THE PATHOLOGIC CONTINUUM OF PLASMIN ACTIVITY IN SEVERE INJURY

By

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To the survivors and future victims of trauma

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ABBREVIATIONS

%BV/TV = % bone volume/total volume

a2AP = a2-antiplasmin

ADP = adenosine diphosphate

AGE = Advanced Glycation Endproducts

APR = acute phase response

ASO = antisense oligonucleotide

AUC = area under the curve

BMD = bone mineral density

BMI = body mass index

C5a = complement 5a

CIA = collagen-induced arthritis

CTX = cardiotoxin

DAMP = damage-associated molecular pattern

DC = dystrophic calcification

DIC = disseminated intravascular coagulation

DPI = days post-injury

DXA = Dual-energy X-ray absorptiometry

EBL = estimated blood loss

EHR = electronic medical record

ELISA = enzyme-linked immunosorbent assay

ERCM = estimated red cell mass

FBG = fibrinogen

FFP = fresh frozen plasma

FTP = fibrin-targeted peptide

H&E = hematoxylin & eosin

HO = heterotopic ossification

HR = heart rate

IL-6 = interleukin-6

LBS = lysine binding site

mCT = micro computed tomography

MG = methylglyoxal

MMP = matrix metalloproteinase

MODS = multiple organ dysfunction syndrome

MSB = Martius Scarlett Blue

nBPT = normalized blood product transfused

NET = neutrophil extracellular trap

NFkB = nuclear factor kB

NGL = NFkB-GFP-Luciferase-expressing

NLR = Neutrophil-to-lymphocyte ratio

PAI-1 = plasminogen activator inhibitor-1

PAP = plasmin-antiplasmin

PAR = protease-activated receptor

PBS = phosphate-buffered saline

Plg = plasminogen

POD = post-operative day

PPP = platelet-poor plasma

PSF = posterior spinal fusion

PSGL-1 = P-selectin glycoprotein ligand-1

PXE = Pseudoxanthoma elasticum

RA = rheumatoid arthritis

RBC = red blood cell

rhPlg = recombinant human plasminogen

ROI = region of interest

SERPIN = Serine protease inhibitor

SIRS = systemic inflammatory response syndrome

SK = streptokinase

SOFA = sequential organ failure assessment

STiCSS = Soft Tissue Calcification Scoring System

T2DM = Type 2 diabetes mellitus

TAFI = thrombin-activated fibrinolytic inhibitor

TAT = thrombin-antithrombin

TBSA = total body surface area

TBV = total blood volume

TEG = thrombelastography

TIC = trauma-induced coagulopathy

TLR = toll-like receptor

tPA = tissue plasminogen activator

TXA = tranexamic acid

uPA = urokinase plasminogen activator

uPAR = urokinase plasminogen activator receptor

WT = wild-type

εACA = ε-aminocaproic acid

SCIENTIFIC CONTRIBUTIONS

Article 1: Breakthrough Plasmin Activity Correlates with Blood-loss, Transfusion, and Inflammation in Posterior Spinal Fusion Surgery Treated with Antifibrinolytics

For this prospective review, I worked alongside the clinician co-authors on this manuscript to design the study, and I wrote the IRB protocol for the study. During the study, I collected and analyzed samples with the assistance of my co-authors. I was responsible for manuscript preparation, including drafting the paper, analyzing the data, and developing figures from the data. I submitted this paper, revised it according to reviews from the journal and resubmitted the final version.

Article 2: Plasmin drives burn-induced systemic inflammatory response syndrome

For this translational study in both human patients and an animal model, I was involved in all steps of the study. For the humans, I assisted in blood collection and analysis from human burn patients at VUMC. I designed the animal studies with Dr. Schoenecker and executed them, including the injury models and specimen collection and analysis. I drafted both the original and final drafts of the manuscript, analyzed the data, developed the figures, conducted statistical analyses, submitted the paper, and made revisions for the final draft.

Article 3: Severe injury-induced osteoporosis and skeletal muscle mineralization: Are these related complications?

For this study, I was responsible for conducting all animal experiments, collecting tissues, including bone and muscle, and analyzing tissue specimens by micro-computed tomography (μCT), and histology. I wrote 2 of the original drafts of the manuscript, developed several

figures, provided critical revisions prior to submission of the article, and assisted with responding to reviewer comments.

Article 4: The plasminogen activation system in the musculoskeletal acute phase response: injury, repair, and disease.

For this comprehensive review article, I completed the literature review (including all background research) developed the figures, and drafted both the original and the revised manuscript with Jon Schoenecker. I incorporated critical revisions from all co-authors, and following peer-review, I completed final document revisions and resubmission.

Article 5: Plasmin regulates injury-induced soft tissue mineralization and heterotopic ossification in a multi-step, activator-dependent manner

For this study, I conducted animal experiments, collected tissues, and analyzed tissues by radiograph, μCT, and histology with my co-author, Dr. Satoru Egawa. For the manuscript, I reviewed background literature for premise, constructed the original draft, developed figures, ran statistical analyses, provided critical revisions, and I will assist with the final submission of the paper.

CHAPTER OVERVIEW

In chapter 1, I provide a brief overview on the acute phase response and the history of plasmin. In this section, I discuss the structure and function of plasmin(ogen), the enzyme response for degrading blood clots and the web-like protein deposited in injured tissues, within the repair phase of the physiologic acute phase response.

In chapter 2, I describe a pathologic acute phase response as it occurs in severe injury, specifically, and the change observed in plasmin activation following a severe injury compared with a more minor, isolated injury and how plasmin is therapeutically manipulated in certain pathologic conditions. Article 1 includes a study assessing pathologic activation plasmin during posterior spinal fusion surgery: an elective surgery to correct scoliosis. From this, I determined the best measures of plasmin activity that associate with negative outcomes, including blood loss and inflammation. Then, Article 2 includes a clinical and preclinical study demonstrating the role of plasmin in trauma-induced inflammation an accidental trauma in the absence of bleeding: burns.

In chapter 3, I describe plasmin activity's role in tissue repair later within the same continuum following severe injury. I describe a loss of plasmin activity seen later in the acute phase response when it is required to modulate tissue repair and how this precedes muscle calcification and the inappropriate formation of bone within muscle. Article 3 describes a mouse model in which simultaneous osteoporosis and injured muscle calcification occur following burn. Article 4 provides a comprehensive review of plasmin's paradoxical roles in both musculoskeletal repair and degeneration in disease conditions. I concluded this chapter using the model from Article 3 to determine the role of plasmin in burn-induced muscle calcification and subsequent bone formation in muscle, a common complication see in polytraumatic burns.

In chapter 4, I discuss other forms of plasmin deficiency, including congenital deficiencies and acquired deficiencies beyond trauma, including diabetes. Here, I demonstrate modification of plasminogen by glucose byproduct, methylglyoxal, found in uncontrolled type 2 diabetes mellitus, reduces its capacity to be activated and to degrade fibrin. I conclude the chapter by demonstrating a mild loss of plasmin activity observed in plasminogen isolated from patients with varying degrees of type 2 diabetes mellitus.

In chapter 5, I describe plasmin activation in moderate exercise and the possible effects of daily exercise on potential for plasmin activation and coagulation. I conclude the chapter with the description of a new output for wellness: unforced exercise. In this model, I demonstrate that mice with severe injuries and those deficient in plasmin both exhibit deficits in unforced exercise. The goal of this work is to use this model as an output along with outputs of tissue repair and classic neurobehavioral assessments to determine the role plasmin plays in long-term wellness and recovery following severe injury.

In chapter 6, I provide a conclusion for the work within my dissertation, including future directions and the clinical implications for this work.

CHAPTER 1: PLASMINOGEN AND THE ACUTE PHASE RESPONSE

Survival

Following an injury, such as a fracture or muscle tear, the body activates a coordinated process by which to sustain and resolve the injury: the acute phase response (APR)(1,2). Previous definitions of the APR describe it as a hepatic upregulation of damage control genes, including clotting factors, inflammatory mediators, and growth factors(3,4). However, the APR is a temporally regulated, localized response involving multiple organs and cell types.

Coagulation

In the initial survival phase of the APR (survival-APR) coagulation is activated at the site of injury to prevent bleeding and to contain the injury from pathogen invasion. The coagulation system is composed of an extrinsic pathway, and intrinsic pathway (contact), and a common pathway. When damage occurs within a blood vessel due to acute injury or chronic damage from hypertension, damaged endothelial cells and exposed subendothelial cells and matrices activate different elements of the coagulation cascade. Concurrently, circulating platelets aggregate at the site of vascular damage along with fibrinogen to seal off the breach in the vessel. The intrinsic and extrinsic pathways of coagulation converge on the platelet, which exposes coagulation factors, catalyzing the generation of terminal coagulation protease thrombin. Thrombin cleaves web-like protein fibrinogen into biomechanically strong fibrin polymers and activates the platelet to release paracrine and autocrine molecules which further fuel coagulation(5,6). The result of an effective coagulation response is a strong, hemostatic clot formed along the vessel wall at the site of damage which does not occlude the vessel. In order to contain coagulation to the site of damage, endogenous anticoagulants protein C, protein S, and antithrombin prevent coagulation from occurring beyond the zone of injury(5,7). While coagulation is typically considered an

intravascular process, coagulation also occurs outside of the vessels within damaged tissues to recruit inflammatory cells to the site of injury and to provide temporary relief from strain(2) (Figure 1.1).

Survival Inflammation

In concurrence with the activation of coagulation, a survival inflammatory response is initiated by damaged tissue. Release of inflammatory cytokines and chemokines from damaged tissues facilitates leukocyte recruitment to the site of injury. Neutrophils are the first to respond to tissue damage and infection, and they are activated by various damage-associated molecular patterns (DAMPs), including fibrinogen, ATP, and endothelial matrix proteins, to release neutrophil extracellular traps (NETs) composed of DNA, which wall off invading pathogens along with fibrin and platelets and contribute to clot strength(8–10). Tissue resident mast cells are also activated immediately following injury, releasing histamines, anticoagulants, and leukotrienes to stimulate recruitment and activation of other leukocytes to the damaged milieu (11–13)(Figure 1.1A). The coagulation and survival inflammatory responses synergize with one another to effectively contain the damage and prevent infection. Platelets and neutrophils exhibit bidirectional positive feedback upon one another, and coagulation factors, including fibrin, further activate neutrophils, macrophages, mast cells, and other leukocytes(10,14). Furthermore, inflammatory cytokines released during the survival APR, especially IL-6, induce transcription of multiple clotting factors and regenerative growth factors in the liver, referred to as acute phase reactants(4,15). While inflammation is often incorrectly identified as always problematic, an acute, local inflammatory response is an essential part of the APR(16) (Figure 1.1).

In a physiologic APR, the survival coagulation and inflammatory responses are contained locally to the zone of injury. A defect in the survival APR can provoke life-threatening complications, including bleeding, thrombosis, organ failure, and infection(1,2,17).

Repair: Inflammation, Remodeling, Angiogenesis, Regeneration

Once the injury has been adequately contained, the body transitions to the repair phase of the APR (repair-APR). During the repair-APR, a reparative inflammatory response is activated to promote tissue repair, and to remove the matrices established during the survival-APR to contain the damage, including fibrin, DNA (NETs), and a mixture of platelets and neutrophils to terminate the survival inflammatory response within the zone of injury(1,2). To accomplish this, proteases, including MMPs are activated to degrade these matrices, and pro-inflammatory (or M1) macrophages are recruited to the zone of injury to remove temporary matrices and necrotic tissue(18). Once the debris is cleared, these macrophages can undergo a phenotypic change to anti-inflammatory (M2), or new M2 macrophages can infiltrate the injured tissues to release growth factors and to promote remodeling(19). Growth factors then facilitate stem cell migration and angiogenesis, alleviating hypoxia caused by the injury and allowing cellular regeneration, stem cell differentiation, and restoration of tissue function(1,2,20) (Figure 1.1B). The temporal aspect of the repair process is different for each tissue type: muscle and skin complete this process within days to weeks of the injury(20,21), while bone can take several months to fully recover in healthy individuals(2,22). An effective repair-APR is essential to prevent chronic complications within the injured tissue, including loss of function, fibrosis, pain, and tissue degeneration.

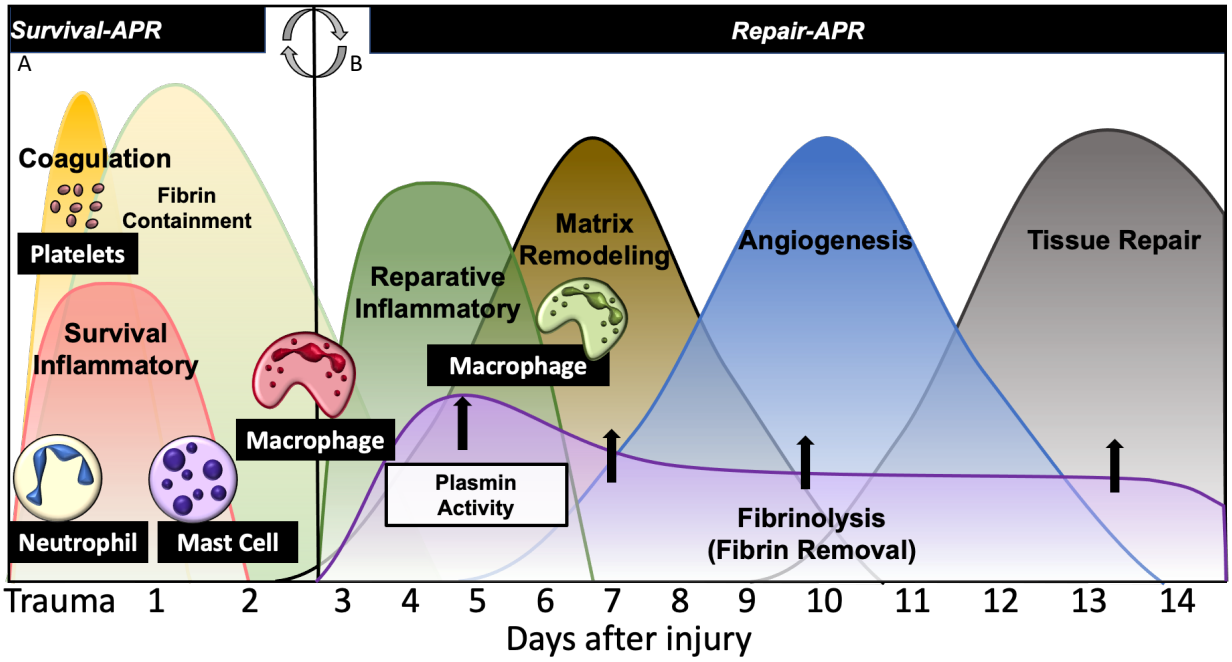

Figure 1.1: **The non-severe acute phase response (APR)** is comprised of A) an initial survival response marked by acute activation of coagulation and survival inflammation involving multiple cell types and proteins, including fibrin, at the site of injury to contain the damage and prevent infection. B) Once the injury is contained, the tissue transitions to the repair-APR where macrophages remove necrotic tissue, plasmin removes fibrin, and angiogenesis and tissue regeneration occur to restore tissue function.

Plasminogen Structure and Function

The protease plasmin plays essential biologic roles in both the repair-APR and tissue maintenance(1). Plasmin is primarily expressed in hepatocytes and circulates as a closed-conformation zymogen, plasminogen. Glu-plasminogen, the major form of plasminogen in circulation, has a carboxy-terminal serine protease domain, an amino-terminal PAN (plasminogen-

apple-nematode) domain, which blocks the protease domain and keeps plasminogen in a closed conformation(23), and 5 kringle domains (Figure 1.2). Each kringle domain except one contains an aspartate-rich core which has a high affinity for lysine residues(24,25). Plasmin(ogen) has a high affinity for lysine-rich proteins, including its major substrate, fibrin(ogen), and many of the extracellular receptors with which it interacts. In its closed conformation, plasminogen has 1 exposed lysine binding site (LBS) on Kringle 1 which, once bound to lysine in fibrin or a receptor, induces a slight conformation shift to the structure of plasminogen to expose another LBS. This process continues until plasminogen is in a fully open. Once fully open, plasminogen's activation cleavage site is exposed, allowing it to be activated by various proteases to plasmin. Plasmin can also cleave the PAN domain on other Glu-plasminogen, producing Lys-plasminogen that is in open conformation and susceptible to activation(25,26). Plasmin's primary activators are tissue plasminogen activator (tPA) and urokinase plasminogen activator (uPA), and these serine proteases cleave plasminogen at the Arg561-Val562 peptide bond to produce active plasmin and release a biologically active peptide known as angiostatin (Figure 1.2). Active plasmin can then proteolyze fibrin to which it is bound (fibrinolysis), or it may activate other proteases, and cell surface receptors, triggering both extra- and intra-cellular signaling events. Because plasmin has a wide range of targets, its activity is regulated by serine protease inhibitors (SERPINs) α2-antiplasmin (α2AP) and α2-macroglobulin, which irreversibly bind and inhibit free plasmin in an LBS-dependent manner(24,26). The primary and specific inhibitor of plasmin is α2AP. Plasmin-antiplasmin (PAP) complexes circulate in the blood for several hours before being cleared hepatically. Because plasmin activity is transient and on its own is relatively unstable in blood, PAP complexes are often used to specifically detect if plasmin was activated(27). Another secondary marker of plasmin activity is fibrin degradation product (FDP), of which the most

commonly measured is crosslinked degradation product D-dimer(5,27). Plasmin(ogen) bound to fibrin and cellular receptors is mostly protected from inhibition by α2AP so that specific plasmin activity can occur without free plasmin wreaking havoc. Along with α2AP, activity of plasminogen activators, tPA and uPA, are inhibited by SERPINs plasminogen activator inhibitor-1 (PAI-1) and sometimes PAI-2(24). The different plasminogen receptors and their downstream targets are discussed within *Article 4.* The most recognized role of plasmin(ogen) in biology is fibrinolysis both in intravascular clots and in extravascular tissues at sites of injury. However, plasmin has a wide range of fibrin-dependent and -independent functions, including modulation of inflammation, matrix remodeling, hematopoiesis, angiogenesis, and cellular differentiation and migration(1,24,28). These functions make plasmin essential for the repair and maintenance of tissues, but in disease conditions in which plasmin activity is poorly regulated, these functions may cause plasmin to take on pathologic roles.

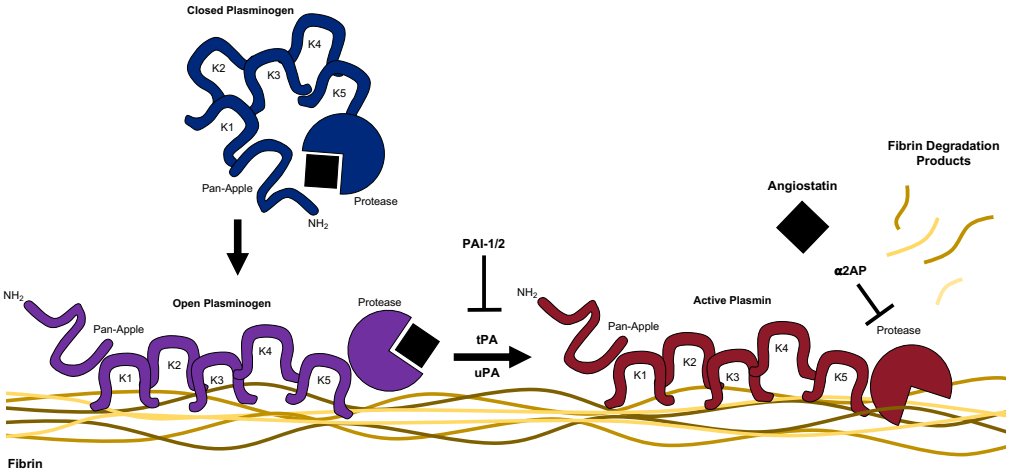

Figure 1.2: Plasminogen structure, activation, and inhibition. Plasminogen circulates in a closed conformation, and upon binding to lysine-rice fibrin, it opens and exposes proteolytic activation site for tPA and uPA. Cleavage of Arg561-Val562 releases angiostatin and produces active plasmin which can degrade fibrin and cleave other targets before being inhibited by α2AP.

The Discovery of Plasminogen and Fibrinolysis

The first recorded descriptions of plasmin function were by surgeon John Hunter and anatomist Giovanni Morgagni in the 1700s. Hunter noticed that patients with severe penetrating traumas bled profusely and that even post-mortem, the blood of these patients was liquefied. Morgagni observed a similar phenomenon in blood isolated from deceased trauma victims: their blood would clot and then liquify. While neither Hunter nor Morgagni knew the cause, they both hypothesized that something was being activated by a severe injury that did not prevent clotting but was causing blood clots to rapidly break down after they had formed(29). In 1843, Albert Dastré determined that a protease was being activated in clotted blood, leading to fibrinolysis and reliquification. He called this protease (or group of proteases) "fibrinolysin," and later this enzyme was named "plasmin" after Rosemary Biggs and Robert MacFarlane demonstrated the role of a specific enzyme in *in vitro* clot lysis(30). It wasn't until the 1940s that plasminogen was purified and its mechanisms of activation by tPA, uPA, and bacterial streptokinase (SK) were demonstrated by L. Christensen and Colin MacLeod(31). Since, scientists and clinicians continue to rigorously study both the beneficial and detrimental effects of plasmin activity in order to therapeutically target the plasminogen activation system in disease.

Plasminogen in the Physiologic APR

During a physiologic APR, plasmin is primarily activated within injured tissues during the repair-APR to promote tissue regeneration (Figure 1.1A). Plasmin(ogen) is localized to the site of

injury through binding to fibrin deposited during the survival-APR, necrotic tissue, and receptors on damaged cells and infiltrating leukocytes, including neutrophils and macrophages(1,2) Injured tissues can express either tPA or uPA in the days following injury, depending on the tissue type, to activate the localized plasminogen to plasmin(32). Once active, plasmin executes a wide range of functions to promote tissue repair, including removal of deposited fibrin and stimulation of macrophage activity, angiogenesis and regenerative signaling (Figure 1.1B). Consequently, plasmin is essential for the physiologic repair of all tissues, including muscle, skin, bone, and nervous tissue(33–36). A comprehensive review of plasmin's many functions in tissue repair are all highlighted in detail within Article 4. While some plasmin is activated in response to the coagulation activation in the survival-APR, the majority of plasmin activity occurs following the survival-APR to remove clots and damaged tissue (Figure 1.1B)

Chapter 1 Summary:

The APR is a complex process by which the body sustains and resolves injury, whether that is physical trauma, infection, or psychologic injury. The APR can be temporally and phenotypically divided into 2 phases: survival (acute coagulation and inflammation to contain the injury) and repair (remove the temporary containment and resolve the damage). The APR is tightly regulated in a temporal and spatial manner to remove potential pathogens and restore function to injured tissues in a timely manner without provoking significant complications.

Plasmin is a multifunctional enzyme that not only degrades fibrin clots but plays critical roles in tissue regenerative processes. Zymogen plasminogen circulates in a closed conformation activation until it encounters fibrin or receptors on the surface of damaged cells, opening its conformation to allow for activation to plasmin by tPA or uPA. In a physiologic APR, plasmin is

activated locally at the site of injury during the repair-APR to direct regenerative processes and resolve inflammation.

CHAPTER 2: Plasmin in Survival: Plasmin provokes Trauma-Induced Coagulopathy and SIRS

Severe Injuries

Severe injuries, such as severe burns, traumatic brain injuries, and polytraumas, are a leading cause of death and disability worldwide(37). A severe injury is one that provokes a systemic response, affecting multiple tissues, including major organs. In the United States severe injury or accidental trauma is the leading cause of death in individuals under the age of 46, killing 150,000 individuals and affecting over 3 million individuals per year(38). A major reason for this is that severe injuries provoke early, life-threatening complications, including bleeding, thrombosis, infection, and organ dysfunction or failure(39). Medical advances in intubation, blood transfusions, anticoagulant medications, and surgeries have allowed trauma patients to survive the initial complications of severe injuries. However, these patients often proceed to endure long-term complications, including tissue fibrosis, chronic pain, loss of limb function, and tissue degeneration (e.g. osteoporosis)(40–42). Unintentional trauma is estimated to cost up to $400 billion in medical costs and work loss costs, and these complications are not only medically costly but account for up to 30 disability-adjusted life years lost(38), highlighting both the financial burden and loss of quality of life imposed by these types of injuries.

The complications provoked by severe injuries are driven by a complex, pathologic response to the injury. While it's understood that severe injury-provoked complications are rooted in a dysregulated APR, the key molecular mechanisms driving this dysfunction are not well understood. Consequently, most treatments for severe injuries are focused on fluid resuscitation, surgical intervention, and blood transfusions—none of which adequately address the root cause of trauma complications. An understanding of the elements of the APR that go wrong following

severe injury is critical for the development or purposing of therapeutics to target the disease pathophysiology and improve patient outcomes.

Coagulopathy

"Coagulopathy" is a broad term used to describe dysfunctional coagulation associated with disease. Coagulopathy can refer to a state of hypocoagulability, hypercoagulability, or both in concurrence. During acute states of disease, including infection or severe injury, coagulation is pathologically altered by these conditions(43–45) In the case of a severe injury with bleeding, coagulation is activated to an immense magnitude in effort to wall of the wound and prevent bleeding. In major penetrating traumas, coagulation is continually activated until hemostasis is achieved, but many of these injuries require surgery for cessation of bleeding(46). Consequently, the coagulation system is exhausted in the process, consuming platelets and clotting factors, including endogenous anticoagulants protein C/S and antithrombin and other procoagulants. The net result of this is an increased risk for venous thrombosis with a simultaneous risk for bleeding at the site of the wound where platelets and clotting factors have been expended(43,47). This phenomenon also occurs in infections as the innate immune response and coagulation system fuel one another to quarantine and destroy pathogens. In disseminated infections, this often results in diffuse, intravascular activation of coagulation fueled by inflammation(7,44,45,48). The pathologic clinical result of this is disseminated intravascular coagulation (DIC), which also expends platelets in the thrombi so patients have a paradoxical occurrence of clotting and bleeding. For this reason, many studies have demonstrated that low platelet counts in these patients upon admission are predictive of thrombosis(49).

Systemic Inflammatory Response Syndrome (SIRS)

SIRS is a non-specific clinical condition that may arise from severe injury, infection, or autoimmune disease for which the clinical criteria include dramatic changes (both increases or decreases) in body temperature, heart rate, respiration rate, and white blood cell count(50). Although not measured clinically, SIRS is also marked by significant increases in plasma levels of pro-inflammatory cytokines, including IL-6, IL-1β, TNFα, and IFNγ—a phenomenon termed the "cytokine storm." Furthermore, an increase in circulating neutrophils and a decrease in circulating lymphocytes is often observed during the peak of the inflammatory response(44) (Figure 2.1).

Both coagulopathy and SIRS can provoke complications on their own, but in concurrence, they produce an "immunocoagulopathic" condition that greatly increases the risk of bleeding, thrombosis, and organ failure(7,44) (Figure 2.1).

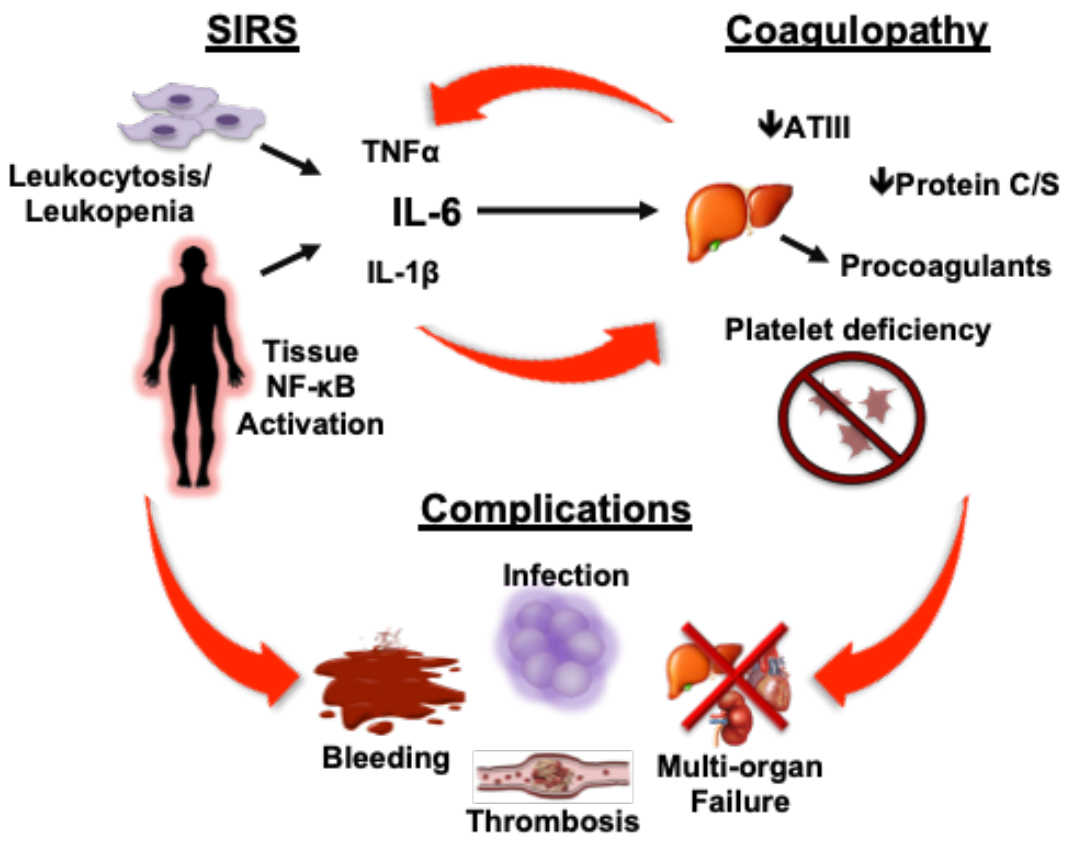

Figure 2.1: Clinical and research criteria for SIRS and coagulopathy. The concurrence of SIRS and coagulopathy in trauma and infection greatly increase the risk of fatal complications.

Plasminogen in the Pathologic APR

Severe injuries and infections can trigger a pathologic APR that provokes systemic, life-threatening complications. Severe injuries are those which provoke a systemic response that has pathologic effects on multiple organ systems(38,51). Some examples of severe injuries include severe burns, traumatic brain injuries and penetrating traumas with significant blood loss(52,53) While the magnitude of the APR should be proportionate to the magnitude of the injury in order to effectively contain the injury or pathogens, a pathologic APR is marked by too great of a

response that fuels itself out of control. Following a severe injury, immense coagulation and inflammatory responses are initiated to stop bleeding and infection, and these responses can become dysregulated, resulting in coagulopathy and SIRS(29,47,54). Within this significant survival-APR, plasmin activity is aberrantly altered: rather than a local activation of plasmin at the site of injury during repair, severe injuries provoke systemic activation of plasmin during the survival-APR (46,52,53)(Figure 2.2A). The vascular response to hypoxia and hypotension instigated by severe injury triggers a release of tPA from the endothelium, activating plasmin within the blood. Plasmin activation in response to hypoxia is an effective adaptive mechanism to break down thrombi occluding vessels and to remove fibrin and tissue from injured tissues requiring oxygen, however, in this case, hypoxia-induced plasmin activation has detrimental effects(43,55). The most well-described consequence of early acute plasmin activation during the survival-APR is blood loss; plasmin is rapidly activated during coagulation, degrading hemostatic fibrin clots as they are being formed. In response to progressive bleeding exacerbated by plasmin, coagulation is continually activated as plasmin breaks down fibrin clots. Bleeding due to excess plasmin activation is a significant source of morbidity and mortality in both trauma and surgical fields. This phenomenon is termed “hyperfibrinolysis,” and the prognosis for trauma patients who present with significant hyperfibrinolysis is poor(52,55).

Following the survival-APR of a severe injury, a period of poor plasmin activity is observed, which is sometimes termed “fibrinolytic shutdown” or hypofibrinolysis(53,56) (Figure 2.2B). Studies have attributed this to a wide range of causes, and this phenomenon may be multi-factorial. This is thought to increase the risk of thrombosis and organ failure due to poor fibrinolytic potential, and studies have demonstrated that patients admitted to the trauma unit can

present with hyper-, hypo- or eufibrinolysis depending on the diagnostic test used, but it's unclear if these phenotypes are the result of a differential response to injury types or if they all exist on the same continuum at different time points(53). There have been several proposed mechanisms for fibrinolytic shutdown, the majority of which appear to be an adaptive response to a severe-APR. Following activation of coagulation and fibrinolysis, built-in negative feedback mechanisms regulate the system. For example, activation of thrombin during coagulation facilitates the activation of protein C, an endogenous anticoagulant that shuts down thrombin generation on the platelet by proteolytically inactivating clotting factors (FV and FVIII). Studies have suggested that following endothelial release of a tPA bolus into the blood, a delayed release of PAI-1 from endothelial vesicles is observed, overwhelming remaining tPA and inhibiting uPA released 7-9 hours following the injury(57–59). Other studies have suggested that plasminogen is consumed during its mass activation to plasmin, depleting the plasma until hepatic expression of plasminogen can restore blood to physiologic levels(54,60). Additionally, activation of proteases, such as neutrophil elastase, during a severe survival-APR, may result in plasminogen degradation(61). The fibrinolytic shutdown phenotype has been used to predict lethal thrombotic outcomes, but the effects of poor plasmin activity during the repair-APR on tissue regeneration and recovery are not well understood.

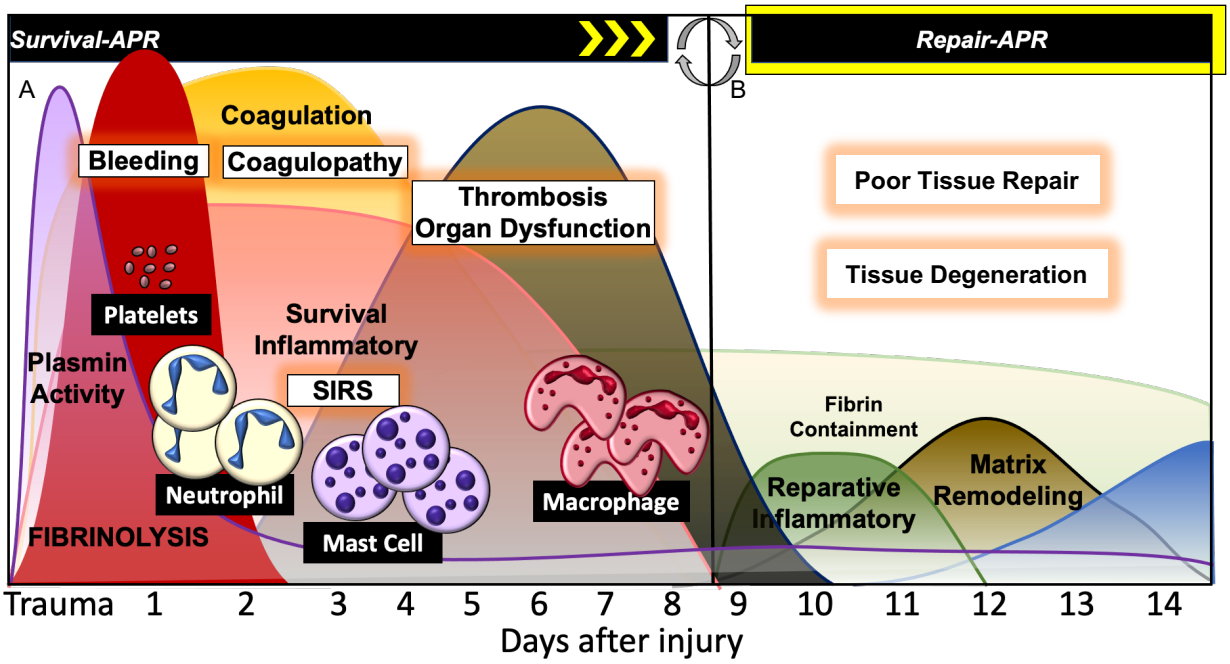

Figure 2.2: The APR following a severe injury. A) A severe injury provokes a systemic, dysregulated survival-APR marked by excess inflammation and uncontrolled coagulation, predisposing patients to bleeding, thrombosis, and organ dysfunction. B) If patients survive the initial response to the injury, they often exhibit delayed transition to the repair-APR with failure to resolve inflammation, poor fibrin removal, and inefficient repair mechanisms, leading to long-term disease in both injured and uninjured tissues.

Early Complications of Severe Injuries: Bleeding, Thrombosis, Organ Dysfunction

The resultant effect of an unbridled cycle of coagulation, inflammation, plasmin activation, clot degradation, bleeding, and further coagulation activation are fatal complications, including blood loss, thrombosis, and organ dysfunction(39,47). Constant clot degradation by plasmin and a consumption of platelets and clotting factors can provoke bleeding, while platelet-neutrophil interactions and consumption of endogenous clotting factors can promote the development of venous thromboses. Furthermore, activation of coagulation in microvasculature within tissues

provokes and inflammatory, hypoxic condition, driving organ dysfunction and failure(44) (Figure 2.2A). These complications were not survivable prior to the development of safe blood transfusions, steroids, and anticoagulant drugs. Although these treatments can facilitate symptom management and survival in patients with severe injuries, they often fail to treat the root cause of the condition and can provoke further complications. For example, the concurrent paradoxical hyper/hypocoagulable conditions observed in coagulopathy are notoriously difficult to treat as anticoagulants for thromboprophylaxis increase the risk of bleeding, and blood products to treat bleeding or hypocoagulability may increase the risk of a thrombotic event. Steroids are effective to reduce the inflammatory response to an injury, but they may exacerbate the existing risk of infection(50,62). Therefore, improved diagnostics and therapeutics to both predict and treat these complications respectively are warranted.

Later Complications of Severe Injuries: Pathologic Tissue Repair

Beyond complications within the survival phase of the APR, severe injuries provoke chronic complications that may affect patients for years, including tissue fibrosis, bone formation in soft tissues (heterotopic ossification, HO), wound dehiscence and infection, bone loss (osteopenia/osteoporosis), muscle wasting (sarcopenia), and chronic pain(20,42,63,64). Overall, these complications greatly diminish functionality and quality of life. The mechanisms driving these long-term complications are poorly understood, but they have been linked to an aberrant survival-APR that fails to appropriately transition to the repair-APR, prolonging pro-inflammatory signaling and crippling the removal of necrotic tissue by macrophages, angiogenesis, and regenerative mechanisms in injured tissue(65) (Figure 2.2B).

Therapeutics

Several therapeutics target plasmin or the plasminogen activation system to either inhibit or activate plasmin. Antifibrinolytic drugs are a class of drugs that inhibit plasmin activity or plasmin activation, and they fall into 2 categories: protease inhibitors and lysine analogues.

Protease inhibitors:

Only one protease inhibitor was approved for the antifibrinolytic use, and it is no longer used clinically due to its association with organ failure-related deaths. Aprotinin, also known as bovine pancreatic trypsin inhibitor, inhibits plasmin at 125 KIU/mL and is far more effective at preventing fibrinolysis and associated blood loss during surgical procedures. However, aprotinin was withdrawn from use in 2007 due to increased risk of organ failure and death associated with its use(66,67). While it's unclear which mechanisms provoke these adverse effects, aprotinin is not a specific protease inhibitor, and as such, inhibits clotting and inflammatory proteases, including clotting FXII, kallikrein, and members of the trypsin family of proteases. Furthermore, as a bovine protein, it carries a higher risk of anaphylaxis associated with its use(67). Currently, it is still used in rare cases, but it is not used as an antifibrinolytic clinically.

Lysine analogues

Lysine analogues are small molecules that are considered structurally similar to lysine. As lysine analogues, they competitively bind to LBS on plasminogen preventing it from binding to fibrin and being activated to plasmin and preventing plasmin from binding to fibrin and degrading it. E-aminocaproic acid (εACA) is essentially a lysine molecule without the amino group on the 2-carbon of the molecule. EACA was discovered in 1957 and reported in the early 1960s by Utako

and Shosuke Okamoto in Japan for the treatment of conditions of excessive bleeding(68–70). It's been applied in the treatment of dental and surgical bleeding, menorrhagia, and gastrointestinal bleeding(70). However, εACA has poor efficacy with approximate effective concentrations in blood at just under 1mM(71). Because of this, the Okamoto lab focused on finding another molecular candidate with better efficacy with the goal of developing an effective, safe treatment for post-partum hemorrhage, which was, at the time, a significant source of morbidity and mortality in young women(69).

Several years after the discovery of εACA, the Okamotos discovered a new molecule, tranexamic acid (TXA). While TXA is not structurally similar to lysine, it also binds to LBS on plasmin and plasminogen, preventing plasmin activation and fibrinolysis. TXA is more efficacious than εACA with effective plasma concentrations at 60-600 μM dependent on the indication(67,72), although it likely wouldn't be approved today with this efficacy. Both lysine analogues are cleared renally (and therefore require adjusted doses in renally impaired patients), and they both have short plasma half-lives of 2-3 hours(73,74). In critically ill, hypercoagulable patients, antifibrinolytic drugs may increase the risk of thrombosis. Furthermore, because of their similar structures lysine analogues have the potential to induce seizures at high plasma concentrations (~>800 μM)(75). Specifically, studies have demonstrated TXA-mediated inhibition of GABA receptors and agonism of glycine receptors in the brain are likely responsible for seizures(75). If dosed appropriately, lysine analogues should not reach concentrations high enough to result in seizures in healthy individuals. Both εACA and TXA are considered relatively safe with few contra-indications, and therefore, they are used frequently in trauma, surgery, obstetrics, gynecology, and rare hematologic disease(68,70,76).

The findings of the global WOMAN clinical trial conducted in the 2010s strongly suggested that TXA was effective to reduce bleeding and prevent death in post-partum hemorrhage with little risk of adverse effects(77). The study was published in 2017, one year following the death of Utako Okamoto, and TXA is now standard of care world-wide for the treatment of post-partum hemorrhage.

Thrombolytics:

As a fibrinolytic protease that degrades clots, plasmin's activity is often harnessed to break down thromboses. Recombinant tPA is administered intravenously to patients undergoing strokes or is applied locally at the site of venous thromboembolism during a thrombectomy to activate plasmin, degrade fibrin, and dissolve the thrombus. Like endogenous tPA, recombinant tPA has a short plasma half-life of several minutes to an hour. Recombinant and endogenous tPA are both irreversibly bound and inhibited by PAI-1 and then rapidly cleared by hepatocytes(78,79). The critical adverse event associated with tPA use is bleeding, especially intracranial hemorrhage when used for strokes. Recombinant tPA is considered a life-saving drug for the treatment of venous and arterial thrombosis(78).

Diagnostics

Functional assays:

Functional assays to measure coagulation and fibrinolytic (plasmin) are a measure of potential to activate these pathways either in whole blood or plasma. A common clinical diagnostic test for plasmin activity is thrombelastography (TEG) or thrombelastometry (TEM). TEG/TEM is run in whole blood in which a coagulation activator is added (usually contact activator, kaolin),

and then a small wire measures viscoelastic changes as the clot forms. TEG measures the rates of coagulation activation (R), fibrin formation (K), amount of fibrin (α-angle), and overall maximum clot strength (max amplitude). Once the maximum amplitude is reached, if plasmin is active or activated in the sample upon clot formation, the torsion wire measures percent clot degradation over 30 minutes (LY30)(80,81). This technique is widely used to detect coagulopathy as well as changes in fibrinolysis. It is very commonly used in liver transplant surgeries and in trauma patients to identify hyperfibrinolytic states and hypocoagulable states(82). While TEG is a useful diagnostic to predict bleeding in these specific clinical circumstances, it is another marker of fibrinolysis, similar to D-dimer. Because it uses whole blood, it is sensitive to changes in platelet count and plasmin fibrinogen(49). Consequently, it is less sensitive to changes in fibrinolytic potential or plasmin activation and more sensitive to changes in coagulation factors, especially in coagulopathic conditions(52). Furthermore, because TEG is a functional assay, it can only detect plasmin activity and clot lysis during the short period in which plasmin is activated (or there is a high concentration of plasminogen activator in the blood). In trauma patients, this is often only several hours, so retrospective detection of plasmin activation using TEG is not possible.

