrin content, fibrin structure, platelet concentration and platelet function	TEG: 51–69 mm ROTEM: 50–70 mm
HAS			
FOT	Force onset time	The time that it takes for thrombin to be generated in whole blood	3.0–8.0 min
PCF	Platelet contractile force	Represents the force produced by platelets (at 20 min) during clot retraction and therefore a measure of platelet function during clotting. It is dependent on thrombin, platelet concentration and metabolic status	4.5–9.5 kdynes
CEM	Clot elastic modulus	Represents the strength of the clot after 20 min and it is measured simultaneously with PCF. It is dependent on fibrin content, fibrin structure, red cell flexibility, platelet concentration and PCF	14.0–35.0 kdynes cm^2

Adapted from Brophy 2011.[197]

ROTEM®

The ROTEM analyzer (Pentapharm GmbH, Munich, Germany) is a modified form of thromboelastography which measures viscoelastic properties of whole blood (WB) clotting under low shear.[200] TEG and ROTEM parameters are relatable, and can be found in Table 1.5. The ROTEM is also used to test the effectiveness of the intrinsic and extrinsic pathways individually through activation agonist changes. The commonly used tests in this lab include Na-TEM (native WB clotting after recalcification), In-TEM (intrinsically activated with thromboplastin-phospholipid), and Ex-TEM (extrinsically activated with rabbit brain thromboplastin).[104]

Hemodyne HAS®

The Hemodyne HAS (Hemodyne, Richmond, VA, USA) is a whole blood clotting assay used to measure platelet interactions in a clotting system. The basis of testing has been previously described in detail.[201,202] The HAS determines platelet contractile force (PCF), clot elastic modulus (CEM), and force onset time (FOT). PCF is a measurement of the forces generated by platelets during clot retraction. Normal range for health human subjects is 4.5-9.5 kdyne.[200] CEM is used to express the clot stiffness that develops over a 20 minute time period. The normal range for humans is 14-35 kdyne/cm.[200] FOT is a measure of the time it takes for the sample to start clotting after recalcification. Normal human range for FOT is 3-8 minutes. A breakdown and further explanation of these parameters can be found in Table 1.5.

Flow Cytometry

The Accuri C6 flow cytometer (BD Biosciences, San Jose, CA, USA) was used for all flow experiments performed in this lab. The unit measures variables over a

very large dynamic range, and is compact enough to fit on a benchtop.[203] It uses a pulse method to excite samples concurrently with both 488 nm and 640 nm lasers. The filter configuration for all measurements was 530/30 nm for FL1, 585/40 in FL2, 670 LP in FL3, and 675/25 in FL4. Quality control is performed daily with 6 and 8 bead validation as per manufacturer's instruction.

Whole Blood Platelet Analysis

Platelets are commonly identified with the constitutive marker CD41. Other antibodies are used to identify various aspects of platelet activation. Commonly observed markers of platelet activation include: CD62-P (P-selectin), CD63 antigen, PAC-1 (activated GPIIb/IIIa), increased intracellular ROS (reduced fluorescein and calcein derivatives), intracellular Ca++ elevation (BAPTA derivatives), bound fibrinogen (streptavidin derivatives), and PS exposure (Annexin-V).

Microparticles

Platelet derived microparticles are small phospholipid microvesicles normally shed by activated cells, and are defined by both size as well as surface receptor staining.[101] The total circulating microparticles (cMPs) are determined using standardized sized polystyrene beads and FSC/SSC (0.01-1.0 μm). Size alone cannot be used as microparticles from endothelial cells, leukocytes, erythrocytes, and smooth muscle cells can all be found in the blood. A monoclonal antibody targeting a constitutive platelet marker (CD41) is used to identify platelet-derived microparticles (pMPs) out of this population of cMPs. Other antibodies can be used to determine the origin of other cMPs (CD45=Leukocytes, CD31+ / CD41- = Endothelial cells) as well as surface expression of PS (Annexin-V), TSP1

(thrombospondin-1), CD62P (P-selectin), CD142 (TF), and PAC-1 for functional characterization.[104]

Chapter 2:

Evaluation of an Aseptic Spray-Dried Plasma Product for Prompt Reversal of Coagulopathy

2.1 Introduction

Uncontrolled hemorrhage and resulting shock represent the major causes of preventable deaths on the battlefield.[2,4] Multiple avenues are being approached to increase protection for the warfighter, but severe traumatic injury will be a reality in battle for the foreseeable future. Improvements in the treatment of extremity bleeding have reduced its impact on mortality[2], but the new generation of tourniquet technology will never be applicable to head and torso wounds. This makes arresting hemorrhage inside the parameters of trauma-induced shock a complex undertaking. Development of ACoT and trauma induced DIC suggest a consumption disorder, as the loss of function of key plasma proteins complicates hemostasis. This intertwines the need for volume correction with the need to reverse coagulopathy when developing resuscitation methods.

Current US combat care protocols define three distinct stages in the treatment of casualties each with varying degrees of available intervention. Care under fire is the first stage of treatment, and occurs while still under direct fire from the enemy. Current military doctrine demands that the suppression of fire take precedence over rendering care, so the individual injured often performs this level of treatment (self aid). Management of bleeding is often the only aid rendered at this time.[204]

Once immediate threats are subdued treatment moves into the tactical care stage. This stage often involves aid from fellow soldiers (buddy aid) as well as medics/corpsman embedded with combat units, and increases the options for treatment and intervention. During this time the possibility of further combat action is considered eminent. Treatment focuses on treating any remaining hemorrhage and limited breathing management. Fluid administration lines through intravenous access are possible during this stage if needed. All treatment is limited to what can be transported organically within the combat unit.[204]

The final stage involves the evacuation to a forward medical support or surgical hospital, introducing a high level of care and access to blood components. While treatment at this stage may parallel treatment available in the civilian world, it can take hours to secure an area and evacuate the casualty.[204]

Due to the need for both volume replacement as well as the reversal of coagulopathy, the ability to deliver whole blood (WB) or blood components during the tactical care stage could prove to be vital to the treatment of combat casualties. Issues with stability and shelf life with the transport of WB as well as the possibility

for immunoreaction events and viral transmission related to blood product administration are complexities that have yet to have been solved.

Fresh frozen plasma (FFP) offers both coagulation factor replacement as well as volume replacement due to high osmotic pressure. FFP lowers the space restrictions and increases shelf life to up to a year, but samples must be stored at -18° C.[205] Freeze-drying of plasma has been shown to create a lyophilized product that maintains coagulation activity. Treatment allowed the freeze dried plasma (FDP) to be stored at ambient temperature.[205] This process did reduce space requirements for storage even further, but the requirement to reconstitute at full volume and the lack of addressing the viral issue highlight the shortcomings of this process.[206]

Recent development of a novel aseptic, hypertonic, hyperosmotic spray-dried plasma (Entegrion Inc., Research Triangle Park, NC, USA) addresses two key shortfalls of FDP. The spray dried plasma (SDP) process allows for both the destruction of viral contamination as well as reconstitution at one third of original volume. The increases in sterility combined with a possible 2/3 decrease in fluid volume are clear advantages over current choices.

The goal of these experiments was to compare the effects of SDP to current treatments (WB, FFP) in swine model of traumatic shock and coagulopathy with focus on treatment of hypovolemia and coagulopathy. In order to accomplish this goal a model of trauma induced hypovolemic shock and coagulopathy that was repeatable was required. As part of the model development and verification process resuscitation protocols were evaluated as well.

During the resuscitation protocol compatibility issues between the swine and the human SDP product were severe and caused the suspension of the study before the main goal could be met. Data generated during the model development did reveal a reproducible model of hypovolemic shock and trauma induced coagulopathy, but no treatment analysis could be made. An *in-vitro* assay of the effects of SDP and FFP on human blood samples was used for a preliminary comparison of the two products while model species selection was evaluated.

2.2 Materials and Methods

Materials

SDP was provided in powder form (Entegrion, Research Triangle Park, NC, USA) and reconstituted within 1 hour of intended use using provided buffer. The product is generated from pooled human plasma, which undergoes a proprietary treatment to produce a lyophilized product. Reconstituted it resembles pooled fresh citrated plasma in protein concentration and pH.

FFP was obtained from Virginia Blood Services (Richmond, VA, USA) and was all type A. All volunteer blood used from this study was type A to control for immunoreactions.

Human type A blood was obtained from healthy volunteers via direct peripheral venipuncture by trained staff directly into standard vacutainers. All blood samples were performed one time only and drawn into a combination of sodium citrate, EDTA, and heparinized vacutainers with the total volume per sample point $\leq$ 25 mL. Blood was obtained from excess controls drawn for the protocol "**Defining Platelet Function During Polytrauma.**"

Large Animal Model

General Preparation/ Instrumentation

The Virginia Commonwealth University Institutional Animal Care and Use Committee approved the experiments described in this section. Juvenile crossbred Yorkshire swine were used for all animal experiments. All animals were shipped to, inspected by, and quarantined within the VCU animal care facility. Animals were allowed to acclimate to their new environment for a minimum of 72 hours in order to limit stress influences on experimental outcome. During their entire stay in the animal care facility the swine were provided with food and water *ad libitum.*

Immediately prior to the experiment the animals were sedated with intramuscular ketamine (20 mg/kg) and xylazine (2 mg/kg), then transported to the procedure room. Once in the procedure room anesthesia induction was achieved via inhalation with 3-5% isofluorane. After induction the animals were placed in the supine position and orally intubated. Anesthesia was maintained using 1-3% isofluorane in 30% oxygen. Minute ventilation (Draeger Trilo, Telford, PA, USA) was adjusted to produce an end-tidal CO_2 ($etCO_2$) value of 35-40 mmHg (Biopac Systems Inc. Goleta, CA, USA). Animals were placed on a warming blanket and kept at a core temperature between 37-38.5°C via readout from a rectal probe connected to the heating apparatus (Blanketrol II, Cincinnati Sub-Zero Products, Cincinnati, OH, USA).

During the preparation period and surgical instrumentation blood gases were monitored to ensure that the partial pressure of carbon dioxide (pCO_2) was maintained at 35 ± 5 mmHg, and continuous oxygen consumption was monitored

using indirect calorimetry (Biopac Systems Inc. Goleta, CA, USA). Oxygen debt was calculated from the same indirect calorimetric data.

Continuous electrocardiograph (ECG) monitoring was performed using a standard 3-lead configuration (Biopac Systems Inc. Goleta, GA, USA). Mean arterial pressure (MAP) and arterial blood samples were performed via an arterial catheter placed surgically in the right carotid artery. Mixed venous hemoglobin saturation ($ScvO_2$), central venous pressure (CVP), pulmonary artery pressure (PAP), and continuous cardiac output (CCO) were performed via an oximetric pulmonary artery catheter (Edward Lifesciences, Irvine, CA, USA) inserted into the pulmonary artery through the right internal jugular vein. This catheter was also used for mixed venous blood sampling. Blood samples for coagulation were drawn via catheter inserted into the left external jugular vein. This line was also used for fluid administration. The left femoral artery was exposed via a large surgical incision in the groin area and cannulated with a 4.8 mm internal diameter catheter used for the controlled arterial hemorrhage during the injury model.

To ensure the highest level of accuracy in oxygen consumption monitoring the animals was given intravenous pancronium bromide (0.1 mg/kg) every 45 minutes to induce and maintain paralysis. This is necessary as the animal's physiological response to shock includes hyperventilation even under adequate surgical anesthesia, and this would skew oxygen consumption data via changes in minute volume.

All animals were given a 20-minute stabilization period, during which blood gases, hemoglobin, and hematocrit were rechecked. At the end of this stabilization

period baseline coagulation, blood chemistry, and hematology samples were drawn directly into appropriate vacutainers. After baseline samples animals were given an additional 10 minutes to stabilize prior to the injury protocol.

Injury Model

The goal of injury design was to simulate a combination of injuries that may occur in the battlefield. Soft tissue injury and a long bone fracture were combined to the hemorrhage to create a dynamic injury. This type of additional injury has been shown to cause a significant adverse effect on oxygen transport when compared to hemorrhage alone.[207,208] Bilateral hind limb skeletal muscle injury was created by firing a 7 cm long, 1 cm diameter captive bolt device (Accles & Shelvoke, Ltd., Birmingham, England) two times into each limb with care taken to avoid any major vasculature. The captive bolt device was also placed directly against the right femur and fired to generate a comminuted femur fracture.

Animals were then subjected to a controlled hemorrhage until 40% of their estimated total blood volume had been removed. The 40% goal was chosen for reproducibility, injury severity (near fatal), and the fact that it was hypothesized that it would produce hemorrhagic shock severe enough to create a repeatable and measurable coagulopathy. Hemorrhage occurred at a rate of 1% estimated blood volume per minute using a computerized pump (Masterflex pump and pump-head, Cole Palmer, Vernon Hills, IL, USA) to a target MAP of 30 mmHg. Shed blood was collected in 3-bag blood collection sets (Terumo Products, Somerset, NJ, USA) to allow for subsequent use of whole blood as a resuscitation fluid to maintain MAP > 30 mmHg if needed.

Indirect calorimetry was employed to monitor continuous oxygen consumption (VO_2) at the airway in a breath-by-breath manner. Oxygen debt (OD) was calculated (200 measurements per minute) and expressed in ml/kg. The calculation involved the cumulative difference between VO_2 during the injury period and the baseline VO_2 integrated the time before and after the injury and hemorrhage.

During the injury period the animals were ventilated using room air and 2% isofluorane, and the animal's temperature was allowed to spontaneously drift by removal of the warming blanket. MAP was maintained at 30 mmHg using small aliquots of saline (or whole blood if absolutely required) until oxygen debt reached 80 mg/kg (D80). This level has been used extensively in this lab and is associated metabolic acidosis (lactate levels 8-12 mmol/L and base excess levels -10 to -15 mEq/L), oxygen extraction ratios of 80% or higher, and greater than 50% mortality.[207,208] Once oxygen debt goals were reached blood samples were once again obtained for coagulation, blood gas, hematology, and blood chemistry analysis.

Physiological Monitoring

Systematic blood gas, metabolite, and electrolyte analysis was performed using the Stat Profile Critical Care Xpress Bedside Analyzer (Nova Biomedical Corp., Waltham, MA, USA). Samples were obtained simultaneously from the arterial and venous sampling catheters described above at all time points during the experiments.

Hematology analysis was performed using the VetScan HM_2 Hematology System Bedside Analyzer (Abaxis, Union City, CA, USA). Samples were obtained

from the venous sampling catheter and drawn directly into standard EDTA anticoagulated vacutainers (BD Biosciences, San Jose, CA, USA).

A combined blood chemistry panel was performed using the VetScan Chemistry System (Abaxis, Union City, CA, USA). Samples were obtained from the venous sampling catheter and drawn directly into standard Li-Heparin vacutainers (BD Biosciences, San Jose, CA, USA).

Resuscitation

After the end of the injury period after sampling animals were then utilized to test various resuscitation fluids as part of the approved protocol. This study was to test the effectiveness of a spray dried plasma product for treatment of the shock-induced coagulopathy. It was compared to FFP, NS, and fresh whole blood (FWB) to determine both coagulation as well as resuscitation properties. Once this protocol had been completed, all surviving animals were euthanized via IV injection of Potassium Chloride while still under anesthesia.

***In-Vitro* Model**

Treatment Protocol

Due to adverse reactions between the swine and the human plasma product testing was suspended prior to reaching a standardized administration protocol. An *in-vitro* experiment was designed to compare the coagulation effects of SDP to those of fresh frozen plasma (FFP) when added to whole human blood. Type A FFP was obtained from Virginia Blood Services, and type A whole blood was obtained by venipuncture from healthy volunteers.

Samples were divided into 3 treatment groups receiving normal saline (NS), FFP, or SDP in a volume required to bring the treatment up to 30% total concentration. This concentration was chosen to mimic the final conditions that were to be present in the animal prior to the decision to terminate the swine experiments. Samples were incubated at 37° C for 30 minutes, inverted 3 times gently to avoid platelet activation prior to testing.

Coagulation Testing

All coagulation samples were drawn directly into Na-Citrate 1:9 standard vacutainers (BD Biosciences, San Jose, CA, USA) from the venous sampling line. Samples were delivered to the lab within 30 minutes of draw, and all experiments were completed within 2 hours.

