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TITLE: Developing a Novel Therapy for Rhabdomyolysis

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14. ABSTRACT

Purpose: The purpose of this project is to determine the optimal dose and timing of cilastatin therapy to determine strategies to mitigate acute kidney injury (AKI), and to determine whether or not the pragmatic administration of cilastatin prevents rhabdomyolysis induced AKI (rhAKI), hyperkalemia, and mortality caused by a combat-relevant model of severe musculoskeletal trauma.

Scope: To determine the optimal dose and timing of cilastatin administration and strategies to mitigate rhAKI, we will use the glycerol injection model of rhabdomyolysis to determine the dose response relationship and assess cilastatin's protective effect. We will test glomerular filtration rate (GFR) as the primary outcome of renal function in addition to rigorous documentation of rhabdomyolysis severity, renal inflammation, and renal histologic injury.

Major Findings: Inducible proximal tubule-specific deletion of megalin is highly protective in a mouse model of induced rhAKI. Findings indicate that urine output and GFR were not altered by cilastatin administration. Further, cilastatin-dependent protection from rhAKI is absent in mice without proximal tubule megalin. Further study determined that caffeine does not worsen rhAKI, but administered ibuprofen has an exacerbative effect. In swine, it was discovered that hemorrhagic shock (HS) causes myoglobinemia. Administered cilastatin does not exacerbate the harm in animals with combined rhabdomyolysis and HS, and may in fact be beneficial in that it ameliorates hyperkalemia. However, cilastatin was not shown to have a beneficial effect in AKI caused by mixed HS and myoglobinemia. Cilastatin does ameliorate AKI due to muscle-predominant injury with myoglobinemia. Administering Vitamin D (as calcitriol) has little to no effect on animals with rhabdomyolysis.

15. SUBJECT TERMS

Rhabdomyolysis, acute kidney injury, rhabdomyolysis induced acute kidney injury, chronic kidney injury, megalin, proximal tubule epithelial cells, cilastatin, glomerular filtration rate, hyperkalemia, renal, myoglobinemia.

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1. INTRODUCTION:

Rhabdomyolysis is a lethal musculoskeletal disorder. Destruction of skeletal muscle by crush trauma, blast injury, burn injury, or excessive activity causes release of muscle protein into the systemic circulation where it is toxic to the kidney. The purpose of this proposal is to test and develop an intervention exploiting novel molecular biology (discovered in the PI's lab), which prevents released muscle protein from injuring the kidney. The scope of the research is to utilize animal models to create a critical body of data supporting translation to a clinical trial for an FDA-approved medication, including effectiveness data in a combat relevant model, understanding of real-world modifiers of injury and recovery, optimal dose and timing, and submission of an investigational new drug application allowing clinical trial and use.

2. KEYWORDS:

1. Musculoskeletal	11. C57BL/6 mice
2. Rhabdomyolysis (rh)	12. Swine
3. Acute Kidney Injury (AKI)	13. Hyperkalemia
4. Chronic Kidney Injury (CKI)	14. Multitrauma
5. Megalin (Lrp2)	15. Injury (blast/burn/crush/musculoskeletal)
6. Proximal tubule epithelial cells (PTEC)	16. Renal
7. Cilastatin	17. Ibuprofen
8. Glomerular Filtration Rate	18. Caffeine
9. Myoglobin	19. Legal Performance Enhancing Drugs
10. Kidney	20. Myoglobinemia

3. ACCOMPLISHMENTS:

What were the major goals of the project?

The major goals of the project are first: to determine the optimal dose and timing of cilastatin therapy and determine strategies to mitigate real-world accelerators of AKI and second, to determine whether pragmatic administration of cilastatin prevents RIAKI, hyperkalemia and mortality caused by combat-relevant model of severe musculoskeletal trauma.

- Milestones for Goal 1, Year 1 and 2:
- Obtain IACUC approval from the PVAMC for mouse experiments. Obtain ACURO approval. Recruit and hire staff. Initiate Aim 1 experiments. 100% complete.
- Complete GFR for experiment 1.1, therapeutic timing. Select timing for experiment 1.2. 50% complete, delayed.
- Complete remaining assays of 1.1. Start 1.2, optimal dose Started. 25% complete, delayed.
- Complete GFR analysis for 1.2 and select dose for further experiments. First submission for publication. Submit yearly report. 66% complete.
- Complete Accelerant experiment, aim 1.3-1.5: 100% complete
- Complete analysis for accelerant experiment, aim 1.3-1.5: 25% complete
- Milestones for Goal 2, Year 1 and 2:
- Obtain IACUC approval from OHSU and ACURO approval. Enter FDA pre-IND consultation program. >100% completed. IACUC and ACURO approvals were achieved. Amended IACUCs and ACURO approvals received.
- Pre-IND program not available for this drug.
- Cilastatin identified as a candidate for orphan drug status (<200,000 cases of /year in US)

- We are seeking industry partners interested in seeking this status with us in order to pursue a clinical trial.
- Multiple contacts made and meetings held in the last 2 months as of time of this submission.
- Train Schreiber lab personnel in sample acquisition and storage methods for kidney tissue, blood, and urine samples. Generate experimental guidance materials and manuals. Complete FDA pre-consultation (initial) and alter Experiment 2.2 if necessary. 100% complete.
- Randomize swine to receive vehicle, cilastatin, or cilastatin+calcitriol. Initial swine experiments (Aim 2.1, first 9 animals). Initial GFR measurements in OHSU bioanalytical core. 100% completed
- Complete interim analysis after 9 animals. Adjust 2.1 goals if necessary. Continue randomized experiments. 100% completed

Early findings were published in JASN as *Cilastatin ameliorates acute kidney injury due to rhabdomyolysis in mice* (this paper reported as accepted in 2021 progress report and was published during the term relevant to the present progress report).

Additional findings have formed part or whole of the following list of submitted or accepted abstracts and papers:

1. *Cilastatin inhibits renal myoglobin endocytosis and acute kidney injury following rhabdomyolysis*. Abstract. American Society of Nephrologists Kidney Week, November, 2021. San Diego, CA.
2. *Rhabdomyolysis-Induced Acute Kidney Injury and Treatment Are Impacted by Legal Performance-Enhancing Drugs*. Abstract, Military Health Systems Research Symposium. August 2022, Kissimmee, FL.
3. *Rhabdomyolysis-Induced Acute Kidney Injury in Reproductive-Age Mice Causes Delayed Pregnancy and Offspring Abnormalities*. Abstract, Military Health Systems Research Symposium. August 2022, Kissimmee, FL.
4. *Specific therapy for myoglobin-induced acute kidney injury in a combat-relevant, large animal polytrauma model*. Abstract, Military Health Systems Research Symposium. August 2022, Kissimmee, FL.
5. *Applying clinical research data science methods to a collaborative translational study*. Abstract. OHSU APOM Research day. June, 2022. Portland, OR.
6. *Rhabdomyolysis-induced acute kidney injury and treatment are impacted by legal performance enhancing drugs*. Abstract. OHSU research week, May, 2022. Portland, OR.
7. *Pregnancy following recovered acute kidney injury results in placental insufficiency and perinatal mortality*. Abstract. OHSU research week, May, 2022. Portland, OR.
8. *Pregnancy following recovered acute kidney injury results in placental insufficiency and perinatal mortality*. Abstract. OHSU Department of Medicine Research Symposium, June, 2022. Portland, OR.
9. *Pregnancy after AKI results in placental insufficiency and perinatal mortality*. Abstract. World DOHAD Congress. August, 2022. Vancouver, BC.
10. *Pregnancy following resolved AKI results in growth restricted offspring in mice*. Abstract, Society for Reproductive Investigations. March, 2022. Denver, CO.
11. *Molecular mechanisms of rhabdomyolysis-induced kidney injury: from bench to bedside*. Manuscript. Submitted

Data from this work has been included as part of the following submitted and/or pending grant proposals:

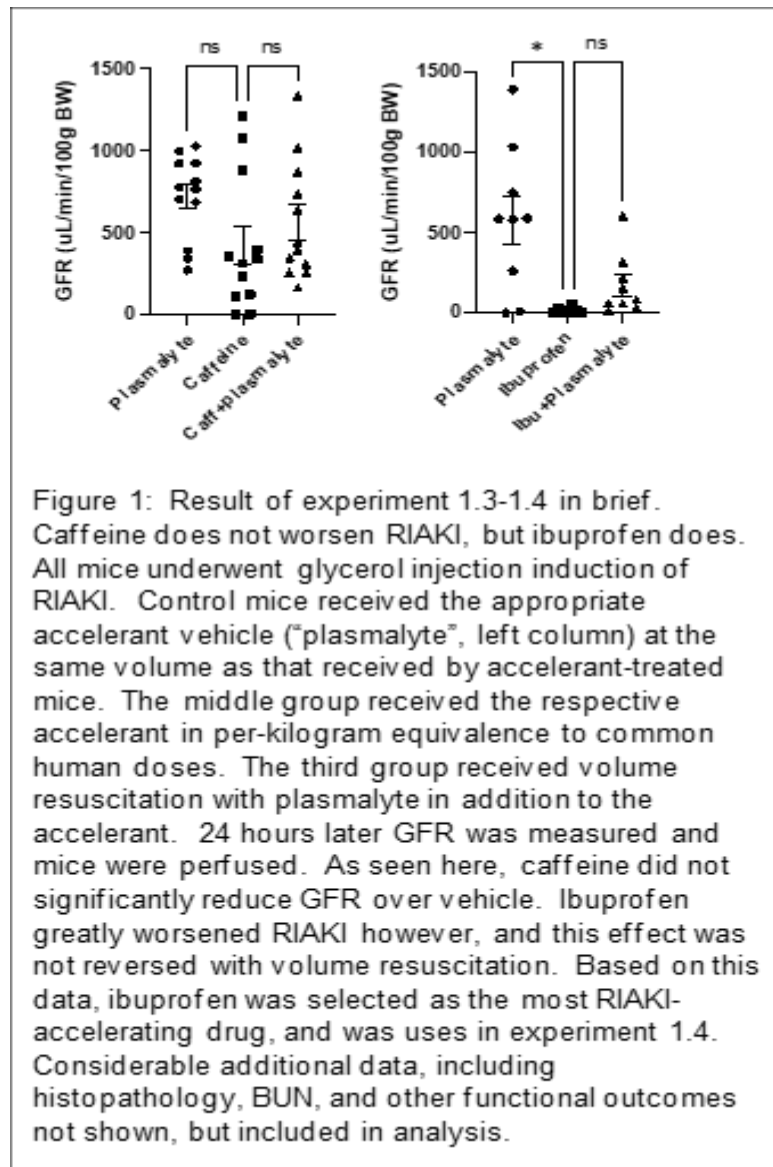
1. W81XWH-22-PRMRP-TTDA-GG/Administratively withdrawn
Nanotherapy for acute and long-term sequelae of rhabdomyolysis
2. PA-20-185/R01DK134505/pending IRG review
Proximal nephron-specific drug therapy for nephrotoxic, ischemic, and crush syndrome induced acute kidney injury
3. PA-20-185/R01DK135528
Role of megalin-dependent kidney injury in placental insufficiency

