Hibernation and Thrombo-protection: Exploring Fibrinolytic Mechanisms in the American Black Bear

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SPT Thesis

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Abstract

Research Question: Are there differences in fibrinolytic regulatory mechanisms in naturally hibernating American black bears (Ursus americanus) that may serve as a basis for "thromboprotection" from stasis? Specifically, how do tissue plasminogen, urokinase plasminogen activator, and plasminogen activator inhibitor-1 expression patterns differ in hibernating vs active bears?

Background, Significance, and Rationale for the Question:

Thrombotic complications are a common pathology following days of stagnation, periods of immobility, and hospital admissions. The commonality of thrombotic events is lethal and contributes to one in four deaths worldwide. Although deep vein thrombosis (DVT) cases reduced from 264 per 100,000 to 167 per 100,000 from 1999 to 2010, researching control and therapeutic measures continue to expand our understanding. Rudolf Virchow in 1847 published a triad of factors describing stasis, a period of inactivity, as a contributing factor to venous thrombosis. Yet, hibernating animals, and bears in particular, are dormant and inactive for three to four months with minimal thrombotic complications. These mammals provide an opportunity to expand our scientific knowledge of fibrinolysis mechanisms in relationship to stasis from Virchow's Triad.

Materials and Methods:

This cross-translational study will entail recruitment of hibernating and non-hibernating American black bears (*Ursus americanus*). Hibernation was determined by level of inactivity and monitoring of body temperature with specific parameters determined. We have a professional working relationship with the Michigan Department of Natural Resources; a state agency with

ongoing *Ursus americanus* research. After IUACA and animal husbandry approval at the Michigan resource, and per their established methods, blood draws were completed under anesthesia via the femoral or jugular veins following strict protocols demonstrated by Friedrich et al. during active summertime and hibernation. Samples were frozen to -80 degrees to maintain consistency of factors. Cell count, hemostasis testing, and clotting factor assays are to be performed at Baylor All Saints pathology laboratory in Fort Worth, Texas.

Results

Previous studies conducted by Friedrich et al. demonstrated marked reductions in Factor XI, Factor XII, vWF, a prolonged PT and aPTT, and an increase in antithrombin in blood samples from hibernating bears, and we attempted to confirm these findings. We hypothesize that urokinase and tissue plasminogen activator levels will be increased to enhance fibrinolysis to maintain coagulation homeostasis. In partnership with the Michigan Department of Natural Resources, we collected 16 live bear samples during the winter and spring of 2023. Our samples have been successful stored at negative 80-degrees Fahrenheit and await delivery to Baylor All Saints in Fort Worth, Texas. We have sufficient sample collection to run appropriate assays and adequate data to test our hypothesis in spring of 2024.

Conclusion

Our study has opened the door to further explore cross-translational opportunities. American black bears have enabled us to see further than human studies and understand the interrelatedness that is visible in everyday life. We look forward to processing of 16 samples of bear specimens, and irrespective of the results, we have demonstrated through execution of this project that we could execute a meaningful multi-institutional collaboration delivering samples for scientific

analysis of which there are only two other global examples. I have learned how to effectively establish partnerships to complete seemingly impossible work and know this will impact my career as a clinician and professional.

Research Question

The objective of our project was to answer the big "dreamer" question: how do bears hibernate for 4 to 6 months and not encounter any thrombotic pathologies? To answer our overarching question, we delegated our project into important pillars hoping to define if there are differences in fibrinolytic regulatory mechanisms in naturally hibernating American black bears (*Ursus americanus)* that may serve as a basis for "thrombo-protection" from stasis. Specifically, how do tissue plasminogen, urokinase plasminogen activator, and plasminogen activator inhibitor-1 expression patterns differ in hibernating vs mobile bears? We focused on the fibrinolytic cycle as a unique avenue that has yet to be described in the American black bear. We hypothesize that within our bear samples that hibernating bears will have the ability to sequester platelets, bolster the fibrinolytic cycle including urokinase and tissue plasminogen activator, and decreased plasminogen activator inhibitor – 1.