Thromboelastography was performed on citrated whole blood samples using the TEG® 5000 Hemostasis Analyzer (Haemoscope Corp., Niles, IL, USA) using kaolin and $CaCl_2$ as per manufacturer's instructions. Use of this technique has been described by this lab before.[193,209,210]

Modified rotational thromboelastography was performed on the ROTEM® analyzer (Pentapharm GmbH, Munich, Germany). The standard Na-TEM (simple recalcification), In-TEM (intrinsically activated), and Ex-TEM (extrinsically activated) tests were all completed according to manufacturer's specifications. These tests have been described in detail by this lab before.[197,200]

The plasma coagulation cascade pathway tests of Prothrombin Time (PT) and activated partial thromboplastin time (PTT) as well as functional fibrinogen concentration were all measured in platelet poor plasma using the Start-4®

coagulation analyzer (Diagnostica Stago, Asnières, France). All tests were performed according to the manufacturer's recommendation.

Samples were stained for flow cytometry with primary antibodies for CD62p-PE, PAC-1-FITC, CD41-PE-Cy5 and appropriate isotypic controls (BD Biosciences, San Jose, CA, USA). After a 30-minute incubation samples were fixed and analyzed with an Accuri C6 cytometer (BD Biosciences, San Jose, CA, USA). A total of 20,000 cells were analyzed in each sample.

Platelet aggregation was measured on the Chrono-log Aggregometer (Chrono-log Corp., Havertown, PA, USA) on citrated whole blood as described by previous work in this lab.[209] Samples were activated using collagen (2μg/mL) or ADP (10 μM final concentration) according to manufacturer's recommendations. Aggregation was measured as impedance increases in whole blood after stimulation with agonist.

Whole blood cell counts were performed on the ABX Micros 60 (Horiba Medical, Irvine, CA, USA). Tests were performed on EDTA anticoagulated whole blood drawn at the time of patient sampling according to manufacturer's instruction.

Data Analysis

Data distributions were checked using QQ plots, and descriptive statistics (counts, percentages, mean ± SD, median (IQR)) were used to summarize data for all experiments. All parameters for *in-vitro* studies were measured as fold change from control (NS treatment group). Different Treatment group parameters were compared using the student's t-test, analysis of variance (ANOVA), or the non-

parametric Kruskal-Wallis test as appropriate. Multiple comparisons were made using either Tukey-HSD method or Wilcoxon test as appropriate. All statistical analyses were performed using JMP statistical software, version 10.0.0 (SAS Institute, Cary, NC). The level of significance for all statistical tests was $p < 0.05$.

Results

Large Animal Model

Observed Differences in Arterial and Venous Coagulation

During the model development phase of the experiments citrated whole blood was obtained in simultaneous samples at baseline from both the arterial and venous sampling catheters. Blood was drawn directly into standard Na-Citrate vacutainers (BD Biosciences, San Jose, CA, USA) and subjected to plasma protein assays as well as mechanical whole blood coagulation assays. Samples showed no difference in PT, PTT, or functional fibrinogen measurements (data not shown). Venous blood showed significant quickening in TEG clotting kinetics (Table 2.1), with R (mean decrease of 27%) and k (mean decrease of 23%) values both reflecting faster clotting. This significant kinetic increase was also apparent during HAS force onset time (FOT) analysis, with a mean decrease of 32%. Maximum Amplitude (MA) also showed significant increases in venous blood suggesting a small increase in clot strength. As composition of protein components is constant between the samples, these differences appear to be platelet oriented.

Table 2.1: Mechanical Clotting Analysis of Matched Arterial and Venous Blood Samples

		Unit	Mean (STD DEV)		p ≤ 0.05
			Arterial	**Venous**	
Thromboelastography					
		n	12	12	
TEG					
	R	**min**	**7.76 (0.93)**	**5.70 (0.93)**	**Y**
	K	**min**	**1.68 (0.23)**	**1.29 (0.22)**	**Y**
	MA	**mm**	**66.78 (2.99)**	**68.57 (2.98)**	**Y**
Modified Thromboelastography					
		n	12	12	
HAS					
	FOT	**min**	**5.58 (0.85)**	**3.77 (0.83)**	**Y**
	PCF	kdyne	8.96 (2.06)	9.15 (2.06)	N
	CEM	kdyne/cm^2	25.44 (5.88)	27.96 (5.68)	N

All data presented as mean (Standard Deviation). Bold type indicates parameters with significant differences. min; minute, TEG; Thromboelastograph, HAS; Hemostasis Analyzer System, R; R time, K; kinetic time, MA; maximal amplitude, FOT; force onset time, PCF; platelet contractile force, CEM; clot elastic modulus

Verification of Clinically Relevant State of Hypovolemic Shock when Oxygen Debt Reaches 80 mg/kg (D80)

In order to show a clinical relevance of the injury mechanism, a clear state of shock must be produced in response. To confirm the creation of a state of trauma-induced shock a wide range of physiological parameters were monitored. To this end these parameters will be compared to accepted clinical definitions of shock states. Of the 17 animals used in this development study only 1 animal did not survive until D80, indicating that at the point of resuscitation the injury was severe, but not so severe it would overwhelm resuscitation efforts.

Epidemiological studies have resulted in a better understanding of clinical states of hypovolemic shock.[211] Using this relatively new method of stratifying shock base excess (BE) is first converted to base deficit (BD). This is a simple transformation from a negative value to a positive value (BD= BE x -1). Stratification then allows for characterization of four classes: no shock (BD≥ 2.0), mild shock (BD> 2.0 to 6.0), moderate (BD> 6.0 to 10.0), and severe (BD> 10.0).[211] Using this classification arterial blood gas results show a state of moderate shock (mean 6.54 ± 3.94) in the population at D80 (Table 2.2). This level is significantly higher than the mean baseline BD of -5.76 ± 2.3.

Lactic acidosis (serum lactate > 5 mg/dL) is also a common marker of metabolic dysfunction and systemic hypoxia and it has been shown that it can be viewed independently from BD.[212] Lactic acidosis has also been shown to cause increased INR values, and recombinant Factor VIIa (rFVIIa) has been shown to reverse this coagulopathy.[213] After the injury period the animals displayed a mean

Table 2.2: Blood Gas Parameters at Baseline and Compared to End of Hemorrhage

	Unit	Mean (STD DEV)		p ≤ .05
Arterial Blood Gas		**Baseline**	**End of Injury**	
pH		**7.43 (0.07)**	**7.32 (0.06)**	**Y**
pCO_2	mmHg	**43 (2)**	**35.11 (5.7)**	**Y**
pO_2	mmHg	124 (20)	119.7 (24.5)	N
Hb	g/dL	**9.9 (0.61)**	**8.32 (1.31)**	**Y**
sO_2	%	98.4 (0.74)	97.7 (2.1)	N
COHb	%	0.16 (0.39)	0.48 (0.69)	N
MethHb	%	0.51 (0.2)	0.62 (0.62)	N
K+	mmol/L	**3.78 (0.21)**	**5.06 (0.733)**	**Y**
Na+	mmol/L	**137.9 (2.8)**	**135.6 (3.2)**	**Y**
Ca++	mEq/L	1.2	1.17 (0.095)	N
Cl-	mmol/L	**105.7 (4)**	**108.8 (5.13)**	**Y**
Glucose	mg/dL	**141.7 (37.5)**	**224.3 (94.3)**	**Y**
Lactate	mmol/L	**1.08 (0.3)**	**7.85 (2.25)**	**Y**
ctO_2	Vol%	**13.7 (0.83)**	**11.5 (1.7)**	**Y**
BE	mEq/L	**5.76 (2.3)**	**-6.54 (3.94)**	**Y**
Mixed Venous Blood Gas				
pH		**7.39 (0.027)**	**7.17 (0.07)**	**Y**
pCO_2	mmHg	**54.6 (3.93)**	**65.4 (3.7)**	**Y**
pO_2	mmHg	**42.1 (3.74)**	**21.96 (20.8)**	**Y**
Hb	g/dL	**9.96 (0.82)**	**8.89 (1)**	**Y**
sO_2	%	**62.5 (6.4)**	**10.86 (5.74)**	**Y**
COHb	%	0.011 (0.45)	0.194 (0.18)	N
MethHb	%	**0.65 (0.3)**	**1.24 (0.22)**	**Y**
K+	mmol/L	**3.87 (0.18)**	**5.19 (0.77)**	**Y**
Na+	mmol/L	138 (3.9)	136.3 (4.1)	N
Ca++	mmol/dL	1.21 (0.076)	1.22 (0.08)	N
Cl-	mmol/L	103.56 (3.8)	105.5 (4.4)	N
Glucose	mg/dL	**143.8 (37)**	**228.7 (98.3)**	**Y**
Lactate	mmol/L	**1.11 (0.34)**	**7.71 (2.1)**	**Y**
ctO_2	Vol%	**8.73 (1.3)**	**1.31 (0.55)**	**Y**
BE	mEq/L	**7.62 (2.6)**	**-3.85 (4)**	**Y**

All data presented as mean (Standard Deviation). Bold type indicates significant difference ($p < 0.05$). pCO_2; partial pressure CO_2, pO_2; partial pressure O_2, Hb; hemoglobin, sO_2; O_2 saturation, COHb; carboxyhemoglobin, MethHb; methemoglobin, ctO_2; total blood O_2

arterial lactate level of 7.85 ± 2.25 mg/dL, placing them solidly within the clinical definition of lactic acidosis.

Also of interest is the mean mixed venous sO_2 fell significantly by 83%. This further indicates that the animals were under severe oxygen deficit conditions, and that a supply dependent shock condition was obtained.

Hemodynamic parameters also reinforce the creation of a shock state. Continuous cardiac output (CCO) is routinely monitored as part of resuscitation protocols[214,215], and quantifies the volume of blood that the heart moves over time. Mean CCO fell significantly 66% after injury when compared to baseline measurements (Table 2.3) Mean Arterial Pressure also fell dramatically and significantly from 93.6 ± 15.6 mmHg to 37.8 ± 10.6 mmHg at the end of the injury period. Reduced end tidal CO_2 ($etCO_2$) has been correlated with poor outcome in trauma patients, and been suggested as a indicator of the need for aggressive resuscitation.[216] $etCO_2$ dropped significantly during the injury period, falling 24% when compared to baseline values. Taken together these data confirm the state of a supply dependent condition of shock, and further characterize the severity of shock present in this model.

Liver dysfunction has been characterized by many other groups during states of traumatic shock.[217–219] Using blood chemistry analysis (Table 2.3) significant drops in albumin and alanine aminotransferase (30% and 17% respectively) indicate that the injury did reduce liver function in these animals.

Table 2.3: Physiological Parameters at Baseline and Compared to End of Hemorrhage

	Unit	Mean (STD DEV)		p ≤ 0.05
		Baseline	**End Hemorrhage**	
	n	17	16	NA
Hemodynamics				
$etCO_2$	mmHg	**44.6 (2.6)**	**34 (6.3)**	**Y**
CCO	L/min	**5.19 (.97)**	**1.77 (0.63)**	**Y**
MAP	mmHg	**93.6 (15.6)**	**37.8 (10.6)**	**Y**
Hematology				
WBC	10^9/L	**13.3 (3.6)**	**18.1 (9.8)**	**Y**
LYM	10^9/L	**9.8 (2.6)**	**10.8 (2.8)**	**Y**
MON	10^9/L	0.28 (0.25)	0.24 (0.28)	N
GRA	10^9/L	3.3 (2.2)	7.1 (8.3)	N
RBC	10^12/L	**6.17 (0.32)**	**5.44 (0.52)**	**Y**
HGB	%	**9.72 (0.71)**	**8.55 (1.06)**	**Y**
HCT	%	**25 (1.6)**	**22.3 (2.8)**	**Y**
PLT	10^9/L	**253.3 (52)**	**220.1 (43.4)**	**Y**

All data presented as mean (Standard Deviation). Bold type indicates significance. $etCO_2$; end tidal CO_2, CCO; continuous cardiac output, MAP; mean arterial pressure, WBC; white blood cells, LYM; lymphocytes, MON; monocytes, GRA; granulocytes, RBC; red blood cells, HGB; hemoglobin, HCT; hematocrit, PLT; platelets.

Kidney function was also evaluated for changes occurring in response to the injury period. Blood urea nitrogen and creatinine are commonly used clinical markers of kidney function, and have been shown to be elevated in periods of kidney hypoperfusion.[220,221] These parameters both show significant elevation at D80, with an 18% and 47% respectively. To further strengthen this conclusion of organ dysfunction, total blood protein was found to drop significantly from 5.73 ± 0.49 g/dL to 4.43 ± 0.66 g/dL. This 23% drop indicates a global decline in kidney and liver function. Taken together these results show an aspect of hypoxic and ischemic effects on both the liver and kidneys.

Glucose measurements all indicate a hyperglycemic response to injury (Table 2.2 and Table 2.4) that is consistent with traumatic shock. It has been shown that the development of hyperglycemia after injury correlates with a poor prognosis when compared to those with normal glucose metabolism.[222]

Whole blood cell counts also reveal changes after the injury period. Mean white blood cell (WBC) counts rose significantly (44%) at D80 when compared to baseline (Table 2.3). Lymphocyte (LYM) counts also rose significantly (10%) when comparing these time points. Increased WBC counts are well recognized in trauma populations, and can be correlated with severity of injury.[223,224] Red blood cell (RBC) counts also reveal significant changes in response to the injury period. This agrees with prior findings in both human and swine trauma studies.[208,209,225–228]

Table 2.4: Blood Chemistry Values at Baseline and Compared to End of Hemorrhage

	Unit	Mean (STD DEV)		p ≤ 0.05
		Baseline	**End Hemorrhage**	
	n	17	16	NA
ALB	**g/dL**	**3.3 (0.4)**	**2.3(0.6)**	**Y**
ALP	IU/L	140 (27)	136 (35)	N
ALT	**IU/L**	**34 (7.8)**	**28.1 (5.9)**	**Y**
AMY	**IU/L**	**1460 (405)**	**1140 (330)**	**Y**
TBIL	mg/dL	0.31 (0.05)	0.29 (0.07)	N
BUN	**mg/dL**	**9.1 (2.7)**	**10.7 (2.5)**	**Y**
Ca++	**mg/dL**	**10.38 (0.48)**	**10.17 (1.31)**	**Y**
Phos	**mg/dL**	**8.97 (0.92)**	**10.9 (1.4)**	**Y**
Cre	**mg/dL**	**0.94 (0.25)**	**1.38 (0.26)**	**Y**
Glucose	**mg/dL**	**139 (38)**	**220 (96)**	**Y**
Na+	mmol/L	133 (2)	133 (2)	N
K+	**mmol/L**	**4.89 (0.8)**	**5.64 (1.02)**	**Y**
TP	**g/dL**	**5.73 (0.49)**	**4.43 (0.66)**	**Y**
GLOB	**g/dL**	**2.6 (0.87)**	**1.99 (0.66)**	**Y**

All data presented as mean (Standard Deviation). Tests performed in Li-Heparinized blood. Bold type indicates parameters with significant differences. ALB; Albumin, ALP; alkaline phosphatase, ALT; alanine aminotransferase, AMY; amylase, TBIL; bilirubin total, BUN; blood urea nitrogen, Cre; creatinine, TP; total protein, GLOB; globulin.

Observed Trauma Induced Coagulopathy in a Swine Model

To determine the effects of the injury on coagulation a comparison was performed of blood drawn at baseline with blood drawn at D80. A summarization of the testing performed can be found in Table 2.5. In response to shock a PT increase of 13% ($p < 0.05$) was found for the D80 samples. Unexpectedly, both PTT and functional fibrinogen levels were decreased after injury, although they were not significantly different between the two groups.

Platelet counts fell significantly during the injury period, dropping 13% in response to the injury (Table 2.3). This drop, while common among trauma populations[229–231], did not reach levels to indicate a clinical state of thrombocytopenia. This drop does however need to be considered when evaluating tests of whole blood platelet function.