In platelet poor plasma (PPP), clotting potential, thrombin generation, and plasmin generation can be measured later following blood collection, but these tests are not routinely used clinically. These assays consist of adding a clotting initiator, such as lipidated tissue factor, and a plasmin activator (tPA) to plasma. Clot formation and lysis is measured by turbidity on a standard plate reader. To measure thrombin and plasmin generation specifically, thrombin- and plasmin-specific fluorogenic substrates are added to the wells, and fluorescent signal over time is measured by the plate reader. Stable forms of plasmin and thrombin are used to generate a standard curve to calculate the amount of plasmin and thrombin generated respectively(83,84). These assays are

useful to determine how different factors in the plasma affect the generation of these active proteases but then also the functional ability of those proteases to generate and subsequently degrade the fibrin clot, but like TEG, they cannot determine if significant plasmin or thrombin has been activated in the patients.

Reporter assays:

Reporter assays are those which described downstream markers of a process, such as secondary markers of plasmin activity or activation. Plasmin activity is often measured by secondary reporters of plasmin activity, such as fibrinolytic measures. Clinically, plasmin activity is often measured by quantified D-dimer, a crosslinked-fibrin degradation product described above, by ELISA. This marker is also used to identify venous thromboses as plasmin is activated at the site of thromboses to degrade them. While D-dimer is an accurate, reliable marker of fibrinolysis, it is dependent on fibrin formation and subsequent plasmin activation on the surface of fibrin. D-dimer has a plasma half-life of approximately 8 hours, making it a good retrospective marker of plasmin activity(85). In some cases, significant plasmin activation can occur without fibrin, resulting in a lower D-dimer(86).

Finally, another way to measure plasmin activation that is not commonly used clinically but is fibrin-independent is to quantify PAP complexes by ELISA. Once plasmin is activated, if it is unbound to a receptor or fibrin, it's rapidly bound and inhibited by α2AP to form a plasmin-antiplasmin (PAP) complex(24). PAP complexes circulate in the blood for roughly 3-4 hours following activation, so they may be used retrospectively to detect if plasmin was activated, but they are not useful far beyond an injury(87). This method may be used to detect plasmin activation

in cancers or diseases in which high levels of plasminogen activator are released, activating plasmin without fibrin.

Plasminogen activators tPA and uPA can be quantified by activity assay or ELISA, but the release of these into the blood is transient (minutes) and does not predict how much plasmin will be activated. Also, tPA and uPA are rapidly inhibited by their endogenous inhibitors, PAI-1 and PAI-2, making them difficult to detect by antibody-based methods(79). Recent studies have implicated an endothelial receptor-associated protein, S100A10 (p11) in catalyzing plasmin activation(88), but it is unclear if increases in plasma levels of p11 are causative or associated with plasmin activation.

Article 1: Breakthrough Plasmin Activity Correlates with Blood-loss, Transfusion, and Inflammation in Posterior Spinal Fusion Surgery Treated with Antifibrinolytics

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INTRODUCTION

The use of antifibrinolytics during invasive high blood-loss spine surgery, such as posterior spinal fusion (PSF), has significantly improved clinical outcomes by reducing blood-loss and transfusion (89,90). Clinical trials have investigated optimal dosing of antifibrinolytics based on blood-loss and transfusion rates(89,91–93). Despite the impact of these findings, recent studies have suggested that measures of breakthrough plasmin activity, the main protease of the fibrinolytic system, may better reflect antifibrinolytic efficacy(92,94). Furthermore, studies have indicated that effective antifibrinolytic dosing reduces surgery-induced coagulopathy and systemic inflammatory response syndrome (SIRS), which may not be reflected by outcome measurements of blood-loss and transfusion rates (58,91,95). Therefore, to optimize antifibrinolytic dosing, a comprehensive analysis of plasmin activation and the biological processes affected by its activation (Figure 2.3) is required. The goal of this study was to determine the most sensitive, clinically relevant, diagnostic of breakthrough plasmin activity during PSF treated with an antifibrinolytic, such as tranexamic acid (TXA). This information is essential for future studies designed to determine the optimal dosing of antifibrinolytics during spine surgery.

A significant barrier to determining antifibrinolytic efficacy by measuring plasmin activity is the uncertain clinical significance of the various methodologies used to measure plasmin activity. These diagnostics fall broadly into three categories: plasmin activity assays, plasmin reporter assays, and factor level measurements. ***Plasmin activity assays***, including thromboelastography (TEG), measure the potential to activate plasmin *ex-vivo* and vary according to how plasmin activity is measured. ***Plasmin reporter assays*** are antibody-based assays that measure either plasmin-inhibitor complexes (e.g., plasmin-antiplasmin (PAP)) or markers of

fibrinolysis, such as d-dimer. ***Factor level measurements*** are designed to determine the concentration of plasminogen system factors in plasma. Although these assays are available for clinical use, their clinical significance is not well understood.

In this study, we hypothesized that plasmin reporter assays are the most sensitive diagnostic of clinically relevant *in-vivo* plasmin activity in the presence of an antifibrinolytic, correlating better with adverse outcomes of PSF: blood-loss, transfusion, and measures of SIRS. To test this hypothesis, we conducted a prospective study in patients undergoing PSF surgeries treated with a high dose of TXA and examined breakthrough plasmin activity measured by all three methods relative to both downstream pathways activated by plasmin, including coagulation, fibrinolysis, inflammation, and to clinical outcomes, including blood-loss and transfusion.

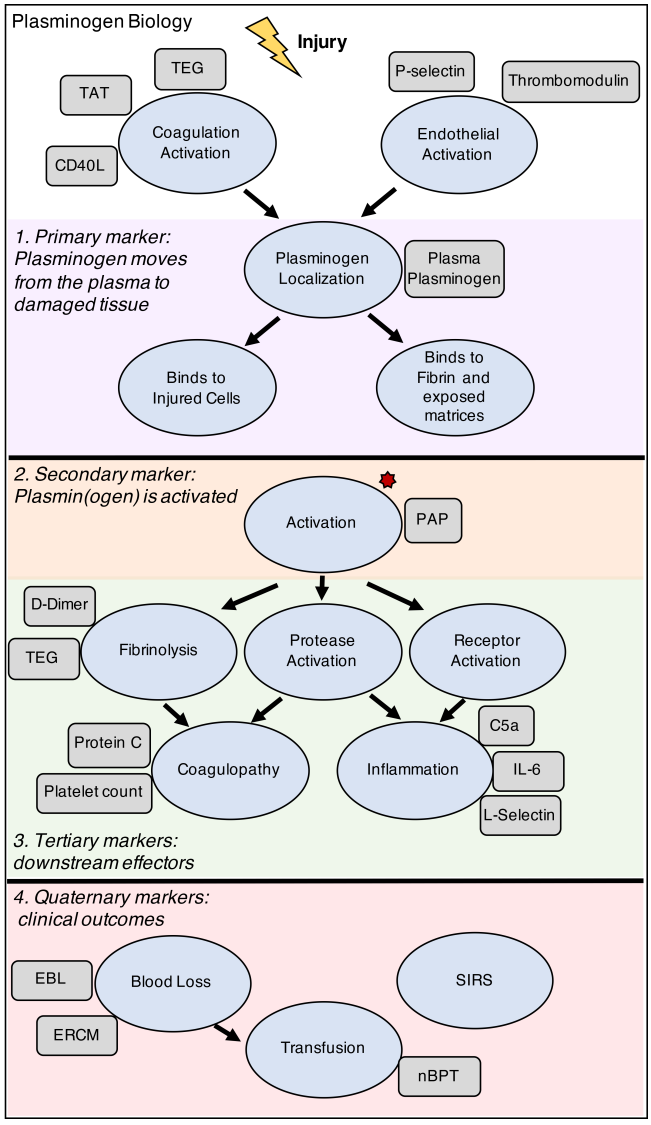

Figure 2.3: Plasminogen Biology, Markers of Plasmin Activation, and Outcomes. Following injury, coagulation is activated to contain the damage, and plasminogen is localized to deposited fibrin and injured cells. Once plasmin is activated from plasminogen, it cleaves its downstream targets, including fibrin, complement 5, and extracellular receptors involved in inflammatory and coagulation signaling. The result of excess plasmin during surgical procedures is fibrinolysis-related blood-loss, possibly requiring transfusions. TAT= thrombin-antithrombin, TEG= thrombelastography, PAP= plasmin-antiplasmin, ERCM= estimated red cell mass deficit, EBL = estimated blood-loss, nBPT= normalized blood product transfused.

METHODS

Study Design

Following approval from the Institutional Review Board (#181982, NCT03741023), patients aged 7-18 years old with either neuromuscular or idiopathic scoliosis undergoing elective PSF were recruited to quantify changes in intra-operative plasmin activation in a heterogeneous population. All procedures occurred between December 2019 and March 2021 at a single pediatric medical center. Blood was collected during hospitalization as outlined in Figure 2.4. Collected blood was centrifuged at 1500x*g* and 13,000x*g* for 15 minutes each to isolate platelet-poor plasma (PPP) for further analysis. Patient demographic information, including age, sex, weight, height, ethnicity, and comorbidities were collected from the electronic health record (EHR). Additionally, procedural information, including the number of vertebrae fused, number of osteotomies, operative time, and routine lab blood and coagulation measurements associated with the procedure were collected from the EHR.

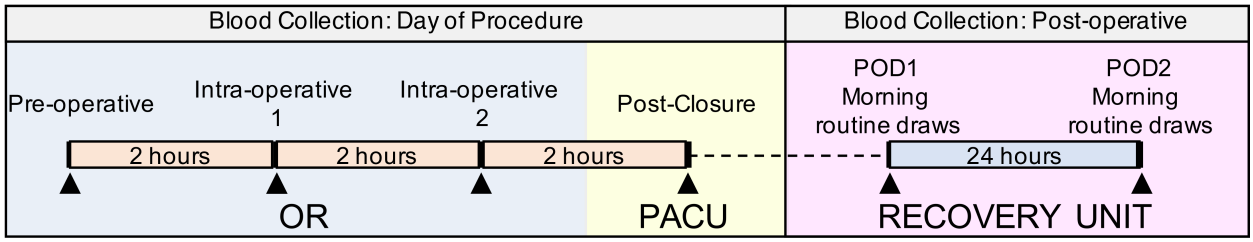

Figure 2.4: Blood collection procedures for the PSF study

Patient Care and Routine TXA Administration

PSF surgeries were performed by 2 orthopaedic surgeons and 1 anesthesiologist. Prior to incision, patients received an intravenous (IV) bolus dose of 100mg/kg TXA for patients <20kg and 2000mg TXA for patients ≥20kg and a maintenance TXA IV infusion of 10mg/kg/hr for

patients <50kg or 500mg/hr for patients ≥50kg. Red blood cell salvage was routinely given back when possible. Idiopathic scoliosis patients who underwent PSF followed a standardized anesthesia protocol consisting of lidocaine, propofol, and sufentanil infusions to allow for neuro-monitoring. Anesthesia protocols for neuromuscular scoliosis patients were adjusted based on specific surgical needs. If neuro-monitoring was not required in neuromuscular scoliosis patients, general anesthetic consisted of inhaled anesthetic agents and muscle relaxant rocuronium.

Plasmin Activity Assays:

Streptokinase Plasmin Activation

To measure SK-mediated plasmin activity patient plasma samples and control pooled plasma (George King Bio-Medical Inc., Overland Park, KS) were diluted 1:5, 1:10, and 1:20 in HEPES buffer. Increasing concentrations of TXA (Pfizer, New York, NY) were added to control plasma or purified plasminogen (Haemtech, Essex Junction, VT). Diluted plasma (or purified plasminogen) and fluorogenic substrate (H-D-Val-Leu-Lys-AFC, Anaspec, Fremont, CA) were added in triplicate to a 96-well plate. Plasmin activation was initiated by the addition of 0.5U/μL bacterial streptokinase (SK) (MilliporeSigma, Burlington, MA) and fluorescence was measured every 30 seconds for 1 hour using a Synergy 2 plate reader (Biotek, Winooski, VT). Rates of plasmin generation were calculated based on change in fluorescence over time and were reported as a percent of control pooled plasma(96).

Thrombelastography (TEG)

For a subset of patients enrolled in this study (N=8), TEG (TEG 5000, Haemonetics, Boston, MA) was immediately performed in whole blood in duplicate at each time point as previously described(97).

Plasmin Reporter/Antigen, Coagulation Antigen, Endothelial Activation, and Inflammasome Assays

ELISA and Multiplex Analysis

PPP samples diluted 1:2 were analyzed by Luminex-based custom multiplex to detect D-dimer, P-selectin, CD40L, IL-6, IL-10, IL-1β, thrombomodulin, L-selectin, uPAR, uPA (R&D, Minneapolis, MN). Plasmin-antiplasmin (PAP) complexes were measured by ELISA at a 1:10 dilution (Technozym Diapharma, West Chester, OH). Plasminogen antigen levels were measured by ELISA (Molecular Innovations, Novi, MI) at a 1:10,000 dilution.

Blood-loss Calculations

Blood-loss was calculated using both a hematocrit-based estimated red cell mass (ERCM) deficit normalized as a percent of total blood volume (% TBV) and normalized blood product transfused (nBPT)(98,99). nBPT was calculated as the volume of intraoperative RBC product, both autologous (cell salvage) and allogeneic (packed RBCs), normalized to patient weight (mL/kg). The anesthesia post-operative record of estimated blood-loss (EBL) was also noted from the patient charts.

Statistical Analysis

Paired, two-tailed Mann-Whitney U tests were used to evaluate differences between sample timepoints from the pre-operative values with Dunnett's post-hoc correction for multiple

comparisons. Pearson correlations were used to evaluate associations between patient variables. All statistical calculations and figures were generated with GraphPad Prism version 8.0.0 (www.graphpad.com).

RESULTS

Cohort Description

Across the 17 patients enrolled in this study undergoing PSF, 9 (53%) patients were diagnosed with neuromuscular scoliosis, while the remaining 8 (47%) patients were diagnosed with idiopathic scoliosis. This cohort included 13 females (76%) and 4 males (26%) ranging from 10-18 years of age with a median BMI of 19.8 (range:16.5-36.2). The average length of the procedure for these patients was 4.9 hours (range:3.2-7.6), and the median number of levels fused was 13 (range:8-16), with a median of 4 (range:0-14) osteotomies performed per patient. Cohort demographics are described in Table 2.1.

Table 2.1: Posterior Spinal Fusion Cohort Demographics

	Total (N=17)	Idiopathic (N=8)	Neuromuscular (N=9)
Age	13(10-18)	15(11-18)	12(10-17)
Sex (male/female)	4/13	1/7	3/6
Body Mass Index (kg/m^2)	19.8(16.5-36.2)	22.2(16.7-32.9)	19.8(16.5-36.2)
Length of procedure (min)	292 ± 71.3†	262 ± 66.4†	319 ± 67.6†
Levels fused	13(8-16)	11.5(8-16)	16(9-16)
Osteotomies	4(0-14)	4(0-8)	3(0-14)
Length of Stay (days)	3.8 ± 1.8†	2.8 ± 0.7†	4.7 ± 1.9†

Median (range), unless otherwise indicated, †Mean ± SD

Blood-loss Measurements and Transfusions

The mean ERCM deficit was 17.9% TBV (range:7.7-51.8) and the mean nBPT was 6.0 mL/kg (range:0-17.9). On average, patients with idiopathic scoliosis lost less blood than those with neuromuscular scoliosis (17.0% vs 19.7% TBV, respectively), consistent with previous studies(100). Across all 17 patients, 16/17 (94.1%) received autologous red blood cell salvage during the procedure, and 3/17 (17.6%) received allogeneic packed red blood cell (pRBC) transfusion. Pre-surgical lab values, blood-loss measurements, and transfusions are highlighted in Table 2.2.

Table 2.2: Posterior Spinal Fusion Lab Values and Blood Loss

	Total (N=17)	Idiopathic (N=8)	Neuromuscular (N=9)
Pre-surgical Lab Values:			
PT (s)	13.7 ± 0.6	13.7 ± 0.7	13.6 ± 0.5
aPTT (s)	30.6 ± 2.9	30.8 ± 3.1	30.3 ± 2.7
Fibrinogen (mg/dL)	311 ± 82.6	323 ± 98.2	298 ± 68.0
Platelet Count(L^{-1})	273 ± 72.5	284 ± 79.3	262 ± 69.0
EBL (mL)	583.5 ± 274.4	519.6 ± 186.5	633.2 ± 329.7
EBL (% TBV)	20.5 ± 10.8	12.2 ± 4.1	23.6 ± 5.1
ERCM Deficit (% TBV)	17.9 ± 10.0	17.0 ± 14.3	19.7 ± 4.4
Received Cell Salvage N(%)	16(94)	8(100)	8(89)
Cell Salvage (mL)	166 ± 101	175 ± 117	157 ± 88.3
Received pRBC N(%)	3(18)	1(13)	2(22)
nBPT (mL/kg)	6.0 ± 8.0	5.7 ± 10.2	6.2 ± 6.1

Mean ± SD unless otherwise indicated. ERCM = estimated red cell mass, EBL = estimated blood loss, cell salvage = autologous red cells, pRBC = allogeneic packed red blood cells, nBPT=normalized blood product transfused, TBV= total blood volume.

Intraoperative and Post-operative Coagulation

All patients exhibited significant coagulation activation indicated by intra-operative increases in thrombin-antithrombin (TAT) complexes and decreases in platelet count following the surgery (Figure 2.5A-B). TEG analysis of whole blood in a subset of patients (N=8) indicated a mild decrease in coagulation potential parameters within normal range throughout the procedures (Figure 2.6A-D). Significant blood-loss occurred in both idiopathic and neuromuscular scoliosis patients with an average blood-loss of >17% TBV (range:8-52%) (Figure 2.5C). Markers of platelet and endothelial activation (P-selectin, CD40L, thrombomodulin) and levels of endogenous anticoagulant protein C did not change significantly throughout hospitalization (Figure 2.7).

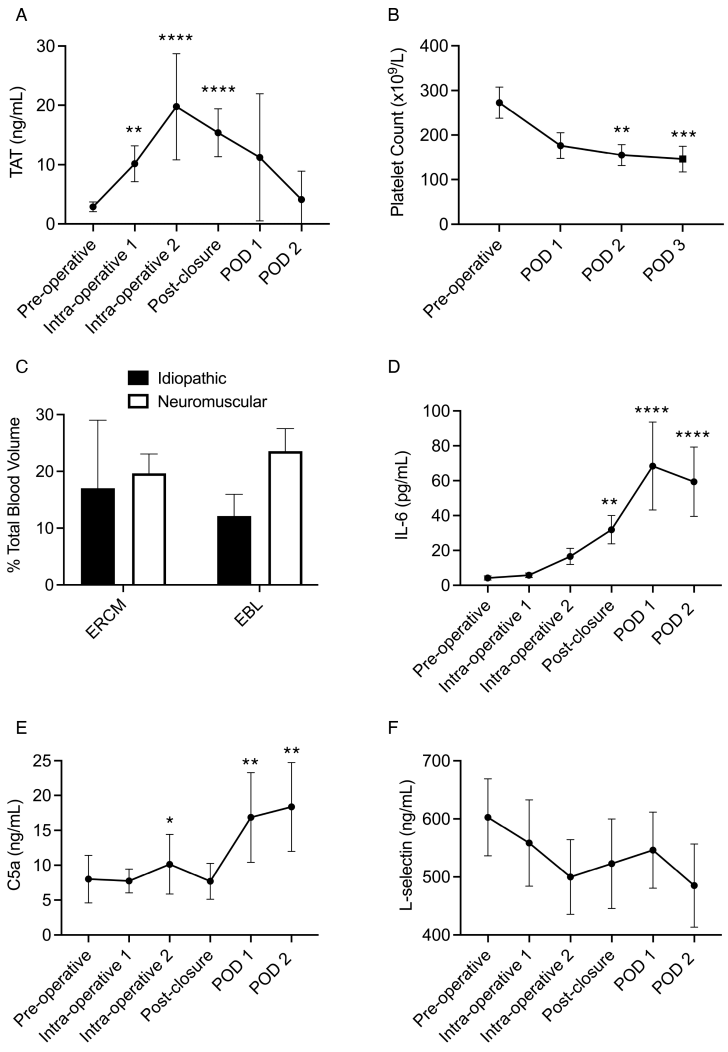

Figure 2.5: Coagulation, inflammation, and blood loss following PSF surgery. Posterior spinal fusion surgery significantly activates coagulation, causing a detectable increase in A) TAT throughout the case, B) a post-operative drop in platelet count. C) Both idiopathic and neuromuscular patients exhibited significant blood-loss throughout the procedures. D) Pro-inflammatory cytokine IL-6 peaked by POD1 while E) Complement activation peaked intra-operatively with reactivation occurring on POD1 and POD2. F) No significant changes in leukocyte activation marker, L-selectin, occurred during hospitalization. N= 17, *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001 compared with pre-operative values. Points represent the mean with 95% confidence intervals.

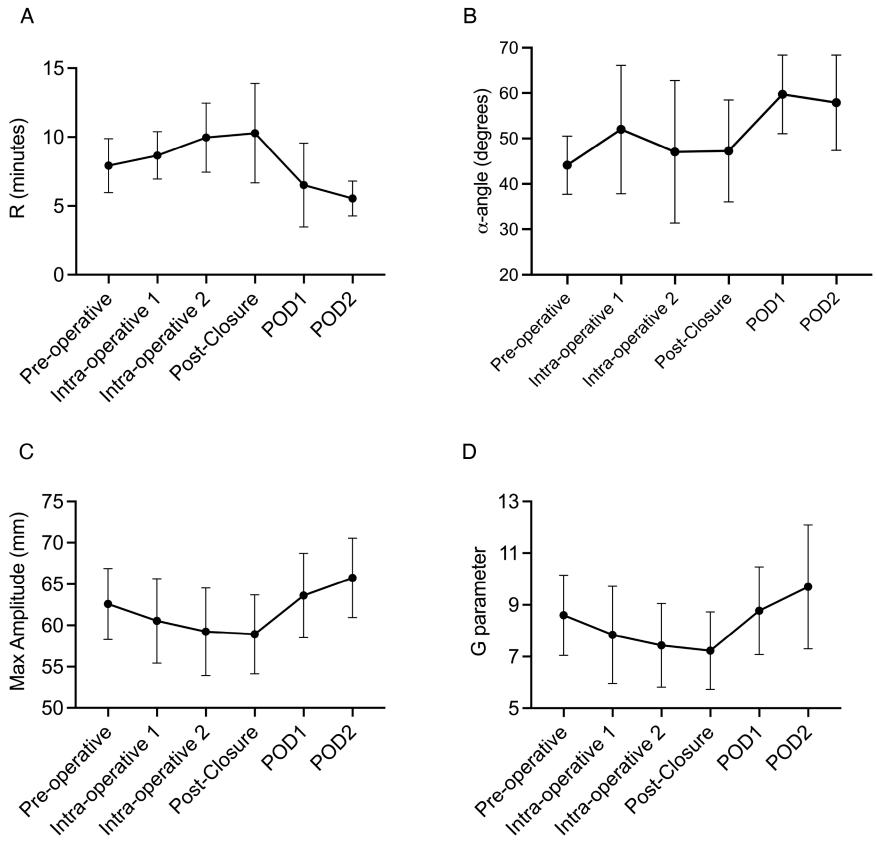

Figure 2.6: TEG Measurements following posterior spinal fusion. TEG coagulation values demonstrated a mild, nonsignificant decrease in clotting potential intra-operatively by A-B) increased reaction (R) and clotting time (K) and C-D) decreased max amplitude and overall clot strength (G parameter). N=8, points represent the mean with 95% confidence intervals.

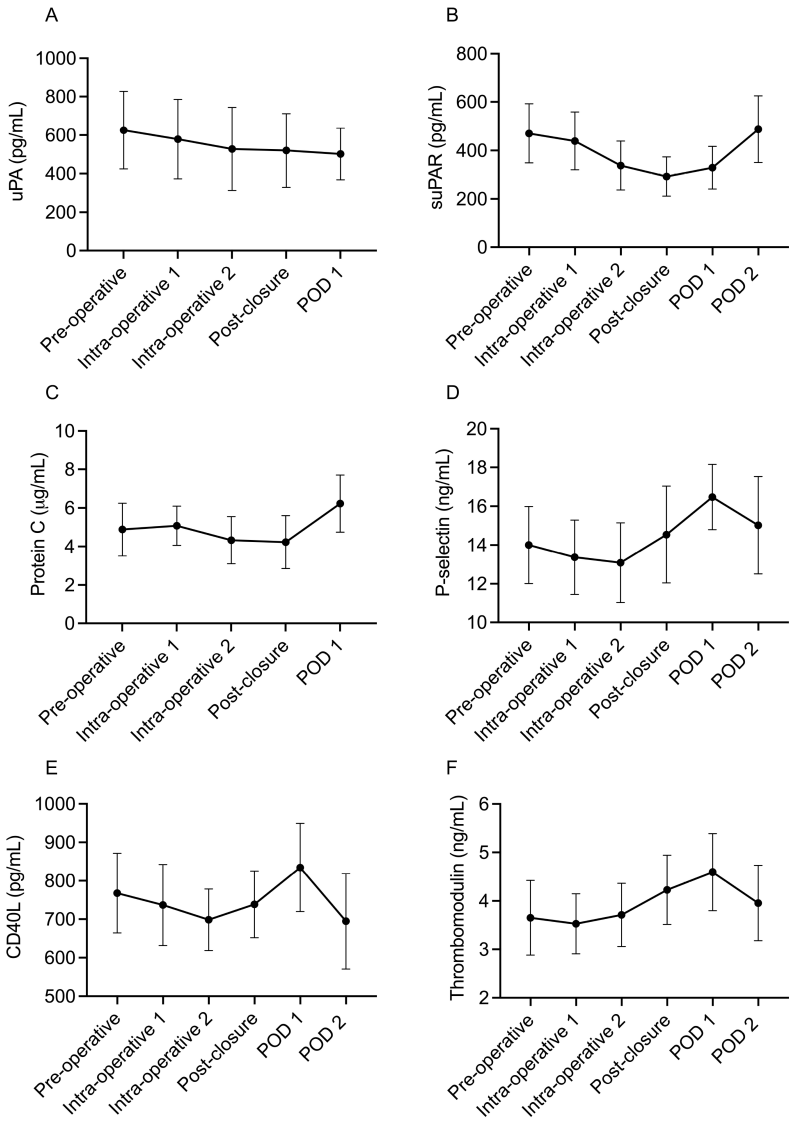

Figure 2.7: Measures of coagulopathy and plasmin activators following posterior spinal fusion. A) uPA levels did not change significantly throughout surgery, and B), uPA receptor (uPAR), decreased slightly intra-operatively and returned to baseline in the post-operative monitoring period. Markers of coagulopathy, C) protein C antigen and platelet activation, D) P-selectin, and E) CD40L and endothelial activation F) thrombomodulin, do not change significantly during or after PSF. N=17, points represent the mean with 95% confidence intervals.

Inflammation

In this cohort, inflammatory cytokine IL-6 peaked at POD1, before beginning to return to baseline by POD2 (Figure 2.5D), consistent with previous studies(91). PSF patients exhibited a transient peak of complement activation intraoperatively and prolonged elevation of C5a at POD1 and POD2 (Figure 2.5E). Soluble L-selectin, a common marker of leukocyte activation, did not change significantly throughout hospitalization (Figure 2.5F).

Intra-operative changes in plasmin(ogen) and fibrinolysis

Throughout the PSF procedure and TXA administration, plasmin activity assays and plasmin reporter assays demonstrated conflicting results. Clot lysis on TEG (LY30) decreased intra-operatively and returned above baseline by POD1 (Figure 2.8A). Furthermore, plasmin activity measured by SK-based assay decreased intra-operatively and did not completely return to baseline by POD2 (Figure 2.8B). In contrast, significant *in-vivo* plasmin activation, measured by PAP, was detected as early as 2 hours post-incision and reached a maximum at approximately 6 hours post-incision (Figure 2.8C). Significant fibrinolysis also occurred, indicated by a 3-6 fold intra-operative increase in D-dimer that remained elevated throughout hospitalization (Figure 2.8D). Despite consistent TXA dosing across all patients, variable breakthrough plasmin activation occurred intra-operatively. Trauma studies have suggested that TXA loses efficacy within hours of the injury due to an increase in circulating urokinase plasminogen activator (uPA). However, in this cohort, we did not see significant differences in free uPA or its receptor (suPAR) (Figure 2.7A-B).

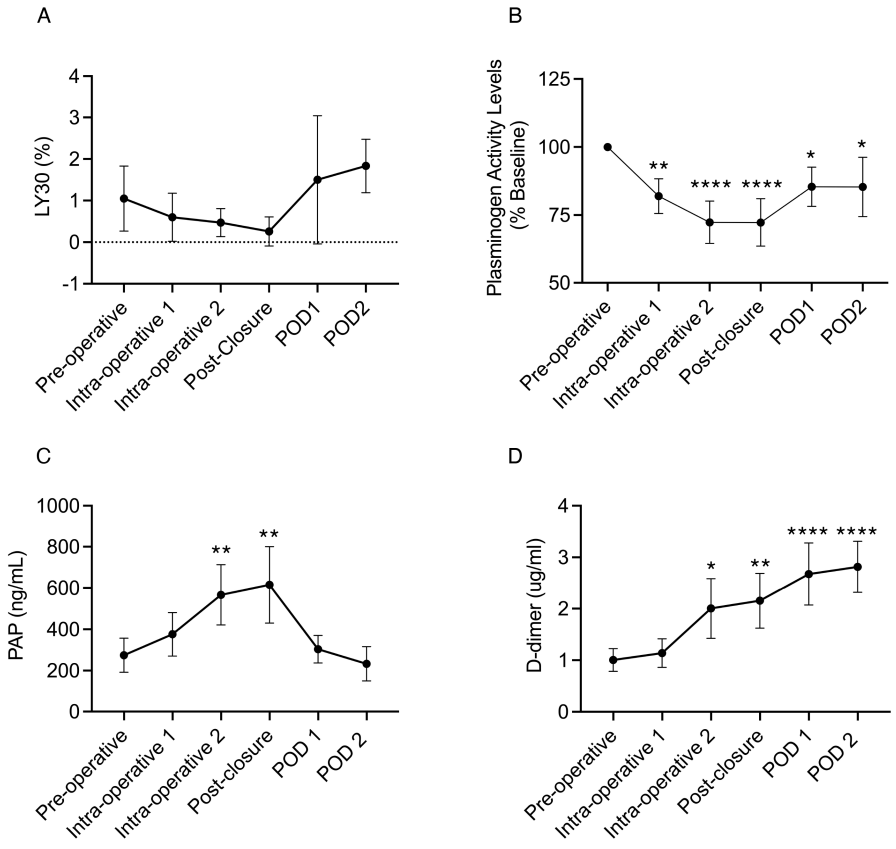

Figure 2.8: Measures of plasmin activation and fibrinolysis following posterior spinal fusion surgery. A) Fibrinolysis measured by TEG clot lysis (LY30, N=8) and B) SK-based plasmin activity decreased in the intra-operative period during TXA administration. C) Plasmin activation (PAP) and D) fibrinolysis (D-dimer) increased significantly during the procedure. N=17, *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001 compared with pre-operative values. Points represent the mean with 95% confidence intervals.

TXA and plasminogen concentration alter plasmin activity assays

TXA has been shown to inhibit clot lysis on TEG measurements(92,101). TXA has a dose-dependent inhibitory effect on SK-based plasmin activity assays, however, plasma concentrations of TXA above 200µg/mL begin to lose this effect (Figure 2.9A). This was not observed in purified

plasminogen (Figure 2.9B), suggesting that a plasma component may alter TXA's inhibitory effect on plasmin activation at supra-therapeutic concentrations of TXA. However, plasminogen antigen levels, which are unaffected by therapeutic TXA (not shown), decreased intra-operatively and were correlated with SK-based plasmin activity measurements (Figure 2.9 C-D). The intra-operative increase in PAP did not correlate with the drop in plasminogen observed (R^2=0.180), indicating that the loss of plasminogen may not be due to activation-associated consumption alone but is likely due to a combination of factors including consumption, redistribution, and protease-mediated degradation(61,102).

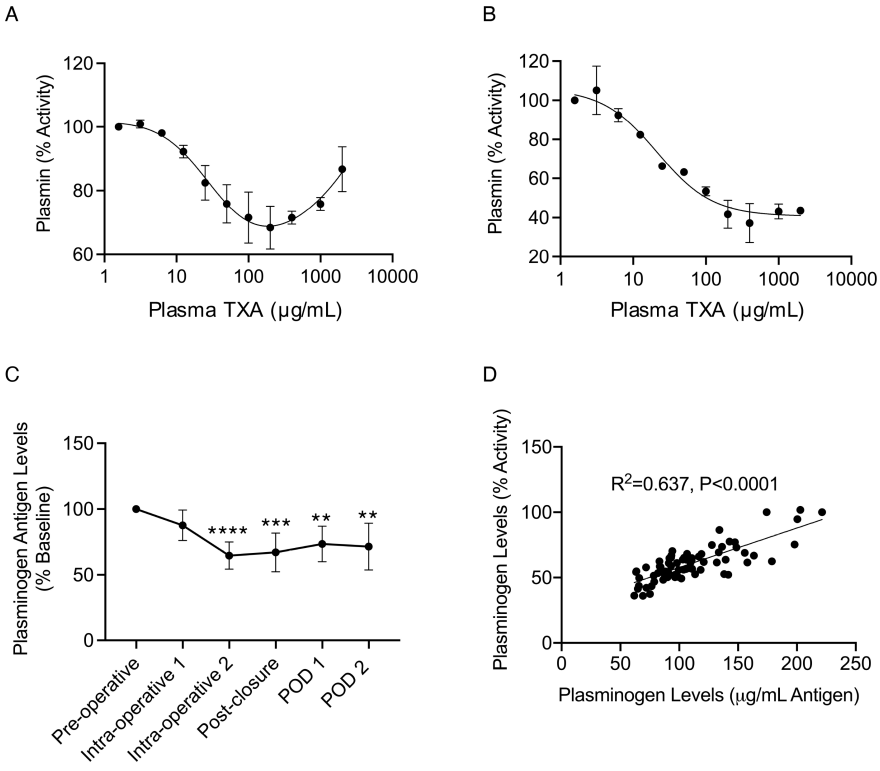

Figure 2.9: Plasminogen levels correlate with plasmin activity. A) In plasma, TXA has a dose-dependent inhibitory effect on the SK-based plasmin activity assay that is lost at high concentrations, but B) in purified plasminogen, TXA has an inhibitory effect on the assay at any concentration above 6.3µg/mL. (N=3 per point, points represent mean with SD). C) Plasminogen antigen levels decrease intraoperatively and remain decreased through POD2 N=17, *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001 compared with pre-operative values (points represent the mean with 95% confidence intervals), and D) plasminogen antigen levels strongly correlated with SK-based plasmin activity quantification (R^2=0.637, P<0.0001).

Intra-operative plasmin activity and outcomes

The magnitude of the intra-operative increase in plasmin activation (PAP) correlated with nBPT (R^2=0.388, P=0.007) and ERCM deficit (R^2=0.400, P=0.006). Furthermore, the intra-operative increase in D-dimer weakly correlated with ERCM deficit (R^2=0.264, P=0.035) (Table

2.3), and D-dimer levels significantly correlated with activation of the complement pathway (C5a) throughout hospitalization (R^2=0.364, P=0.0001).

Table 2.3: Plasmin Activity Measures, Blood Loss, and Inflammation in Posterior Spinal Fusion

	nBPT	ERCM	C5a
PAP	R^2=0.388 **P = 0.008**	R^2=0.400 **P=0.007**	R^2=0.021 P=0.402
D-dimer	R^2=0.146 P=0.131	R^2=0.264 **P=0.035**	R^2=0.346 **P=0.0001**
Plasmin Activity	R^2=0.114 P=0.186	R^2=0.129 P=0.157	R^2=0.022 P=0.781

*P value indicates slope of the correlation is significantly different than zero for each variable. nBPT = normalized blood product transfused, ERCM= estimated red cell mass deficit, C5a= complement 5a (active), PAP= plasmin-antiplasmin.

DISCUSSION

Controlled musculoskeletal injury in PSF leads to significant activation of coagulation and inflammation, resulting in adverse outcomes such as excessive bleeding and SIRS. A key pathophysiologic event that instigates this process is the intra-operative activation of the fibrinolytic protease plasmin. Following injury, plasminogen binds to injured tissue(1,16,103) where it is later activated to plasmin to support repair of musculoskeletal tissue(1,33,34,36). However, at a specific threshold of injury severity, early, excessive plasmin activation occurs contributing to activation of coagulation, inflammation and their associated adverse outcomes (43,104–107). Consequently, the use of antifibrinolytics has revolutionized elective surgical

procedures, reducing the effects of pathologic plasmin activity on surgical outcomes. However, the diagnostics required to optimize antifibrinolytic therapy have remained elusive. Specifically, it is unknown how to determine if the antifibrinolytic administered was efficacious at inhibiting its target, plasmin. In this detailed analysis, we determined that the plasmin reporter assays (PAP and D-dimer) are the most sensitive, and clinically correlative, at measuring plasmin activity during PSF with an antifibrinolytic.

Many different tests are used in clinical and research settings to assess the activity of the fibrinolytic system to predict blood-loss and antifibrinolytic efficacy. Plasmin activity assays, such as TEG, are useful to detect dramatic changes in coagulation and fibrinolysis in certain patient groups(46,97,108). In this study, measurement of clot lysis on TEG (LY30) demonstrated a decrease in *ex-vivo* fibrinolytic potential intra-operatively during TXA administration while direct measures of plasmin activation and fibrinolysis (PAP and D-dimer) increased (Figure 2.10). These results are contradictory, and while some have interpreted reduction in LY30 to reflect the efficacy of antifibrinolytics(92,109), the findings within demonstrate that plasmin activity assays are subject to a multitude of variables that may cloud the clinical interpretation of these results. This is not the first time this has been reported; different plasmin assays are often contrary to one another regarding their interpretation as to whether there is excess or diminished plasmin activity *in-vivo*(52). In a recent study of patients undergoing PSF with intra-operative TXA administration, measurement of LY30 demonstrated decreased *ex-vivo* fibrinolytic potential intra-operatively from baseline, suggesting sufficient dosing of TXA, while plasmin activity reported by D-dimer suggested augmented intraoperative plasmin activity(92). These contradictory findings indicated that despite minimal fibrinolytic potential in blood, there was still significant activation of plasmin

during surgery, creating a conundrum as to which measures are optimal for assessing antifibrinolytic efficacy in spine surgery.

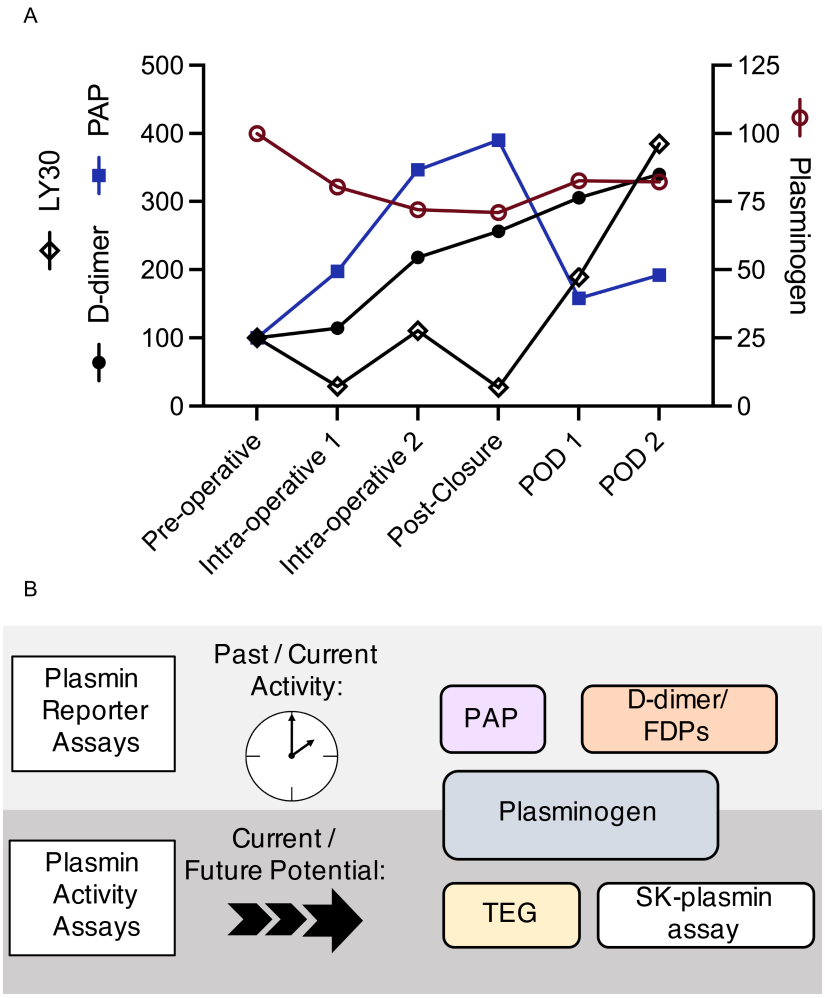

Figure 2.10: Plasmin Assay Differences A) While LY30 and plasminogen decrease or remain level during PSF procedure, PAP and D-dimer increase significantly. PAP and D-dimer also follow different time courses during and after surgery. PAP increases prior to fibrinolysis (D-dimer) but is undetectable in the post-operative period until POD2, while D-dimer increases intra-operatively and remains elevated in the post-operative period. (values expressed as a percent of baseline values for each patient.) B) Different assays used to measure plasmin activity and fibrinolysis depict different aspects of plasmin biology.

To clarify this finding, we used an SK-based plasmin activity assay with fewer confounding variables than TEG. Similar to TEG, we observed a drop in plasmin throughout the surgery, but this phenomenon was shown to be due to a loss of plasma plasminogen. This is the first study to demonstrate that plasma plasminogen levels may significantly redistribute from the blood during and after surgery to the extent that they become an independent factor in *ex-vivo* plasmin activation assays. We use the term 'redistribute' to indicate that circulating plasminogen is diminished in the plasma, most likely due to both plasmin activation and plasminogen localization to damaged tissues (Figure 2.10). It is clear from these studies that the amount of tissue damage incurred during PSF surgery is sufficient to lead to a significant reduction of circulating plasminogen. This finding has an impact on the use of plasmin activity assays to make clinical decisions in orthopaedics as the amount of plasminogen redistribution is proportional to the amount of injured tissue(102) – therefore, in the setting of a large surgery, such as PSF, the apparent drop in plasmin activity may indicate altered fibrinolytic potential in the blood that does not reflect the local environment at the site of tissue damage. Interestingly, plasminogen redistribution may prove useful as a measure to quantify surgical tissue damage in the future. From these findings, we strongly recommend that plasmin activity assays, such as TEG, not be used as the sole determinant of the efficacy of antifibrinolytics as they are subject to confounding variables such as the antifibrinolytic itself, as well as plasminogen redistribution.

We observed consistent, progressive elevation of PAP and D-dimer during PSF, indicating breakthrough plasmin activation with the TXA dosing regimen that was not detected by TEG. However, an intra-operative increase in PAP or D-dimer in isolation does not predict adverse outcomes, and thus, we set out to determine if this change in plasmin activity was clinically meaningful. We determined that an intra-operative increase in plasmin activation, measured by

PAP and D-dimer, was associated with measures of blood-loss in this cohort. Additionally, we found that increases in D-dimer were associated with complement activation, which is consistent with what is known about plasmin roles in inflammation(58,95,106). Together, these associations suggest that breakthrough plasmin activation may contribute to blood-loss and inflammation.