Introduction & Significance

Rudolf Virchow in 1847 published a triad of factors directly attributing venous thrombosis to abnormalities of blood vessel wall, abnormalities of blood constituents, and abnormalities of blood flow[1]. This epoch-making publication continues of significant importance in the complex understanding of thrombotic events and complications. Stasis, a period of inactivity is described as a contributing factor to venous thrombosis and a significant portion of Virchow's triad. However, before toying with the effects of stasis it's important to have a firm grasp of the coagulation processes. Hemostasis is balance of tightly regulated processes including platelet activation, blood clotting, and vascular repair[2]. This process allows for an organism to encounter endothelium damage and prevent excessive blood loss. Primary hemostasis allows for the closing off of damaged blood vessels, preventing pathological thrombosis and restore vascular integrity. The history of coagulation is expansive with the first categorization of description of blood clotting described by Plato as forming fibers when it leaves the heat of the human body. Discovery of platelets in 1865, description of tissue factor in 1905, and full description of the coagulation pathway in the late 1950s[2].

Unstable platelet plugs are vital to the stopping of bleeding and activation of the coagulation cascade. It begins with endothelial damage which leads to vasoconstriction via the neural stimulation reflex and release of endothelin by the damaged cell. Subendothelial extracellular matrix which includes collagen becomes exposed and allows for binding of Von Willebrand factor (vWF) found within Weibel-Palade bodies of endothelial cells and alpha granules of platelets to platelet surface receptor glycoprotein Ibα (Gp1b)[3]. Stable platelet formation is a result of an intricate process described in a systematic process of tethering, rolling, activation, and firm adhesion[4]. This process permits the slowing down of fast flowing platelet in

the blood stream and eventually firm adhesion to the vessel injury. Platelet adhesion to Gp1b initiates multiple signal transduction pathways including a cascade of tyrosine kinases and eventual release of calcium. These newly activated platelets release of calcium leads to an increase in cytosolic calcium levels that begins a plethora of events. Increased calcium levels lead to a conformational change of platelet shape, release of Adenosine diphosphate (ADP), thromboxane A_2 (TxA_2) and ultimately further adhesion. Thromboxane A_2 is a potent vasoconstrictor and further amplifies platelet activation via paracrine manner. ADP released by dense granules of platelets following activation results in a secondary activation amplification loop and binding to $P2Y_{12}$ receptors. Through G-protein coupled receptor signaling there is an increase in the expression of GpIIb/IIIa on the surface of bound platelets. Fibrinogen found within the blood binds these expressed GpIIb/IIIa receptors and links multiple platelets together[4]. This process is deemed primary hemostasis and results in the formation of a temporary platelet plug.

Balance between the mechanisms of pro-aggregation (TxA_2 and decreased blood flow) and anti-aggregation factors (prostaglandin I_2 and nitric oxide and increased blood flow) determines the efficacy of plug formation. Following platelet plug formation the coagulation pathway allows for the development of a permanent and stable plug. It is suggested that the coagulation pathway contains two different pathways that converge in development of a permanent clot restoration of hemostasis. These pathways were described as the intrinsic pathway due to the presence of these factors in blood and extrinsic due to tissue factor being released by endothelium following damage. Described as a cascade due to its 'waterfall' appearance, the coagulation cascade begins with the exposure of tissue factor (TF) to the blood stream following endothelial injury[3]. TF binds to factor VII (FVII) and promotes cleavage of FVII to FVIIa[3]. This complex proteolytically cleaves small amounts of FX into FXa allowing

association of FXa with FVa and activation of FII (prothrombin) to FIIa (thrombin). Thrombin allows for activation of FI (fibrinogen) to FIa (fibrin) and eventually stabilization of platelet plug with assistance of crosslinking by FXIIIa. The cascade of FX to FIa is described as the common pathway. Increasing amounts of thrombin plays a role in amplification of platelet activation, conversion of FV into FVa further increasing activation of itself. Models of coagulation support that the extrinsic pathway, collagen, and high molecular weight kininogen leads to intrinsic pathway activation specifically FXII. Factor XIIa is leads to cleavage of FXI to FXIa. FXIa leads to activation of FIX to FIXa. With FVIIIa as a cofactor the intrinsic pathway integrates with the common pathway and activates FXa[3]. This highly extensive process of platelet stabilization, activation of the coagulation cascade, and eventual platelet plug stabilization is regulated by both procoagulant and anticoagulant factors.