4. PA-20-188/K99DK134812
Determining mechanisms of longitudinal reproductive and developmental impact following parental recovered acute kidney injury
5. NRSA F32/F32DK134127
Long-term impact of acute kidney injury: reproductive, pregnancy, and offspring health
6. BIRCWH K12
Acute kidney injury impacts reproductive function
7. Oregon Clinical & Translational Research Institute Biomedical Innovation Program (BIP) Drug Discovery
Novel megalin inhibitors to enhance cancer and antimicrobial chemotherapy, and treat traumatic acute kidney injury
8. PA-20-185/R01DK132675 Megalin targeted delivery of renal-clearable nanomedicine

What was accomplished under these goals?

Goal 1: determine the optimal dose and timing of cilastatin therapy and determine strategies to mitigate real-world accelerators of AKI.

1. Major activities:
 - a. Mouse experiments to characterize awake GFR and cilastatin response
 - b. Mouse experiments to assess timing of cilastatin administration (aim 1.1)
2. Specific objectives:
 - a. Obtain approvals
 - b. Analyze GFR from all aim 1.1 animals
 - c. Analyze GFR from all aim 1.2 animals
 - d. Select dose and timing based on objective b and c.
3. Significant results/Key outcomes:
 - a. Approvals obtained during prior funded year.
 - b. Initial GFR and other data accrued for 2 time points, 2 doses, sham, and vehicle groups.
 - c. This data revealed that the mean 24h awake GFR in vehicle treated animals exposed to rhabdomyolysis is only about 30% reduced compared to that of shams. This is a far smaller reduction than previously recorded under anesthesia. This result is due to a change in the model (asleep to awake GFR) and is not an effect of the treatment or reflective on the hypothesis.
 - d. Cilastatin tested under these circumstances was not “effective” at any dose by our definition (50% improvement in reduction of GFR) because data scatter was greater than the difference between shams and vehicle treated animals.
 - e. On careful analysis, the lack of difference between groups was primarily because of the combination of not enough injury and increased variation in the new lab with new personnel as compared with prior experiments.



- f. The postdoctoral fellow who performed previous experiments is no longer available to us to consult.
- g. We therefore conducted model development trials to determine influence of injection location (all within the hindlimb muscles as approved) on glycerol deposition, magnitude of injury, and inter-animal variation.
- h. We have established that the source of inappropriately high GFR (i.e. "not enough injury") and excess variation (i.e., high standard deviation in GFR) was injection into a single, small hindlimb muscle, which did not adequately elevate the plasma myoglobin. This procedural change occurred as a result of departure of the prior postdoc, and is therefore model shift.
- i. Prior to correcting the model shift, the mean GFR in vehicle-treated animals was 1073 ± 150 uL/min/100g. After correction of the technique, the mean GFR is 263 ± 340 uL/min/100g. The increased standard deviation here is because of zero values. Animals with higher values (i.e. treated) would be expected to have less variation.

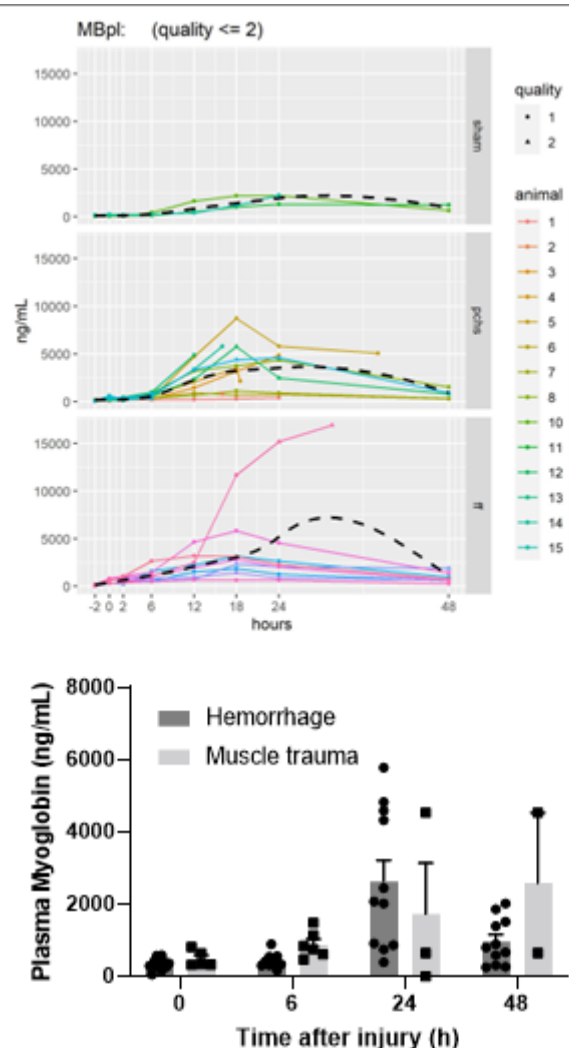
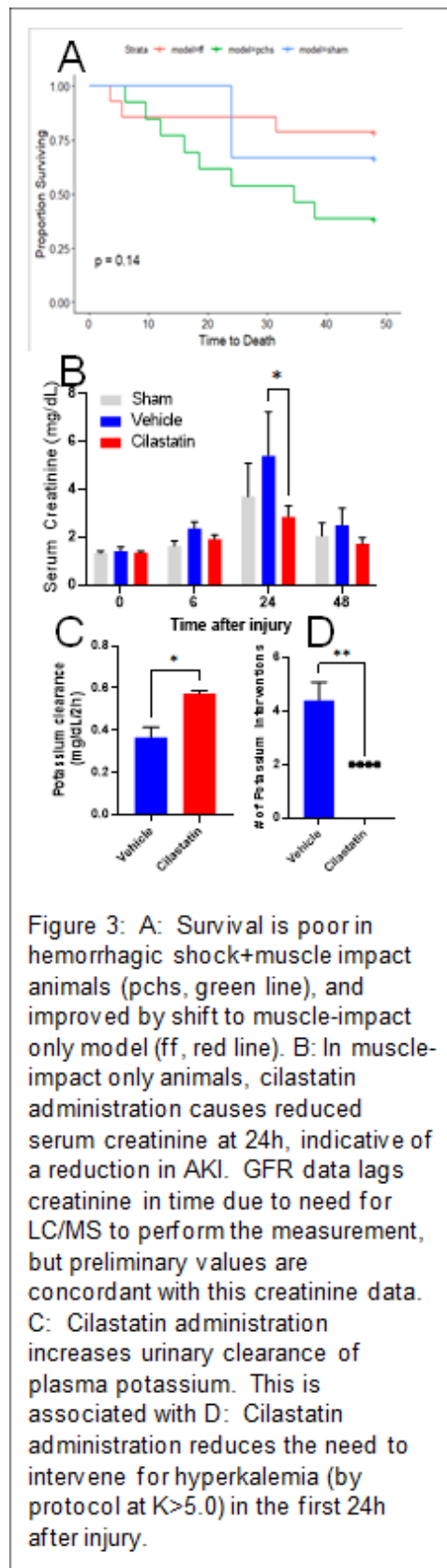


Figure 2: TOP: Myoglobinemia in sham (top panel), chest impact with hemorrhagic shock (middle panel, PCHS) and large muscle impact-exposed pigs (bottom panel, FF). Each colored line represents values from one animal over 48 hours of general anesthesia and ICU-level care. The dashed black line represents the mean value for all animals in the group. Time zero is the time of drug or vehicle administration, approximately 30 mins after injury. Sham and -2h animals demonstrate normal (zero) plasma myoglobin levels. Some elevation in sham occurs because of immobility during anesthesia. Note that muscle-impact only animals demonstrate earlier rise and greater maximal plasma myoglobin than other groups, but overall, both injury models demonstrate similar plasma myoglobin. BOTTOM: Mean plasma myoglobin quantified in both injury models is overall similar, with earlier rise in muscle-impact only animals.

- j. Accordingly, in a test experiment, vehicle treated mice demonstrated GFR 190 ± 380 , while cilastatin-treated mice demonstrated GFR 409283 uL/min/100g. These data are consistent with our prior, published data, and we are now proceeding with experiment 1.1 to determine optimal timing; experiment 1.2 will follow.
- k. We have trained an expert surgeon through these optimization experiments who will remain available to us for the remainder of experiments 1.1 and 1.2, and no further impediments are expected. We fully expect to complete 1.1 and 1.2 by December 31, 2022.
- l. Due to the delay in experiment 1.1 and 1.2, we proceeded with experiments 1.3-1.5, testing whether cilastatin therapy would reverse RIAKI worsened by kidney injury-accelerant drugs often consumed by Warfighters. This experiment is successfully completed and results are in analysis. Abstracts have been submitted to MHSRS and other meetings and a manuscript, tentatively planned for submission to Military Medicine, is in preparation (see **fig 1**).