A limitation to this study is that only a single antifibrinolytic dosing schematic was used in this cohort, but despite this, we observed significant differences in breakthrough plasmin activation between patients, which correlated with clinical outcomes. Had outcomes in this study been correlated to the dose of antifibrinolytics given, the results would remain inconclusive. This study included AIS and neuromuscular-scoliosis patients. This selection was intentional as these patients are known to have differing clinical outcomes, providing a heterogeneous population to determine the clinical significance of breakthrough plasmin activity(100). Additionally, this study was limited to a small cohort; however, the purpose of this study was to identify clinically significant diagnostic markers of breakthrough plasmin activity in order to inform the design of future, large-cohort studies to effectively optimize antifibrinolytics to limit intra-operative plasmin activation.

CONCLUSION

The intra-operative change in plasmin reporter assays (e.g., quantitative D-dimer or PAP) provides more sensitive detection of plasmin activity compared with plasmin activity assays, such as TEG, to better determine the efficacy of antifibrinolytic dosing. Significant changes in PAP or D-dimer may indicate breakthrough plasmin activation, however, these findings should be measured in relation to adverse outcomes (e.g., blood-loss, SIRS) to determine if detected plasmin activation is clinically meaningful before altering the dosing regimen. This prospective study provides rationale for the use of specific diagnostics to detect breakthrough plasmin activity in

future clinical trials examining antifibrinolytic efficacy in high blood-loss spine surgery to determine its clinical efficacy in attenuating coagulopathy, SIRS, blood-loss and transfusion.

The role of plasmin in burn-induced coagulopathy

Plasmin and coagulation positively feedback upon one another, allowing regulation of both "opposing" systems by one another. However, in a state in which both pathways are activated, such as a severe injury or disseminated infection, they have the potential to drive a coagulopathic state. Studies have implicated plasmin in coagulopathy, and the most well-understood role for plasmin is in hyperfibrinolysis. As discussed in Chapter 2, excess activation of plasmin following injury results in continual fibrin degradation, consuming clotting factors, platelets, and endogenous anticoagulants. This has been well described, but there are other mechanisms by which plasmin can mediate coagulopathy.

The contact activation pathway of coagulation, or the intrinsic pathway, both activates and is activated by plasmin. Studies have demonstrated that plasmin and clotting and inflammatory proteases FXII and kallikrein can activate one another as part of a positive feedback system, activating fibrinolysis in response to coagulation(110)(106). A downstream target of kallikrein is high molecular weight kininogen, which is cleaved to bradykinin. Bradykinin plays a role in the vascular response to hypoxia and has been implicated in pathologic conditions associated with infection and trauma. Bradykinin has been associated with angioedema, and through binding to the $G_{q/i}$-coupled B_2 bradykinin receptor on endothelial cells, it triggers the release of tPA, activating more plasmin(111). This pathologic cycle has been implicated in organ dysfunction and thrombosis most recently in patients with COVID19 from SARS-CoV-2 infection(111). Aberrant

function of the contact pathway has been implicated in thrombotic events, but the contact pathway is not essential for hemostasis, and therefore, it has been suggested as a preferrable target for thromboprophylaxis(112,113). In coagulopathic conditions, it's unclear if plasmin is a driving mechanism behind dysfunction of the contact pathway or if contact activation initiates pathologic roles for plasmin.

Plasmin has direct and indirect effects on platelet function. Because plasmin degrades fibrin, a key binding partner for both GpIIb/IIIa and GpVI on the platelet, plasmin can quickly render platelets less functional by removing their anchor. Furthermore, a study in trauma patients suggested that D-dimer generated by fibrinolysis can bind to GpIIb/IIIa and GpVI on free platelets in circulation(114). This occupation of GpIIb/IIIa and GpVI not only prevents platelets from binding to fibrin(ogen), but it can also activate inhibitory signaling pathways within the platelet(115). During a survival-APR, this plasmin-induced platelet dysfunction can perpetuate lethal bleeding, and as platelets serve critical roles in immunity through interactions with leukocytes, platelet dysfunction may potentially increase the risk of infection(10). Another way in which plasmin can alter platelet function is through protease-activated receptor (PAR) activation or desensitization on the platelet. Human platelets express multiple PARs, which are primarily targeted by thrombin to activate platelets, triggering platelet spreading and release of platelet granule contents(116). In high concentrations, plasmin has been shown to activate PAR4, a $G_{q/12}$-coupled receptor(117). Although this may prevent plasmin-associated bleeding, systemic activation of plasmin has the potential to trigger inappropriate activation of platelets outside of the zone of injury. Alternatively, plasmin has been shown to cleave and inactivate PAR1, reducing platelet response to thrombin(118). Therefore, plasmin can pathologically alter platelet function, rendering them non-functional or hyperactive during a survival-APR.

Burn-induced coagulopathy (BIC) is a serious condition marked by platelet dysfunction, thrombocytopenia, and endotheliopathy(119). We hypothesized the plasmin may play a role in burn-induced platelet dysfunction. To test this hypothesis, we investigated the effects of burn patient plasma on healthy platelet function.

Methods: In this prospective study, plasma samples were collected from 20 adult patients with burns >10% TBSA at a regional burn center. To assess the effects of burn plasma on platelet function, platelets isolated from healthy individuals were incubated with heparinized plasma from burn patients or control plasmas. Burn patient plasmas were analyzed by custom multiplex for markers of inflammation (IL-6, IL1β, TNFα), platelet activation (P-selectin, CD40L), and plasmin activation/fibrinolysis (PAP, D-dimer) (R&D). Upon activation, functional platelets express P-selectin, which binds P-selectin Glycoprotein Ligand -1 (PSGL-1) expressed on endothelium and leukocytes, and dimerized GpIIb/IIIa. These outputs were used to measure platelet activation following stimulation with platelet agonists. Platelet rich plasma (PRP) was isolated from healthy control subjects as previously described (REF). Citrated burn patient plasma containing 4U/mL heparin was recalcified to 17mM $CaCl_2$ and incubated with PRP at a ratio of 1:1 for 2 hours at room temperature. Following incubation with burn patient plasma, platelets were incubated with antibodies against GpIIb/IIIa (PAC-1, BD Biosciences) and P-selectin/CD62 (BD Biosciences) for 20 minutes. The platelets were then stimulated with 100 μM PAR1-activating peptide, 200μM PAR-4-activating peptide, or 3.16μM convulxin, which stimulates collagen receptor GPVI on the platelet, for 30 minutes. Finally, the platelets with fixed in 1% paraformaldehyde and analyzed by flow cytometry for P-selectin expression and GpIIb/IIIa activation (Figure 2.11A).

Results: Platelets incubated with burn plasmas exhibited a reduction in response to stimulation compared with those incubated with control plasma. No significant differences were seen in GPVI-induced signaling, but both PAR1 and PAR4-induced GPIIb/IIIa activation and P-selectin expression were significantly reduced in platelets incubated with burn patient plasma, suggesting a defect in platelet signaling (Figure 2.11B-C). This dysfunction did not strongly correlate with TBSA affected or modified Baux score for each burn patient but was significant when compared to healthy controls ($P< 0.05$ Figure 2.11D). However, measurement of markers of inflammation, coagulation, and fibrinolysis in burn plasmas revealed a significant correlation of both plasma D-dimer ($P< 0.02$) and soluble P-selectin ($P< 0.05$) with induced platelet dysfunction (Figure 2.11E-F). This suggests circulating factors indicative of coagulation, fibrinolysis, and endothelial dysfunction may play a role in platelet dysfunction observed in BIC.

Conclusions: These data suggest a role for circulating markers of coagulation and fibrinolysis (secondary to plasmin activity) in burn-induced platelet dysfunction. Burn resuscitation often involves fluid replacement and administration of fresh frozen plasma (FFP) to replace pathologic components within burn patient plasma. Another therapeutic approach being used in burn patients in plasmapheresis to remove toxic metabolites and proteins from the plasma(80,120). The results of this study suggest a possible benefit to these therapeutic approaches, and they also suggest a possible new role for plasmin-mediated fibrinolysis in BIC, though this data is only correlated and not causative. A previous study has shown that D-dimer has direct inhibitory effects on platelet function, which may be a protective mechanism to prevent inappropriate platelet activation. Further studies are required to examine specifically how plasmin and other markers of coagulation, such as P-selectin, may affect platelet signaling and function.

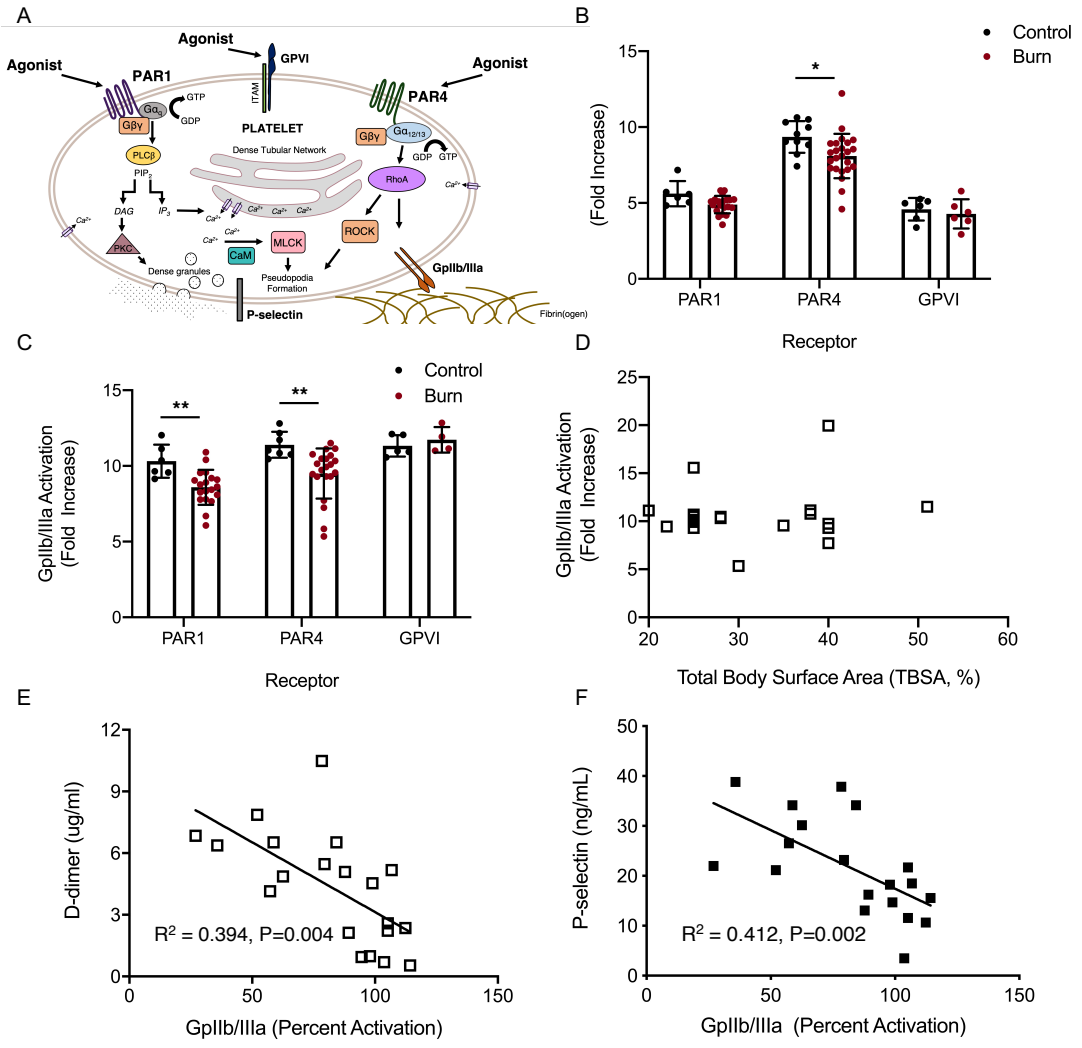

Figure 2.11: Burn plasma-induced platelet dysfunction. A) Human platelet function assay consisting of stimulating platelets with agonists for PAR1/4 and GpVI, measuring P-selectin surface expression and GpIIb/IIIa activation by flow cytometry. Both B) P-selectin expression was reduced following stimulation with PAR4-AP, and C) GpIIb/IIIa activation was significantly reduced following PAR1 and PAR4 stimulation in platelets incubated with burn patient plasma. D) GpIIb/IIIa expression in platelets following PAR4 stimulation was not correlated with the burn severity of the different patient plasmas, but E) D-dimer, and F) P-selectin levels in the plasma were positively associated with a loss of GpIIb/IIIa activation on the platelet.

Intra-operative plasmin activation during posterior spinal fusion surgery is associated with a loss of platelet function

We previously investigated how burn plasma altered platelet function in an *in vitro* assay and found that D-dimer levels in the plasma were associated with the magnitude of platelet dysfunction in platelets incubated with each plasma. Studies in trauma have suggested that D-dimer causes platelet dysfunction through binding to GpIIb/IIIa and GPVI, altering platelet signaling(114,115). In continuation of this, we hypothesized that in whole blood, the magnitude of plasmin activation in a significant injury is associated with the magnitude of platelet dysfunction. We investigated this hypothesis in a model of controlled traumatic injury: spinal fusion surgery, in which significant bleeding occurs.

Methods: In a prospective study of 11 patients undergoing posterior spinal fusion to correct scoliosis, we collected blood beginning pre-operatively for a baseline value, every 2 hours intra-operatively, immediately post-operatively, and at post-op days 1 and 2. Citrated whole blood was diluted 1:20 in heparinized Tyrode's buffer (as described above). Platelets were incubated with antibodies against GpIIb/IIIa (PAC-1, BD Biosciences) and P-selectin/CD62 (BD Biosciences) for 20 minutes. Platelets in the diluted whole blood were stimulated with 3.16μM convulxin, 100 μM ADP, or 10 μM thrombin for 30 minutes, fixed in 1% paraformaldehyde and measured by flow cytometry as described above (Figure 2.11A). PPP was isolated from the same blood sample, and blood markers of plasmin activation (PAP, D-dimer) were measured by ELISA (Diapharma Technozym, Asserachrom Diagnostica Stago, respectively) along with markers of inflammation and coagulation (IL-6, IL-1b, TNFα, P-selectin, CD40L).

Results: Patients exhibited a variable platelet response to the surgical procedure. On average, platelet GpIIb/IIIa activation in response to stimulation by thrombin, ADP, and convulxin was

decreased intraoperatively, and by post-operative day 1, platelet function had increased to above baseline levels (Figure 2.12A-C), although we did not reach significance due to variability. P-selectin expression was not significantly altered in any conditions (not shown). The intra-operative decrease in GpIIb/IIIa activation in response to thrombin stimulation strongly correlated with the intra-operative increase in fibrinolysis, measured by D-dimer (R^2=0.879, P<0.0001, Figure 2.12D) and weakly (not significantly) correlated with plasmin activation (PAP, R^2=0.431, P=0.054, not shown).

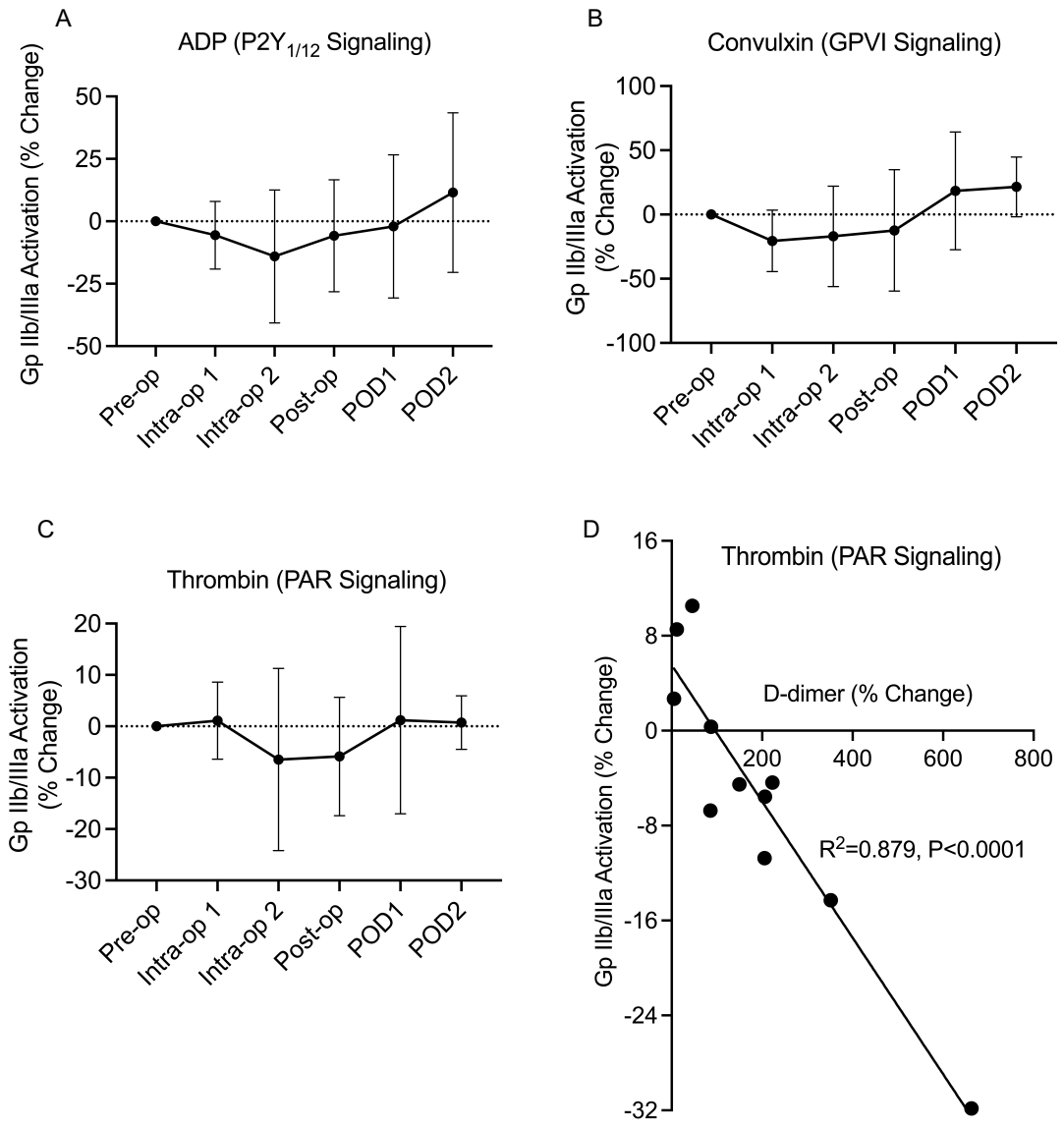

Figure 2.12: Human platelet dysfunction induced by posterior spinal fusion surgery. GpIIb/IIIa activation was reduced in platelets intra-operatively following stimulation with A) ADP, B) convulxin, and C) thrombin and returned to just above baseline at post-op day (POD) 1 and POD2. D) The percent decrease in thrombin-induced GpIIb/IIIA activation was associated with the magnitude of intraoperative fibrinolysis (D-dimer).

Conclusions: Intraoperative fibrinolysis is associated with platelet dysfunction in the GpIIb/IIIa pathway. Because D-dimer is capable of binding to GpIIb/IIIa, it's possible that D-dimer is

preventing detection of active GpIIb/IIIa by antibodies on flow cytometry, however, the different responses observed with different agonists suggest a possible alteration to the signaling pathway which may occur through D-dimer binding to GpIIb/IIIa, GPVI, or other glycoproteins. While it was not significant, PAP trended towards a correlation with platelet dysfunction, and it's possible that plasmin directly reduces platelet stimulation to thrombin as plasmin can cleave or inactivate PARs(117,118). Future studies are needed to examine the effects of plasmin and D-dimer separately on platelet dysfunction using this assay.

Developing a novel murine model of burn-induced platelet dysfunction

Platelet dysfunction has been associated with a wide range of clinical disorders, including poor hemostasis, increased susceptibility to infection, and poor tissue repair. Beyond hemostasis, platelets serve critical roles in immune function and tissue regeneration(121–123).

Platelets have been shown to express toll-like receptors that recognize damage- and pathogen-associated molecular patterns (DAMPs/PAMPs), including cell-free DNA (cfDNA), bacterial membrane proteins, and glycans(123). TLR signaling in platelets can activate the platelets to release their α-granule contents, including leukocyte chemoattractants, and to express surface receptors for leukocytes(124,125). Upon activation, platelets have been shown to aggregate to neutrophils through binding of platelet P-selectin to neutrophil PSGL-1. Once aggregated, neutrophils and platelets exert paracrine effects upon one another, increasing both platelet release of granule contents to fuel coagulation and neutrophil NETosis(10). These interactions have been

shown to play a critical role in containing and eliminating pathogens, including influenza viruses and infected cells(123,124,126).

Another key function of platelets is the release of growth factors from their α-granules at the site of injury. Because platelets are activated in injured tissues to promote hemostasis, they not only contain the damage but initiate the beginning of repair. Platelet α-granules contain platelet-derived, vascular-endothelial, insulin-like, transforming, and epidermal growth factors, all of which contribute to leukocyte migration, angiogenesis, and stem cell migration and differentiation(127,128). Therefore, platelets have been implicated in the repair of virtually all tissues, hence the clinical use of PRP for treatment of tissues that are difficult to heal, such as tendons and scar tissue(129).

Patients with both genetic platelet defects or acquired platelet defects due to pharmacologics, chronic inflammatory and autoimmune disease, or trauma have been shown to be at a much greater risk of bleeding, infection, and poor tissue repair(114,126) The mechanisms behind acquired platelet dysfunction, especially in trauma, are unclear and therefore difficult to target therapeutically. Furthermore, there are few good, feasible models in which to study injury-induced platelet dysfunction. In this brief study, I, alongside Dr. Matt Duvernay, developed a murine model of burn-induced platelet dysfunction for future studies on mechanism.

Methods: Using an established murine 30% TBSA burn injury model described above in Article 2, we investigated burn-induced platelet dysfunction. Sham animals received identical preparation and resuscitation without the burn injury. Blood was collected into 3.2% citrate at 6, 24, and 48 hours post-burn for measurement of platelet function (N=5 per group per time point). Following blood collection, whole blood was diluted 1:20 in Tyrode's buffer (134mM NaCl, 2.68 mM KCl, 1.8mM $CaCl_2$, 1.05mM $MgCl_2$, 417μM NaH_2PO_4, 11.9mM $NaCO_3$, 5.56mM

glucose, pH 7.4) with 200U/mL heparin. Diluted whole blood was incubated with anti-mouse GpIb (CD42b), GpIIb/IIIa, (JON/A), and P-selectin (CD62) (BD Biosciences) for 30 minutes. Then diluted blood was then stimulated with either 10 μM ADP or 400μM PAR4-AP for 30 minutes and fixed in 1% paraformaldehyde. Flow cytometry was performed, gating for CD42b+ cells (platelets), and P-selectin expression and GpIIb/IIIa activation was measured on the platelets of each treatment group and time point (Figure 2.13A).

Results: Within 6h following the burn, mouse platelets in whole blood exhibited a slight increase in expression of P-selectin and activation of GpIIb/IIIa following stimulation with either ADP or PAR4-AP (Figure 2.13B-E). GpIIb/IIIa activation was reduced in response to ADP or PAR4-AP at 24h and at 48h in the PAR4-stimulated platelets(Figure 2.13B-C). While P-selectin expression following ADP stimulation of the $P2Y_1$ receptor demonstrated little difference between burn and sham throughout the time course, P-selectin expression following PAR4 stimulation exhibited significant differences at 24 and 48h post-burn compared with shams(Figure 2.13D-E). No differences were observed in unstimulated platelets from either group at any timepoint (not shown). Overall, maximum platelet dysfunction in multiple signaling pathways affecting both P-selectin expression and GpIIb/IIIa activation occurred 24h post-burn.

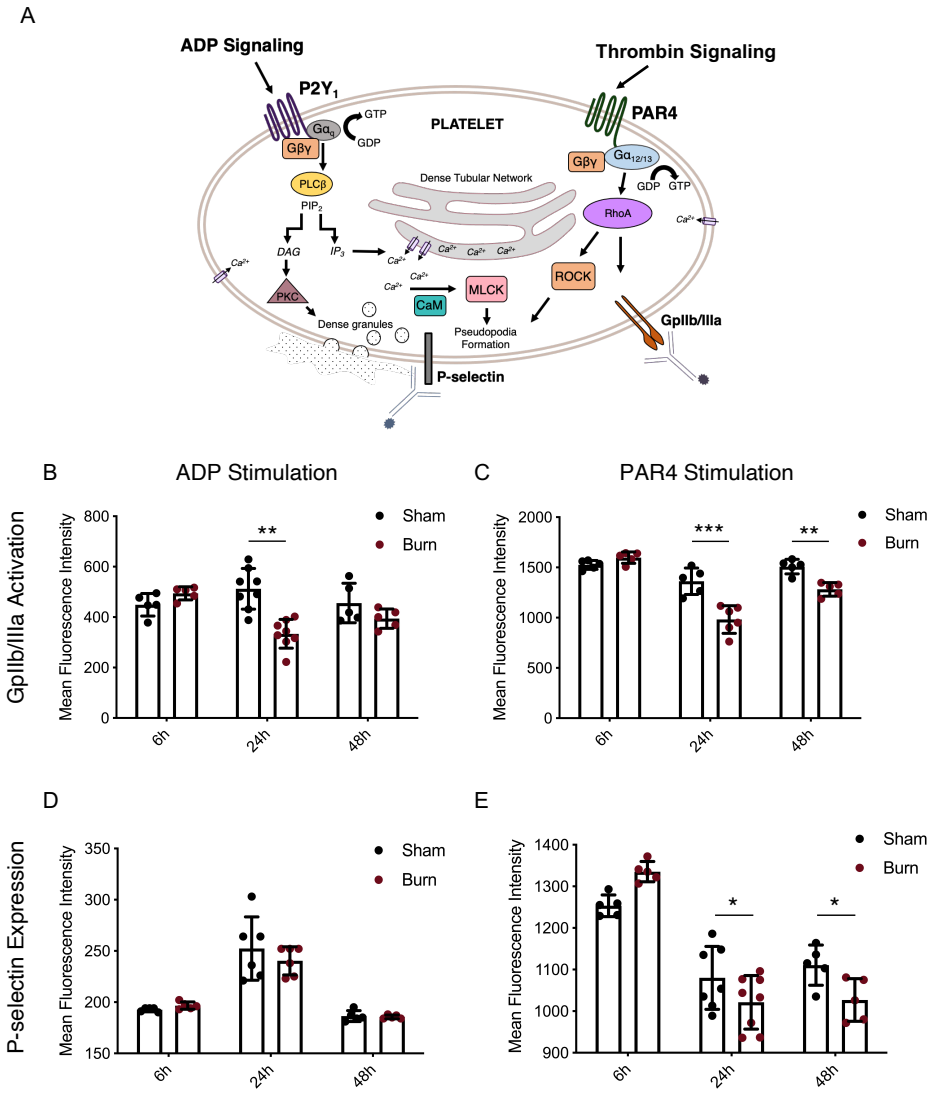

Figure 2.13: Burn-induced platelet dysfunction in a murine model. A) Mouse platelet function assay consisting of stimulating platelets with agonists for PAR4 and $P2Y_1$, measuring P-selectin surface expression and GpIIb/IIIa activation by flow cytometry. B) GpIIb/IIIa activation was decreased at 24h post-burn following ADP stimulation and C) 24h and 48h following stimulation with PAR4-AP. D) P-selectin expression following ADP stimulation was not significantly affected by the burn, but E) PAR4-AP-mediated P-selectin expression was significantly reduced at 24h and 48h post-burn. *P<0.05, **P<0.01 compared with matched shams at each time point. N=5-7 per time point.

Conclusions: In this study, we developed a novel murine model of burn-induced platelet dysfunction. Other studies have used platelet aggregation assays to test this, but these assays cannot isolate dysfunction of specific signaling pathways. Therefore, this model can be used in the future to determine a) which factor(s) are involved in burn-induced platelet dysfunction and b) the effects of this on other outcomes, including bleeding time and tissue repair, in a small scale, affordable animal model. The goal of developing this model was to then manipulate plasmin and fibrin(ogen) in different ways to determine how these affect platelet function following severe injury. This is something that we hope to examine in the future.

Article 2: Plasmin drives burn-induced systemic inflammatory response syndrome

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KEYWORDS: plasmin, SIRS, trauma, burn, coagulopathy, antifibrinolytics

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INTRODUCTION

The acute phase response (APR) is the physiologic process by which the body survives and resolves tissue injury. Following a non-severe injury, the APR is comprised of initial, synergistic activation of coagulation and inflammation to contain the damage and prevent infection at the site of injury. Once adequate containment is achieved, repair mechanisms are activated to remove the damaged tissue and promote regeneration to restore tissue function(1). A severe injury provokes a deranged APR that imposes global, pathologic effects on distant organ systems. Consequently, severe injuries are a leading source of morbidity and mortality due to critical adverse outcomes(130). The hallmark of a pathologic, severe injury-induced APR is dysregulated inflammation and coagulation(131,132). The inflammatory pathology is referred to as systemic inflammatory response syndrome (SIRS) highlighted by a 'cytokine storm.'(133) The coagulopathy is referred to as a trauma-induced coagulopathy (TIC) which includes coincident paradoxical bleeding and thrombotic phenotypes(134,135). Together, SIRS and TIC result in an "immunocoagulopathic" condition which affects the vasculature and peripheral tissues, increasing the risk of multiple organ dysfunction syndrome (MODS)(132,136,137), and impeding tissue repair later in convalescence(37,138,139) A critical knowledge gap is molecular mechanisms that initiate this detrimental immunocoagulopathy in a severe injury-induced APR.

In the context of a non-severe injury APR, the protease plasmin is activated locally at the site of the damage within days following injury, where it removes fibrin (fibrinolysis) and other matrices and activates growth factors, which together stimulate tissue regeneration(1). While

plasmin's conventional role is intravascular and extravascular fibrinolysis, plasmin also modulates inflammation through a plethora of mechanisms, including cellular signaling, matrix removal, and activation of cytokines and other proteases(106,140–142). In the context of a maladaptive APR following a severe injury, excess, early, systemic activation of plasmin has been shown to perpetuate lethal clinical outcomes. The most characterized adverse outcome of systemic pathologic plasmin activation following severe injury is blood loss due to inappropriate clot degradation (hyperfibrinolysis)(143,144). Clinical trials investigating the use of antifibrinolytic drugs have clearly demonstrated that timely prevention of plasmin activation reduces both blood loss, transfusion, and death(145,146). In addition, these studies also demonstrate reduction in markers of inflammation, but it is unclear if the anti-inflammatory effects of antifibrinolytics are secondary to a reduction in bleeding(147). Taken together, these data suggest a context-dependent contribution for plasmin activation following severe injury, exacerbating inflammation in severe injury but functioning to resolve inflammation following mild injuries. In disease states, such as cancer, arthritis, and infection, plasmin has been identified as a pathologic instigator of inflammation(148–150) but its isolated role in severe injury-induced SIRS, including the cytokine storm and activation of inflammatory pathways within injured tissue, is unknown.

In this study, we investigated the role of plasmin in the deranged inflammatory component of a pathologic APR, assessing its effects on cytokine storm and tissue-specific inflammatory signaling. We hypothesized that severe injury-induced excess plasmin activity is a key molecular driver of SIRS. A major barrier to investigating the relationship between injury-induced plasmin activity and inflammation is the presence of bleeding because of hyperfibrinolysis, which is often fatal and may instigate a secondary inflammatory response. To circumvent bleeding as a confounding variable we investigated plasmin activation following severe injury and its causative

role in SIRS in both clinical and preclinical models of severe burn in which pathologic systemic inflammation occurs without the risk of lethal blood loss.

RESULTS

Clinical Cohort Description

As a part of a prospective study, 31 patients with partial and full-thickness cutaneous burns affecting 5-95% of their total body surface area (TBSA) and 15 healthy control subjects were enrolled following informed consent (Table 2.4) for blood collection. Flash burns were the most common mechanism of injury (77%, 24/31), with 42% (13/31) of patients experiencing concomitant inhalation injury (Table 2.5). Patients experiencing burn injury were found to have a median revised Baux Score of 79 (range 9-119) at the time of admission, with in an in-hospital mortality rate of 19% (6/31) (Table 2.5). Throughout hospitalization, 71% of patients (22/31) required surgical intervention for treatment of their burn injuries. Within the first week of hospitalization 87% (27/31) of burn patients developed markers of organ dysfunction based on the Sequential Organ Failure Assessment (SOFA) scoring system(151), and 42% (13/31) developed multiple organ dysfunction defined as a SOFA score ≥6 (Table 2.5).

Table 2.4: Burn Subject Demographics

	Burn Patients (N=31)	Controls (N=15)
Age (years)	45 (12-88)†	25 (20-44)†
Sex (male/female)	22/9	10/5
Ethnicity		
African-American	4 (13)	0 (0)
Non-hispanic White	27 (87)	10 (66.7)
Hispanic	0 (0)	1(6.67)
Asian/PNI	0 (0)	4 (26.7)
Body Mass Index (kg/m^2)	25.3 (18-35)†	21.5 (19-26)†

N (%) unless otherwise indicated
†Represents median and range

Table 2.5: Burn Characteristics & Outcomes

Mechanism	
Flash burn	24 (77)
Scald	2 (6)
Other	5 (16)
Inhalation Injury	13 (42)
SIRS	27 (87)
TBSA (%)	27 (5-95)†
Revised Baux Score	79 (9-119)†
Surgical Intervention	22 (71)
SOFA Score	5 (0-13)†
MODS (SOFA Score ≥ 6)	13 (42)
Length of Hospital Stay (days)	21 (0-81)†
Mortality	6 (19)

N(%) unless otherwise indicated
†Represents median with range

Burn-induced SIRS and Coagulopathy

Severe burns are unique injuries that provoke SIRS and coagulopathy without a significant risk of bleeding prior to surgical intervention(152,153). In this cohort, 87% (27/31) of patients developed SIRS within the first 72 hours of the burn based on clinical criteria, including dramatic changes in heart and respiratory rate, temperature, and white blood cell count(154) (Table 2.15). In addition to clinical criteria, the burn patients we analyzed exhibited other indications of SIRS, including high plasma concentrations of inflammatory cytokines IL-6 and IL-1β(133,155) (Figure 2.14A-B), consistent with previous findings in burn patients(156,157). Another hallmark of systemic inflammation closely linked with coagulation activation and plasmin activation is complement activation(58,106,158). In our cohort, burn patients exhibited high levels of C5a, indicative of complement activation, at the time of admission. Throughout hospitalization, C5a levels fluctuated relative to surgical interventions, which represent additional instances of tissue injury and introduce the risk of bleeding (Figure 2.14C). Interestingly, soluble L-selectin, a common marker for leukocyte activation, decreased, although not significantly, during hospitalization (Figure 2.14D), consistent with previous findings in trauma patients(159).

In addition to changes in SIRS, burn patients also exhibit hallmarks of coagulopathy, including decreased platelet count, excess thrombin generation, and markers of platelet activation due to injury severity and high-volume crystalloid fluid resuscitation(153,160,161). Upon admission and prior to surgical intervention, burn patients exhibited signs of coagulation activation marked by significantly decreased platelet counts within 1.5 days post-burn, with the average platelet count nadir occurring at 2 days post-burn, and elevated soluble P-selectin and CD40L in the plasma, indicative of platelet activation. (Figure 2.14E-G). Previous studies have demonstrated increased circulating thrombomodulin as an indicator of endothelial dysfunction in patients with

severe burns(37), but in this heterogeneous cohort, we observed only a minor, non-statistically significant increase in plasma thrombomodulin on average (Figure 2.14H).

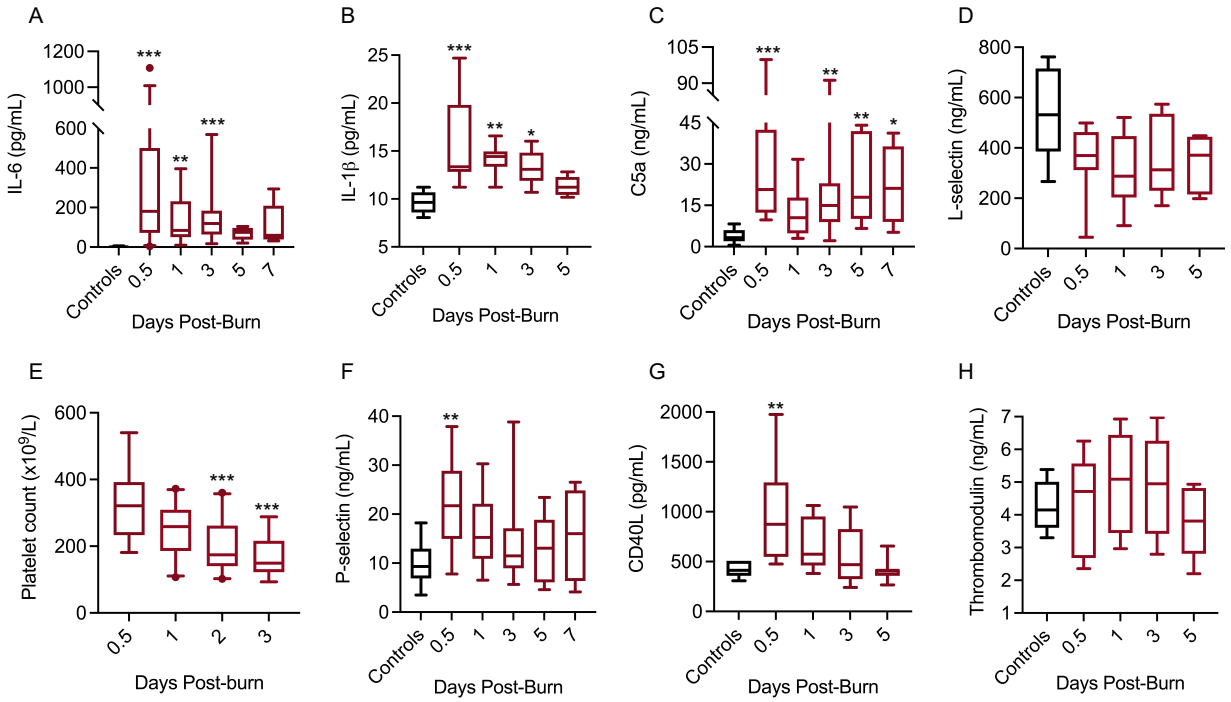

Figure 2.14: Inflammation and coagulopathy in human burn patients. SIRS and coagulopathy in burn patients. Burn patients exhibit markers of inflammation, including A-B) significantly elevated inflammatory cytokines (IL-6, IL-1β) and C) markers of complement activation (C5a) upon admission, and D) a reduction in leukocyte activation marker soluble L-selectin throughout hospitalization. A) Burn patients exhibit hallmarks of coagulopathy including E) decreased platelet count with high levels of platelet activation markers, F) P-selectin, G) CD40L and H) endothelial activation marker thrombomodulin. (*P<0.05, **P<0.01, ***P<0.001 compared with controls. A Kruskal-Wallis with Dunn's post-hoc correction for multiple comparisons was performed; N=24-31 for 0.5, 1, 3, days, and 7-15 for 5 or 7 days due to mortality or discharge, N=15 for controls. Boxes represent median with 5-95% percentile)

Plasmin activation and fibrinolysis with reduced circulating plasminogen levels in burn patients.

Current data on plasmin activation in burn is controversial with some studies suggesting patients do not exhibit hyperactivation of plasmin, as measured by clot lysis on thrombelastography (TEG)(80), while other studies employing alternative measurements of plasmin activity suggested significant plasmin activation immediately following burn(60). In this study, we found that burn patients exhibited significant activation of plasmin activation. D-dimer levels were significantly elevated in all patients upon admission and normalized within 1-2 days of admission if no surgical intervention was initiated (Figure 2.15A). Furthermore, the burn patients in this study were found to exhibit circulating plasmin-antiplasmin (PAP) levels 10- to 50-fold greater than normal values within the first 12 hours following burn injury (Figure 2.15B). Therefore, burn does provoke significant, early activation of plasmin and fibrinolysis.

Furthermore, levels of proteins previously suggested to play a role in aberrant plasmin activation following trauma—circulating soluble urokinase plasminogen activator (uPA), soluble urokinase plasminogen activator receptor (suPAR), and soluble endothelial protein p11 (S100A10) (88,164)—were significantly elevated within the first 1-3 days post-burn (Figure 2.15C-D). However, detection of free uPA decreased throughout hospitalization following rapid plasmin activation (Figure 2.15E).

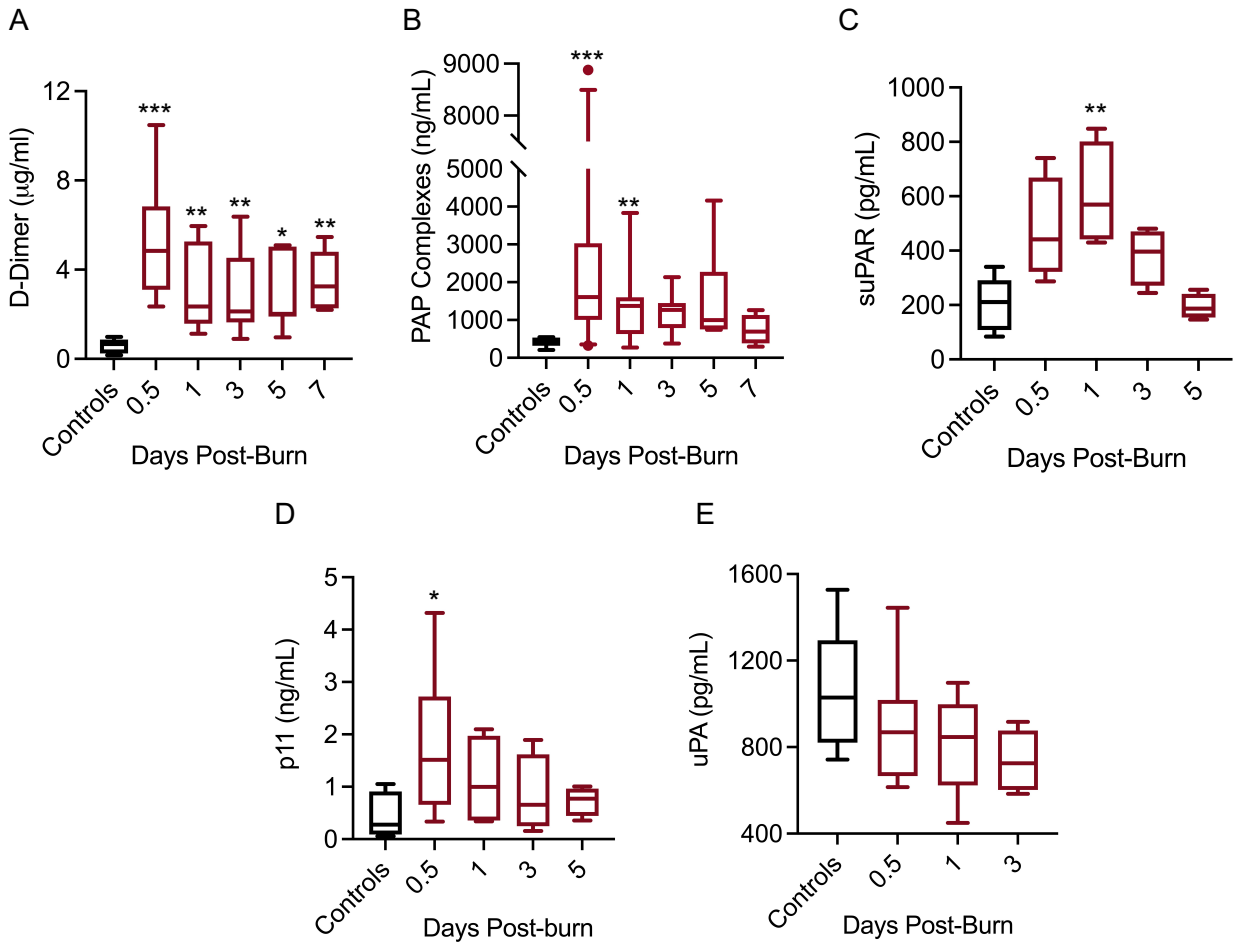

Figure 2.15: Plasmin activation and fibrinolysis in burn patients. Burn patients exhibit significant A) plasmin activation and B) fibrinolysis within the first day of the burn. C) Soluble uPAR was elevated within the first 3 days of the burn, and D) Soluble endothelial p11 was elevated upon admission prior to approaching baseline around 3 days post-burn while E) free uPA levels decreased throughout hospitalization (Kruskal-Wallis with Dunn's post-hoc correction for multiple comparisons was performed: *P<0.05, **P<0.01, ***P<0.001 compared with controls; for A-B: N=24-31 for 0.5, 1, 3, days, and 7-15 for 5 or 7 days for C-D, N-15. N=15 for controls. Boxes represent median with 5-95% percentile).