Fibrinolytic mechanisms allow for the degradation of platelet plugs, return of hemostasis, and anti-coagulation. Protein C and Protein S have anti-coagulant activity in their proteolytic inactivation of FVIIIa and FVa. This cleavage results in halting of activation of thrombin and halting of the common pathway. Protein C is activated by binding of thrombin to thrombomodulin found within endothelial cells to become activated. Protein S is required to bind Protein C to perform a similar role. Recent research indicates that activated Protein C is restricted to the endothelium of vascular beds and primarily plays a role of preventing clotting reactions in uninjured vessels [2]. Antithrombin (AT) has affinity towards FVIIa, IXa, Xa, XIa, and XIIa but has high affinity towards three key coagulation factors; FIXa, FXa, and thrombin. AT is a serine protease inhibitor (SERPIN) and is strongly activated by the presence of heparin-like molecules (HLM). HLM's bind to antithrombin, FIXa, FXa, and thrombin bring the molecules in close proximity further amplifying antithrombin's effects[2]. As shown in figure 1, a decrease in plasminogen activator inhibitor I (PAI-1) results in an increase of tissue plasminogen activator I

(tPAI) and urokinase plasminogen activator I (uPAI) which both play a role in the activation of plasmin and the degradation of fibrin. This process sways the scales towards anti-coagulation.

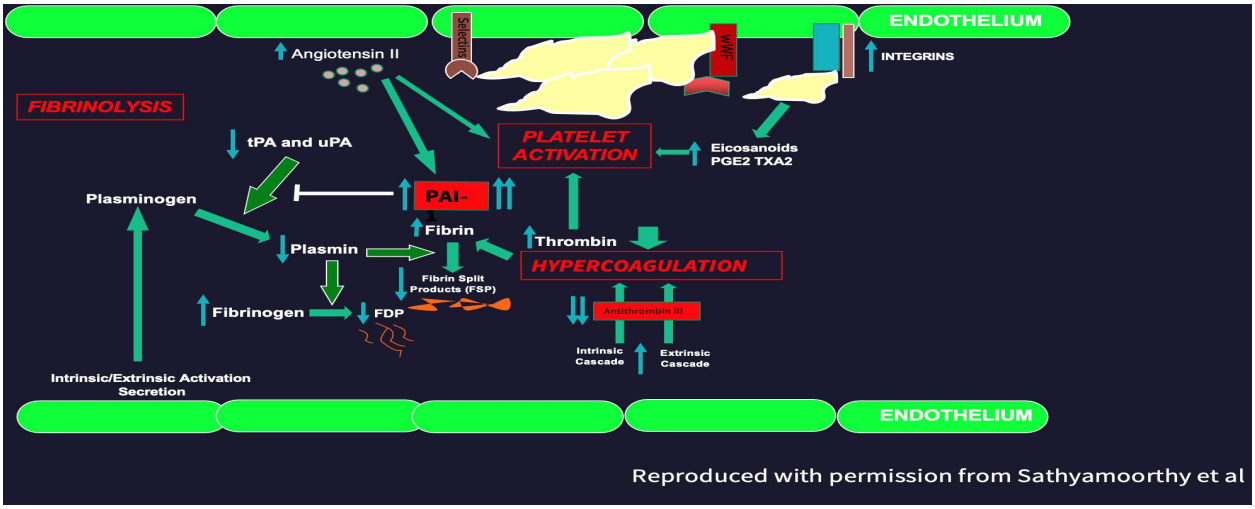

Figure 1: This graphic describes the intricate interaction balance between pro-coagulant and pro-fibrinolytic processes with the American black bear. Reproduced with permission from Dr. Sathyamoorthy.

Significance and Impact

Thrombotic complications are a common pathology following days of stagnation, periods of immobility, and hospital admissions. The commonality of thrombotic events is lethal and contributes to one in four deaths worldwide[5]. The significant incident rates pose a financial burden on the United States healthcare sector with cumulative treatment cost of seven to ten billion USD per year[6]. Although DVT cases reduced from 264 per 100,000 to 167 per 100,000 from 1999 to 2010, researching control and therapeutic measures continue to expand our understanding[7]. Periods of inactivity are of direct correlation to length of hospital stays and post-operative recovery hospital admissions[8]. Critically ill and hospitalized patients encounter increased thrombotic pathologies secondary to many factors. These factors include surgical interventions, invasive test and treatments, prolonged immobility and vascular injury. Studies

have shown that the prevalence of a deep vein thrombosis in the intensive care unit is near ten percent of patients[9]. The same studies suggested that hospital stay is significantly prolonged following occurrence of DVT as well as the risk of pulmonary embolism and mortality. Prolonged hospital stays and further pathologies means more cost of care required of the patient as well extended time away from their occupation. This financial and social stress is significant and warrants prophylactic measures to reduce prevalence. Past epidemiology studies suggest that there are 500,000 annual venous thromboembolism (VTE) cases world-wide and 52 percent of those cases are related to a recent hospitalization[10]. This clinical burden was estimated to cost 13.5 billion and 27.2 billion for total healthcare cost and treatment of venous thromboembolism. VTE is a significant healthcare problem that affects patients across all socioeconomic, ethnic, and demographic characteristics.

