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TITLE: Evaluation of a Novel Dual Antifibrotic and Proregenerative Strategy to Facilitate Improved Functional Outcomes in the Treatment of Volumetric Muscle Loss

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CONTRACTING ORGANIZATION: The Henry M. Jackson Foundation for the Advancement of Military Medicine Inc.
Bethesda, MD

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13. SUPPLEMENTARY NOTES

14. ABSTRACT

The overall objective of the proposed work is to evaluate the safety and efficacy of administration of mCAR513 for: (1) mitigating the fibrotic wound healing response to volumetric muscle loss (VML), and (2) to enhance the efficacy of leading, near term regenerative therapies in promoting reconstitution of skeletal muscle volume and end-organ neuro-musculoskeletal function following VML injury thereby facilitating improved outcomes, namely sustained readiness and lethality of the fighting force. During this reporting period, we encountered unexpected issues in accessing mCAR513 due to supply chain issues as a result of the COVID-19 pandemic. In response, we are pursuing alternative options to replace mCAR513 as the antifibrotic intervention for this proposal. These studies are currently ongoing and expected to be completed early next year. We have included preliminary results in this report.

15. SUBJECT TERMS
None listed.

16. SECURITY CLASSIFICATION OF:			**17. LIMITATION OF ABSTRACT**	**18. NUMBER OF PAGES**	**19a. NAME OF RESPONSIBLE PERSON**
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1. INTRODUCTION:

Primary Goal & Overarching Hypothesis:

An overarching objective of our research program is to develop individualized, patient-specific (i.e., personalized medicine) therapies capable of providing the highest level of functional performance and optimal QoL for SMs and veterans with traumatic extremity injuries. However, before this can be achieved, numerous aspects of those injuries, including VML injuries, require further investigation. Thus, pre-clinical studies involving a dual anti-fibrotic and pro-regenerative therapeutic, which seek to elucidate these key questions, particularly those, focused on VML injuries, are crucial to understanding the fundamental mechanisms related to the interplay between anti-fibrosis and tissue regeneration. Thus, the primary goal of this study is to develop a dual anti-fibrotic and pro-regenerative treatment strategy, which facilitates optimal functional outcomes following VML injury. The proposed work will test the overarching hypothesis that optimization of duration and timing of administration of an anti-fibrotic will improve the efficacy of a comprehensive pro-regenerative medicine treatment strategy and thus facilitate improved skeletal muscle form and function compared to either the anti-fibrotic or pro-regenerative interventions in isolation.

2. KEYWORDS:

Volumetric Muscle Loss, Antifibrotic, Regenerative Therapy, Skeletal Muscle, Wound Healing

3. ACCOMPLISHMENTS:

- **What were the major goals of the project?**
    - Specific Aim 1: The *objective* of this aim is to determine if mCAR513 will enhance the efficacy with which regenerative medicine therapies mediate regeneration and functional restoration of a VML affected muscle. The *hypothesis* to be tested in this aim is that mCAR513 will promote an anti-fibrotic microenvironment within the VML wound bed thereby enhancing the efficacy regenerative medicine therapies resulting in a greater density of contractile tissue and improvements in functional capacity
    - Specific Aim 2: The objective of this aim is to determine the efficacy of a dual treatment strategy within the more challenging large animal model. The hypothesis to be tested in this aim is that an optimized dual treatment strategy will facilitate improved functional outcomes compared to either treatment in isolation. We will test this hypothesis using an established DoD relevant, large animal (porcine) model of VML with a well characterized fibrotic wound healing response that results in extensive collagen deposition that is representative of the human condition to evaluate functional outcomes following an optimized treatment strategy.