All patients with burns presented with decreased circulating levels of plasminogen, and those with severe burns (>20% TBSA, N=20/31) exhibited 50-75% decreases in plasminogen (Figure 3A). Upon admission, circulating plasminogen levels were inversely associated with the percent TBSA affected by the burn (R^2=0.487, P=<0.0001). Across the first 7 days following burn injury, plasminogen levels remained 40-60% of normal levels. In addition to redistribution, other studies have demonstrated that significant plasmin activation can result in lower circulating plasminogen levels(60). The significant increase in PAP (Figure 2.15B) suggests that at least a portion of the observed decrease in plasminogen is due to plasmin activation and subsequent clearance from the blood. While the clinical significance of diminished plasminogen is currently unknown, this study found that plasminogen depletion from the plasma at the time of admission, strongly correlated with the development of organ dysfunction in the first week of hospitalization (R^2=0.343, P=0.0013) (Figure 2.16B).

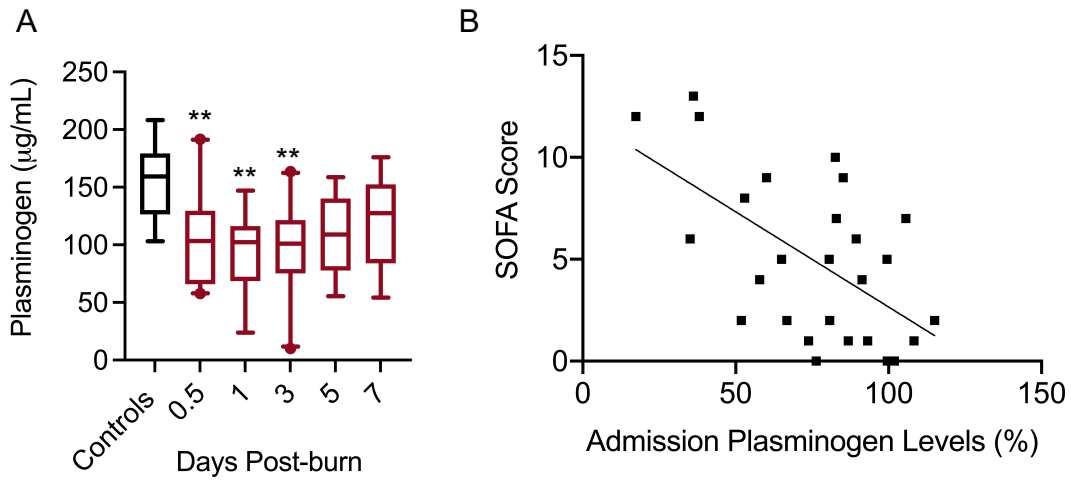

Figure 2.16: Decreased plasminogen levels in burn patients are associated with organ failure. A) Plasminogen levels in burn patients were below normal values upon admission and remained low throughout hospitalization. (Kruskal-Wallis with Dunn's post-hoc correction, *P<0.05, **P<0.01, ***P<0.001, compared with controls; N=24-31 for 0.5, 1, 3, days, and N=15 for 5 or 7 days, N=15 for controls. Boxes represent median with 5-95% percentile). B) Admission plasma plasminogen levels were inversely correlated with the magnitude of organ failure incurred within the first week of hospitalization (Pearson correlation, R^2=0.343, P=0.0013).

Plasmin in Burn-Induced SIRS

Previously, the significance of early, exuberant plasmin activity in severe injuries without bleeding was unknown. In this study, early PAP values positively correlated with peak plasma IL-6 values (R^2=0.445, P=0.0018) and negatively correlated with the lowest platelet count in patients prior to surgical intervention (R^2=0.425, P=0.0014); thereby associating with both inflammation and coagulopathy markers, respectively (Figure 4A). Furthermore, admission PAP values >3000pg/mL were found to associate with mortality within 7 days of burn injury (P=0.0012, HR=14.73) (Figure 2.17B). However, these correlative

findings do not confirm a causative role for plasmin in burn-induced inflammation and may represent an association between factors within the severe APR. Therefore, we implemented murine models to investigate the mechanistic relationship between early plasmin activation and burn-induced SIRS.

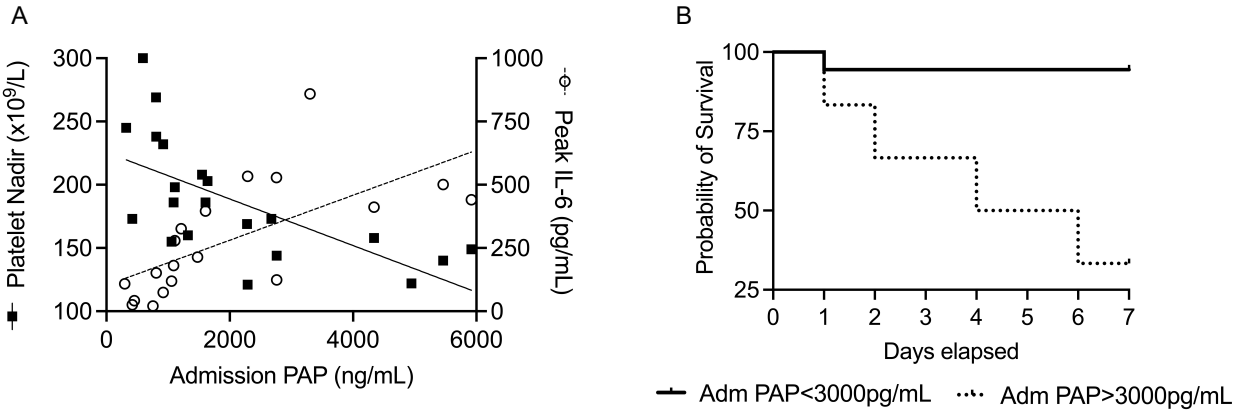

Figure 2.17: The magnitude of early plasmin activation is associated with markers of SIRS, coagulopathy, and in-hospital mortality. A) Plasmin activation within 12h of the burn correlated with patient platelet nadir prior to surgical intervention (R^2=0.425, P=0.0014) and peak plasma IL-6 (R^2=0.445, P=0.0018) by Pearson correlation. B) Admission PAP levels >3000pg/mL were significantly associated with 7-day mortality (Log-rank test, P=0.0012, HR=14.73). N=24 patients admitted within 12h of the burn.

Mouse Model of Burn-induced SIRS

To investigate the mechanistic role of plasmin in burn-induced SIRS, we used a well-established murine severe burn model, in which SIRS is known to occur(165). Although SIRS is typically measured by circulating markers and basic vital signs, the inflammation provoked by severe injuries is systemic, affecting distant organs and other injured tissues. Many severe burn injuries are not isolated to a dermal wound alone, but also involve concurrent injuries to muscle,

bone, and nerves(166). Therefore, we used a validated model of burn with concomitant muscle injuries remote from the site of burn(65). To provoke a non-severe APR, isolated calf muscle injuries were administered. In contrast, to provoke a severe APR, a 30% TBSA dorsal burn was conducted in combination with the same isolated calf muscle injuries (Figure 2.18A). On a cytokine panel, we observed the greatest difference in plasma between muscle injury alone and burn with muscle injury in IL-6 measurements (Figure 2.19) which has been shown to be an accurate predictor of SIRS and MODS(133,155,167). Therefore, to measure systemic markers of SIRS in the blood, we quantified plasma IL-6 and neutrophil-to-lymphocyte ratio (NLR), as markers of inflammation and innate immune response. Aligning with a more severe APR, mice with the burn injury developed a significant increase in circulating IL-6 and NLR that peaked within 6 hours post-injury and resolved within 24-48 hours compared with those who received muscle injuries alone (Figure 2.18B-C).

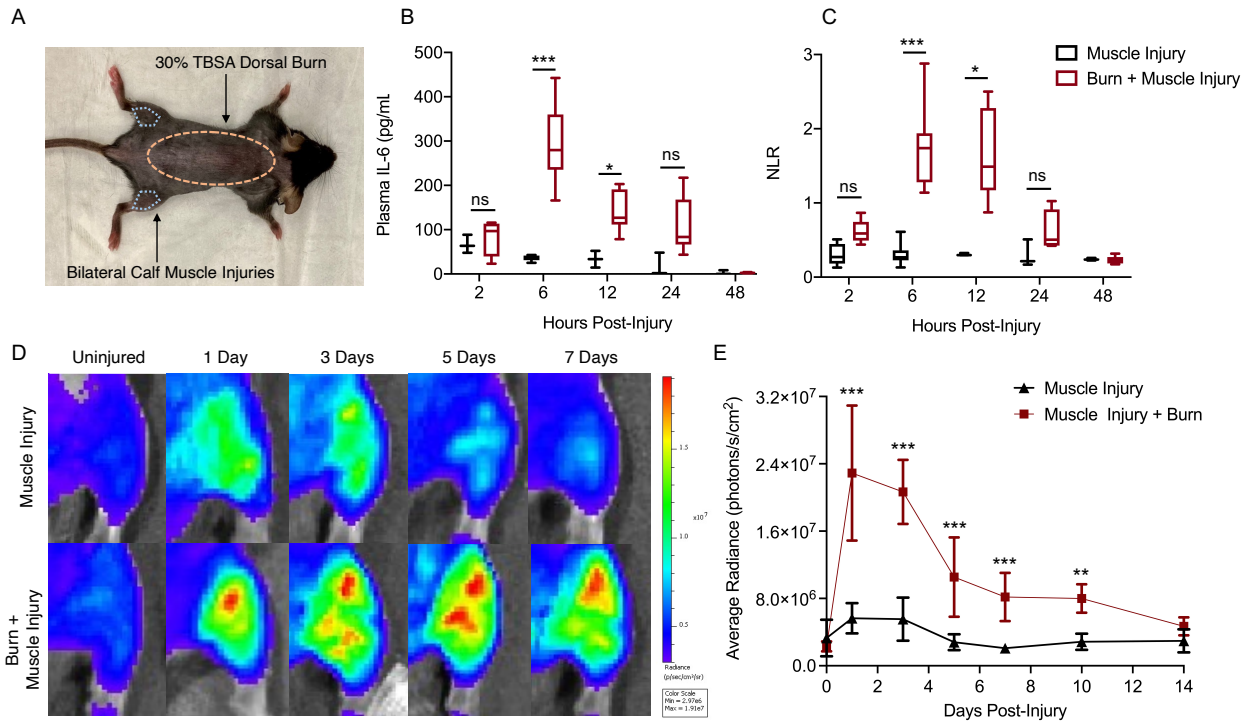

Figure 2.18: A mouse model of burn polytrauma provokes a systemic inflammatory response, affecting circulating and tissue markers of inflammation. A) Mouse model of 30% TBSA dorsal burn injury with concomitant, bilateral calf muscle injuries. B-C) Plasma IL-6 and neutrophil to lymphocyte ratio (NLR) were significantly increased within 6-12h of the burn injury compared with the muscle injury alone, which does not significantly increase either marker. D-E) NFκB signaling in an isolated calf muscle injury is increased 1-3 days post-injury and is resolved by 7 days, (N=5/group, boxes represent 5-95th percentile; multiple Mann-Whitney U tests at each time point with Holms-Sidak correction for multiple comparisons, *P<0.05, ***P<0.001, compared with muscle injury alone). The presence of a dorsal burn distant from the site of muscle injury increases and augments NFκB signaling in injured muscle. (Repeated measures ANOVA, *P<0.05, **P<0.01, ***P<0.001 compared with muscle injury alone, N=7 mice/group, points represent mean with 95% CI).

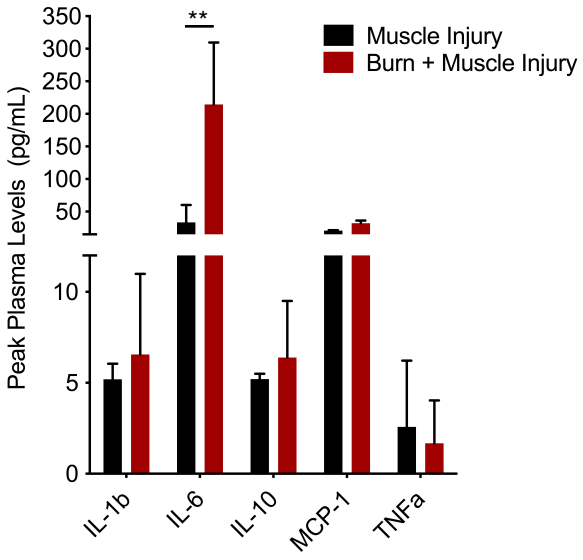

Figure 2.19: Peak cytokine response post-burn in a murine model. Of the cytokines measured, IL-6 exhibited the strongest response to a sterile, 30% TBSA burn injury with calf muscle injuries compared with muscle injuries alone.

Burn injury augments and prolongs NF-κB signaling at a site of injury remote to the burn

To measure the effects of burn on tissue-specific inflammation at sites of injury remote to the site of burn, we implemented the burn model with concurrent, anatomically remote, bilateral muscle injuries in mice expressing an NF-κB-inducible luciferase reporter(168). Transcription factor NF-κB, a central mediator of many convergent cellular inflammatory pathways, is activated within muscle immediately following injury and is resolved within 5 days following injury (Figure 2.18D-E). The addition of the dorsal burn injury not only augmented NF-κB activity in the injured muscle but also prolonged it up to 14 days post-injury (Figure 2.18D-E), suggesting that burns provoke a systemic change that alters inflammatory signaling locally at sites of injury distant to the burn wound. Because the burned tissue becomes necrotic almost immediately after the injury,

NF-κB activity was not significantly detected by bioluminescence at the wound in this model (Figure 2.20).

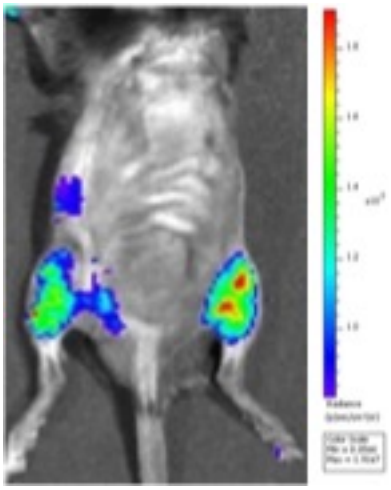

Figure 2.20: Bioluminescent signal in mouse with burn and bilateral calf muscle injuries.

Inhibition of early plasmin activation reduces burn-induced systemic inflammation

To determine the role(s) of plasmin in burn-induced systemic inflammation, we used pharmacologic and genetic tools to manipulate plasmin activity following burn injury in mice. Low level PAP, plasma IL-6, and NLR were detected following an isolated muscle injury only (i.e., non-severe APR). However, when the muscle was injured in conjunction with a burn (severe APR), significant plasmin activation (PAP) was observed in the plasma of mice at 2 hours post injury with resolution by 6 hours post injury (Figure 2.21A), aligning with clinical observation from burn patients (Figure 2.15B), although with different timing.

To assess the role of plasmin activity in burn-induced systemic inflammation, we enhanced plasmin activity or plasmin activation prior to causing a burn injury in wild-type animals. This was achieved through the administration of an inhibitor of plasminogen activator inhibitor-1 (PAI-1)

in combination with an intravenous bolus of tissue plasminogen activator (tPA) at the time of injury or a validated antisense oligonucleotide (ASO) against plasmin's primary inhibitor, α2-antiplasmin (α2AP)(33). PAP was not detectable in mice treated with a2AP ASO, as expected. However, an increase in PAP was observed in mice treated with a PAI-1 inhibitor in combination with tPA (Figure 2.21B). Regardless of the pharmacologic mechanism employed to enhance plasmin activity, no significant changes in plasma levels of IL-6 or NLR were observed at 6 hours post injury (Figure 2.21C-D). To reduce early plasmin activation following injury, a burn injury was applied to mice with a 50% plasminogen deficiency ($Plg^{+/-}$) or wild-type mice treated with clinical antifibrinolytic TXA immediately following the burn. In mice with either genetic or pharmacologic reduction of plasmin(ogen), we observed a significant reduction in PAP at 2 hours post burn injury (Figure 2.21B). Furthermore, plasma levels of IL-6 and NLR at 6 hours post injury were significantly reduced by up to 50% compared with non-treated burn injuries (Figure 2.21C-D). No significant differences were observed between groups at 24h (Figure 2.22). There were also no differences observed between these groups in animals that received a muscle injury alone (not shown). Together, these data suggest that plasmin activity exacerbates systemic inflammation following a severe burn, but that its role in burn-induced inflammation is maximized by the magnitude of the injury rather than the amount of plasmin activity.

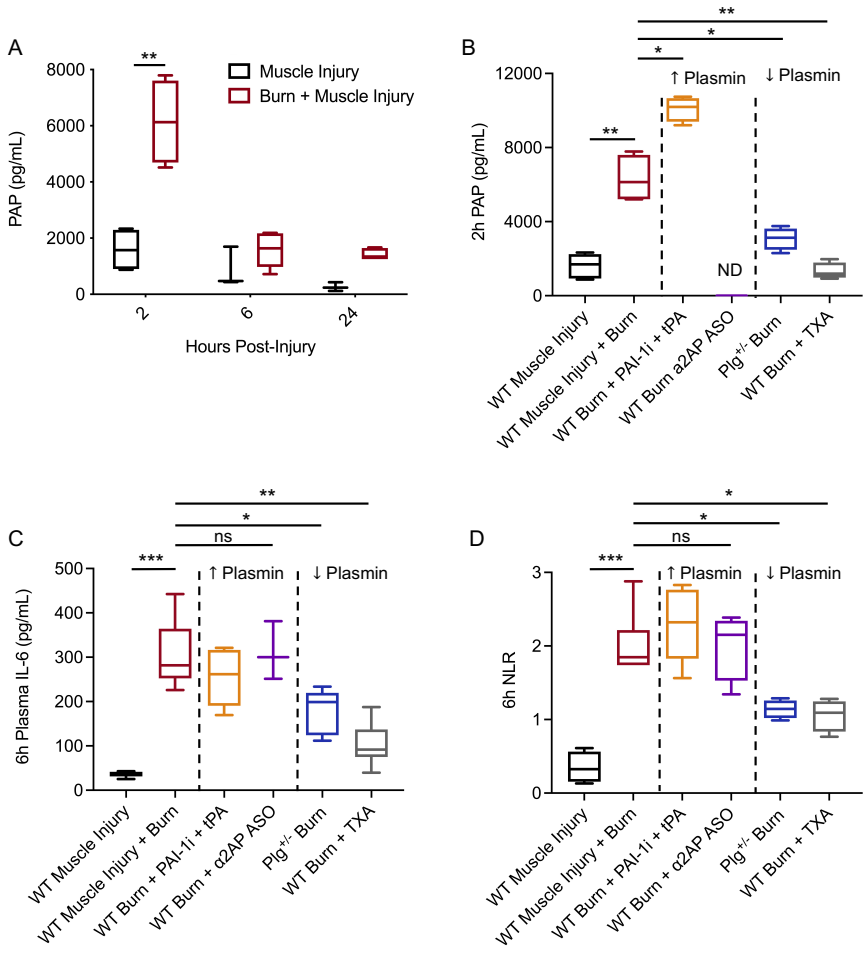

Figure 2.21: Inhibition of early plasmin activation reduces circulating inflammatory markers following burn. A) Plasmin activity peaks within 2h of the injury and remains slightly elevated 24h following the burn injury. B) PAI-1i + tPA was effective at increasing plasmin activation, while $Plg^{+/-}$ mice and WT mice treated with TXA had reduced plasmin activation. As a part of the PAP complex, α2AP ASO resulted in PAP values below the limit of detection (ND= not detected). C-D Treatments to enhance plasmin activity (PAI-1i + tPA or α2AP ASO) did not alter inflammatory markers in the blood following burn, while $Plg^{+/-}$ mice or WT mice treated with TXA exhibited reduced circulating markers of inflammation (IL-6 and neutrophil-to-lymphocyte-ratio (NLR)). Kruskal-Wallis with Dunn's post-hoc correction, *P<0.05, **P<0.01, ***P<0.001, N=4-5/group, box and whiskers represent the 5-95th percentile.

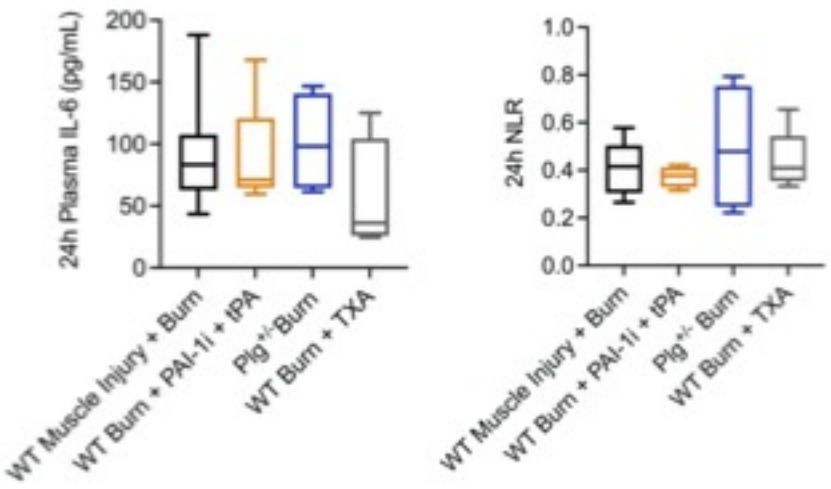

Figure 2.22: Inflammatory markers 24 hours post-burn in mice. Enhancement or inhibition of plasmin did not significantly alter A) plasma IL-6 or B) NLR at 24h following the burn injury. (Kruskal-Wallis was performed)

Inhibition of plasmin reduces the magnitude of NF-κB signaling in a remote muscle injury following burn

To determine if plasmin plays a role in tissue inflammation at sites of injury remote to the burn, mice expressing an NFκB-inducible luciferase-GFP reporter gene (NGL) were challenged with either a muscle injury alone or a muscle injury with a burn and treated with α2AP ASO, TXA, or a validated plasminogen antisense oligonucleotide (Plg ASO) to transiently knock down plasminogen expression. Enhancement of plasmin activity by α2AP ASO did not alter the magnitude of NF-κB activity in injured muscle (Figure 2.23A). While burned animals treated with TXA or Plg ASO still exhibited augmentation of NF-kB activity within injured muscle compared with the muscle injury alone, both methods of early plasmin inhibition significantly reduced the magnitude of the muscle NF-κB activity (Figure 2.23B-C). These data suggest that plasmin activity

following burn injury also exacerbates local inflammation within tissue at sites of injury remote to the burn, but this effect could not be exacerbated further with pharmacologic enhancement of plasmin activity in this model.

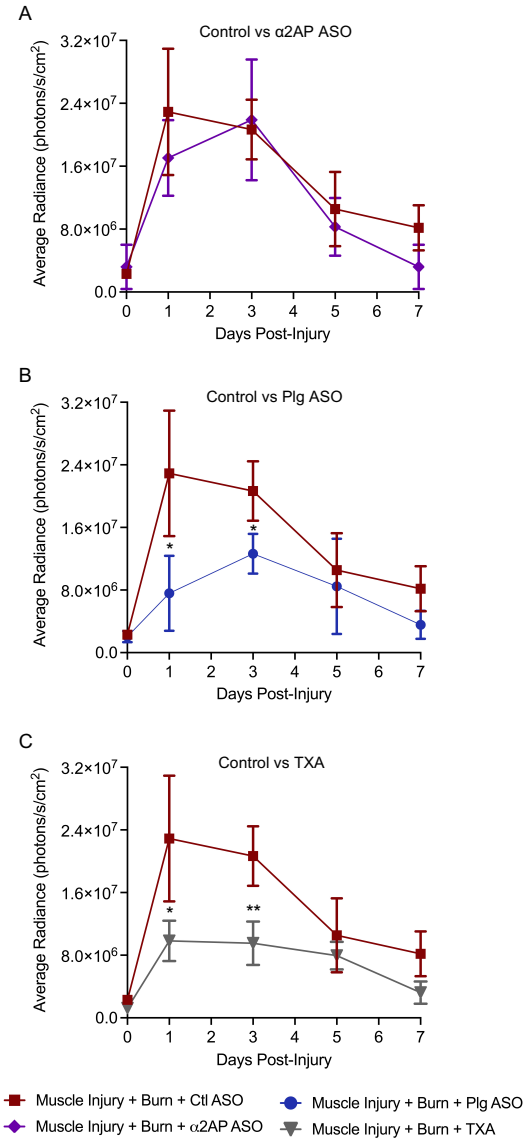

Figure 2.23: Inhibition of early plasmin activation following burn reduces NFκB signaling in injured muscle. A) Enhanced plasmin activity by α2AP ASO had no significant effect on muscle NFκB activity, but inhibition of plasmin activity by B) Plg ASO or C) TXA reduced NFκB signaling in injured muscle following burn. No differences were observed across groups with muscle injuries alone (not shown) (Repeated measures ANOVA, *P<0.05, **P<0.01 N=5/group, data points represent mean with 95% CI).

DISCUSSION

Previous studies suggested that burn injury does not provoke significant plasmin activation except in extreme cases of coagulopathy(80). However, we demonstrated significant burn-induced plasmin activation and fibrinolysis based on markers of *in vivo* plasmin activation and fibrinolysis. The clinical significance of severe injury-induced plasmin activation in the absence of bleeding was previously unclear. In this study, we observed a clinical association between markers of immunocoagulopathy and plasmin activation following burn injuries. To investigate a causative relationship between burn-induced plasmin activity and inflammation specifically, we implemented a mouse model of polytrauma which includes a severe burn injury with a remote muscle injury. The results of this study suggest that SIRS, measured by both circulating cytokines and tissue inflammatory signaling, occurs following burn and that the magnitude of this inflammatory response is, in part, mediated by plasmin. Therefore, documenting that plasmin plays a functional role in the dysregulated APR following severe injury has important clinical implications. The results of this study suggest that pharmacologically blocking early plasmin activity following burn attenuates SIRS—a key driver of thrombosis and MODS in injured patients.

Trauma-related deaths have been observed to occur in different peaks over time: immediate, early, and late deaths(169). Recent research has demonstrated that medical advances have greatly reduced the late deaths, but those within the first day of injury due to hemorrhage, brain injury, and early organ dysfunction remain unchanged(39,170). Although immediate deaths cannot be prevented with medical treatment, there is room for significant improvement in the reduction of the ~50% of deaths that occur within the first hours to days of the injury. Currently, approximately 30-40% of trauma deaths occur within several hours of the injury, and 10-20% of

deaths occur beyond 24 hours post-injury. The first of these waves is mostly attributed to hemorrhage and brain injury, and the later deaths are attributed to MODS and infection. In burn specifically, the waves of mortality are shifted later as there is little risk of early hemorrhage, however, the majority of burn-related deaths within the first week occur due to SIRS-associated MODS or sepsis(171,172). This study and others have suggested that inhibition of plasmin may reduce injury-associated SIRS, and interestingly, recent studies in surgery have demonstrated that antifibrinolytic drugs reduce injury-associated immunosuppression by inhibiting plasmin's effects on immune cells, reducing the risk of post-surgical infection(173,174). In this study, we examined the early inflammatory response, and while infection and endotoxemia can drive an inflammatory response, this occurs within days in humans and in animal models of burn(175,176) rather than within the first 12 hours, suggesting that the inflammatory response was due to the injury specifically. While antifibrinolytics are often used to reduce the risk of bleeding-associated mortality within hours of the injury(59), the findings of this study suggest that antifibrinolytics may be used to reduce the risk of mortality in both the early and later peaks following the injury.

Burn-induced SIRS has been shown to affect other tissues, and the consequences of severe burn-provoked inflammation on tissue health have been extensively demonstrated by the phenomenon of burn-induced osteoporosis and muscle wasting(177–179). Although we used a remote muscle injury to investigate how the systemic response to burn affects inflammatory signaling in distant tissues, tissue inflammation is often observed in a multitude of other organs following burn injuries, resulting in MODS(136,180). Patients with severe injuries often present with polytraumas or require surgical intervention involving skin and muscle. These combinatory injuries, such as blast injuries, are frequently seen in military populations as well as motor vehicle accident victims(166,181). Furthermore, the majority of patients with severe burns undergo early

surgical intervention, including excision and grafting, which introduces further tissue damage(182). Therefore, severe injury-induced inflammation in peripheral tissues is a critical target to reduce its pathologic effects on other healthy or injured tissues. Future studies are required to determine if the effect of plasmin on tissue inflammation following burn is due systemic changes in circulating inflammatory mediators or if plasmin acts specifically at the site of the injury to augment inflammatory signaling.

Plasmin has a wide range of effects on inflammatory signaling through multiple mechanisms. Plasmin(ogen) interacts with many receptors on the surface of macrophages, neutrophils, and dendritic cells to regulate cellular migration, phagocytosis, NETosis, and inflammatory signaling(142)(183). Following the early APR, plasmin has been shown to resolve inflammation by removing fibrin, inducing neutrophil apoptosis, and reprogramming macrophages within the injured tissues to anti-inflammatory phenotypes(1,183–185). Alternatively, plasmin has also been shown to activate inflammation through matrix metalloproteinase (MMP) activation(148,186,187), complement activation and bradykinin pathway activation(58,106), macrophage chemokine release(188), protease-activated receptor (PAR) signaling(142,189) and activation of NF-κB-mediated cytokine and chemokine production in neutrophils and macrophages(28,150,186,190–192) in a time- and concentration-dependent manner. Plasmin can also exert pro-inflammatory effects through fibrinolysis: fibrin degradation products have been shown to increase release of inflammatory cytokines from monocytes(28)(193). Each of these pathways has been shown to be acutely activated at the site of significant injury, and while beneficial during tissue repair, these mechanisms have the potential to exacerbate inflammation during the early, severe-APR, driving SIRS. Studies in severe injuries have shown that inhibition of plasmin to reduce bleeding has the “off-target” effect of reduced inflammation(147), and here

we have shown that, independent of bleeding, early inhibition of plasmin following severe injury reduces markers of SIRS. Although we did not identify one clear mechanism by which burn-induced plasmin activation exacerbates SIRS, this work puts forward the potential of using antifibrinolytic therapeutics to prevent SIRS- related MODS and possibly death—not just bleeding. Building upon this study, future mechanistic studies are warranted to determine the pathway(s) by which plasmin fuels different aspects of burn-induced inflammation.

Along with burn-induced plasmin activation, we observed a prolonged, acquired hypoplasminogenemic state in the plasma of burn patients. Although this state was preceded by a transient burst of plasmin activation, the loss of plasma plasminogen in humans was directly correlated with the magnitude of tissue damage, suggesting that this is likely composed of both redistribution and activation-mediated consumption. It's possible that this is due to binding of plasminogen to the immense volume of fibrin deposited within the zone of coagulation of a burn. The damaged tissue milieu presents multiple binding sites for plasminogen in both the intra-and extravascular spaces, which provides an evolutionary advantage of localizing plasmin activation to the site of coagulation or tissue injury. However, in polytraumas, this could potentially cause circulating plasminogen to preferentially bind to larger areas of tissue damage, while smaller injuries requiring plasmin for repair, such as muscle, may become transiently deficient in plasmin. Currently, the clinical significance of this plasminogen redistribution is unknown, but in this study, the loss of circulating plasminogen retrievable by blood draw also correlated with the degree of organ failure incurred in burn patients within the first week of hospitalization, which may reflect the magnitude of tissue damage.

Previous studies have reported a range of fibrinolytic potentials, measured by clot lysis on TEG, in trauma patients from hyperfibrinolysis to fibrinolytic shutdown(46,104). Injury-induced,

blood hypoplasminogenemia has the potential to affect some diagnostic assays measuring fibrinolytic potential in the blood and may falsely indicate a hypofibrinolytic condition, even though *in vivo* markers of plasmin activation and D-dimer indicate differently. This phenomenon may reflect a transfer of plasminogen from blood to large areas of tissue damage where it is activated, rather than a systemic activation of plasmin throughout the blood that can lead to severe hyperfibrinolytic phenotypes and subsequent blood loss in certain types of severe injuries. This may, in part, account for differences in "fibrinolytic phenotypes" observed across different trauma patients(104) as well as timing of admission.

Clinically, the APR may be measured by markers that are typically elevated within 1-2 days of the injury, and these include C-reactive protein (CRP), fibrinogen, ferritin, and procalcitonin. These measures are often taken serially in patients with infection(17), along with complete hematologic and metabolic panels, while trauma patients are more likely to have just receive hematologic and metabolic panels (complete blood count, glucose, PT-INR, etc.) and imaging taken during hospitalization(194,195) Because over 75% of trauma-related deaths occur within the first 24 hours(170), early markers of the APR beyond the common hematologic and metabolic measure may provide better indication of where in the APR a patient is and how their condition will progress. More recently, IL-6 has been used to measure the magnitude and timing of the APR in patients with severe injuries as it is a critical initiator of the APR in the liver(196,197). In this study, early plasmin activation, IL-6, and platelet counts were used to measure the magnitude and timing of the APR in burn patients. Although plasmin activity is commonly measured by TEG in patients with bleeding-associated severe injuries, it would be beneficial to measure plasmin activation with other markers of the APR even in the absence of bleeding in order to predict the risk of adverse outcomes.

Another issue encountered in this study is the possibility that, in burn, plasmin activation is missed due to timing of admission. Notably, PAP levels were only slightly elevated in later samples or when patients were admitted to the hospital more than 12 hours post-burn regardless of burn severity, and consistently, PAP levels rise and fall within 6 hours of the burn injury in mice. This data suggests that plasmin activation is a rapid and transient occurrence following burn that may be poorly detected using standard measures of plasmin activity, such as TEG. Because burns represent a large surface area of tissue damage with little to no risk of bleeding, in contrast to a penetrating trauma, it is possible that pathologic plasmin activation is occurring more locally without a systemic release of plasminogen activator into the blood that would result in significant clot lysis by TEG measurements. Although TEG is clinically useful to predict bleeding and inform resuscitation in certain types of injury and disease(80,97,198) in order to detect plasmin activation in different types of severe injury without bleeding risk, other markers of plasmin activation, such as D-dimer or PAP, may be required in addition to TEG measurements.

Based on a collective of studies, plasmin does appear to play a paradoxical role in inflammation following injury, depending on timing and location, and the inflammatory response to injury is an important, complex sequence of events(58,60). Human studies in trauma have shown a time-dependent effect of TXA on patient outcomes: early administration of antifibrinolytics reduces blood loss, inflammation, and risk of infection, but later administration increases the risk of bleeding and/or thrombosis(56,145,199). While plasmin has been shown to exacerbate an inflammatory response in infection and injury, it has conversely been shown to be equally important in resolving the inflammatory response(150,200), making plasmin essential later for the repair of all tissues(1,16,34,201). In this study, while early inhibition of plasmin activity reduces markers of inflammation, enhancing plasmin activity early in convalescence did not increase

inflammatory markers, suggesting that plasmin activity may be maximized in this model or plasmin's pro-inflammatory effects may be dependent on the magnitude of injury. Alternatively, enhancing plasmin activity later following a severe injury may promote repair, while inhibiting plasmin later may have pathologic effects on tissue repair, but this requires further study. Collectively, this highlights the potential therapeutic benefit to inhibiting plasmin following trauma is time-dependent, and therefore, accurate *in vivo* diagnostic measures of plasmin activity are required to inform antifibrinolytic therapy.

While this study aimed to provide a comprehensive translational study, there were limitations. The human burn patients analyzed had variable burn severities in order to measure the heterogeneous responses to the injuries, however, some of these patients had minor, secondary injuries and differing surgical interventions along with the burns that may have added variability to the cohort. Alternatively, while the mouse burn-polytrauma model was consistent, it did not reach the severity of some of the human burns and therefore did not provoke as severe of a systemic response. Future studies are needed to evaluate the benefits of therapeutically targeting plasmin to reduce inflammation and organ dysfunction in larger animal models with greater injury severity.

Conclusion:

Severe burn injuries provoke clinically significant plasmin activation. Although hyperfibrinolysis has been the area of primary concern for plasmin activation following severe injury, plasmin also plays a role in severe injury-induced SIRS in the absence of bleeding. The results of this study suggest that early inhibition of plasmin following severe injury by antifibrinolytics may provide further therapeutic benefit in patients with severe injuries by reducing SIRS and the risk of SIRS-related complications.

METHODS

Human Burn Patients

Burn patients admitted to the Vanderbilt University Medical Center burn unit with ≥5% total body surface area (TBSA) burns were prospectively enrolled into this study upon consent from either the patient or closest family member. Patients with electrical burns, multiple severe injuries, or Stevens-Johnson syndrome were not included in this study. Thirty-one burn patients were recruited in total. Blood samples were collected from burn patients at 0.5, 1, 3, 5, and 7 days post-burn or until discharge or mortality. Demographic data was collected from patient charts including age, sex, and body mass index (BMI). Study-related data collected from the charts included TBSA affected, the presence of inhalation injury, routine complete blood counts throughout hospitalization, relevant surgical procedures, and mechanism of burn. Burn severity was calculated using the revised Baux score as previously described(202) to account for patient age, TBSA affected by the burn, and the presence of inhalation injury. Additionally, serial creatinine, bilirubin, FiO_2, PaO_2, platelet counts, and blood pressure values taken from routine laboratory tests and Glasgow Coma Scores were collected for calculation of Sequential Organ Failure Assessment (SOFA) as previously described(151). A single blood sample was collected from 15 healthy individuals to serve as controls.

Animals

For all experiments, 6-8-week-old, 18-21g male and female mice on a C57BL/6J background were used with matched animals for each treatment group. Wild-type mice were obtained from Jackson Laboratory (Bar Harbor, MN), and plasminogen (Plg)-deficient mice background were obtained originally from the lab of Dr. Jay Degen. For *in vivo* quantification of NF-κB activity, transgenic mice expressing GFP and luciferase downstream of an NF-κB-activated 5'HIV-LTR promoter

(NGL) were used as previously described(168). Wild-type and NGL mice were provided standard laboratory chow, and Plg-deficient mice and comparable wild-type littermate controls were provided synthetic laboratory chow (Research Diets Inc., New Brunswick, NJ) and water ad libitum. All mice were housed in a 12-hour light-dark cycle within a designated animal facility at Vanderbilt University.

Burn Injury Model:

To assess the systemic effects of severe burn on local and systemic inflammation, a well-established model of burn injury(165) was employed. Thirty minutes prior to the burn, mice were administered 0.1mg/kg subcutaneous buprenorphine. Under 3.0% isoflurane general anesthesia, mice were shaved along the dorsum, and 1mL of sterile saline was injected subcutaneously along the spine to prevent injury to deep tissues during the burn. Bilateral calf muscle injuries were administered by intramuscular injections of 10μM cardiotoxin, as previously described(65). Following, the mice were placed in a heat-resistant template with a cutout to expose the dorsum and submerged in 100°C water bath for 10 seconds to create an approximately 30% total body surface area (TBSA), full thickness burn to the dorsum. Immediately following the burn, a resuscitative intraperitoneal injection of 2mL Lactated Ringer's solution was administered, and mice were monitored for 30 minutes following the injury. A maintenance dose of buprenorphine was administered every 8-12 hours for 72 hours, and all animals were housed in individual sterile cages following the burn injury. Experimental controls received the same treatment and muscle injuries in the absence of burn. Blood was collected at 2, 6, 24, and 48 hours post-burn for serological analysis.

Pharmacologic manipulation of plasmin activity

To inhibit plasmin activation, clinical antifibrinolytic drug tranexamic acid (TXA) was administered at 1000mg/kg intraperitoneally beginning immediately after the burn and every 3 hours up to 9 hours post-burn, and control animals received injections of saline. Alternatively, mice received a validated antisense oligonucleotide (ASO) against plasminogen (IONIS Pharmaceuticals, Carlsbad, CA) to knockdown plasminogen expression, or a non-targeted control ASO(33). All antisense oligonucleotides (ASOs) were developed and provided by IONIS Pharmaceuticals (Carlsbad, CA). Plasminogen (AGTGATGGTCTATTGTCACA) and control ASOs (CCTTCCCTGAAGGTTCCTCC) were administered at 330mg/kg by subcutaneous injection weekly beginning 2 weeks prior to injury and continuing for the duration of the study. These ASOs were synthesized, purified, and tested for efficacy as previously described(33).

To enhance existing plasmin activity provoked by the burn, animals received a validated ASO against plasmin's primary inhibitor, α2-antiplasmin (α2AP) (CACTGGTGATGGTCCTTCCG), or control ASO as described above. To increase the amount of plasmin activated at the time of injury, a combination approach was implemented to significantly increase plasmin activation at the time of burn. Mice were placed on synthetic laboratory chow supplemented with a specific inhibitor of plasminogen activator inhibitor-1 (PAI-1), MDI-2517 (MDI Therapeutics, Inc, Novi, MI) received from Dr. Daniel Lawrence at University of Michigan. The PAI-1 inhibitor was administered at 500mg/kg of food beginning one week prior to the procedure, and the mice also received an intravenous injection of 10mg/kg recombinant human tissue plasminogen activator (Activase™, Genentech, San Francisco, CA) as previously described(203) at the time of burn. Control animals received synthetic chow without PAI-1 inhibitor one week prior to the procedure and were administered intravenous saline at the time of burn.

In vivo Imaging

Imaging of injured muscle for bioluminescence was conducted using the Xenogen IVIS Spectrum (PerkinElmer, Waltham, MA). Under brief anesthesia, NGL mice received a retro-orbital injection of 75mg/kg D-luciferin (PerkinElmer, Waltham MA) in sterile saline. Mice were imaged within 5 minutes of injection with an exposure time of 1.0 s. Image quantification and analysis were conducted using IVIS Living Image 4.3.1 software (PerkinElmer, Waltham, MA). All mice were imaged prior to injury and 1, 3, 5, and 7 days post-injury, and a subset of mice were imaged at 10 and 14 days post-injury.

Blood and Plasma Measurements

For human plasma measurements, citrated blood was centrifuged at 1,500xg and then 13,000xg for 15 minutes each to produce platelet poor plasma (PPP). Commercially available and validated ELISAs were used to measure plasma plasminogen, plasmin-antiplasmin (PAP) complexes, and thrombin-antithrombin (TAT) complex (Molecular Innovations, Novi, MI). Plasma P-selectin, CD40L, IL-6, IL-1β, C5a, and D-dimer were measured on a custom multiplex panel using validated, commercially available antibodies at a 1:2 plasma dilution (R&D Systems, Minneapolis, MN). Plasma plasminogen levels were measured using a fluorogenic plasmin activity assay (adapted from Abcam (ab204728) Cambridge, MA). Plasma samples and control pooled plasma (George King Bio-Medical Inc., Overland Park, KS) were diluted 1:10, 1:20, and 1:50 in HEPES buffer. Diluted plasma and fluorogenic substrate (420μM final) (H-D-Val-Leu-Lys-AFC, Anaspec, Fremont, CA) were added in duplicate to the wells of a 96 well plate. Plasmin activation was initiated by the addition of 0.5U/μL streptokinase (MilliporeSigma, Burlington, MA) and fluorescence (excitation at 380nm, emission at 500nm) was measured every 30 seconds for 1 hour using a Synergy 2 plate reader (Biotek, Winooski, VT). Initial rates

of plasmin generation were calculated and plasminogen levels were reported as a percent of pooled control plasma.