Rationale

Hibernating animals are dormant and inactive for three to four months without any thrombotic complications. Interestingly enough, mammals can reduce their metabolism, body temperature, and blood flow greater than ten percent during hibernation[11]. American black bears (*Ursus americanus*) hibernation is short lived, they enter their den for hibernation in early December and exit late March. Their innate ability to control homeostasis is frequently studied due to weight and body temperatures being comparable to humans. With a better understanding of the incidence of thrombotic complications and the coagulation pathways, American black bears provide an expansive number of opportunities for discovery and growth of coagulative knowledge. *Ursus americanus* are sparingly studied secondary to difficulties in harvesting blood samples. Yet, Friedrich et al. who worked in conjunction with the Michigan Department of Natural Resources collected and analyzed the blood samples of hibernating bears in Michigan's

upper peninsula. The Michigan Department of Natural Resources is a state agency with ongoing Ursus americanus. This collaboration and experiment is one of the first to consider analyzing clotting factors of American black bears in hibernation[12]. Their study involved 32 American black bears including 23 active summertime bears and 9 in hibernation. Data suggested decreased platelets, prothrombin time and activated prothrombin time. Assays for specific clotting factors include data supporting decreased fibrinogen, plasminogen, antithrombin, antiplasmin, protein C and vWF levels. This data suggests there are fibrinolytic mechanisms that are preventing coagulopathies during hibernation. As we began processing our first samples and collecting data, a paper discovered down-regulation of HSP47 which serves as a thromboinflammation regulator. This heat shock protein resides in the SERPIN family similar to tPA and antithrombin we are evaluating in our study. Their study collected 13 subadult Scandinavian black bears and through mass spectrometry identified HSP47 as a major discrepancy between active summertime and hibernating bears[11]. Naturally, we hope to continue evaluating these mechanisms with cross-comparison of hibernating and nonhibernating American black bears. These mammals provide an optimal comparative biology research opportunity with hopes to expand our scientific knowledge of fibrinolysis regulation in bears versus humans with reference to depressed core temperatures and blatant provoking of Virchow's triad.

Materials and Methods

American black bears provide a novel opportunity to expand our current understanding and advance the literature of thrombotic pathologies. This novel comparative biology research project with evaluate the homeostatic mechanisms and fibrinolytic cascade of hibernating black bears while comparing to their active summertime counterparts. Hibernating American black bears were recruited with the help of the Michigan Department of Natural Resources (DNR). After establishment of a working relationship with state department of Michigan we were able to successfully plan for collection of our first samples in the spring of 2023. The Michigan DNR performs frequent studies on large animal species including mountain lions, black bears, and wolves to improve conservation efforts. They have established a geolocation method in which targeted animal species are appropriately sedated allowing for GPS collar placement. Black bears enter hibernation in early December and exit their dens late March. Proper recruitment of naturally hibernating bears required timely blood draws and sample collection. In the winter of 2022, 12 hibernating black bears were identified. Utilizing this newly established technology we were able to accurately locate the black bears den in which they had entered to hibernate. Through careful organization, we planned for sedation and collection of blood samples from the 12 hibernating black bears in the winter of 2023. An additional 4 bears samples were collected in the spring of 2023 to allow for cross-comparison of blood samples. Determination of the subject's hibernation status was completed by evaluating inactivity levels, season, time of collection, and the bear's core body temperature. Bear cubs were excluded from this study because their fibrinolytic mechanisms may differ from the adult bear.

Extraction methods

After IUACA and animal husbandry approval at the Michigan Department of Natural Resources, and per their well-established methods, blood draws were completed under anesthesia via the femoral or jugular veins following strict protocols demonstrated by Friedrich et al. during active summertime and hibernation[12]. Anesthesia was performed with intramuscular injection of tiletamine hydrochloride and zolazepam 7 mg/kg of body mass into hibernating bears. Blood draws were completed following anesthesia via access to the femoral or jugular veins. Ethylenediaminetetraacetic acid tubes and sodium citrate tubes (3.2 % sodium citrate blue-top) was used as primary collection testing vials as both will help prevent rapid clotting following collection of samples. Within 60 minutes of collection of the blood samples, the blue top samples were centrifuged and supernatant removed for storage. Being that these samples were collected with the help of the Michigan DNR in the upper peninsula of Michigan, these samples was refrigerated, frozen, and shipped overnight to Fort Worth. Prior to shipping and within 60-minute from collection, these blood samples placed in sodium-citrate collection tubes were centrifuged at appropriate speed and time as recommended per Geisinger medical laboratories[13]. Establishment of a working partnership with the Baylor All Saints pathology laboratory allowed us to receive shipment of our 16 samples from Michigan and appropriate analysis.