TEG testing revealed significant increased clotting kinetics after injury as R decreases from 5.68 (±1.49) to 4.16 (±0.97) minutes (Table 2.5). The kinetic time (K) also showed significant quickening, falling from 1.32 (±0.328) to 1.03 (±0.151) minutes. Significant decrease was seen in the angle (α) in the D80 samples, falling from 70.35 (±4.39) to 68.12 (±5.32) degrees. The clot strength was significantly decreased as well, falling from 68.12 (±5.798) to 66.94 (±5.318) mm.

ROTEM analysis was performed to judge the individual cascade pathways' contributions to the whole blood clotting process. There were significant differences found in kinetic parameters found in both In-TEM and Ex-TEM testing. Significant increases in median CFT were seen at 12% and 10% respectively. While

Table 2.5: Coagulation Values at Baseline and Compared to O^2 Debt= 80 mL/kg

	Unit	Mean (STD DEV)		p ≤ 0.05
		Baseline	**D80**	
Plasma Clotting Tests				
	n	14	11	
PT	**s**	**12.9 (0.85)**	**14.6 (1.74)**	**Y**
PTT	s	25.4 (5.73)	23.6 (4.41)	N
FIB	mg/dL	162 (60.58)	138.8 (59.88)	N
Thromboelastography				
	n	17	16	
R	**min**	**5.68 (1.49)**	**4.16 (0.97)**	**Y**
K	**min**	**1.32 (.328)**	**1.03 (0.151)**	**Y**
Angle	**degrees**	**70.35 (4.39)**	**68.12 (5.32)**	**Y**
MA	**mm**	**68.12 (5.798)**	**66.94 (5.318)**	**Y**
Modified Thromboelastography				
	n	8	8	
In-TEM				
CT	s	130.9 (22.4)	121.6 (24.5)	N
CFT	**s**	**39.4 (4.8)**	**44.3 (6.4)**	**Y**
Rα	**degrees**	**82.2 (1)**	**81.1 (1.3)**	**Y**
MCF	mm	67.4 (4.7)	65.5 (4.3)	Y
Ex-TEM				
CT	s	51.2 (10.7)	48.3 (9.7)	N
CFT	**s**	**45.1 (4.8)**	**49.6 (7.6)**	**Y**
Rα	**degree**	**81 (1.1)**	**80.2 (1.6)**	**Y**
MCF	mm	70 (4.6)	68.9 (4)	N

All data presented as mean (Standard Deviation). Bold type indicates significant differences. PT; prothrombin time, PTT; activated partial thromboplastin time, FIB; Functional fibrinogen concentration, R; R time, K; k time, MA; maximum amplitude, CT; clotting time, CFT; clot formation time, MCF; maximum clot firmness.

the parameters of Rα in both tests and MCF in In-TEM did show significant differences, the magnitude of these differences is relatively small (<3%).

This data viewed together does indicate that a mild coagulopathy has begun to develop at the end of the injury period. Kinetic increases seen in the TEG agree with current observations by various other groups.[5,186] The increases in PT indicate a dysfunction in the extrinsic system, and this parameter is the driving factor behind the clinical diagnosis of ACoT. Determination of ACoT in these samples is hindered by the lack of a standardized system of reporting these scores in swine. Due to this an INR is impossible to calculate at this time, however the dysfunction measured in the PT pathway was also observed in the In-TEM and Ex-Tem data. This leads to a more probable theory that the observed dysfunction is present in the final common pathway. Factor assays would be needed to clearly pinpoint the source.

It is possible that a more comprehensive coagulation panel to include both platelet function testing as well as complete plasma proteomic analysis would be able to better define this mild coagulopathy seen in these animals.

SDP *In-Vitro* Testing

Effects of Plasma Product Treatment on Whole Blood Coagulation and Platelet Function

There were a multitude of differences among the treatment groups, however the SDP and FFP treatments showed agreement in the 11 of the 14 (78.5%) differences when compared to the NS group (Table 2.6). There were 5 direct significant ($p<0.05$) differences between the two treatment groups, and 80% of these differences were observed during Na-TEM testing. The other significant

difference between the groups was PT. To further highlight the differences between the two groups there were also 3 indirect differences between the FFP and SDP groups. An indirect difference can be observed when an individual treatment group differs from control, but the other treatment group does not follow the same trend. The indirect differences observed in the clotting initiation times (R and CT) show a quicker response in SDP treated samples (45% and 32% respectively) than control, but FFP was not significantly different than control. FFP treatment caused an increase in clot strength (15%) generated intrinsically (In-TEM MCF), but this was not reflected in the SDP treatment group. Due to the small number of *in-vitro* experiments, clinical relevance is not clear at this time.

Platelet function was also assessed by flow cytometry. After treatment with SDP there was over a two-fold increase in the number of platelets expressing PAC-1 (Figure 2.1). A decrease in the cells expressing PAC-1 after FFP treatment appeared to be trending towards significance ($p = 0.0534$). SDP treated platelet PAC-1 expression was significantly higher than FFP when compared directly.

Table 2.6: SDP *In-Vitro* Coagulation Comparison

	Unit	Fold Change from control		p ≤ 0.05
Treatment Group		**FFP**	**SDP**	
TEG				
R	min	1.09 ± 0.089	0.54 ± 0.089	b,c
K	min	0.74 ± 0.14	0.54 ±0.139	a, b, c
Angle	degrees	1.15 ± 0.236	1.38 ± 0.289	ns
MA	mm	1.07 ± 0.084	1.09 ± 0.084	ns
Na-TEM				
CT	s	1.17 ± 0.172	0.68 ± 0.172	b,c
CFT	s	0.71 ± 0.035	0.56 ± 0.025	a, b, c
Rα	degrees	1.11 ± 0.0154	1.18 ± 0.0153	a, b, c
MCF	mm	1.14 ± 0.041	1.15 ± 0.043	a, b
In-TEM				
CT	s	1.02 ± 0.099	0.97 ± 0.100	ns
CFT	s	0.70 ± 0.070	0.72 ± 0.070	a, b
Rα	degrees	1.09 ± 0.037	1.10 ± 0.038	a, b
MCF	mm	1.15 ± 0.081	1.13 ± 0.080	a
Ex-TEM				
CT	s	0.92 ± 0.095	0.87 ± 0.095	ns
CFT	s	0.69 ± 0.037	0.72 ± 0.034	a, b
Rα	degrees	1.12 ± 0.019	1.15 ± 0.020	a, b
MCF	mm	1.17 ± 0.047	1.11 ± 0.042	a, b
Aggregometry				
Collagen	Ohms	0.94 ± 0.361	1.07 ± 0.360	ns
ADP	Ohms	1.66 ± 0.870	1.77 ± 0.870	ns
Plasma				
PT	s	0.73 ± 0.012	0.85 ± 1.013	a, b, c
PTT	s	0.91 ± 0.076	0.87 ± 0.076	ns
FIB	mg/dL	1.97 ± 0.150	1.71 ± 0.149	a, b

All data presented as mean fold change ± Standard Deviation, p ≤ 0.05 was used to determine significance. ns; no significant differences between any groups, a; significant difference between FFP group and NS control group, b; significant difference between SDP group and NS control group, c; significant difference between FFP and SDP groups. PT; prothrombin time, PTT; activated partial thromboplastin time, FIB; Functional fibrinogen concentration, R; R time, K; k time, MA; maximum amplitude, CT; clotting time, CFT; clot formation time, MCF; maximum clot firmness

2.4 Discussion

Large Animal Model

During the model development period arterial and venous samples were analyzed for coagulation differences. This kind of sampling is difficult in healthy human volunteers, as the discomfort and risk of arterial sampling often outweigh its benefit. In this case matched samples from 12 animals showed a clear separation of the two sources by kinetic measures. There were no differences in the function of the plasma cascade proteins, yet increases were seen in both kinetic measures and clot strength. These data as a whole suggest that the increases in the generation of thrombin and increases in strength are platelet driven, although platelet-fibrin interactions measured via HAS analysis did not confirm these results. More tests would need to be performed to define exactly where this difference in function lies.

Validation of both a state of shock, as well as the development of an endogenous coagulopathy in response to this shock was vital to moving forward to examine treatment differences between the products. A moderate state of hypovolemic shock was achieved in a relatively quick manner. Development of metabolic and respiratory acidosis, kidney and liver dysfunction, increased WBC counts, and hyperglycemia strengthen this assessment. Care must be used when determining injury severity in a model like this, as some biological markers and symptoms do not appear immediately. Reaching a point of "no return" where no matter resuscitation efforts the animal cannot survive. We have extensive experience in this model and the severity was set to a level found to be approximately 50% lethal in past studies.[207–209] The loss of an animal before D80

was reached strengthens the argument that the model was severe enough in this aspect.

The data regarding coagulation did reflect the development of a moderate coagulopathy by D80. PT indicates a significant drop, as did ROTEM pathway assays. This indicates a probable issue in the common pathways, with labile factor being the most likely to drop in levels quickly. In order to determine this exactly a full panel of factor function tests and thrombin generation profiles in platelet poor plasma would be needed. The coagulopathy that was present did not follow simple hypocoagulability trends. Kinetic increases can be attributed to changes in platelet function and their ability to mediate thrombin generation. The magnitude of platelet function increase should be viewed in the context that it occurred after a 10% decrease in the amount of circulating platelets. To completely define the contributions of platelet function a more targeted approach would need to be undertaken, but the current data suggests the presence of a more complex coagulopathy than originally anticipated.

***In-Vitro* Model**

The problems associated with the transportation and storage of FFP on the battlefield has caused interest in the development of more shelf stable blood products. The spray-dried plasma undergoes a form of lyophylization dramatically reducing volume and increasing shelf life. The theory is that it can be reconstituted upon need, fulfilling both a volumetric requirement due to osmotic pressure as well as supplying coagulation factors that may be lost in a patient due to consumption.

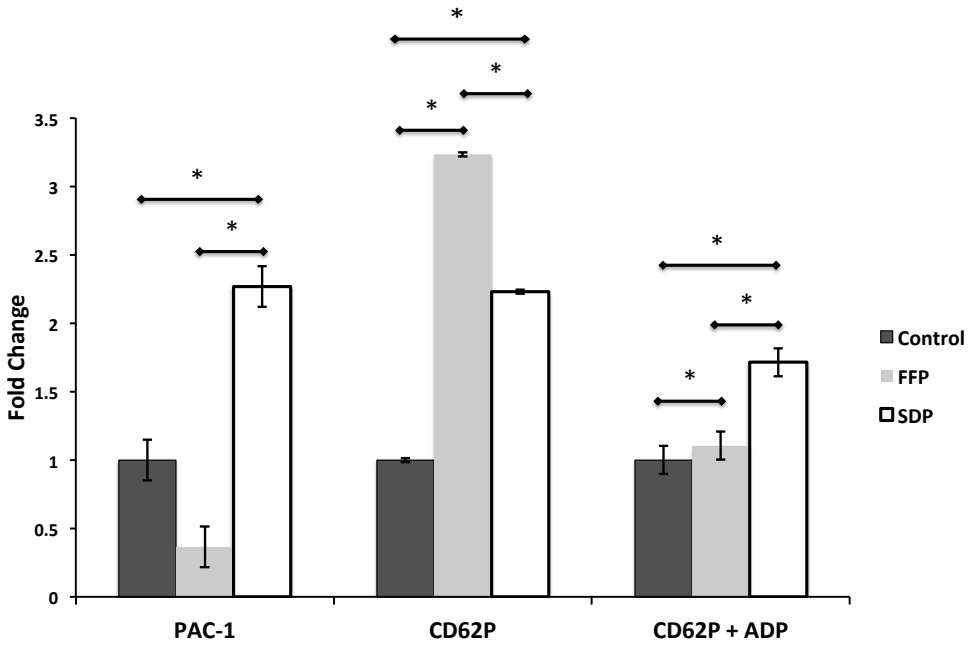

Figure 2.1: Platelet Activation Marker Analysis between Treatment Groups. Percentage of positive cells measured by flow cytometry and expressed as fold change. * $p \leq 0.05$, PAC-1; antibody identifying activated GP IIb/IIIa, CD62P; P-selectin, FFP; fresh frozen plasma, SDP; spray dried plasma.

shock, interspecies reactions between the human SDP and swine were considered a viable possibility. Due to these reasons the goal of testing the SDP to treat the coagulopathy were never fully realized. In an attempt to generate meaningful analysis this *in-vitro* experiment was developed.

Due to elevated levels of fibrinogen in all treatment groups, rises observed in clotting strength were expected when compared to control. The parameters of MCF and MA in normal recalcification assays showed no differences between the two treatment groups directly, suggesting that this strength increase is similar in response to both (Table2.6). Kinetic parameters show an increase in reaction speed and decrease in initiation time, as well as increased PAC-1 expression. This suggests platelet activation in response to SDP as compared to control. There were some signs of platelet activation in response to FFP as well, including increased CD62P expression (secretion) and faster kinetics than control samples. No clear distinction can be generalized from this data however, as the trends do not separate and define different populations in most direct and indirect comparisons (76% of parameters show no significant differences, and only 86% of the parameters show no indirect differences).

As a relatively small sample size perhaps the most reliable conclusions that can be drawn from this work is that FFP and SDP do have *in-vitro* effects on platelet function when compared to NS treated samples. These effects can be viewed generally as increasing activity in the extrinsic pathway and increasing clotting kinetics mechanically, but more work must be done to specifically define these parameters in the context of platelet function.

Chapter 3:

Examining Platelet Function During Polytrauma

3.1 Introduction

It is well known that multisystem injury has an impact on hemostatic function and this impairment of the normal coagulation system impacts patient survival. During the current military conflicts uncontrolled truncal hemorrhage has been identified as the leading cause of potentially survivable death.[2] While combat operations very likely produce a higher incidence of traumatic injury than most individuals are faced with during civilian life, uncontrolled bleeding has also been identified as the second leading cause of death in civilian trauma.[3] As the amount and number of injuries increases in trauma coagulopathy presents earlier and is more common. [232] This earlier presentation of hemostatic dysfunction is found in almost 40% of combat casualties that require blood product administration, and has been related to a six fold increase in mortality.[4] All these findings highlight a need for a better understanding of the mechanisms responsible for platelet dysfunction and coagulopathy in polytrauma victims.

The main objectives of this study were to clearly define platelet function in the polytrauma victim and to understand the impact of injury and treatment in this context. The hypothesis developed was that platelet function is altered during multisystem injury and has significant implications for hemostasis and damage control resuscitation and surgery. There were two specific aims that were pursued in order to verify this hypothesis. The first aim was to define platelet function over time in response to trauma using a comprehensive and multipronged panel of accepted and emerging coagulation tests. The second aim was to define the relationships between platelet function and injury severity, tissue hypoperfusion, hypothermia, acidosis, transfusion, traumatic brain injury, and clinical outcomes to include the need for immediate surgery and survival.

3.2 Materials and Methods

Enrolment

All patients that presented over a 24-month period to the Virginia Commonwealth University Medical Center (VCUMC) Emergency Department meeting immediate trauma team activation criteria were screened for study inclusion. All patient participants and healthy volunteers were screened under the following criteria.

Inclusion:

- Age $\geq$ 18 years
- Acutely injured patients meeting predetermined mechanistic, vital sign, and physical exam related criteria for immediate trauma team activation in the Emergency Department.
- Time of injury within 3 hours of initial sample procurement
- Healthy uninjured volunteer

Exclusion Criteria:

- Pregnancy
- Documented do not resuscitate order
- Intentional self inflicted injury
- Recent (within two weeks) use of anticoagulants including heparins, aspirin, clopidogrel, prasugrel, or warfarin as confirmed by patient report or medical record.
- Prisoner
- Non-English speaker
- Refusal to participate

Blood Sampling Protocol

Blood samples were obtained via non-heparinized peripheral catheters or direct peripheral veinipuncture directly into standard vacutainers by VCUMC trained staff. All blood samples were drawn into a combination of sodium citrate, EDTA, and heparinized vacutainers with the total volume per sample point $\leq$ 25 mL. Healthy volunteers provided one sample only, while the trauma population was sampled serially at admission and then 8, 24, 48, and 72 hours post admission. Precaution was taken to ensure that sample procurement was not a significant risk to the patient population, and samples were not obtained if the patient's last clinical hemoglobin level was below 7 g/dL.