	Timeline	USUHS	Status
Specific Aim I: Characterize the spatiotemporal dynamics of skeletal muscle wound healing associated with administration of mCAR513 as an adjunct anti-fibrotic therapy to regenerative medicine treatment strategies for volumetric muscle loss.			
Sub-Aim 1A: Assess the ability of mCAR513 to dampen the fibrotic pathobiology associated with VML	**Months**	**POC**	**--**
Subtask 1A-1: Obtain IACUC & ACURO Approvals	1-3	Dearth	**Complete**
Subtask 1A-2: Perform Rodent Surgeries	3-12	Dearth / Goldman	**Ongoing**
Subtask: 1A-3: Perform In-vivo Functional Analyses	3-13	Dearth / Goldman	**Ongoing**
Subtask 1A-4: Perform Cellular, Molecular, & Histological Analyses	4-15	Dearth / Goldman	**Ongoing**
Subtask 1A-5: Data Reduction, Interpretation & Dissemination	6-16	Dearth / Goldman	**Ongoing**
Sub-Aim 1B: Evaluate the ability of mCAR513 to enhance the efficacy with which regenerative medicine therapies facilitate restoration of skeletal muscle form and function following VML	**Months**	**POC**	**--**
Subtask 1B-1: Obtain IACUC & ACURO Approvals	1-3	Dearth	**Pending**
Subtask 1B-2: Perform Rodent Surgeries	15-22	Dearth / Goldman	**Pending**
Subtask 1B-3: Perform In-vivo Functional Analyses	15-23	Dearth / Goldman	**Pending**
Subtask 1B-4: Perform Cellular, Molecular, & Histological Analyses	16-25	Dearth / Goldman	**Pending**
Subtask 4: Data Reduction, Interpretation & Dissemination	17-26	Dearth / Goldman	**Pending**
Milestone(s) Achieved: Determination of an optimized treatment strategy, consisting of an anti-fibrotic and pro-regenerative materials, which facilitates improved outcomes following VML injury.			

	Timeline	USUHS	Status
Specific Aim II: To characterize the ability of an optimized dual anti-fibrotic & pro-regenerative strategy to facilitate improved functional outcomes within a clinically relevant, large animal model of volumetric muscle loss			
Subtask 2-1: Obtain IACUC & ACURO Approvals	14-17	Dearth	**Pending**
Subtask 2-2: Perform Porcine Surgeries	25-30	Dearth / Goldman	**Pending**
Subtask 2-3: Perform In-vivo Functional Analyses	25-31	Dearth / Goldman	**Pending**
Subtask 2-4: Perform Cellular, Molecular, & Histological Analyses	28-35	Dearth / Goldman	**Pending**
Subtask 2-5: Data Reduction, Interpretation & Dissemination	30-36	Dearth / Goldman	**Pending**
Milestone(s) Achieved: Evaluation of the efficacy of an optimized combination therapy at facilitating the highest level of function following VML injury in a large animal model.			

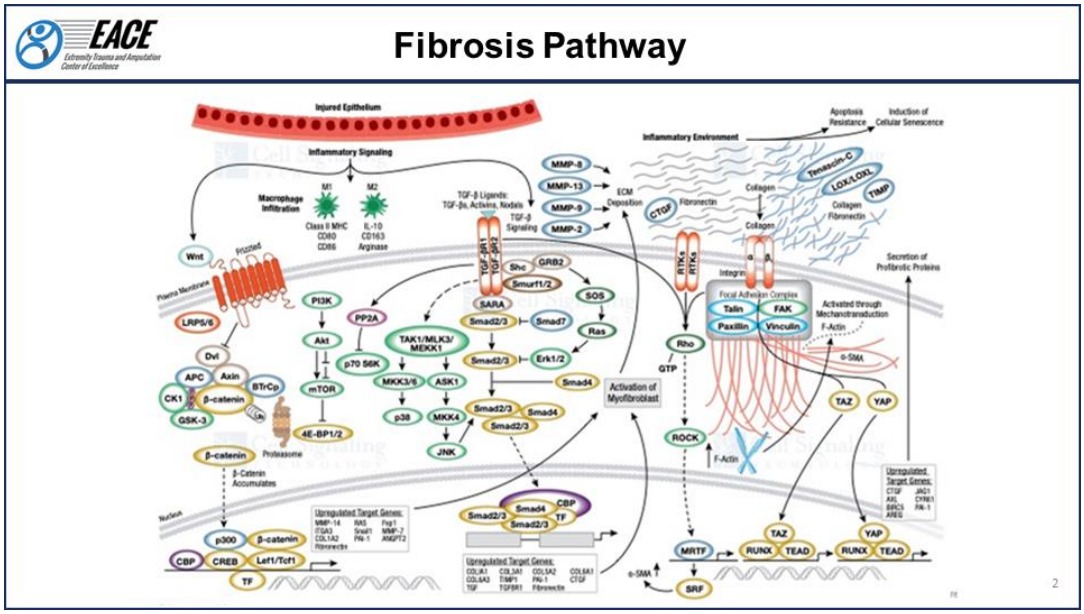
- **What was accomplished under these goals?**