Analysis of Samples

Cell count and evaluation of samples will be performed in the laboratories of Sathyamoorthy laboratory collaborators in the Baylor All Saints Pathology Laboratory. Samples will be received on dry ice and dethawed only prior to the day of experimental assays, as PAI-1

and tPA are highly thermolabile and susceptible to degradation with multiple freeze thaw cycles. Automated coagulation instruments will be utilized to determine clotting time, chromogenic substrate assays, and D-dimers. Activated thromboplastin time (aPTT) was calculated using automated techniques for evaluation of factors I, II, V, VIII, IX, X, XI and XII. Specific factor assays was utilized for proper evaluation of tPAI, uPAI, protein C, protein S, fibrinogen, plasminogen, antithrombin, antiplasmin and PAI-1.

Statistical Analysis

Our data will be analyzed utilizing *t*-test and differences considered significant at a $p < 0.05$. We did not determine the statistical power for our study.

Results

Through GPS tracking systems we were able to identify 12 hibernating bears and 4 active bears for collection (Figure 2). Following the described collection methods, we successfully obtained blood samples from these 16 individual bears. Each bear sample was divided into 3 separately identified collection tubes allowing for adequate processing. These tubes included an Ethylenediaminetetraacetic acid tube and 2 sodium citrate tubes (3.2 % sodium citrate blue-top) per bear. Within 60 minutes of collection, the samples in sodium citrate blue-tops were centrifuged and supernatant was removed. All but one sample were immediately stored on dry ice before being transferred for storage in negative 80 degrees Fahrenheit. This sample was collected on an island and due to resources was unable to be quickly stored on dry ice.

Date	Bear ID	Age	Sex	Blood -Light Blue	Blood -Purple	Comments
1/24/23	**BB0201**	Adult	F	Y	Y	N/a
1/24/23	**BB0202**	Juvenile	M	Y	Y	1 year old.
1/24/23	**BB0203**	Juvenile	F	Y	Y	1 year old.
1/24/23	**BB0204**	Juvenile	M	Y	Y	1 year old.
2/27/23	**BB6902**	Adult	F	Y	Y	N/a
2/27/23	**BB6903**	Juvenile	M	Y	Y	1 year old.
2/28/23	**BB6808**	Adult	F	Y	Y	N/a
2/28/23	**BB6809**	Juvenile	M	Y	Y	1 year old.
2/28/23	**BB6810**	Juvenile	F	Y	Y	1 year old.
3/1/23	**BB8303**	Adult	F	Y	Y	N/a
3/2/23	**BB6804**	Adult	F	Unknown	Y	Light bluetop not recorded on datasheet, will have to check freezer.
3/10/23	**BB4201**	Adult	F	Y	Y	N/a
6/1/23	**BB5203**	Adult	F	Y	Y	N/a
6/2/23	**BB5204**	Adult	M	Y	Y	N/a
6/14/23	**BB1701**	Adult	M	Y	Y	Freezing of samples was delayed due to being on

						island, may be compromised.
6/24/23	**BB5205**	Adult	M	Y	Y	N/a

Figure 2: Samples (16) collected to date from collared bear subjects, Michigan Department of Natural Resources.

Discussion and Innovation

Thromboembolism is a serious and important threat to people, particularly those who are immobilized for an extended period of time. The fibrinolytic cycle is an intricate balance between pro- and anti-coagulative regulatory processes. With immobilization this balance is shifted towards coagulation. Virchow's triad continues to hold true and with immobilization and stasis, coagulation will occur[14]. Studies have shown that thromboembolism is believed to originate in the venous valves. Our veins rely on valves that are provoked and function along with muscular contraction. As humans, the muscular contraction of our most distal muscle groups are activated during mobilization such as walking, running, or jumping. With periods of immobilization, specifically hospitalizations, the muscular contractions of our lower legs are minimal. Therefore, the contraction of our muscles is minimal and pooling of blood within our veins increase. It has been demonstrated that the most common factor between people with episodes of thromboembolism is a recent hospitalization[9].