Goal 2: determine whether pragmatic administration of cilastatin prevents RIAKI, hyperkalemia and mortality caused by combat-relevant model of severe musculoskeletal trauma.

- 1. Major activities:
 - a. Maintain and revise as needed IACUC/ACURO protocols and maintain/obtain approvals.
 - b. Operationalize swine protocols
 - c. Supervise training of new personnel in Schreiber lab during personnel changes
 - d. Monitor data acquisition in Schreiber lab
 - e. Validate data obtained
 - f. Operationalize data into “tidy” format
 - g. Build comprehensive data acquisition, storage and analysis system (**7843** lines of R code with **56242** data points at the time of this submission)
 - h. Develop curve-fitting automation for LC/MS iohexol concentrations to determine glomerular filtration rate (GFR) measurements.
 - i. Randomize, blind, and support swine experiments
 - j. Prepare drug solutions for administration to pigs
 - k. Maintain supply of critical reagents and drugs during supply chain disruptions
 - l. Conduct swine experiments in the Schreiber lab
 - m. Analyze data in real time to assess effectiveness of cilastatin for major outcomes and assure successful completion of major objectives
 - n. Modify experimental model as needed to assure high-quality results, in discussion with Schreiber lab and SO as needed.



2. Specific objectives:

- Operationalize and continue swine experiments
- Develop real-time analysis system from data management system

- c. Modify experimental model when excess noise:signal prevented assessment of results in original model
- 3. Significant results/Key outcomes:
 - I. Approvals for IACUC/ACURO obtained, maintained, sought again for modifications and obtained again.
 - II. Experiments on pigs began in June of 2021, 35 pigs complete at this submission.
 - III. In the results and figures below, we present data from 3 groups. **Preliminary group names in the preliminary figures are based on surgical model names. Hemorrhage with chest muscle impact is designated as “PCHS.” Large muscle impact only (thigh muscle) is designated as “FF.”**
 - IV. Results:
 - a. Hemorrhagic shock causes myoglobinemia similar to that caused by simultaneous muscle impact (see **fig 2**).
 - b. Survival is poor in the combined hemorrhagic shock/muscle impact model, worsening signal:noise, and imperiling study power. This resulted in an ACURO-approved change in model (**fig 3a**).
 - c. Cilastatin does not cause harm in animals with combined rhabdomyolysis and hemorrhagic shock.
 - i. We do not show the data here for brevity, but mean arterial pressure, oxygenation, heart rate, coagulation status, and all other physiologic metrics checked for evidence of harm show no difference between cilastatin and vehicle treatment to date.
 - d. Cilastatin ameliorates AKI due to muscle-predominant injury with myoglobinemia (**fig 3a**).
 - e. Cilastatin reduces the need to intervene for hyperkalemia after muscle-predominant injury with myoglobinemia (**fig 3c-d**).
 - f. Administration of Vitamin D (calcitriol) with cilastatin likely has minimal to no beneficial or detrimental effect, (data not shown).

Discussion of goals not met:

There are three goals of significance not met. First, we have not completed experiments 1.1 and 1.2, which were designed to delineate dose and timing for cilastatin. Because of experimental variability we chose to re-characterize the model system and proceed with 1.3 through 1.5; these experiments are ahead of schedule. 1.1 is now underway and we expect to complete 1.1 and 1.2 by December 31, 2022. Second, we identified excess mortality in the original swine model, which necessitated a change of model and slowed aim 2 experiments. Aim 2 experiments have resumed following the change in model with much improved signal to noise, and we are currently demonstrating results consistent with the experimental hypothesis. We are on track to complete these experiments on time and within budget. Third, we have not entered the FDA pre-IND process, because we discovered this is not available for drugs in our field. In addition, we have identified a number of barriers to IND development. However, we have determined that cilastatin use for rhabdomyolysis may qualify for orphan drug status, which is financially incentivized and attractive to industry. We are pursuing this and several drug combination and novel preparations (see “grant applications” above) in order to ensure promulgation of the discoveries in this work. The major goal is to establish groundwork for a clinical trial; we are currently engaged in outreach to experts to ensure this goal is met.

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

All staff are provided with 1:1 mentoring with the PI on a weekly or biweekly basis depending on the trainee’s skill set and room for growth. Two research assistants learned 2 new animal models. RA2 Megan Nickerson wrote and submitted her first abstract, which was accepted by the MHSRS. She was taught new lab skills in Western blots, ELISAs, tissue staining and cryostat sectioning, as well as lysate preparation, brush border

isolation, urine desalting, and IHC. Most of these skills were demonstrated to her by the Senior Lab Assistant, Adam Munhall, for whom teaching is in of itself a demonstration of professional development. Mr. Munhall has applied computer programming for data management to improve efficiency and accuracy of accumulated lab results, which has resulted in opportunities to train ancillary staff in data system development.

All staff participate in and attend weekly Anesthesiology and Perioperative Medicine Research Seminars. Three members of the lab presented during OHSU-APOM Research Day as well as OHSU Research Week. All staff participate in professional development by completing the following workshops: Information and Privacy Security Essentials, Emergency Preparedness at Work and Home, Respect at the University, COVID-19 Core Training, Unconscious Bias Foundations, Laboratory Safety, Bloodborne Pathogen Training, Fraud, Waste, and Abuse, Compliance Program Training, VA Biosafety and VA General Lab Safety, VA Information and Privacy Security.

In addition, select members of the lab have completed Q-fever awareness training, Laboratory Animal Occupational Health, Animal Care and Use – Working with Mice, Unconscious Bias Hiring for Managers and Supervisors, Mouse handling lab (OHSU & VA), LinkedIn Learning Public Speaking Foundations, Interpersonal Communications, Emotional Intelligence, and Conflict Resolutions trainings. Conferences at which lab members presented science or attended: Society for Reproductive Investigations (presented, March 2022), Military Health System Research Symposium (presented August, 2021), OHSU Research Week (May 2022), APOM Research Day (June, 2022).

The post-doctoral fellow on this project was awarded an Oregon Students Learn and Experience Research (OSLER) TL1 grant. In the first 12 months of the grant period, the fellow conducted the proposed experiment under the mentorship of the PI; exposing dams and sires to rhabdomyolysis-induced acute kidney injury (RIAKI) nearly immediately upon funding. Mice were successfully bred, and Dr. Hebert assessed placental, pregnancy, birth, and adult offspring outcomes. These outcomes were surprisingly, and significantly divergent, which Dr. Hebert correctly interpreted as implicating recovered AKI as a potential cause of adverse pregnancy outcomes. She has presented these results widely at OHSU, and at national meetings, including the Society for Reproductive Investigation. Abstracts have been accepted for 2 more national meeting presentations. She has been highly productive, submitting four proposals for future funding, including an F32, a K99/R00, a K12, and an additional foundation proposal. A manuscript describing the work is in preparation. This year she has published a first-author, peer-reviewed manuscript describing sexual dimorphism in uteroplacental angiogenesis. In addition, Dr. Hebert first-authored a published review paper and a second (currently under review). Dr. Hebert has also received extensive didactic research training as part of the OHSU Human Investigations Program. Trainings completed (or in progress) are as follows: Leadership Skills in Team Science, OHSU Vollum Writing Class, Proposal Development.

How were the results disseminated to communities of interest?

- A. Our lab's work was presented at the Society for Reproductive Investigation in March of 2022 (Denver, CO), wherein we presented *Pregnancy following resolved AKI results in growth restricted offspring in mice*.
- B. Three abstracts have been accepted for presentation at the Military Health System Research Symposium in August.
 - 1. *Rhabdomyolysis-Induced Acute Kidney Injury and Treatment Are Impacted by Legal Performance-Enhancing Drugs*
 - 2. *Rhabdomyolysis-Induced Acute Kidney Injury in Reproductive-Age Mice Causes Delayed Pregnancy and Offspring Abnormalities*
 - 3. *Specific therapy for myoglobin-induced acute kidney injury in a combat-relevant, large animal polytrauma model*

The following abstract was accepted for the virtual Kidney Week Conference that took place in November of 2021. *Cilastatin inhibits renal myoglobin endocytosis and acute kidney injury following rhabdomyolysis.*

An abstract titled *Pregnancy after AKI results in placental insufficiency and perinatal mortality*, has been accepted for presentation at the World DOHAD Congress in Vancouver, Canada in August of 2022.

The following abstracts were presented at research symposiums hosted by Oregon Health & Science University.

1. *Rhabdomyolysis-induced acute kidney injury and treatment are impacted by legal performance enhancing drugs* (OHSU Research Week, May 2022)
2. *Pregnancy following recovered acute kidney injury results in placental insufficiency and perinatal mortality* (OHSU Research Week, May 2022; & OHSU Department of Medicine Research Symposium)
3. *Applying clinical research data science methods to a collaborative translational study* (OHSU APOM Research Week, May 2022)

C. The manuscript describing our findings related to *Rhabdomyolysis-Induced Acute Kidney Injury and Treatment Are Impacted by Legal Performance-Enhancing Drugs* is in draft and will be submitted to Military Medicine this Fall.

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

In the next reporting period, the goals and objectives we plan to accomplish first are the partially completed aims from Year 1 and 2, and to commence the activities described in the milestones for Year 3.

Aim 1 Year 1 or 2 milestones to complete

- Complete GFR for experiment 1.1, therapeutic timing. Select timing for experiment 1.2.
- Complete remaining assays of 1.1. Start 1.2, optimal dose
- Complete GFR analysis for 1.2 and select dose for further experiments.

Aim 1 Year 2 milestones to complete:

- **Months 25-28:** Complete assays for 1.2.
- **Months 28-31:** Complete all assays and data analysis for 1.1.
- **Months 31-34** Complete 1.4, initiate 1.5.
- **Months 33-36:** Complete 1.5, initiate 1.6.