  During the current reporting period (Yr1), the investigative team devoted significant effort towards several activities have been undertaken/completed towards study set up/initiation, including (but not limited to):

  - Internal study kickoff meeting completed
  - Award account set up at HJF
  - BPO account initiated at USU
  - IACUC protocol submitted/approved
  - Ordering of necessary supplies
  - Outreach to obtain mCAR513 from CALIBR/SCRIPPS

  Unfortunately, in the time that passed from when the proposal was written/funded to when the funding selection was made/award made, the investigative team is now unable to gain access to the proposed anti-fibrotic treatment – mCAR513. Specifically, the investigative team was able to virtually meet with the CALIBR/SCRIPPS team shortly after the award was issued to reacquaint everyone with the study and to discuss the amount, and associated timing, of mCAR513 needed for the study. However, following that initial meeting, the investigative team has since not been able to successfully contact the CALIBR/SCRIPPS team despite numerous outreach efforts. *Thus, in an unexpected turn of events the investigative team has not been able to acquire the mCAR513 material as previously anticipated, and thus the study needed to pivot to an alternative anti-fibrotic intervention.*

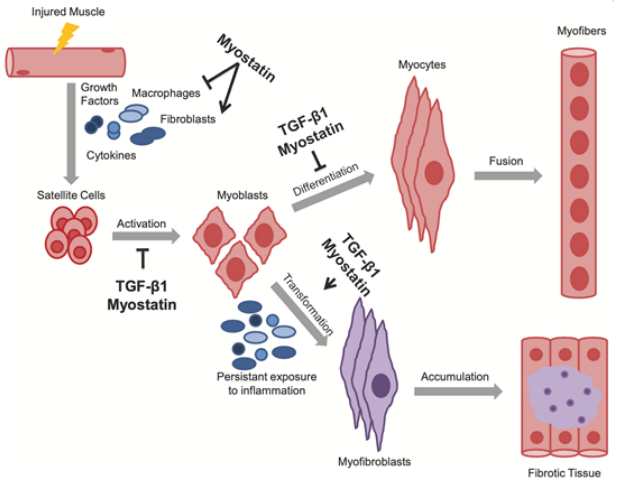
  To that end, the investigative team has conducted a thorough examination of the literature base to determine the best potential anti-fibrotic therapies to utilize. Particular emphasis was placed on the following topics: prior documented efficacy within skeletal muscle, and specifically VML; cost effectiveness; route of administration, clinical translation availability/potential, etc. The outcome of these analyses revealed agents which block TGF-β signaling as the primary target of opportunity. To that end, three candidates rose to the top – Nintedanib, Pirfenidone, & Saracatinib --- details of each are provided within the slides below.



TGF-beta Role in Skeletal Muscle Injury

In mature adult muscle, TGF-b negatively affects skeletal muscle regeneration by **inhibiting satellite cell proliferation**, **myofiber fusion**, and expression of some muscle-specific genes

Furthermore, TGF-b1 ***induces*** the **transformation of myogenic cells into fibrotic cells** after injury



https://skeletalmusclejournal.biomedcentral.com/articles/10.1186/2044-5040-1-19

Nintedanib

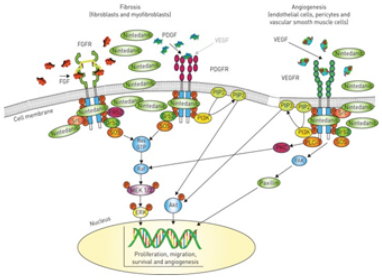
Pharmacological Mitigation of Fibrosis in a Porcine Model of VML Injury

Corona BT et al. Tiss Eng Part A. (2020)

Objective:
- Does Nintedanib mitigate fibrotic deposition in VML?