For mouse plasma measurements, PPP was isolated as described above, and IL-6 (1:2 plasma dilution) (Abcam, Cambridge, MA) and PAP (1:100 plasma dilution) (Biotang USA, Albuquerque, NM) were measured by validated ELISA. Mouse complete blood counts were measured using the Forcyte Hematology Analyzer (Oxford Science, Oxford, CT) at the Vanderbilt University Medical Center Translational Pathology Shared Resource.

Statistics

To compare groups at different time points with a control group in both humans and animals, Kruskal-Wallis tests were performed with post-hoc Dunn's test to correct for multiple comparisons. Pearson's correlation was used to determine a linear associative relationship between variables, including plasmin activation, inflammation, coagulopathy, and patient outcomes. For Kaplan-Meier analysis, a log-rank test was used to determine predictors of mortality within 7 days of burn. To compare treatment groups with control treatment over time in animal experiments, multiple Mann-Whitney U tests were performed with Holms-Sidak correction for multiple comparisons between groups over time. All tests were performed in GraphPad Prism version 9.1.2.

Study Approval

All human study procedures were approved by the Vanderbilt University Medical Center Institutional Review Board (Protocol #150751). All animal study procedures were approved by the VUMC Institutional Animal Care and Use Committee (Protocol # M1800154).

Chapter 2 Summary:

Because plasmin holds diverse roles in physiology, including the modulation of inflammation and coagulation, a dysregulation of plasmin activity in a pathologic APR has the potential to drive pathologic effects, the sum of which drive complications of severe injuries, including bleeding, thrombosis, and organ dysfunction.

In a controlled trauma, elective posterior spinal fusion surgery, "breakthrough" plasmin activation in the presence of antifibrinolytic treatment was associated with greater blood loss and inflammatory markers. Further, the magnitude of intra-operative fibrinolysis was associated with a loss of platelet function during the procedure. In an uncontrolled trauma without immediate bleeding risk (severe burn), plasmin activation was associated with markers of coagulopathy, inflammation, and mortality. Early inhibition of plasmin following a severe burn injury reduced not only circulating markers of inflammation, but also inflammatory signaling within injured tissue. The results of this work collectively suggest that excess plasmin activity during a survival-APR is a mechanistic driver of systemic inflammation, a significant instigator of thrombosis and organ dysfunction. Plasmin activity during the survival-APR was also associated with different markers of coagulopathy, including platelet dysfunction, platelet loss, and blood loss. Because the pathologic effects of plasmin are dependent upon the magnitude of plasmin activity provoked by the injury and the severity of the injury, accurate diagnostics will be required to measure early plasmin activity and effectively treat it to reduce the risk of adverse outcomes in patients with severe injuries or those undergoing invasive elective surgical procedures.

CHAPTER 3: PLASMIN IN MUSCLE REPAIR FOLLOWING BOTH NON-SEVERE AND SEVERE INJURIES

Murine Models of Severe Injury and Repair

Severe injuries provoke a systemic response that has global pathologic effects on multiple organ systems, including the musculoskeletal system. Many severe injuries also occur as polytraumas, meaning that they may occur with other minor or severe injuries(38,197). Consequently, severe injuries often result in poor repair of even minor injuries and degeneration within peripheral tissues, the consequences of which include chronic pain, tissue fibrosis, bone loss (osteoporosis), and loss of limb function, provoking significant morbidity for years following the injury(42,204–206). Because plasmin is essential for musculoskeletal repair through multiple mechanisms and severe injuries often provoke a period of poor plasmin activity during repair(1), we examined models that could be implemented to assess the global effects of severe injury on muscle and bone health with manipulation of plasmin. Our particular studies required a polytraumatic injury that includes a severe injury. Validated murine models of severe injury are difficult due to the size of the animals and a lack of consistency. There are many polytrauma models used in rodents, including blast, traumatic brain injury, or spinal cord injury. In our model, we wanted to include a severe injury with a concomitant minor (muscle) injury completely remote to the site of burn to observe how the burn altered the microenvironment and repair within the injured muscle. Another obstacle we faced is that mice recover and repair from injuries typically faster and without complication compared with human patients. Therefore, we developed a model of severe burn (30% TBSA) with concurrent, bilateral calf muscle injuries, and control animals received only the bilateral calf muscle injuries. We used this model in Article 2 to assess how

severe injury alters global inflammation and inflammation within injured tissues, and then we used the same model in Articles 3 and 5 to investigate how a severe burn or various plasmin deficiencies affect repair of the remote muscle injury.

Article 3: Severe Injury-Induced Osteoporosis and Skeletal Muscle Mineralization: Are these Related Complications?

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1. Background:

Due to advances in critical care medicine, patients now survive severe injuries, such as burn, blast, or polytraumatic injuries, that were once considered fatal. While being fortunate to survive, many patient experience complications during convalescence, including poor tissue repair, and hemostasis(1,207–209). While severely injured[1] patients are beleaguered by a variety of systemic derangements, one of the more paradoxical complications involves the dysregulation of biomineralization(179,210–212). Specifically, patients can experience a loss of mineralization from the skeleton, referred to as severe injury-induced osteoporosis, that diminishes skeletal integrity, delays rehabilitation, increases length of hospital stays, and increases the risk of fragility fractures(42,213). Furthermore, severely injured patients can also experience the accrual of mineralization in soft tissues such as skeletal muscle, resulting in complications such as dystrophic calcification (DC) and heterotopic ossification (HO). A recent report in *CTI* demonstrated that DC, if persistent within damaged skeletal muscle, is sufficient to promote HO formation(65), which clinically is associated with pain, joint dysfunction, and, in severe cases, may necessitate amputation of the affected limb(212,214). Although estimates vary, it has been reported that up to 80% of patients develop HO after a severe injury. Furthermore, severe injury-induced HO has also complicated more than 60% of the severe orthopaedic extremity injuries during the Afghanistan and Iraq conflict(63,204).

[1] "Severe injury" or "severely injured patients" refers to injuries that have a high risk for mortality. Examples include major mono- or poly-traumatic injuries, burns, blast, spinal cord injuries, traumatic brain injuries, etc.

Following an injury, the acute phase response (APR) is activated in proportion to the injury severity(1,2,45). To ensure survival, the APR first activates coagulation and an acute inflammatory response to stop bleeding and prevent infection. Proportional to the amount of tissue injury, an exuberant survival inflammatory response following a severe injury can drive systemic changes in the musculoskeletal system(1,2,215). Both pre-clinical and clinical studies have examined the molecular connections between traumatic injuries and hypermetabolism, skeletal muscle wasting, and rapid bone turnover(215–220). However, few studies have examined the relationship between severe injury-induced bone turnover, resulting in osteoporosis, and soft tissue calcification of injured tissues.

The overarching hypothesis of this study was that a shared pathophysiology exists, such that a "transfer" of mineralization from the bone to soft tissue compartments can occur. To determine if severe injury-induced osteoporosis and pathologic soft tissue calcification are mechanistically linked, we utilized a combined burn and skeletal muscle injury to model of severe injury. Unlike other preclinical severe injury models, burn injuries have a low risk of hemorrhage, thereby allowing for consistent investigation of the late complications of convalescence. This study aimed to examine if severe injury-induced osteoporosis and pathologic soft tissue calcification were temporally coincident. If found true, this temporal relationship may indicate a unifying pathophysiology between events and thereby provide the basis for future mechanistic and pharmacologic studies to help minimize soft tissue calcification in severely injured patients, such as combat veterans.

2. Materials and Methods:

2.1 Experimental Overview: The overarching goal of this study was to examine, in a murine model of severe injury, if severe injury-induced osteoporosis and soft tissue calcification were temporally coincident, and therefore potentially pathologically connected. To examine this hypothesis, following a burn and/or skeletal muscle injury, bone quality and the presence of soft tissue calcification were assessed using both longitudinal and endpoint measures (**Figure 3.1**).

2.2 Animal Husbandry: All animal procedures were approved by Vanderbilt University IACUC and performed in accordance with ethical standards of the institution (M/15/024, M1800154). All studies were conducted in 6-week-old male C57BL/6J mice, weighing 20–25g. Mice were individually housed in a regulated facility with 12-h light-dark cycle and provided food (5LOD chow) and water *ad libitum*.

2.3 Cardiotoxin-Induced Skeletal Muscle Injury Mouse Model: Skeletal muscle injury was induced by an intramuscular injection of 40 μL of 10uM Cardiotoxin (CTX, Accurate Chemical and Scientific Corp; Westbury, NY) in the posterior compartments of the left and right lower limbs, as previously described(33,65,221).

2.4 Burn Injury Mouse Model: This model was adapted from a separate burn wound model(222) and modified here to be conducted in conjunction with or without a skeletal muscle injury to the lower extremity to model severe injury. Following a subcutaneous injection of buprenorphine (0.5-mg/kg) 30 min prior to the burn procedure, mice were anesthetized with isoflurane and the dorsal hair was removed **(Figure 3.1A).** Following hair removal, if the mouse was designated to receive a focal skeletal muscle injury, the gastrocnemius and soleus muscles were injected with CTX at

this time (**Figure 3.1B**). 1ml of saline was injected subcutaneously posterior to the spine to protect the underlying spinal column from thermal injury (**Figure 3.1C**). The mouse was then placed in a heat-resistant template (**Figure 3.1D&E**) and partially submerged in a 100°C water bath for 10sec to create a full thickness cutaneous burn covering approximately 30% of the total body surface area (**Figure 3.1F**). Immediately following the burn, the mouse was dried and injected with 2ml of intraperitoneal fluid resuscitation with lactated Ringer's solution.

2.5 Radiographs: At 7, 14, 21, and 28 days post injury (DPI), mice were analyzed by digital radiography for the development of mineralization within the injured lower extremity as previously described (**Figure 3.1G**)(33,65,221). Soft tissue mineralization was quantified using the previously validated soft tissue calcification scoring system (STiCSS)(221). Briefly, the operational definitions of each score are based on the percentage area of soft tissue calcification observed in the posterior compartment of the lower extremity: 0 (0%), 1 (1-25%), 2 (25-49%), 3 (50-75%) and 4 (>75%).

2.6 Longitudinal Dual energy X-ray absorptiometry (DXA) Imaging: Immediately prior to injury and at 1, 2, 5, 7, 14, 21, and 28 DPI, DXA imaging (Faxitron, LX-60) was obtained with an average of 3 images (**Figure 3.1G**). Mice were laid in the prone position with their muzzle placed in a nose cone to maintain anesthetization. Legs of the mouse were placed laterally away from the body of the animal to obtain a clear image of the femurs. Scans were checked for quality, as movement of the mouse can result in aberrant measure. The whole femur was selected utilizing the arbitrary ROI analysis tool, and bone mineral density (BMD) values were obtained in g/cm^2. Measures from the left and right femurs from a single mouse were averaged together and reported as a single value

within. As mice were not fasted as part of this study, vertebral measures were found to be unreliable due to the presence of food pockets(223).

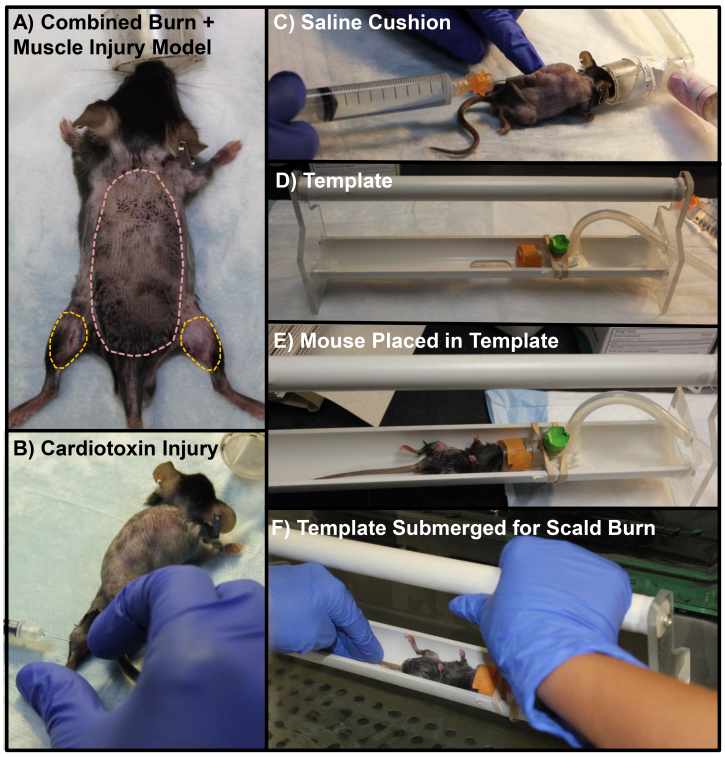

Figure 3.1: Combined burn and skeletal muscle injury to model severe injury. To phenocopy the clinical condition of a severe injury, a A) 30% total body surface area full-thickness cutaneous burn was applied in combination with a remote focal skeletal muscle injury. Prior to burn injury, B) an intramuscular injection of cardiotoxin (CTX) is applied to the gastrocnemius and soleus muscles to induce a focal muscle injury. A medial approach is utilized to injure both the left and the right lower extremities prior to burn injury. C) Placement of a 1mL subcutaneous saline cushion to protect the spine during burn injury. D) To control the burn region on the dorsum of the mouse, a heat resistant template is utilized. The mouse is placed in the template (E), and the template is partially submerged in a 100°C water bath (F) for 10 seconds. G) Both longitudinal and end point assessments were carried out at 1, 3, 5, 7, 14, 21, or 28 days post injury as noted in the experimental timeline. Mice were sacrificed at 1, 3, 7, or 28 days post injury.

2.7 Micro-Computed Tomography (μCT): To more sensitively assess changes in bone quality, μCT imaging was performed on the distal femur, distinct from the site of skeletal muscle mineralization at 1, 3, 7, and 28 DPI (**Figure 3.3G**), as previously described(33,65,221). The bone volume/tissue volume (BV/TV) ratio, trabecular thickness, trabecular space, and trabecular number were assessed within the metaphyseal region. Average cortical thickness, total cross sectional area, and tissue mineral density were assessed within the diaphyseal region(224). To visualize skeletal muscle mineralization, μCT imaging of the gastrocnemius and soleus muscles were conducted at 7 DPI as previously described(33,65,221).

2.8 Histologic Analysis of Skeletal Muscle Mineralization: Injured skeletal muscle was isolated at 7 and 28 DPI (**Figure 3.3G**) and routinely fixed in 10% neutral-buffered formalin, processed, and embedded(65). 6μm sections were stained with hematoxylin and eosin (H&E) or von Kossa to visualize mineralization(33,65). At least 5 mice were analyzed per cohort/timepoint with >3 sections per mouse. Images included in manuscript represent the average result per cohort.

2.9 Statistical Analysis: Differences in BMD from DXA assessment were assessed utilizing a 2-way ANOVA corrected for multiple comparisons. Differences in endpoint μCT parameters were assessed individually at each time point between cohorts and between 1 and 28 DPI in the same cohort by a non-parametric Mann-Whitney U test given that they are discrete samples. Difference in STICSS Score were analyzed with a non-parametric 1-way ANOVA corrected for multiple comparisons. Statistical analyses were performed in GraphPad Prism (v6, GraphPad Software, La Jolla, CA) with $\alpha=0.05$. Two-sided testing was applied throughout. N>3 for all timepoints assessed. Significance reported was relative to adjusted P values where appropriate.

3. Results

3.1 Severe Injury-Induced Osteoporosis: In mice receiving a combined burn and CTX muscle injury (model of severe injury), significant bone loss was observed compared to mice receiving only a CTX muscle injury (lesser injury)(**Figure 3.2A).** Longitudinal DXA imaging detected a significant reduction in average BMD of the left and right femurs by 7 DPI. Given that the mice examined are still undergoing bone development, a significant change in BMD between 5 and 28 DPI ($p=0.050$) was observed in mice receiving a CTX injury, indicative of longitudinal bone growth. Alternatively, over the same time period, no change in BMD was observer in mice that received CTX + Burn injury ($p=0.627$), leading to the greatest magnitude change in BMD between cohorts at 28 DPI (**Figure 3.2A**). To more sensitively measure changes in bone quality and bone architecture, samples were collected and analyzed by μCT. By 3 DPI, a significant loss of percent metaphyseal bone volume/tissue volume, reduced trabecular thickness and trabecular number, and increase in trabecular space was detected in mice receiving CTX + Burn injury.(**Figure 3.2B&C**, **Table 3.1**). Assessment of cortical bone volume at 28 DPI between cohorts revealed a significant loss in the average cortical bone thickness in mice receiving CTX + Burn injury compared to unburned controls (CTX-0.157 ± 0.013; Burn + CTX-0.135 ± 0.006; $p=0.001$), with no marked changes in total cross-sectional area of the diaphysis ($p=0.279$) or tissue mineral density ($p=0.493$).

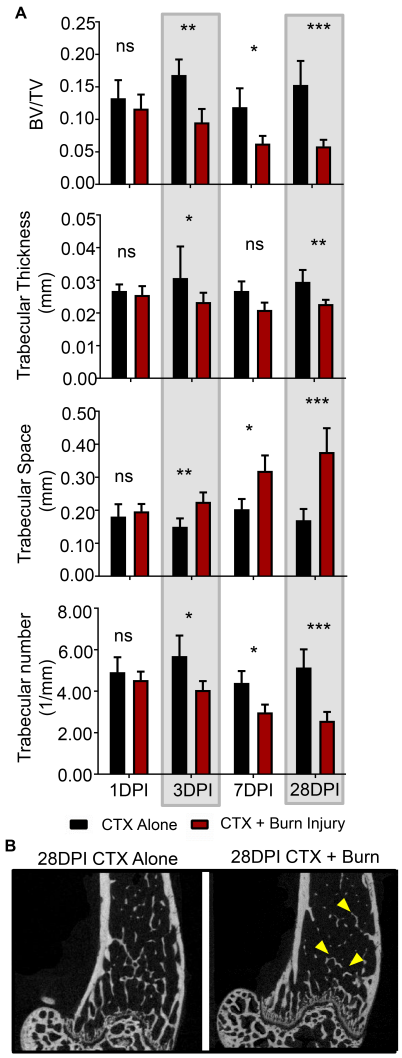

Figure 3.2: Assessment of severe injury-induced osteoporosis in a murine model of combined burn and skeletal muscle injury. A) Pre-injury and post-injury DXA measures from 1 through 28DPI. DXA measures of the left and right femurs were averaged and reported per mouse. B) To more sensitively assess bone architecture and bone loss, mice were sacrificed at 3, 5, 7, and 28 DPI, and the distal metaphysis was assessed for changes in trabecular bone, measured by %BV/TV, trabecular thickness, space between trabeculae, and trabecular number. C) 2D cross section of the distal metaphysis of the femur in the XY plane. Yellow arrows indicate areas of reduced trabecular bone. Error bars represent mean + SD in all images. * notes significance (p) less than 0.05, ** P<0.01, *** P<0.001.

	1 DPI		3 DPI		7 DPI		28 DPI		*Analysis between 1 and 28 DPI*	
	CTX (N=5)	**CTX ± Burn (N=7)**	**CTX (N=5)**	**CTX ± Burn (N=6)**	**CTX (N=3)**	**CTX ± Burn (N=6)**	**CTX (N=10)**	**CTX ± Burn (N=10)**	**CTX**	**CTX ± Burn**
%BV/TV	13.3 ± 2.8	16.6 ± 2.2	16.8 ± 0.024	9.5 ± 2.1 **	11.9 ± 2.9	6.3 ± 1.2 *	15.3 ± 3.7	5.8 ± 1.0 ***	ns, p=0.636	***, P<0.001
Trabecular Thickness (µm)	27 ± 2	26 ± 3	31 ± 10	23 ± 3 *	27 ± 3	21 ± 2	30 ± 4	23 ± 1 **	ns, p=0.723	ns, p=0.622
Trabecular Space (µm)	181 ± 37	197 ± 22	150 ± 26	225 ± 29 **	203 ± 31	319 ± 47 *	170 ± 34	376 ± 72 ***	ns, p=0.998	***, P<0.001
Trabecular Number (1/mm)	4.92 ± 0.72	4.54 ± 0.41	5.69 ± 0.99	4.06 ± 0.43 *	4.40 ± 0.57	2.98 ± 0.38 *	5.14 ± 0.88	2.57 ± 0.43 ***	ns, p=0.988	***, P<0.002

Table 3.1: Statistical analysis of bone loss following burn in mice. Detailed statistical analysis of trabecular and cortical bone loss between mice receiving a combined burn and skeletal muscle injury (Burn + CTX; model of severe injury) or muscle injury alone (CTX; lesser injury) measured by endpoint µCT analysis. * notes significance (P) less than 0.05, ** P<0.01, *** P<0.001.

3.2 Skeletal Muscle Mineralization: By 7 DPI, marked skeletal muscle mineralization was observed in the gastrocnemius and soleus muscles by radiographic analysis and μCT reconstruction in mice that received CTX + Burn injury. Importantly, no significant mineralization was observed at any timepoint in mice that received either a focal skeletal muscle injury (CTX) or a burn injury alone (**Figure 3.3A**). Histological analysis confirmed that the skeletal muscle mineralization observed at 7 and 28 DPI was morphologically indicative of DC (**Figure 3.3B**). Longitudinal radiographic analysis demonstrated that the deposited DC, while significantly greater in mice receiving CTX + Burn injury at 7DPI, progressively regressed from injured muscle between 7 and 28 DPI resulting in comparable levels of mineralization to CTX controls by 28 DPI(**Figure 3.3C**).

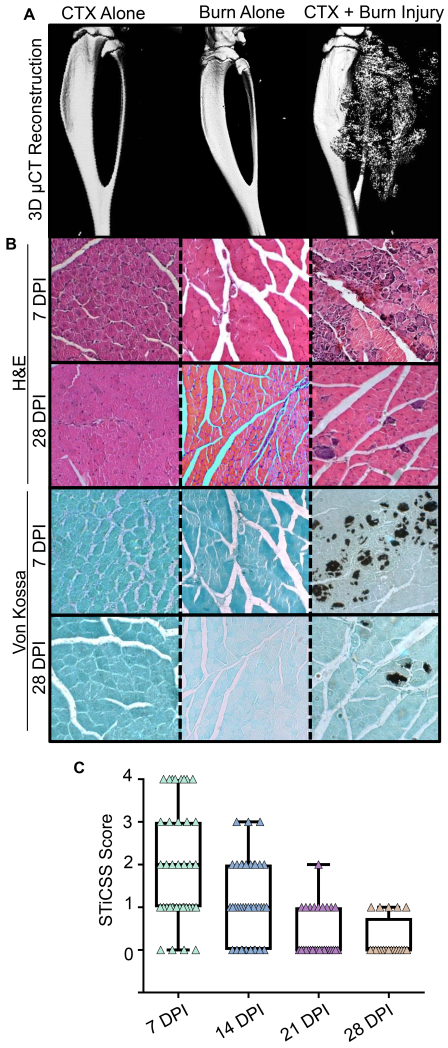

Figure 3.3: Assessment of skeletal muscle mineralization in a murine model of combined burn and skeletal muscle injury. A) By 7 DPI, marked skeletal muscle mineralization was radiographically evident by 3D µCT reconstruction. B) Representative histologic analysis is morphologically indicative of dystrophic calcification. N≥3 for each cohort. C) Longitudinal radiographic assessment and STiCSS scoring in mice undergoing CTX or CTX + Burn injury. Given that both the left and right lower limb is injury, each point upon the graph represents a single limb. STICSS: CTX: 7DPI- N=12 limbs assessed. CTX + Burn injury: 7DPI- N= 38 limbs assessed, 14 DPI- N= 38 limbs, 21 DPI- N=30 limbs, 28 DPI- N= 20 limbs. Error bars represent median + interquartile range. * notes significance (p) less than 0.05, ** P<0.01, *** P<0.001.

4. Discussion:

This study identified that a temporal concordance exists in which both severe injury-induced osteoporosis and soft tissue mineralization occur. Specifically, progressive bone loss was observed as early as 3 DPI with μCT assessment and a subsequent gain in DC that was radiographically evident by 7 DPI. These results demonstrate the plausibility that a unifying pathophysiology may exist between these complications in which a "transfer" of mineralization from the bone to soft tissue compartments may occur, similar to what has been described previously in cardiac literature(225).

Under a homeostatic state, plasma contains near-saturating levels of calcium and phosphate(226). These levels are essential to allow for proper cellular signaling and to maintain musculoskeletal integrity. Yet, soft tissues are therefore, likewise exposed to these near-saturating levels and can experience pathologic mineralization if a nidus, such as tissue injury, occurs. Therefore, throughout life the body employs protective mechanisms, such as plasmin, pyrophosphate, and osteopontin, to block the mineralization within soft tissues(33,214,226). As we age, in addition to accruing microtissue injures, the body likewise experiences a diminished capacity to regulate biomineralization, leading to conditions such as calcific artery disease and osteoporosis(227–231). Given these temporal changes under homeostatic conditions, it is also possible that age may influence the body's capacity to regulate biomineralization following a severe injury. This is observed in both burn patients and those with traumatic injuries, where the incidence of HO is estimated to be lower in pediatric patients compared to adults with injuries of similar severity(211,232–235). Interestingly, reports from veterans suffering polytraumatic injuries indicates that a lower age, specifically <30 years of age, was independently predictive of

heterotopic ossification development on multivariate analysis(63). While many mechanisms can influence this change in incidence, further studies characterizing the capacity for protection mechanisms to regulate biomineralization throughout life may provide critical insight and pharmacologic direction.

Severe injuries such as burn or polytraumas can lead to the systemic derangement of multiple body systems including coagulation, inflammation, and the immune system(210,215,219,236,237). Given the quick onset of bone loss following severe injuries, prior studies have attributed bone loss to a variety of mechanisms including endogenous glucocorticoids, production of pro-inflammatory cytokines, progressive vitamin D deficiency, and disruption of calcium homeostasis(215,238). Independent of the mechanism, numerous studies have illustrated that release of calcium, phosphate, and magnesium from the bone can have systemic effects on muscle physiology, regulation of inflammation, and glucose handling. To date, few investigations have examined how severe injuries can predispose soft tissues to pathologic mineralization. Given the temporal concordance between the development of osteoporosis and soft tissue calcification, it is plausible that a severe injury can 1) drive bone-turnover, thereby increasing the availability of calcium and phosphate in the plasma (215,239) and 2) simultaneously diminish soft tissue protection mechanisms against calcification(60,102,119,240); thereby establishing an environment in damaged skeletal muscle where DC formation is favorable. Given the plausibility of these complications sharing a common pathophysiology, further experiments investigating a "transfer of compartment" hypothesis is warranted. If found to be true, pharmacologic interventions aimed at reducing bone turn over may likewise reduce the risk of soft tissue calcification; thereby adding to the growing body of literature illustrating the potential clinical

benefit of administering anti-catabolic agents in patients following severe injuries later in convalescence(241,242).

While previously thought to be distinct pathologies, a prior study demonstrated that DC formation within skeletal muscle, if persistent, is sufficient to form HO(65). Here, progressive regression of DC from skeletal muscle was observed over 28DPI, indicating that that the regressive mechanisms, previously determined to act through macrophage phagocytosis(65), are still functioning in our model. However, if the severity of the injury was increased or comorbidities were added to the model, both of which are linked to reduced macrophage function, DC regression may be impaired, leading to the possible formation of HO.

Injury is an essential component of skeletal muscle mineralization and the formation of HO. While clinical studies have detected the development of mineralization in muscle following a burn injury(214), the location of these mineralized deposits is often unpredictable, making them difficult to reliably detect and track longitudinally. Unlike patients clinically, the ability to control essential variables is possible in a pre-clinical animal model. For example, the location of injury can be controlled in a pre-clinical animal model, allowing researchers to better predict the location of skeletal muscle mineralization. Yet, when utilizing an animal model of disease, one should consider the ability of the model to phenocopy the clinical condition and outcomes to support translation of associated findings.

5. Conclusion:

By utilizing a murine model of combined burn and skeletal muscle injury as a model of severe injury, this study effectively examined late complications of convalescence, specifically dysregulated biomineralization. This study found that a temporal concordance exists in which both severe injury-induced osteoporosis and soft tissue mineralization occur. Going forward, future studies are required to investigate potential mechanistic links between severe injury-induced osteoporosis and soft tissue mineralization, as well as potential therapeutic interventions for mitigating dysregulated biomineralization. The murine model presented within this manuscript can be utilized in such mechanistic studies, providing the foundation for future clinically translatable work.

Plasmin in tissue repair

Plasmin is essential for the repair of all tissues, including musculoskeletal tissues. One way in which plasmin promotes musculoskeletal health is by regulating bone formation: plasmin prevents bone formation in soft tissues but promotes healthy bone formation during development and during fracture repair(33,36). The mechanisms behind this paradox, and the plasminogen activators and receptors responsible for this were previously unknown. Plasminogen activators, tPA and uPA, play different roles in biology, although they can, at times, compensate for one another. The plasminogen activation system in musculoskeletal health is detailed in the following Article 4.

Article 4: Plasminogen activation in the musculoskeletal acute phase response: injury, repair, and disease

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Introduction

The musculoskeletal system serves primarily to counteract gravity, protect vital structures, and allow locomotion. These functions rely on the synergistic interactions of bones and muscles. Muscles attach to bones through tendons and lever them through bone articulations, or joints(2,243,244). Additionally, the musculoskeletal system serves as a primary hematopoietic

center and a systemic regulator of essential minerals, including calcium and phosphate (243,244). Proper function and maintenance of the musculoskeletal system is therefore essential for these processes.

The musculoskeletal system is repeatedly injured as the physical demands of daily life outweigh the capacity of the tissue to absorb strain(245). Injuries to the musculoskeletal system differ in nature, severity, and immediate impact but can all lead to chronic complications without timely repair(2,245,246) For instance, everyday engagement of the musculoskeletal system results in daily microinjuries, instigating reparative processes imperceptible to the individual(2,247) Alternatively, a significant acute injury, such as a muscle tear or a bone fracture, immediately obviates limb or organ function until function is restored(247). When musculoskeletal injuries occur in the context of disease, the mechanisms of repair are compromised, and debilitating complications persist. For example, inflammatory diseases, such as diabetes, obesity, and autoimmune conditions, in addition to natural aging result in the loss of muscle (sarcopenia) and bone (osteopenia) as a result of unresolved daily microinjuries(246,248–251). Unresolved injuries or degenerative diseases of the musculoskeletal system cause poor range of motion, chronic pain, and loss of limb function, ultimately resulting in significant disability(252–254). In the U.S., musculoskeletal disease or injury affects every 1 of 2 persons over the age of 18 and accounts for approximately two-thirds of the 68,000 disease conditions listed in the International Classification system of Diseases (ICD-10)(250,255). Therefore, both acute and chronic damage to the musculoskeletal system requires effective and timely repair to preserve function and quality of life.

Coagulation is Critical for Musculoskeletal Integrity and Repair

The high physical demands of the musculoskeletal system require an abundant supply of energy. Consequently, musculoskeletal tissues are highly vascularized(2,243). Regardless of the severity, injuries to musculoskeletal tissue result in the activation of coagulation and fibrinolytic factors that promote the formation and degradation of fibrin for the primary purpose of hemostasis(2,27,246). However, discoveries over the last two decades clearly indicate that the roles of the coagulation and fibrinolytic systems in musculoskeletal injury extend beyond their canonical roles in hemostasis to include proper repair and regeneration of tissues(2,245,256). This review summarizes current knowledge on the roles of fibrin and the fibrin-dependent and -independent roles of plasmin in musculoskeletal repair and degeneration, highlighting the temporal-spatial context of their functions and considerations for pharmacological manipulation of each.

Plasmin and The Acute Phase Response

The enzyme plasmin, classically known for its ability to maintain blood flow by cleaving fibrin and breaking down intravascular clots or thrombi, is also essential for tissue repair through both fibrin-dependent and independent mechanisms(24,33,36,257,258). Since the first observations of plasmin activity by John Hunter and Giovanni Morgagni in the late 1700s(29,259) and Albert Dastre in 1893(259,260), the fibrinolytic properties of plasmin have been recognized as both dangerous and beneficial based on context. Consequently, plasmin has been inhibited by antifibrinolytic drugs to prevent blood loss during trauma or harnessed through administration of its activator, tissue plasminogen activator (tPA) for thrombolysis in ischemic stroke patients(30,54,259,261). In recent decades, additional plasmin functions have been documented that extend beyond fibrinolysis and include promotion of angiogenesis, chemotaxis,

hematopoiesis, and cellular differentiation(34,184,262–264), revealing an essential role in musculoskeletal repair and maintenance. Importantly, because of these diverse functions, plasminogen activation requires tight regulation based on spatiotemporal context following injury.

Following an acute injury to musculoskeletal tissues the body must rapidly respond to resolve bleeding, risk of infection, and tissue hypoxia(2,27,45). To manage these afflictions, a systemic response known as the acute phase response (APR) is initiated(3,265). The APR can be segregated into two phenotypically distinct phases: survival and repair. In the survival phase, coagulation system activation prevents bleeding and restores hemostasis. Simultaneously, an acute inflammatory response functions to prevent infection, effectively containing the initial damage(2,27) (**Figure 3.4A**). During survival-APR, fibrin is deposited in both the intra- and extra vascular spaces(27). Intravascular fibrin stabilizes clots to prevent bleeding, and extravascular fibrin is deposited within injured tissues to form a temporary matrix that prevents pathogen invasion and absorbs strain(2,27). Once the temporary or provisional fibrin matrix is in place and survival is ensured, the body transitions into the repair-APR. Locally generated plasmin is critical during the repair phase to clear fibrin and initiate both the resolution of hypoxia and restoration of tissue function(16,24,184). First, plasmin degrades deposited fibrin and stimulates a reparative inflammatory response to promote the removal of damaged and necrotic tissue(142,185). Once the fibrin and damaged tissues are removed, plasmin activity continues in the damaged zone to promote angiogenesis or revascularization to help prevent sustained tissue hypoxia(184,266). Additionally, the plasmin-dependent release of growth factors contributes to remodeling and cellular regeneration, restoring function to the injured tissue(34,267) (**Figure 3.4B**). Effective tissue repair is dependent upon a coordinated sequence of fibrin deposition and plasmin activation, with a specific timing, location, and magnitude for each. Conversely, an imbalance of these

elements in the musculoskeletal system can prevent repair following injury and drive degeneration of these tissues in disease conditions (**Figure 3.6)**.

The diverse functions of plasmin make it both essential and dangerous, depending on the context. Many diseases pathologically alter plasmin's functions, causing both acute and chronic complications due to an excess or lack of plasmin activity(45,53). Excess plasmin activity, either locally or systemically, can exacerbate a plethora of disease conditions. For example, traumatic injuries and invasive surgeries provoke systemic plasmin activation during the survival phase of the APR, causing aggressive fibrinolysis at the site of injury and subsequent bleeding complications(46,54,82,268) (**Figure 3.4C**). Interestingly, more recent research in trauma has suggested that reduced fibrinolysis based on thromboelastography is associated with poor outcomes, including thrombosis and organ dysfunction (43-44). However, it is unclear if this suggested change in fibrinolysis and possible change in plasmin activity is a causative factor in these outcomes or if it reflects the general dysfunction in inflammation, coagulation, and fibrinolysis that occurs following traumatic injuries. (**Figure 3.4D)**. Furthermore, disease including infection, diabetes, cancer and autoimmune disorders have reported pathologic changes in plasmin activity and/or function (45,53,267,269). Therefore, while plasmin is essential for proper tissue repair, inappropriate plasmin activity can greatly exacerbate pathology in disease conditions.

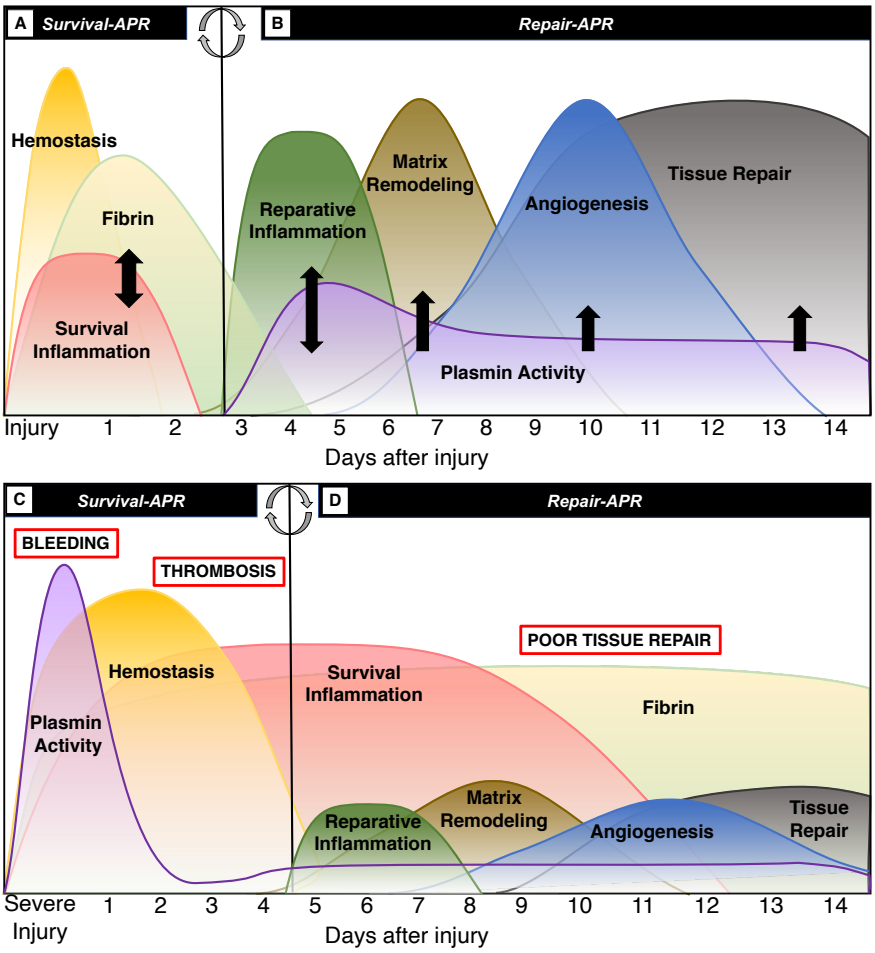

Figure 3.4: **The physiologic and pathologic acute phase response.** A) Following an isolated injury to the musculoskeletal system, the survival phase of the APR exists to contain the injury by activating coagulation and the innate immune system to prevent bleeding and infection, respectively. B) Once the injury is contained, the body enters the repair phase of the APR, during which plasmin is activated to remove fibrin deposited during survival, promote macrophage function, and to stimulate matrix remodeling and angiogenesis. A normal APR resolves the injury without complications. C) A severe or traumatic injury provokes a pathologic APR in which plasmin is activated during survival causing bleeding complications, or it is shut down, increasing risk of thrombosis and D) provoking persistent fibrin deposition and poor tissue repair later in convalescence.

Plasmin and Fibrin in Skeletal Repair

Following a musculoskeletal injury, such as a fracture, thrombin is activated at the site of injury to form a fibrin and platelet sealant that both prevents blood loss and provides a temporary matrix to absorb the strain of the injury(27). Neutrophils are recruited to the fracture where they deposit neutrophil extracellular traps (NETs) which bolster the fibrin matrix against sterile and non-sterile pathology (47). In addition to protecting against microbial invasion, neutrophils also function to promote to tissue repair by augmenting localized inflammation at the site of injury(270–272). Following fibrin deposition and neutrophil activation, infiltrating macrophages present heterodimeric $\alpha_M\beta_2$ integrin on their surface that stimulates an acute inflammatory response upon binding fibrin(273). $\alpha_M\beta_2$ integrin activation stimulates the production of inflammatory cytokines, including IL-1β, IL-6, and TNF-α, promoting further recruitment and activation of pro-inflammatory, anti-microbial (M1) macrophages at the site of the injury(273–275). Plasminogen bound to both fibrin at the site of the fracture and immobilized on the cell surface of infiltrating macrophages is activated to plasmin(2,36). Plasmin facilitates the transition to the repair phase by removing provisional fibrin matrices and stimulating reparative, anti-inflammatory macrophage function(185,191,276). While its role in NET removal remains unclear, plasmin is present in NET matrices and degrades proteins in both NETs and protein aggregates presented on the surface of necrotic cells(103,272,277,278) Plasmin has been shown to reprogram macrophages from a pro-inflammatory (M1) phenotype, to an anti-inflammatory, reparative (M2) phenotype(19,183) and to stimulate chemotaxis and efferocytosis(185,279) in these macrophages. A number of reports have implicated both the interaction of plasmin(ogen) with the Plg_RKT receptor and α-enolase and plasmin-mediated activation of the PAR1 receptor as being critical for plasmin-mediated macrophage function(19,262,279,280). Specifically, plasmin activation of

PAR1 and co-receptors, including integrin $\alpha_9\beta_1$, on macrophages initiates ERK1/2 signaling and expression of CCL2, augmenting phagocytosis and macrophage migration at the site of tissue injury(262,281). Plasminogen binding to Plg_RKT facilitates activation of plasmin on the surface of macrophages, leading to activation of pro-MMP9 to promote macrophage migration(282). In contrast, plasmin increases phagocytosis but inhibits production of inflammatory cytokines and cell migration in dendritic cells, providing an alternative pro-reparative, anti-inflammatory effect of plasmin on phagocytic cells(283). However, one recent study demonstrated that fibrinolysis itself enhances macrophage ingress and egress by preventing macrophage tethering to fibrin through $\alpha_M\beta_2$ binding(284). In addition, the plasminogen activators tPA and urokinase plasminogen activator (uPA) bind to the Annexin A2/S100A10 heterotetrameric complex and the uPA receptor (uPAR) respectively to facilitate activation of plasmin on the surface of macrophages and damaged cells(285–287). Reparative macrophages remove necrotic tissue and fibrin degradation products(288,289), while facilitating the migration of mesenchymal stem cells(290). As plasmin and macrophages remove the fibrin and dead or necrotic tissue, plasmin activates pro-MMP9 and VEGF-A at the site of the fracture to stimulate matrix remodeling and angiogenesis respectively(184,266). With the damaged tissue cleared of fibrin and remodeled, angiogenesis restores the vascularity disrupted by the fracture, allowing bone to form around the fracture site. Within weeks to months, the newly formed bone is remodeled into strong, functional bone capable of withstanding significant force(2,246) (**Figure 3.5A**). Therefore, plasmin provokes a reparative response from macrophages through fibrin-dependent and fibrin-independent mechanisms.