Aim 1 Year 3 milestones to complete:

- **Months 25-27:** Complete experiment 1.6.
- **Months 28-30:** Complete assays and data analysis for experiments 1.3-1.6.
- **Months 31-33:** Third manuscript for publication. Cilastatin field care white paper basic science component drafted.
- **Months 34-36:** Cilastatin field care white paper presented at MHSRS. Submit final report.

Aim 2 Year 1 milestones to complete:

- **Months 25-26:** Investigate orphan drug pathway to identifying a pharmaceutical partner for RCT.
- **Months 25-27:** Initial GFR measurements in OHSU bioanalytical core.
- **Months 25-36:** Continue randomized experiments.

Aim 2 Year 2 milestones to complete:

- **Months 25-27:** Continue GFR mass spec analysis contemporaneously.

- **Months 25-31:** Continue randomized experiments in experiment 2.2. Continue GFR mass spec analysis contemporaneously.
- **Months 31-36:** Complete molecular biology, GFR, molecular biology assays, and other non-pathology data.

Aim 2 Year 3 milestones to complete:

- **Months 25-27:** Complete tissue processing from 2.1; complete pathologic analysis from 2.1
- **Months 28-30:** Complete experiment 2.2. Write and submit IND application.
- **Months 31-33:** Complete experiment 2.3. Proteomics data available. Begin analysis. Cilastatin field care white paper translational (swine model) component drafted. Complete proteomics data analysis.
- **Months 34-36:** Submit final manuscripts and conference abstracts. Submit final report. Cilastatin field care white paper presented at MHSRS.

4. IMPACT:

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

Findings from this project have been accepted for presentation at multiple conferences. Below is a list of abstracts that have been accepted in the current grant reporting period.

1. Pregnancy following resolved AKI results in growth restricted offspring in mice (Society for Reproductive Investigations, March 2022, Denver, CO.)
2. Rhabdomyolysis-induced acute kidney injury and treatment are impacted by legal performance enhancing drugs (Military Health System Research Symposium (MHSRS, August 2022, Kissimmee, FL.)
3. Rhabdomyolysis-induced kidney injury in reproductive-age mice causes delayed pregnancy and offspring abnormalities (MHSRS, August 2022, Kissimmee, FL).
4. Specific therapy for myoglobin-induced acute kidney injury in a combat-relevant, large animal model (MHSRS, August 2022, Kissimmee, FL.)
5. Pregnancy after acute kidney injury results in placental insufficiency and perinatal mortality (DOHAD, World Congress, August 2022, Vancouver BC.)
6. Cilastatin inhibits renal myoglobin endocytosis and acute kidney injury following rhabdomyolysis (American Society of Nephrologists, (ASN Kidney Week, November 2021, Virtual Meeting).

What was the impact on other disciplines?

Postdoctoral fellow Dr. Jessica Hebert started and completed her examination of *Long-Term Impact of Acute Kidney Injury: Reproductive, Pregnancy, and Offspring Health* as intended thanks to the funding she received as a recipient of the Oregon Students Learn and Experience Research (OSLER) TL1 award. Dr. Hebert's project was conducted as an amendment to the IACUC approved protocol that guides this Department of Defense funded project. Discoveries made as a result of Dr. Hebert's study serve as preliminary data for future studies and grant proposals. In the past year Dr. Hebert has submitted an F32, a K99, a BIRCWH (finalist), a Baxter (funded), and a Tartar trust (funded). She also presented findings from this project at the Society for Reproductive Investigations conference in Denver (March 22), and has been invited to present at The Military Health System Research Symposium (Kissimmee, August 2022), as well as World DOHAD (Developmental Origins of Health and Disease) Congress (Vancouver, August 2022). The findings from the OSLE also led to a published manuscript; first authored by Dr. Hebert. *Male fetal sex affects uteroplacental angiogenesis in growth restriction mouse model* was published in the journal *Biology of Reproduction* in April of 2021.

What was the impact on technology transfer?

In May of 2022, we submitted a proposal for a Biomedical Innovations Program (BIP) award at OHSU. This program is supported by OHSU's technology transfer office. As part of the submission we disclosed novel technology, "drug combinations to inhibit megalin in rhabdomyolysis". Our submission, *Novel megalin inhibitors to enhance cancer and antimicrobial chemotherapy, and treat traumatic acute injury*, was supported by preliminary data derived from this DOD funded endeavor. Our submission described our two-drug approach to implementing a new kidney-specific therapy to reduce cost and mortality in trauma and improve existing antibiotics and cancer treatments by blocking megalin, a kidney specific receptor. While we did not receive funding through the BIP mechanism, we continue evaluating therapeutic drug combinations to reduce kidney injury.

What was the impact on society beyond science and technology?

Nothing to Report.

5. CHANGES/PROBLEMS:

Changes in approach and reasons for change

Nothing to Report.

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

Most of the challenges disclosed during the first reporting period related to personnel staffing obstacles due to Covid-19 precautions. These obstacles have not been a factor in the second year of the grant period. In fact, we have added a research assistant to our project staff. In addition, while not a direct employee supported by the grant, we have been utilizing the skills of an animal surgeon who is an employee of the OHSU APOM department. These changes have accelerated progress in our mouse model. Additional staffing has also been contributing to the swine model team, although turn over in animal surgeons has caused some slowing in productivity due to the necessity of onboarding and training replacements. However, as before, supply chain issues persist. We have developed a moderately secure supply chain for the principal test drug, cilastatin at the time of this writing.

Three goals have not been met, but we have mitigated the relevant delays and are making progress. See "Discussion of goals not met" in "Accomplishments" for details. Briefly, we have not completed experiments 1.1 and 1.2, which were designed to delineate dose and timing for cilastatin. Because of experimental variability we chose to re-characterize the model system and proceed with 1.3 and 1.4; these experiments are ahead of schedule. Second, we identified excess mortality in the original swine model, which necessitated a change of model and slowed aim 2 experiments. Aim 2 experiments have resumed following the change in model with much improved signal to noise, and we are currently demonstrating results consistent with the experimental hypothesis. Third, we have not entered the FDA pre-IND process, because we discovered this is not available for drugs in our field. The major goal is to establish groundwork for a clinical trial; we are currently engaged in outreach to experts to ensure this goal is met.

Changes that had a significant impact on expenditures

At the writing of this report we are forecast to be below our proposed spending for Year 2. This surplus is partially due to carryover from the first grant year, and the general slow start due to COVID-19 precautions that impeded the commencement of our projects in year 1. The aforementioned challenge of finding a reliable cilastatin source has resulted in orders made and cancelled. As this an essential and significant portion of the supplies budget we see some expenditures below projections.

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

Not Applicable.

Significant changes in use or care of vertebrate animals

Nothing to Report.

Significant changes in use of biohazards and/or select agents

Nothing to Report.

6. PRODUCTS:

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

Journal publications.

1. Jessica F. Hebert, Jess A Miller, Rahul Raghavan, Amie Romney, Jason E Pobradsky, Monique Y Rennie, Allison M Felker, Perrie O'Tierney-Ginn, Maya Morita, Elizabeth A DuPriest, Terry K Morgan. *Male fetal sex affects uteroplacental angiogenesis in growth restriction model*. Biology of Reproduction. Published 2021, April 1; 104-(4):924-934. Acknowledgement of federal support: yes.
2. Jessica F. Hebert, Kevin Burfeind, Darren Malinoski, Michael P Hutchens. *Molecular mechanisms of rhabdomyolysis-induced kidney injury: from bench to bedside*. Kidney 360. Publication status: submitted. Acknowledgement of federal support: yes.

Books or other non-periodical, one-time publications.

Nothing to Report.

Other publications, conference papers and presentations.

Abstracts/Presentations

Presentations:

1. Pregnancy following resolved AKI results in growth restricted offspring in mice (Society for Reproductive Investigations, March 2022, Denver, CO.)

Abstracts:

1. Rhabdomyolysis-induced acute kidney injury and treatment are impacted by legal performance enhancing drugs (Military Health System Research Symposium (MHSRS, August 2022, Kissimmee, FL.)*Intent to publish submitted to MHSRS.
2. Specific therapy for myoglobin-induced acute kidney injury in a combat-relevant, large animal model (MHSRS, August 2022, Kissimmee, FL.)
3. Pregnancy after acute kidney injury results in placental insufficiency and perinatal mortality (DOHAD, World Congress, August 2022, Vancouver BC.)
4. Cilastatin inhibits renal myoglobin endocytosis and acute kidney injury following rhabdomyolysis (American Society of Nephrologists, (ASN Kidney Week, November 2021, Virtual Meeting).

Poster Presentations

1. Rhabdomyolysis-induced acute kidney injury and treatment are impacted by legal performance enhancing drugs (OHSU Research Week, May 2022)
2. Pregnancy following recovered acute kidney injury results in placental insufficiency and perinatal mortality (OHSU Research Week, May 2022; & OHSU Department of Medicine Research Symposium)
3. Cilastatin inhibits renal myoglobin endocytosis and acute kidney injury following rhabdomyolysis (ASN Kidney Week, November 2021)
4. Applying clinical research data science methods to a collaborative translational study (OHSU Research Week, May 2022)

- **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**

"DATAHUB database and analysis software (see "Accomplishments"), containing >7000 lines of code in R and >50,000 individual datapoints, including all data acquired from pigs in this work.

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project?