Patient Data Acquisition

Demographic and descriptive data was collected from the patients' medical record. This data was needed to delineate groups according to injury severity, degree of tissue hypoperfusion, and presence and severity of head injury. This mined data was extensive and included:

- Age, sex, height, and weight
- Mechanism of injury

- Vital signs (blood pressure, pulse, temperature, respiratory rate, and pulse oximetry data)
- Pre-Admission use of aspirin, non-steroidal anti-inflammatory medications, warfarin, heparins, or other specific anti-platelet drugs
- Major injuries as identified by physical examination, computed tomography, or during surgical intervention by anatomic distribution for calculation of the Injury Severity Score (ISS)
- Significant past medical history to include history of coronary artery disease, cerebrovascular accident, diabetes mellitus, coagulopathy, medication history, and medications used during current hospitalization
- Volume and type of blood products transfused and crystalloid resuscitation fluids administered
- Glascow Coma Scale and presence and type of intracranial injury
- Basic Metabolite and electrolyte profile
- Complete cell count with differential and platelet count
- Venous blood gas analysis with base excess
- Lactate concentration
- PT, PTT, INR
- Blood typing
- Serum alcohol level
- Serum Toxicology screen for drugs of abuse

Data was also collected from medical records regarding the subsequent time points including new medications and interventions as well as any clinical tests performed within two hours of the follow up time points.

Coagulation Testing

High shear platelet adhesion and aggregation was quantified using the PFA-100 (Siemens Healthcare Diagnostics, Tarrytown, NY, USA). This technology has been described before[192], but briefly uses two disposable cartridges to test platelet response to collagen and ADP (Coll/ADP) or collagen and epinephrine (Coll/EPI). The amount of time needed for the blood to form a plug and completely occlude the cartridge aperture is expressed as the closure time (CT), and was recorded for each patient.

Platelet aggregation was measured on the Chrono-log Aggregometer (Chrono-log Corp., Havertown, PA, USA) on citrated whole blood as described previously by this lab.[209] Samples were activated using collagen (2µg/mL) or ADP (10 µM final concentration) according to manufacturer's recommendations. Aggregation was measured as impedance increases in whole blood after stimulation with agonist.

Kinetics of thrombin generation were assessed in platelet poor plasma (PPP), as described by Hemker[233], by measuring the cleavage of a fluorogenic substrate using the Calibrated Automated Thrombogram® (CAT®; Thrombinoscope BV, Masstricht, The Netherlands). A Fluoroscan Ascentâ fluorometer (Thermolab Systems OY, Helsinki, Finland) was used to measure the resultant fluorescence.

Thromboelastography was performed on citrated whole blood samples using the TEG® 5000 Hemostasis Analyzer (Haemoscope Corp., Niles, IL, USA) using kaolin and $CaCl_2$ as per manufacturer's instructions. Use of this technique has been described by this lab in previous work.[193,209,210]

Further mechanical whole blood coagulation analysis was performed on whole blood using the Hemodyne Hemostasis Analysis System (HAS Hemodyne Inc., Richmond, VA, USA) using citrated whole blood. In depth assay procedures have been described by this lab before.[209] Parameters for both the TEG and the HAS have been reviewed in depth in prior work by this lab. [193]

The Platelet Mapping® assay (Haemoscope Corp., Niles, IL, USA) utilizes modified TEG based thromboelastography to determine functionality of the arachidonic acid (AA) and ADP response pathways in platelets. The assay was

described in depth earlier in this work. Multiple groups have recently demonstrated significant correlation between platelet mapping response and optical platelet aggregometry, suggesting further diagnostic value to this assay.[194,234] Briefly involves isolating the platelet contribution to the whole blood clotting process. Heparinized samples are used to eliminate thrombin response, and then reptilase, Factor XIII (FXIII), and either ADP, AA, or collagen are added to initiate these respective pathways. The resulting MA is compared to a matched citrated sample activated by kaolin and recalcification (full MA potential) after subtracting the fibrin contributions measured by a sample with only reptilase and FXIII. The data is expressed at % inhibition of the individual agonist pathways examined.

ELISA was used to quantify additional plasma proteins related to coagulation. Citrated whole blood was centrifuged to produce platelet poor plasma as per laboratory protocol. Plasma was then aliquoted and frozen at -80° C. All ELISA assays were commercially obtained as kits and performed to manufacturer's instructions on freshly thawed samples. Thrombin-Antithrombin (TAT) complex was measured using the Enzygnost® TAT micro kit (Siemens Healthcare Diagnostics, Tarrytown, NY, USA). D-dimer concentrations were determined using the TECHNOZYM® D-Dimer ELISA Kit (Technoclone GH, Vienna, Austria).

The plasma coagulation cascade pathway tests of Prothrombin Time (PT) and activated partial thromboplastin time (PTT) as well as functional fibrinogen concentration were all measured in platelet poor plasma using the Start-4® coagulation analyzer (Diagnostica Stago, Asnières, France). All tests were performed according to the manufacturer's recommendation.

Whole blood cell counts were performed on the ABX Micros 60 (Horiba Medical, Irvine, CA, USA). Tests were performed on EDTA anticoagulated whole blood drawn at the time of patient sampling according to manufacturer's instruction.

Samples were stained for flow cytometry with primary antibodies for CD62p-PE, PAC-1-FITC, CD41-PE-Cy5 and appropriate isotypic controls (BD Biosciences, San Jose, CA, USA). After a 30-minute incubation samples were fixed and analyzed with an Accuri C6 cytometer (BD Biosciences, San Jose, CA, USA). A total of 20,000 platelets were analyzed in each sample.

Clinical Interpretation

Point of care tests performed at VCUMS at time of admission were used to determine the variables of systolic blood pressure (BPS), diastolic blood pressure (BPD), temperature, base excess (BE), respiratory rate (RESP RATE), O_2 saturation (sO_2), pulse, lactate, Glasgow Coma Scale (GCS), injury severity score (ISS), and Traumatic Brain Injury (TBI). Emergency response fluid administration data was also obtained from the patient records at VCUMS.

ACoT was defined by accepted guidelines adopted by the American Society of Anesthesiologists and summarized by Yuan.[235] Briefly patients presenting with an International Normalized Ratio (INR) ≥ 1.5 were classified as presenting with ACoT. Early coagulopathy of trauma (ECoT) was defined as an INR > 1.2 but < 1.5, as defined by past work by other labs.[185]

Hypercoagulability was defined by an TEG R-time of <3.7 minutes, and was based on recent work performed by Shreiber.[5]

DIC classification was based on currently accepted guidelines developed by the International Society for Thrombosis and Hemostasis (ISTH).[230,236] Briefly the parameters of platelet count, plasma D-Dimer levels, fibrinogen concentration, and prothrombin time are assigned values based on standardized levels. These scores are tabulated, with any score over the cutoff level considered overt DIC. Further explanation of this calculation can be found in Table 3.1.

Hypothermia classification was based on currently accepted definitions[229], with a temperature of < 35° C at admission considered a state of hypothermia.

Hypovolemic Shock classifications were based on the work of Mutschler utilizing Base Deficit (BD) at the time of admission to the Emergency Department.[211] Briefly shock is stratified into four categories; no shock (BD ≤ 2.0 mmol/L), mild (BD > 2.0 to 6.0 mmol/L), moderate (BD > 6 to 10.0 mmol/L), and severe (BD > 10.0 mmol/L).

Data Analysis

Data distributions were checked using QQ plots, and descriptive statistics (counts, percentages, mean ± SD, median (IQR)) were used to summarize patients' data for each set of categorical groups. Different groups' parameters were compared using the analysis of variance (ANOVA) or the non-parametric Kruskal-Wallis test as appropriate. Multiple comparisons were made using either Tukey-HSD method or Wilcoxon test as appropriate. Pearson's Chi-square test was used for categorical variables. All statistical analyses were performed using JMP statistical software, version 10.0.0 (SAS Institute, Cary, NC). The level of significance for all statistical tests was $p < 0.05$.

3.3 Results

Demography

Across the course of the study there were 105 patients enrolled for which ISS score was available. Table 3.2 summarizes the intake data on all patients used for this analysis. There were more men enrolled by a 4:1 ratio when compared to women. The majority of patients had blunt injuries (73%), 22% presented with blunt and penetrating injuries, and 6% of the patients suffered from penetrating injuries alone. The median ISS score was 18 (IQR 10 to 29) indicating a diverse trauma severity population. TBI was indicated in 26% of the patient population. Hypovolemic shock was indicated in 56%, and the majority of the shock cases were mild (57%).

Coagulopathy was observed in varying degrees. One third of the subjects presented with hypofibrinogenemia, 21% with ACoT, and 17% with ECoT. 6% of the patients showed overt signs of DIC. Pre-hospital saline was administered to 85% of the subjects, and 14% received blood products prior to admission.

Table 3.1: Definition of Overt DIC

Criterion	Points
(1) Baseline Platelet Count	
≥ 100 000 μL^-1	0 point
50 000 to < 100 000 μL^-1	1 point
< 50 000 μL^-1	2 points
(2) Baseline D-Dimer	
≤ 390 ng/mL	0 point
> 390 to ≤ 4 000 ng/mL	2 points
> 4 000 ng/mL	3 points
(3) INR	
≤ 1.4	0 point
> 1.4 to ≤ 2.3	1 point
> 2.3	2 points
(4) Fibrinogen Level	
≥ 100 mg/dL	0 point
< 100 mg/ dL	1 point
Sum: 1 + 2 + 3 + 4	**≥ 5 points: Overt DIC**

DIC; disseminated intravascular coagulation, INR; International Normalized Ratio. Adapted from Kienast et. al. (2006).[237]

Table 3.2: Demography, Injury Severity, Pathophysiology, and Treatment Parameters Upon Admission of 105 Trauma Patients Investigated

		Units	Patients
n			105
Age		YRS	35 (24, 52.5)
Sex	MALE	% (n)	82 (86)
	FEMALE	% (n)	18 (19)
Race	W	% (n)	56 (59)
	NW	% (n)	47 (49)
Injury Type	B	% (n)	73 (76)
	P	% (n)	22 (23)
	B/P	% (n)	6 (6)
ISS		SCORE	18 (10, 29)
GCS		SCORE	15 (4.5, 15)
TBI		% (n)	25 (26)
Hypothermia		% (n)	3 (3)
Shock	Total	% (n)	56 (59)
	Mild	% (n)	32 (33)
	Moderate	% (n)	12 (13)
	Severe	% (n)	12 (13)
Hypofibrinogenemia		% (n)	33 (35)
ACoT		% (n)	21 (22)
DIC		% (n)	6 (6)
Blood Products (PH)		% (n)	14 (15)
Saline (PH)		% (n)	85 (89)

All data presented as mean (IQR) or % (n). ISS; Injury Severity Score, GCS; Glasgow Coma Scale, TBI; Traumatic Brain Injury, ACoT; Advanced Coagulopathy of Trauma, DIC; Disseminated Intravascular Coagulation, PH; Pre-Hospital, W; White, NW; Non-White, B; Blunt, P; Penetrating, B/P; Blunt and Penetrating.

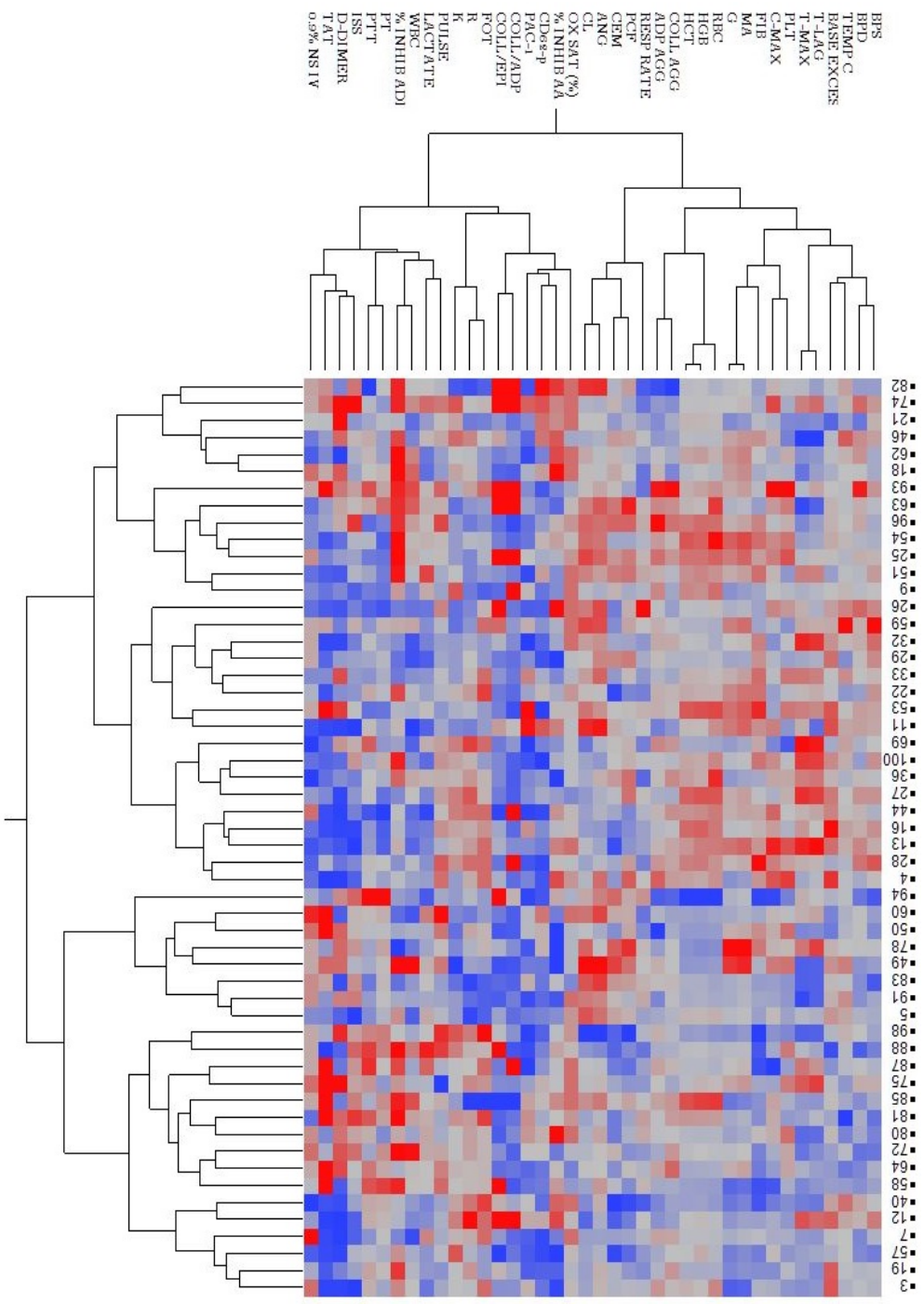

Figure 3.1: Heat Map and Dendrogram of Polytrauma Patients. Generated by Two Ways Hierarchical Cluster Analysis. The variables are clustered in at least 4 main groups as well as the patients

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Classification of Patient Population by Injury Severity

The patient data from VCUMS were combined with the platelet function and coagulation assay data for analysis. The combination of physiological and clinical parameters with laboratorial coagulation tests has the potential to result in a better characterization of the patient state and lead to better treatment decisions and improved outcome. Using this database an exploratory analysis with hierarchical two ways clustering using 40 different variables was performed. This statistical method was adapted from previous work by Cohen et. al.[238] Figure 3.1 shows the heat map and dendrogram resulting from this analysis suggesting the existence of at least 4 clusters of patients with different combination of measurements values.

Only half of the patients from the study, with no missing data, could be used for this analysis, limiting the use of these grouping techniques. Nonetheless, the heat map suggests that the Injury Severity Score (ISS) could be used as a method of grouping the trauma patients and helps to understand the pattern of physiological response.

ISS is clustered with the clinical parameters pulse, lactate, volume of IV normal saline and white blood cells count as well as the laboratory variables PT, PTT, % of ADP inhibition, D-dimer and TAT complexes, indicating association of parameters not usually used for triage in the ER. Although these parameters look to be the best predictors of cluster allocation the complex relationship of variables supports a physiologic response to trauma that is more subtle and intricate.