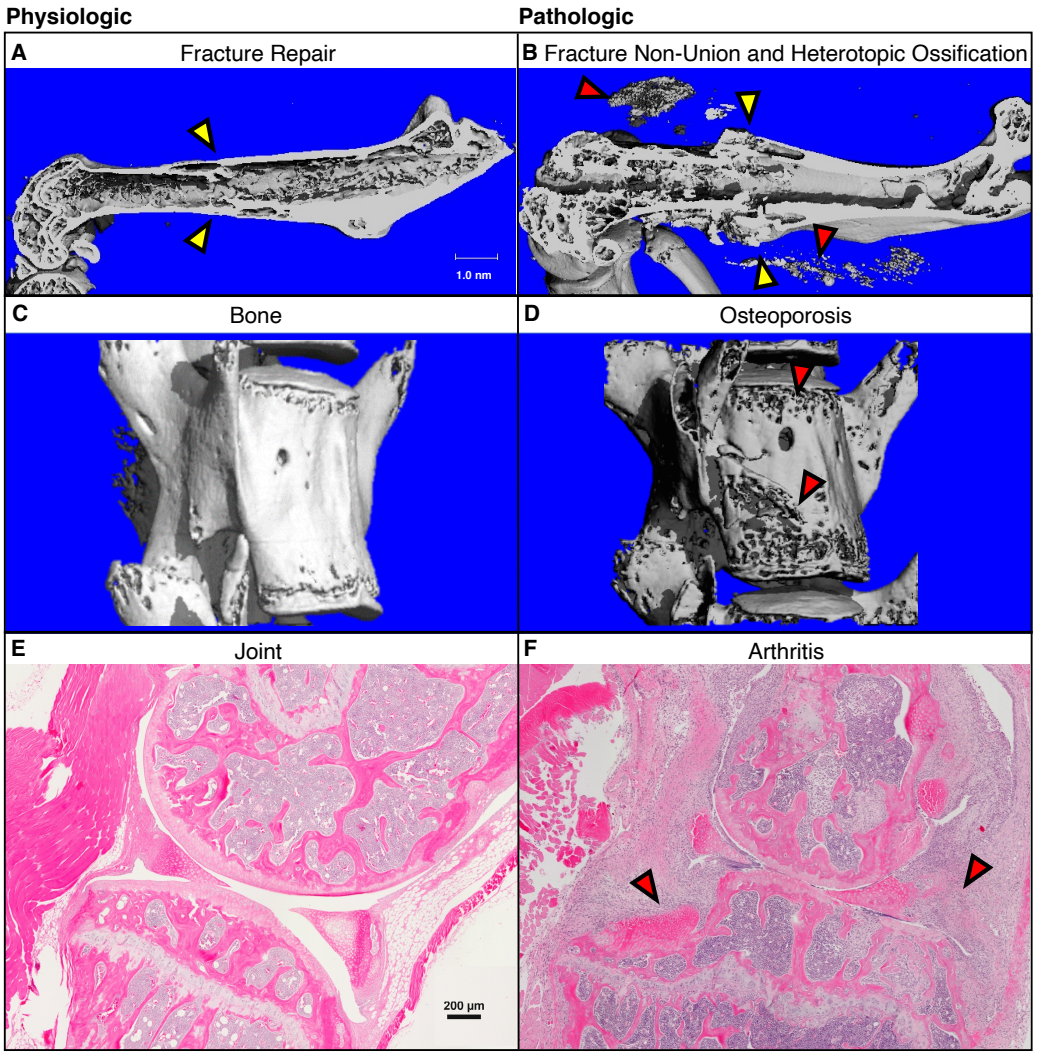

Figure 3.5: Healthy and diseased murine musculoskeletal tissues. A) Physiologic musculoskeletal health results in effective muscle and fracture repair (yellow arrows), B) Pathologic musculoskeletal repair may result in poor fracture repair, including fracture non-union (yellow arrows), and poor muscle repair, including bone formation in muscle (heterotopic ossification-red arrows). Physiologic maintenance of musculoskeletal organs promotes healthy bone (C) and joints (E), while pathologic musculoskeletal maintenance and inflammation provoke degenerative disease, including significant bone loss (osteoporosis (D)), and arthritis (F). Images shown are murine tissues.

Animal models of fracture have revealed the specific roles for both coagulation and fibrinolysis in fracture repair(36,246). While it was widely presumed that fibrin is essential for fracture repair(291), fibrinogen-deficient ($FBG^{-/-}$) mice maintained the ability to heal a fracture normally, despite significantly more fracture-related blood loss. In contrast, plasminogen-deficient ($PLG^{-/-}$) mice, which are unable to clear fibrin from the injury site, had poor fracture repair in both drill hole and femur fracture murine models of bone injury. In the drill hole model, $PLG^{-/-}$ mice had reduced cartilage matrix and bone formation at the site of injury(258). Similarly, in a transverse femur fracture model with significantly more bone and vascular disruption than the drill hole model, $PLG^{-/-}$ mice had little to no bridging of vascularity across the fracture callus(36). Without union of vascularity across a fracture callus, the bone does not unite, referred to as a non-union, or remodel (2,36,246,256)(**Figure 3.5B**). Collectively, the phenotypes displayed by fibrin and plasmin-deficient animals post-injury confirm the temporal nature of fibrin and plasmin's respective roles in healing: fibrin is critical for the initial containment of the injury through hemostasis, while plasmin is essential later during bone repair. These studies, however, are incapable of resolving the fibrin-dependent and fibrin-independent nature of plasmin's role in skeletal repair. To answer this question, our lab has demonstrated that fibrinogen deficiency partially restores the normal sequence of fracture repair in $PLG^{-/-}$ mice. Specifically, fibrinogen deficiency in $PLG^{-/-}$ mice resulted in increased angiogenesis and the restoration of bone union at the site of fracture(36). These data are consistent with studies suggesting fibrin independent roles for plasmin in fracture repair, but also clearly demonstrate that a principal role of plasmin is the removal of fibrin.

Studies segregating tPA- and uPA-dependent plasminogen activation suggest that their roles in fracture repair may be non-redundant. Fractures in uPA-deficient ($uPA^{-/-}$) mice exhibit

poor remodeling and large callus formation due to poor macrophage migration and vascular bridging at the site of the fracture(276,292), similar to $PLG^{-/-}$ animals. tPA-deficient ($tPA^{-/-}$) mice, on the other hand, exhibited only a delay in fracture repair. This delay was attributed to reduced proliferation of the bone forming cells, osteoblasts, due to a lack of plasmin-mediated activation of the ERK1/2 pathway(293). Moreover, tPA has been found to induce HIF1α and VEGF-A activity at the fracture site, increasing the rate of neovascularization during repair(293). No such function has been described for uPA.

Plasmin and fibrin in muscle repair

Plasmin is also critical for proper muscle repair and regeneration(33,34,294). Consistent with a normal APR, fibrin is deposited during survival to contain the zone of injury, and it is removed during the repair phase for regeneration to occur(33,34). Work from the Muñoz-Cánoves lab demonstrated that plasmin activity peaks in injured muscle within 3-5 days of the injury to remove fibrin and necrotic tissue and to regenerate new muscle(32,34). Activated plasmin removes fibrin and signals through M2 macrophages to promote the removal of necrotic tissue (19,183,288). Once the provisional fibrin matrix and necrotic debris is removed, plasmin proteolytically activates growth factors and pro-regenerative factors (*i.e.,* VEGF-A, pro-MMPs, etc) released from surrounding, regenerating muscle cells to remodel and revascularize the zone of injury(184,266). In the presence of adequate blood supply and an acute, localized inflammatory response, cells surrounding the injury regenerate, and satellite stem cells differentiate into functional myotubes to replace the area of damage(33,34). As in bone repair, tPA and uPA do not function interchangeably in muscle repair. Studies of plasmin activity in both cardiotoxin and freeze-crush models of muscle injury have demonstrated that uPA activity increases in the muscle following injury, while there is little change in tPA activity(32,34). Furthermore, in vivo muscle

repair and in vitro myogenesis are dependent on uPA-, but not tPA-, mediated plasmin activation(32).

A failure of coordinated repair in muscle results in a persistent state of tissue strain, hypoxia, and inflammation(253). These chronic complications, including the development of muscle fibrosis, muscle calcification, and sarcopenia, can cause significant pain and permanent loss of muscle function in patients(21,40,253). Animal studies have demonstrated that a plasmin deficiency causes ineffective macrophage infiltration and function, persistent fibrin deposition, and chronic inflammation of injured tissues(19,35,183,282,284). In a muscle injury specifically, the absence of plasmin results in fibrosis, skeletal muscle calcification, and bone formation within injured muscle, better known as heterotopic ossification (HO) (**Figure 3.5B**)(32,33). As little as a 50% deficiency in plasminogen and plasmin activity is sufficient to drive calcification of skeletal muscle in mice following injury and the development of HO(33). These studies suggest the possibility that deficiencies in plasmin activity routinely encountered in the clinic, such as those observed in trauma patients, may be sufficient to drive pathologic repair of injured muscle(36),(33). These data establish a paradox for plasmin's role in musculoskeletal repair. The role of plasmin in mineralization appears to be tissue specific: within the context of bone, plasmin is essential for bone formation(36,258), but in skeletal muscle, plasmin activity prevents bone formation (HO)(33). Interestingly, unlike in bone repair, fibrin(ogen) deficiency improves macrophage migration and prevents fibrosis in injured muscle, but it is insufficient to completely restore muscle repair in $PLG^{-/-}$ mice(33). Therefore, plasmin mediates muscle repair through both fibrin-dependent and independent mechanisms.

Musculoskeletal Degeneration: A "chronic wound"

Like the repair of an acute injury, maintenance of musculoskeletal tissue function throughout life requires a delicate balance between fibrin and plasmin. Healthy bones and joints should not contain a significant amount of fibrin given that the tissue is not damaged and therefore does not require hemostasis. In certain inflammatory diseases, such as diabetes and autoimmune conditions, and during aging, the spatiotemporal regulation of fibrin formation and plasmin activation is often disrupted(251,269,274,295). In conditions of poor plasmin activity or excess activation of coagulation, fibrin is deposited throughout tissues, provoking localized survival-APR inflammation and constant tissue remodeling(248,269,274). Consequently, daily microinjuries provoke a persistent cycle of the APR that ultimately leads to musculoskeletal degeneration rather than repair (**Figure 3.6**).

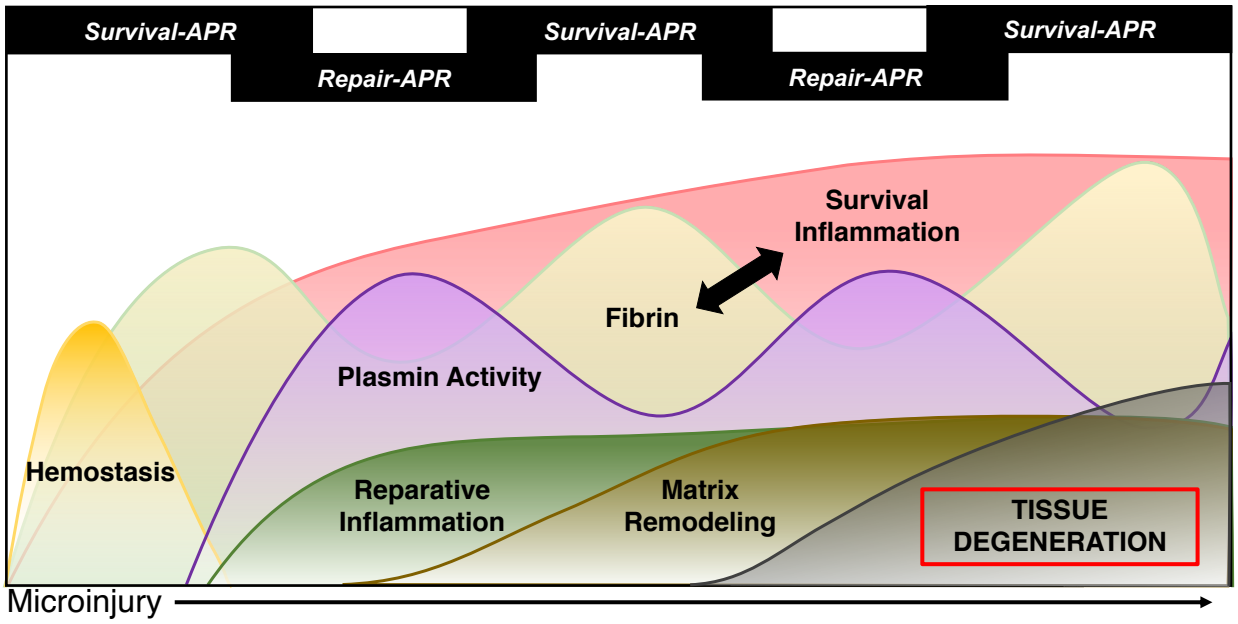

Figure 3.6: The acute phase response in chronic inflammatory disease. In chronic inflammatory conditions and aging, microinjuries sustained during daily movement trigger a persistent APR cycle in which fibrin deposition and plasmin activation are dysregulated in musculoskeletal tissues. The consequence of this cyclical APR is chronic inflammation, inappropriate tissue remodeling, and ultimately, degeneration of the musculoskeletal tissues.

Fibrin Accumulation in Bone Degeneration

Osteoporosis is the debilitating loss of bone resulting in significant costs in both healthcare expenses and quality of life (**Figure 3.5D**). In the United States, osteoporosis afflicts over 10 million individuals, increasing their risk of fractures and loss of mobility(248,255). Age, sex, and body mass index

(BMI) are all factors in the risk of developing osteoporosis(248,250). Inflammation and uncontrolled bone resorption are current therapeutic targets for this disease, but recent data suggest that fibrin may be an initiator of these pathologies, making it a more efficacious target(274).

Studies in $PLG^{-/-}$ deficient mice have demonstrated that the absence of plasmin activity drives the deposition of fibrin in bone(274). Further, fibrin in bone activates resident macrophages through binding the $\alpha_M\beta_2$ integrin, stimulating local production of IL-6, IL-1β, and TNFα. These inflammatory cytokines work synergistically with one remodeling protein RANKL to stimulate the proliferation and differentiation of bone-resorbing osteoclasts(273,274). The net effect of this is increased bone resorption, resulting in decreased bone mass (osteopenia) and bone degeneration (osteoporosis). Consequently, the genetic ablation of fibrinogen ($FBG^{-/-}$) or the $\alpha_M\beta_2$ binding motif on fibrinogen ($Fib\gamma^{390\text{-}396A}$) effectively prevented osteoporosis in $PLG^{-/-}$ mice, demonstrating a direct role for persistent fibrin deposition in bone disease progression. Mechanistically, these studies determined that engagement of MAC-1 on fibrin potentiates the fusion of monocytes to form osteoclasts in the presence of RANKL(274). These studies indicate that persistent fibrin in bone may potentiate osteoporosis by driving osteoclastogenesis thereby uncoupling the bone remodeling unit favoring a catabolic phenotype. Although early clinical studies have identified a relationship to circulating fibrinogen and osteoporosis (296),further studies are required to determine if targeting fibrin(ogen) is a viable therapeutic approach to prevent osteoporosis.

Coagulation and Fibrinolysis in Degenerative Joint Disease

In addition to loss of bone, chronic inflammatory conditions cause degeneration in joints by hijacking thrombin and plasmin activity. Since the 1960s, inappropriate fibrin formation and plasmin activity have been detected in the synovial fluid of arthritic joints(295,297–299). Affecting approximately 1.3 million individuals in the U.S., rheumatoid arthritis is an autoimmune disease marked by a persistent APR: localized inflammation, fibrin formation, cellular infiltration, and plasmin activation within the synovium (**Figure 3.6**), which eventually leads to cartilage and bone degradation(249,300) (**Figure 3.5F**). Similar to osteoporosis, IL-6, IL-1β, and TNFα have

all been implicated in RA, indicating anti-cytokine monoclonal antibodies for treatment(300,301). In a collagen-induced arthritis (CIA) murine model, fibrinogen deficiency reduced the local inflammatory response to the collagen injection and conferred resistance to RA in the paw joints, suggesting that fibrin is a pathologic driver in this disease. In the same model, Fib$\gamma^{390\text{-}396A}$ mice expressing fibrinogen unable to bind to $\alpha_M\beta_2$ on the macrophage, were resistant to CIA(275). Furthermore, the prevention of fibrin crosslinking by removing or inhibiting transglutaminase factor XIII is sufficient to reduce local inflammation and bone erosion in CIA(302). Interestingly, another study using the CIA model demonstrated the seemingly contradictory finding that a plasminogen deficiency was found to protect mice from the development of RA in the paw joints(303). This was further investigated in a more clinically relevant model of RA: transgenic mouse overexpressing human TNFα (Tg197), in which mice spontaneously develop the disease. Work in Tg197 mice demonstrated that while plasminogen deficiency exacerbated disease progression in the paw joints, it reduced disease progression in the knee joints, demonstrating that plasmin has both pro- and anti-arthritic functions based on the specific joint tissue and the model of disease(304). It was found that in Tg197 mice, fibrin drives the disease progression of RA in the paw joints, while in the knee joints, plasmin activation of MMP9 plays a significant role in joint degeneration. Furthermore, combined plasmin(ogen) and fibrin(ogen) deficiency negated both the pro- and anti-arthritic roles of plasmin in RA(304). Collectively, these data suggest that plasmin and fibrin may work cooperatively to promote disease progression through different mechanisms.

More recent research implicates a specific plasminogen activator, receptor, and cell type in RA disease progression. Previous studies in synovial samples from RA patients have demonstrated that cells present in synovial fluid, including fibroblasts and macrophages,

overexpress uPAR(305). Further studies in a CIA model of RA demonstrated that a deficiency or inhibition of macrophages, uPA, or uPAR conferred resistance to CIA(306,307). To investigate if the pathologic effect of plasmin in RA is mediated by uPAR on the surface of macrophages, bone marrow transplants from uPAR-deficient mice into wild-type mice were employed. Transplant of uPAR-deficient hematopoietic cells into wild-type mice prevented the development of arthritis in a CIA model(307). Therefore, uPA-uPAR mediated activation of plasmin on the surface of hematopoietic cells, including macrophages, is a possible mechanism for inappropriate plasmin activation in arthritic joints. In contrast, a tPA deficiency exacerbated arthritis in the same murine model(308). These findings of plasminogen activator specificity in arthritis suggest that tPA drives fibrinolysis in the joints to reduce inflammation, while uPA activates plasmin on the surface of macrophages and fibroblasts via uPAR, propagating inflammation and tissue remodeling. Interestingly, patients with hereditary hemophilia A and B often develop bleeding within the joints, which leads to persistent inflammation, cartilage remodeling and joint degeneration(309,310)Recent research from the Mosnier lab has demonstrated that insufficient thrombin-activated fibrinolytic inhibitor (TAFI) activation in $FVIII^{-/-}$ mice results in unchecked plasmin activation in hemophilic joints. Similarly to the joint pathology observed in RA, unbridled uPA-mediated plasmin generation, secondary to defective TAFI activation within joints, was found to drive hemophilic joint bleeds and ensure arthropathy(310). New research is investigating the possible therapeutic use of uPA inhibitors to treat joint degeneration(311), but it should be applied with caution as uPA also plays a critical role in musculoskeletal repair. Research from the Hamilton lab has demonstrated that while uPA deficiency protects against CIA, if there is a soft tissue injury adjacent to the joint, a uPA deficiency worsens local inflammation and arthritis develops(312,313). While the fibrinolytic function of plasmin prevents fibrin-mediated

inflammation in joints, chronic plasmin-mediated macrophage activation and matrix remodeling in joints drives cartilage degeneration and bone erosion.

Future Perspectives

Plasminogen activation has been implicated in many disease states, including musculoskeletal disease, and future studies in this field may identify therapeutic targets within the coagulation and fibrinolytic systems to diagnose and treat diseases beyond the vascular system. Specifically, there is a great clinical need for improved therapeutics to treat musculoskeletal repair problems and degenerative diseases, and the pharmacologic manipulations of plasmin and/or fibrin may present effective treatment options. Potent amiloride-derived uPA inhibitors developed in the Ranson and Kelso labs show promising potential for the treatment of uPA-mediated pathologies, including joint degeneration(311,314), but further studies are required to determine optimal use without affecting uPA-mediated tissue repair. Additionally, novel inhibitors of plasminogen activator inhibitor-1 (PAI-1) may also provide therapeutic benefit if dosed within the appropriate spatiotemporal context to enhance plasmin activation(315). Likewise, the pathologic effects of persistent fibrin deposition in musculoskeletal repair and degeneration are clear, but anticoagulant or fibrin-targeting drugs carry the risk of bleeding side effects(316) and may not have efficacy in extravascular compartments.

Because macrophages can have both reparative and pathologic roles within the musculoskeletal system, they may represent another target for musculoskeletal disease. Recent research in cancer, cardiovascular disease, and inflammatory conditions have identified macrophage polarization as a possible therapeutic target for treatment of these diseases(317,318). Because much of plasmin and fibrin's respective roles within the musculoskeletal system are mediated or amplified by macrophages, and alternative approaches may target receptors for

plasmin(ogen), plasminogen activators (i.e. uPAR), or fibrin (i.e. $\alpha_M\beta_2$) on the surface of macrophages without dramatically affecting global plasmin(ogen) activation or hemostasis. While macrophages have long been associated with tissue repair mechanisms, new research has elucidated diverse functions for neutrophils in tissue repair and degeneration(270,271) Prolonged tissue inflammation mediated by neutrophils can have deleterious effects on repair, but acute inflammation instigated by neutrophil cytokines and NETs provoke a reparative response(270). Investigations on the roles of neutrophils in thrombosis, inflammation, and tissue repair suggest that NETs within injured tissue may also stimulate tissue repair through macrophage function(271,272). Collectively, the plasminogen activation system and downstream effector cells present promising therapeutic targets for a range of conditions beyond vascular disease, including the treatment of musculoskeletal disease.

Conclusion

Proper musculoskeletal repair is dependent upon coordinated activation of coagulation and fibrinolysis as part of the survival and repair phases of the APR respectively. When a specific tissue or entire system gets stuck in an unresolved, perpetual APR, it drives chronic inflammation and tissue degeneration. The delicate interplay between coagulation and fibrinolysis in musculoskeletal health is dependent upon context, which explains why different models have found seemingly conflicting data on their roles in pathology and repair. Because plasmin is essential for repair of muscle and bone following injury, plasmin-enhancing therapeutics may be an effective strategy to improve musculoskeletal healing in patients with repair problems, but timing, location, and level of plasmin(ogen) activation remain key factors that differentiate plasmin's roles in repair from its pathologic roles in degeneration. As such, therapeutically targeting plasmin or uPA may prevent its pro-arthritic roles, but it should be considered that

inhibition of this system might have deleterious effects on tissue repair after injury. The difference between the beneficial and the pathologic effects of plasmin and fibrin in musculoskeletal health is the spatiotemporal context of each, and therefore, therapeutic efforts to target either plasmin or fibrin must take into consideration the precise etiology of disease, stage of disease, and anatomical location of disease in order to minimize off-target effects.

Article 5: Plasmin regulates trauma-induced heterotopic ossification through multi-step, activator-dependent mechanisms.

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In completion for submission

INTRODUCTION

Severe injuries, such as burns, blasts, and traumatic brain injuries, are not only a significant source of mortality, but they also provoke complications that predispose surviving patients to lifelong disabilities. While recent advances in critical care medicine have allowed patients to survive severe injuries, they have uncovered a novel challenge: poor tissue repair and the chronic complications associated with it. Common complications of tissue repair and homeostasis in patients with severe injuries include infection, osteoporosis, bone fracture non-union, and muscle fibrosis or calcification. Furthermore, burns with concurrent musculoskeletal trauma or blast injuries, which primarily afflict military populations, present with a higher risk of HO development and associated complications(63,319,320). In the United States, it's estimated that these sequelae cost billions of dollars per year and result in significant loss in quality of life(319,320). Burn and blast injuries provoke significant musculoskeletal complications, including poor bone repair and HO(63,234). In the U.S., approximately 500,000 patients are treated for burns annually, costing approximately $1.5 billion in healthcare and $5 billion in additional costs per year(321,322). While the mortality rate for severe burns in the U.S. is less than 1%, the rate of complications remains high(322). Specifically, the development of HO has been reported to occur in up to 20% of severe burn cases, leaving patients with chronic pain, decreased range of motion, and loss of limb function(234). Furthermore, burns with concurrent musculoskeletal trauma or blast injuries, which primarily afflict military populations, present with a higher risk of HO development and associated complications(63). Therefore, burn with concurrent musculoskeletal trauma was selected as a model to investigate our hypotheses in both humans and mice. Although the lasting burden of disease in patients with severe injuries is recognized as a substantial clinical problem, few

therapeutic targets have been identified to effectively improve tissue repair and recovery in these patients.

The protease plasmin (activated plasminogen) is widely recognized for its role in intravascular fibrin degradation and subsequent clot lysis, but more recent studies have extended the role of plasmin beyond intravascular fibrinolysis. Through numerable mechanisms, plasmin has been shown to be essential for repair of virtually all tissues, including skin, nerve, muscle, and bone(33–36). Notably, plasmin's functions in the musculoskeletal system are context-specific: while plasmin is required for physiologic bone formation and fracture union, it is also critical to prevent the pathologic formation of calcification and bone in soft tissues(1). Aberrant bone formation within soft tissues, termed heterotopic ossification (HO), is associated with reduced joint range of motion, loss of limb function, and chronic pain(207,323). These musculoskeletal complications not only impose a significant reduction in quality of life but also notably increase healthcare costs due to recurrent surgical procedures, medication, and physical rehabilitation(319,320).

Studies in patients with severe injuries and invasive surgeries have revealed moderate to dramatic changes in plasmin activation or activity following the injuries. Many mechanisms have been proposed for the observed alterations to plasmin, including increases in circulating plasminogen activators and plasminogen activator inhibitors(57,88). Regardless of the contributing mechanism, altered plasmin activity following significant injuries is well-documented. Based on plasmin's essential roles in muscle repair and the prevention of soft tissue calcification, we hypothesized that plasmin is a pharmacologic target that regulates the formation of DC and HO following severe injury. To test this hypothesis we implemented a model of severe injury with concomitant muscle injury and both genetic and pharmacologic manipulation of plasmin.

METHODS

Animals: For all experiments, 6-week-old male and female mice on a C57BL/6J background were used. Wild-type mice were obtained from Jackson Laboratory (Bar Harbor, MN), and plasminogen (Plg), urokinase plasminogen activator (uPA) and tissue-type plasminogen activator (tPA)-deficient mice background were obtained from the labs of Dr. Matthew Flick (Cincinnati Children's Hospital, OH) and Dr. Jay Degen. Wild-type mice were provided standard laboratory chow and Plg, uPA, and tPA-deficient mice were provided synthetic laboratory chow (Research Diets Inc., New Brunswick, NJ) and water ad libitum. All mice were housed in a 12-hour light-dark cycle within a designated animal facility at Vanderbilt University.

Burn and Muscle Injury Models: To study muscle repair, and validated model of isolate muscle injury was implemented(33). Six week-old mice were administered an isolated muscle injury by local injection of 40μL of 10μM cardiotoxin into the gastrocnemius and soleus muscles. To assess the systemic effects of severe burn on muscle repair, a well-established model of burn injury(222) was administered with concurrent bilateral muscle injuries as described above. Thirty minutes prior to the burn, mice were administered 0.1mg/kg subcutaneous buprenorphine. Under 3.0% isoflurane general anesthesia, mice were shaved along the dorsum, and 1mL of sterile saline was injected subcutaneously along the spine to prevent injury to deep tissues during the burn. Bilateral calf muscle injuries (as described above) were administered immediately prior to the burn. The mice were placed in a heat-resistant template with a cutout to expose the dorsum and submerged in 100°C water bath for 10 seconds to create an approximately 30% total body surface area (TBSA), full thickness burn to the dorsum. Immediately following the burn, a resuscitative intraperitoneal injection of 2mL Lactated Ringer's solution was administered, and mice were

monitored for 30 minutes following the injury. A maintenance dose of buprenorphine was administered every 8-12 hours for 72 hours, and all animals were housed in individual sterile cages following the burn injury. Experimental controls received the same treatment in the absence of the burn injury.

In Vivo Assessment of Muscle Calcification:

Radiographs: To assess muscle calcification following the injury alone or muscle injury with a concurrent burn, radiographic images of the injured muscle were obtained weekly beginning at 7 days post-injury (DPI) until 28 DPI. Under brief anesthesia, mice were placed in a prone position, and each leg was imaged using a Faxitron X-ray (Faxitron Bioptics, Tucson, AZ) within the Vanderbilt University Institute of Imaging Science (VUIIS). Images were taken at 35kV with a 4.0s exposure time. Radiographs were scored using the Soft Tissue Calcification Scoring System (STiCSS), a previously validated ordinal scale to quantify calcification based on the percentage of the injured muscle region with calcification visible by radiograph(221).

Micro-computed Tomography: Following euthanization at 28 DPI, fixed injured hindlimbs were scanned by μCT (μCT40; Scanco Medical AG, Bassersdorf, Switzerland) at 55 kVp, 145 μA, 200 ms integration, 500 projections per 180-degree rotation, with a 20-μm isotropic voxel size. Following three-dimensional reconstruction of the scans, the regions of mineralization in the injured calf muscles were selected as the volume of interest (VOI). A threshold of 132/1000 with a Gaussian noise filter of 0.2 and support of 1 to differentiate soft from mineralized tissue. Scanco software was used to quantify the volume of mineralization within the injured tissues as previously described(33).

Histology: Following euthanization at different time points following the injuries, muscle tissues were collected and fixed in 10% neutral buffered formalin. Fixed tissues were transferred to 70% ethanol prior to perfusion and embedding in paraffin. For histologic analysis of the tissues, 5 μm sections were taken for staining. To assess muscle morphology, sections were deparaffinized and stained with Gill's Hematoxylin and Eosin. Micro-calcification was detected using a standard Von Kossa stain in which deparaffinized sections were stained in 1% aqueous silver nitrate solution for 20 minutes under a UV lamp followed by a Fast Green counterstain. Stains used for bone formation and fibrosis were conducted on tissues that were decalcified in 0.5 M EDTA, pH 7.4 for 1 week prior to paraffinization. A standard Martius Scarlet Blue (MSB) stain was used to detect areas rich in fibrous and collagenous deposits, including decalcified bone, and a standard Safranin-O stain counterstained with Fast Green was used to detect the presence of proteoglycans. To specifically detect fibrin deposits in injured muscle, a goat anti-mouse fibrin IgG (produced in the lab of Jay Degen) was used at a 1:1000 followed by a secondary rabbit anti-goat IgG AlexaFluor647 at a 1:100 dilution. Immunofluorescent stains were costained with DAPI for visualization of cell nuclei within the muscle. All stained slides were imaged using a Zeiss Axio Imager.A1 microscope (Zeiss, Oberkochen, Germany) at 20X magnification.

In vivo imaging of fibrin and plasminogen: To assess localization of plasminogen in vivo, novel recombinant human plasminogen (rhPlg) (developed by Drs. Ruby Law and James Whisstock, Monash University), was labeled with AlexaFluor790 without significantly disrupting structure or function of the protein. At different time points following the injuries, 20mg/kg rhPlg in sterile HEPES buffer was injected intraperitoneally. To assess fibrin deposition in injured tissues, a

fluorescently labeled fibrin-specific targeting peptide (FTP11-Cy5.5, Dr. Jason McCarthy, Masonic Medical Research Laboratory, MD) was used. At different time points following the injuries, 0.2μg/kg FTP in sterile PBS was injected intraperitoneally. Twenty-four hours following injection, under brief anesthesia, mice were imaged at 700nm for FTP and 800nm for rhPlg using the LI-COR Pearl Imager (PerkinElmer, Waltham, MA) within the VUIIS facility (Figure 3.7A). Images were quantified in ImageJ (NIH, LOCI software). The region of injured muscle was selected in each image and measured for mean fluorescence intensity (MFI) per unit of area. Quantifications are expressed as a fold of baseline values measured from uninjured control muscle.

Therapeutic Interventions:

Antisense Oligonucleotides: All antisense oligonucleotides (ASOs) were developed and provided by IONIS Pharmaceuticals (Carlsbad, CA). Plasminogen (AGTGATGGTCTATTGTCACA), α2-antiplasmin (α2AP) (CACTGGTGATGGTCCTTCCG), and control ASOs (CCTTCCCTGAAGGTTCCTCC) were all administered at 330mg/kg by subcutaneous injection weekly beginning 2 weeks prior to injury and continuing for the duration of the study. These ASOs were synthesized and purified as previously described (REFS). Efficacy of each ASO was initially tested in mouse primary hepatocytes and has been validated to effectively knockdown mRNA levels and subsequent expression of the hepatic-derived target proteins *in vivo* at the specified dose (REFS).

Tranexamic Acid: (TXA), an FDA-approved, anti-fibrinolytic drug used for the reduction of intraoperative bleeding, was used to inhibit plasmin activation and activity *in vivo*. To reduce plasmin activation long-term following an isolated muscle injury, mice were administered daily

intraperitoneal injections of 1000mg/kg TXA as previously described(324). Injections were administered beginning at the time of injury and up to 1, 3, 5, or 7 DPI.

rhPlg: To restore circulating plasminogen in the setting of plasminogen deficiency, mice received intraperitoneal injections of 1mg unlabeled rhPlg (described above) daily beginning at the time of injury and up to 7 days post-injury. This dosing scheme for human plasminogen has been validated in previous animal studies(263,325).

Statistics: A departmental biostatistician was consulted for all statistical analyses for these studies. Two-way, non-parametric ANOVA (Kruskal-Wallis test) was performed with a Dunn's post hoc multiple comparison test to determine significant differences between groups.

Institutional Approval for Animal and Human Studies: All animal procedures were approved by the Vanderbilt University Medical Center Institutional Animal Care and Use Committee (protocol M1800154).

RESULTS

Fibrinolysis occurs in injured muscle within 3-7 days post-injury.

Near-infrared-labeled recombinant human plasminogen is localized to injured muscle between 1-5 days following an isolated muscle injury (Figure 3.7 B-D). Previous studies using zymography have demonstrated that plasmin activity peaks in injured muscle within 3-7 days post-injury. Consistent with this, fibrin is deposited in injured muscle within 1 day of the injury, and it is

removed between 3-7 days post-injury during the timeframe in which plasmin activity has been shown to peak in injured muscle (Figure 3.7B-D).

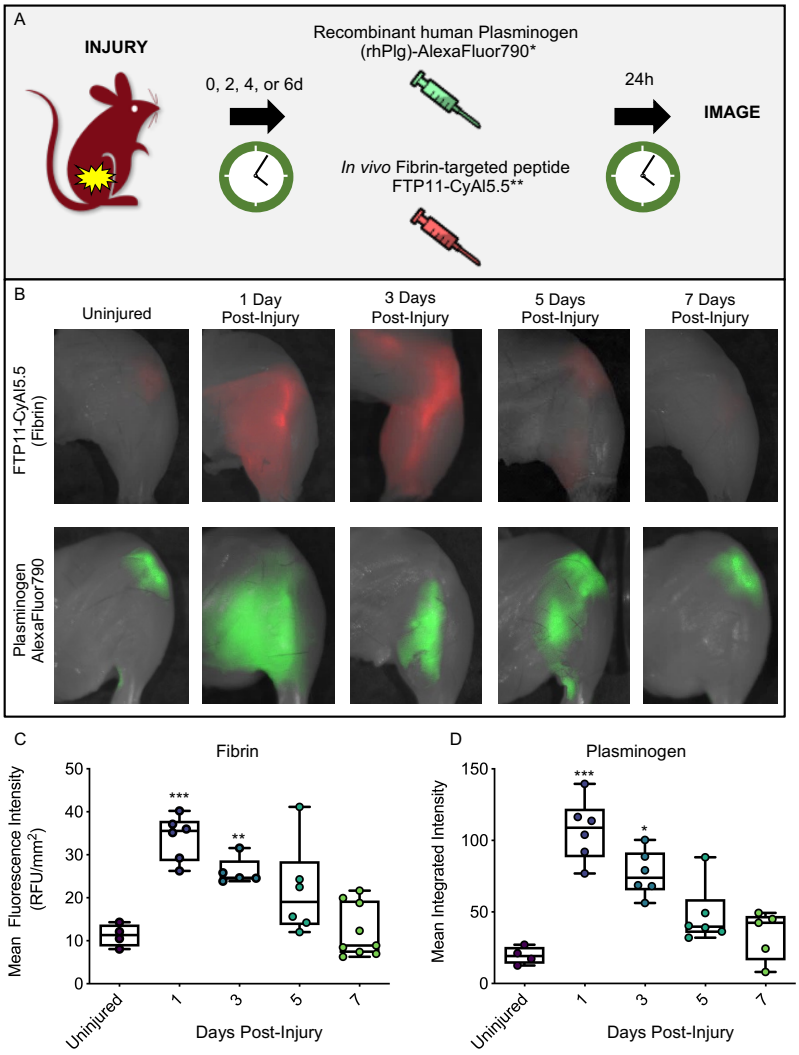

Figure 3.7: Fibrin deposition and plasminogen binding in muscle following acute injury. A) Mice received a calf muscle injury and then 1, 3, 5, or days post-injury they were injected with labeled plasminogen or FTP 2h prior to imaging. B-D) Fibrin is deposited in injured muscle within the first day and persists through 5 days post-injury. Plasminogen is localized to injured muscle up to 5 days post-injury. N=5-6/group, ***P<0.001, **P<0.01, *P<0.05, compared with uninjured animals.

Plasmin activity is critical for fibrin removal and prevention of calcification in injured muscle between 3-7 days post-injury

Previous studies have indicated the plasmin is essential for muscle repair due to both fibrin-dependent and –independent mechanisms(32–34). Muscle repair is particularly sensitive to loss of plasmin activity to the effect that even a 50% loss of plasmin activity, observed in plasminogen heterozygous mice ($Plg^{+/-}$), results in persistent fibrin deposition and fibrin-independent calcification of injured muscle(33). While plasmin activity peaks in injured muscle within 3-7 days of the injury(32,34), we hypothesized that there is a critical timeframe within which plasmin is critical to prevent dystrophic calcification and promote proper regeneration of injured muscle. To investigate the time-dependent effect of plasmin in muscle repair, mice were dosed with intraperitoneal injections of clinical antifibrinolytic drug tranexamic acid (TXA) at 1000mg/kg daily or saline, beginning at the time of injury up until 1, 3, 5, or 7 days post-injury (Figure 3.8A). In mice treated with saline for any length of time or those treated with TXA for 1 day post-injury, muscle followed a normal regenerative process evident at 7 DPI, with no detectable calcification or fibrin present (Figure 3.8B-C). Alternatively, dosing TXA for 3 days or longer following a muscle resulted in muscle calcification and persistent fibrin deposition that increased based on length of TXA administration (Figure 3.8B-C) This collectively suggests that there is a critical window within which plasmin activity is necessary for fibrin removal and the prevention of calcification within injured muscle. The previous studies demonstrated that plasmin activity is increased in muscle between 3-7 days post-injury, but these results demonstrate that plasmin activity plays a critical role in muscle regeneration during this time frame.

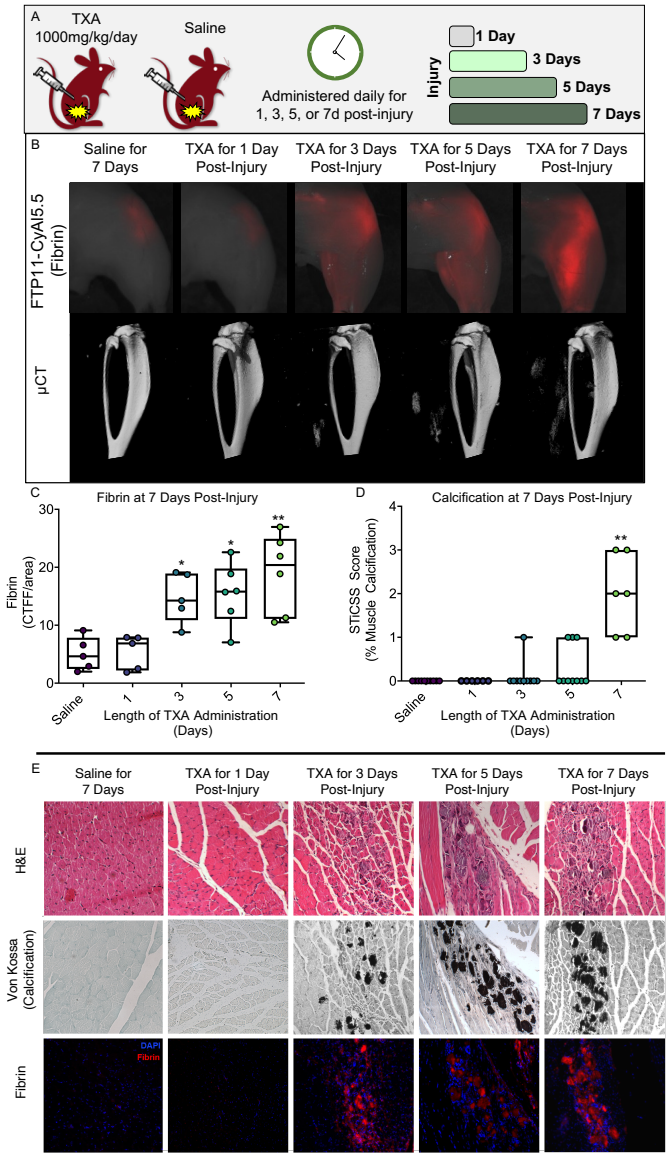

Figure 3.8: Tranexamic acid dosing beyond 1 day post-injury prolongs fibrin deposition and causes calcification in injured muscle. A) Mice received a calf muscle injury and were treated with either TXA or saline daily for 1, 3, 5, or 7d post-injury, beginning immediately following injury. B-E) Inhibition of plasmin up to 1d post-injury does not provoke fibrin deposition or muscle calcification, but dosing of TXA 3d and beyond following the injury resulted in a length-dependent increase in fibrin deposition and muscle calcification. N=5-6/group, ***P<0.001, **P<0.01, *P<0.05, compared with saline-treated animals.

UPA, but not tPA, is essential for the prevention of dystrophic calcification

Previous studies have demonstrated that both plasmin and uPA are necessary for muscle myotube regeneration and muscle repair, while tPA is disposable. At 7 days post-injury (DPI) $uPA^{+/-}$ and $uPA^{-/-}$ mice had significant mineralization present within the injured muscle detected by both radiograph. Conversely, tPA-deficient mice did not develop robust calcification within the injured muscle, similar to wild-type animals (Figure 3.9), suggesting that plasmin prevention of dystrophic calcification is dependent on uPA, but not tPA.

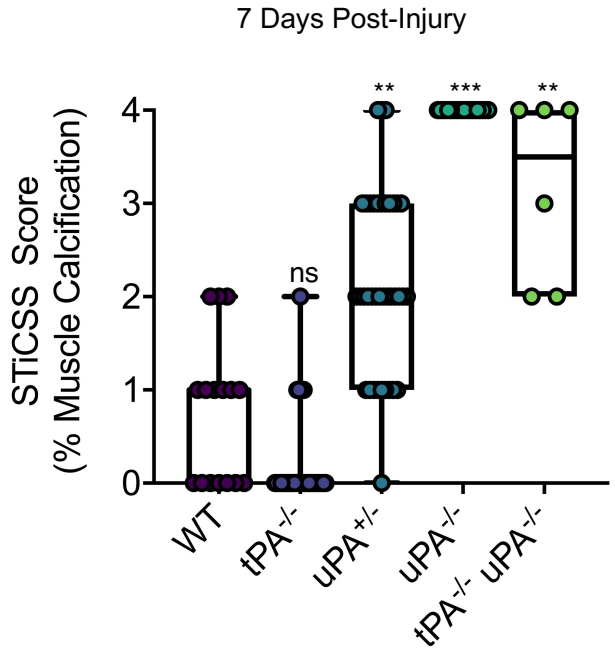

Figure 3.9: The prevention of dystrophic calcification within 7 days following muscle injury is uPA, and not tPA-dependent. Like WT mice, tPA-deficient mice do not develop dystrophic calcification following muscle injury, while mice with partial or full uPA deficiency develop muscle calcification following injury (**P<0.01, ***P<0.001 compared with WT mice, N=6-10/group)

Burn injury provokes persistent fibrin deposition and dystrophic calcification in a remote muscle injury

Severe injuries provoke a systemic response that can dramatically affect organ function and alter the process of repair in injured tissues, and severe injuries often occur as polytraumas, such as blast injuries, involving damage to other tissues(157). Burns are a type of severe injury that have chronic effects on tissue health for months to years following the burn, resulting in tissue fibrosis, osteoporosis, and HO(213,234). In a mouse model of 30% total body surface area (TBSA) dorsal burn with a concomitant injuries to the calf muscles, the burn injury provoked robust calcification and persistent fibrin deposition within the remote muscle injuries by 7 DPI(Figure 3.10). Collectively, these results suggest that a burn provokes a systemic response and a localized change in within the local tissue microenvironment at the site of muscle injury.