Name:	Michael Hutchens, MD, MA
Project Role:	Principal Investigator
Researcher Identifier:	(ORCID) 0000-0001-8583-1812
Nearest person month worked:	4
Contribution to Project:	Dr. Hutchens is the PI and responsible for every component of the project.
Funding Support:	Department of Defense

Name:	Martin A. Schreiber, MD
Project Role:	Co-investigator
Researcher Identifier:	(ORCID)0000-0002-4430-6779
Nearest person month worked:	2
Contribution to Project:	Dr. Schreiber is responsible for the implementation of the swine multitrauma model experiments. He will train the swine model personnel.
Funding Support:	Department of Defense

Name:	Nicole Andeen, MD
Project Role:	Renal Pathologist
Researcher Identifier:	(ORCID) 0000-0002-4882-6640
Nearest person month worked:	1
Contribution to Project:	Dr. Andeen applies rigorous and reproducible methods for assessment of kidney injury in the swine model. Each animal's pathology is estimated using methods described in the research narrative.
Funding Support:	Department of Defense

Name:	Tahnee Groat, MPH
Project Role:	Project Lead
Researcher Identifier:	(ORCID) 0000-0002-8002-7742
Nearest person month worked:	12
Contribution to Project:	Ms. Groat oversees day to day aspects of proposed work. Assures compliance with regulatory bodies, assists in the production of required reports, maintains adherence to projected milestone goals, and assists personnel in administrative and basic science procedures.
Funding Support:	Department of Defense.

Name:	Jessica Hebert, PhD
Project Role:	Post-doctoral fellow
Researcher Identifier:	(ORCID) 0000-0003-2433-8359
Nearest person month worked:	12
Contribution to Project:	Dr. Hebert has learned and practices the rhabdomyolysis model, the glomerular filtration measurement and metabolic cage studies, and conducts all molecular biology assays as described in the research narrative. She has trained the Research Assistant 2 in these methods. She also analyzes data including the confocal microscopy imaging and quantifying injury. She will present at the Military Health Sciences Research Symposium in August.
Funding Support:	Department of Defense.

Name:	Adam Munhall, BS
Project Role:	Senior Research Assistant
Researcher Identifier:	(ORCID) 0000-0002-1960-5486
Nearest person month worked:	12
Contribution to Project:	Coordinates with Schreiber Lab Project Lead to assure proper flow of procedures and data collection. Assists with equipment set up and maintenance, histologic preparation, molecular biology assays, and preliminary data analysis.
Funding Support:	Department of Defense.

Name:	Megan Nickerson, BS
Project Role:	Research Assistant II
Researcher Identifier:	(ORCID) 0000-0001-9334-2656
Nearest person month worked:	12

Contribution to Project:	Ms. Nickerson assists with lab equipment set up, take-down, cleaning, lab assay preparation, assessment, and data acquisition, monitoring, and inventory for Aim 3 of the grant. Ms. Nickerson participates in the rhabdomyolysis model for the swine portion of the grant, running assays and analyzing acquired data. Ms. Nickerson assists in preparing histologic samples.
Funding Support:	Department of Defense.
Name:	Mahaba Eiwaz
Project Role:	Research Assistant II
Researcher Identifier:	(ORCID) 0000-0001-6085-0304
Nearest person month worked:	6
Contribution to Project:	Ms. Eiwaz assists with lab equipment set up, take- down, cleaning, lab assay preparation, assessment, data acquisition and analysis for Aims 1. Ms. Eiwaz performs model development procedures and orients new staff. Ms. Eiwaz participates in the rhabdomyolysis mouse model.
Funding Support:	Department of Defense
Name:	Moqing Liu, PhD
Project Role:	Sr. Research Associate
Researcher Identifier:	(ORCID) 0000-0001-8385-0649
Nearest person month worked:	3
Contribution to Project:	Dr. Liu oversees the intraoperative data collection of experiments in Schreiber's swine multitrauma models. She performs the quality control for samples collected and preliminary processing of these samples before distributing them to team members. Dr. Liu conducts statistical analysis on preliminary data obtained during procedures. Dr. Liu Dr. Appleman oversees laboratory assays and assists with reports.
Funding Support:	Department of Defense.
Name:	Karen Minoza
Project Role:	Schreiber Lab Research Associate
Researcher Identifier:	None Provided
Nearest person month worked:	3
Contribution to Project:	Ms. Minoza assists with animal surgeries and monitoring of swine during experiments. She prepares the surgical area and instruments. She manages logistics for proposed experiments and assists Dr. Liu and the animal surgeon.
Funding Support:	Department of Defense.
Name:	Marissa Beiling, MD
Project Role:	Surgeon
Researcher Identifier:	(ORCID) 0000-0002-0872-7656
Nearest person month worked:	4
Contribution to Project:	Dr. Beiling performs all components of the swine multitrauma surgical model. She makes observations and makes decisions relevant to the procedure based on observable outcomes. He works

with Dr. Garay Appleman and Dr. Liu to ensure measurements are obtained as planned. Dr. Beiling participates in data analysis and the dissemination of findings.

Funding Support: Department of Defense.

Name: James El Haddi, MD
Project Role: Surgeon
Researcher Identifier: (ORCID) 0000-0002-1274-2138
Nearest person month worked: 0.5
Contribution to Project: Dr. El Haddi performs all components of the swine multitrauma surgical model. He makes observations and makes decisions relevant to the procedure based on observable outcomes. He works with Dr. Garay and Dr. Liu to ensure measurements are obtained as planned.

Funding Support: Department of Defense.

Name: Arthur Kim, MD
Project Role: Surgeon
Researcher Identifier: None Provided
Nearest person month worked: 1.5
Contribution to Project: Dr. Kim performs all components of the swine multitrauma surgical model. He makes observations and decisions relevant to the procedure based on observable outcomes. He works with Dr. Garay and Dr. Liu to ensure measurements are obtained as planned.

Funding Support: Department of Defense.

Name: Xiao-Yue Han, MD
Project Role: Surgeon
Researcher Identifier: (ORCID) 0000-0002-4608-6036
Nearest person month worked: 0.5
Contribution to Project: Dr. Han performs all components of the swine multitrauma surgical model. They makes observations and decisions relevant to the procedure based on observable outcomes. He works with Dr. Garay and Dr. Liu to ensure measurements are obtained as planned.

Funding Support: Department of Defense.

Name: Joseph Garay, PhD
Project Role: Research Associate
Researcher Identifier: (ORCID) 0000-0002-2287-3051
Nearest person month worked: 4
Contribution to Project: Mr. Garay is the Project Lead for the swine portion of the project and thereby oversees the day to day aspects of the experiments in Schreiber's swine multitrauma models. He scheduled and managed the procedures. Mr. Garay provides consultation on measurement collection procedures. Dr. Garay has assumed responsibility for all regulatory activities for the swine ACURO and OHSU IACUC.

Funding Support: Department of Defense.

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: VA Portland Medical Center

Location of Organization: Portland, OR

Partner's contribution to the project:

A. Facilities (e.g., project staff use the partner's facilities for project activities)

8. SPECIAL REPORTING REQUIREMENTS

See Award Chart, page 23



W81XWH2010196: Developing a Novel Therapy for Rhabdomyolysis

PI: Michael Hutchens, Oregon Health and Science University, Oregon

Budget: \$3,981,563

Topic Area: FY19 PRMRP-Technology/Therapeutic Development Award

Mechanism: W81XWH-19-PRMRP-TTDA

Research Area(s): 0415, 0813

Award Status: 1 July 2021 – 30 June 2022

Study Goals:

The central hypothesis in this proposal is that the pharmacologic inhibition of megalin, achieved by administering cilastatin ameliorates rhabdomyolysis induced acute kidney injury in mice and a combat relevant, large animal multitrauma model. The objective in this proposal is to repurpose an inexpensive FDA approved drug to prevent rhabdomyolysis-induced kidney injury and therefore mortality during prolonged field care of warfighters with crush, blast, or burn-induced skeletal muscle destruction.

Specific Aims:

Aim 1: To determine the optimal dose and timing of cilastatin therapy and determine strategies to mitigate real world accelerators of acute kidney injury.

Aim 2: To determine whether pragmatic administration of cilastatin prevents rhabdomyolysis acute kidney injury, hyperkalemia, and mortality caused by a combat-relevant model of severe musculoskeletal trauma in swine.

Key Accomplishments and Outcomes:

Publications: Matsushita K, Mori K, Saritas T, Eiwaz MB, Funahashi Y, Nickerson MN, Hebert JF, Munhall AC, McCormick JA, Yanagita M, Hutchens MP. Cilastatin Ameliorates Rhabdomyolysis-induced AKI in Mice. J Am Soc Nephrol. 2021 Oct;32(10):2579-2594. doi: 10.1681/ASN.2020030263. Epub 2021 Aug 2. PMID: 34341182; PMCID: PMC8722809.

Patents: none to date

Funding Obtained: Oregon Clinical and Translational Research Institute (OCTRI) Oregon Students Learn and Experience Research(OSLER)T1Award.Long-term Impact of Acute Kidney Injury: Reproductive, Pregnancy, and Offspring Health.

9. APPENDICES:

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3. Specific therapy for myoglobin-induced acute kidney injury in a combat-relevant, large animal polytrauma model August, 2022, MHSRS	26
4. Rhabdomyolysis-Induced Acute Kidney Injury and Treatment are Impacted by Legal Performance-Enhancing Drugs August, 2022. MHSRS	27
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Abstract 1: Cilastatin inhibits renal myoglobin endocytosis and acute kidney injury following rhabdomyolysis

Authors: Jessica F. Hebert, PhD; Kevin Burfeind, MD, PhD; Katsuyuki Matsushita, MD, PhD; Megan Nickerson, BS; Mahaba Eiwaz, BS; Tahnee Groat, MA; Yoshio Funahashi, MD, PhD; Michael P. Hutchens, MD, MA

Background: Muscle-derived myoglobin, a renal toxin, causes rhabdomyolysis-induced acute kidney injury (AKI) via megalin-mediated endocytosis into proximal tubule cells (PTs). Endocytosis kinetics and inhibition are poorly understood. We characterized myoglobin uptake *in vivo*, hypothesizing cilastatin, a putative megalin inhibitor, would prevent endocytosis akin to megalin knockouts.

Methods: Procedures in wild-type (WT) male C57BL/6 mice and inducible, PT-specific megalin-deleted mice (iMegKO) were approved by OHSU or PVAMC IACUC. FITC-myoglobin (FMb) and cilastatin (200 mg/kg) were injected retroorbitally. Experimental rhabdomyolysis (ER) was induced via intramuscular injection of 50% glycerol (8 mL/kg). Glomerular filtration rate (GFR) was measured 24h later, and immunofluorescence and single-cell RNASeq were performed on renal tissue.

Results: FMb was observed in PTs 15 min post-injection in control mice; in iMegKO mice, FMb puncta were nearly absent ($p < 0.0001$). FMb puncta were reduced in cilastatin-treated WT without ER vs vehicle controls ($p = 0.0012$). iMegKO prevented AKI following ER, with 24h GFR 5x control ($p < 0.001$). Cilastatin injection did not affect GFR in iMegKO ($p = 0.89$), but had a significant effect on ER-induced AKI in wild-type mice: GFR was 8x vehicle ($p = 0.03$) while PT uptake of endogenous myoglobin decreased ($p < 0.05$). A composite score of endocytosis related genes (endoscore) showed significant alterations (Figure 1).

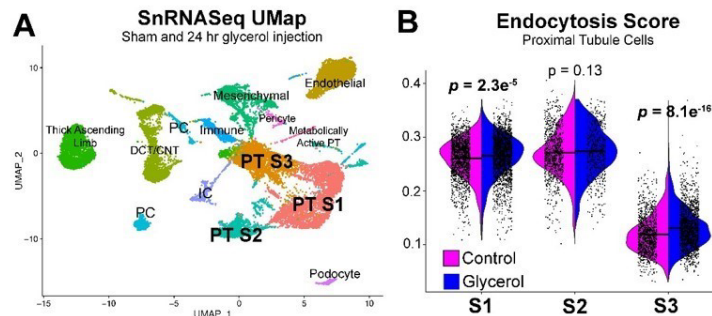


Figure 1. Kidney SnRNASeq after RIAKI. A) Umap clustering of cells isolated from whole kidney tissue 24 hrs after glycerol or vehicle injection (n=2 kidneys and approximately 10,000 cells per group). Data generated in Seurat V4 using Rstudio. PT = proximal tubule, PC = principal cells of the collecting duct, IC = intercalated cells of the collecting duct. DCT = distal convoluted tubule. B) Split violin plots of different PT segments depicting “endocytosis score”, generated by calculating average expression of 657 genes in gene ontology term “endocytosis” (GO:0006897). Line = median endocytosis score of all cells within group. P values generated from student’s t-test.

Conclusions: Megalin interference prevents ER-induced AKI by reducing myoglobin endocytosis; cilastatin recapitulates the effect in a megalin-inhibitory fashion. Alteration of endocytosis-related genes confirms this process is critical in rhabdomyolysis, and may suggest additional therapeutic targets. Future studies will also include cilastatin delivery timing, dosage, and formulation.

Abstract 2: Pregnancy after acute kidney injury results in placental insufficiency and perinatal mortality

Authors: Jessica F Hebert PhD, Yoshio Funahashi MD, PhD , Jackie Emathinger PhD, Adam Munhall BS, Megan Nickerson BS, Tahnee Groat MPH, Susan Gurley, MD, Michael P Hutchens MD, MA

Introduction: Rhabdomyolysis-induced acute kidney injury (RIAKI) is the sudden onset of renal cell injury following muscular trauma. RIAKI is a model of AKI particularly relevant to people of childbearing age as it may occur following crush trauma from accidents, combat, or overexertion. Although functional recovery in a few days is common, future onset chronic kidney disease and preeclampsia have been linked to prior AKI.

Objective: We modeled RIAKI in mice to test the hypothesis that recovered parental AKI mediates critical determinants of adult disease in offspring.

Methods: Procedures in 8-week-old male and female C57BL/6 mice were approved by institutional IACUC. Rhabdomyolysis was induced via intramuscular injection of 50% glycerol (8 mL/kg) while shams were untreated. AKI was assessed 24 hours later via glomerular filtration rate (GFR; $\mu\text{L}/\text{min}/100\text{g}$ body weight). GFR was repeated 2 weeks post-recovery to establish functional renal recovery, followed by sham/sham and RIAKI/RIAKI matings for 1) fetal and placental metrics at day 16.5 (term 19) gestation and 2) post-birth offspring success as measured by GFR, fetal/placental weight, offspring weights, and ELISA analysis of tissues for angiotensin II (AngII), a marker of vasoconstriction and renal dysfunction.

Statistics were assessed by t-test for 2 group analyses, and two-way ANOVA with posthoc tests for breeding and fetal sex analyses.

Results: At gestational day 16.5, pups from RIAKI pairings had lower fetoplacental ratios than those from sham pairings ($p=0.05$). There was no difference in mating efficiency or litter size between RIAKI and sham pairs. However, perinatal death was higher in pups from RIAKI pairings ($p<0.05$), with a relative risk of death before weaning at 3.14 times that of offspring of sham pairings.

Pups from RIAKI pairs were born smaller ($p<0.01$) and grew less rapidly in the first week ($p<0.01$) than pups from sham pairs. By two weeks old, pup weights were no longer different between groups. Ang II kidney tissue content varied by offspring sex. Females from sham pairings had more AngII than males ($p<0.05$), but female offspring of RIAKI breedings had comparatively lower AngII than sham females ($p<0.05$).

Conclusions: This is the first report of placental insufficiency and fetal growth restriction following recovered RIAKI. A history of parental RIAKI results in growth-restricted offspring and placental insufficiency similar to other models of fetal growth restriction, suggesting the possibility of similar long-term disease in offspring of parents with recovered AKI. Despite apparent recovery, individuals with RIAKI may have risk of future gestational disease that affects offspring. This study suggests that recovered RIAKI poses a risk for future gestational and offspring disease and demonstrates that the post-injury period represents an opportunity to mitigate this risk to reproductive health.

Abstract 3: Specific therapy for myoglobin-induced acute kidney injury in a combat-relevant, large animal polytrauma model

Authors: Marissa Beiling MD, Adam C. Munhall BS, Joseph Garay PhD, Megan N. Nickerson BS, Martin Schreiber MD, Michael P. Hutchens MD, MA

Introduction: Hyperkalemia is a frequent and potentially lethal complication of AKI in critically injured warfighters. Rhabdomyolysis-induced acute kidney injury (RIAKI) induces hyperkalemia due to increased potassium release from injured muscle and decreased potassium clearance due to myoglobin induced kidney injury. Polytrauma induces AKI by multiple mechanisms, and in the absence of visible large muscle crush injury, the contribution of myoglobinemia to AKI has not been defined. We quantified plasma and urine myoglobin, creatine kinase, and kidney injury markers and tested the effect on hyperkalemia interventions of cilastatin, a renal myoglobin uptake inhibitor which may be a specific therapy for myoglobin- induced kidney injury.

Methods: Anesthetized swine (38-45kg) sustained either thigh injury (TI) or a pulmonary contusion with liver injury (PCLI). Under general anesthesia maintained with ketamine and isoflurane, animals were prepared with arterial and venous catheters, an endotracheal tube, and a urinary catheter placed by midline laparotomy. Musculoskeletal injury was induced with an explosive-driven captive bolt. Thigh injury was produced with up to four total impacts to the muscular portion of the inner hind leg. PCLI included an impact to the chest followed by chest tube placement and laparotomy with subsequent grade V liver laceration causing standardized hemorrhagic shock prior to packing and hemostasis. 30 minutes after injury, animals received a bolus of either cilastatin sodium (100 mg/kg in 500 mL PlasmaLyte) or placebo followed by protocolized resuscitation and initiation of maintenance intravenous fluids (IVF.) The surgical team was blinded to study drug/placebo assignment and drug bags were opacified. Animals were kept under general anesthesia for 48 hours while undergoing blood draws and urine collection at 2, 6, 12, 18, 24 and 48 hours. Hyperkalemia was treated for potassium levels over 5.0 with a standard regimen (1g calcium, 10 units of insulin, and 50g dextrose) and hypotension (MAP<40) was treated with a 10mL/kg bolus of lactated Ringer's solution. Sham procedure consisted of identical surgical preparation without impact or liver laceration.

Glomerular filtration rate (GFR) was measured at 6, 24, and 48 hours after injury by liquid chromatography/mass spectrometry quantification of iohexol in serial timed blood draws following iohexol bolus injection. CK was determined using a commercial kinetic assay. Plasma myoglobin was measured by ELISA. Kidney samples were formalin-fixed and paraffin-embedded prior to staining with anti-myoglobin antibody for immunofluorescence studies. Statistical analysis was performed in R and Prism, using Student's t-test for parametric data and Mann-Whitney for categorical, non-parametric data (hyperkalemia interventions).

Results: Animals subjected to PCLI or TI developed AKI (mean 6h GFR in PCLI/TI 74.5 ± 27.1 vs Sham: 117.59 ± 9.34 mL/min, $p < 0.01$). AKI was similar between models. 6-hour GFR was similar between TI and PCLI (108.8 ± 49.0 mL/hr in TI, 69.6 ± 17.2 in PCLI $p = 0.46$), as was 24h serum creatinine (2.5 ± 0.3 in TI vs. 2.6 ± 0.7 in PCLI, $p = 0.7$). Renal immunofluorescence demonstrated myoglobin in proximal tubular cells and within medullary casts in both models. Peak plasma Mb and Plasma CK and were similar in both models and occurred at similar time points. Peak Mb in TI was 2512.4 ± 930.7 vs. PCLI 2999.8 ± 2697.6 ng/mL, $p = 0.65$. Peak CK was also similar: 294.5 ± 52.1 IU/L in TI and 192.6 ± 134.1 in PCLI, $p = 0.15$. Additionally, the number of hyperkalemia interventions in TI was similar to that in PCLI (3 ± 1.4 in TI, 3.4 ± 1.8 in PCLI, $p = 0.76$). In both models, cilastatin administration 30 minutes after injury resulted in reduced number of hyperkalemia interventions in the first 12 hours (vehicle 4.4 ± 1.5 interventions, cilastatin 2.0 ± 0.0 interventions, $p = 0.008$ by Mann-Whitney).

Conclusions: Thigh impact and pulmonary contusion with liver injury both elevate plasma Mb and CK and cause similar levels of AKI, associated with deposition of myoglobin in kidney tissue. These data suggest that myoglobinemia with AKI may be a feature of polytrauma which includes blunt impact. In both models, cilastatin reduced the need for hyperkalemia interventions, suggesting this drug may have translational utility for this lethal complication of polytrauma. Study characterizing this role in large animals is ongoing.

Abstract 4: Rhabdomyolysis-Induced Acute Kidney Injury and Treatment are Impacted by Legal Performance-Enhancing Drugs

Authors: Jessica F Hebert PhD, Megan N Nickerson BS, Tahnee Groat MPH, Michael P Hutchens MD

Introduction: Caffeine and ibuprofen improve exertional performance and are commonly used by warfighters to enhance readiness. Excess physical exertion and crush, burn or blast injury can cause

rhabdomyolysis and induce acute kidney injury (AKI). AKI strongly associates with mortality in evacuated/critically injured soldiers. Both caffeine and ibuprofen mediate renal function. Therefore, investigation of mitigating and exacerbating factors in AKI is vital to survival, return to service, and readiness. As part of ongoing investigation into putative specific therapy for rhabdomyolysis, we tested the hypothesis that caffeine and ibuprofen worsen rhabdomyolysis-induced AKI (RIAKI) in a mouse model. We also tested whether supportive care for RIAKI is impacted by caffeine or ibuprofen use.

Methods: Procedures in 8-10-week-old wild-type (WT) male C57BL/6 mice were approved by institutional IACUC. Experimental rhabdomyolysis was induced via intramuscular injection of 50% glycerol (8 mL/kg) to the bilateral hindlimbs. Administration of caffeine (CAF, 3 mg/kg, n=12, approximate human equivalent: 12-ounces of strong coffee) was performed via subcutaneous injection 30 min before induction of rhabdomyolysis, and caffeine controls (n=12) received an equal volume of the diluent subcutaneously. In a separate group, administration of ibuprofen (IBU, 10 mg/kg, n=8, approximate human equivalent 3-4 200 mg tablets, a commonly used and widely-recommended dose), was performed via intraperitoneal injection 30 min before induction of rhabdomyolysis, and controls (n=9) received intraperitoneal corn oil and subcutaneous fluid equivalent to that given to caffeine-treated mice to control for the subcutaneous injection. Resuscitation/treatment groups received resuscitation fluid at the initial dose recommended by current clinical guidelines (PlasmaLyte-A, 20 mL/kg) via subcutaneous injection in addition to caffeine (n=12) or ibuprofen (n=9). Urine was collected for 24h in metabolic cages. Measurement of glomerular filtration rate (GFR) was performed 24h after injury using a fluorescence detector (Medibeacon), following retroorbital injection of FITC-Sinistrin.

Statistical analysis was performed in Prism using ANOVA with post-hoc Sidak or Brown-Forsyth and Welch ANOVA with Dunnett's T3 post-hoc test as appropriate. To control for missed glycerol injections (which fail to cause kidney injury), unbiased/blind statistical outlier removal was performed using the Motulsky/ROUT/FDR method with Q=0.01, removing a maximum of 2 values per 3-group dataset. Values are presented as mean $\pm$ standard deviation.

Results: Glycerol injection caused AKI in all mice, reducing GFR by 50% (1307 $\pm$ 72 in sham, 657 $\pm$ 352 μ L/min/100g in glycerol-injected mice, p<0.001) and increasing blood urea nitrogen by more than two-fold (BUN, 24.0 $\pm$ 0.2 in sham, 69 $\pm$ 44 mg/dL, p=0.02 in glycerol-injected mice). Caffeine administration did not worsen RIAKI, modestly increasing BUN, but not significantly altering GFR or urine output (BUN 108 $\pm$ 64 in CAF vs. 68 $\pm$ 25 mg/dL in respective control, p<0.01). Treatment of caffeine-treated RIAKI mice with 20 mL/kg PlasmaLyte did not significantly alter the elevated BUN (mean BUN in CAF+PLAS 108 $\pm$ 64 mg/dL, not significant compared with CAF).

Ibuprofen administration greatly worsened RIAKI, reducing GFR by 98% and causing significant oliguria and elevation of BUN (GFR 14.3 $\pm$ 19.5 vs 577.4 $\pm$ 454.6 μ L/min/100 g in control, urine output 0.5 $\pm$ 0.4 in IBU vs 2.7 $\pm$ 1.7 mL/24h in control, p=0.2., BUN 264 $\pm$ 201 in IBU vs 66 $\pm$ 21 mg/dL in control, p=0.02). Treatment with PlasmaLyte improved oliguria, but did not alter GFR or BUN, indicating that RIAKI treatment efficacy is mitigated in the presence of IBU (GFR 169.5 $\pm$ 190.1 μ L/min/100g, p=0.08 compared with IBU alone, urine output 2.4 mL/24h, p=0.03 compared with IBU alone, BUN 189 $\pm$ 120, p=0.45 compared with IBU alone).

Conclusions: Taken together these data indicate that ibuprofen given at commonly used doses worsens RIAKI, although caffeine does not. Additionally, supportive treatment for RIAKI is ineffective in reversing the second hit conferred by ibuprofen, suggesting that IBU may pose challenges for resuscitation and treatment of warfighters with RIAKI. Although this study is limited to a single dose of each drug, and by being performed in a small animal model, study of putative RIAKI therapies may consider this data as suggesting a possible mechanism for translational challenge.

Abstract 5: Rhabdomyolysis-induced acute kidney injury in reproductive-age mice causes delayed pregnancy and offspring abnormalities

Authors: Jessica F. Hebert PhD, Yoshio Funahashi, PhD, MD, Jackie Emathing PhD, Adam Munhall BS, Megan Nickerson BS, Tahnee Groat MPH, Susan Gurley MD, Michael P Hutchens, MD, MA.

Introduction: Rhabdomyolysis-induced acute kidney injury (RIAKI) is the sudden onset of renal cell injury and functional impairment following muscular trauma as a result of renal cell interaction with released muscle myoglobin. RIAKI can be caused by excessive physical training or crush, burn or blast injury and is associated with mortality in critically injured war fighters. Apparent functional renal recovery is common in a matter of days following aggressive fluid resuscitation. However, future onset chronic kidney disease and preeclampsia have been linked to prior AKI. Mitigation of these hazards would have implications for return to service and to the reproductive health of both male and female military veterans. Therefore, we modeled RIAKI in mice to test the hypothesis that recovered parental AKI mediates critical determinants of adult disease in offspring.

Materials and Methods: Procedures in 8-week-old wild-type (WT) male and female C57BL/6 mice were approved by institutional IACUC. Rhabdomyolysis-induced AKI (RIAKI) was generated via intramuscular injection of 50% glycerol (8 mL/kg), while shams were untreated. Urine was collected for 24h in metabolic cages to analyze RBP4 (ELISA, Abcam), a molecular marker for renal injury, then AKI was assessed via glomerular filtration rate (GFR; $\mu\text{L}/\text{min}/100\text{g}$ body weight). Briefly, fluorescein isothiocyanate (FITC)-Sinistrin elimination was measured transcutaneously using a fluorescence detector (MediBeacon). Data were acquired for 90 min and GFR was calculated using the half-life ($t_{1/2}$). GFR was repeated 2 weeks post-recovery to establish functional renal recovery, followed by establishing sham/sham and RIAKI/RIAKI matings for 1) fetal and placental metrics at day 16.5 (term 19) gestation and 2) post-birth offspring success.

1. Gestational Day 16.5 (near term): sham/sham and RIAKI/RIAKI pairings were bred overnight to ensure day of pregnancy. Pregnancy was confirmed by visualizing plug after mating and weight shift of 3+ grams by day 13.5. 24 hours before euthanasia, pregnant dams were placed in metabolic cages for urine collection, and GFR was assessed the day of procedure. At day 16.5, dams were sacrificed by tribromoethanol injection. Maternal blood, kidney, and heart were collected. Fetus and placenta were separated and weighed; placental insufficiency was calculated by fetoplacental ratio: fetal weight (g)/placental weight (g), with a smaller ratio indicating insufficiency. Placenta, fetal liver and kidneys were collected for later analysis.

2. Birth to 12 weeks postnatal: pairs were evaluated for mating efficiency by determining days between mating and birth. Perinatal loss was considered a dead or missing pup between birth and weaning (3 weeks). Pups were weighed at birth and once per week thereafter for 12 weeks. Offspring renal function was assessed by GFR and blood, kidney, heart, and brain saved for later study. 12-week-old offspring renal tissue was processed for proteins and angiotensin II (AngII), a marker of vasoconstriction and renal dysfunction, was assessed by ELISA. Statistics were assessed by t-test for 2 group analyses, and two-way ANOVA with posthoc tests for breeding and fetal sex analyses.

Results: GFR was reduced in RIAKI breeders ($n=6$ each: males: 618 vs 1304, $p=0.005$; females: 688 vs 1367 $\mu\text{L}/\text{min}/100\text{g}$, $p=0.003$) 24h post-procedure with high levels of urinary RBP4 also observed ($p<0.001$ vs. shams). Recovery was demonstrated in all animals; at 2 weeks, GFR in RIAKI animals was not different from that of shams. At gestational day 16.5, pups from RIAKI pairings had lower fetoplacental ratios than those from sham pairings ($p=0.05$). There was no difference in mating efficiency or litter size between RIAKI and sham pairs. However, perinatal death was higher in pups from RIAKI pairings ($p<0.05$), with a relative risk of death before weaning at 3.14 times that of offspring of sham pairings.

Pups from RIAKI pairs were smaller ($p<0.01$) and grew less rapidly in the first week ($p<0.01$) than pups from sham pairs. By two weeks old, pup weights were no longer different between groups. Angiotensin II (angII) kidney tissue content varied by offspring sex. Females from sham pairings had more AngII

than males ($p < 0.05$), but female offspring of RIAKI breedings had comparatively lower AngII than sham females ($p < 0.05$).

Conclusions: This is the first report of placental insufficiency, and fetal growth restriction following recovered RIAKI, a model of AKI relevant to people of childbearing age. A history of parental RIAKI results in growth-restricted offspring and placental insufficiency similar to other models of fetal growth restriction, suggesting the possibility of similar long-term disease in offspring of parents with recovered AKI. Despite apparent recovery, individuals with RIAKI may have risk of future gestational disease that affects offspring. The differences in AngII in 12-week-old offspring kidneys suggest a potential for a sex-dependent compensatory mechanism to be explored further. This study suggests that recovered RIAKI poses a risk for future gestational and offspring disease and suggests that the post-injury period represents an opportunity to mitigate this risk to reproductive health, thus having implications for return to service and force readiness.

Mahaba Eiwaz
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EXPERIENCE

5/25/2018 – PRESENT

Research Assistant II, Portland VA Research Foundation (PVARF), in Dr. Michael P Hutchens lab.

The Hutchens lab studies muscle-kidney interactions in critical illness, focusing on novel signaling and transport mechanisms that contribute to acute and chronic kidney disease.

EDUCATION

06/2018

Bachelor of Science, Portland State University.

Post-baccalaureate: 4.0 GPA

Skills 2018-2022

- GFR Measurement with retroorbital injection: 4 years.
- Small animal surgical preparations: 4 years
- Perfusion Fixation, terminal exsanguination: 4 years
- Measurement of urine volume and protein content: 4 years
- Perform mice autopsy: 4 years
- Breed and maintain mice population: 3 years
- Protein extraction from biological samples: 4 years
- Cell culture technique, transfections, stable cell line development (primary cells): 4 years
- Experimental and analytic laboratory techniques: 4 years
- Running PCR, Performing DNA/RNA detection using fully automated and semi-automated systems: 4 years
- Perform immunoassays: 4 years
- Gel electrophoresis and Blotting techniques Ex: Western Blot, urine electrophoresis: 4 years
- Quantification of tubulointerstitial fibrosis markers: 4 years
- Cell culture technique, transfections, stable cell line development (primary cells): 4 years
- Maintains inventories of chemicals, supplies, equipment, office supplies, and other items: 4 years
- Electron Microscopy
- Trained and oversee much more experienced and junior staff on the team
- Analyze problems, diagnose root cause, and apply corrective actions, and provide alternative strategies :4 years

List of Published Work

Papers

- Wakasaki R, **Eiwaz M**, McClellan N, Matsushita K, Golgotiu K, Hutchens MP. Automated systematic random sampling and Cavalieri stereology of histologic sections demonstrating acute

tubular necrosis after cardiac arrest and cardiopulmonary resuscitation in the mouse. *Histol Histopathol.* 2018;33(11):1227-1234. doi:10.14670/HH-18-012

- Wakasaki R, Matsushita K, Golgotiu K, Anderson S, **Eiwaz MB**, Orton DJ, Han SJ, Lee HT, Smith RD, Rodland KD, Pichowski PD, Hutchens MP. Glomerular filtrate proteins in acute cardiorenal syndrome. *JCI Insight.* 2019 Feb 21;4(4): e122130. doi: 10.1172/jci.insight.122130. PMID: 30829647; PMCID: PMC6478405.
- Matsushita K, Saritas T, **Eiwaz MB**, McClellan N, Coe I, Zhu W, Ferdaus MZ, Sakai LY, McCormick JA, Hutchens MP. The acute kidney injury to chronic kidney disease transition in a mouse model of acute cardiorenal syndrome emphasizes the role of inflammation. *Kidney Int.* 2020 Jan;97(1):95-105. doi: 10.1016/j.kint.2019.06.022. Epub 2019 Aug 1. PMID: 31623859; PMCID: PMC6957079.
- Matsushita K, Mori K, Saritas T, **Eiwaz MB**, Funahashi Y, Nickerson MN, Hebert JF, Munhall AC, McCormick JA, Yanagita M, Hutchens MP. Cilastatin Ameliorates Rhabdomyolysis-induced AKI in Mice. *J Am Soc Nephrol.* 2021 Oct;32(10):2579-2594. doi: 10.1681/ASN.2020030263. Epub 2021 Aug 2. PMID: 34341182; PMCID: PMC8722809.
- Funahashi Y, Chowdhury S, **Eiwaz MB**, Hutchens MP. Acute Cardiorenal Syndrome: Models and Heart-Kidney Connectors. *Nephron.* 2020;144(12):629-633. doi: 10.1159/000509353. Epub 2020 Aug 19. PMID: 32814315.
- Burfeind KG, Funahashi YF, **Eiwaz MB**, Munhall A, Hutchens MP. Natural killer lymphocytes mediate renal fibrosis due to acute cardiorenal syndrome. *Kidney International.* (In preparation)

Published abstracts.

- Hutchens MP, **Eiwaz MB**, Matsushita K. Myocardiocyte transcription factor cardiac LIM protein (CSRP3) mediates renal tubular transcription. American Society of Nephrology. Kidney Week. Accessed November 9, 2019.
- Hutchens, M., Gurley, S. B., **Eiwaz, M. B.**, Lu, T., & Lim, K. (n.d.). Elevated left ventricular cardiac LIM protein (CSRP3) in ESRD. American Society of Nephrology. Kidney Week. Accessed November 9, 2019.
- Hebert, J., Burfeind, K. G., Nickerson, M. N., Funahashi, Y., **Eiwaz, M. B.**, Groat, T., Hutchens, M. (n.d.). Cilastatin inhibits renal myoglobin endocytosis and AKI following rhabdomyolysis. American Society of Nephrology. Kidney Week. Accessed November 04, 2021.
- Burfeind, K. G., Funahashi, Y., Munhall, A. C., **Eiwaz, M. B.**, Hutchens, M. P. (n.d.). Immune cells as drivers of kidney myofibroblast formation and fibrosis after acute cardiac dysfunction. American Society of Nephrology. Kidney Week. Accessed November 06, 2021.
- Yoshio, F., **Mahaba, E. B.**, Jessica, H., Kevin, B., Adam, M., Megan, N., Tahnee, G., Michael P Hutchens Role of megalin and sex in AKI-CKD transition due to cardiorenal syndrome type 1. IARS, International Anesthesia Research Society.

Presentations.

- Breeding Lrp2-floxed Ndr1-cre mice. **Mahaba B Eiwaz**, Michael P Hutchens.
- Deletion of renal megalin does not alter the severity of acute kidney injury in a cardiorenal syndrome type 1 mouse model. Yoshio Funanashi, **Mahaba B Eiwaz**, Michael P Hutchens

Posters (selected)

- Hutchens, M., Gurley, S. B., **Eiwaz, M. B.**, Lu, T., & Lim, K. (n.d.). Elevated left ventricular cardiac LIM protein (CSRP3) in ESRD. American Society of Nephrology. Kidney Week. Accessed November 9, 2019.

- Funahashi, Y., **Eiwaz, M. B.**, Hutchens, M. P. (n.d.). Deletion of renal megalin does not affect AKI to CKD transition in a cardiorenal syndrome type1 mouse model. Research week, Department of Anesthesiology & Perioperative Medicine, Oregon Health & Science University (OHSU).
- Yoshio, F., **Mahaba, E. B.**, Jessica, H., Kevin, B., Adam, M., Megan, N., Tahnee, G., Michael P, H. (n.d.). Role of megalin and sex in AKI-CKD transition due to cardiorenal syndrome type1. International Anesthesia Research Society.
- F. Hebert, J., Matsushita, K., Nickerson, M., **Eiwaz, M. B.**, Groat, T., Funahashi, Y., McCormick, J., Hutchens, M. P. (2021). Rapid interference with crush syndrome-induced myoglobin endocytosis in the kidney. Department of Anesthesiology & Perioperative Medicine, Oregon Health & Science University (OHSU).
- F. Hebert, J., Burfeind, K., Matsushita, K., Nickerson, M., **Eiwaz, M. B.**, Groat, T., Funahashi, Y., Hutchens, M. P. (2021). Cilastatin inhibits renal myoglobin endocytosis and acute kidney injury following rhabdomyolysis. American Society of Nephrology. Kidney Week.

Activities (selected)

- Mater chemistry volunteer in the Oregon Museum of Science and Industry (OMSI) chemistry lab. Out of six hundred volunteers, museum lab leaders elected me as the spotlight volunteer of the month in October 2016, and in 2020 I was recognized for my efforts with the 110% award. Every weekend, wearing my red lab coat, I get to interact with tourists and Oregonians who visit the museum. At the same time, I teach middle schoolers chemistry, performing public demonstrations in the lab. I also teach new volunteers how to run the chemistry lab. This work has allowed me to pursue my passion for improving access to science labs for young students from low-income homes.
01/2015-08/2021. 1304.460 hrs.
- I worked as a learning assistant for my general chemistry professor, Dr. Eric Sheagley. Dr. Shealey teaches general chemistry course and is the general chemistry lab coordinator at Portland State. Learning Assistant: Teaching classes 09/2015-04/2017. 120 hrs.
- I worked as a chemistry workshop leader under the supervision of Dr. Eric Sheagley. In class, I had the flexibility to use different instructional approaches. Workshop mentor: 05/2016-05/2018. 108 hrs.
- I worked as a professor's assistant with Professor Cliff Breedlove in the Department of World Languages at Portland state university. Knowing the challenge of learning new languages firsthand, I wanted to help others learning new languages as adults. I taught undergraduate and graduate students Arabic. Leading lectures during reading and communication sessions taught me there is common ground no matter where people come from. 05/2017-05/2018. 55 hrs.