In response to the clustering results patients were stratified into 4 different groups based on their ISS score as described before by Copes in 1988[239] and further

adapted by Bolorundo.[231] The groups were: mild injury (ISS ≤ 8), moderate injury (ISS 9 - 15), severe injury (ISS 16 - 25) and profound injury (ISS > 25). Using the four-group stratification admission variables were once again tabulated in Table 3.3. From this analysis the emergence of trends begins to appear.

There appears to be an increase in the both the number of cases as well as the severity of hypovolemic shock as the level of Injury severity increases (Table 3.3). TBI also trends upwards as shock level increases. ACoT and hypofibrinogenemia also appear to trend higher as injury severity increases, but the simple appearance of hypofibrinogenemia did not indicate ACoT. This suggests consumption of additional factors other than fibrinogen in the development of coagulopathy. Both DIC and hypothermia were relatively rare. 3 cases of hypothermia were seen between the severe and profound categories combined, and the 6 cases of DIC were only present in the profound category.

To explore these qualitatively observed trends further data was examined for correlation between ISS categories and presentation of clinical disorders. These results can be found in Table 3. Strong positive correlation was found between ISS severity and GCS ($p<0.0002$), hypofibrinogenemia ($p<0.0001$), and ACoT ($p<0.0001$). Hypovolemic shock occurrence also strongly correlated with ISS category positively ($p<0.0001$), but sample size was insufficient ($n=22$) to determine if individual shock severity categories showed significant correlation with shock severity levels using the Pearson's Chi-square test.

Table 3.3: Demography, Pathophysiology, and Treatment Parameters Upon Admission Based on ISS Classification of 105 Trauma Patients

		Unit	ISS Classification				Correlation
			Mild	**Moderate**	**Severe**	**Profound**	
ISS Score			1 to 8	9 to 15	16 to 24	≥ 25	
n			17	27	25	36	NA
Age		YRS	27 (21, 52.5)	39 (27, 52)	39 (25, 53)	31 (23, 53)	NA
Sex	MALE	% (n)	14 (82)	24 (89)	21 (84)	27 (75)	NA
	FEMALE	% (n)	3 (18)	3 (11)	4 (16)	9 (25)	
Race	W	% (n)	10 (59)	12 (44)	14 (56)	23 (64)	NA
	NW	% (n)	7 (41)	15 (56)	11 (44)	13 (36)	
Injury Type	B	% (n)	11 (65)	18 (67)	18 (72)	29 (81)	NA
	P	% (n)	5 (29)	8 (30)	5 (20)	5 (14)	
	B/P	% (n)	1 (6)	1 (4)	2 (8)	2 (6)	
GCS		**SCORE**	**15 (14.5, 15)**	**15 (14, 15)**	**14 (8, 5)**	**8.5 (3, 15)**	**Negative (a)**
TBI		% (n)	0 (0)	4 (15)	7 (28)	15 (42)	**Positive (a)**
Hypothermia		% (n)	0 (0)	0 (0)	1 (4)	2 (6)	
Shock	Total	% (n)	4 (23)	10 (37)	17 (68)	28 (78)	**Positive (b)**
	Mild	% (n)	3 (16)	8 (30)	7 (28)	15 (42)	
	Moderate	% (n)	0 (0)	1 (4)	8 (32)	4 (11)	
	Severe	% (n)	1 (6)	1 (4)	2 (8)	9 (25)	
Hypofibrinogenemia		% (n)	1 (6)	3 (11)	8 (32)	23 (64)	**Positive (b)**
ACoT		% (n)	0 (0)	1 (4)	6 (24)	17 (47)	**Positive (b)**
DIC		% (n)	0 (0)	0 (0)	0 (0)	6 (17)	**Positive (b)**
Blood Products (PH)		% (n)	0 (0)	2 (7)	4 (16)	9 (36)	NS
Saline (PH)		% (n)	16 (94)	18 (67)	21 (84)	31 (86)	NS

All data presented as % (n) or median (IQR). Bold text indicates significance ($p \leq 0.05$). a; $p \leq 0.05$, b; $p \leq 0.0001$, ISS; Injury Severity Score, GCS; Glasgow Coma Scale, TBI; Traumatic Brain Injury, ACoT; Advanced Coagulopathy of Trauma, DIC; Disseminated Intravascular Coagulation, PH; Pre-Hospital, W; White, NW; Non-White, B; Blunt, P; Penetrating, B/P; Blunt and Penetrating.

Table 3.4 shows a cumulative tabulation of all variables that were found to be outside normal ranges. White blood cell counts were elevated in the severe (13.8 (11.05, 18.7) 10^9 cells/L) and profound (13.95 (11.025, 19.125) 10^9 cells/L) categories. Red blood cells were lower than normal values in the moderate (4.3 (3.7, 4.89) 10^12 cells/L), severe (3.8 (3.5, 4.4) 10^12 cells/L), and profound (3.85 (3.1, 4.275) 10^12 cells/L) categories. High shear adhesion and aggregation was higher in response to collagen and ADP in both the moderate (62 (53, 84) seconds) and severe (61 (51.5, 77.5) seconds) groups, while in response to collagen and epinephrine only the severe group (89 (81.5, 130.5) seconds) was outside normal ranges. Inhibition of the ADP pathway was elevated in mild (75.7 (42.1, 82.3)%), severe (85.9 (61.675, 98.425)%), and profound (97 (79.75, 99.75)%) injury groups. Prothrombin time is elevated in only the profound group (17.4 (15, 20.5) seconds), and D-dimer levels are higher in the moderate (1065.05 (302.4, 3684.45) ng/mL), severe (1986.15 (363.975, 4252.95) ng/mL), and profound (3448. 75 (778.525, 4897.925) ng/mL) categories.

Coagulation Based Classification

While ISS is still the gold standard in trauma evaluation, this method may not identify subtle differences in platelet function when measured across a global population. Two other standards were chosen to separate the population based on observed coagulopathies in an attempt to better define possible changes. ACoT is a recognized and relatively severe consumption disorder, but recent work suggests the development of an early coagulopathy as well (ECoT). Using these new parameters data was again analyzed (Table 3.5).

Table 3.4:Hematology and coagulation parameters outside normal range at admission based on ISS classification

	Unit	ISS Classification				Normal Range
		Mild	**Moderate**	**Severe**	**Profound**	
ISS Score		1 to 8	9 to 15	16 to 24	≥ 25	NA
WBC	10^9/L	8 (6.3, 8.95)	10.4 (7, 13.7)	**13.8 (11.05, 18.7)**	**13.95 (11.025, 19.125)**	4 to 11
RBC	10^12/L	4.58 (3.93, 4.85)	**4.3 (3.7, 4.89)**	**3.8 (3.5, 4.4)**	**3.85 (3.1, 4.275)**	4.5 to 5.8
Closure Time (PFA 100)						
Coll/ADP	s	81 (58.5, 113)	**62 (53, 84)**	**61 (51.5, 77.5)**	71 (53.75, 114)	70 to 125
Coll/EPI	s	115 (97.5, 159)	89 (81.5, 130.5)	**89 (81.5, 130.5)**	125 (91, 288.5)	90 to 200
Aggregation	Ohms	14 (10.75, 16.25)	15.5 (12, 19)	14.5 (12, 17.5)	**12.25 (7.75, 15)**	14 to 28
ADP Inhibition	%	**75.7 (42.1, 82.3)**	65.4 (42.85, 92.25)	**85.9 (61.675, 98.425)**	**97 (79.75, 99.75)**	< 70
PT	s	13.75 (12.55, 14.28)	14.4 (13.3, 15.3)	14.8 (13.75, 17.15	**17.4 (15, 20.5)**	11 to 15
D-Dimer	ng/mL	143.1 (87.8, 537.25)	**1065.05 (302.4, 3684.45)**	**1986.15 (363.975, 4252.95)**	**3448.75 (778.525, 4897.925)**	< 400

All data presented as mean (IQR). Bold type indicates variables outside normal range. ISS; injury severity score, WBC; white blood cell count, RBC; red blood cell count, Coll; collagen, ADP; adenosine di-phosphate, EPI; epinephrine, PT; prothrombin time.

Table 3.5: Demography, Pathophysiology, and Treatment Parameters Upon Admission Based on Consumption Coagulopathy

		Unit	Coagulopathy Classification		
			Normal INR < 1.2	**ECoT INR 1.2 to < 1.5**	**ACoT INR ≥ 1.5**
n			61	18	24
ISS Score			14 (9,22)	20.5 (11.25, 29.25)	33.5 (22,42.5)
Age		YRS	37 (23.5, 53)	47 (26.5, 61.5)	29.5 (23.25, 44.5)
Sex	MALE	% (n)	90 (55)	72 (13)	71 (17)
	FEMALE	% (n)	10 (6)	28 (5)	29 (7)
Race	W	% (n)	52 (32)	44 (8)	21 (5)
	NW	% (n)	48 (29)	56 (10)	79 (19)
Injury Type	B	% (n)	72 (44)	78 (14)	71 (17)
	P	% (n)	7 (4)	0 (0)	4 (1)
	B/P	% (n)	21 (13)	22 (4)	25 (6)
GCS		SCORE	15 (13, 15)	12.5 (3, 15)	9 (3, 15)
TBI		% (n)	21 (13)	33 (6)	33 (8)
Hypothermia		% (n)	0 (0)	14 (2)	8 (1)
Shock	Total	% (n)	58 (35)	39 (7)	13 (3)
	Mild	% (n)	30 (18)	33 (6)	38 (9)
	Moderate	% (n)	7 (4)	22 (4)	21 (5)
	Severe	% (n)	5 (3)	6 (1)	29 (7)
Hypofibrinogenemia		% (n)	10 (6)	33 (6)	88 (21)
Blood Products (PH)		% (n)	5 (3)	18 (3)	36 (8)
Saline (PH)		% (n)	82 (50)	72 (13)	88 (21)

All data presented as mean (IQR) or % (n). ISS; Injury Severity Score, GCS; Glasgow Coma Scale, TBI; Traumatic Brain Injury, ACoT; Advanced Coagulopathy of Trauma, ECoT; Early Coagulopathy of Trauma, DIC; Disseminated Intravascular Coagulation, PH; Pre-Hospital, W; White, NW; Non-White, B; Blunt, P; Penetrating, B/P; Blunt and Penetrating.

Recent work by many groups has also indicated a state of hypercoagulability (HC) measured by thromboelastography[5,186], so the classification developed by Schreiber[5] for HC (TEG R < 3.7) was also used. This particular coagulopathy is not expected to be consumption driven. The data from this analysis can be found in Table 3.6.

These two tables then can be compared to the data generated to the ISS stratification to see if similarities exist. The consumption disorder grouping shows that 68% of the patients for whom a reportable INR and R time were found (103) developed some level of coagulopathy. ISS scores appear to rise as level of coagulopathy increases, agreeing with the observation that ISS categorical values were correlated with development of ACoT (Table 3.3). Other parameters were observed to have similar responses to the ISS table as well. Increased shock appears to lead to increased coagulopathy, and intuitively functional fibrinogen concentration falls as consumptive coagulopathy persists. When looking at the hypercoagulability data there appears to be a disconnect between ISS score and development of this disorder. 30% of the patients showed signs of HC. Median ISS was 20 (9,30) in the HC+ group compared to the HC-. Interestingly of the 18 females used for this examination only 2 (11%) developed HC. This is in stark contrast to the 52% (29 out of 56) of the men whom developed this pathology. When comparing this same female population 66% (12 out of 18) developed some sort of consumptive coagulopathy, and 7 reached the level of ACoT (29%). 30 men (55%) developed either ECoT or ACoT matching the HC data well. 36% of ACoT and 18%

of ECoT subjects received pre-hospital blood products, where only 23% of HC received them.

To investigate apparent trends in the two competing data tables Chi-squared correlations were analyzed between the two types of coagulopathy and hypovolemic shock, ISS categories, and hypofibrinogenemia. The parameters of ECoT and ACoT were both considered as consumption coagulopathy (INR driven) and were compared to HC (R driven) coagulopathy (Table 3.7). Individuals that presented with both ACoT and HC were excluded from this analysis in order to examine the individual disorders separately. Chi-squared analysis revealed a positive association when comparing ACoT with ISS ($p < 0.0001$) and hypovolemic shock ($p < 0.05$) as well as a negative correlation with hypofibrinogenemia. ($p < 0.0001$). HC does not appear to correlate with shock ($p=0.3586$), ISS categories ($p=0.9831$), or hypofibrinogenemia ($p=0.5947$)

To better define the relationships seen in the categorical correlations a set of constant variable continuous correlations were performed focusing on the parameters that drive the two examined coagulopathy categories. INR and R time were selected, as they are the defining parameter for ACoT/ECoT and HC classifications respectively. They were compared to BD (defining parameter for hypovolemic shock), Fibrinogen, and ISS (Table 3.8). INR showed strong positive correlation with BD ($p<0.05$, $r=0.54$) and ISS ($p<0.0001$, $r=0.447$), and a strong negative correlation with fibrinogen. In support of the categorical Chi-squared testing, HC does not appear to be correlated with BD ($p=0.98$), ISS ($p=0.5436$), or fibrinogen ($p=0.318$).

Table 3.6: Demography, Pathophysiology, and Treatment Parameters Upon Admission Based on Hypercoagulability

		Unit	TEG Classification	
			HC R < 3.7	**Normal R>=3.7**
n			31	72
ISS Score			20 (9, 30)	17.5 (12, 29)
Age		YRS	40 (27, 53)	33 (23, 52.75)
Sex	MALE	% (n)	94 (29)	22 (16)
	FEMALE	% (n)	6 (2)	78 (56)
Race	W	% (n)	52 (16)	42 (30)
	NW	% (n)	48 (15)	58 (42)
Injury Type	B	% (n)	58 (18)	79 (57)
	P	% (n)	29 (9)	19 (14)
	B/P	% (n)	13 (4)	1 (1)
GCS		SCORE	14 (9, 15)	15 (3, 15)
TBI		% (n)	23 (7)	28 (20)
Hypothermia		% (n)	0 (0)	6 (3)
Shock	Total	% (n)	61 (19)	55 (40)
	Mild	% (n)	32 (10)	32 (23)
	Moderate	% (n)	19 (6)	10 (7)
	Severe	% (n)	10 (3)	13 (9)
Hypofibrinogenemia		% (n)	27 (8)	36 (26)
ACoT		% (n)	23 (7)	24 (17)
Blood Products (PH)		% (n)	23 (7)	10 (7)
Saline (PH)		% (n)	80 (25)	82 (59)

All data presented as mean (IQR) or % (n). ISS; Injury Severity Score, GCS; Glasgow Coma Scale, TBI; Traumatic Brain Injury, ACoT; Advanced Coagulopathy of Trauma, DIC; Disseminated Intravascular Coagulation, PH; Pre-Hospital, W; White, NW; Non-White, B; Blunt, P; Penetrating, B/P; Blunt and Penetrating.

Table 3.7: Correlation Comparison of Coagulopathy Categories

	Category			
	ACoT		HC	
	correlation	p value	correlation	p value
Shock	positive	< 0.05	none	0.3586
ISS Category	positive	< 0.0001	none	0.9831
Hypofibrinogenemia	negative	< 0.0001	none	0.5947

HC; hypercoagulability, ISS; International Normalized Ratio.

Table 3.8: Correlation Comparison of Coagulopathy "Driving Factors"

	Driving Parameter			
	INR		R	
	correlation (p value)	r value	correlation (p value)	r value
BD	positive (< 0.05)	0.54	none (0.98)	NA
ISS	positive (< 0.0001)	0.447	none (0.543)	NA
FIB	negative (< 0.0001)	-0.5034	none (0.318)	NA

INR; International Normalized Ratio, R; R time, BD; Base Deficit, ISS, injury severity score, FIB; functional fibrinogen concentration.

The correlations suggest two non-related disorders, but do not define individual parameters. In order to define the two groups by their platelet function components coagulation data was compiled and analyzed for differences. Mean platelet counts were not significantly different between HC (188.9 ± 74.80) and the ECoT/ACoT group (213.9 ± 63.80).

PFA testing demonstrated no significant differences between the two classification groups in high shear conditions. Interestingly in the HC group collagen and ADP activation resulted in a median CT relatively close to the lower limit (60 s) of normal for this test (61 (53,67) s) (Table 3.9).

