AWARD NUMBER: W81XWH-18-1-0653

TITLE: Diagnostic and Therapeutic Strategy to Prevent Trauma-Induced Upper-Extremity Muscle Fibrosis and Heterotopic Ossification

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REPORT DATE: October 2020

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved OMB No. 0704-0188

1. REPORT DATE
October 2020

2. REPORT TYPE
Annual

3. DATES COVERED
30Sep2019-29Sep2020

4. TITLE AND SUBTITLE

Diagnostic and Therapeutic Strategy to Prevent Trauma-Induced Upper-Extremity Muscle Fibrosis and Heterotopic Ossification

5a. CONTRACT NUMBER
W81XWH-18-1-0653

5b. GRANT NUMBER

5c. PROGRAM ELEMENT NUMBER

5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

6. AUTHOR(S)

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8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)

U.S. Army Medical Research and Development Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT

Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT

Heterotopic ossification (HO), the formation of ectopic bone in soft tissue, and muscle fibrosis, severe scarring of muscle tissue, often result due to traumatic injury. The military population has been shown to be particularly as risk of these afflictions following battlefield trauma. Here, we investigate a class of hypoxia-targeting drugs as treatments for HO and traumatic muscle fibrosis. We also develop a high frequency spectral ultrasound imaging system (SUSI) to detect early HO lesions and muscle fibrosis in order to facilitate early treatment.

15. SUBJECT TERMS
Heterotopic ossification, rapamycin, Amphotericin, fibrosis, ultrasound, SUSI, mTOR/HIF1α, trauma, muscle crush

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	**b. ABSTRACT**	**c. THIS PAGE**			USAMRMC
Unclassified	Unclassified	Unclassified	Unclassified	33	**19b. TELEPHONE NUMBER** *(include area code)*

Standard Form 298 (Rev. 8-98)
Prescribed by ANSI Std. Z39.18

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1. INTRODUCTION:

The central goal of this grant is to demonstrate the efficacy of timed, image guided mTOR/ HIF-1α inhibitors (Rapamycin and Amphotericin) to attenuate muscle fibrosis, HO and joint contracture development without negatively affecting wound healing processes. We aim to change the current treatment paradigm of fibrosis and HO management in military, veteran and civilian populations at risk from one of delayed diagnosis and excision to one of early detection and timed, precise prevention. We hypothesize that early mTOR/HIF-1α signaling is critical for fibrosis and HO formation and that this destructive process can be mitigated through imaged guided delivery of mTOR/HIF-1α inhibitors. Specifically, we will deploy Rapamycin and Amphotericin B for timed, image guided drug delivery to block HO and fibrosis.

2. KEYWORDS:

Heterotopic ossification, skeletal muscle, rapamycin, Amphotericin, fibrosis, ultrasound, SUSI, mTOR/HIF1α, trauma, blast injury

3. ACCOMPLISHMENTS:

What were the major goals of the project?

Regulatory Tasks:
Subtask 1: IACUC/ACURO Approval
Status: Status: USU IACUC protocol number: SUR-18-065 approved 2018-12-26, UM IACUC protocol number: PRO00007930 approved 2017-09-20. ACURO protocol number: OR170174 approved at UM and USU.

Specific Aim 1: *To validate timed, image guided prevention of heterotopic ossification with FDA approved inhibitors of hypoxic signaling.*
Status: Completed Y2Q4

Subtask 1: Validate imaging and histology and demonstrate that image guided Hif1α/mTOR inhibition with our therapeutics prevents HO.
Status: Completed Y2Q4

Subtask 2: Define the time point at which treatment is best administered.
Status: Completed Y2Q4

Subtask 3: Validate timed treatment starting when early HO is detected by imaging and histology.
Status: Completed Y2Q4

Milestone: Completion of validation of timed, image guided prevention studies and data analysis. Preparation and submission of peer-reviewed manuscripts.
Status: In progress Y2Q4

Specific Aim 2: *To mitigate post-traumatic muscle fibrosis through timed inhibition of mTOR/HIF-1α signaling with Amphotericin and Rapamycin separately or as combined therapy.*
Status: In progress Y2Q4

Subtask 1: Demonstrate that mTOR/HIF-1α inhibitors decrease post traumatic extremity muscle fibrosis.
Status: In progress Y2Q4

Subtask 2: Validate mTOR/HIF-1α inhibitors as separate and combined treatment modalities in extremity muscle fibrosis.
Status: In progress Y2Q4

Subtask 3: Define the time point at which treatment is best administered.
Status: In progress Y2Q4

Milestone: Completion of muscle fibrosis timed inhibition studies and data analysis. Preparation and submission of peer-reviewed manuscripts.
Status: In progress Y2Q4

What was accomplished under these goals?

Specific Aim 1: To validate timed, image guided prevention of heterotopic ossification with FDA approved inhibitors of hypoxic signaling.

1. Treatment with Hif1α inhibitors and high frequency spectral ultrasound imaging in two rodent models of post-traumatic heterotopic ossification

Heterotopic ossification (HO) is a devastating condition in which ectopic bone forms inappropriately in the soft tissue following large surface area burns, musculoskeletal trauma, and many orthopedic surgeries. The military population has been shown to be particularly at risk of

HO following battlefield wounds, with occurrences in approximately 63-65% of blast-related extremity amputations and approximately 62% of limb sparing procedures (1-3). Previously, rapamycin has shown efficacy in inhibiting HO in rat model of blast trauma and in a mouse burn-tenotomy model by inhibition of Hif1α (4,5).

Here, we expand upon previous studies to investigate treatment with the Hif1α inhibitors rapamycin, Amphotericin B, and a combination of rapamycin and Amphotericin B to prevent trauma-induced HO formation.

1a. Effect of Rapamycin and Amphotericin B Treatment on HO in a Combat Trauma Rat Model of HO formation

Adult male Sprague Dawley rats (450-500 g) were housed in clean plastic cages and kept on a 12-hour light/dark cycle with unlimited access to food (standard rodent chow) and fresh water ad libitum. The study protocol (**SUR-18-065**) was reviewed and approved by the Institutional Animal Care and Use Committee of the Uniformed Services University of the Health Sciences, in compliance with all applicable Federal regulations governing the protection of animals in research. We used our established rat model of blast-related HO which incorporates the critical injury patterns associated with combat-related extremity injury including 120 ±7 kPa systemic blast overpressure exposure (120kPa), followed by femur fracture, quadriceps crush injury, tourniquet application for 3 hours prior to trans femoral amputation through the zone of injury. Animals were single housed post-surgery.

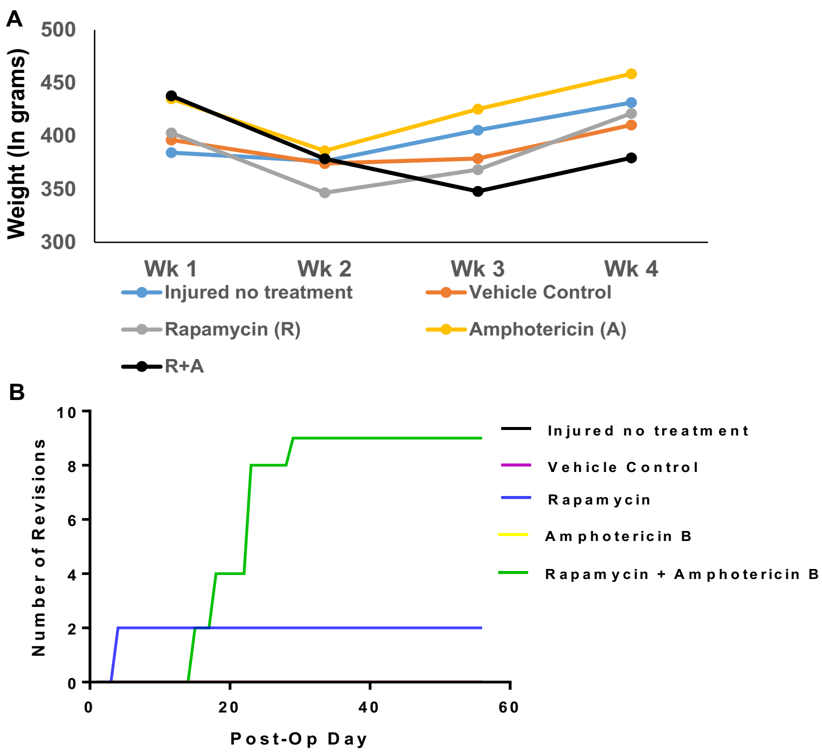
Treatments with either **rapamycin** (**R, 2.5 mg/kg**; Cayman Chemicals), **Amphotericin B** (**A, 2 mg/kg**; water-soluble formulation from X-Gen) or **both** (**R+A**, 1.25 mg/kg **(R)** and 1 mg/kg **(A)**, respectively) were started on post-operative day 1 (POD1) and administered i.p daily. Rapamycin was administered for 14 days and Amphotericin B for 10 days. Vehicle control group was included only for rapamycin (3% DMSO in saline), since Amphotericin B was dissolved in water. **Table 1** details the cohorts for this study.

Table 1. Experimental design for testing the effects of rapamycin and Amphotericin B on HO formation.