Method:
- Nintedanib (oral tablets twice daily) + VML + Pigs
- Timepoint = 4 weeks
- N=3 per group

Findings:
- Nintedanib reduced fibrosis in VML at 4 weeks
- Loss of muscle strength in Nintedanib-treated limbs compared to non-treated
- TGF-beta pathway limited upregulation in Nintedanib group



Pirfenidone

Pirfenidone
recombinant human protein

Mechanism of Action:
- TGF-beta1 antagonist

Web of Science Search:
- Pirfenidone AND skeletal muscle
- Results 3 publications

Dosing:
0.75 mg/kg/day

******FDA-approved medication******

Although the precise mechanism of action of pirfenidone and its specific molecular targets have yet to be elucidated[6,7], the molecule has demonstrated anti-fibrotic, anti-inflammatory, and antioxidant activity.[3,5] One vital anti-fibrotic mechanism involves suppression of TGF-β1 (transforming growth factor-β1), a key cytokine involved in fibrogenesis and extracellular matrix production.[3,4,5,6]

There is also evidence to suggest that pirfenidone has the ability to downregulate the expression of potent pro-inflammatory cytokines including TNF-α, interleukin-1, and interferon gamma.[3,5] In animal models, pirfenidone can inhibit both the influx of inflammatory cells and the increased pulmonary vascular permeability induced by bleomycin.[3]

MMP-9 mRNA were significantly elevated relative to control, although the response was muscle specific. Pirfenidone treatment resulted in a significant reduction in the level of hydroxyproline concentration across all muscles, although the effect was small. Results from this study reveal intrinsic dis-similarities in collagen metabolism between functionally different skeletal

glycolic Acid) (PLGA) Nanoparticles

Pirfenidone loaded PLGA nanoparticles

previously described [17]. Briefly, 50 mg PLG

a dose dependent manner. Pirfenidone could be loaded successfully within PLGA nanoparticles, which entered the corneal fibroblasts within 5 minutes. Pirfenidone nanoparticles but not free pirfenidone significantly ($P<0.05$) reduced collagen I level, corneal haze and the time for corneal re-epithelialization following alkali burn.

Saracatinib

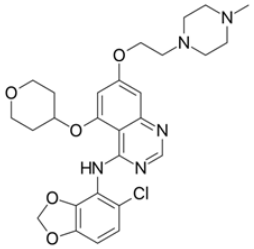
Saracatinib
Tyrosine Kinase Inhibitor

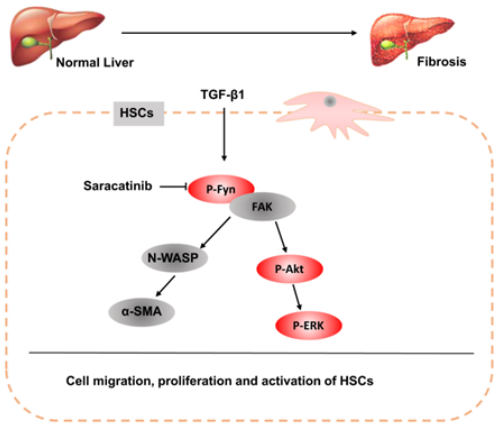
Mechanism of Action:
- SRC inhibitor

Solubility:
- DMSO = 200mg/mL
- Water = Insoluble

Web of Science Search:
- Saracatinib AND muscle injury
- Results 0 publications

Dosing:
- 3.0 mg/kg/day (~350g rat)





(right image) https://www.nature.com/articles/s41419-020-2229-2
(left image) https://en.wikipedia.org/wiki/Saracatinib

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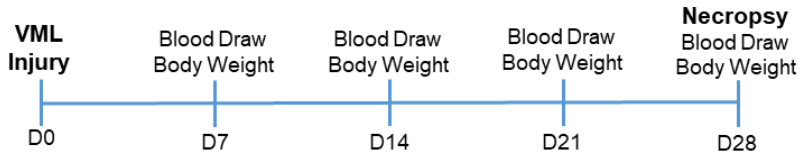
In an effort to avoid any further delays incurred by the study, the investigative team has: 1) generated and received approval for a modification of the USUHS IACUC to include the additional anti-fibrotic agents described above, and 2) initiated a pilot study to evaluate the effectiveness of the 3 aforementioned anti-fibrotic candidates at reducing fibrosis within a VML injury model. The slides below summarize these ongoing experiments.