Therapeutic enhancement of plasmin, but not plasminogen supplementation, prevents burn-induced DC and prolonged fibrin deposition within injured muscle

Because plasmin plays several roles in muscle repair in a time-dependent manner, we further hypothesized that pharmacologic enhancement of plasmin would restore muscle repair following a burn. To assess the efficacy of two therapeutic options—recombinant human plasminogen (rhPlg) and an antisense oligonucleotide (ASO) against plasmin's primary inhibitor, α2-antiplasmin (α2AP)—in an isolated calf muscle injury in $Plg^{+/-}$ mice. Both rhPlg and α2AP ASO were effective for restoring muscle regeneration in a model of poor plasmin activity due to plasminogen deficiency(33). Wild-type mice that received both a burn and a muscle injury, rhPlg did not prevent calcification detected by radiograph (Figure 3.10B). Alternatively, treatment with α2AP ASO completely restored muscle regeneration following a burn (Figure 3.10A-C). This

suggests that either the burn provokes poor plasmin activity in the injured muscle, or enhancement of existing plasmin activity is sufficient to prevent dystrophic calcification.

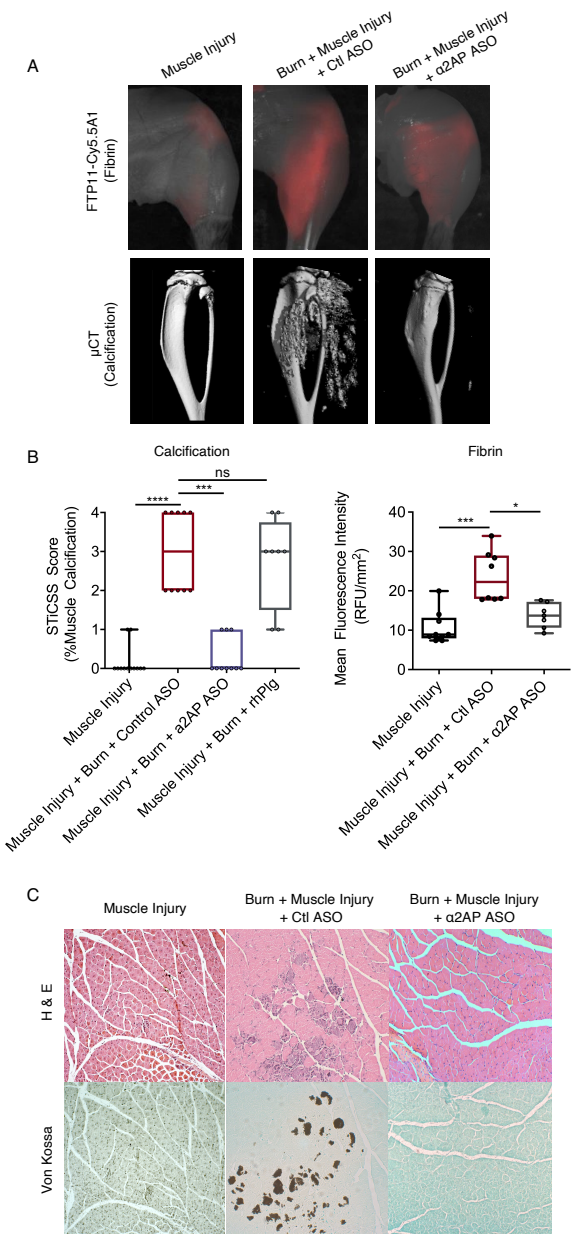

Figure 3.10: Burn injury provokes muscle calcification and fibrin deposition. A-C) Burn injury provokes calcification and prolonged fibrin deposition within a remote muscle injury detected at 7 days post-injury by in vivo fibrin-targeted peptide (FTP), μCT, and histology analysis. Therapeutic enhancement of plasmin activity by α2AP ASO, but not plasminogen supplementation, prevents burn-induced muscle calcification and fibrin deposition (*P<0.05, ***P<0.001, ****P<0.0001, N=8-10/group).

Plasmin is essential for the removal of DC

Following a burn, injured muscle develops significant calcification. Previous work has demonstrated that macrophages are able to resorb calcification within muscle (dystrophic calcification, DC) up to a certain point. Regardless of the cause of DC, if it is not removed, the tissue eventually gets remodeled and forms disorganized and lamellar bone within the muscle, termed heterotopic ossification (HO). To investigate this removal of DC that develops in muscle after bone, we administered a burn with an isolated muscle injury as shown above. Longitudinal radiographs demonstrated that while significant DC develops after a burn, covering the majority of the calf muscle, this DC is resorbed within 4 weeks of the burn (Figure 3.11). Consistent with our previous findings(326), clodronate-mediated macrophage ablation prevented the resorption of DC, preceding the formation of HO within the muscle by between 4-6 weeks after the burn(Figure 3.11). Plasmin is known to exert its reparative effects through stimulating macrophage migration, phagocytosis, and phenotype switching(Figure 3.11). Therefore, we hypothesized that plasmin is essential for the macrophage-mediated resorption of DC. Because a plasmin deficiency itself will provoke DC, we administered an ASO against plasminogen (Plg ASO) to deplete plasmin(ogen) after muscle calcification was already present within the muscle. The ASO depletes circulating plasminogen within 3-4 days, and therefore, 300mg/kg/week of either plasminogen or a control ASO were administered beginning at 3 days following a burn with a muscle injury or a muscle injury alone. Plasminogen ASO takes 3-4 days to completely diminish plasma levels of plasminogen(33). Treatment with plasminogen ASO 3 days following a muscle injury alone did not provoke DC or subsequent HO, suggesting that the dosing did not have an effect on the initial muscle regeneration after the injury (not shown). In the mice that received a burn with a muscle injury, those treated with a control ASO developed DC that was resorbed by 4 weeks post-burn,

but those treated with plasminogen ASO following the burn developed DC that was not resorbed and developed into bone by 4 weeks post-burn as confirmed by histology (Figure 3.11). Therefore, plasmin is not only essential to prevent calcification of injured muscle, but it is also essential for the resorption of DC formed within injured muscle.

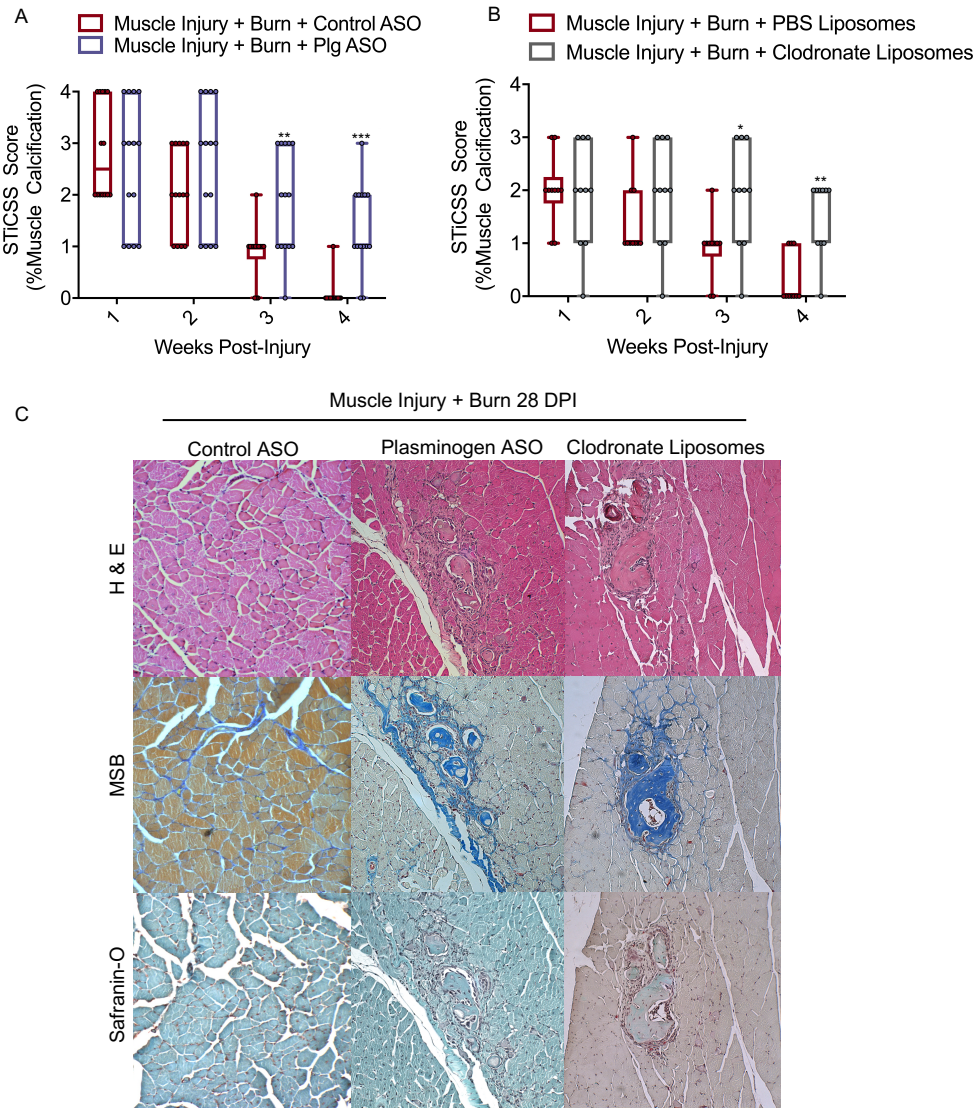

Figure 3.11: Burn-induced dystrophic calcification matures to heterotopic ossification in the absence of plasmin or macrophages. Burn-induced muscle calcification regresses within 4 weeks of the injury, but a deficiency in plasmin by Plg ASO (A) or macrophages by clodronate treatment (B) following the formation of dystrophic calcification prevents regression and leads to the maturation of the DC in lamellar bone within the injured muscle (HO). C) Histologic analysis of HO formation by H&E, MSB (collagen and fibrosis stain), and Safranin-O (cartilage stain). (*P<0.05, **P<0.01, ***P<0.001 compared with control/vehicle animals at each time point, N=5-6/group.

Regression of dystrophic calcification is dependent upon uPA

Because dystrophic calcification, once formed, can be regressed in a macrophage- and plasmin-dependent manner, we aimed to determine which plasminogen activators and receptors present in macrophages may be responsible. In uPA-deficient mice that develop DC following calf muscle injury, $uPA^{+/-}$ mice exhibit regression of the DC within 28 DPI, but $uPA^{-/-}$ mice to not regress the DC within 28 DPI (Figure 3.12C-D). $TPA^{-/-}$ mice with a burn and a muscle injury developed DC which regressed normally over 4 weeks (Figure 3.12E). All mice except $uPA^{-/-}$ and $tPA^{-/-}/uPA^{-/-}$ animals exhibited DC regression over 28 DPI (Figure 3.12), suggesting that plasmin's role in regression is uPA-dependent.

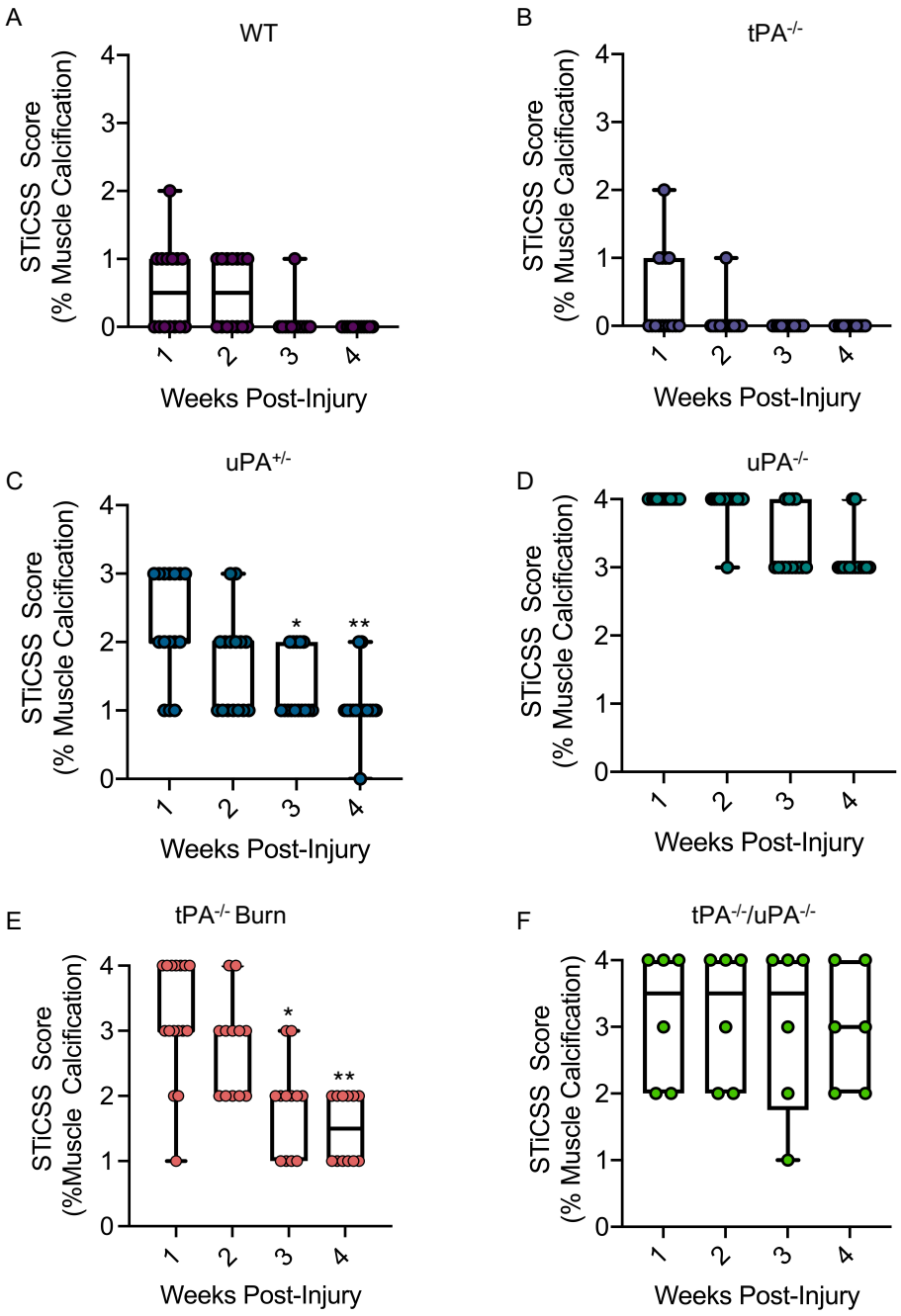

Figure 3.12: Regression of dystrophic calcification in tPA- or uPA-deficient mice. A) Dystrophic calcification following isolated muscle injury is regressed within 4 weeks and B-F) regression of dystrophic calcification in injured muscle is uPA- and not tPA-dependent (*P<0.05, **P<0.01 compared with 1 week post-injury, N=6-10/group).

Discussion:

Plasmin has both pathologic and beneficial roles in disease depending on time, location, and magnitude of plasmin activation(1,147,150,327). Like many other mechanistic players in the APR, physiologic plasmin activation is subject to tight regulation. Recent research on plasmin's functions across physiology are beginning to suggest that early plasmin activity during a survival-APR may have deleterious effects on not just bleeding but also a proper inflammatory response as fibrin containment of injuries stimulates a reparative response from macrophages, neutrophils, and mesenchymal stem cells(173,328). Conversely, plasmin generated on the surface of infiltrating leukocytes, fibrin, and necrotic tissue is critical to incite the removal of the necrotic tissue and fibrin, angiogenesis, mesenchymal stem cell differentiation, and cellular regeneration during a repair-APR(1). Therefore, diseases or injuries that alter dramatically alter the timing or magnitude of plasmin generation produce plasmin-mediated pathologies. A severe injury, such as a burn, shifts the timing and magnitude of plasmin activation from a moderate level during a repair-APR to a high level during a survival-APR. Not only is the dramatic increase in plasmin activation during a survival-APR exacerbate bleeding, inflammation, and coagulation dysfunction, but the subsequent diminished plasmin activity during a repair-APR has the potential to negatively affect repair of sensitive tissues, such as muscle.

Plasmin is not only essential for muscle and bone repair, but it also critical in regulating a multi-step process during repair so that bone does not form within injured muscle. Previous work from our lab has shown that plasmin is essential to prevent the formation of dystrophic calcification within muscle(33), and this study has demonstrated that pharmacologic enhancement of plasmin is sufficient to prevent burn-induced calcification in injured muscle. Furthermore, the removal of DC formed in injured muscle following a burn injury is both plasmin and macrophage-dependent.

In the absence of either of these, DC remains in the injured muscle and is gradually remodeled into bone, or HO, within 6 weeks of the injury. While the roles of plasmin in the prevention and removal of DC are fibrin-independent, plasmin exerts multiple effects on the macrophage through many receptors, including fibrin(183,279,329), and both plasmin and macrophages are necessary for the removal of DC and the prevention of HO formation in injured muscle.

With the survival of severe injuries, such as burns, patients and clinicians face the challenge of repair complications, including fibrosis, HO, and degeneration of injured and peripheral tissues(157). The systemic derangements provoked by these injuries have persisting effects, and few viable treatment options are available to improve recovery in these patients. Here, we have shown that severe burn injuries cause calcification of injured muscle remote from the site of burn, suggesting that burn causes systemic changes that alter tissue repair. Specifically, a severe burn alters the time-course of plasmin activation following injury, resulting in poor plasmin activity during 3-7 days post-injury between which plasmin is critical for muscle repair. Further, if a state of poor plasmin activity persists, calcification within injured muscle remains and may remodel into HO, leading to chronic pain and lack of mobility. Unlike mice, patients with severe injuries often undergo multiple surgical procedures to correct the damage(214), causing transient, recurring APRs that may prolong plasmin deficiencies and systemic inflammation.

Severe injuries involving polytraumas often present with paradoxical defects in bone repair with increased risk of pathologic bone formation within injured muscle, and few ideal therapeutics exist to prevent the trauma-induced HO while maintaining physiologic bone repair and maintenance. Bisphosphonates have proven effective to prevent HO and to stabilize bone, preventing trauma-induced osteoporosis(214,226,241). However, bisphosphonates can have

deleterious effects on bone repair and remodeling, making them an inferior therapeutic option(330). Plasmin, however, is beneficial for both inhibiting aberrant soft tissue mineralization and promoting healthy bone repair(33,36). Therefore, enhancing plasmin following severe injury may represent a superior therapeutic option to promote physiologic musculoskeletal repair. Importantly, enhancement of plasmin should be carefully considered with respect to timing following the injury. Early activation of plasmin may drive bleeding and inflammation-associated complications, making TXA an effective therapeutic to prevent early complications in patients with severe injuries(145). However, based on the findings of this study and other trauma studies, the beneficial effects of TXA are lost within hours following the injury, and TXA may increase the risk of thrombosis and repair complications if dosed beyond the therapeutic window(199,327). Therefore, enhancement of plasmin later in convalescence during tissue repair may significantly improve long-term outcomes in patients with polytraumatic severe injuries.

Enhanced plasmin prevents calcification in a plasmin-independent genetic predisposition to soft tissue calcification

Plasmin is essential for prevent calcification within injured muscle, but calcification of soft tissues (including muscle) can arise from a variety of sources beyond plasmin deficiency, including rare genetic diseases. Pseudoxanthoma elasticum (PXE) is a rare disease affecting 1 in 100,000 to 1 in 25,000 individuals that presents an increased risk for calcification of soft tissues, including skin, muscle, major internal organs, and vasculature(331). PXE is attributed to a mutation in *ABCC6*, a gene encoding the Abcc6 ATP transporter(332). The Abcc6 transporter pumps ATP into the extracellular environment where it is cleaved to AMP and pyrophosphate by membrane-bound ectonucleosidases, such as Enpp1(326). Pyrophosphate is composed of two phosphates linked by a phosphoanhydride bond, and its most common biologic role is preventing the formation of calcium phosphate, which forms calcific deposits in soft tissues as hydroxyapatite. Pyrophosphate acts as a competitive antagonist, binding calcium ions and preventing their interaction with phosphate molecules, thereby preventing calcification(226). In PXE patients with mutations in *ABCC6,* there is less extracellular pyrophosphate, predisposing these individuals to the pathologic soft tissue calcification observed(326).

The disease pathophysiology of PXE has been studied in the Schoenecker Lab extensively by my colleague. Dr. Stephanie Moore-Lotridge. As a part of my research, I assisted her in studying pharmacologic methods to prevent soft tissue calcification in a mouse model of PXE. Plasmin is a potent mediator of tissue repair, including the prevention of muscle calcification following injury(33). Therefore, we hypothesized that pharmacologic enhancement of plasmin activity would prevent calcification in injured muscle in Abcc6-deficient mice.

Methods: Six-week-old wild-type or Abcc6-deficient mice were administered calf muscle injuries by local injection of 10μM cardiotoxin as previously described(221). Mice received either a control ASO or an ASO against plasmin inhibitor α2-antiplasmin (α2AP) at 330mg/kg/week, beginning 2 weeks prior to injury to enhance plasmin activity. Mice received radiograph images of their injured hindlimbs at 7 days post-injury using Faxitron Imaging with 4s exposure at 35kV to assess muscle calcification. Following euthanization, injured hindlimbs were analyzed by μCT analysis as previously described(326).

Results: Abcc6-deficient mice exhibited dose-dependent calcification of their injured muscles at 7 days post-injury, whereas WT mice exhibited little to no muscle calcification (Figure 3.13A-B). Enhancement of plasmin with α2AP ASO prevented with formation of muscle calcification in $Abcc6^{+/-}$ and $Abcc6^{-/-}$ mice (Figure 3.13A-B).

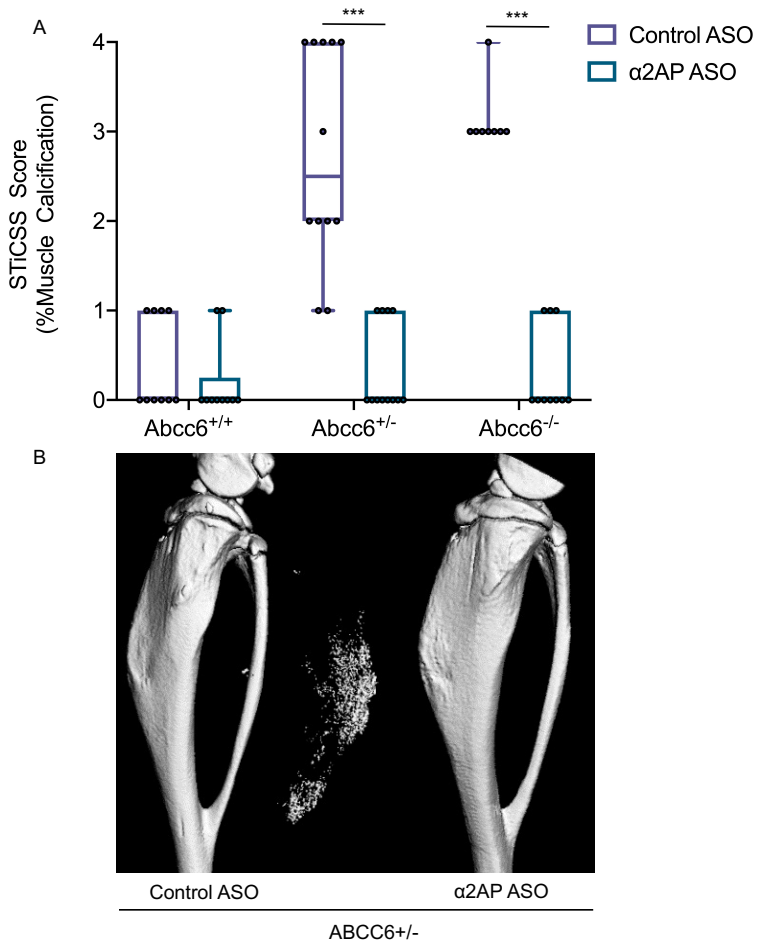

Figure 3.13: Enhanced plasmin prevents dystrophic calcification in a mouse model of Pseudoxanthoma Elasticum. A) Abcc6-deficient mice develop robust muscle calcification within 7 days post-injury compared with WT mice ($Abcc6^{+/+}$), and enhancement of plasmin activity by α2AP ASO prevents DC in Abcc6-deficient animals (***P<0.001, N=8-10/group). B) Representative μCT images of $Abcc6^{+/-}$ mice with control or α2AP ASO.

Discussion: Currently, there are few effective treatments to prevent calcification of soft tissues in PXE patients without compromising bone integrity. Bisphosphonates are pyrophosphate analogs that can prevent soft tissue calcification, but some classes of these drugs also interfere with bone repair and maintenance(REF), making them a less than ideal therapeutic option. Plasmin has been

shown to exhibit paradoxical effects on bone formation with tissue-specific tropism: plasmin prevents calcification of soft tissue but promotes healthy bone formation. The results of this study suggest that plasmin overcomes the increased hydroxyapatite formation in Abcc6 deficiency and may present a therapeutic option for tissue repair and the prevention of soft tissue calcification in PXE patients. While my dissertation work is focused on manipulating plasmin following a severe injury to promote recovery, its therapeutic potential in musculoskeletal repair goes beyond severe injury to various genetic diseases that predispose patients to pathologic tissue repair.

Chapter 3 Summary:

Within this chapter, I've highlighted the different roles plasmin plays in musculoskeletal development, repair, and degeneration in the form of a comprehensive review article. Plasmin plays paradoxical roles within the musculoskeletal system based on timing, location, and concentration.

In the context of bone, while plasmin is essential for physiologic bone formation and maintenance within the defined skeletal system, it prevents aberrant bone formation within soft tissues by preventing the formation of dystrophic calcification and promoting the macrophage-mediated regression of DC that does form. Plasmin's roles in HO are dependent upon concentration. A physiologic concentration of plasminogen is required to sustain appropriate muscle repair and to prevent DC formation; a 50% deficiency or greater promotes poor muscle repair, fibrosis, and the formation of DC within injured muscle. However, bone repair and formation (even in a pathologic capacity) remain intact with a 50% loss of plasminogen. Therefore, 50% normal plasmin levels are insufficient to prevent DC or promote DC regression within injured muscle but are sufficient to drive the maturation of persistent DC to HO (Figure 3.14). A near

complete or complete loss of plasminogen provokes DC with insufficient bone repair or formation mechanisms to drive the maturation of DC to HO (Figure 3.14).

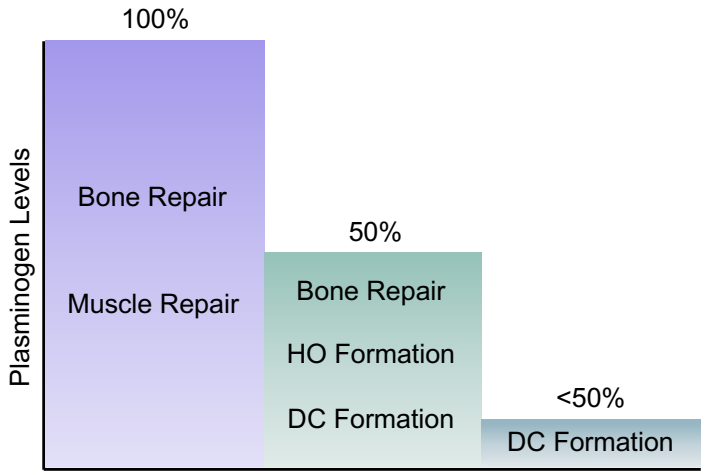

Figure 3.14: The seemingly paradoxical roles of plasmin(ogen) in bone repair and the prevention of bone outside of the skeletal system are concentration-dependent.

In addition to plasmin deficiencies and trauma-induced pathologic musculoskeletal outcomes, plasmin enhancement therapy may be applied to other genetic disease which predispose patients to certain pathologic muscle repair, such as dystrophic calcification observed in PXE patients with mutations in *ABCC6.* Because most therapeutics to prevent soft tissue calcification interfere with physiologic bone maintenance and repair, plasmin therapy may provide a safer alternative to prevent DC. While complete plasminogen deficiency is rare in humans, acquired deficiencies in disease states can provoke a partial loss of plasminogen, making these individuals susceptible to problems of muscle repair. If this plasminogen deficiency is chronic and concurrent with inflammatory disease, this can begin to provoke complications of bone repair and overall bone health. Acquired deficiencies are discussed in the following Chapter 4.

CHAPTER 4: PLASMINOGEN DEFICIENCY

Congenital Plasminogen Deficiency

While congenital plasminogen deficiency types I and II—hypoplasminogenemia (loss of expression) and dysplasminogenemia (loss of function)—are rare (less than 2 in 1,000,000 individuals) acquired hypoplasminogenemia and dysplasminogenemia can occur in severe injuries and chronic disease, respectively(333). Transient periods of plasminogen deficiency often follow severe injury due to consumption and redistribution to large sites of injury, as highlighted in Articles 1 and 2. Alternatively, chronic inflammation and metabolic disease can induce structural changes to circulating plasminogen, diminishing its ability to a) bind to fibrin and cellular receptors, b) be activated or c) proteolytically engage with its downstream targets. This phenomenon is often observed in many different circulating proteins in patients with poorly controlled blood glucose levels, such as patients with type 2 diabetes mellitus(334).

Methylglyoxal modification of plasminogen reduces *in vitro* and *in vivo* function

Diabetic patients with uncontrolled hyperglycemia often experience a wide range of pathologic conditions of both tissue repair and tissue homeostasis. Studies have clearly demonstrated that chronic hyperglycemia and insulin resistance alter cell signaling and protein function, causing systemic dysregulation. This condition provokes delayed or insufficient repair of tissues including skin, muscle, and bone, and over time, they cause degeneration of these tissues, such as osteoporosis(335–337). There are a multitude of factors known to contribute to poor repair and tissue degeneration in diabetic patients, including hypercoagulability and chronic

inflammation(338,339). While studies have elucidated some mechanisms behind these problems, few ideal therapeutic targets have been identified to treat poor repair in these patients.

Plasmin plays a critical role in regulating coagulation and inflammation in both the intravascular and extravascular environs. Our group and others have demonstrated that plasmin is essential for both tissue repair and the prevention of osteoporosis(1,2,274). Specifically, plasmin is essential for repair of skin wounds, bone fractures, and muscle regeneration through fibrinolysis, stimulation of angiogenesis, migration of mesenchymal stem cells, and controlled regulation of inflammatory signaling(1,28,106). Notably, a 50% reduction in plasmin activity can have physiologically significant consequences in repair. A common problem identified in diabetic patients is that of poor fibrin degradation, or hypofibrinolysis. This has been attributed to excess circulating fibrinogen and fibrin deposition, inappropriate fibrin crosslinking, and high serum levels of PAI-1(338,340–343). Furthermore, studies have shown that plasmin(ogen) is vulnerable to modification by carbohydrate metabolism byproducts at lysine and arginine residues, possibly reducing its function in fibrinolysis, tissue repair and tissue homeostasis(334).

Prolonged hyperglycemia, consistent with uncontrolled diabetes mellitus, precedes the breakdown of carbohydrates into reactive groups, such as dicarbonyls, that participate in the Maillard reaction with amino acids in proteins, irreversibly modifying them. While some proteins can retain reasonable function following adduction by these dicarbonyls, these reactions can not only disrupt protein function through structural changes, but they can also produce modified proteins with strong inflammatory signaling capacity. Advanced Glycation Endproducts (AGEs), proteins heavily modified by glycation, can activate downstream inflammatory signaling through activation of the NF-κB pathway and stimulating ROS production by mitochondria through the receptor for AGEs (RAGE), exacerbating diabetic complications(344,345).

Methylglyoxal (MG), a common dicarbonyl byproduct of carbohydrate breakdown that is elevated in chronic hyperglycemia, modifies many proteins, such as plasminogen, inhibiting their function and increasing their inflammatory potential(346). Previous studies of plasminogen isolated from patients with uncontrolled diabetes demonstrated that plasminogen is glycated at specific lysine residues, resulting in poor plasminogen binding, reduced plasmin activation, and impaired protease function(334). While these studies examined plasmin generation *in vitro*, they did not include functional assays and models of tissue repair to assess how glycation of plasminogen by MG affects its function *in vivo*. We hypothesized that MG modification of plasminogen will alter its function in *in vitro* fibrinolytic assays as well as *in vivo* tissue repair applications.

Methods:

Plasminogen Purification:

Twenty-five units of human plasma were added to an 8L flask and warmed to 30°C. H-D-Phe-Phe-Arg-chloromethylketone 1μM (FFR-CK), H-D-Phe-Pro-Arg-chloromethylketone (FPR-CK) 1μM, and phenylmethylsulfonyl fluoride (PMSF) 144μM were added to the plasma and allowed to stir in for 20 mins. Plasma was then centrifuged at 2796 RFC for 20 mins to remove any clots or precipitants and then loaded onto a Lysine Sepharose 4B column (GE Healthcare, Chicago, IL) at room temperature followed by 2L of start buffer (0.15M NaH_2PO_4, 3mM EDTA, 0.02% NaN_3 pH 7.4) until the absorbance at 280nm was stable below 0.05. The column was then eluted with 1L of step buffer (0.3M NaH_2PO_4, 200 mM εACA, 3mM EDTA, 0.02% NaN_3 pH 7.4) collecting 15 ml fractions. Fraction absorbance was measured at 280 nm and all samples with a large amount of protein were pooled. Again, 1 μM FFR-CK, 1 μM FPR-CK, and 144 μM PMSF were added

to the plasma and the pooled samples were dialyzed vs. 24L of start buffer with 4 changes. After dialysis, the sample was again loaded onto a lysine sepharose column equilibrated at 4^0C followed by the addition of 2L of start buffer until the absorbance at 280 nm of the fractions was zero or stable. Plasminogen was eluted off the column using a 2 liter gradient consisting of 1liter of start buffer and 1 liter of limit buffer (0.3M NaH_2PO_4, 3mM εACA, 3mM EDTA, 0.02% NaN_3 pH 7.4). Fractions were again read at 280nm with the first peak of protein being Glu plasminogen-1 and the second peak being Glu Plasminogen-2. The plasminogen was concentrated down using a centrifugal filter with a molecular weight cut-off of 10kDa (Amicon). The sample was then dialyzed vs 12L of storage buffer storage buffer (50mM HEPES, 0.125M NaCl, pH 7.4) with 3 changes.

MG-Plasminogen Incubations

MG incubations were carried out in 50 mM Hepes, 125mM NaCl, pH 7.4 buffer. 20 uM Glu-plasminogen was incubated with MG (Sigma-Aldrich, St. Louis, MO) at physiologic and supraphysiologic molar ratios of: 1:1, 1:2, 1:4, 1:8, 1:16, and 1:32 for 24 hours at 37°C. Plasminogen that was treated with phosphate buffered saline under the same conditions served as control plasminogen for all experiments.

Simultaneous Turbidity and Plasmin Generation Assay

A simultaneous turbidity-plasmin generation assay was performed in a 96-well culture plate with black sides and a clear bottom (Corning, Corning, NY). Each well contained 100nM Glu-plasminogen, 70pM tissue plasminogen activator (tPA) (Merck, Darmstadt, Germany), 2.4μM fibrinogen (Enzyme Research Laboratories, South Bend, IN) 5mM $CaCl_2$, and 210 μM fluorescent

plasmin substrate (H-D-Val-Leu-Lys-AFC, Anaspec, Inc, Fremont, CA) are added to assay buffer (50mM Hepes and 125 mM NaCl, at pH 7.4). 0.2units/mL bovine thrombin (King Pharmaceuticals, Bristol, TN) was injected automatically by the Synergy 2 Microplate reader for a final volume of 200 uL. Reactions were read every 30 seconds for 5 hours. Clot formation and lysis were measured by absorbance (OD) at 350nm every 30 seconds. Plasmin activity was directly monitored via fluorescent (RFU) excitation wavelength of 380nm and emission wavelength of 560nm. All experiments run in duplicate with two wells run in the absence of fibrinogen. Additionally, another two wells were run in absence of plasminogen. To ensure that the presence of methylglyoxal did not affect the assay, methylglyoxal was added to 2 control wells. Control wells run without fibrinogen or plasminogen served as baseline controls for turbidity and plasmin generation, respectively, and were subtracted from the measurements. Clot lysis time was calculated as the T_{50} or the time to reach half-maximal OD_{350}. Plasmin generation was calculated as the change in fluorescence over time (first order derivative). The area under the curve of the first derivative was then calculated and expressed as arbitrary units of plasmin activity. All data calculations were performed in GraphPad Prism 5.

Inhibition of Plasminogen Activation by εACA

The sensitivity of the assay to plasminogen inhibition was validated using the known therapeutic plasminogen inhibitor εACA. The reaction was prepared as described above and final concentrations of εACA (0.1 uM -10 mM) were added just prior to starting the reaction.

Plasminogen Gel Shift assays

Plasminogen gel shift assays were performed on control plasminogen and plasminogen treated with methylglyoxal (molar ratio of 1:16). Samples were incubated in the presence and absence of

1 uM tPA for 24 hours. Samples were then run on a 4-20% gradient gel under reducing conditions and stained with simple blue for identification of protein bands.

Plasminogen binding studies on MG-treated plasminogen

Plasminogen binding studies were conducted using a 5 ml lysine-sepharose column. Two milligrams of control plasminogen or plasminogen treated with methylglyoxal (molar ratio of 16 ;1) were loaded on to the column and 1 ml fractions were collected at a flow rate of 1 ml/min continuously adding a phosphate buffer (0.15 M NaH_2PO_4, 3 mM EDTA, 0.02% NaN_3 pH 7.4) continually. Fractions 1-28 were collected during loading and fractions 29-51 were collected after the addition of limit buffer to elute the plasminogen. Protein levels were determined by absorbance at 280nm on a Synergy 2 Microplate reader.

In vivo measurement of modified plasmin activity in injured skeletal muscle

Plasminogen incubated with MG at 1:1, 1:8, or 1:16 molar ratios were dialyzed in 3 changes of 4L 50mM HEPES, 125mM NaCl, pH 7.4 to remove MG from the samples. Plasminogen heterozygous mice ($Plg^{+/-}$) with poor muscle repair received bilateral injuries to the calf muscle by intramuscular injection of 40µL 10µM cardiotoxin as previously described(221). Mice were administered a resuscitative dose of intraperitoneal plasminogen previously incubated with different molar ratios of MG at 1mg per day or equivolume saline, beginning on the day of injury and up to 7 days post-injury. $Plg^{+/-}$ mice developed robust calcification and fibrosis of injured muscle, and therefore, muscle repair was assessed at 7 days post-injury by radiographic analysis, and following euthanasia, µCT and histologic analysis. Radiographs were scored using the validated Soft Tissue Calcification Scoring System (STiCSS) as a percent of the hindlimb calcified(221).

Results:

Functional Evaluation of Adducted Plasminogen: To determine the functional effect of adductions to plasminogen by methylglyoxal, we developed a novel assay that simultaneously measures fibrin clot formation, fibrin clot lysis, and plasmin activity in a 96-well plate (Figure 4.1 A&B) optimized.

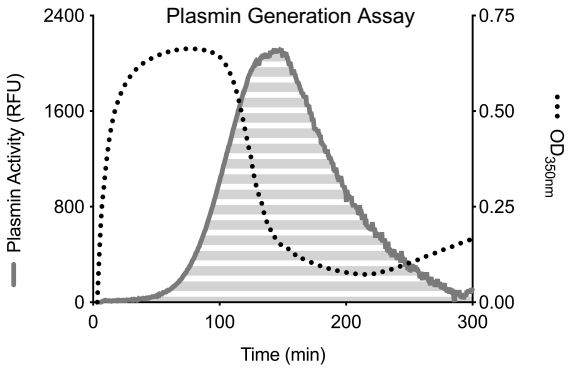

Figure 4.1: The plasmin generation assay. Measurement of clot formation and lysis as well as plasmin generation measured by fluorogenic plasmin substrate cleavage.

To validate that simultaneous turbidity and plasmin generation assays were sensitive in detecting inhibition of plasminogen activation and fibrinolysis experiments were performed in the presence of increasing concentrations of aminocaproic acid, a known inhibitor of plasmin activation. These results confirm that this assay is sensitive to changes in fibrinolysis and plasmin activation, with a half maximal inhibitory concentration (IC_{50}) of 44uM of aminocaproic acid for clot lysis, and an IC_{50} of 73uM of aminocaproic acid for plasmin activity (Figure 4.2A&B). Having established the ability of our simultaneous turbidity plasmin generation assay to detect changes in plasmin activity and fibrinolysis, we next determined if adduction of plasminogen by MG inhibits fibrinolysis and plasminogen activation.

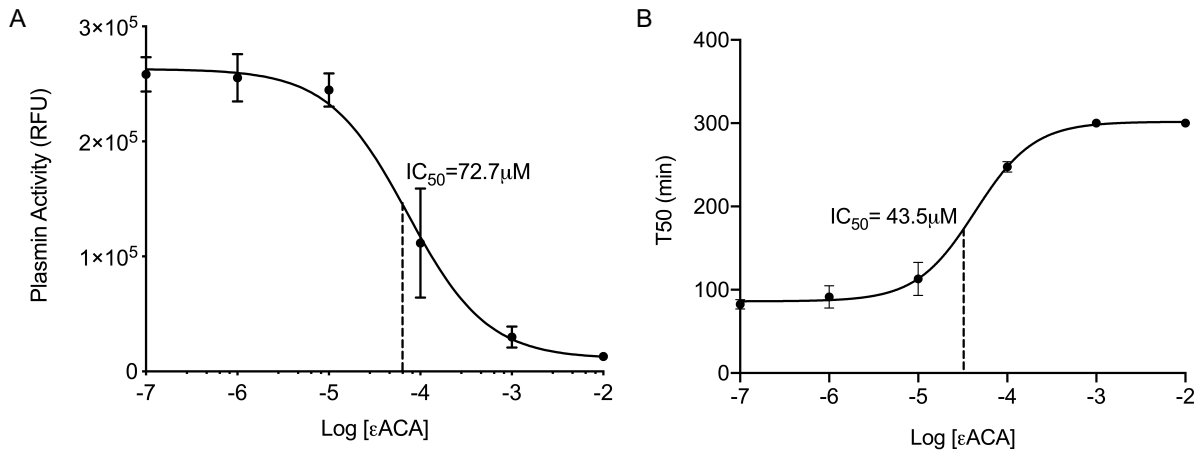

Figure 4.2: The effects of aminocaproic acid on plasmin generation. Aminocaproic acid (εACA) causes a concentration-dependent inhibition of A) plasmin generation and B) clot lysis.

Plasminogen was treated with increasing molar ratios of MG for 24 hours and then used in plasmin generation assays to assess the effects on fibrinolysis and plasminogen activation. Treating plasminogen with MG resulted in a concentration-dependent decrease in plasmin activation (Figure 4.3A) and clot lysis(Figure 4.3B). Having determined that MG treated with plasminogen is functionally inhibited, the MG-treated plasminogen was tested in the plasmin generation assays in with variable tPA concentrations that do not require fibrin for plasminogen activation (>100pM). Increased concentrations of tPA were capable of activating MG-treated plasminogen. However, MG-treated plasminogen exhibited a right-ward shift and a reduction in the maximal amount of plasmin activity compared to control plasminogen, indicating that MG-treated plasminogen is poorly activated. The concentration-dependent decrease in plasmin activity in the presence of fibrin and tPA, and the decreased total plasminogen activation even at maximal doses of tPA (10nM), suggest that functional impairment of plasminogen is likely the result of both an inability to bind fibrin as well as an inability to be convert to active plasmin by tPA (Figure 4.3C).