Treatment groups	**Animal number**
Rapamycin alone **(R, 2.5 mg/kg)**	8
Amphotericin B alone **(A, 2 mg/kg)**	8
Rapa+ Ampho **(R@ 1.25 mg/kg + A@ 1 mg/kg)**	8
Vehicle Control **(VC, 3% DMSO in saline)**	8
Injured no treatment **(IN)**	8
Total animal number	**40**

Weight loss was observed for all injury groups in the first 2 weeks post injury. The R+A drug combination group demonstrated the highest and persistent weight loss among all groups. However, all cohorts, except the combination group regained their post-operative weights by week 4 post injury **(Fig. 1A)**, thereby establishing the safety of the drugs and corresponding dosage used. The combination group also demonstrated recurrent wound dehiscence, which needed multiple revisions **(Fig. 1B)**, indicating that the combination of rapamycin and

Amphotericin B potentially interferes with wound healing and would not be a suitable therapeutic option in complex polytraumatic injuries, such as those seen in combat-wounded. The total number of surgical site wound dehiscences and subsequent revision procedures amongst the 8 animals in each treatment group was 10 in the Amphotericin+ rapamycin treatment group, 2 in the rapamycin treatment group and none in the other treatment groups. As illustrated in Figure



1B, wound dehiscence in the combination treatment group most often occurred between 14-30 days post injury/surgery and following the last treatment dose.

Figure 1. Weight monitoring and wound closure complications post-treatment. A). Longitudinal post-operative weight monitoring for the four cohorts; Injured no treatment, Vehicle control, Rapamycin, Amphotericin, and Rapamycin + Amphotericin from post-operative week 1 through post-operative week 4. B). Number of revisions needed at the zone of amputation due to wound dehiscence, over a period of 2 months' post-surgery for each cohort

This study also investigated HO formation after treatment with rapamycin, Amphotericin B, and rapamycin and Amphotericin B in combination. MicroCT imaging was carried out for each cohort every 2 weeks for 8 weeks. Analysis of the mean ectopic bone volume at 8 weeks demonstrated significant reduction (~60%; p-value 0.022) in the volume of HO only in the rapamycin treated group in comparison to vehicle control treated rats as shown in **Figure 2**. The reduced attenuation of HO formation in the combined rapamycin+Amphotericin (R+A) treatment group may be due prolonged and exaggerated inflammation within the zone of injury as the result of a higher incidence of wound dehiscence and surgical revision complications post drug treatment.

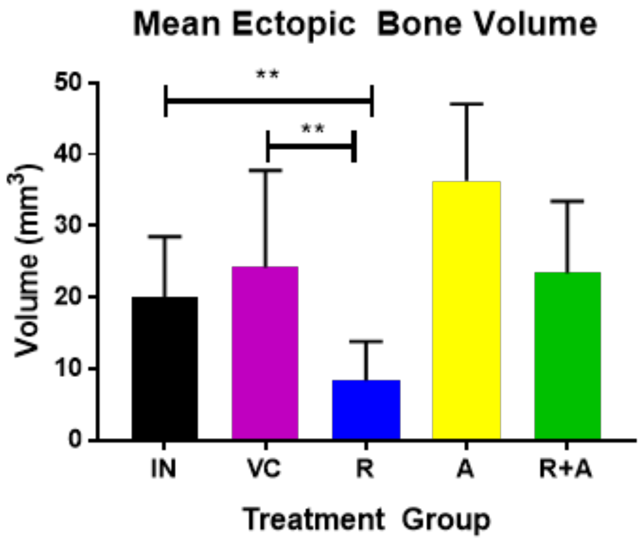


Figure 2. Quantification of ectopic bone volume as assessed by micro-CT imaging at 8 weeks post surgery in five cohorts; namely injured no treatment (IN), vehicle control (VC), or the treatment groups, rapamycin (R), Amphotericin B (A), and the combination group of rapamycin and Amphotericin B (R+A).

In these studies, a rat model of blast-related HO was used to investigate the safety and efficacy of administration of rapamycin, Amphotericin B, and combination rapamycin + Amphotericin B treatments to prevent HO formation following traumatic injury. Rapamycin (2.5 mg/kg) and Amphotericin B (2 mg/kg) were shown to be safe for administration in the rat model for 14 days and 10 days, respectively. However, the combination treatment of rapamycin (1.25 mg/kg) and Amphotericin B (1 mg/kg) showed weight loss that had not been regained by four weeks post injury and recurrent wound dehiscence, which needed multiple revisions, indicating that the combination of rapamycin and Amphotericin B potentially interferes with wound healing and would not be a suitable therapeutic option in complex polytraumatic injuries, such as those seen in combat-wounded.

This study also analyzed the volume of ectopic bone formation every two weeks for 8 weeks using microCT imaging. **Analysis of the mean ectopic bone volume at 8 weeks demonstrated significant reduction (~60%; p-value 0.022) in the volume of HO in rats treated with rapamycin in comparison to vehicle control treated rats**.

1b. SUSI distinguishes native anatomical features and detects early HO formation in a combat related model of post-traumatic HO formation

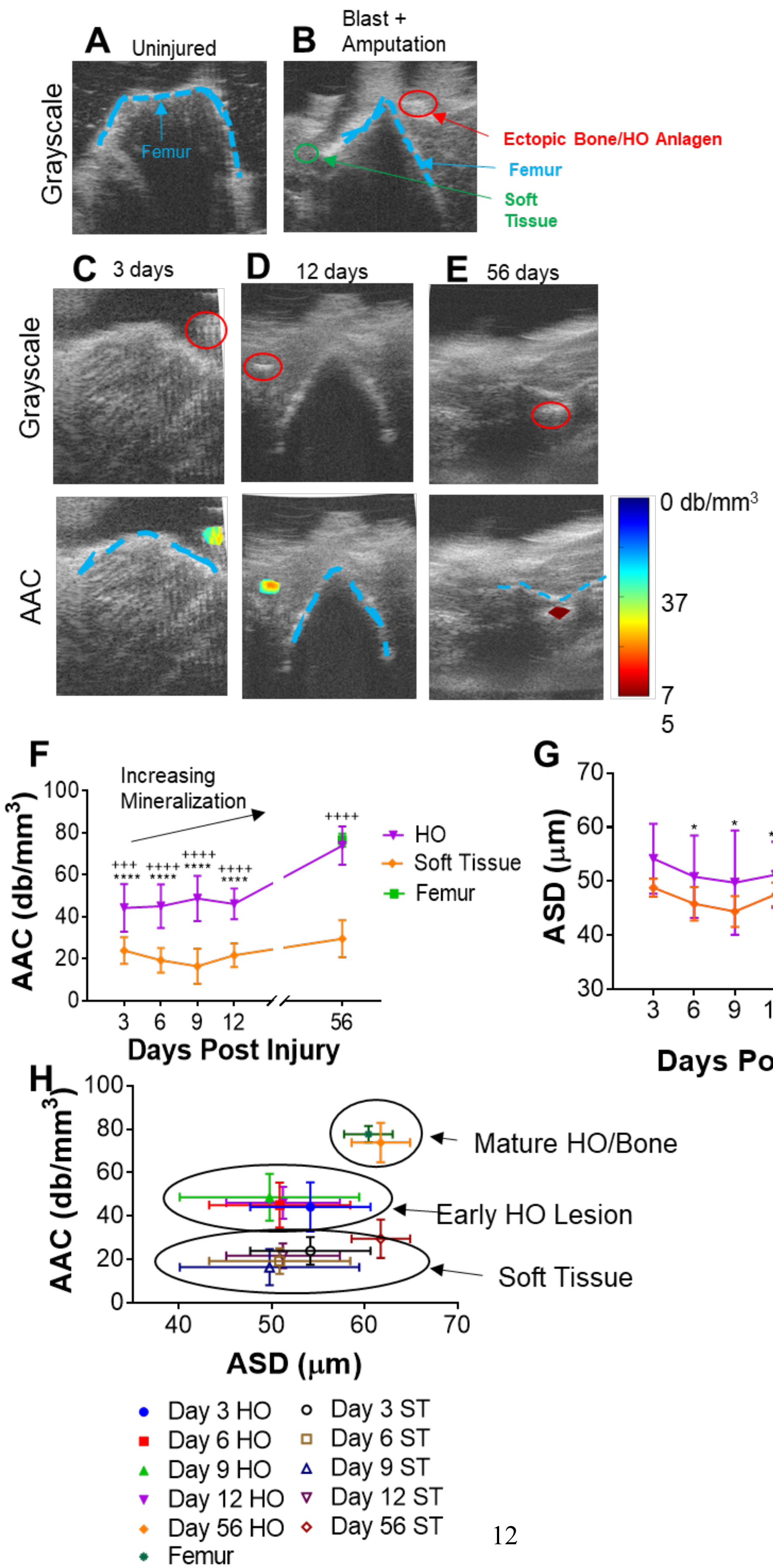
Despite potential pharmacological treatments, clinical detection of HO in patients is currently limited by poor visualization at early time points. At present, HO is often only visualized after it has formed, as diagnosis relies on the use of CT and MRI to identify ectopic lesions (6,7). Early diagnosis would allow for pharmacological treatment in order to arrest bone development, instead of surgical excision, often the only treatment strategy currently available, which often leads to additional ectopic bone formation (8). We have previously shown that high frequency spectral ultrasound imaging (SUSI) can effectively visualize traumatic HO formation in our burn-tenotomy model of HO at early time points (9).