Whole blood aggregometry revealed a higher response to collagen in the HC group (13.98 ± 4.412 Ohms) than in the ACoT group (9.79 ± 4.687 Ohms). ADP stimulation generated a stronger response in HC platelets (10.28 ± 4.487 Ohms) than in ACoT (6.29 ± 3.016 Ohms) as well.

Flow cytometry analysis showed no significant differences between the two groups, and fell within the normal reportable ranges for this lab.

Care must be exercised when drawing any conclusions from the mechanical testing in this particular situation. A strong case for bias can be made due to two separate and compelling reasons. Kinetics appear increased in both HAS (FOT) and TEG (R, K, Angle) analysis. HC classification criteria is defined by a decreased R time, so significant mathematical bias is imparted on the mean/median of this group as well as K and Angle which are directly related to the same kinetic processes (thrombin generation). FOT is a comparable measure to R time, and must also be

Table 3.9: Comparison of Platelet Function Tests between Coagulopathy Groups

	Unit	Coagulation Status		p value
		HC	**ACoT**	
	n	24	17	
Aggregation				
High Shear				
COLL/ADP	s	61 (53, 67)	67 (53, 80)	0.1997
COLL/EPI	s	92 (77, 121)	123 (97.25, 153)	0.1879
Low Shear				
COLL	**Ohms**	**13.98 (4.412)**	**9.79 (4.687)**	**0.0068**
ADP	**Ohms**	**10.28 (4.487)**	**6.29 (3.016)**	**0.0015**
Flow Cytometry				
CD62P	%	3.8 (2, 15.5)	2.6 (1.15, 6.15)	0.1264
PAC-1	%	4.9 (0.9, 17.5)	1.55 (0.35, 8.725)	0.0896
Mechanical Testing				
HAS				
FOT	**min**	**2.76 (0.898)**	**4.21 (1.300)**	**0.0005**
PCF	**kdynes**	**8.61 (1.825)**	**5.13 (2.279)**	**< 0.0001**
CEM	**kdyne/cm2**	**33.27 (7.348)**	**23.39 (11.499)**	**0.0095**
TEG				
R	**min**	**3.12 (0.378)**	**5.10 (0.996)**	**< 0.0001**
K	**min**	**1.25 (0.297)**	**2.06 (1.187)**	**< 0.0001**
Angle	**degrees**	**72.38 (3.879)**	**62.01 (10.000)**	**0.0006**
MA	**mm**	**62.64 (4.500)**	**54.15 (8.332)**	**0.0009**
Platelet Mapping				
ADP Inhibition	%	71.92 (25.113)	85.80 (26.041)	0.1168
AA Inhibition	%	48.10 (25.404)	46.01 (27.865)	0.8224

All data presented as mean (SD) or median (IQR). Bold type indicates significant differences. HC; hypercoagulability, ACoT; Acute Coagulopathy of Trauma, COLL; collagen, ADP; adenosine di-phosphate, EPI; epinephrine, FOT; force onset time, PCF; platelet contractile force, CEM; clot elastic modulus, R; R time, K; K time, MA; maximum amplitude.

viewed with equal skepticism. This same logical argument can be used to rule out strength measurements as well. Consumption and dysfunction in the plasma cascade is the driving factor behind elevations of INR, which are used to define ACoT.

Platelet mapping showed no significant differences between the groups. AA inhibition was normal in both the HC group (48.10 ± 25.404 %) and ACoT group (46.01 ± 27.865 %). ADP inhibition was slightly higher than normal (< 70%) in the HC group (71.92 ± 25.113 %) and ACoT patients fell squarely in the above normal range (85.80 ± 26.041).

3.4 Discussion

Current practice in trauma care is to classify individuals based on ISS. ISS does strongly correlate with development of ACoT but this does not tell the whole story behind coagulation changes in this patient population. The development of a more targeted method of evaluation of the coagulopathy induced by traumatic injury may possibly lead to improved treatment parameters as well as better outcomes compared to current practice.

If broken into 4 severity classifications ISS does correlate strongly with the physical appearance of TBI and lower GCS. The Coagulation disorders hypofibrinogenemia and ACoT also appear to be strongly associated with the ISS stratification proposed. If one were to examine only the commonly used clinical coagulation tests (PT, PTT, [Fibrinogen]) this classification system does appear to be effective.

If the clustering analysis is revisited however it suggests a complex interplay between traumatic injury and the systematic physiological coagulation response. An alternate approach was then employed in which patients were classified according to coagulation disorders, in an attempt to identify more subtle changes. INR driven consumption coagulopathy analysis data does show strong association with ISS, and when classified by severity this stratification resembles ISS stratification in its association with shock and hypofibrinogenemia.

Using mechanical kinetic testing to identify a hypercoagulable population a competing classification system was developed based on TEG R time. This population has been reported by many groups in recent years, and as been consistently apparent in our recent animal shock studies. This classification system does not show correlation with development or severity of shock, and appears to effect women at a lower rate than men in this population.

The driving forces behind the classification systems were examined to determine if the stratification of variables introduced bias into these associations as well. When looking at continuous variable correlations the associations between INR (driving force behind ACoT diagnosis) and BD, ISS, and functional fibrinogen concentration maintain the relationships observed during categorical analysis. On the other hand R time (driving force behind HC diagnosis) shows no significant correlation with these same variables, further strengthening the theory that these two populations are distinct.

Direct comparison of the ACoT+ population with the HC+ population shows distinct differences in platelet aggregation response, and suggests the possibility of

two separate conditions. Categorical data supports the distinction when looking at the incidence of ACoT within the HC grouping. The rate of occurrence of ACoT in the HC+ population (23%) is comparable to the rate in the HC- population (24%), suggesting that while separate in many ways, HC and consumption coagulopathy are not mutually exclusive.

Interestingly female patients were found to develop HC at a much lower rate than men (11% to 52% respectively) suggesting a sex related component to this disorder.

All the data presented in this analysis suggest a disconnect between current clinical treatment classification and the full extent of trauma induced coagulopathy. A more focused approach to delineate treatment based on in depth platelet function and coagulation evaluation.

The analysis of the data obtained during this study is in the very early stages, and only admission variables have been analyzed at this point. As more in depth examination of the data occurs it is hoped that a better picture of not only the presentation of coagulopathy becomes apparent, but also the progression and resolution of these conditions over time lead to better strategies for care in the severely injured.

Chapter 4:

In-Vitro Effects of Reactive Oxygen Species on Coagulation and Platelets in Whole Blood

4.1 Introduction

Oxidative stress has been shown extensively to be a component of a variety of disease states as well as ischemic and hypoxic injury. It is also well known that many disease states such as cardiovascular disease, sepsis, diabetes, and traumatic shock all involve the development of a coagulopathy to some extent. Due to this the examination of the effects the exposure of platelets to oxidative stress warrants investigation. In this set of experiments a novel model of ROS exposure on platelets in whole blood was developed in order to elucidate the effects that oxidative stress has on platelet function and coagulation.

Reactive Oxygen Species *In Vitro* Exposure Model

While many groups have examined the effects of various reactive oxygen species on platelets, the artificial environments employed in these experiments introduces considerable variation. The use of washed platelet models have been shown to introduce considerable activation in the process due to repeated washes and the high forces associated with multiple high speed centrifugation steps. The washing process decreases aggregation in response to collagen as well as increases basal CD62P expression in both basal and collagen activated platelets (Figure 4.1 and Figure 4.2 respectively).[240] The use of platelet poor plasma as a buffer has also been attempted in order to avoid the activation associated with the washed platelet model. Unfortunately the results have been divergent, and little consensus has been reached.[241] These inconsistencies may be attributed to increases in protein concentration and subsequent buffering ability increases associated with PPP composition. To best mimic the physiological environment that platelets encounter during *in vivo* ROS exposure the use of whole blood in required.

The use of hydrogen peroxide (H_2O_2) as a ROS for cellular exposure is possibly the easiest method to explore their effects. It is relatively cheap and available as a reagent. It also passes freely through cell membranes allowing diffusion driven intracellular exposure. It does not however mimic natural exposure conditions, which involve superoxide generation (O_2^-) and its subsequent enzymatic

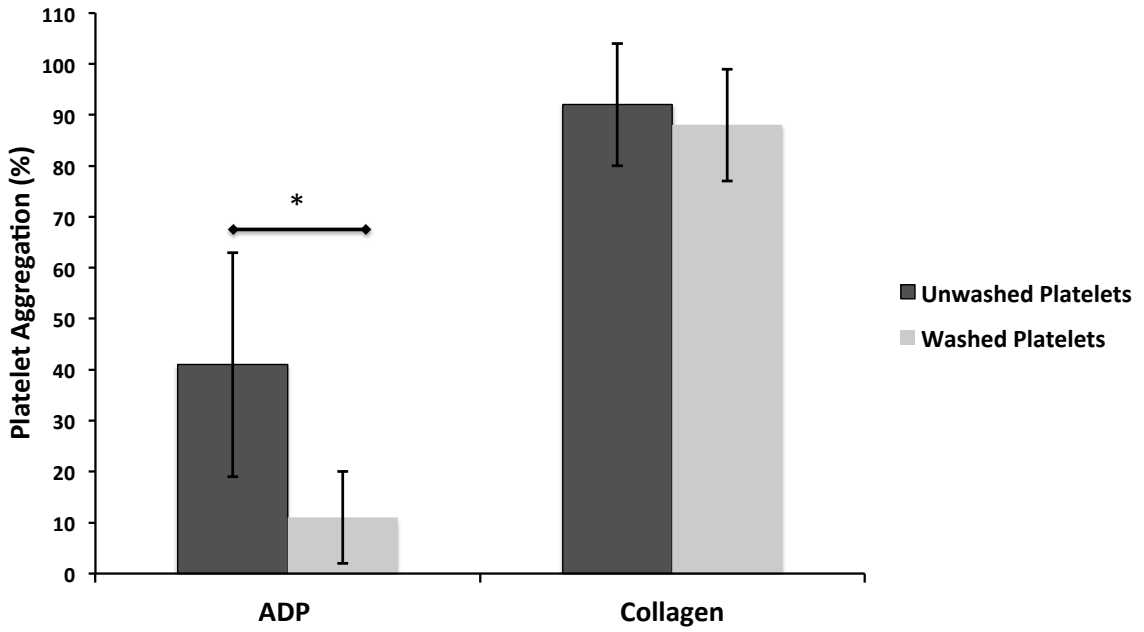

Figure 4.1: Differential Aggregation Responses in Washed Platelets. Adapted from Schoenfeld et al. (2004)[240]

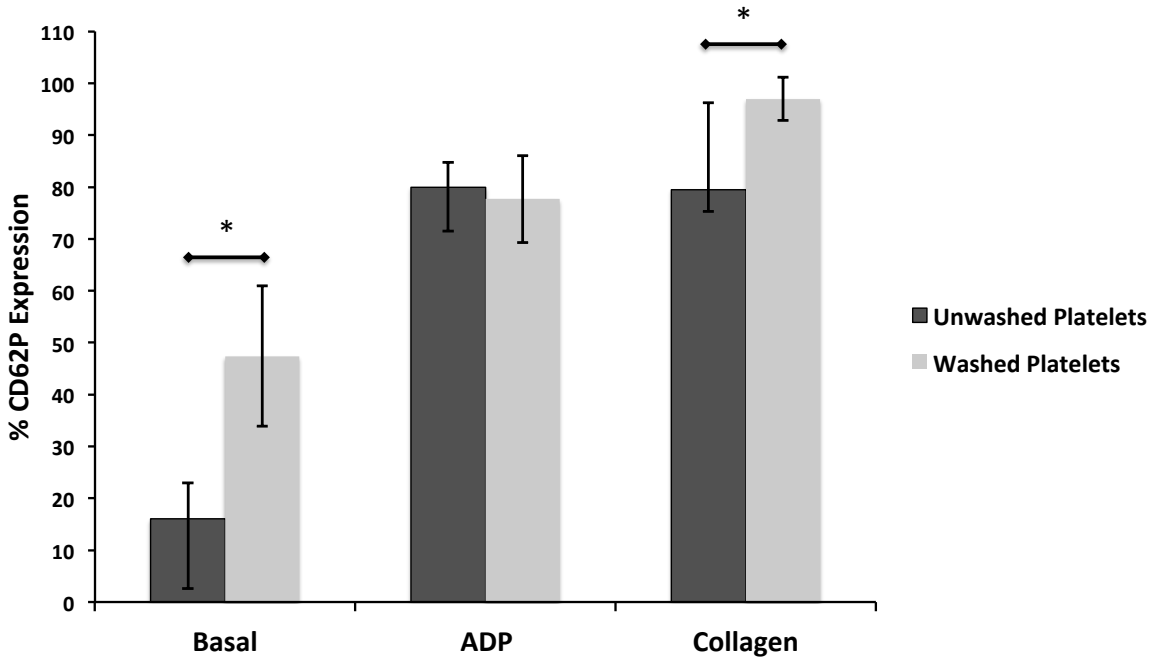

Figure 4.2: CD62P Expression in Washed and Unwashed Platelets. Adapted from Schoenfeld et al. (2004)[240]

and spontaneous conversion to other species including H_2O_2 as well as various reactive nitrogen species (RNS).[241] To better mimic a pathological state the use of xanthine and xanthine oxidase was utilized to generate O_2^-.

Hypoxanthine is generated in ischemia, cardiovascular disease, and traumatic shock.[242,243] Hypoxanthine is converted first to xanthine (X) and then to uric acid by xanthine oxidase (XO), generating an O_2^- at each step. This X/XO system utilizes the final step in this process, and has been shown to generate oxidative stress in washed platelet models in a consistent and dose dependent manner.[244]

Using Handin's work an *in vitro* model of ROS exposure on platelets in whole blood has been developed. 75 μU/mL of XO combined with .15 mM X has been shown to generate approximately 19.2 nmol O_2^- / mL/ min. [244] While this set of experiments was performed using washed platelets, the generation of superoxide via the X/XO system is known to occur in whole blood. This level of exposure has been shown by past work to cause increased platelet sensitivity to thrombin without causing complete platelet activation.[244]

4.2 Materials and Methods

Blood Sampling

All blood samples used to develop this model were surplus from healthy volunteers that enrolled in a separate approved study: "**Defining Platelet Function in Polytrauma**". This data was intended to serve as a feasibility test for a grant proposal. All blood was drawn via direct veinipuncture into standard sodium citrate vacutainers by trained staff in accordance to guidelines approved by the VCU IRB. All experiments were begun within 30 minutes of sample acquisition.

Treatment Protocol

Blood samples were aliquoted into two treatment groups. A superoxide generation system was created in the test group by addition of 75 μU/mL xanthine oxide (XO) in phosphate buffered saline (PBS). A vehicle control was used, with matching amounts of PBS added based on blood volume to match the spiking volume of the treatment group. In order to assure adequate supply of substrate both samples were given a dose of xanthine to bring their exogenous xanthine concentration to 0.15 mM. Immediately following addition of XO or vehicle the samples were gently inverted three times to ensure mixing and then incubated for 30 minutes at 37°C. After incubation analysis was performed immediately to analyze the effects of ROS exposure on the plasma coagulation cascade, platelet function, and whole blood clotting.

Coagulation Testing

Thromboelastography was performed on citrated whole blood samples using the TEG® 5000 Hemostasis Analyzer (Haemoscope Corp., Niles, IL, USA) using kaolin and $CaCl_2$ as per manufacturer's instructions. Use of this technique has been described by this lab before.[193,209,210]

The plasma coagulation cascade pathway tests of Prothrombin Time (PT) and activated partial thromboplastin time (PTT) as well as functional fibrinogen concentration were all measured in platelet poor plasma using the Start-4® coagulation analyzer (Diagnostica Stago, Asnières, France). All tests were performed according to the manufacturer's recommendation.

Flow Cytometry

Samples were stained for flow cytometry with primary antibodies for CD62p-PE, PAC-1-FITC, CD41-PE-Cy5 and appropriate isotypic controls (BD Biosciences, San Jose, CA, USA). After a 30-minute incubation samples were fixed and analyzed with an Accuri C6 cytometer (BD Biosciences, San Jose, CA, USA). A total of 20,000 cells were analyzed in each sample.

