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TITLE:
Plasmin Therapy to Prevent Post-Traumatic Heterotopic Ossification in the Upper Extremity After Severe Injury

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CONTRACTING ORGANIZATION:
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14. ABSTRACT
The purpose of this prospective animal study is to investigate new treatments to improve upper extremity joint function and prevent heterotopic ossification (HO, pathologic bone formation in muscle) follow severe injury. Plasmin is a critical reparative protease, essential for tissue regeneration following injury. The work proposed in this application will delineate the ideal timing for prophylactic plasmin therapy needed for clinical trials in both military and civilian trauma patients at risk for developing HO and associated impaired joint function. If our overarching hypothesis is proven true, the clinical impact is of most importance in the upper extremity as even partial prevention of a shoulder or elbow joint contracture can provide a wounded soldier or civilian with independence in activities of daily living. Importantly, as we have established that plasmin is essential both for preventing HO and promoting fracture repair/bone health, this would be the first therapy that does not compromise bone biology in order to prevent HO.

15. SUBJECT TERMS
Heterotopic Ossification, Upper extremity function, joint contracture

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1. INTRODUCTION:

It's estimated that over 50% of combat-related injuries sustained by military personnel affect the musculoskeletal system, with musculoskeletal injuries being one of the most common reasons for medical discharge. Heterotopic ossification (HO) is a significant source of morbidity related to these injuries due to loss of joint and muscle function and chronic pain. HO of the elbow and shoulder joints hinders normal joint function and the ability to perform daily activities. Combat-related amputation from injuries to the extremities can also increase the risk of HO development post-surgery. Chronic musculoskeletal conditions not only prevent return to duty, but they can also increase the risk for future injuries. As such, combat-related HO presents a substantial medical burden to the military with long-term consequences.

Treatments for HO include prophylactic drugs, surgical intervention, physical therapy, and radiation therapy, but all of these treatments either lack efficacy or instigate significant adverse effects. Surgical removal of HO is effective, but this is only beneficial if intervention occurs after the HO has matured and it institutes a high risk of hemorrhage and infection, both of which may increase morbidity and medical costs. While prophylactic NSAID therapy remains largely ineffective for the prevention of HO, bisphosphonate therapy, while effective, negatively affects fracture healing and bone remodeling. As such, an effective prophylactic therapy that does not interfere with bone healing or maintenance will not only prevent HO and long-term sequelae, but it will also circumvent the medical complications associated with the current therapeutic interventions.

The overall objective of this prospective animal study is to investigate new treatments to improve upper extremity joint function and prevent HO follow severe injury. Plasmin is a critical reparative protease, essential for tissue regeneration following injury. Findings from our laboratory have demonstrated that in severely injured patients, such as individuals experiencing burn injuries, plasmin is depleted in relation to the severity of injury (as measured by total body surface area burned). Aligning with these clinical results, we observed a marked depletion of plasmin activity in our murine model of thermal injury. Furthermore, when mice received a concomitant burn and tissue injury to their elbow, we observed HO formation and impaired elbow function, akin to results observed in genetically plasminogen deficient animals.

From these results, this proposal is focused on the application of plasmin therapy, as a means to reduce HO formation and improve upper extremity function following injury. Specifically, the work proposed in this application will delineate the ideal timing for prophylactic plasmin therapy. The proposed research will be conducted using a validated dorsal thermal injury (to represent severe injury) combined with a targeted skeletal muscle injury at the elbow. Importantly, this combination of injuries results in calcification around the elbow with functional deficits of that upper extremity that do not occur without the concomitant thermal injury.

If our overarching hypothesis is proven true, the clinical impact is of most importance in the upper extremity as even partial prevention of a shoulder or elbow joint contracture can provide a wounded soldier or civilian with independence in activities of daily living. Importantly, as we have established that plasmin is essential both for preventing HO and promoting fracture repair/bone health, this would be the first therapy that does not compromise bone biology in order to prevent HO.

The Aims to be examined include:

Aim 1: Determine the therapeutic window of restoring plasminogen consumed by thermal injury with recombinant plasminogen to prevent upper extremity heterotopic ossification and loss of function.

Aim 2: Determine the therapeutic window of enhancing plasmin activity by targeting plasmin's inhibitor (alpha2-anti-plamsin) to prevent upper extremity heterotopic ossification and loss of function.

Aim 3: We postulate that utilizing both methods, within their critical therapeutic windows determined in Aim 1 and 2, will provide the most efficacious treatment that prevents elbow heterotopic ossification and loss of function after a severe thermal injury with a concomitant injury of the elbow.

2. KEYWORDS:

Heterotopic Ossification, Upper extremity function, joint contracture, plasminogen, elbow

3. ACCOMPLISHMENTS:

What were the major goals of the project?

Specific Aim 1: **Determine the optimal timing of enhancing plasmin activity by targeting its inhibitor α2AP that i) restores plasmin activity, ii) prevents HO and elbow joint contracture.**	**Timeline**	**Percent Compleation**
Major Task 1: Conduct the combined thermal/muscle injury model with/without a2AP ASO administration	**Months**	
Subtask 1: Obtain ACURO approval	1-3	100%
Subtask 2: Obtain IACUC approval	1-3	100%
Subtask 3: Treatment administered beginning at 0,1,2,3,4,5,6, or 7 days post injury. 1mg/day for 3 days. 5 mice per group.	3-9	30%
Subtask 4: Synthesis of fibrin targeting peptide (FTP) for weekly in vivo imaging of fibrin resolution by plasmin	1-6	50%
Milestone Achieved: Local IACUC and ACURO Approval	3	100%
Milestone: Synthesis of fibrin targeting peptide completed	6	100%
Major Task 2: Longitudinal Assessment of HO	**Months**	
Subtask 1: Radiographic Analysis weekly: 7, 14, 21, and 28 DPI	3-9	30%
Subtask 2: MicroCT analysis Endpoint: 28 DPI.	6-12	30%
Subtask 3: Histological Analysis Endpoint: 28 DPI.	6-12	0%
Milestone(s) Achieved: Completed HO assessment with a2AP ASO treatment	12	20%
Major Task 3: Longitudinal Assessment of Elbow Function	**Months**	
Subtask 1: Grip Strength Analysis weekly: 7, 14, 21, 28 DPI	4-9	30%

Subtask 2: Wire Hang Analysis weekly: 7, 14, 21, and 28 DPI	4-9	0%
Subtask 3: Treadscan Analysis weekly: 7, 14, 21, and 28 DPI	4-9	0%
Milestone Achieved: Completed elbow function assessment with a2AP ASO treatment	9	30%
Major Task 4: Longitudinal Assessment of Plasminogen Levels	**Months**	
Subtask 1: Serologic Analysis weekly: 7, 14, 21, and 28 DPI	4-12	0%
Subtask 2: Weekly assessment of fibrin resolution by plasmin using in vivo imaging of FTP: 7, 14, 21, and 28 DPI	4-9	0%
Milestone Achieved: Completed assessment of plasminogen levels and plasmin activity with a2AP ASO treatment	12	0%
Major Task 5: Data Analysis	**Months**	
Subtask 1: Data Analysis	6-12	30%
Milestone Achieved: Completion of all data collection and analysis within Aim 1	12	30%
Specific Aim 2: Determine the optimal timing of restoring plasminogen by administering recombinant plasminogen that i) restores circulating plasminogen levels, ii) prevents heterotopic ossification (HO) and elbow joint contracture	**Timeline**	
Major Task 1: Conduct the combined thermal/muscle injury model with/without recombinant plasminogen administration	Months	
Subtask 1: Treatment administered at 0,1,2,3,4,5,6, or 7 days post injury. 5 mice per group.	12-18	0%
Subtask 2: Synthesis of FTP	12-18	0%
Milestone Achieved: Completion of model with recombinant plasminogen administration and FTP synthesis	18	0%
Major Task 2: Longitudinal Assessment of HO	**Months**	
Subtask 1: Radiographic Analysis weekly: 7, 14, 21, and 28 DPI	12-18	0%
Subtask 2: MicroCT analysis Endpoint at 28 DPI.	15-21	0%

Subtask 3: Histological Analysis Endpoint at 28 DPI.	15-21	0%
Milestone Achieved: Completion of HO assessment following recombinant plasminogen administration	21	0%
Major Task 3: Longitudinal Assessment of Elbow Function	**Months**	
Subtask 1: Grip Strength Analysis weekly: 7, 14, 21, and 28 DPI	13-18	0%
Subtask 2: Wire Hang Analysis weekly: 7, 14, 21, and 28 DPI	13-18	0%
Subtask 3: Treadscan Analysis weekly: 7, 14, 21, and 28 DPI	13-18	0%
Milestone Achieved: Completed elbow function analysis following recombinant plasminogen administration	18	0%
Major Task 4: Longitudinal Assessment of Plasminogen Levels	**Months**	
Subtask 1: Serologic Analysis weekly: 7, 14, 21, and 28 DPI	15-21	0%
Subtask 2: Weekly assessment of fibrin resolution by plasmin using in vivo imaging of FTP: 7, 14, 21, and 28 DPI	13-18	0%
Milestone Achieved: Completed assessment of plasminogen levels and plasmin activity with recombinant plasminogen	21	0%
Major Task 5: Data Analysis		
Subtask 1: Data Analysis	16-24	0%
Milestone Achieved: Completion of all data collection and analysis within Aim 2	24	0%
Specific Aim 3: Determine the optimal timing and duration of administration of a2AP ASO + recombinant plasminogen that prevents HO and elbow joint contracture following combined thermal/muscle injury.	**Timeline**	
Major Task 1: Conduct the combined thermal/muscle injury model with/without a2AP ASO + recombinant plasminogen administration	**Months**	
Subtask 1: Treatment administered based on optimal dosing points determined in aims 1 & 2. 5 mice per group.	24-30	0%
Subtask 2: Synthesis of FTP	24-30	0%

Milestones Achieved: Completion of burn model with combined dosing of therapeutics	30	0%
Major Task 2: Longitudinal Assessment of HO.	**Months**	
Subtask 1: Radiographic Analysis weekly: 7, 14, 21, and 28 DPI	24-30	0%
Subtask 2: MicroCT analysis Endpoint at 28 DPI.	27-33	0%
Subtask 3: Histological Analysis Endpoint at 28 DPI.	27-33	0%
Milestone Achieved: Completion of HO assessment following combined therapy	33	0%
Major Task 3: Longitudinal Assessment of Elbow Function	**Months**	
Subtask 1: Grip Strength Analysis weekly: 7, 14, 21, and 28 DPI	24-30	0%
Subtask 2: Wire Hang Analysis weekly: 7, 14, 21, and 28 DPI	24-30	0%
Subtask 3: Treadscan Analysis weekly: 7, 14, 21, and 28 DPI	24-30	0%
Milestone Achieved: Completion of elbow function assessment following combined therapy	30	0%
Major Task 4: Longitudinal Assessment of Plasminogen Levels		
Subtask 1: Serologic Analysis weekly: 7, 14, 21, and 28 DPI	27-33	0%
Subtask 2: Weekly assessment of fibrin resolution by plasmin using in vivo imaging of FTP: 7, 14, 21, and 28 DPI	25-30	0%
Milestone achieved: Completed assessment of plasminogen levels and plasmin activity with both plasminogen and a2AP ASO therapies	33	0%
Major Task 5: Data Analysis and Manuscript Preparation		
Subtask 1: Data Analysis	30-36	0%
Subtask 2: Manuscript Preparation	30-36	0%
Milestones Achieved: Completion of all data collection and analysis within Aim 3 and manuscript preparation of data collected from this proposal	36	0%

What was accomplished under these goals?

Subtask 1: Obtain ACURO approval 1-3
Subtask 2: Obtain IACUC approval 1-3

Both ACURO and IACUC approval were obtained during the first year of the grant. In addition to these approvals, we completed the Necessary subtask of modifying the SOW to conduct experiments with a2AP ASO prior to examining recombinant plasminogen. Modifications were discussed with granting agency and submitted for approval.

Subtask 4: Synthesis of fibrin targeting peptide (FTP) for weekly in vivo imaging of fibrin resolution by plasmin 1-6

Dr. McCarthy has produced the first batch of FTP for use in our animal model durong the first year of this proposal. Subsequant batches will be produced as part of Aims 2 and 3 of the proposal.

Specific Aim 1: Conduct the combined thermal/muscle injury model with/without a2AP ASO administration

To accomplish this Aim, we began the following:

Begin conducting the combined thermal/muscle injury model with and without a2AP ASO administration
Begin Radiographic analysis weekly.
Begin Endpoint μCT assessments on conducted samples
Begin Functional Assessments

After receiving the necessary approval, we conducted our first round of experiments investigating the efficacy of a2AP ASO administered at the time of injury to prevent calcification of skeletal muscle in the upper extremity (**Specific Aim 1**). These experiments were conducted in our mouse model of combined burn injury + upper extremity injury (UEI) (**Major Task 1**). Through the use of radiographic analysis, we found that administration of a2AP ASO at the time of injury significantly reduced the amount of calcification in injured skeletal muscle by 7 days post injury (7DPI) (Figure 1)(**Major Task 2**).

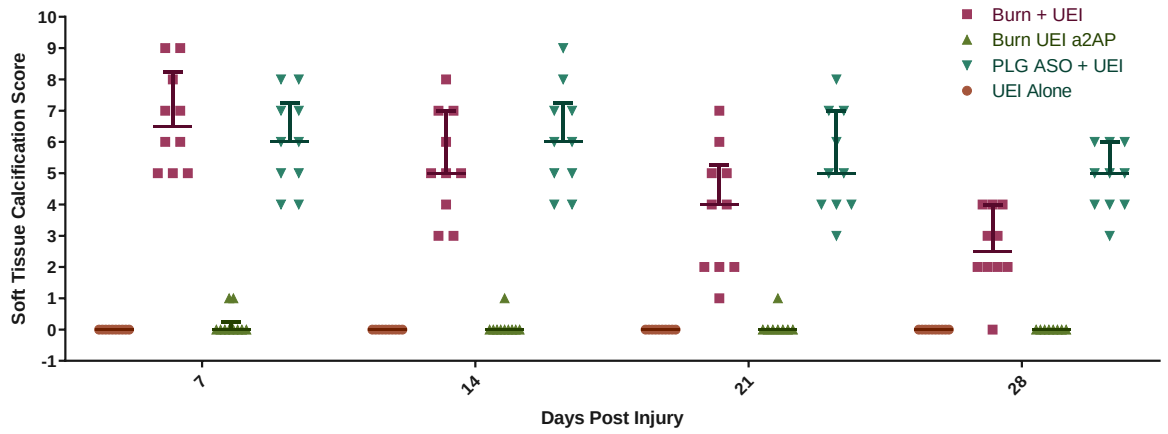
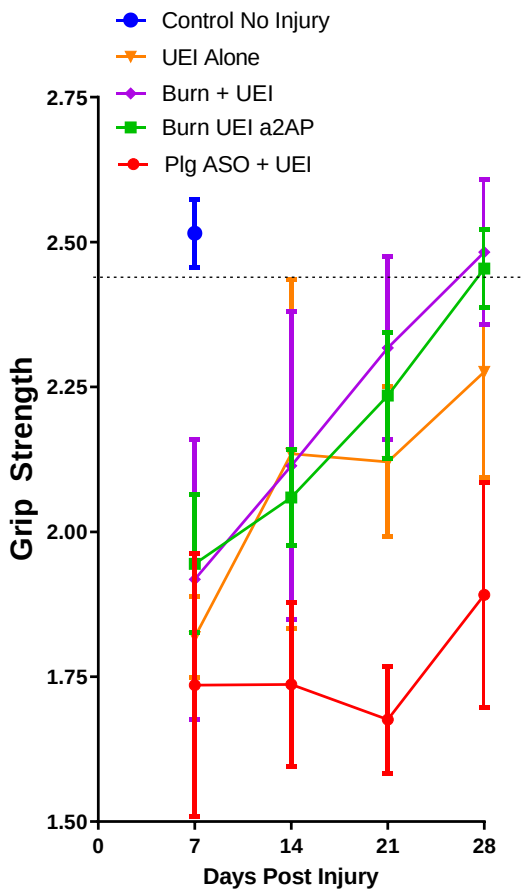


Figure 1

As we assessed calcification in the upper extremity over time, we detected a regression of calcification from damaged tissues in control animals (Burn + UEI) (**Major Task 2, Sub Task 1**). Alternatively, if plasminogen was reduced throughout the regeneration period (between 7 and 28 DPI), we observed impaired regression of calcification from damaged tissues. These results demonstrate the importance of plasmin(ogen) in the healing process, tissue regeneration, and regression of calcification from soft tissues. These plasminogen depletion experiments are important internal controls to demonstrate the true effect of loss of plasminogen on impaired tissue regeneration, muscle calcification, and impaired upper extremity function.

As a secondary output, we assessed the effect of a2AP ASO administration at the time of injury of the function of the upper extremity by measuring grip strength each week following injury (**Major Task** 3). We observed a comparable decrease in upper extremity function in all cohorts assessed, including mice treated with a2AP ASO. Over time, we observed a comparable increase in function between the control group (Burn + UEI) and a2AP ASO treatment group, reaching pre-injury function by 28 DPI (**Major Task 3, Sub Task 1).** Interestingly, in mice with reduced plasminogen (PLG ASO treatment), we did not observe an improvement in elbow function over time (Figure 2)- further demonstrating the importance of plasminogen in UE repair.



Together, these findings suggest that a2AP ASO treatment at the time of injury can effectively prevent the formation of calcification in the injured muscle tissues (**Specific Aim 1**). Furthermore, we observed a comparable trend in regaining grip strength following injury, suggesting that a2AP ASO therapy did not adversely impact this measure of joint function (**Major Task 3**).

Going forward, alternative measure of upper extremity function may be necessary to determine if a2AP ASO treatment improves function at a greater rate than control treated animals (**Improve upone Major Task 3**).

Given the success of a2AP ASO administration at the time of injury, we will next begin to assess the therapeutic window in which a2AP ASO is effective at inhibiting upper extremity calcification following injury (**Major Task 1**).

What opportunities for training and professional development has the project provided?
Dr. Stephanie Moore-Lotridge through this project has had the oppertuinty to mentor Ms. Breanne Gibson (Graduate student) and Mr. Zachary Backstrom (Research assistant) in matter pertaining to mouse work, imaging, and functional testing. As she anticipates persuing a career in research education, these experience are integral to her professional development.

How were the results disseminated to communities of interest?
Nothing to Report

What do you plan to do during the next reporting period to accomplish the goals?
During the next reporting period, we plan to continue our studies investigate the therapeutic window in which a2AP ASO is effective at inhibit muscle calcification and the associated effects on upper extremity function. In addition to grip strength, we will be looking into new novel methods that may better reflect upper extremity function and translatability to human activities of daily living.

4. **IMPACT:**
What was the impact on the development of the principal discipline(s) of the project?
Nothing to Report

What was the impact on other disciplines?
Nothing to Report

What was the impact on technology transfer?
Nothing to Report

What was the impact on society beyond science and technology?
Nothing to Report

5. CHANGES/PROBLEMS:

Changes in approach and reasons for change
The only change made to the approach during this period was the transition from beginning with Aim 2 instead of Aim 1. No changes to the science to be conducted were made.

In the approved proposal, we had anticipated beginning with Specific aim 1, where administration of recombinant plasminogen would be administered to rescue heterotopic ossificaiton formation and elbow joint contracture. Due to a delay in the production of recombinant plasminogen from our collaborator, we propose to instead begin with specific Aim 2 and examine the effect of enhancing plasmin activity on preventing HO and elbow joint contracture. Given that these aims are entirely independent, they are therefore interchangeable and we anticipate no delay in completion of the proposed work. We have discussed synthesis of the recombinant plasminogen with our collaborators and will begin to have ample supplies of this protein in 2019, therefore setting us up well to conduct these experiments in year 2 of the proposal (2020).

This change was discussed with the awarding agency grants office and was submitted for approval by our OSP offices at Vanderbilt university medical center by the end of January. We receive notification that this was sent to the sponsoring program on January 30th, 2019. In October 2019, we received notification from our grants office that a restriction was noted on our proposal pertaining to animal studies. In discussions with Dr. Mariam Redington, our science officer, it is indicated that all ACRUO approval has been met. We will be continuing to follow this up and ensure that all approval is appropriate.

Actual or anticipated problems or delays and actions or plans to resolve them
During this reporting period, prior to the beginning of the first round of experiments, the x ray cabinet necessary to conduct weekly radiographic analysis of animals malfunctioned. Initially it was believed that the x ray tube had expired, thus precluding us from obtaining radiographic images. Therefore, we were not able to begin experiments since without this imaging modality, as we would be unable to obtained critical output data on calcification of the muscle.

Upon this news, Dr. Moore-Lotridge and Dr. Schoenecker began working with other members of the Bone Center and Imaging Center at Vanderbilt University and Vanderbilt University Medical

center to explore additional equipment options. During the time of these discussion (Early April), the current x ray cabinet was able to be fixed and it was determined that the malfunction was due to broken door seal. Therefore, the equipment is now functioning and was available for us to gather data- though this malfunction did cause us to have a delay in the experimental timeline.

Given that the current x ray system is older and beginning to experience technical problems, to avoid any further delays in our experiments, Dr. Schoenecker in collaboration with the bone center moved towards obtaining a new piece of equipment to ensure that this imaging capacity is available throughout this funding. The new faxitron has now been delivered to VUMC and will be available for use during the next granting period.

Changes that had a significant impact on expenditures
Due to equipment malfunctioning as part of our core facility, we experienced a delay in mouse experiments being conducted in this period, and thus a reduction in expenditure for both core cost and animal cost. Now that all equipment has been remedied, we do not anticipate any further delays in the conduction of our experiments.

Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents
Nothing to Report

Significant changes in use or care of human subjects
Nothing to Report

Significant changes in use or care of vertebrate animals.
Not Applicable

Significant changes in use of biohazards and/or select agents
Not Applicable

6. PRODUCTS: List any products resulting from the project during the reporting period. If there is nothing to report under a particular item, state "Nothing to Report."

- **Publications, conference papers, and presentations**
  Nothing to Report
  Journal publications. Nothing to Report
  Books or other non-periodical, one-time publications. Nothing to Report
  Other publications, conference papers, and presentations. Nothing to Report
- **Website(s) or other Internet site(s)**
  Nothing to report
- **Technologies or techniques**
  Nothing to report
- **Inventions, patent applications, and/or licenses**
  Nothing to report
- **Other Products**
  Three technical progress reports were completed this year.

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project?

Name: *Jonathan G Schoenecker*
Project Role: *PI*
Researcher Identifier (e.g. ORCID ID): 0000-0002-3097-5496
Nearest person month worked: *2 months*
Contribution to Project: *Dr. Schoenecker oversaw the IACUC and ACURO submission process and directed the planning of upcoming experiments. Dr. Schoenecker worked with Dr. Moore-Lotridge to analyze all data obtained from this proposal period.*

Name: *Stephanie Moore-Lotridge*
Project Role: *Post-doctoral Fellow*
Researcher Identifier (e.g. ORCID ID): 0000-0002-3045-4199
Nearest person month worked: *3 months*
Contribution to Project: *Dr. Moore-Lotridge conducted the proposed experiments to produce the reported data on the efficacy of a2AP ASO administration at the time of injury for preventing muscle calcification.*

Name: *Breanne Gibson*
Project Role: *Graduate Student*
Researcher Identifier (e.g. ORCID ID): N/A
Nearest person month worked: *1 month*
Contribution to Project: *Ms. Gibson reviewed the ACURO application and assisted experiments conducted during this funding period.*

Name: *John C. Reese*
Project Role: *Research Assistant*
Researcher Identifier (e.g. ORCID ID): N/A
Nearest person month worked: *3 months*
Contribution to Project: *Mr. Reese is responsible for mouse colony maintenance, laboratory management, and ordering of products needed for experiments.*

Name: *Zachary K. Backstrom*
Project Role: *Research Assistant*
Researcher Identifier (e.g. ORCID ID): N/A
Nearest person month worked: *5 months*
Contribution to Project: *Mr. Backstrom helped to conduct the proposed mouse model and experimental outputs in collaboration with Dr. Moore-Lotridge.*

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?

Nothing to report

What other organizations were involved as partners?
Organization Name: Masonic Medical Research Institute (Dr. Jason McCarthy)
Location of Organization: Utica, NY
Partner's contribution to the project: Production of fibrin imaging agents for use in our animal models

8. SPECIAL REPORTING REQUIREMENTS

COLLABORATIVE AWARDS: N/A

QUAD CHARTS: Submitted