Many hospitals have implemented venous thromboembolism (VTE) preventative measures such as chemical prophylaxis, spontaneous compression devices that simulate muscle contraction, and mobility when hospitalized. These measures continue to battle against the rise of VTE, but it is still estimated that there over 500,000 hospitalizations for VTE per year[9]. Our study was designed with finding a reasonable explanation for bears blatant violation of Virchow's Triad and a novel chemoprophylaxis for VTE. As we continue to learn more about the coagulation cycle, various regulatory processes, and potential avenues for discovery, we often neglect to explore solutions that are evident in everyday life. Nature provides answers to

questions that we have yet to ask or even ponder. In this case, the hibernation and stagnancy that bears complete during winter may be the breakthrough in VTE prevention we need. Our study is just the beginning of what can be accomplished by looking beyond the microscope and viewing wildlife.

Cooperation is the single most important pillar to significant research. Through collaboration and the combining of various minds, vital discoveries are possible. Our efforts to find a suitable set of wild American black bears was the most difficult process we encountered. There are few agencies within the United States who have the skillset and training to perform research on dangerous and large animals. We discovered that with enough tenacity and denial of failure, you can find a willing partner to bolster your research endeavors. The journey towards our working partnership with the Michigan Department of Natural Resources (DNR) was full of roadblocks. Through detailed exploration of previous research, we were able to contact a large animal expert in Connecticut who had worked with Michigan DNR. Though he was no longer involved in large animal research, he was able to provide us with a contact in Michigan. After months of phone calls and empty emails, we finally received an email detailing a possible lead for black bears in Michigan. Rapidly our relationship blossomed and we were two years into discussion when the opportunity arose to capture live hibernating bears in the Upper Peninsula of Michigan. In the winter and spring of 2023, the large animal experts in Michigan captured and collected 16 individual bear samples for processing. Then came the second most difficult task we encountered during this period of study, finding a location for sample processing. Fortunately, our laboratory has previous relationships with working clinical laboratories who are willing to complete are necessary assays. Our pathology partners at Baylor All Saints in Fort Worth are processing our bear samples and our results will be completed in the spring of 2024. Valuable partnerships and friendships are built with trust, and the development of that trust takes time.

Though we often want instantaneous satisfaction or results, the practice of patience has proven to be the most vital exercise.

Future Directions

Nature provides inherent limitations including access to safe, humane, collection practices. Though this project, took over 4 years to develop we have established a strong connection with the Michigan DNR and foresee a fruitful future. Together we were able to make giant strides towards a discovery that benefits both bears and people. We expect more samples in the coming seasons and the more samples we are able to collect, the stronger our evidence will become. The agreement of our entities includes an understanding that scientific breakthroughs take time and through collaboration we stride towards discovery.

The Michigan DNR is an entity that has an important role in the conservation of large animal species throughout the state. They are able to document trends in migration patterns, breeding, and the growth of endangered populations. Our study plays an adjunct role and our goal is never to deter the team from their mission. Benefitting from the advancement of technology, black bears are collared for further understanding of hibernation and important population control. We utilized this technology to be able to geolocate exactly where bears are hibernating and when they awake in the spring. We have plans to expand the collection of bear samples to 10 or more active bear samples in 2024-2025. Having this data will allow us to have a stronger comparison when evaluating the hibernating bear samples.

This study will proceed as a legacy piece for the Sathyamoorthy laboratory. Major discoveries take time to form partnerships; with this project we have accomplished a noteworthy goal and will transition remaining work to other members of our laboratory to finalize this

project. If we confirm the null hypothesis, we could provide yet another key piece of evidence to motivate development of PAI-1 inhibitors for clinical application.

Conclusions

Our study has opened the door to further explore cross-translational opportunities. American black bears have enabled us to see further than human studies and understand the interrelatedness that is visible in everyday life. We look forward to processing of 16 samples of bear specimens, and irrespective of the results, we have demonstrated through execution of this project that we could execute a meaningful multi-institutional collaboration delivering samples for scientific analysis of which there are only two other global examples. I have learned how to effectively establish partnerships to complete seemingly impossible work and know this will impact my career as a clinician and professional.

Compliance

TCU IACUC approval # #2022-9, June 2022. Animal subject husbandry/management by the Michigan Department of Natural Resources per their protocols.

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