Experimental Design

Study Timeline:



Groups:

1) Vehicle (10% DMSO + Saline) - **Complete**
2) Pirfenidone (0.75 mg/kg/day) - **Ongoing**
3) Nintedanib (2.7 mg/kg/day) - **Ongoing**
4) Saracatinib (3.0 mg/kg/day) - **Ongoing**

**All groups have a consistent cohort size of N=10*

Antifibrotic Analyses
Routine Histology: Hematoxylin & Eosin, Picrosirius Red, WGA
Weight Measurements: Kidney, Liver, Muscle, Body (over time)
Clinical Chemistry: Alb, Alp, Alt, Ast, Bun, Ca, Cl, Crea, ECO2, Glu, K+, Na+, Tbil, TP, Chol, Alkp, GGT, Phos, CK, Trig, LDH, dHDL, Amyl, Lip
Quantitative Histomorphometry: Percent Fibrotic Area, Mean Myofiber Diameter, Distribution of Myofiber Areas, Total Number of Myofibers
**Vehicle controls will be administered at equivalent volumes as the treatments* *Note: All groups will have a consistent cohort size (n=10).*

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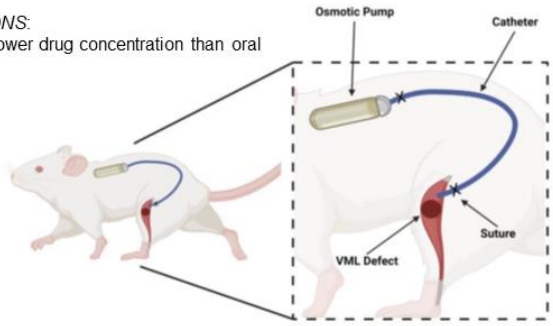
Experimental Design

Drug Delivery Method:

- Osmotic pump with catheter extension to VML injury

PROS:
- Local continuous drug delivery
- Eliminates repeat administration
- Enables higher throughput

CONS:
- Lower drug concentration than oral



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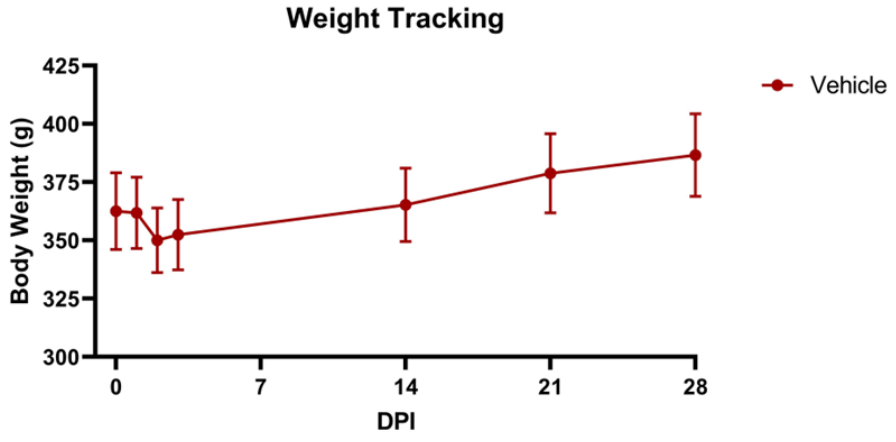
Preliminary Results