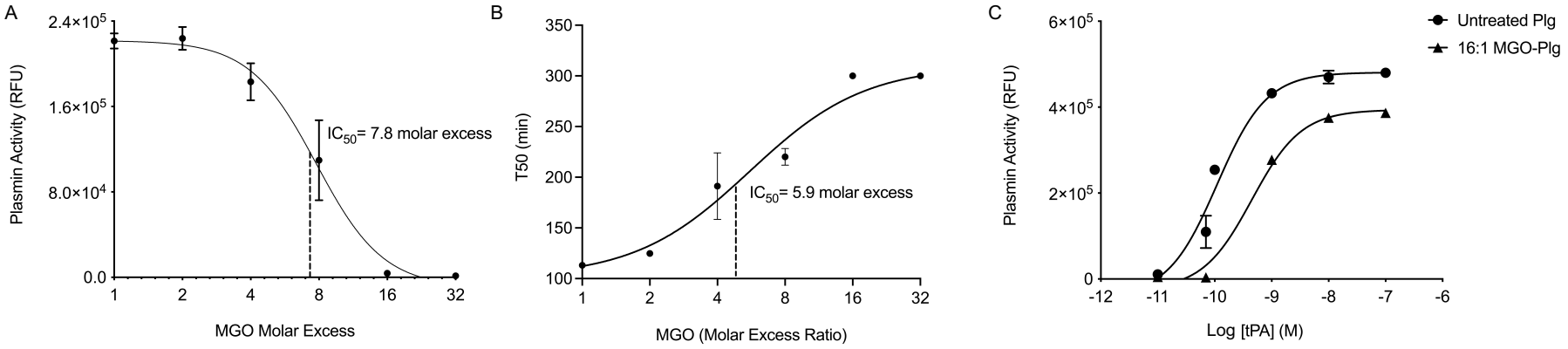

Figure 4.3: The effects of methylglyoxal on plasmin generation. MGO incubation causes a dose-dependent reduction in A) plasmin activation and B) clot lysis and C) a reduced potential for tPA-mediated plasmin generation, even at tPA concentrations that do not require fibrin. More datapoints are required to accurately determine IC_{50} for publication.

Methylglyoxal Pre-incubation Impairs Plasminogen Activation and Lysine Binding: To confirm that plasminogen treatment with methylglyoxal at a 16:1 molar ratio modified plasminogen in a manner that impaired its activation, we performed a gel shift assay in which methylglyoxal-treated plasminogen and untreated control plasminogen were incubated with 1uM of tPA, a concentration at which fibrin is not required for plasmin activation. After 24 hours in the presence of tPA, methylglyoxal-treated plasminogen showed bands at molecular weights of both plasminogen and plasmin, indicating that a population of plasminogen was not activated. In contrast, the untreated control plasminogen incubated with tPA resulted in single band at the molecular weight of plasmin, indicating that all plasminogen had been converted to the active form, plasmin (Figure 4.4A). These results demonstrate that methylglyoxal can render plasminogen incapable of being activated. Physiologic activation of plasminogen by tPA requires that both plasminogen and tPA bind to lysine residues on a fibrin matrix, allowing for open conformation plasminogen and tPA to be co-localized, resulting in plasminogen activation. Using a lysine-sepharose affinity column to simulate lysine residues on a fibrin matrix, we examined whether the ability of plasminogen to bind lysine residues was impaired by methylglyoxal treatment. These data demonstrate that during loading of methylglyoxal-treated plasminogen onto the lysine column an increase in absorbance was detected in fractions 5-10, indicating that a population of the methylglyoxal-treated plasminogen was unable to bind the column (Figure 4.4B). The remaining methylglyoxal-treated plasminogen and control untreated plasminogen were eluted from the column in fractions 32-50 (Figure 4.4B).

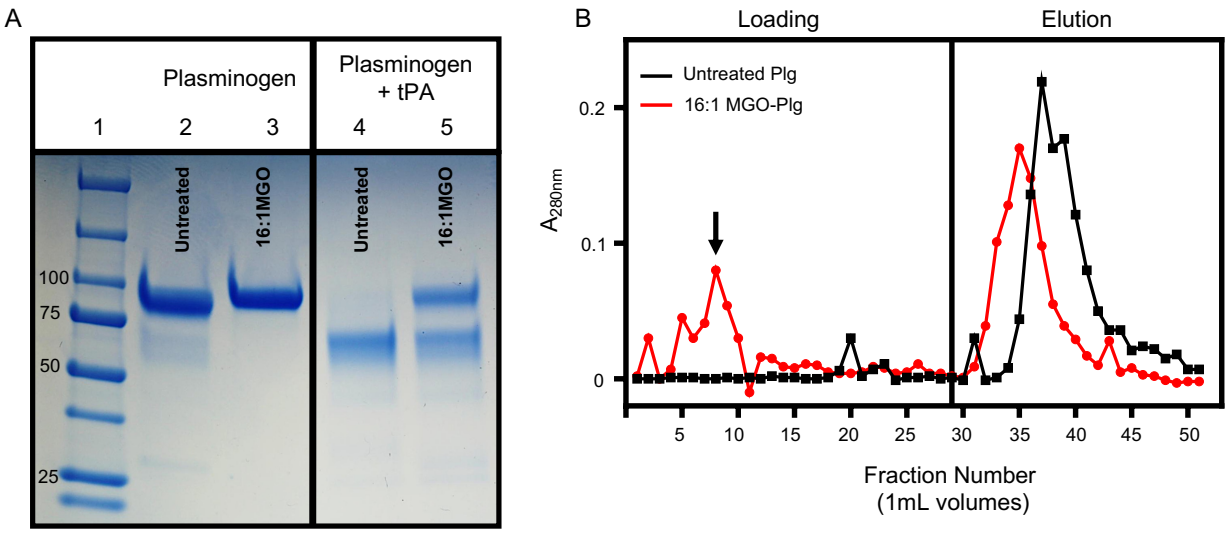

Figure 4.4: The effects of methylglyoxal on plasminogen binding and activation. A) MG-treated plasminogen is poorly cleaved to plasmin by tPA and B) exhibits impaired binding to a lysine-sepharose column.

MGO-modified plasminogen is insufficient to rescue muscle repair in Plg$^{+/-}$ mice

Plasminogen is essential for repair of all tissues, and therefore, plasminogen-deficient mice (Plg$^{-/-}$) have poor tissue repair(33–36). Muscle repair is particularly sensitive to plasminogen levels such that a 50% loss of circulating plasminogen observed in Plg$^{+/-}$ mice results in defective muscle repair that causes muscle calcification. To test the *in vivo* functionality of MG-modified plasminogen, Plg$^{+/-}$ mice were administered calf muscle injuries, and recombinant human plasminogen (rhPlg) was dosed therapeutically to restore muscle repair. Beginning at the time of injury, mice received 1mg of plasminogen modified at 1:1, 1:8, or 1:16 MGO, as described above, per day. At 7 days post-injury, the Plg$^{+/-}$ mice treated with 1:1 and 1:8 modified plasminogen had little to no calcification, while those treated with plasminogen modified at 1:16 MGO developed

robust calcification (Figure 4.5A&B), suggesting that the 1:16 MG-incubated plasminogen has little *in vivo* function for muscle repair.

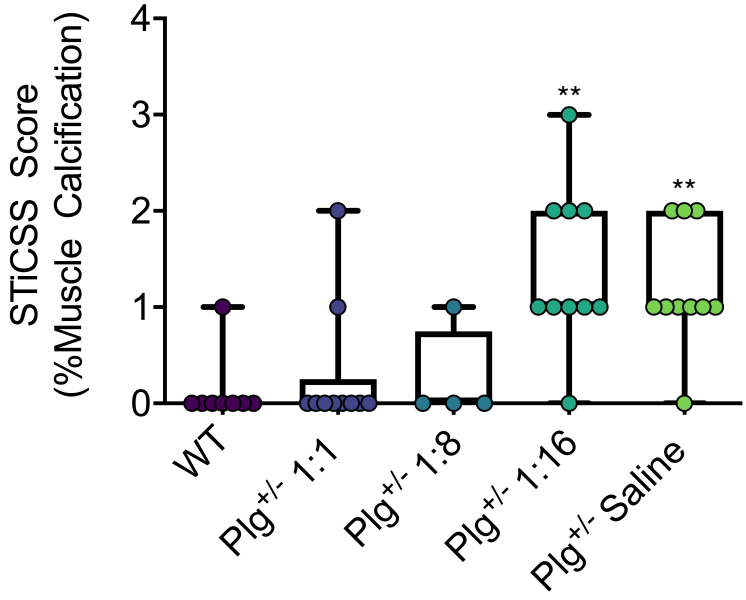

Figure 4.5: Methylglyoxal-incubated plasminogen in muscle repair. A-B) Plasminogen incubated with a 16-fold molar excess of MG failed to prevent muscle calcification, while plasminogen incubated with an equal concentration of MG retained ability to prevent muscle calcification in $Plg^{+/-}$ mice 7 days following injury (**P<0.01 compared with WT mice, N=4-10/group).

Discussion:

Our findings reveal that modification of plasminogen by MG, a reactive dicarbonyl reported to be elevated in diabetes, decreases plasmin activation and impairs fibrinolytic activity *in vitro* and impairs plasmin's ability to promote muscle repair *in vivo.* Though other investigations have demonstrated that plasminogen can be adducted by reactive metabolites such as MG(334), this is

the first study to confirm that methylglyoxal's inhibitory action on plasminogen occurs in a concentration-dependent manner. Our data suggest that modification of plasminogen by MG may contribute to poor fibrinolysis or acquired dysplasminogenemia in T2DM, and this could potentially affect plasmin's ability to promote tissue repair through fibrin-dependent and independent mechanisms.

In addition to the clinical implications of our findings, this study also demonstrates that both fibrinolysis and plasmin generation can be measured simultaneously. Using near physiologic concentrations of activators, we were able to initiate the conversion of plasminogen to plasmin in a fibrin-dependent manner. The use of near physiologic concentrations more closely replicates *in vivo* fibrinolysis. Therefore, our assay was dependent upon the co-localization of tPA and plasminogen on exposed lysine residues that were generated during the formation of the fibrin clot. This interdependence of both tPA and plasminogen on fibrin for co-localization may prove invaluable for screening of new anti-fibrinolytic drugs, or new pharmacological strategies aimed at preventing the formation of or modulating the stability of a fibrin clot.

Previous investigations have shown that diabetics have elevated blood levels of reactive metabolites, such as MG, that correlate with the magnitude of hyperglycemia and act to modify proteins by adduction of positively charged amino- and sulfhydryl groups on amino acids(339,344,345). Additionally, studies have reported an acquired hypofibrinolytic state in diabetics is associated with chronic hyperglycemia. Given these associations and that fibrinolysis and fibrin-independent plasmin activity have both been shown to be essential for the repair of all tissues, including muscle, skin, nerve, and bone, much investigation is now focused on the etiology of the acquired hypofibrinolytic state in diabetic patients. Analysis of plasma from diabetic

patients has demonstrated a significant elevation in the fibrinolytic inhibitor PAI-1(340). This has led to the hypothesis that hypofibrinolysis is the result of elevation of fibrinolytic inhibitors. Our data demonstrates that an impaired fibrinolytic system may also occur in patients with diabetes through ROS modification. Our hypothesis is further supported by investigations demonstrating that: decreased plasmin activity is due to is post-translational modifications and that these modifications 1) are correlate with glycemic control and 2) increased levels of reactive metabolites, specifically MG, occur in patients with diabetes. Here, we demonstrated a functional impairment in MG-adducted plasminogen to promote repair of injured muscle. However, future studies are needed to determine if the acquired hypofibrinolytic state correlates with adduction of plasminogen and poor tissue repair outcomes in patients with T2DM.

Plasminogen is not the only component of the fibrinolytic system that could be affected by adduction and thus capable of contributing to in an acquired hypofibrinolytic state. Fibrinogen, which circulates in adults at a concentration of 4 mg/ml, the second highest protein concentration in blood, contains many lysine and arginine residues susceptible to adduction(341). *In vivo* and *in vitro* investigations have demonstrated that fibrinogen is susceptible to methylglyoxal adduction. However, future investigations are needed to determine the functional impact of adducted fibrinogen on fibrinolysis in patients with T2DM.

The results presented in this investigation demonstrate that reactive byproducts of glycolysis, such as MG, are capable of modifying plasminogen in a concentration dependent manner to decrease plasminogen binding, activation, and function. Furthermore, this study suggests that the modification of plasminogen reduces or diminishes its function in tissue repair, dependent on the level of modification.

Functional *in vitro* testing of plasminogen isolated from patients with T2DM

Background/Methods: While *in vitro* modification of plasminogen with MG caused a dramatic loss of plasminogen binding, activation, and functional capacity, it's unclear how plasminogen is affected *in vivo* in patients with poorly managed T2DM. In continuation with the previous study, we received purified plasminogen from 40 patients with T2DM and 30 age-matched control patients from Drs. Ruby Law and James Whisstock at Monash University, Melbourne, Australia. Following crystallographic analysis to determine which patient proteins were most heavily affected by modification, the purified plasminogen was run in a plasmin generation assay with turbidity as described above.

Results: There was a trend towards prolongation of 50% clot lysis time (T50) in wells with plasminogen isolated from T2DM patients compared with controls. However, there was not a significant difference between either plasmin generation or T50 between the control and the diabetic groups (Figure 4.6A-B). Significant variability occurred within groups, and normalization for inter-assay variability or blood sugar measurements such as hemoglobin A1C did not correct this. For each sample run, plasmin activity and T50 correlated with one another (Figure 4.6C).

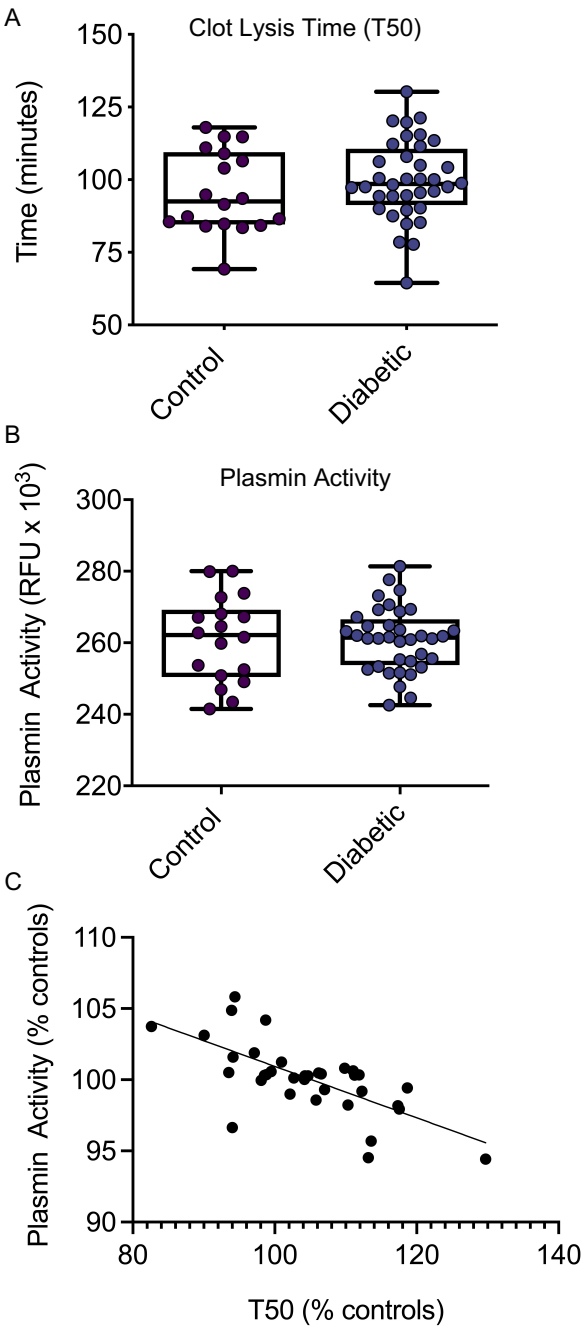

Figure 4.6: Plasmin generation in with plasminogen isolated from patients with Type 2 Diabetes. A-B) Plasminogen purified from patients with T2DM exhibited a trend in prolonged clot lysis and reduced plasmin activation, but it did not reach significance. C) Plasmin activity and clot lysis were strongly correlated with one another (R^2=0.469, P<0.0001).

Conclusions: Plasminogen isolated from patients with T2DM appear to have a reduced capacity to degrade fibrin clots in an isolated system. It's unclear what the *in vivo* implications are for this, but in combination with the potential of elevated or adducted fibrinogen and excessively crosslinked fibrin, this loss of fibrinolytic potential may add to the risk of fibrin-mediated thrombosis, inflammation, and tissue repair problems.

Chapter 4 Summary:

Plasmin(ogen) deficiencies come in many different forms, all with pathologic effects on physiology. Congenital deficiencies in plasminogen are extremely rare and can successfully be treated with protein replacement therapies. Acquired deficiencies—either hypoplasminogenemia or dysplasminogenemia—are poorly characterized and are currently not treated. In Chapter 2, acquired hypoplasminogenemia was demonstrated following surgical or burn injury.

Patients with T2DM notoriously have problems with thrombosis and poor tissue repair. In this chapter, I've discussed how reactive dicarbonyl MGO that has been shown to accumulate in blood during periods of prolonged hyperglycemia has the potential to structurally modify plasminogen, making it resistant to activation. This modification also prevents its roles in muscle repair in a mouse model of muscle injury. In plasminogen isolated from patients with T2DM, there appears to be a slight prolongation of clot lysis in a purified assay system, but hypofibrinolysis in T2DM patients is most likely a combination of inflammation, protein modification, and hypercoagulability. More studies are needed to the future to determine if plasmin activity can be safely enhanced in these patients to treat this hypofibrinolytic condition.

CHAPTER 5: EXERCISE, PLASMIN ACTIVATION, AND WELLNESS

Regular exercise causes microinjuries that provoke a local APR, transiently stimulating both containment and repair mechanisms. This is, in part, why exercise can have beneficial effects on tissue health and overall wellness. Studies have shown that short bursts of coagulation, fibrinolysis, and inflammation can cause mild downregulation of coagulation and inflammatory signaling, resulting in reduced overall coagulation and inflammatory potential(347,348). Animal models have shown that exercise prior to injury reduces the local and systemic inflammatory response to aging(349,350). In humans, individuals who participate in consistent exercise routines have an increased coagulation potential but a reduced risk of thrombosis, suggesting a robust coagulation response in an appropriate setting (vascular breach) but a resistance to inappropriate coagulation activation(348). Exercise is also a commonly implemented measure of wellness, and diseased animals and humans that cannot exercise do not receive the benefit of it.

During my graduate work, I studied coagulation, fibrinolysis, and inflammation following exercise of different intensities in humans in order to quantify the exercise-induced APR. Then, myself along with an undergraduate mentee, Zack Backstrom, developed a mouse model of unforced exercise to measure wellness in mice with severe injuries or deficiencies in the plasmin activation system. Because exercise activates plasmin, we approached the concept with the hypothesis that moderate exercise provides short-burst, daily doses of plasmin. In this chapter, I highlight these experiments in contrast to the APR in response to a severe injury and also demonstrate how a pathologic APR can results in inability to exercise.

Moderate exercise provokes plasmin activation and increased coagulation potential without systemic inflammation.

The ideal method by which to quantify current and previous plasmin activity is unclear. Often, reporter assays and functional assays are not in agreement with one another due to time. In circumstances in which activation of coagulation or fibrinolysis are transient, functional assays might miss these events. A physiologic event that instigates acute, mild activation of coagulation and plasmin/fibrinolysis is daily exercise. Studies have shown intensity-dependent activation of clotting pathways and endothelial tPA release following exercise. Because exercise is a relevant, safe model to study minor injury incurred during daily exercise, we conducted a prospective study in young, healthy individuals to observe how different types of exercise affect coagulation and plasmin activity. We hypothesized that reporter assays PAP and thrombin-antithrombin (TAT) complexes would be more indicative of plasmin and coagulation activation respectively than function clot lysis assays with plasmin generation.

Methods: This study was conducted as a prospective, nonrandomized study. Participants for the study were recruited via informational flyer and screened for enrollment eligibility. Study inclusion criteria included healthy individuals between the ages of 18-29 with a healthy BMI measurement (between 18.5-30.0) capable of moderate exercise twice weekly were enrolled in the study. Exclusion criteria for the study included pregnancy, history of recurrent drug use or smoking, history of infection, inpatient admission, or serious injury within the past 6 months, and chronic or recent (10 days prior) anticoagulant or NSAID use. Additionally, any volunteers with pre-existing conditions that limit the ability to safely participate in exercise were excluded from the study. Fifteen eligible healthy volunteers were enrolled in the study upon their consent. Sex,

date of birth, ethnicity, weight, height, maximum heart rate (HR) (calculated as 220 – age), and typical exercise frequency was recorded for each participant based on self-reporting.

Participants refrained from moderate or intense exercise for 24 hours prior to the experiments. Each participant received a baseline HR measurement prior to each exercise period. VO2 max was approximated using the HR ratio method ((HR_{max}/HR_{rest}) ×□15.3mL/kg□minute). While wearing a heart rate monitor, participants underwent a period of either walking at ~50% VO_2 max, or running at ~80% VO_2 max for 20 minutes at a time. Data was collected for both exercise intensities for all participants, and experiments in the same participant were not conducted within less than 1 week of the previous experiment. Blood was drawn immediately prior to and following each 20-minute exercise session and centrifuged at 1500xg and 30,000xg to produce platelet-poor plasma. This study and all procedures therein were approved by the Vanderbilt University IRB (protocol 171641).

Plasma was analyzed for markers of inflammation (IL-6, R&D) and coagulation (TAT, Molecular Innovations) and plasmin activation(PAP, Diapharma; p11, Abbexa) by ELISA, and clot formation and lysis assays were conducted with plasmin generation as previously described (REF). Because baseline values vary between individuals, all plasma measurements were compared with baseline values for each individual and expressed as a fold change from baseline.

Results: Following both a 20 minute walk and a run, there were no significant changes in plasma IL-6 or TAT (Figure 5.1A-B not to power). However, we observed significant increases in PAP and p11 following the 20 minute run, while we only observed a minor increase in either following the walk (Figure 5.1C-D), suggesting an intensity-dependent activation of plasmin during exercise. Interestingly, the walk did not alter coagulation potential measures by maximum clot amplitude and turbidity area under the curve (AUC), but the 20 minute run induced a

moderate increase in clotting potential (Figure 5.1E). Conversely, neither the walk nor the run significantly altered potential to generate plasmin or degrade clots (Figure 5.1F).

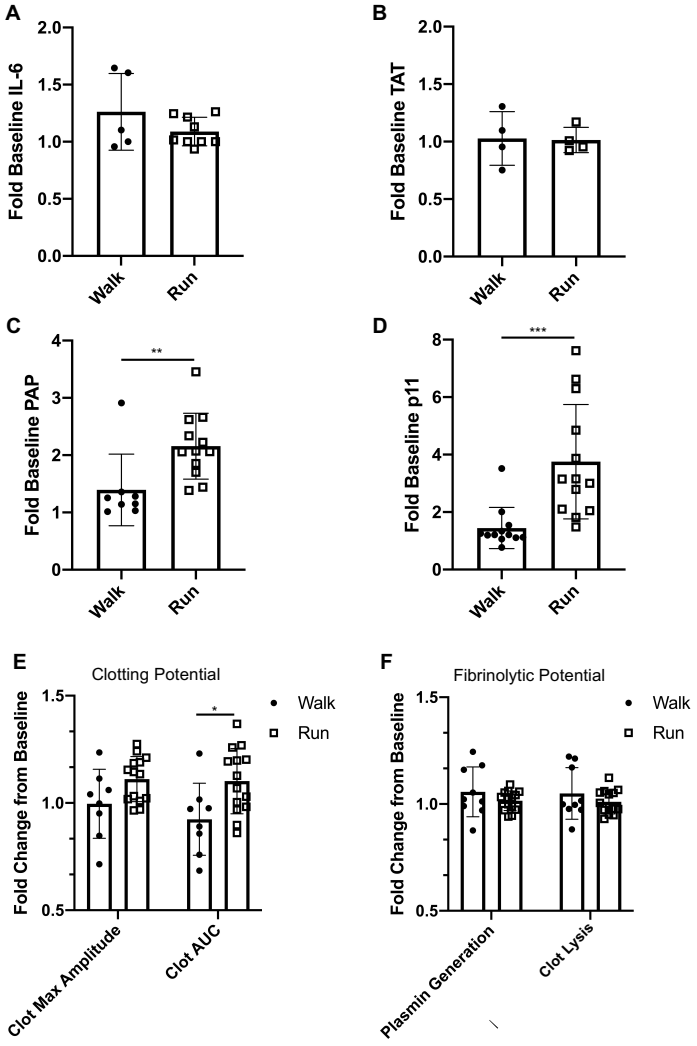

Figure 5.1: Inflammation, coagulation, and plasmin activation following graded exercise. A) A 20 minute walk or run does not provoke significant immediate inflammation measured by IL6 or B) coagulation activation measured by TAT. C) A 20 minute run provokes a significant increase in circulating PAP, D) p11, and E) clotting potential measured by turbidity, but it does not alter plasmin/fibrinolytic potential (F) compared with baseline values. (*P<0.05, **P<0.01, ***P<0.001, N=8-15/group)

Conclusions: These results suggest a difference in coagulation and plasmin activation during exercise of differing intensities. While reporter assays failed to detect coagulation activation (or inflammation), they detected previous plasmin activity by PAP. Alternatively, the functional assays detected an increase in coagulation potential but no change in plasmin/fibrinolytic potential. This may be because plasmin is activated and inhibited very quickly, so while it is being picked up by PAP complexes that remain in circulation for hours following the activating event, we do not observe a residual increase in fibrinolytic potential. Other studies have suggested that exercise does not initiate coagulation activation but increases plasma levels of certain clotting factors so that, in the instance of an injury, the system has been primed for efficient hemostasis to occur. Collectively, this study demonstrates that different assays used to measure coagulation and plasmin activity may have conflicting results—not because of a lack of accuracy—but because of timing of blood sample collection and what each assay is fundamentally measuring (previous activation vs potential to activate in the future).

Consequences of a pathologic APR: mice with severe burns or plasmin(ogen) deficiency exhibit long-term deficit in unforced exercise

Exercise is not only recognized as a way to promote wellness but also as a marker of wellness. Preclinical and clinical studies have used unforced exercise as a marker of physical and mental health(347,351). Both severely injured and plasmin-deficient mice exhibit long-term deficits in wellness markers, including body composition and depression(352–354). Here, we used a new model to assess wellness by unforced exercise in plasmin-deficient or severely burned mice.

Methods: Male plasminogen-deficient mice ($Plg^{-/-}$) or wild-type littermates on a C57BL/6J background were placed on a standard laboratory chow (5L0D) and aged to 6 months old. A different group of male wild-type C57BL/6J mice received a 30% TBSA burn to the dorsum with concomitant calf muscle injuries by injection of cardiotoxin at 6 weeks of age, as previously described(354). Control mice only received bilateral calf muscle injuries. Animals with injuries were aged out to 6 months of age. At 6 months of age, either uninjured, $Plg^{-/-}$ mice or mice with burn injuries were individually housed with sensored running wheels that measure distance run. Running data was collected during the nocturnal period in which mice are active (18:00-6:00) for 48 days following a 7 day acclimation period to the new cage and wheel.

Results: Compared with aged, uninjured WT mice, $Plg^{-/-}$ mice ran less over 48 days of monitoring (Figure 5.2A). Further, WT mice gradually ran more over time, while $Plg^{-/-}$ mice progressively ran less (Figure 5.2A). WT mice with a burn and muscle injury versus those with just muscle injuries all exhibited an increase in distance run over time up to 30 days before reaching a plateau. While mice with burns did not exhibit a progressive decline in running like the $Plg^{-/-}$ mice, they did consistently run fewer kilometers per night than mice that received calf muscle injuries without a burn 5 months prior (Figure 5.2B).

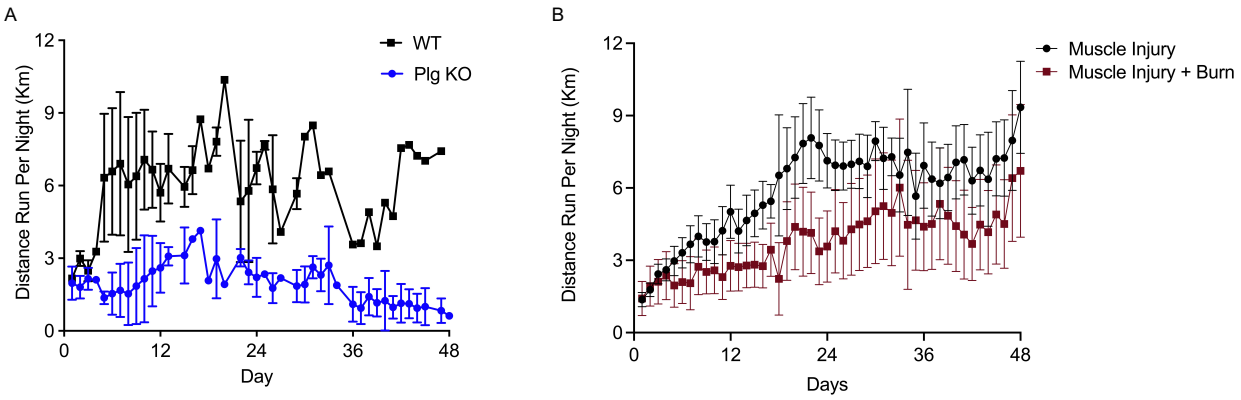

Figure 5.2: Unforced exercise in mice with plasmin deficiency or a severe injury. A) Plasmin deficient mice run less over time than WT mice. B) Mice that received a burn 5 months prior ran less than mice with calf muscle injuries alone. Not to power-N=3-5 mice/group, NS.

Conclusions: Mice with plasmin deficiency exhibit reduced capacity for running and increased fitness measured by unforced exercise. Mice that received burn injuries exhibit a deficit in running capacity compared with mice that did not get a burn injury, suggesting long-term fitness deficits following a severe burn. This study included 3-5 mice per group, but because of variability, the results were not to power. Both experiments were the result of 2 different experiments with similar trends, suggesting that this is a biologically relevant phenotype that needs to be further investigated. Future studies will expand these studies to identify the true exercise deficit in $Plg^{-/-}$ and severely burned mice and will investigate therapeutics that may restore wellness measured by unforced exercise.

Chapter 5 Summary:

Exercise provokes a dose-dependent APR measured by coagulation, plasmin activation, and inflammation. Twenty minutes of moderate exercise is sufficient to cause a burst of plasmin activation and slightly increase coagulation potential without altering systemic markers of inflammation. We hope to continue to work to determine if the acute activation of plasmin in the blood during exercise exerts reparative or protective effects on tissue health based on plasmin's roles in tissue repair and maintenance.

Mice with a plasminogen deficiency or those with severe injuries exhibit prolonged inflammation, osteoporosis, muscle fibrosis, and wasting phenotypes. Here, we demonstrated that plasmin deficiency or severe injury has prolonged effects on unforced exercise. In humans, chronic pain, osteoporosis, and loss of range of motion are considered major factors in reduced willingness to exercise following a severe injury, and our goal is to continue these studies to determine if different therapeutics manipulating the plasminogen activation system or bone turnover may improve willingness to exercise in animals.

CHAPTER 6: CONCLUSIONS

A critical part of science is the collaborative effort of learning from previous work, building upon it, and then providing future directions for the field based on those new findings. In this chapter I'll discuss some future directions for the work within my dissertation and proceed to highlight the current clinical implications of the presented findings.

Future Directions:

In chapter 2, I discussed the dysregulation of the APR following a severe injury and the consequences, including thrombosis, bleeding, and organ failure due to SIRS and coagulopathy. Currently, there are still few good treatment options for these patients other than rigorous transfusion, fluid resuscitation, intubation, and surgical intervention. As we continue to delineate the events of the APR as part of a whole, we're hoping to improve both diagnostic testing and therapeutic development. Currently, coagulopathy is treated with transfusion or standard anticoagulants, such as low molecular weight heparin, and SIRS is occasionally treated with steroids or plasmapheresis to combat inflammation. However, there is still a significant knowledge gap regarding the intersection between coagulation and inflammation that drives complications in a severe APR.

Plasmin has been shown to activate or exacerbate inflammatory immune functions and platelet activation. In chapter 2 I described two studies in which we investigated associations between plasmin activation or fibrinolysis and platelet dysfunction. In both spinal fusion surgeries and in *ex vivo* incubations of healthy platelets with burn patient plasma, the magnitude of platelet dysfunction was associated with markers of plasmin activity. Because plasmin and fibrin degradation products can impede platelet function through different mechanisms, future studies

should examine how plasmin specifically versus D-dimer affect platelet function not only by flow-cytometry based assays but also by functional platelet assays, such as aggregometry. Temporary inhibition of plasmin activation or preventing of plasmin binding to cells may present a new target to preserve platelet function and also prevent unnecessary activation of platelets, which can activate peripheral mononuclear cells, such as neutrophils, leading to an immunothrombotic phenotype. The questions to be studied further on this topic are:

- How does plasmin itself affect platelet function *in vitro* using these assays?
- How do fibrin degradation products affect *in vivo* platelet function, even in the absence of bleeding?
- Does genetic or pharmacologic inhibition of plasmin alter platelet function following severe injuries, including burns?

In Article 1, I've discussed the different ways to measure plasmin activation or activity in surgical patients, and each of them come with significant limitations that make their use for rapid, informative testing suboptimal. Current viscoelastic testing (such as TEG) can miss small changes in coagulation and fibrinolysis that have significant clinical implications, and these measures are very sensitive to the time at which the blood is collected for the analysis. Reporter measurements, such as PAP or D-dimer, give a more accurate picture of the magnitude of plasmin activation and fibrinolysis, but they cannot confirm that a patient is currently experiencing plasmin activation since these markers remain in circulation for hours following plasmin activation. New technology is currently in development to isolate the different elements of coagulation and fibrinolysis, including platelets, fibrin, plasmin, and neutrophils in whole blood that will provide rapid results, but future studies are needed to confirm that these tests sensitively predict outcomes and can accurately inform treatment in patients with severe injuries.

Current antifibrinolytic drugs, aminocaproic acid and TXA, are safe and effective, but they have poor potency and lack distinct molecular characteristics to prevent off-target effects. Because they require dosing at such a high concentration for efficacy, the therapeutic range for dosing in different procedures or injuries is variable, and studies assessing efficacy all use different metrics to assess the pharmacodynamics of the drugs. Furthermore, because the manipulation of plasmin is time-sensitive, any new therapeutics should either have a short half-life or an easy neutralization strategy. Some groups have developed specific antibodies, but these are often costly and increase the risk of an autoimmune reaction or prolonged inhibition of plasmin. In the future, we'd like to work with high throughput screening specialists in collaboration with structural biologists Dr. Ruby Law and Dr. James Whisstock to determine if we can isolate either a small molecule or peptide that can specifically inhibit plasmin at a low concentration and work effectively in our animal models.

In chapter 3, I discussed plasmin's critical roles in tissue repair and demonstrated that enhancement of plasmin during tissue repair restores muscle healing following a burn injury. In this study, we used an ASO against plasmin's primary inhibitor, α2AP, to enhance plasmin activity. The early concern with a treatment of this nature was the risk of bleeding, however, this treatment does not enhance plasmin activation but sustains the activity of plasmin that is already activated. Therefore, this approach may present a viable treatment option to promote tissue repair. Furthermore, in chapter 4, I discussed poor fibrinolytic potential in T2DM patients and its possible implications in poor tissue repair, suggesting another possible application for plasmin therapy. Other therapeutics that are in development to increase plasmin activity for vascular health as well as tissue repair include PAI-1 inhibitors, such as the one used in Article 2. The future directions for this area include:

- Studies examining α2AP ASO's safety profile as it primarily targets hepatocytes and knocks down expression of α2AP for several days.
- Studies examining potential negative effects of enhancing plasmin activity during acute traumatic injury or infection as plasmin can drive both inflammation and bacterial dissemination
- Development of tissue-directed therapy to enhance plasmin activity since PAI-1 inhibitors have global effects
- Testing plasmin-enhancing drugs to determine if these can safely be used to improve tissue repair and maintenance in individuals with acquired forms of hypoplasminogenemia (trauma, etc) or dysplasminogenemia (T2DM, etc).

In Article 2, our work demonstrated that plasmin is a driving factor in systemic inflammation and inflammatory signaling in injured tissue. It's unclear if plasmin exacerbation of systemic inflammation drives the local tissue inflammation or if these are coincidental events both driven by plasmin by different mechanisms. Because plasmin has multiple pro-inflammatory mechanisms, these should be tested in the future to determine the mechanism(s) by which plasmin drives an increase in circulating inflammatory markers and tissue NFκB signaling.

In Chapter 5, I briefly discuss the profibrinolytic effects of moderate, brief exercise and the possible benefits of this on wellness. It's well known that exercise is beneficial for tissue health, and future studies will examine what role exercise-induced plasmin activation may play in this, specifically examining which type(s), intensities, and timing provoke a beneficial response without provoking significant tissue damage.

Clinical Perspectives:

A continuum: survival-APR problems drive complications during both survival and repair. Previously, severe injuries carried a high mortality rate, and although medical advances have greatly improved chances of survival, they've also uncovered further challenges to be addressed: complications of survival and repair. Severe injuries provoke a pathologic time-course of plasmin activity, shifting it from a prolonged, local activation at the site of injury towards acute, systemic activation during survival. This not only places patients with severe injuries at risk for early complications, including bleeding and organ dysfunction, but also increases the risk of later complications, including wound dehiscence, fibrosis, and HO. Within the field of critical care, early and later complications are often treated as two separate disease pathologies with different etiologies. However, a plethora of studies have implicated elements of a dysregulated survival-APR, such as SIRS, in *both* survival and repair complications. Increasing evidence has suggested that patients that experience these survival complications are at a greater risk of repair complications, which begs the question: are these related as a part of the same underlying pathologic continuum within the severe APR?

Plasmin mediates critical physiologic and pathologic functions.

In the field of biology, we have been swift to identify a protein or cellular target as "good" or "bad" based on its role in a particular disease, such as bone-resorbing osteoclasts in osteoporosis, however, this is myopic and neglects the essential roles a target plays in physiology. Within my dissertation work, I have demonstrated times at which plasmin is a pathologic driver of disease, while later, in the same model, it promotes healthy tissue repair. A collective of studies along with my work have demonstrated that plasmin plays beneficial and detrimental roles in biology depending upon timing, location, and concentration.

Diagnostics of plasmin activity.

Currently, the use of certain clinical laboratory tests to measure plasmin activity are rarely used appropriately and often poorly interpreted. Because there is a defined timeline at which plasmin switches from its pathologic to its physiologic roles, it's critical that we improve how we diagnose changes in plasmin activity using clinical laboratory tests. Currently, TEG often misses dramatic changes in plasmin activity unless a patient is actively exsanguinating, and common quantified D-dimer measurements currently have a long turn-around time, impeding its ability to inform critical patient care. The results of my studies in human burn patients and those undergoing high blood loss elective surgeries suggest that TEG and D-dimer, in combination with PAP and plasminogen measurements, could improve the accuracy of testing for trauma-induced plasmin activity. Furthermore, standard tests currently could be greatly improved through the development of point-of-care devices, especially in the trauma unit where timely intervention is an imperative. The limitations of different clinical laboratory tests are poorly understood. My dissertation work has highlighted that *ex vivo* measurements taken in blood or plasma do not necessarily reflect the biology of a local tissue environment as many proteins and cells in blood are localized to the site of injury, altering what's present in circulation, and blood tests are also not always able to depict what has already happened in a patient. Therefore, clinical interpretations of standard laboratory tests, specifically for measuring plasmin activity, should be approached with caution and confirmed using rigorous clinical trials.

Plasmin as a therapeutic target.

Finally, plasmin may be a differential therapeutic target throughout convalescence in patients with severe injuries. Here, we have shown that early inhibition of plasmin following severe injury may not only reduce the risk of bleeding but may also attenuate that uncontrolled survival inflammatory

response to a severe injury, such as a burn. Conversely, plasmin's pro-inflammatory response and anti-inflammatory effects locally at the site of tissue injury are critical *during tissue repair*. Therefore, judicious enhancement of plasmin during the repair-APR may present a good pharmacologic approach to improve tissue repair in patients predisposed to chronic issues of fibrosis, wound dehiscence, and heterotopic ossification(Figure 6.1).

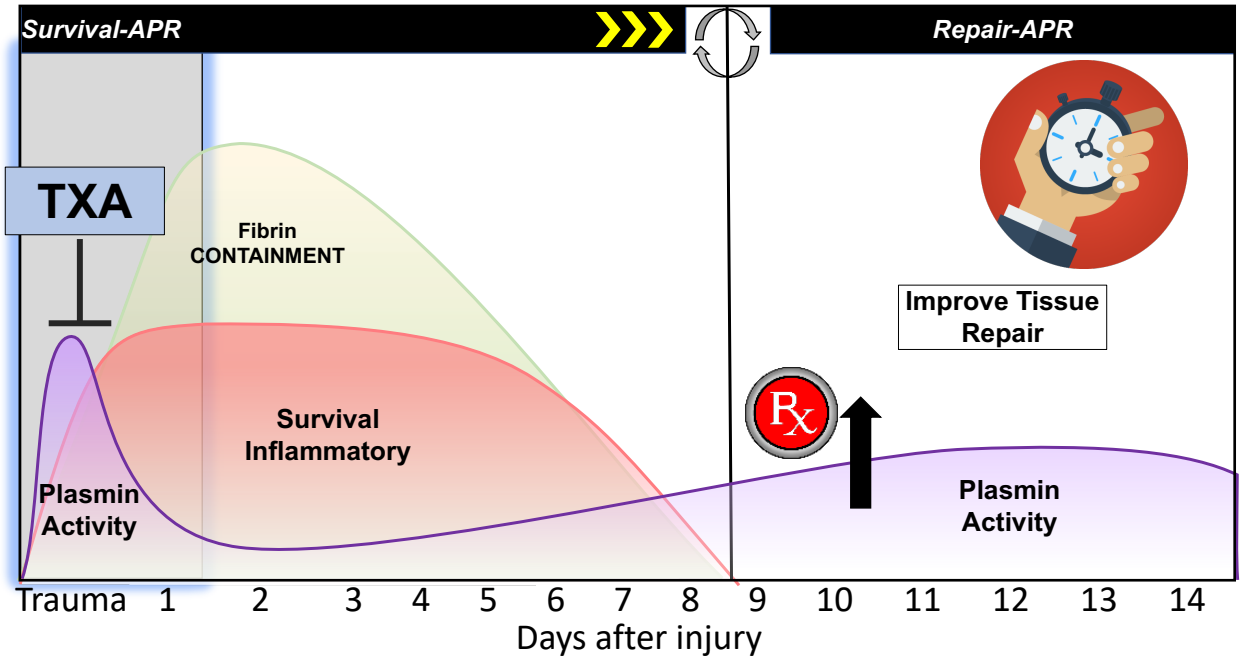

Figure 6.1: Differential therapeutic targeting of plasmin through the acute phase response. Because plasmin plays paradoxical roles in the trauma response and later tissue repair and recovery, judicious, early inhibition of plasmin may reduce SIRS and coagulopathy-related complications early in convalescence, while pharmacologic enhancement of plasmin during tissue repair may present a novel therapeutic approach to promote tissue repair in trauma patients and in those predisposed to tissue repair problems, including individuals with T2DM.

Physician William Osler said,

"He who studies medicine without books sails an uncharted sea, but he who studies medicine without patients does not go to sea at all."

In my dissertation work, *I started with clinical observations* made from studies in burn and surgical trauma patients and taken those into animal and cellular models to manipulate plasmin and determine its roles in both SIRS and muscle repair following burn injury. In this work, myself along with my colleagues have determined differential roles for plasmin in the time-course following a severe injury. Early activation of plasmin during the survival APR can drive deleterious complications stemming from hyperfibrinolysis, coagulation dysfunction, and systemic inflammation. Conversely, poor local plasmin activity at the site of damage following a muscle injury results in pathologic tissue repair that develops into fibrosis and calcification or bone formation within the injured muscle. I was fortunate to work with exceptional scientists and clinicians on a project that has clear clinical implications in trauma medicine. My hope for this work is that it will inform 1) future animal studies on the mechanisms of plasmin in inflammation and well as tissue regeneration and 2) well-designed clinical trials aimed at improving outcomes in patients with severe injuries.

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