Intracellular ROS detection was performed using Invitrogen CM-H2DCFA (Life Technologies, Grand Island, NY, USA) For this measurement citrated PRP was prepared by centrifuging citrated whole blood at 130g for 10 minutes. PRP was incubated with monoclonal antibodies directed against CD41a and CM-H2DCFA, for 15 minutes at 37°C, and then immediately analyzed. Platelets were identified using CD41a and levels of ROS quantified by mean fluorescent intensity in the FL1 channel. Platelet aggregates were defined by shifts in FSC/SSC described in prior work by prior groups.[245–247] Agonist stimulation was performed with ADP (10mM) or convulxin (500 ng/mL).

Data Analysis

Data was analyzed using a paired student's t-test and matched pairs analysis. Each individual sample was reported as relative fold change from baseline. All statistical analyses were performed using JMP statistical software, version 10.0.0 (SAS Institute, Cary, NC). The level of significance for all statistical tests was $p < 0.05$.

4.3 Results

ROS Exposure Increases Intracellular ROS Levels

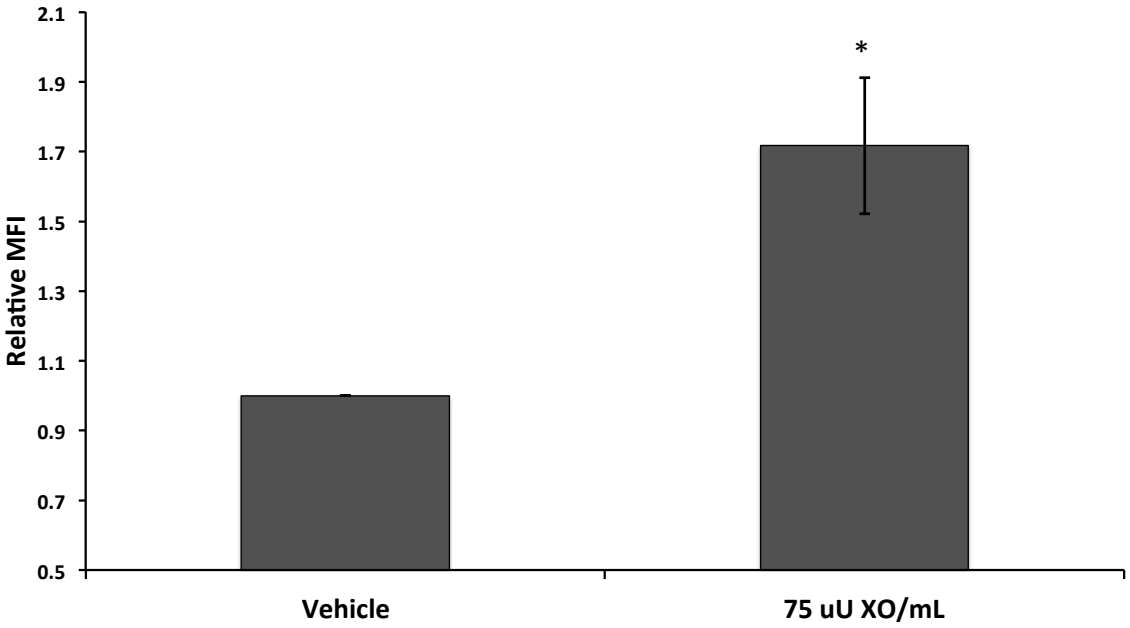

Figure 4.3: Increased Intracellular ROS Levels in Platelets After X/XO Treatment. Cellular Staining performed via CM-H2DCFA and flow cytometry. Results expressed in relative fold change of mean fluorescent intensity. n=3, 20,000 platelets analyzed per run. *; $p \leq 0.05$, X; xanthine, XO; xanthine oxidase.

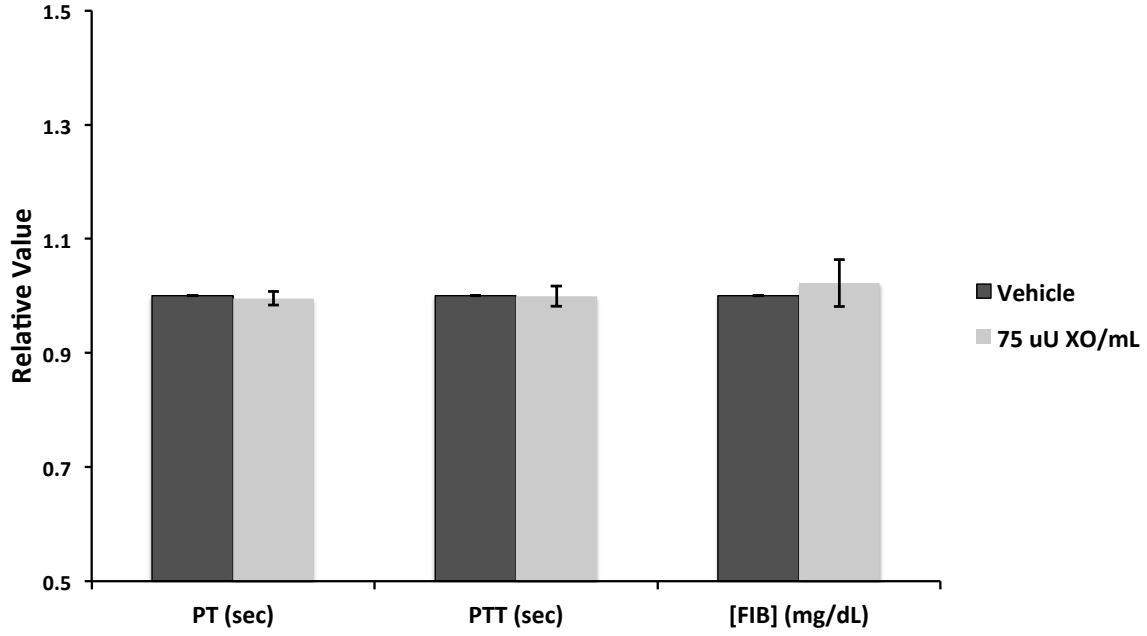

Figure 4.4: Classical Plasma Clotting Tests Unchanged After ROS Exposure. Results expressed in fold change compared to vehicle control. n=4 in all. PT; prothrombin time, PTT; activated partial thromboplastin time, [FIB]; functional fibrinogen concentration, XO; xanthine oxidase, sec; seconds.

The X/XO system generates extracellular O_2^- in the blood sample. To ensure that this extracellular superoxide flux is capable of generating the internal ROS elevation required for examining ROS effects on intracellular signaling pathways cells were quantified for intracellular ROS levels via flow cytometry. Treatment of whole blood with the X/XO system at the 75 μU/mL XO level significantly increased the level of intracellular ROS in platelets ($p < .05$) as measured by CM-H2DCFA staining for flow cytometry (Figure 4.3). This shows the X/XO system is a valid option for increasing platelet ROS levels in a whole blood model.

Reactive Oxygen Species Exposure Does Not Effect Coagulation Protein Pathways

While platelets are the main driving factor in coagulation and hemostasis, the proper function of the plasma protein enzymatic cascades are paramount for the conversion of fibrinogen to fibrin, and hence a stable clot. Due to the use of a global coagulation-monitoring tool (TEG) to evaluate whole blood clotting, the proper function of the clotting cascades must be evaluated. Immediately after incubation with the X/XO system plasma was analyzed for PT, PTT, and functional fibrinogen concentration. There was no significant difference in either the intrinsic or extrinsic pathways, and fibrinogen levels were normal in all samples both pre and post ROS exposure (Figure 4.4)

Reactive Oxygen Species Exposure Increases Clotting Kinetics

Clotting kinetic properties of whole blood are an important measure of the ability of the whole coagulation system to progress at a normal rate. While this measure does not often delineate an exact point at which the analyzed sample may

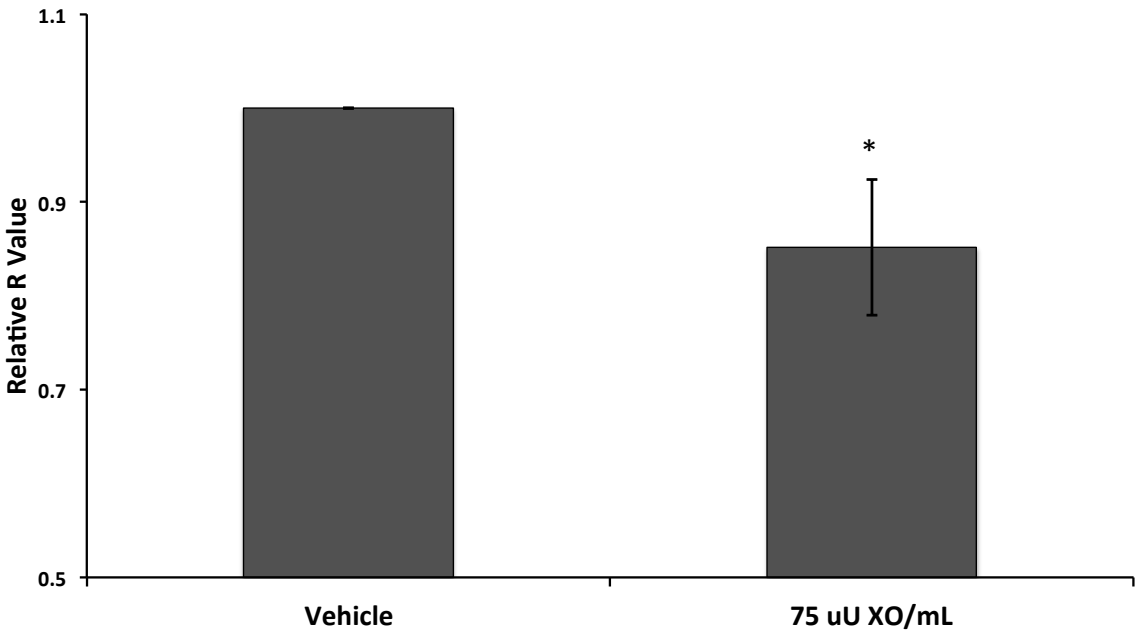

Figure 4.5: Decrease in Relative R Time After ROS Exposure. Results expressed in fold change compared to vehicle control. n=3. *; $p \leq 0.05$, XO; xanthine oxidase.

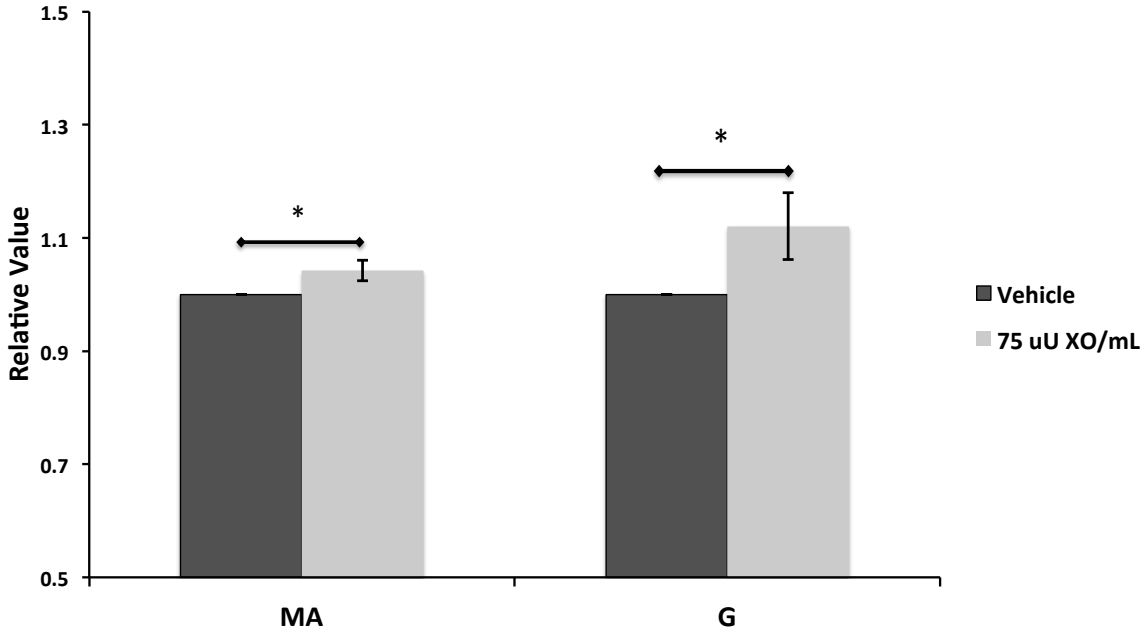

Figure 4.6: Increase in Relative Clot Strength After ROS Exposure. Results expressed in fold change compared to vehicle control. n=3, *; $p \leq 0.05$, XO; xanthine oxidase.

have a deviation, it allows for the monitoring of the systems normal progression on a global level. This data demonstrated a significant ($p < .05$) drop in the relative R values of the sample after ROS treatment when compared to a matched vehicle sample (Figure 4.5). While other measures of clotting kinetics such as k and angle displayed no change (data not shown) this decrease in kinetics suggests an increase in the platelet's ability to mediate thrombin generation.

Reactive Oxygen Species Exposure Increases Clot Strength

The measure of clot strength in whole blood is a combination of functional fibrinogen concentration as well as the platelet's ability to aggregate and attach to the fibrin network and contract.[193] As shown earlier there was no change in functional fibrinogen concentration between the ROS treatment group and the vehicle control group. It is then logical to attribute changes in the measures of clot strength to platelet function when comparing the groups. Analysis of both the (MA) as well as G TEG values both indicate a significant ($p < .05$) increase in clot strength in the ROS exposed platelets when compared to the control group (Figure 4.6). This data suggests an increase in platelet function, as functional fibrinogen can be viewed as constant between the samples.

Reactive Oxygen Species Exposure Effects on Basal Platelet Activation

Two measures of platelet activation were examined by flow cytometry. CD62P surface expression is widely used to identify platelet secretion. CD62P is an integral part of the α-granule membrane, and is exposed after the granules are secreted. Aggregation is examined in two separate ways. The PAC-1 antibody binds to the activated fibrinogen receptor, GPIIb/IIIa. Once activated, GP IIb/IIIa binds

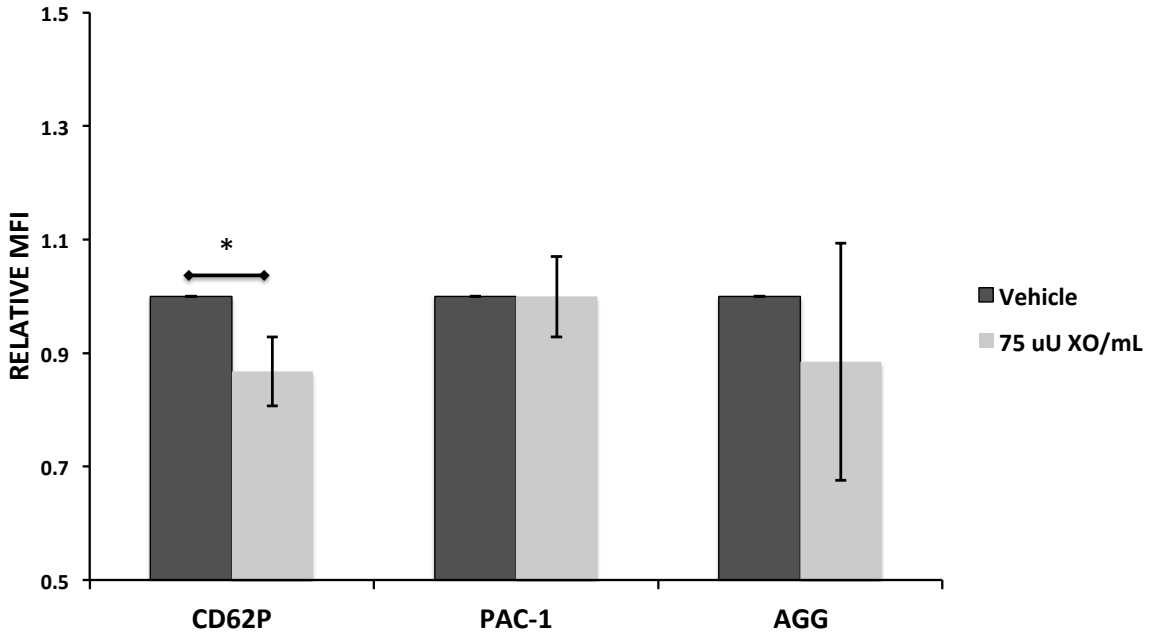

Figure 4.7: Basal Platelet Activation Analysis After ROS Exposure. Results expressed in fold change compared to vehicle control. n=3. *; $p \leq 0.05$, XO; xanthine oxidase, MFI; mean fluorescent intensity.

fibrinogen, aggregating with other activated platelets bound through the same mechanism. Measuring both PAC-1 binding as well as the formation of platelet aggregates using changes in forward scatter and side scatter gives a thorough picture of the functionality of the platelet aggregation process. After treatment with 75 μU/mL XO for 30 minutes there was a slight significant ($p < .05$) decrease in basal CD62P expression (Figure 4.7). It should be noted that while a significant decrease in relative expression was noted, the levels in both treated and untreated samples were well within the expected range. All other basal markers showed no differences in response to the ROS treatment.