Body Weights Tracking up to 28 DPI



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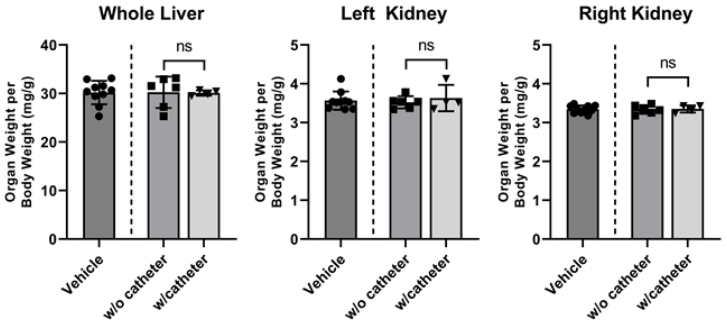
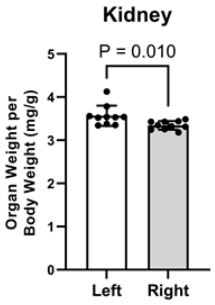
Organ Weight Evaluation





No differences in liver or kidney weights between subset data.

Left kidneys are significantly ***heavier*** than right kidneys.

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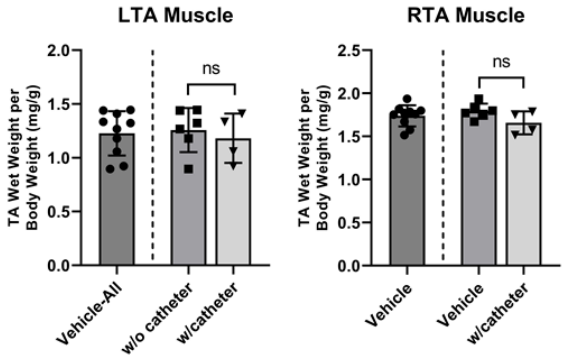
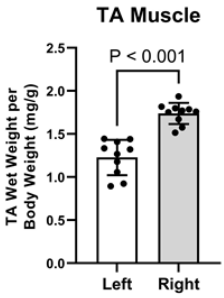
Muscle Weight Evaluation





No differences in right or left TA weights between subset data.

Left TAs are significantly ***smaller*** than right TAs.

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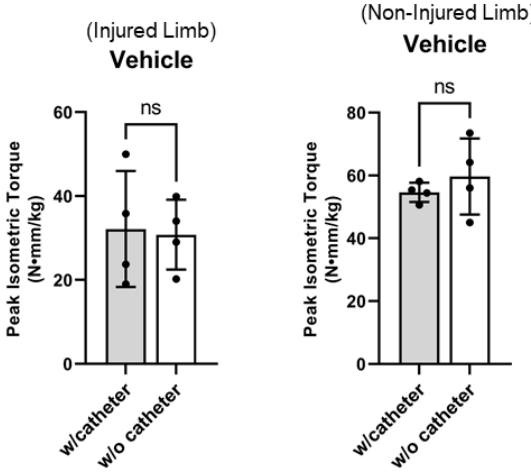
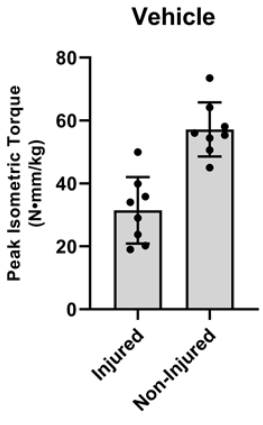
Muscle Function





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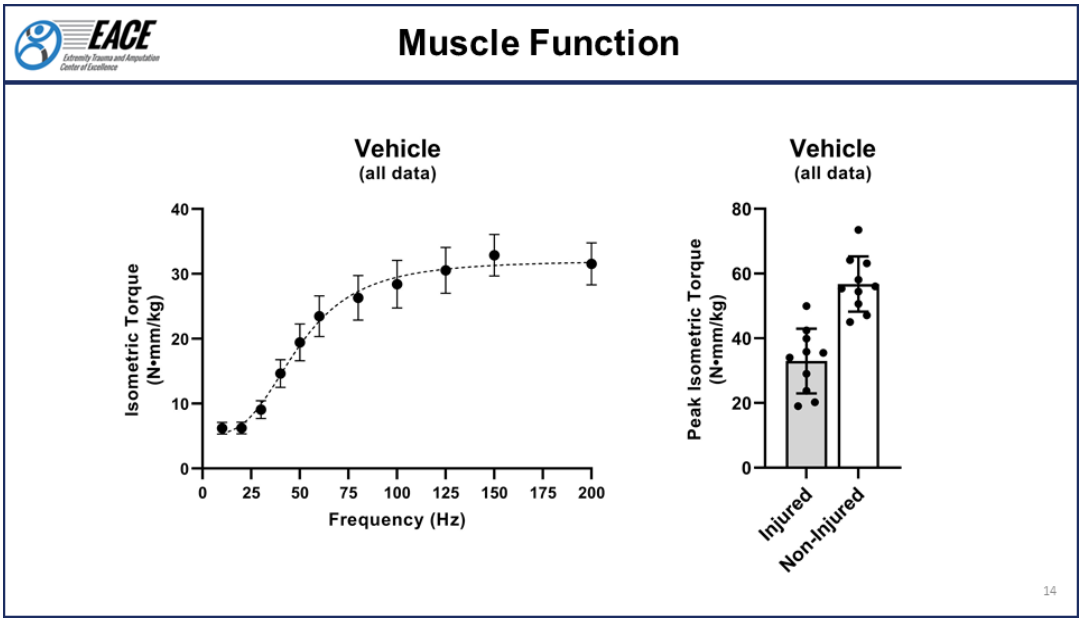