We aim to further develop this SUSI imaging system as a method to detect HO formation at early time points and then appropriately deliver timed, guided treatments to prevent bone development. SUSI is an ultrasound technique that allows for more in-depth analysis of the conventional grayscale B-mode imaging. This technique uses spectra based on radio-frequency backscattered signals to collect additional data parameters to more fully quantify and characterize the tissue. (9). Here, we investigate SUSI technology to identify early HO lesions in the rat model of combat related post-traumatic HO formation. This model involves substantial soft tissue and muscular injury. The muscle niche releases substantial cytokines, chemokines, and other signal modulators after injury, which affect the immune response and wound healing following injury, all of which impact HO formation (10). Soft tissue involvement is also present in most clinical populations at high risk for HO, such as after traumatic injury, amputation, and hip replacement. Thus, we investigated the ability of SUSI to distinguish HO lesions from the surrounding injured soft tissue in a model of combat-related traumatic injury involving soft and hard tissue damage via blast injury and amputation (11).

A blast + amputation injury was performed on Sprague-Dawley rats as previously described previously. Rats were untreated. Limbs were collected at 3, 6, 9, 12, and 56 DPI, fixed in formalin, and imaged. Skin and dense connective tissue was removed before imaging. All imaging was performed using a VisualSonic VEVO770 high resolution small animal ultrasound imaging unit (Visualsonics, Inc., Toronto, CA). All imaging was done with B-mode and radiofrequency mode to acquire backscattered ultrasound signals for SUSI analysis. Injured rat limbs were imaged in the region of the amputation site and for imaging of the contralateral uninjured limb, the limb was cut proximal to the knee joint through the femur, so the exposed end was imaged, which anatomically matched the amputation site of the injured limb. A section of the femur was always in the image in order to provide a point of reference. Prior to imaging, skin and subcutaneous fat was removed from the surface. Samples were submerged in phosphate buffered saline to act as the coupling agent. Three types of tissue were identified and quantified separately for each animal: soft tissue (away from the femur), femur, and suspected HO. HO was identified as bright regions in the grayscale image that were not connected to native bone (femur) and were not another expected anatomical feature (tendon, ligament, connective tissue, etc.). The femur was identified based on bone's high echogenicity and expected femur anatomy. Soft tissue was identified as tissue with low echogenicity not connected to the femur.

The average acoustic concentration (AAC) and average scatter diameter (ASD) were calculated as previously described using our SUSI analysis techniques. These parameters and tissue-specific. We used grayscale images to identify normal anatomy (femur) in the uninjured, amputated limb (**Fig 3A-B**), and to identify femur, surrounding soft tissue in the zone of injury, and HO lesions in the injured limb at 3, 6, 9, 12 and 56 DPI (**Fig. 3C-E**) in separate animals for each time point. In this model, the AAC of the HO lesion was significantly different than that of the surrounding soft tissue and femur by 3 days post injury (44.2 ± 11.4 db/mm^3 vs 24.0 ± 6.3 db/mm^3, vs 76.5 ± 3.1 db/mm^3 HO anlagen vs soft tissue vs femur, $p < 0.01$ both analyses) allowing for early-stage diagnosis (**Fig 3E, F)**. The AAC of the HO anlagen was also significantly different than that of surrounding soft tissue and the femur at 6-12 days post injury ($p<0.001$), as shown in **Fig 3D, F**.

By 56 days post injury, the AAC of HO was similar to that of the femur (73.8 ± 9.1 db/mm^3 vs 76.5 ± 3.1db/mm^3, HO vs femur, $p>0.05$), indicating a mature state of mineralization of the ectopic bone (**Fig 3E, F).** When the ASD was analyzed, ASD of the HO anlagen was significantly different from that of the femur at 6-12 days post injury, but similar to that of the surrounding soft tissue (**Fig 3G**). By 56 days post injury, ASD of the mature HO was similar to that of the femur, but now significantly higher than that of soft tissue (**Fig. 3G**). Plotted together, three distinct clusters were again present in the rat model: soft tissue, early HO anlagens, and mature HO/bone, allowing for improved characterization of tissue type (**Fig. 3H**). **In the traumatic model of HO with soft tissue injury involvement, HO was detected as early as 3 days post injury, allowing for early-stage diagnosis and effective treatment.**



A Uninjured
B Blast + Amputation
Grayscale
Femur
Ectopic Bone/HO Anlagen
Femur
Soft Tissue
C 3 days
D 12 days
E 56 days
Grayscale
AAC
0 db/mm3
37
7
5
F
Increasing Mineralization
AAC (db/mm3)
Days Post Injury
HO
Soft Tissue
Femur
G
ASD (μm)
Days Post Injury
H
AAC (db/mm3)
ASD (μm)
Mature HO/Bone
Early HO Lesion
Soft Tissue
Day 3 HO
Day 6 HO
Day 9 HO
Day 12 HO
Day 56 HO
Femur
Day 3 ST
Day 6 ST
Day 9 ST
Day 12 ST
Day 56 ST

Figure 3: SUSI distinguishes HO anlagen at 3 days post injury in rat model of blast + amputation injury. Grayscale images of A) uninjured and B) blast + amputation injured rat limbs and identification of anatomical features. Grayscale images and average acoustic concentration (AAC) values overlayed on grayscale images at C) 3 days, D) 12 days, E) 56 days post injury. Blue dashed lines indicate femur. F) AAC values of HO and soft tissue at 1-56 days post injury and femur. G) Average scatter diameter (ASD) values of HO and soft tissue at 1-56 days post injury and femur. H) ASD vs AAC values of HO lesions, femur, and soft tissue at 3-56 days post blast + amputation injury. +/* denotes statistical difference compared to soft tissue and femur, respectively. ++++/**** $p<0.0001$; ++ $p<0.002$; + $p<0.05$.

1c. SUSI detects HO formation at 2 weeks post injury in mouse model of traumatic HO formation

In previous reports, we have shown that the mouse model of burn + tenotomy (B/T) also results in HO formation. This model incorporates tendon injury and a 30% total body surface area burn, both of which injuries are common in battlefield trauma. Male C57BL/6J mice were obtained from The Jackson Laboratory (Bar Harbor, ME, USA; stock no. 000664). Briefly, mice were anesthetized with inhaled isoflurane. Dorsal hair was shaved and a partial-thickness scald burn injury that comprised approximately 30% of total body surface area was administered on the dorsal surface using an aluminum block heated to 60°C for 18 seconds. The mouse also received a tenotomy of the Achilles tendon and closure of the skin over the tenotomy site with one 5-0 vicryl suture. Buprenorphine (Buprenex, Reckitt Benckiser Pharmaceuticals) at 0.06 mg/kg given subcutaneously was administered pre-operatively and every 12 hrs for 2 days. A total of n=6 per group per time point were assessed. Mice included the longitudinal SUSI study were treated with 3% dimethyl sulfoxide in phosphate buffered saline (PBS), or the same volume of 1:1 PBS:5% dextrose solution in order to mimic standard drug carriers (n=3 per treatment) daily for seven days starting on the day of injury.

In this longitudinal study, we sought to use SUSI to identify trauma-induced HO lesions at early stages after injury. As early as 1 wk post B/T, an HO anlagen was identified in the grayscale images (**Fig 4A,** red outline in grayscale image). As expected, at 1 week post B/T, the AAC of the HO anlagen was significantly lower than that of the calcaneus, which matches previous reports showing that the HO anlagen at 1 week post injury is largely cartilaginous without mineralized bone (9). Additionally, the AAC of the HO anlagen was not significantly different than that of the surrounding edema at 1 week post B/T, making definitive HO diagnosis based on AAC difficult, as shown in **Fig. 4 A, E** (AAC values: 50.5 ± 7.3 db/mm^3 vs. 37.2 ± 6.9 db/mm^3 vs. 77.5 ± 6.3 db/mm^3 HO anlagen, edema, calcaneus, respectively; n=6/group; one-way ANOVA with Tukey's test, p<0.0001 for HO compared to calcaneus, p>0.05 for HO compared to edema). By 2 weeks post B/T, the AAC of the HO anlagen was significantly different than that of surrounding soft tissue and calcaneus (52.1 ± 6.0 db/mm^3 vs 34.3 ± 6.8 db/mm^3 vs 77.5 ± 6.3 db/mm^3; HO anlagen, soft tissue, calcaneus, respectively; n=6/group; p<0.001 both analyses; one-way ANOVA with Tukey's test), indicating that by using quantitative analysis, the HO lesion can be distinguished from the surrounding soft tissue in the injury zone and the calcaneus as early as 2 weeks post B/T (**Fig 4 B, E**). Similarly, the AAC of the HO anlagen was also significantly different than surrounding soft tissue (p<0.0001) and calcaneus (p<0.0001) at 3 weeks post injury. By 6 weeks post injury, the AAC of HO was similar to that of the calcaneus (**Fig. 4 C-E**), indicating more advanced mineralization of the ectopic bone (70.4 ± 5.5 db/mm^3 vs 77.5 ± 6.3

db/mm^3, HO vs calcaneus at 6 weeks post injury, $p>0.05$) and remained similar to that of the calcaneus at 9 weeks post injury ($p>0.05$). Formation of ectopic bone was confirmed in the injured leg compared to the contralateral uninjured leg at 6 weeks post B/T by x-ray (**Fig. 4F**) and at 9 weeks by microCT (data not shown). The increase in AAC shown here matches the development of HO formation via endochondral ossification, a process of bone formation in which cartilage is systemically replaced with bone tissue. Indeed, past characterization of the B/T model of HO detected cartilaginous HO anlagen at 1 week post injury (9), with mineralized bone present at 9 weeks post B/T (9). Analyzing the ASD, we find that the ASD of the HO lesion is significantly different from that of soft tissue at every week analyzed, including week 1, however the ASD of the HO anlagen is similar to that of the calcaneus at every week analyzed (**Fig. 4G**). Therefore, the ASD may be a useful diagnostic tool early in HO development when a visual grayscale identification can be used to distinguish HO lesions from native bone and ASD parameters are able to distinguish HO lesions from surrounding edema and injured soft tissue. When the ASD and AAC values are plotted together, as shown in **Fig. 4H**, three clearly demarcated groups are present. Soft tissue, early HO lesions, and mature HO/bone are clustered together based on AAC and ASD values, allowing for additional tissue characterization. **Here, we have shown that SUSI is able to distinguish ectopic bone lesions from surrounding injured soft tissue and native bone as early as 2 weeks post injury based on the AAC and ASD, which are unique parameters inherent to the tissue.** This is substantially earlier than current CT and MRI modalities and will allow for more timely treatment to arrest HO development.

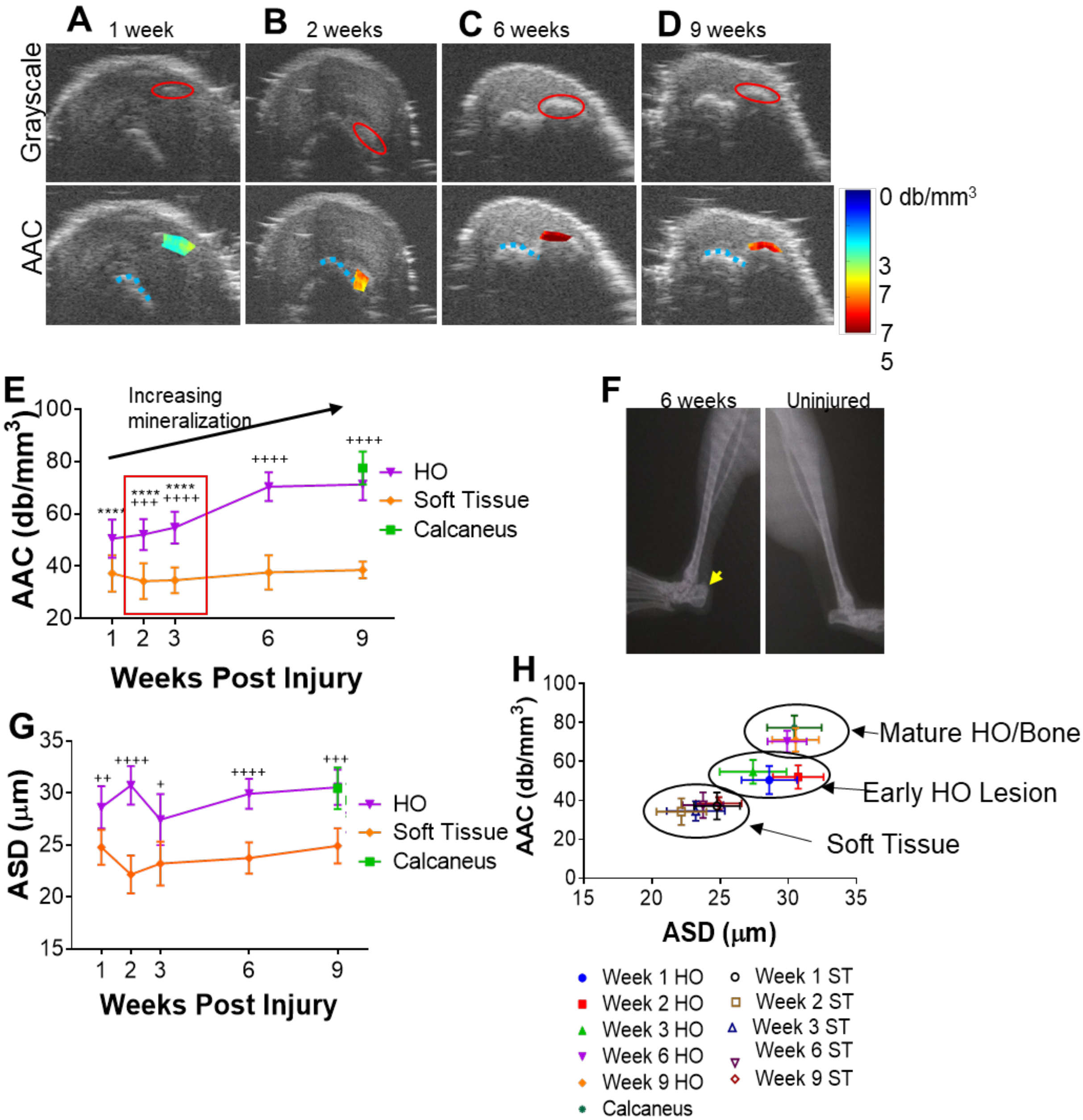


Figure 4: SUSI distinguishes HO anlagen at 2 weeks post injury from surrounding injured soft tissue and native bone. Grayscale images and average acoustic concentration (AAC) values overlayed on grayscale images at A) 1 week, B) 2 weeks, C) 6 weeks and D) 9 weeks post burn-tenotomy. Red circles indicate HO lesions. E) AAC values of HO and soft tissue at 1-9 weeks post injury and calcaneus. Blue dashed lines indicate calcaneus. F) X-ray identifies ectopic bone formation in injured mice at 6 weeks post injury. Yellow arrow indicates ectopic bone formation. G) Average scatter diameter values (ASD) of HO and soft tissue at 1-9 weeks post injury and calcaneus. H) ASD vs AAC values of HO lesions, calcaneus, and soft tissue at 1-9 weeks post burn-tenotomy. ST: soft tissue in zone of injury. +/* denotes statistical difference compared to soft tissue and calcaneus, respectively. ++++/**** $p<0.0001$; +++/*** $p<0.0002$; ++ $p<0.002$; + $p<0.05$.

In previous reports, we have shown that treatment with rapamycin, Amphotericin B, or combination of rapamycin + Amphotericin B does not significantly reduce HO formation in the mouse model of B/T at 9 weeks post injury (**Fig 5a**). These mice were also imaged weekly with SUSI and the AAC was calculated. Significant differences were not found between treatment groups at any time point, as shown in **Fig. 5b**.

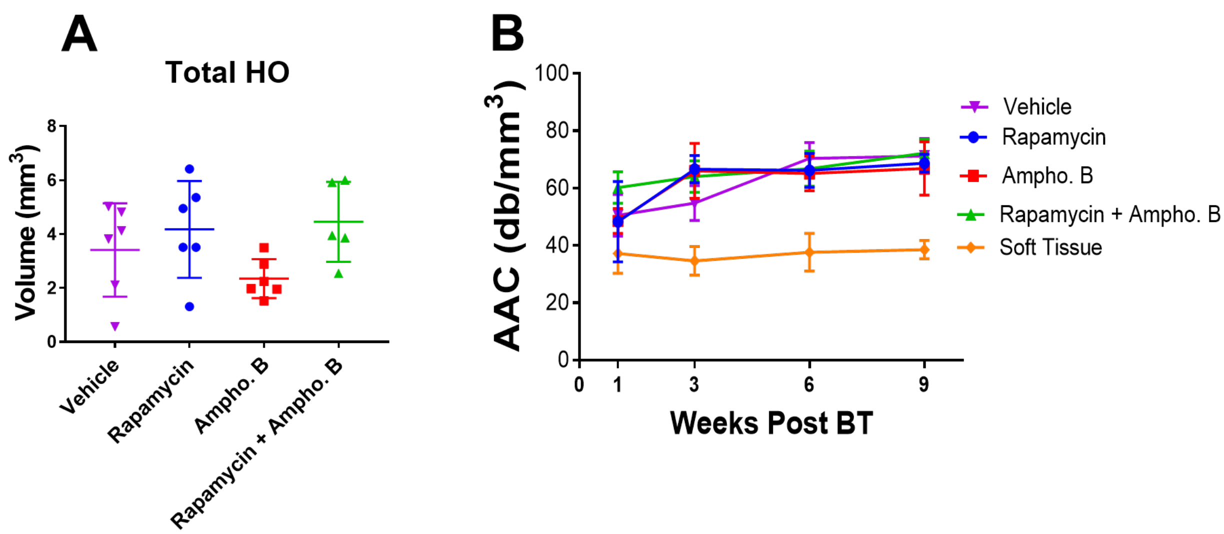


Figure 5: SUSI does not detect differences in AAC between treatment groups. A) Mice treated with rapamycin, Amphotericin B, combination rapamycin + Amphotericin B or vehicle control for 7 days did not present with significant differences in HO formation. B) SUSI does not detect significant difference in AAC at any week between treatment groups of the same mice.

Key Findings or Accomplishments for Specific Aim 1

First, we have shown that daily treatment with rapamycin for 14 days starting on the day of injury decrease total HO formation in a rat model of combat-related post traumatic HO formation. Next, we developed and validated a novel high frequency spectral ultrasound imaging method (SUSI) for the detection of early HO lesions. By calculating the average acoustic concentration (AAC) and average scatter diameter (ASD), SUSI distinguishes HO lesions as early as 3 days and 2 weeks post injury in the rat and mouse models, respectively. This is significantly earlier than current CT or MRI based imaging, and will allow for patient identification and timely treatment to arrest HO formation.

Specific Aim 2: To mitigate post-traumatic muscle fibrosis through timed inhibition of mTOR/HIF-1α signaling with Amphotericin and Rapamycin separately or as combined therapy.

2. Effect of Rapamycin and Amphotericin B Treatment on Muscle Fibrosis in a rat model of post-traumatic fibrosis

Muscle crush injuries occur when heavy debri or blasts compress skeletal muscle. This often results in muscle swelling, interruption of blood flow leading to ischemia reperfusion injury, and in severe cases, remote organ damage due to the buildup of excess myoglobin. Muscle crush injuries also often result in significant fibrosis in the affected muscle, which results in decrease in muscle function and range of motion, and decrease in quality of life. Combat-related muscle crush injuries of the extremities are common in wounded warriors and often require evacuation from theater (12).

2a. Rat Muscle Crush Injury Study Design

Adult male Sprague Dawley rats (450-500 g) were housed in clean plastic cages and kept on a 12-hour light/dark cycle with unlimited access to food (standard rodent chow) and fresh water ad libitum. The study protocol (**SUR-18-065**) was reviewed and approved by the Institutional Animal Care and Use Committee of the Uniformed Services University of the Health Sciences, in compliance with all applicable Federal regulations governing the protection of animals in research. Animals were sedated with a Ketamine/Xylazine cocktail, following which right, lower limb was fully shaved. To induce muscle injury, a 5 minute soft tissue crush injury was performed generating 20lbs/in^2 of pressure using the Chatillion DF Series digital force gauge. Following the crush, a superficial 1.5cm incisional wound was made over the medial quadriceps to the fascial plane, followed by wound closure. Animals were fully recovered post wound closure.

Starting post-operative day 1, treatments were initiated with either **rapamycin** (**R, 2.5 mg/kg**; Cayman Chemicals), **Amphotericin B** (**A, 2 mg/kg**; water-soluble formulation from X-Gen) or vehicle control for rapamycin (since Amphotericin B is dissolved in water). Since we observed weight loss and wound dehiscence issues with the combination group, this group was not tested for the fibrosis study. Rapamycin was administered daily i.p for 14 days and Amphotericin B was administered daily i.p for 10 days. Vehicle control group for rapamycin (3% DMSO in saline) administered daily i.p for 14 days. **Table 2** details the cohorts for this study.

Table 2. Experimental design for testing the effects of rapamycin and Amphotericin B on muscle fibrosis.

Treatment groups	**POD 1**	**POD 7**	**POD 14**	**POD 28**
Injured no treatment **(IN)**	8			
Rapamycin alone **(R, 2.5 mg/kg)**	-	8	8	8
Amphotericin alone **(A, 2 mg/kg)**	-	8	8	8
Vehicle Control **(VC, 3% DMSO in saline)**	-	8	8	8
Total animal number	**8**	**24**	**24**	**24**

Eight animals from each cohort were euthanized at the indicated time points. Tissues were collected for histopathology assessment, SUSI imaging to detect early changes in tissue architecture leading to fibrosis and the effect of therapeutic intervention on this architecture, expression of fibrotic gene targets and complete blood counts to assess possible hematological complications (CBC), as detailed in **Table 3**. Intact injured and the control contralateral limbs will be used for SUSI imaging, followed by histological assessment of fibrosis by Picrosirius Red and Masson Trichrome stains.

Table 3. Details of tissues harvested post necropsy with the corresponding downstream assays.

Tissue harvested	**Harvesting medium**	**Downstream Assay**	**Number of samples (n)**
Injured limb (intact) Contralateral limb (intact)	10% formalin	Histopathology of fibrosis markers, SUSI imaging to detect changes in tissue architecture	4
Injured limb (biopsy) Contralateral limb (biopsy)	RNA Later	Fibrotic gene expression	4
Injured limb (biopsy) Contralateral limb (biopsy)	Snap frozen in liquid N_2	Proteomics to validate mTOR and Hif1α inhibition (p-4EBP1, Akt, pS6K)	4
Whole Blood for Plasma	K2-EDTA tubes	Circulating proteomics (cytokines, chemokines, growth factors), CBC	8

To determine the impact of drug interventions on early attenuation of fibrotic cell signaling on crush-incisional wound healing, we first conducted an extensive literature search on cellular pathways and key proteins related to muscle fibrosis. We worked with Bio-Rad to design low-density custom array plates, comprising of custom primers for 41 target genes **(Table 4)**, 2 housekeeping genes (*Hprt* and *PP1A*), and 5 assay controls specific to rats. We have since ordered and received 15 plates needed to run all samples and have begun the RNA isolation and sample preparation.

Table 4. List of target genes on the low-density array to assess the effect of drugs on attenuation of muscle fibrosis.

Adm	Agt	Akt1	Akt2	Akt3	Atf3	Bad	Bmp7
Ccl11	Ccl12	Ccl3	Cebp	Col1a2	Col3a1	Ctgf	Cxcr4
Il10	Il6	Jun	Mapk1	Mapk3	Mmp14	Mmp1a	Mmp8
Mmp9	Myc	Myd88	Nfkb1	Pdgfa	Pdgfb	Smad2	Smad3
Smad7	Smad4	Tgfb1	Tgfb2	Tgfb3	Tgfbr2	Tlr9	Vegfa
Mmp13							

2b. Rat Muscle Crush Injury Complete Blood Count Analysis Following Hif1α Inhibitor Treatment

Complete blood count analysis using whole blood demonstrated a significant increase in the frequency of white blood cells (WBCs) and lymphocytes for both carrier and rapamycin groups between post-operative day (POD) POD7 and POD14 **(Fig. 6A and D)**. At POD14, the frequency of WBCs was significantly higher in the rapamycin group when compared to Amphotericin treated group, while the lymphocyte frequency at POD14 was significantly higher in the carrier treated group when compared to rapamycin treated group. Circulating levels of neutrophils and monocytes were significantly higher at POD7 and POD14 (p < 0.5) and remained elevated at POD-28 in the rapamycin group **(Fig. 6B-C)**. There was a significant increase in the red blood cell (RBC) count at POD14, only with rapamycin treatment **(Fig. 6E)**, while platelet counts increased significantly at POD7 and POD14 only in the carrier group **(Fig. 6F)**.

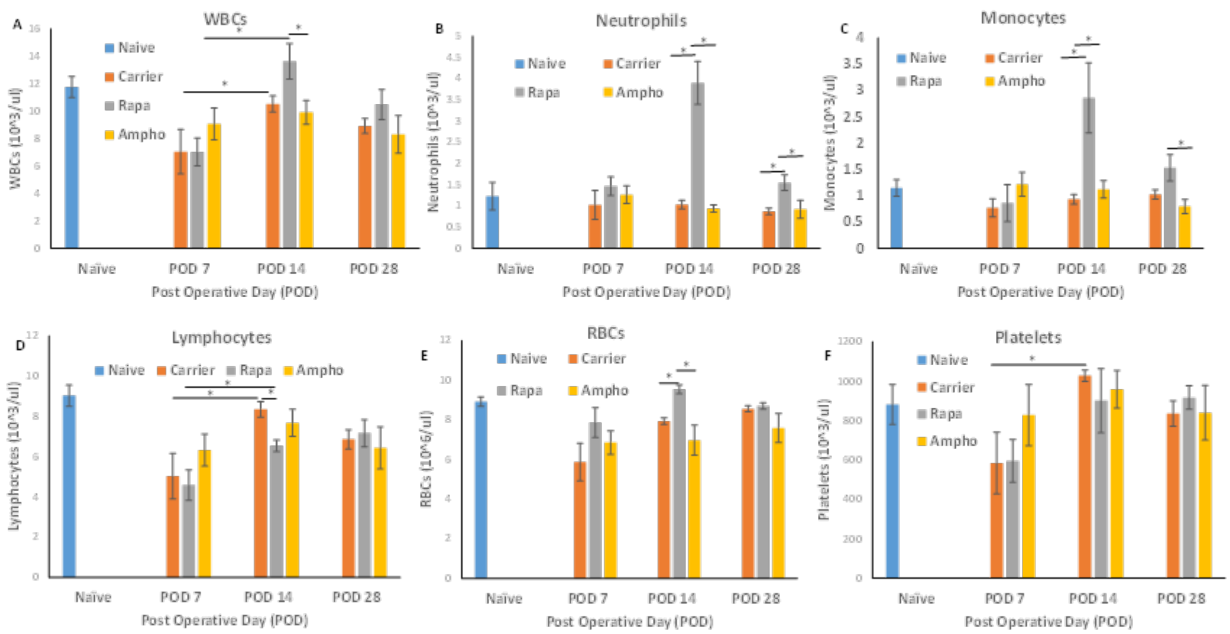


Figure. 6. Circulating leukocyte counts following injury and drug treatment. A) White blood cells (WBCs), B) Neutrophils C) Monocytes D) Lymphocytes E) RBCs and F) Platelets at different time points post treatment with carrier, rapamycin or Amphotericin B. Values are shown as mean±SEM. * p-value<0.05 using Student's t-test.

These findings suggest low dose rapamycin treatment following traumatic extremity injury in the rat results in marked increases in circulating cells of the innate immune system (leukocytosis) dominated by neutrophils (neutrophilia) and monocytes (monocytosis). We will next investigate how these changes in hematological parameters correlate with the development or attenuation of fibrosis, based on histological and gene expression data.

2b. Gene expression in rat muscle tissue post crush-incisional injury

Next, gene expression studies in rat muscle tissue following crush injury were performed to assess the impact of drug interventions on signaling pathways involved in fibrosis, cell death/survival, metabolism, ECM remodeling, tissue repair and regeneration.

Injured quadriceps muscle was harvested from the treatment groups at different time points post crush-incisional muscle injury, as detailed in the **Table 5** below.

Total RNA was isolated from the harvested muscle using RNeasy mini kit (Qiagen) and first strand cDNA was generated from 1 ug total RNA using the Reaction Ready First Strand cDNA synthesis kit (Applied Biosciences, Frederick, MD). We worked with Bio-Rad to design custom low-density array plates, comprising of custom primers for 41 target genes, as detailed in the previous report, 2 housekeeping genes (Hprt and PP1A), and 5 assay controls specific to rats. The target genes can be classified into 4 broad categories: **A.** signaling pathways involved in fibrosis and epithelial-mesenchymal transition (TGFβ-related), osteo-chondrogenesis (BMP-related), adipogenesis, angiogenesis (CEBP, VEGF) **B.** cell metabolism, survival, stress and cell death specific genes **C.** cytokines and chemokines **D.** extracellular matrix (ECM) and ECM modulators.

RT-PCR was conducted using the QuantStudio 7Pro Real-Time PCR System (Rockville, MD) and the ΔCT method was used to assess the gene expression between naïve and treated conditions with hypoxanthine-guanine phosphoribosyltransferase (Hprt) as the housekeeping gene.

Table 5. Experimental design for testing the effects of rapamycin and Amphotericin B on muscle fibrosis.

Treatment groups	**Number of animals**		
Injured no treatment **(IN)**	8		
Injured + treatment	**POD* 7**	**POD 14**	**POD 28**
Rapamycin alone **(R, 2.5 mg/kg, daily i.p for 14 days)**	8	8	8
Amphotericin alone **(A, 2 mg/kg, daily i.p for 10 days)**	8	8	8
Vehicle Control **(VC, 3% DMSO in saline)**	8	8	8
Naive			
Total animal number	**24**	**24**	**24**

**POD: Post-Operative Day.*

Analysis of gene expression changes at the three time points, normalized to naïve tissue demonstrated no significant changes between the treatment groups in most of the gene categories at POD 7. However, there was a marked transcriptional suppressive effect on the majority of the target genes at POD 14, exclusively in the rapamycin treatment group, as shown in **Figures 7-9**. This was clearly reflected in the downregulation in the 'Rapa group', of TGF-β pathway mediators (**Figure 7**), a pathway classically known to be involved in initiation and development of muscle fibrosis. This same trend was also seen in differentiation and stem cell related pathways (**Figure 8A),** cytokines and immune related genes (**Figure 8B**), metabolic and cell death / stress related pathways (**Figure 9A**), and ECM-ECM modulatory genes (**Figure 9B**).

While the carrier group did not show any transcriptional suppression at POD 14, the Amphotericin B treated group demonstrated upregulation of multiple TGF-β pathway mediators and cell death genes, indicating that at the dosage used, Amphotericin B did not have an optimum therapeutic efficacy. By POD 28, the transcriptional suppression for most of the genes was overcome in the rapamycin group. Importantly, there was downregulation of several TGF-β pathway mediators (Smads 2, 3, 4, 7, TGF-β1 and TGF-βr2), osteogenic gene, Bmp7 and immune mediators (Ccl11, IL6, Myd88) at POD 28 for the Amphotericin treated group. Majority of the genes in the carrier group did not demonstrate significant changes in expression between POD 14 and 28. **Based on these results, the second week of low dose rapamycin treatment appears to induce a pan-transcriptional suppression, without any associated adverse events.**

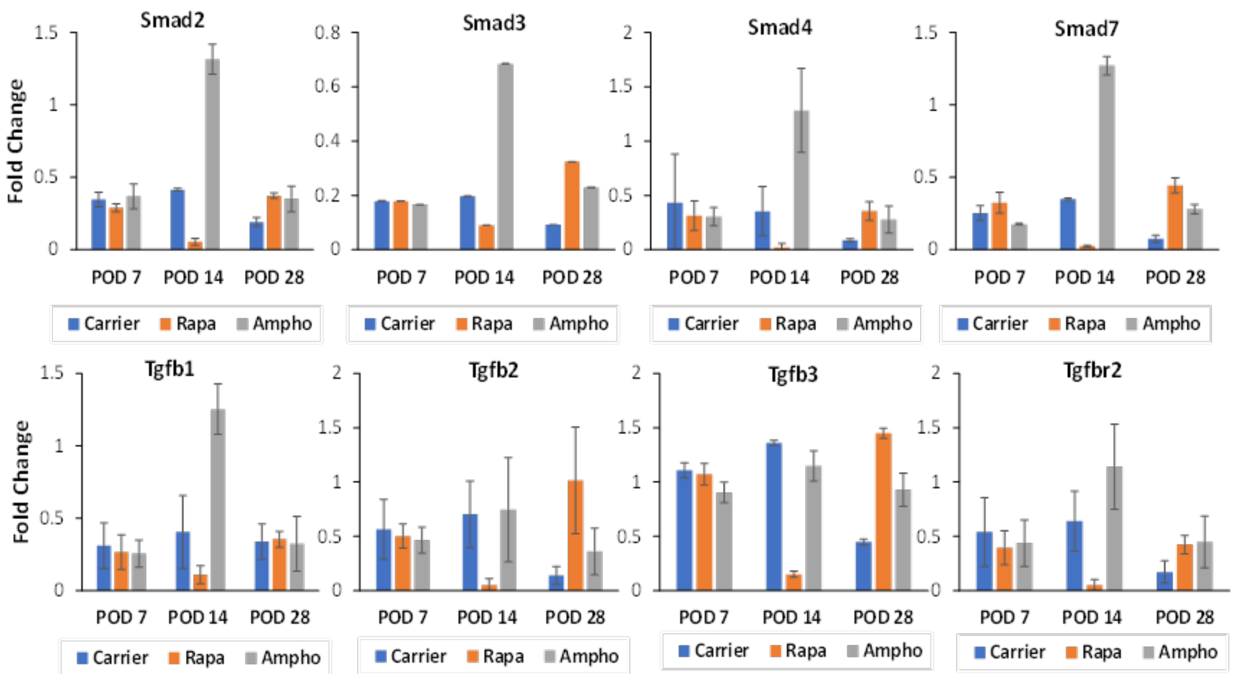


Figure 7. Gene expression analysis of TGFβ pathway members at different timepoints post treatment with either rapamycin, Amphotericin B or carrier post crush-incisional injury in a rat model

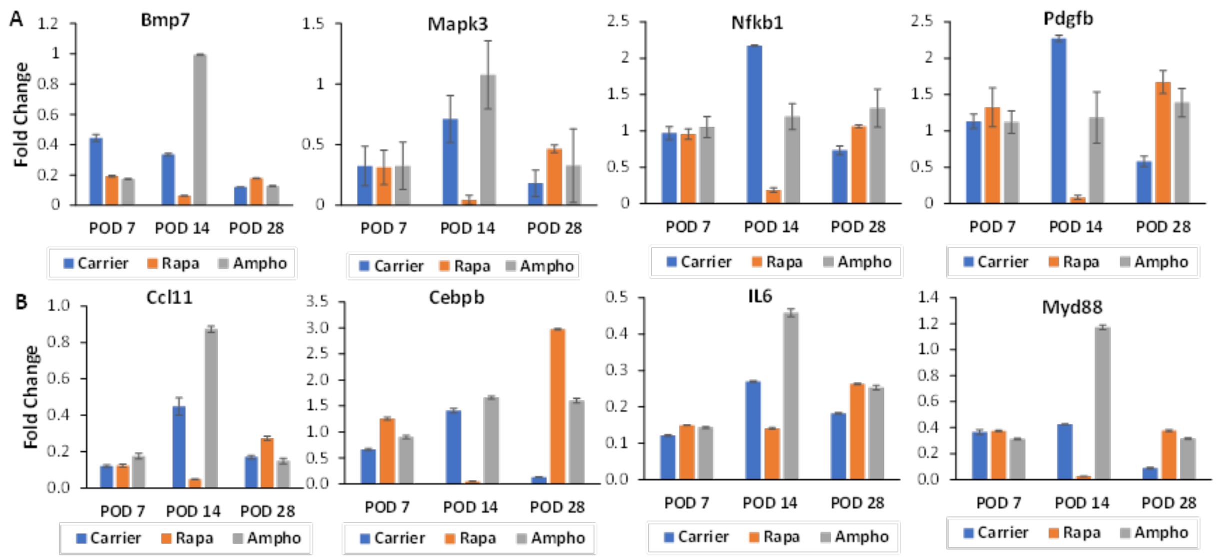


Figure 8. Gene expression analysis of A. osteogenic and stem cell-associated pathways and B. Cytokines and immune cell-specific molecules at different time points post treatment with either rapamycin, Amphotericin B or carrier post crush-incisional injury.

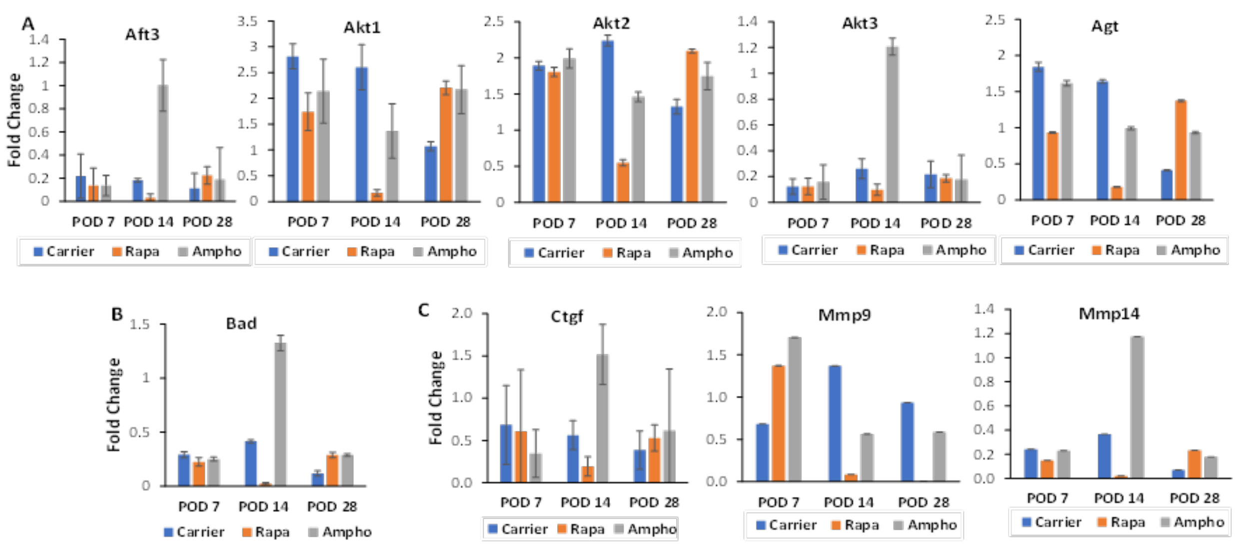


Figure 9. Gene expression analysis of A. cell-stress and metabolic pathways B. pro-apoptotic pathway and C. ECM and ECM modulators at different time points post treatment with either rapamycin, Amphotericin B or carrier post crush-incisional injury.

Key Findings or Accomplishments for Specific Aim 2

First, we have developed a combat relevant model of muscle crush injury in the rat. Next, we treated rats post-muscle crush injury with rapamycin and Amphotericin B to assess the complete

blood count and gene expression. Interestingly, we found that low dose rapamycin treatment following traumatic extremity injury in the rat results in marked increases in circulating cells of the innate immune system (leukocytosis) dominated by neutrophils (neutrophilia) and monocytes (monocytosis). Gene expression analyses were also performed on injured muscle treated with rapamycin, amphotericin and carrier. In the rapamycin group, a marked transcriptional suppressive effect on the majority of the target genes analyzed was noted compared to other treatments. This included genes of the TGF-β pathway mediators, a pathway classically known to be involved in initiation and development of muscle fibrosis. This same trend was also seen in differentiation and stem cell related pathways, cytokines and immune related genes, metabolic and cell death / stress related pathways, and ECM-ECM modulatory genes. These gene expression patterns support our hypothesis that rapamycin treatment will decrease traumatic muscle fibrosis. Further analyses of muscle fibrosis are currently in progress.
Next, limbs post-muscle crush injury will be imaged using SUSI to detect muscle fibrosis and histologically stained to assess for evidence of fibrosis using picrosirius red and Masson's trichrome stains.

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What opportunities for training and professional development has the project provided?

This project has provided training in animal research and handling methods, laboratory techniques, data analysis, and project management skills for the post-doctoral fellows and research personnel that have worked on this project.

How were the results disseminated to communities of interest?

Data from this project was shared with colleagues in orthopaedic surgery, trauma surgery, immunology, cell and developmental biology, biomedical engineering, and other applicable fields through research presentations, research collaboration meetings, and joint laboratory meetings. This work has been published as an abstract at the Military Health Science Research Symposium 2020 annual meeting. Work related to this project has also been submitted to the 2021 annual meeting of the Orthopaedic Research Society.

What do you plan to do during the next reporting period to accomplish the goals?

During the next reporting period, SUSI imaging and analysis will be completed for the muscle crush studies in the rat model. These limbs will also be dissected, processed, cryo-sectioned, and histologically stained to assess for fibrosis. A model of traumatic muscle crush injury will also be performed in a mouse model and assessed for fibrosis using SUSI and histology. Animals treated with rapamycin, Amphotericin B, or vehicle control will be compared to assess for changes in muscle fibrosis.

4. IMPACT:

What was the impact on the development of the principal discipline(s) of the project?

Currently, ectopic bone formation is not detected early enough for effective treatment. Here, we have shown that high frequency spectral ultrasound imaging (SUSI) technology can be used to detect heterotopic ossification in two models of post-traumatic heterotopic ossification (HO). This technology calculates tissue specific parameters that are inherent to the tissue to reduce user bias. This technology will allow for timely treatment in order to arrest HO development and to identify patients that need to be treated. Additionally, we have shown that 14-day treatment regime of rapamycin in the rat model of combat-related traumatic HO formation is adequate to decrease total HO formation.

What was the impact on other disciplines?

These findings will also have impacts on development of medical imaging technology and the use of rapamycin and Amphotericin B as Hif1α inhibitors for other indications.

What was the impact on technology transfer?

These findings may lead to the use of a novel ultrasound imaging system for clinical detection of early heterotopic ossification.

What was the impact on society beyond science and technology?

Nothing to report.

5. **CHANGES/PROBLEMS:** The Project Director/Principal Investigator (PD/PI) is reminded that the recipient organization is required to obtain prior written approval from the awarding agency Grants Officer whenever there are significant changes in the project or its direction. If not previously reported in writing, provide the following additional information or state, "Nothing to Report," if applicable:

Changes in approach and reasons for change

None

Actual or anticipated problems or delays and actions or plans to resolve them

Dr. Levi accepted a new position as the endowed Lee-Hudson Professor with tenure at the University of Texas Southwestern Medical Center (UTSW) as well as the Director of the Center for Organogenesis and Trauma. The award was relinquished by the University of Michigan on June 9, 2020. UTSW is awaiting an invitation to transfer the award. Additionally, labs at the University of Michigan and Uniformed Services University of the Health Sciences were shut down during 03/2020 – relinquishment due to the COVID-19 pandemic. The inability to perform wet laboratory research during this shutdown significantly impacted new data generation for this project during this time period. However, researchers continued to analyze data collected for this project prior to shut down remotely. During this time, abstracts and manuscripts based on this project were also been prepared and/or submitted for peer review.

Changes that had a significant impact on expenditures

None.

Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents

Significant changes in use or care of human subjects

Human subjects are not included in this project.

Significant changes in use or care of vertebrate animals.

None.

Significant changes in use of biohazards and/or select agents

None.

6. **PRODUCTS:**

- **Publications, conference papers, and presentations**

  Journal publications.

- “Novel Ultrasound Imaging Method for Early Identification of Combat Trauma-Induced Heterotopic Ossification” *Abstract accepted at Military Health Science Research Symposium 2020*
- “Novel FDA Approved Therapy to Mitigate Ischemia-Reperfusion Induced Fibrosis” *Abstract accepted at Military Health Science Research Symposium 2020*
- Edwards NJ, Hwang C, Marini S, et al. The role of neutrophil extracellular traps and TLR signaling in skeletal muscle ischemia reperfusion injury. *The FASEB Journal*. 2020 *In Press*
- “High Frequency Spectral Ultrasound Imaging Detects Early Post Traumatic Heterotopic Ossification in Rodents” *Abstract submitted to Orthopaedic Research Society 2021 Annual Meeting*
- “High Frequency Ultrasound Imaging Detects Early Post Traumatic Heterotopic Ossification in Rodents” *Original research article in Preparation for peer reviewed journal submission*
- Stepien DM, Hwang, C, Marini, S, et al. Tuning Macrophage Phenotype to Mitigate Skeletal Muscle Fibrosis. *J Immunology.* 2020 (204)8:2203-2215. DOI: 10.4049/jimmunol.1900814

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**

This research has developed a novel ultrasound imaging system and analysis methods that will be shared with colleagues via scientific presentations and publications.

- **Inventions, patent applications, and/or licenses**

Nothing to report.

- **Other Products**

Nothing to report.

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project?

Name:	Benjamin Levi
Project Role:	PI
Researcher Identifier (e.g. ORCID ID):	NA

Nearest person month worked: 1
Contribution to Project: Planning of experimentation.

Name: Nicole Edwards
Project Role: Post-doctoral fellow
Researcher Identifier (e.g. ORCID ID): NA
Nearest person month worked: 1-No effort on project as she is being covered by a T32 training grant.
Contribution to Project: Dr. Edwards has been the project lead planning of experimentation.

Name: Kaetlin Vasquez
Project Role: Animal and lab manager
Researcher Identifier (e.g. ORCID ID): NA
Nearest person month worked: 1
Contribution to Project: Assist in coordination of project and regulatory documentation.

Name: Shuli Li
Project Role: Animal and lab manager
Researcher Identifier (e.g. ORCID ID): NA
Nearest person month worked: 3
Contribution to Project: Assist in planning of experimentation and coordination of project.

Name: Thomas Davis
Project Role: Co-PI
Researcher Identifier (e.g. ORCID ID): NA
Nearest person month worked: 1
Contribution to Project: Planning of experimentation.

Name: Devaveena Dey
Project Role: Staff scientist
Researcher Identifier (e.g. ORCID ID): NA
Nearest person month worked: 1
Contribution to Project: Planning of experimentation.

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

Previous awards which closed since the last report:

1.Source: NIH K08-GM109105 (Levi, PI)
Project Title: Novel Pathway and Prevention Strategy for Heterotopic Ossification
Effort: 9 Cal. Months
Project Period: 8/1/2014 – 7/31/2018

2.Source: DoD/Henry M. Jackson Foundation (Forsberg, PI, Levi, Subk-PI)
Project Title: Novel Therapeutic Strategy Targeting Bone Morphogenetic Protein Receptor 1 Signaling to
Prevent Trauma Induced Heterotopic Ossification
Effort: 0.60 Cal. Months (Effort is concurrent with K-award through 7/31/2018)

Project Period: 9/1/2015 – 8/31/2018

3.Source: Department of Defense (Levi, PI)
Project Title: Novel Therapeutic Small Molecule Strategy Targeting TAK1 Signaling to Prevent Upper
Extremity Heterotopic Ossification –OR150139
Effort: 0.90 Cal. Months (Effort is concurrent with K-award through 7/31/2018)

Project Period: 10/1/2016 – 09/30/2018

4.Source: Department of Defense (Wang, PI, Levi, Co-I)
Project Title: Apyrase: A Portable Treatment to Prevent Burn Progression and Infection – MB150237
Effort: 0.45 Calendar Months (Effort is concurrent with Kaward through 7/31/2018)

Project Period: 10/1/2016 – 9/30/2019

5. Source: DODW81XWH-16-PRORP-ARA (PI: Levi)
Project Title: Targeting Neutrophil Extracellular Traps to Mitigate Post-Traumatic Lower Extremity Soft
Tissue Injury
Effort: 1.2 Calendar Months (Effort is concurrent with K-award through 7/31/2018)

Project Period: 09/30/2017 – 9/29/2019

Active awards since last report:

1. (this award) Source: Dept. of Defense W81XWH-18-1-0653 (PI: Levi)

Project Title: Diagnostic and therapeutic strategy to prevent trauma induced upper extremity muscle fibrosis and heterotopic ossification – OR170174
Effort: 0.60 Calendar Months

Project Period: 09/30/2018-09/29/2020

2. Source: Dept. of Defense/Massachusetts General Hospital W81XWH-18-1-0608 (PI: Friedstat; Co-I: Levi) Project Title: A Within Scar, Randomized Control Trial Comparing Fractional Ablative Carbon Dioxide Laser to Non-Energy Based, Mechanical Tissue Extraction and No Treatment – MB170043
Effort: 0.39 Calendar Months

Project Period: 09/30/2018 – 09/29/2021

3. Source: Dept. of Defense W81XWH-18-2-0038 (PI: Levi)
Project Title: Continuous, Portable, Non-Perfusion Based 'Short Wave Assessment Tool' (SWAT) Improves Burn Care – MB170041
Effort: 0.60 Calendar Months

Project Period: 09/01/2018 – 08/31/2021

4. Source: International FOP Association (Co-PIs: Levi/Huber)
Project Title: Neutrophil based translational therapies for fibrodysplasia ossificans progressiva Effort: 0 Calendar Months (contributed)

Project Period: 01/01/2020 – 12/31/2020

5. Source: Dept. of Defense CDMRP PRORP-ARA (PI: Levi)
Project Title: Therapeutic small molecule and timed limb stabilization strategies to prevent complications of extremity trauma and enhance return to duty (OR190048)
Effort: 1.2 Calendar Months

Project Period: 09/30/2020 – 09/29/2022

6. Source: Dept. of Defense CDMRP PRORP-ARA / subcontract to Johns Hopkins Univ (PI: James)
Project Title: Improving tendon repair via sensory nerve NGF-TrkA signaling for return to duty (OR190116) Effort: 1.2 Calendar Month
Project Period: 09/30/2020 – 09/29/2022

7. Source: National Institutes of Health/National Institute of General Medicinal Sciences R61 AR078072 (PI: Levi)
Project Title: Neutrophil Biomarker and neutrophil targeted therapy to predict and prevent heterotopic ossification
Effort: 1.8 CM

Project Period: 07/01/2020 – 06/30/2023

What other organizations were involved as partners?

Nothing to report.

8. SPECIAL REPORTING REQUIREMENTS

COLLABORATIVE AWARDS:

QUAD CHARTS:

Diagnostic and therapeutic strategy to prevent trauma induced upper extremity muscle fibrosis and heterotopic ossification

PI: Benjamin Levi **Org: University of Michigan, Ann Arbor, MI**
Contract Investigator: Thomas Davis PhD **Org:** Uniformed Services University of the Health Sciences **Award Amount:** $750,000

Study/Product Aim(s)

Primary Goal: The goals of this grant are to establish the efficacy of hypoxic signaling inhibition for heterotopic ossification (HO) and muscle fibrosis prevention, and to optimize these therapies using a non-invasive, clinically translatable diagnostic method to precisely identify the optimal timing and duration of treatment.

Research Idea: In this grant, we will demonstrate the ability of two therapeutics that target mTOR/HIF-1α signaling to prevent muscle fibrosis, HO and subsequent joint contractures. We will identify the optimal timing and deploy these image guided therapies after high risk injuries for to maximize prophylaxis and limit delayed wound healing.

Approach

- **Specific aim 1:** To validate timed, image guided prevention of heterotopic ossification with FDA approved inhibitors of hypoxic signaling.
- **Specific aim 2:** To mitigate post-traumatic muscle fibrosis through timed inhibition of mTOR/HIF-1α signaling with Amphotericin and Rapamycin separately or as combined therapy.

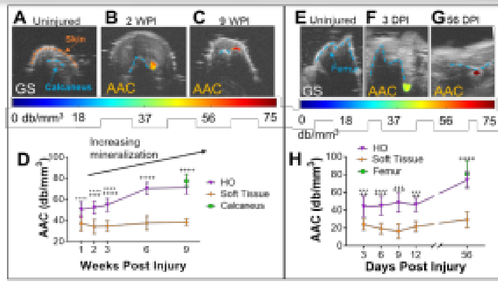

Figure 1: High frequency spectral ultrasound imaging (SUSI) distinguishes HO from surrounding injured soft tissue and native bone: A) Grayscale (GS) image of uninjured mouse. B-C) Average acoustic concentration (AAC) overlayed on GS of HO lesions at 2, 9 weeks post injury (WPI) in mouse model. D) AAC of HO lesions, surrounding soft tissue, and calcaneus at 1-9 WPI. E) GS image of uninjured amputation site in rat model. F-G) AAC overlayed on GS of HO lesions in rat model at 3, 56 days post injury (DPI). H) AAC of HO lesions, surrounding soft tissue, and femur at 3-56 PDI. Blue dashes indicate native bone. +/* denotes statistical difference compared to soft tissue and calcaneus or femur, respectively. ++++/**** $p<0.0001$; +++/*** $p<0.0002$; ++ $p<0.002$.

Timeline and Cost

Activities CY	18-19	19-20
Validate timed, ultrasound guided delivery of mTOR/HIF-1α inhibitors to mitigate post-traumatic HO.	■■■■	■■
Demonstrate inhibitory efficacy of Amphotericin b and Rapamycin on muscle fibrosis.	■	■■■■
Verify efficacy and minimal toxicity of treatment strategies.	■	■■■■
Requested Budget ($750,000)	**$371,000**	**$379,000**

Goals/Milestones

CY18-19 Goals

- ❑We will use our Burn/tenotomy and Blast/Amputation/MRSA-infection rodent models to validate the effect of image guided mTOR/HIF-1α inhibitors (Rapamycin and Amphotericin) on HO *in vivo*. (USUHS, UM)
- ❑Demonstrate that Rapamycin or Amphotericin B delivered during abbreviated periods of time, or guided by spectral ultrasound (SUSI) minimizes treatment duration and off target effects. (USUHS,UM)

CY19-20 Goals

- ❑Demonstrate that our treatment strategy does not alter wound healing or cause off target toxicity
- ❑We will demonstrate that inhibitors of mTOR/HIF-1α (Rapamycin and Amphotericin) mitigate post-injury muscle fibrosis using proven animal models. (USUHS,UM)

Comments/Challenges/Issues/Concerns: N/A

Budget Expenditure to Date

Projected Expenditure: $750,000

Actual Expenditure: UM $304,725