Reactive Oxygen Species Exposure Causes Differential Effects on Platelet Aggregation

After ROS exposure there was a significant ($p < .05$) decrease in the relative % of platelets that stained positive for PAC-1 in response to ADP stimulation (Figure 4.8). The relative % of PAC-1 + platelets after stimulation with the collagen mimetic convulxin (CVX) remained the same. Not surprisingly the relative mean fluorescent intensity (MFI) of PAC-1 was significantly ($p < .05$) decreased in the ADP stimulated platelets, which is consistent with the lower percentage of cells that were able to bind the antibody (Figure 4.9). Interestingly the relative PAC-1 MFI rises significantly ($p < .05$) in CVX stimulated platelets after ROS exposure. This suggests that while the ROS treatment does not increase the number of platelets that can be activated by collagen, these platelets that are activated are expressing more activated GPIIb/IIIa per cell than the untreated platelets. It therefore can be argued

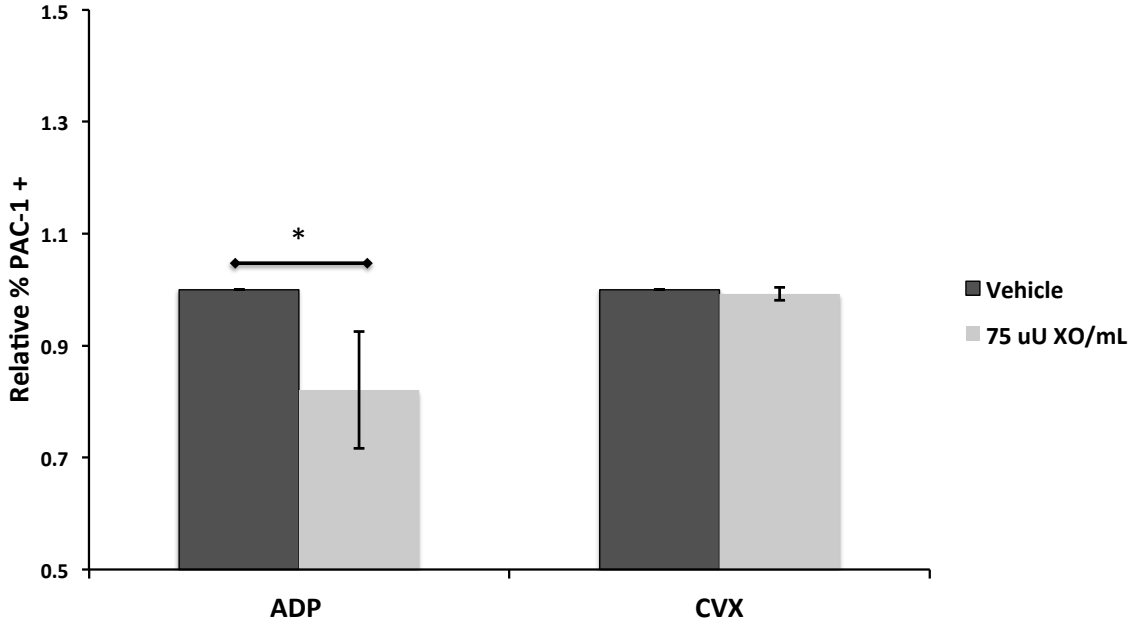

Figure 4.8: Differential Agonist Induced PAC-1 Expression After ROS Exposure. Results expressed in fold change of positively stained cells compared to vehicle control. n=3. *; $p \leq 0.05$, XO; xanthine oxidase, CVX; convulxin.

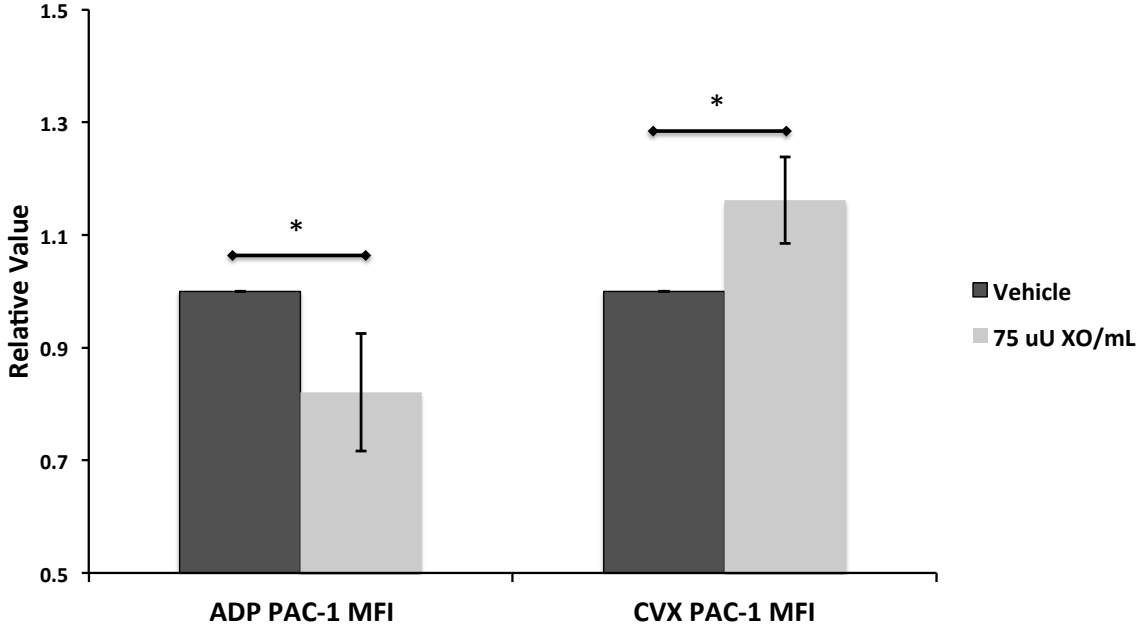

Figure 4.9: Differential Agonist Induced Absolute PAC-1 Expression After ROS Exposure. Results expressed in fold change compared to vehicle control. n=3. *; $p \leq 0.05$, XO; xanthine oxidase, MFI; mean fluorescent intensity, CVX; convulxin

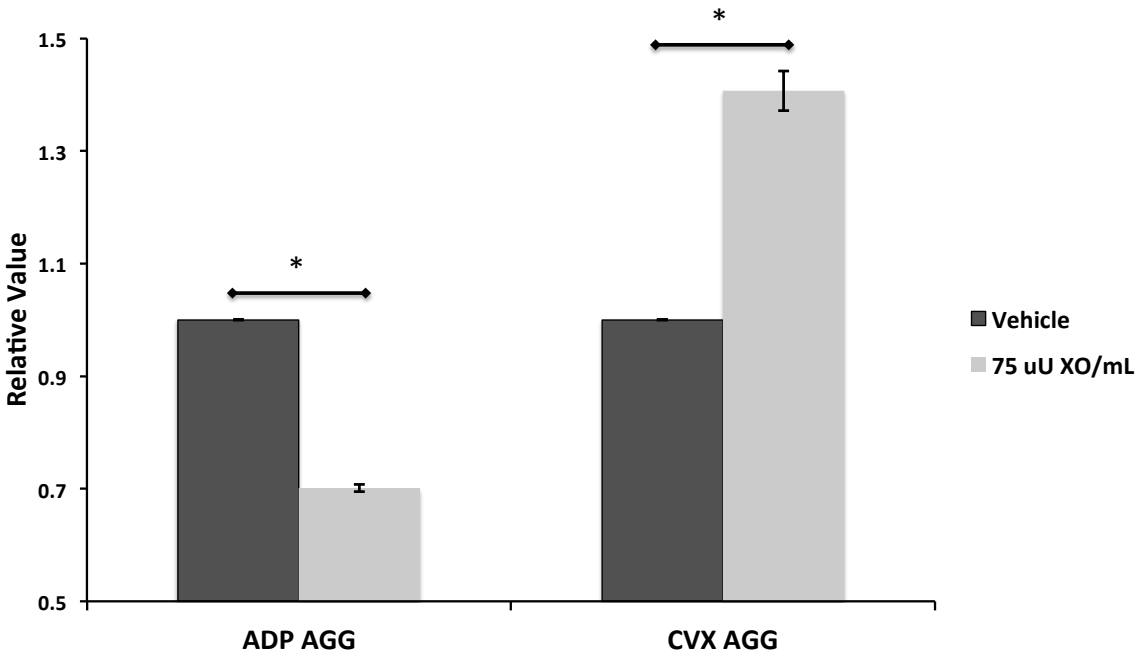

Figure 4.10: Differential Aggregation Response After ROS Exposure. Results expressed in fold change compared to vehicle control. n=3. *; $p \leq 0.05$, XO; xanthine oxidase, MFI; mean fluorescent intensity, CVX; convulxin, AGG; aggregates.

that the treated platelets can become more fully activated by CVX after ROS exposure. When examining the formation of platelet aggregates the data agrees with the PAC-1 binding data. Relative ADP induced platelet aggregate formation significantly decreased ($p < .05$) in ROS treated platelets compared to vehicle control samples. After CVX stimulation there was a significant increase in relative platelet aggregate formation ($p < .05$) when compared to the control group (Figure 4.10)

4.4 Discussion

The major proof of concept for the model was the demonstrated rise in intracellular ROS levels. The main goal was to develop a system to determine the effects ROS exposure has on platelet activity, and in order to reach this goal the rise in ROS internally had to be observed.

The lack in differences seen in the functional plasma clotting pathways indicates that the effects of the model are unable to influence them at these levels. This data becomes more interesting when examining the TEG data together. The decrease in R time suggests a quickening in kinetics of the entire whole blood clotting reaction. By excluding the plasma pathways this kinetic quickening may be considered more platelet driven. The increases in clot strength observed after treatment when compared to a fixed fibrinogen level and identical platelet counts begin to implicate platelets as well. Clot strength measurements on the TEG are the sum of effects of the fibrinogen/fibrin concentration, platelet count, and platelet function.[197] When fibrinogen and platelet counts are removed from the equation the main driver of MA becomes platelet function.

When examining platelet function on the cellular level, basally there are little difference between the two groups. CD62 expression was significantly reduced in the platelets exposed to ROS, but the absolute value of these measurements did not indicate widespread activation in any sample before activation.

Activation response was observed to be differential according to the individual agonist used. ADP was used as a GPCR agonist, and CVX as an integrin agonist. ADP response was lower across the board in the treatment groups when compared to vehicle, displaying dysfunction in one or more of the autocrine signaling pathways. CVX response on the other hand increased aggregate formation dramatically, and while it did not change the % of cells expressing PAC-1, MFI was significantly increased suggesting that more individual receptors on each cell were becoming activated.

It appears that ROS exposure does cause significant differences in coagulation. Using a combined classical, mechanical, and flow cytometric analysis the changes appear to be increased activity in platelets of the treatment group. These become more apparent in response to the collagen mimetic CVX, yet while at the same time there seems to be a concurrent decrease in the activity of the ADP pathway. It should be understood that the agonist responses can be specific to the agonist itself or multiple layers of control asserted downstream.

It cannot then be determined if there is an actual ADP response issue, an autocrine signaling dysfunction, or a problem related to the GCPR response in general compared to integrin response (PLCβ and PLCγ2 for example). Regardless of this uncertainty the conclusion that there is a measurable coagulopathy in the

ROS treatment group is solid. More testing needs to be done however to fully define the precise location and magnitude of the changes occurring.

Chapter 5:

Discussion: Multiple Levels of Trauma Induced Coagulopathy

When considering all data presented in this volume of work certain conclusions can be drawn about the pathology and development of trauma induced coagulopathy. While the animal, human, and *in-vitro* experiments shared certain goals (defining coagulopathy under conditions of trauma, shock, and oxidative stress), they were designed discretely without the expectation of any unified conclusions. Evaluation of the main conclusions drawn from these studies appear to converge around the idea that there are two competing forces in trauma related coagulation changes, and that these changes may possibly be unrelated.

Animal studies suggest than in response to moderate hypovolemic shock there are significant and complicated conditions that arise. While decreased plasma clotting factor activity suggests the beginning of a consumption related disorder, mechanical testing indicates increased kinetic and strength properties. This

disconnection between variables in a relatively controlled setting of traumatic shock suggests that while plasma clotting function decreases, platelet activity increases.

The data collected from the human polytrauma study suggests that injury severity is highly correlated with both the development of hypovolemic shock as well as the development of consumption based coagulopathies (ACoT/ECoT). Additionally there was the development of a hypercoagulopathy after trauma in a significant population that was platelet function related, and appeared at a much higher rate in men than women. These two conditions were not mutually exclusive, but the incidence of ACoT in the HC+ population seemed to remain consistent between HC+ and HC- groups (23% and 24% respectively). HC was not correlated to injury severity, shock, or hypofibrinogenemia suggesting that it is a separate response. This data again highlights a disconnect between platelet function (increased) and factor function (decreased).

In-Vitro experiments were designed to recreate the oxidative stress seen during trauma and subsequent blood loss. The X/XO system utilizes the same ROS generating machinery involved in endogenous hypoxia and ischemic injury. After exposure to X/XO generated O_2^- resulted in increased intracellular ROS levels and increased platelet function. This becomes of increased interest due to the fact that none of the plasma protein pathways were affected. Taken together this data shows the development of a hypercoagulable state in absence of a consumption disorder. Increased response to collagen pathway stimulation while maintaining an abover normal level of inhibition in the ADP pathway suggests differential effects in the

response of platelets to ROS exposure that needs to be investigated more completely.

All three experiments were able to identify what appears to be two distinct and competing forces involved in the coagulation response to trauma. Consumption disorders have been characterized for decades, and current treatment protocols revolve around INR for plasma administration. This is expected, as this disorder appears more apparent in the clinical setting as it involves current standard test panels (PT, PTT, [Fibrinogen]) and correlates well with injury severity and the development of hypovolemic shock. On the other hand HC classifications currently require the use of TEG testing, which is not commonplace in the clinical setting in regards to trauma. If that fact is combined with the data showing that this condition does not correlate with shock, ISS, or INR it is easy to understand why this pathology is commonly not recognized.

The relationship of sex with HC poses more questions, as it is known that estrogen has a protective effect in the context of hypoxia and oxidative stress.[248–250] Taken together with data generated in the *in-vitro* X/XO experiments this suggests that ROS may be one of the driving factors behind the development of HC. A much more in depth look at this is required before any solid conclusion can be drawn however.

Regardless of the causes of HC, it does appear that there are two different types of coagulopathies present in the response of trauma. There is a plasma related loss of function and a platelet related increase in function. These two conditions are not mutually exclusive, but do appear to involve different

parameters. It could be argued that early increases in platelet function cause coagulation and thus consumption, but the lack of a significant drop in platelets when comparing the two groups makes this argument unlikely. It is also possible that the increase in platelet function is an effort to maintain homeostasis triggered in the body due to loss of coagulation factors. This however is not supported by the fact that only 24% of the HC+ population presented with ACoT.

A more likely scenario is that oxidative stress has an effect on platelet function, and this effect is not demonstrated in non-cellular pathways such as the enzymatic plasma coagulation pathways. There are a wide variety of effects that oxidative stress can have on cellular signaling and lipid storage pools, highlighting the need to investigate this phenomena more completely.

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