- **What opportunities for training and professional development has the project provided?**
    - While the overarching intent of this award is not to necessarily facilitate professional development activities, to date, several trainees were provided exposure to this project as part of their development activities. Furthermore, we anticipate that additional opportunities will be made available in the next reporting period.
- **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 accomplish the items listed below:
        - Complete the evaluation of the potential anti-fibrotic alternatives
        - Complete all subtasks within Specific Aim 1

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:**

  - We are currently in pursuit of finding an alternative antifibrotic from the original proposal with mCAR513, due to unforeseen issues in acquiring the material in a timely fashion. Thus, we are evaluating alternative antifibrotics (pirfenidone, Nintedanib, saracatinib) that will elicit the anticipated antifibrotic effects of mCAR513.

- **Actual problems or delays and actions to resolve them:**

  - This research project has incurred unanticipated delays due to the COVID-19 pandemic. The investigative team has taken numerous actions to mitigate/resolve the deleterious effects associated with the COVID-19 pandemic, including supply chain issues.

  - Additionally, delays have incurred due to difficulties in obtaining the original antifibrotic intervention (i.e., mCAR513). The investigative team has taken extensive action to identify an alternative (i.e., Pirfenidone, Saracatinib, Nintedanib), gained regulatory approval to do so, and have ongoing rodent experiments to evaluate their efficacy of reducing fibrosis.

- **Anticipated Problems/Issues**

  - Assuming there are no additional COVID-19 associated restrictions/supply chain issues, and/or any additional unexpected scientific outcomes, we do not anticipate any additional problems/issues for this project.

- **Changes that had a significant impact on expenditures:**

  - Nothing to report

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

  - Nothing to report

6. PRODUCTS:

- **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:**
    - Nothing to Report

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS:

Name:	Christopher L. Dearth, PhD
Project Role:	Principal Investigator
Nearest person month worked:	1
Contribution to project:	Dr. Dearth led the coordination of all aspects of the project related activities, including: budgetary management, personnel management, regulatory (IACUC) approvals, and experimental/laboratory activities, among others.
Name:	Stephen M. Goldman, PhD
Project Role:	Co-Investigator
Nearest person month worked:	1
Contribution to project:	Dr. Goldman assisted Dr. Dearth with project related activities, including: personnel management, regulatory (IACUC) approvals, and experimental/laboratory activities.
Name:	Jessica M. Motherwell, PhD
Project Role:	Co-Investigator
Nearest person month worked:	1
Contribution to project:	Dr. Motherwell assisted Dr. Dearth with project related activities, including: regulatory (IACUC) approvals, and experimental/laboratory activities.
***Note: Drs. Dearth, Goldman & Motherwell are GS DoD employees (part of the EACE), thus their efforts are at no cost to the award.	

- **Has there been a change in 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?**

- Nothing to report

8. SPECIAL REPORTING REQUIREMENTS:

- **COLLABORATIVE AWARDS:**
  - Nothing to report
- **QUAD CHARTS:**
  - N/A

9. APPENDICES: