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TITLE: Developing a Novel Therapy for Rhabdomyolysis

PRINCIPAL INVESTIGATOR: Michael Hutchens

CONTRACTING ORGANIZATION: Oregon Health & Science University (OHSU), Portland, OR

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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 mouse 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 glomerular filtration rate were not altered by cilastatin administration. Further, cilastatin-dependent protection from rhabdomyolysis-induced AKI is absent in mice without proximal tubule megalin.		

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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
2. Rhabdomyolysis (rh)
3. Acute kidney injury (AKI)
4. Chronic kidney injury (CKI)
5. Megalin (LRPs)
6. Proximal tubule epithelial cells (PTECs)
7. Cilastatin
8. Glomerular filtration rate (GFR)
9. Myoglobin
10. Kidney
11. C57b1/6 mice
12. Swine
13. Hyperkalemia
14. Multitrauma
15. Injury (blast/burn/musculoskeletal)
16. Renal

3. ACCOMPLISHMENTS:

What were the major goals of the project?

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

Milestones for Goal 1, Year 1:

- Obtain IACUC approval from the PVAMC for mouse experiments. Obtain ACURO approval. Recruit and hire staff. Initiate Aim 1 experiments. *100% complete. IACUC approval for mouse experiments has been achieved. Staff has been recruited and hired. Aim 1 experiments commenced.*
- 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.*

Milestones for Goal 2, Year 1:

- Obtain IACUC approval from OHSU and ACURO approval. Enter FDA pre-IND consultation program. *90% completed. IACUC and ACURO approvals were achieved. We are in preliminary stages with the pre-IND consultation program, the next step is to be assigned a division for oversight.*
- 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. *66% complete. The training of the Schreiber lab has been completed. Experimental guidance materials and manuals have been drafted and are being finalized. Initial FDA pre-consultation is as described above.*
- 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. *33% completed. Randomization scheme for pigs is complete. First 2 of 9 pigs have gone through the model, with measurements of GFR conducted by OHSU bioanalytical core.*
- Complete interim analysis after 9 animals. Adjust 2.1 goals if necessary. Continue randomized experiments. *22% complete. 7 animals remain to study in order to complete this milestone.*

What was accomplished under these goals?

Some findings from the inception of this project have just been accepted to the Journal of the American Society of Nephrology in a manuscript titled, *Cilastatin ameliorates acute kidney injury due to rhabdomyolysis in mice*. See Appendix 1.

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. Author IACUC and ACURO protocols
 - b. Accrue GFR (instant-read data) and samples for additional assays at later time
 - c. Mouse experiments to characterize awake GFR and cilastatin response
 - d. 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 objectives b and c
3. Significant results/Key outcomes:
 - a. Approvals obtained
 - b. GFR and other data accrued for 2 time points, 2 doses, sham, and vehicle groups

- i. 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.
 - ii. Options for redesign of experiments include altering the model (e.g., by increasing the water deprivation time or glycerol dose) or altering the timing of GFR measurement (to an earlier time point). We are currently preparing a revision IACUC/ACURO protocol to allow testing of these changes.
 - iii. 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 (Figure 1).
- c. Data for Aim 1.2 acquired for 2 cilastatin doses (200 mg/kg and 100 mg/kg). Efficacy not different at these doses; experiments paused while ensuring drug purity and *in vitro* efficacy.

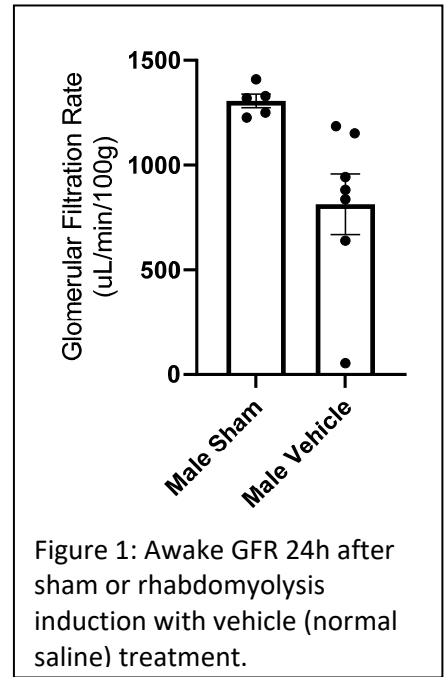


Figure 1: Awake GFR 24h after sham or rhabdomyolysis induction with vehicle (normal saline) treatment.

4. Other achievements:

- a. Because of supply delays, a change in available cilastatin formulations, and challenges with the mouse model, we were required to validate an effective cilastatin preparation mechanistically. Therefore, we:
 - i. Surveyed available cilastatin preparations and tested several, optimizing formulation.
 - ii. Conducted analysis of endocytic puncta in kidneys of cilastatin and vehicle-treated mice. Cilastatin treatment results in smaller endocytic puncta which contain less myoglobin than vehicle treatment (Figure 2).
 - iii. Conducted analysis of urine of cilastatin-treated mice. Cilastatin-treated mice elevate RBP4, myoglobin, and other megalin ligands in their urine at both the 200mg/kg dose and the 100mg/kg dose.

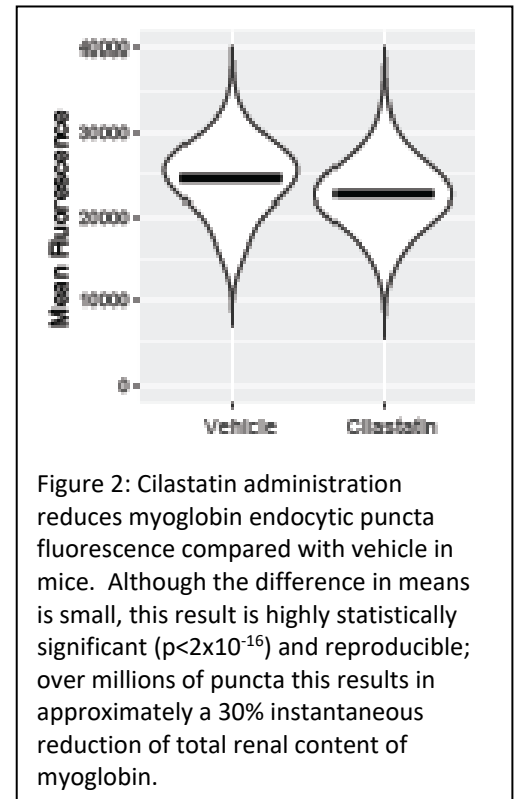


Figure 2: Cilastatin administration reduces myoglobin endocytic puncta fluorescence compared with vehicle in mice. Although the difference in means is small, this result is highly statistically significant ($p < 2 \times 10^{-16}$) and reproducible; over millions of puncta this results in approximately a 30% instantaneous reduction of total renal content of myoglobin.

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

1. Major activities:

- a. Author IACUC/ACURO protocols and obtain approvals
- b. Develop swine protocols for assay acquisition
- c. Author SOPs
- d. Develop workflow including pathology and all other assay workflows
- e. Begin experiments on 1st 9 pigs, training surgical team
- f. Assess adherence to SOPs
- g. Assess and modify SOPs for unexpected challenges
- h. Perform and validate early assays
- i. Prepare and test preparation and method to deliver cilastatin and vehicle

2. Specific objectives:

- a. Obtain approvals from IACUC/ACURO regulatory offices
- b. Establish working protocols in the Schreiber lab.
- c. Finalize SOP's with Andeen lab and generate images
- d. Set randomizing scheme
- e. Develop and communicate workflow for pathology and assays with Dr. Andeen
- f. Begin swine experiments
- g. Assess adherence to SOPs and workflows as project is ongoing
- h. Assess and modify SOPs/workflows when necessary
- i. Perform and validate early assays
 - i. Pig myoglobin immunofluorescence
 - ii. Pig urine protein, urine, and plasma myoglobin, RBP4 assays
 - iii. Prepare and test preparation and method to deliver cilastatin and vehicle
 - iv. LC/MS method for iohexol GFR

3. Significant results/Key outcomes:

- a. Approvals for IACUC/ACURO obtained
- b. Protocols developed for Schreiber team and tested on samples from prior experiments and 2 experimental pigs
 - i. SOP for kidney perfusion/procurement/testing (see Appendix 3)
 - ii. SOP for tissue/blood collection (see Appendices 4a-4c)
- c. SOPs in practice
- d. Workflows for pathology and assays written, tested, and in practice
- e. Surgical and critical care management training completed.
- f. Experiments on pigs began in June of 2021, first 2 of 9 pigs complete
 - i. Each of the first 2 pigs had to be euthanized before the model was complete.

1. Pig 1 was euthanized for respiratory failure/ARDS. All assessments were completed beyond the 24-hour mark (Figure 3).

- a. This pig received vehicle
- b. Data analysis is underway
- c. Post procedure review complete, recommendations agreed to by Schreiber lab, surgical/critical care management training augmented.

2. Pig 2 was euthanized for hyperkalemia and near-cardiac arrest. Baseline assessments and all lab draws up to the 24-hour mark were completed.

- a. This pig received cilastatin 200 mg/kg
- b. Data analysis is underway
- c. Post procedure review complete, recommendations agreed to by Schreiber lab, surgical/critical care management training augmented.
- d. Post procedure review revealed pig had persistent diarrhea despite 7-day acclimatization period and was volume-depleted at the time of surgery, greatly increasing risk of acute kidney injury/hyperkalemia and death.
- e. SOP altered to prevent use of pigs with diarrhea.

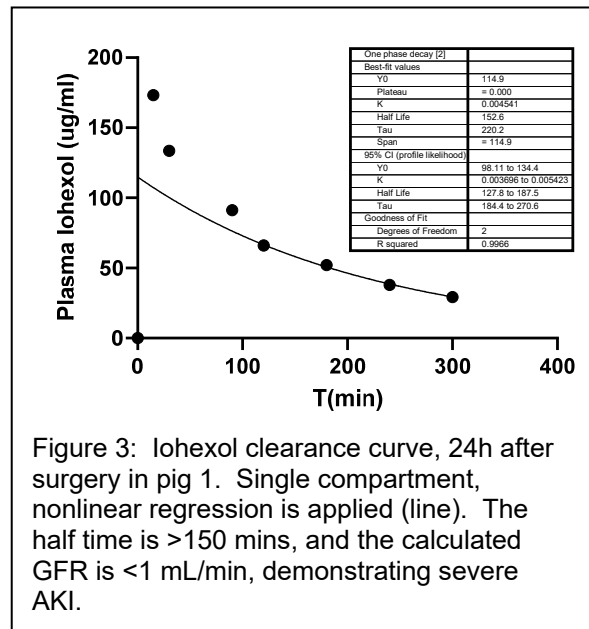


Figure 3: Iohexol clearance curve, 24h after surgery in pig 1. Single compartment, nonlinear regression is applied (line). The half time is >150 mins, and the calculated GFR is <1 mL/min, demonstrating severe AKI.

- ii. Pig urine protein, urine, and plasma myoglobin, RBP4 assay quantification is underway at this writing.
- iii. Plasma myoglobin is greatly elevated in both pigs at some post-surgical timepoints, confirming preliminary data and demonstrating appropriate choice of model.
- iv. All feasibility goals have been met**
- g. Adherence to SOPs assessed and alterations/education addressed to insufficiencies.
- h. Early assays performed
 - i. Pig myoglobin immunofluorescence
 - ii. Pig urine protein, urine, and plasma myoglobin, RBP4 assays
 - iii. Method for preparing/blinding/ testing the delivery of cilastatin and vehicle to pigs established.

4. Other achievements:

An objective was to demonstrate and describe how the administration of the pharmacologic megalin inhibitor cilastatin to wild-type mice recapitulates the renoprotective effects of megalin deletion in the proximal tubule. Cilastatin administration caused selective proteinuria and inhibition of tubular myoglobin uptake similar to that caused by megalin deletion in previous studies (funded by another agency). In the first year of the grant period, we conducted experiments to determine efficacy and mechanism of cilastatin administration. Findings indicate that in the absence of kidney injury, urine output, urine albumin, and glomerular filtration rate were not altered by cilastatin administration. Urine retinol binding protein 4 (RBP4), a plasma protein which is a known megalin ligand, and is greatly increased in the urine of megalin-deleted mice, was nearly doubled by cilastatin administration. These findings are detailed in the appended manuscript:

Matsushita K, Mori K, Saritas T, Eiwaz MB, Funahashi Y, Nickerson MN, Hebert JF, Munhall AC, McCormick JA, Yanagita M, **Hutchens MP**. Cilastatin ameliorates acute kidney injury due to rhabdomyolysis in mice. *Accepted. JASN. 2021*

Discussion of goals not met:

Year 1 achievements were expected to include the completion of the first round of swine and mouse experiments. The SARS-CoV-2 (Covid-19) pandemic had a clear effect on the scientific community as institutions sent personnel home, and then allowed them back only under restrictions. Animal studies at OHSU were paused, and we could not receive pigs to begin the swine model of our study. Additionally, our intended supplier of cilastatin could not meet demands for supply and our lab discovered a discrepancy between the stated formulation of cilastatin and that actually supplied by the original supplier. Fortunately, there are no scientific explanations for goals not met, only logistical. After a pause in the science the project is up and running and at this time, the first two of nine swine have been tested for Aim 2.1 and data analysis is underway for this pragmatic phase of the protocol.

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 bi-monthly basis depending on the trainee's skill set and room for growth. The Postdoctoral Fellow and PI have provided 1:1 hands-on instruction to the Research Assistant to expand mouse-handling skills, specifically, intraperitoneal injections, retroorbital injections, and intramuscular injections. One-on-one mentoring has also provided training in immunofluorescence and histological staining, tissue processing, slide making, microscopy use, and biofluid collection. All staff participate in and attend weekly Anesthesiology and Perioperative Medicine Research Seminars. Three members of the lab presented during OHSU research week, and all members attended. All staff participated in professional development activities 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, and Compliance Program Training.

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, and Conflict Resolutions trainings.

Conferences at which lab members presented science or attended:

Society of Critical Care Anesthesiologists, Association of University Anesthesiologists, International Anesthesia Research Society (presented, May 2021), OHSU Research Week (presented, May 2021), Australian and New Zealand Placenta Research Association (ANZPRA) Virtual Satellite (presented, October 2020), Science Foo Camp (interdisciplinary scientific conference organized by O'Reilly Media, Nature, Google, Digital Science; presented, October 2020).

The Program Manager completed the Winter 2021 Mentoring Intensive Workshop. The Postdoctoral Fellow on this project completed the invitational Vollum Institute Writing Course and was a 2021 Oregon Students Learn and Experience Research (OSLER) TL1 recipient. The OSLER TL1 supports training and career development in clinical and translational research for postdoctoral fellows and is similar to an NIH NRSA T32 grant.

How were the results disseminated to communities of interest?

Pertinent findings to date have been described in a manuscript titled *Cilastatin ameliorates kidney injury due to rhabdomyolysis in mice*, accepted for publication by the Journal of the American Society of Nephrology. An abstract titled *Rapid interference with crush syndrome-induced myoglobin endocytosis in the kidney* has been accepted for presentation at the Military Health System Research Symposium in August, and submissions to the American Society of Nephrology Kidney Week conference are pending.

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 to commence the activities described in the milestones for Year 2. In addition to the milestones laid out in the project narrative, we will also continue testing different cilastatin compounds for efficacy as a means toward identifying a long-term source for cilastatin.

Aim 1 Year 1 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 13-15:** Experiment 1.3 (accelerator administration). Complete assays for 1.2.
- **Months 16-18:** Complete 1.3, initiate 1.4. Complete all assays and data analysis for 1.1-1.3.
- **Months 19-21:** Complete 1.4, initiate 1.5.
- **Months 22-24:** Complete 1.5, initiate 1.6. Second manuscript submission for publication. Submit yearly report.

Aim 2 Year 1 milestones to complete:

- **Months 4-6:** Complete FDA pre-IND consultation (initial) and alter 2.2 if necessary.
- **Months 7-9:** Aim 2.1, complete first animal experimental cohort (final 7 of 9 animals). Initial GFR measurements in OHSU bioanalytical core.
- **Months 10-12:** Complete interim analysis after 9 animals. Adjust 2.1 goals if necessary. Continue randomized experiments.

Aim 2 Year 2 milestones to complete:

- **Months 13-15:** Continue randomized experiments in experiment 2.1. Continue GFR mass spec analysis contemporaneously. Initial cohort to Dr. Andeen for pathologic analysis.

- **Months 16-18:** Continue randomized experiments in experiment 2.1. Continue GFR mass spec analysis contemporaneously. Confirm initial pathology. First manuscript/conference abstracts.
- **Month 19-21:** Complete randomized experiments in experiment 2.1. Start experiment 2.2.
- **Months 22-24:** Complete molecular biology, GFR, molecular biology assays, and other non-pathology data from 2.1. Second manuscript/conference abstracts.

4. IMPACT:

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

Findings from Aim 1 of this project have been accepted for publication by the Journal of the American Society of Nephrology in a manuscript titled, *Cilastatin ameliorates acute kidney injury due to rhabdomyolysis in mice*.

What was the impact on other disciplines?

Postdoctoral fellow Dr. Jessica Hebert was the recipient of an Oregon Students Learn and Experience Research (OSLER) TL1 award for her project, *Long-Term Impact of Acute Kidney Injury: Reproductive, Pregnancy, and Offspring Health*. This grant will cover her salary for one year and affords her myriad professional development opportunities.

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

There have been no changes in approach.

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

There have been challenges due to the Covid-19 pandemic. OHSU instructed all non-essential staff to work from home beginning in March of 2020, and put a hold on hiring, which temporarily delayed filling all of the roles needed for this project. Our department obtained critical employee privileges for staff, enabling us to maintain our mouse colony and commence experiments provided we follow social-distancing protocols; thus limiting the number of people we could have working in the lab simultaneously. Also due to Covid-19, shortages in the raw materials that are essential to every lab (pipette tips, gloves, microcentrifuge tubes, etc.) delayed experiments. In order to manage this obstacle, we recycled any materials that could be autoclaved and repurposed, as well as simple and aggressive stockpiling of resources when available. A significant delay in obtaining ready-made cilastatin solution – the product essential to our experiments—was problematic. Our lab had the intention of relying upon Sigma Aldrich for supplying ready-made cilastatin solution. Two challenges arose. First, one of our lab members identified a discrepancy between the advertised contents of Sigma product “Cilastatin, ready-made for injection” and its actual contents based on the MSDS. This discrepancy was confirmed by direct contact with the company, resulting in a revision of the company web page advertising the product. We then had to re-confirm our preliminary data with the correct vehicle for the product (this experiment successfully confirmed that the formulation change was not relevant to our data or further experiments). Second, early in this reporting period Sigma conveyed a delay with no estimated date of delivery for the cilastatin. These orders began to be partially filled after approximately 6 months, and the

amounts were sufficient to commence the mouse experiments. In the interim we experimented with alternative suppliers of ready-made solution (Cayman Chemical, Toronto Chemical), as well as working with more readily available cilastatin sodium powders in combination with various vehicles to reverse engineer a suitable cilastatin solution. This required considerable drug chemistry work and delayed animal experiments. The interruption in the Sigma Aldrich ready-made solution supply was addressed by project staff comparing the cilastatin products procured from competing suppliers to determine the most suitable option for the forthcoming experiments. At this time, we have adequate cilastatin to continue both mouse and swine experiments and are hopeful there will be no further supply restriction; we are preparing to order the remaining full required amount of cilastatin in early year 2. The swine experiments were delayed due to the pandemic as well, due to a pause in animal experiments at OHSU and a moratorium on receiving animals from the livestock provider.

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 1. This surplus is partially due to the hiring hold put in place by OHSU at the beginning of the stay-at-home orders. Also due to Covid-19 precautions, both mouse and swine experiments were initially delayed and/or slowed down to accommodate social distancing directives or to make adjustments for supply chain shortages. We observed delays in invoicing between the Portland VA Medical Center where our physical lab and mouse colony is located, and the grantee institution, OHSU, that suggests funds have been encumbered but are not deducted from our project as of yet. Travel to conferences was prohibited under Covid-19 precautions, and indeed many conferences were cancelled, resulting in unspent travel allotments. Finally, delays in experiments and tissue collection have a downstream, albeit temporary, cost-savings effect on tissue processing and analysis services.

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, nothing to report.

Significant changes in use or care of vertebrate animals:

We previously recorded glomerular filtration rate (GFR) under anesthesia. This is not necessary and is a source of potential bias as the anesthesia requires titration. Therefore, at the beginning of this project we requested approval (and received it) for awake measurements of GFR. An unexpected challenge resulting from this change in methodology has been a reduction in the recorded amount of injury (that is, under anesthesia, the loss of GFR in our model is increased relative to that recorded by awake GFR, because the anesthetic differentially depresses GFR). We are currently determining the most rigorous method to address this challenge and plan to amend our local IACUC and ACURO in the next 60 days with at least one solution.

Significant changes in use of biohazards and/or select agents:

Nothing to report

6. PRODUCTS:

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

1. Matsushita, Katsuyuki; Sanitas, Turgay; Eiwaz, Mahaba; Funahashi, Yoshio; Nickerson, Megan; Hebert, Jessica; Munhall, Adam, McCormick, James; Yanagita, Motoko; Hutchens, Michael. *Cilastatin ameliorates acute kidney injury due to rhabdomyolysis in mice*. Journal of American Society of Nephrology. Accepted for publication. Acknowledgement of federal support: yes.

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

Nothing to report

- **Other publications, conference papers and presentations**

1. Multi-Organ Dysfunction in Prolonged Field Care Scenarios – Rapid interference with crust syndrome-induced myoglobin endocytosis in the kidney.
Abstract #MHSRS-21-03948 Oral presentation. Military Health System Research Symposium (MHSRS). 23-26 August, 2021. Kissimmee, FL.

- **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**

Postdoctoral fellow Dr. Jessica Hebert was the recipient of an Oregon Students Learn and Experience Research (OSLER) TL1 award for her project, *Long-Term Impact of Acute Kidney Injury: Reproductive, Pregnancy, and Offspring Health*. The OSLER program supports training and career development in clinical and translational research for postdoctoral fellows through a TL1 grant and is similar to an NIH NRSA T32 grant.

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: 9
Contribution to Project: Ms. Nickerson assists with lab equipment set up, take-down, cleaning, and maintenance rodent care and observation, lab assay preparation, assessment, and data acquisition, monitoring, and inventory. Ms. Nickerson participates in the rhabdomyolysis model and the glomerular filtration rate and metabolic cage procedures. Ms. Nickerson assists in preparing histologic samples.

Funding Support: Department of Defense.

Name: Maria Luisa Appleman, PhD
Project Role: Schreiber Project Lead
Researcher Identifier: None
Nearest person month worked: 2
Contribution to Project: Dr. Appleman oversees the day to day aspects of the experiments in Schreiber's swine multitrauma models. She scheduled and managed the procedures. Dr. Appleman was responsible for all regulatory activities for the swine ACURO and OHSU IACUC. Dr. Appleman oversees laboratory assays and assists with reports.

Funding Support: Department of Defense.

Name: Andrew Goodman
Project Role: Schreiber Lab Research Assistant 2
Researcher Identifier: None
Nearest person month worked: 1
Contribution to Project: Mr. Goodman assists with animal surgeries and monitoring of swine during experiments. He prepares the surgical area and instruments. He manages logistics for proposed experiments and assists Dr. Appleman and the animal surgeon.

Funding Support: Department of Defense.

Name: James El Haddi, MD
Project Role: Surgeon
Researcher Identifier: (ORCID) 0000-0002-1274-2138
Nearest person month worked: 1
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. Appleman and Mr. Goodman to ensure measurements are obtained as planned.

Funding Support: Department of Defense.

Name: Joseph Garay
Project Role: Research Associate
Researcher Identifier: (ORCID) 0000-0002-2287-3051
Nearest person month worked: 1

Contribution to Project: Mr. Garay 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.

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?

Provide the following information for each partnership:

Organization Name: VA Portland Medical Center

Location of Organization: 3710 SW US Veterans Hospital Rd, Portland, OR 97239

Partner's contribution to the project:

Facilities (project staff use the partner's facilities for project activities)

8. SPECIAL REPORTING REQUIREMENTS:

See Award Chart, page 17.

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 June 2020 – 30 June 2021

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: Accepted for publication: manuscript titled *Cilastatin ameliorates acute kidney injury due to rhabdomyolysis in mice*, Journal of the American Society of Nephrology.

Patents: none to date

Funding Obtained: Oregon Clinical and Translational Research Institute (OCTRI) Oregon Students Learn and Experience Research (OSLER) T1 Award. Long-term Impact of Acute Kidney Injury: Reproductive, Pregnancy, and Offspring Health.

9. APPENDICES:

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Appendix 1: Publication (accepted manuscript)



Cilastatin ameliorates acute kidney injury due to rhabdomyolysis in mice

Journal:	<i>Journal of the American Society of Nephrology</i>
Manuscript ID	JASN-2020-03-0263.R3
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Date Submitted by the Author:	15-Jun-2021
Complete List of Authors:	Matsushita, Katsuyuki; Oregon Health & Science University School of Medicine, Anesthesiology & Perioperative Medicine Mori, Kiyoshi; School of Pharmaceutical Sciences, University of Shizuoka, Department of Molecular and Clinical Pharmacology; Kyoto University Graduate School of Medicine , Medical Innovation Center Saritas, Turgay; University Hospital RWTH Aachen, Division of Nephrology and Immunology Eiwaz, Mahaba; Oregon Health & Science University School of Medicine, Anesthesiology & Perioperative Medicine Funahashi, Yoshio; Oregon Health & Science University School of Medicine, Anesthesiology & Perioperative Medicine Nickerson, Megan; Oregon Health & Science University School of Medicine, Anesthesiology & Perioperative Medicine Hebert, Jessica; Oregon Health & Science University School of Medicine, Anesthesiology & Perioperative Medicine Munhall, Adam; Oregon Health & Science University School of Medicine, Anesthesiology & Perioperative Medicine McCormick, James; Oregon Health & Science University, Nephrology & Hypertension Yanagita, Motoko; Kyoto University Graduate School of Medicine, Department of Nephrology Hutchens, Michael; Oregon Health & Science University School of Medicine, Anesthesiology & Perioperative Medicine; Portland VA Medical Center, Operative Care Division
Keywords:	rhabdomyolysis, endocytosis, acute renal failure, chronic kidney disease, renal protection



Authors: Matsushita, Katsuyuki; Mori, Kiyoshi; Saritas, Turgay; Eiwaz, Mahaba; Funahashi, Yoshio; Nickerson, Megan; Hebert, Jessica; Munhall, Adam; McCormick, James; Yanagita, Motoko; Hutchens, Michael

Title: Cilastatin ameliorates acute kidney injury due to rhabdomyolysis in mice

Running title: Cilastatin and megalin in rhabdomyolysis

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Study Group/Organization Name: CUST_STUDY_GROUP/ORGANIZATION_NAME :No data available.

Study Group Members' Names: CUST_STUDY_GROUP_MEMBERS :No data available.

Total number of words: 4126

Abstract: Background: Rhabdomyolysis, the destruction of skeletal muscle, is a significant cause of acute kidney injury (AKI) and death due to natural disaster and armed conflict. Rhabdomyolysis may also initiate chronic kidney disease (CKD). Development of specific pharmacologic therapy is desirable because supportive care is nearly impossible in austere environments. Myoglobin, the principal cause of rhabdomyolysis-related AKI, undergoes megalin-mediated endocytosis in proximal tubule cells, which are specifically injured.

Methods and Results: Here, we show that inducible proximal tubule-specific deletion of megalin (iMegKO) is highly protective in a mouse model of rhabdomyolysis-induced AKI. iMegKO mice demonstrated preserved glomerular filtration rate, reduced proximal tubule injury (as indicated by kidney injury molecule-1), and reduced renal apoptosis 24h after injury. These effects were accompanied by increased urinary myoglobin clearance. Unlike littermate controls, iMegKO mice also did not develop progressive decline in glomerular filtration rate and persistent new proteinuria. Administration of the pharmacologic megalin inhibitor cilastatin to wild-type mice recapitulated the renoprotective effects of megalin deletion. This effect was dependent on megalin. Cilastatin administration caused selective proteinuria and inhibition of tubular myoglobin uptake similar to that caused by megalin deletion.

Conclusions: We conclude that megalin plays a critical role in rhabdomyolysis-induced AKI, and megalin interference and inhibition ameliorate rhabdomyolysis-induced AKI. Further investigation of megalin inhibition may inform translational investigation of a novel potential therapy.

Significance Statement

Rhabdomyolysis causes severe acute kidney injury (AKI) and death in earthquakes and armed conflict. Specific treatment is not available and current care is difficult in austere environments. Skeletal muscle myoglobin is a renal toxin which causes AKI in this syndrome. Proximal tubular megalin participates in myoglobin endocytosis and may be an AKI mediator. Here, a mouse model is used to demonstrate a critical role for proximal tubular megalin in rhabdomyolysis-induced AKI. Proximal tubule-specific megalin deletion ameliorates this AKI and this effect is recapitulated by administration of cilastatin, a megalin inhibitor. This translational study therefore identifies megalin as a mediator of rhabdomyolysis-induced AKI and suggests a novel mechanism by which it may be possible to ameliorate it.

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7 **Cilastatin ameliorates acute kidney injury due to rhabdomyolysis in mice**
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47 Running title: *Cilastatin and megalin in rhabdomyolysis*
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Abstract

Background: Rhabdomyolysis, the destruction of skeletal muscle, is a significant cause of acute kidney injury (AKI) and death due to natural disaster and armed conflict. Rhabdomyolysis may also initiate chronic kidney disease (CKD). Development of specific pharmacologic therapy is desirable because supportive care is nearly impossible in austere environments. Myoglobin, the principal cause of rhabdomyolysis-related AKI, undergoes megalin-mediated endocytosis in proximal tubule cells, which are specifically injured.

Methods and Results: Here, we show that inducible proximal tubule-specific deletion of megalin (iMegKO) is highly protective in a mouse model of rhabdomyolysis-induced AKI. iMegKO mice demonstrated preserved glomerular filtration rate, reduced proximal tubule injury (as indicated by kidney injury molecule-1), and reduced renal apoptosis 24h after injury. These effects were accompanied by increased urinary myoglobin clearance. Unlike littermate controls, iMegKO mice also did not develop progressive decline in glomerular filtration rate and persistent new proteinuria. Administration of the pharmacologic megalin inhibitor cilastatin to wild-type mice recapitulated the renoprotective effects of megalin deletion. This effect was dependent on megalin. Cilastatin administration caused selective proteinuria and inhibition of tubular myoglobin uptake similar to that caused by megalin deletion.

Conclusions: We conclude that megalin plays a critical role in rhabdomyolysis-induced AKI, and megalin interference and inhibition ameliorate rhabdomyolysis-induced AKI. Further investigation of megalin inhibition may inform translational investigation of a novel potential therapy.

Introduction

Rhabdomyolysis is the destruction of striated skeletal muscle, which causes systemic circulation of the principal protein in muscle, myoglobin. More than 25,000 cases are reported annually in the USA, caused by crush injury, physical training, or medication. Rhabdomyolysis-induced myoglobinemia causes acute kidney injury (AKI); this form of AKI, termed “crush syndrome”, is a significant cause of mortality in earthquakes and armed conflict (1-3). Clinical and translational studies suggest that crush syndrome can lead to chronic kidney disease, which itself is characterized by excess morbidity and mortality (4, 5). Mortality-reducing treatment is limited to intravenous fluid administration (6) and dialysis; the difficulty and expense of such treatments prevents their effective use in austere environments. Specific, simple therapy which is deliverable in austere environments could therefore have clinical importance.

Myoglobin, a small protein (19kD), is abundantly filtered in the glomerulus and reaches the renal proximal tubule, where it injures renal proximal tubular cells, causing rhabdomyolysis-induced AKI (7). Proximal tubular epithelial cells avidly reuptake filtered proteins, primarily through a proximal tubule-specific endocytic complex composed of megalin and cubilin (8), which has many (>40 known) ligands, including myoglobin (9). Once within the endosomal system, megalin ligands undergo endosomal sorting and can undergo subsequent lysosomal degradation or transcytosis; in particular, transcytosis increases the serum half-life of albumin and likely conserves systemic resources by reducing the need for de novo synthesis of important small proteins which would otherwise be excreted in the urine (10). Since proximal tubule cells are extensively injured in rhabdomyolysis-induced AKI, it is possible that megalin-mediated myoglobin retrieval functions in a maladaptive way when excess myoglobin is present.

Therefore we hypothesized that interference with proximal tubule megalin would ameliorate

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3 acute kidney injury due to rhabdomyolysis. We further hypothesized that cilastatin, recently
4 identified as a megalin inhibitor, would ameliorate rhabdomyolysis-induced AKI through action
5 on megalin.
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10 **Methods**

11 Animals

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14 Animal procedures, performed on 8-12 week-old male C57BL/6 mice (obtained from Jackson
15 Laboratories), and 14-32 week old proximal tubule-specific inducible megalin-deleted mice
16 (iMegKO), were approved by the Oregon Health & Science University Institutional Animal Care
17 and Use Committee (#IP0001188) or the Portland VA Medical Center (#4374-18). iMegKO
18 mice were generated and bred as previously described (11). Briefly, Lrp2^{fl/fl} mice (a generous
19 gift of Professor T. Willnow, Max Delbruck Institut) were bred to mice expressing tamoxifen-
20 dependent cre recombinase in cells which express Ndr1 (Ndr1-CreER^{T2}), which is abundantly
21 expressed in the renal proximal tubule (12). Mice were genotyped using quantitative polymerase
22 chain reaction (PCR) by Transnetyx (Cordova, TN) prior to experimentation. Deletion of
23 megalin in the proximal tubule was induced by injection of tamoxifen (150 mg/kg body weight,
24 intraperitoneally daily for 5 days). 15 days after the first tamoxifen injection, mice were used for
25 experiments. In experiments involving iMegKO mice, cre-negative littermates served as
26 controls, and received tamoxifen identically to experimental mice.
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45 Rhabdomyolysis model

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47 We modeled rhabdomyolysis with a well-characterized model, glycerol intramuscular injection
48 of 50% glycerol in normal saline preceded by a period of water deprivation (13-15). Based on
49 preliminary experiments in wild-type mice which demonstrated consistent reduction in 24h urine
50 output and GFR after 4h water deprivation and 8.0 mL/kg glycerol, this regimen was chosen for
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3 all experiments except the 60-day experiment presented in figure 3. In this experiment 6.5
4 mL/kg was used to ensure 100% survival, because long term survival is only translationally
5 relevant in the setting of 100% short-term survival. Glycerol was injected to the anterior thigh
6 muscle (half the dose to each side).
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11 Cilastatin and vehicle injection

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13 Cilastatin, 200 mg/kg, or equivalent volume of vehicle (<150 μ L per mouse) was administered
14 by retroorbital injection contemporaneously with glycerol injection in rhabdomyolysis
15 experiments, or alone in experiments testing the mechanism of cilastatin.
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22 Measurement of urine volume and proteins.

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24 Urine was collected in urine collection cups pretreated with protease inhibitor for 24h, starting
25 immediately after recovery from experimental anesthesia. Total urine protein was measured
26 using bicinchoninic acid. Equal volumes of urine were loaded for urine gel electrophoresis, and
27 gels were stained with Coomassie blue. Urine myoglobin and retinol binding protein 4 (RBP4)
28 were measured by ELISA (see Supplementary table 1 for specific kits).
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36 Measurement of glomerular filtration rate

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38 GFR was measured by determining elimination of fluorescein isothiocyanate (FITC)-sinistrin
39 transcutaneously as described (16) after FITC-sinistrin (75 mg/kg body weight) bolus injection
40 into the retroorbital plexus under isoflurane anesthesia.
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45 Measurement of plasma urea nitrogen

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47 Whole blood was drawn from the cardiac left ventricle at the time of euthanasia, stored in
48 sodium ethylenediamine tetra-acetic acid containing tubes (BD, Franklin Lakes, NJ), and plasma
49 separated by centrifugation. Plasma urea nitrogen was determined using a commercially
50 available colorimetric assay (Invitrogen, Carlsbad CA).
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Measurement of plasma myoglobin and determination of myoglobin clearance

A separate cohort of mice underwent glycerol injection as described above and underwent left ventricular transcardiac puncture 6h later for plasma myoglobin studies. Myoglobin was quantified in plasma samples using a commercially-available ELISA (Mybiosource.com, San Diego, CA). Myoglobin clearance was calculated using the group (strain) mean 24h plasma myoglobin and 24 urine collections from the same groups according to the formula:

$$Cl_{myoglobin} = \frac{Urine_{myoglobin} * Urine_{Volume}}{Plasma_{myoglobin}}$$

Immunofluorescence, immunohistochemistry, and immunoblotting:

A list of antibodies, probes, kits, buffers, and reagents is in supplemental table S1. Kidneys were perfusion-fixed via the left ventricular apex with 4% paraformaldehyde, and 5 μ m thick, paraffin-embedded sections were stained using Periodic acid-Schiff stain, and α -smooth muscle actin (α SMA)-FITC conjugate. Immunofluorescence staining and immunoblotting were performed as described in supplemental methods.

Imaging and semiautomated unbiased stereology

Fluorescence images were captured an epifluorescence microscope (Axio Imager M2, Zeiss, Jena Germany, and Keyence BZ-X800, Itasca IL). To ensure unbiased quantification of the extracellular matrix component α SMA, kidney injury molecule-1 (KIM-1), cleaved caspase-3, and myoglobin, slides containing 5 sagittal kidney sections, cut at 160 μ m intervals starting at random distance from the caudal renal pole, were scanned using a slide scanner (Axioscan, Zeiss, Jena Germany) and semiautomated unbiased stereology was performed using a custom macro and the Fiji ImageJ distribution as previously described (17, 18).

Quantification of myoglobin endocytosis

We characterized *in vivo* endocytosis of myoglobin first in control and iMegKO mice, and then in wild-type mice treated with vehicle or cilastatin. FITC-myoglobin (0.5 mg) was injected retroorbitally, either alone, or 1h after injection of cilastatin (200 mg/kg, retroorbital). 15 or 30 minutes after injection, mice were killed and perfused, and kidney sections prepared. Sections were scanned. Using ImageJ, FITC-positive puncta were identified and segmented by thresholding (identically across all images and treatments), and area and mean fluorescence for each puncta extracted. The number of puncta per unit tissue area, puncta mean area, and puncta mean fluorescence were compared. To test the hypothesis that cilastatin altered overall renal uptake of endogenous myoglobin we administered vehicle or cilastatin, followed 1h later by intramuscular glycerol to wild-type mice as previously, preparing kidney sections 2h after glycerol injection. To compare overall renal myoglobin content, we performed unbiased stereology as previously described.

Statistical analysis

Statistical analysis was primarily performed using Prism 7.0 (GraphPad, LaJolla CA); analysis of endocytic punctae was performed using R (v4.0.5). Two-group comparisons were performed using Student's t-test, with Welch's correction as appropriate. Multiple-group comparisons were performed with ANOVA (or 2-way ANOVA in the case of before-after comparisons in the same mice) with Holm-Sidak's test as appropriate. For the experiment testing effect of cilastatin in iMegKO mice (depicted in figure 6), *a priori* power analysis (performed using R 4.05, package *pwr*) determined the experimental number. Statistical significance was inferred from $p < 0.05$. Mean and standard error are shown in the figures and text.

Results

Renal effects of proximal tubule-specific megalin deletion.

On day 15 after initiating tamoxifen induction of cre recombinase, megalin was absent in LRP2^{fl/fl}-Ndr1^{CreERT2+} (iMeggKO) mice, but remained abundantly expressed in cortex and not expressed in the medulla of LRP2^{fl/fl}-Ndr1^{CreERT2-} (control) mice (figure 1A and B, supplemental figure 1 and 2). Deletion of proximal tubule megalin did not alter renal abundance of cubilin as determined by immunoblot (OD ratio 1.1±0.3 in control *vs.* 0.9±0.2 in iMeggKO, *p*=0.52, figure 1B). Electrophoresis demonstrated low-molecular weight bands in urine from iMeggKO mice and not that of controls (figure 1C), consistent with prior reports in mice with megalin interference (11, 19). To further evaluate this selective proteinuria, we quantified urine retinol-binding protein 4 (RBP4). RBP4 is a megalin ligand (20) which is specifically upregulated in the urine of megalin-deleted mice (21); it was dramatically increased in the urine of iMeggKO mice compared with controls (controls 25.7±6.4 ng/mL *vs.* 184.6±10.9 in iMeggKO, figure 1D). Body weight and urine output were not different between iMeggKO and controls (urine output 4.6±0.4 mL/24h in iMeggKO *vs.* 4.8±0.4 mL/24h in controls, figure 1E). Surprisingly, iMeggKO status conferred 21% reduced baseline glomerular filtration rate (GFR) compared with that of controls (700.3±34.0 μL/min/100g in iMeggKO *vs.* 882.8±32.7 μL/min/100g, *p*<0.001, *n*=11-12, figure 1F). In accordance with prior reports (11), total urine protein and urine albumin were significantly increased by proximal tubule-specific megalin deletion (protein: 0.4±0.1 mg/24h in controls *vs.* 4.0±0.4 mg/24h in iMeggKO, *p*<0.001, albumin: (30.45±5.4 in controls *vs.* 522.2±57.7 μg/24h in iMeggKO, *p*<0.0001, *n*=11-12/group, Figure 1G).

Proximal tubule-specific megalin deletion abrogates rhabdomyolysis-induced acute kidney injury

Six hours after glycerol injection, all mice demonstrated discolored urine, and serum myoglobin was markedly elevated (81.9 ± 6.2 ng/mL at 6h compared with 6.9 ± 1.1 ng/mL 24h after injection, $p < 0.0001$, $n = 9-10$ /group). All mice survived to the planned endpoint. Twenty-four hours after glycerol injection (experimental design, figure 2A), there was noticeable difference in the urine color and quantity between control and iMegKO mice; the latter demonstrated increased quantity (6.0 ± 0.7 vs. 2.4 ± 0.7 mL/24h, $p = 0.008$, figure 2B) of clearer urine than controls. GFR was completely preserved in iMegKO mice (621.1 ± 27.2 μ L/min/100g at baseline vs 572.4 ± 17.1 μ L/min/100g in 24h after glycerol injection, $p = 0.7$, $n = 4$), but dramatically reduced in controls (864.5 ± 62.7 μ L/min/100g at baseline vs 192.5 ± 43.4 μ L/min/100g in 24h after glycerol injection, $p < 0.001$, $n = 5$, figure 2C and supplemental figure 3). This sharp distinction in GFR was reflected in dramatic differences in serum urea nitrogen (146.5 ± 41.6 mg/dL in controls vs. 37.4 ± 3.5 mg/dL in iMegKO mice, $p = 0.03$, figure 2D). Preserved renal function was accompanied by preservation of renal architecture and reduced immunofluorescence signal for injury-specific molecules. Periodic acid-Schiff (PAS) stained sections from control mice demonstrated extensive necrosis, inflammatory cell infiltrate, and protein casts; these were present but attenuated in iMegKO mice, resulting in reduced blinded injury score (430 ± 43 in controls, 247 ± 28 in iMegKO, $n = 4$ /gr, $p = 0.028$, figure 2E). Similarly, acute tubular injury as demonstrated by kidney injury molecule 1 (KIM-1) and tubular apoptosis (determined by cleaved caspase-3 immunofluorescence) were attenuated by iMegKO status (figures 2F and 2G). There was disruption of normal spatial organization of megalin in control mice, this was not observed in iMegKO, due to lack of megalin (supplemental figure 4). Overall, these data demonstrate that proximal tubule-specific megalin deficiency abrogates AKI due to rhabdomyolysis.

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3 Proximal tubule-specific megalin deletion ameliorates rhabdomyolysis-induced progressive GFR
4 loss and proteinuria
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8 Experimental rhabdomyolysis leads to CKD (4), and there may be association between crush
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10 syndrome and late development of CKD and hypertension (3, 5). Therefore we assessed whether
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12 megalin mediates development of CKD by inducing mild experimental rhabdomyolysis and
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14 following iMegKO and controls for 60 days (experimental design, figure 3A). Survival and
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16 body weight were not different between controls and iMegKO mice (figure 3B and C). In
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18 controls, urine output (figure 3D) and GFR declined steadily after rhabdomyolysis induction
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20 (figure 3E, $864.5 \pm 62.7 \mu\text{L}/\text{min}/100\text{g}$ at baseline, $660.1 \pm 32.0 \mu\text{L}/\text{min}/100\text{g}$ 30d after glycerol
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22 injection, and $659.1 \pm 53.3 \mu\text{L}/\text{min}/100\text{g}$ 60d after glycerol injection, respectively 100.0,
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24 93.8 \pm 5.3, 75.9 \pm 3.4, and 76.2 \pm 6.9 % of baseline). In contrast, iMegKO mice demonstrated stable
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26 urine output and GFR (at 60 days, urine output was the same as baseline and GFR was
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28 106.3 \pm 9.5 % of baseline, $p=0.003$ compared with controls). Controls also developed early
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30 proteinuria and albuminuria on day 2 after glycerol injection (~ 1 mg/day of total protein and 35-
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32 75 μg additional albumin over baseline, figure 3F and G and supplemental figure 5) which
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34 persisted for 60 days. iMegKO mice also developed acute protein and albuminuria, however,
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36 this excess proteinuria resolved to baseline levels by 30 days. At the 60-day endpoint,
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38 consistent with the relatively small difference in GFR (22.1% between groups), serum urea
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40 nitrogen was not different between groups (figure 3H). Unblinded assessment of injury score on
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42 PAS-stained histologic sections was not different, and immunostaining for the fibrosis marker α -
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44 smooth muscle actin was sparse and not different between groups (figure 3H-J and supplemental
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46 figure 6). Together these data support progression of acute injury with loss of GFR, progressive
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48 oliguria, and persistent proteinuria in control mice; this progressive injury did not occur in mice
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3 with proximal tubule-specific megalin deletion. Mice had similar indices of histologic injury at
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5 60 days and little α -smooth muscle proliferation. Therefore we conclude that proximal tubule
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7 megalin mediates mild chronic kidney injury following rhabdomyolysis-induced AKI and may
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9 contribute to long-term change in kidney function.
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11 Pharmacologic inhibition of megalin with cilastatin ameliorates rhabdomyolysis-induced AKI

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13 Recapitulation of the protective effect of megalin interference with drug therapy could be
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15 clinically important. Therefore we administered cilastatin, recently identified as a megalin
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17 inhibitor (22), to wild-type mice. When administered at the time of induction of rhabdomyolysis
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19 (figure 4A), cilastatin was well tolerated; 24h survival was 100% in the cilastatin-treated group
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21 and 80% in the vehicle-treated group. Vehicle-treated mice were oliguric, while cilastatin-treated
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23 mice demonstrated greater urine output (vehicle: 0.4 ± 0.1 mL/24h vs 1.8 ± 0.3 mL/24h, $p=0.01$,
24
25 figure 4B). Cilastatin administration increased both overall proteinuria (figure 4C) and
26
27 albuminuria (figure 4D), paralleling findings in iMegKO mice. Mice treated with cilastatin
28
29 demonstrated 8x greater GFR 24h after glycerol injection than vehicle-treated mice (525.9 ± 125.6
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31 vs. 66.7 ± 31 μ l/min/100g respectively, $n=4-5$ /group, $p=0.03$, figure 4E). Accordingly, plasma
32
33 urea nitrogen was also lower in cilastatin-treated mice than in vehicle-treated mice (37.4 ± 3.5
34
35 mg/dL vs. 146.5 ± 41.6 mg/dL, $p=0.03$, figure 4F). Both groups demonstrated histologic injury
36
37 scores similar to those observed in prior experiments, trending lower in cilastatin-treated mice
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39 (378 ± 30 in vehicle-treated mice vs. 276 ± 46 in cilastatin-treated mice, $p=0.12$, figure 4G). Renal
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41 KIM-1 immunopositivity was modestly reduced by cilastatin treatment, indicating reduced
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43 cellular injury (figure 4H); however, renal apoptosis as indicated by cleaved caspase-3
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45 immunopositivity, although similar to prior experiments, was not different (not shown). Overall,
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47 these results indicate that administration of the renal megalin inhibitor cilastatin mitigated
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3 rhabdomyolysis-induced renal functional decline, with modest effects on overall renal acute
4 tubular injury, in a manner similar to that seen in mice with proximal tubule-specific megalin
5 interference.
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Myoglobin clearance after glycerol injection is similarly mediated by megalin and cilastatin

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12 To test the hypothesis that megalin interference altered myoglobin clearance we assessed urine
13 and blood myoglobin concentration, myoglobin clearance, and renal myoglobin retention. In
14 iMegKO mice and controls, urine myoglobin was not detectable prior to glycerol injection, but
15 was greatly elevated in 24h urine collections begun after glycerol injection, and dramatically
16 increased in iMegKO compared with control (figure 5A). Figure 5B illustrates that 6h after
17 glycerol injection, plasma myoglobin was elevated (95.1 ± 6.7 and 68.8 ± 6.2 ng/mL in controls
18 and iMegKO respectively, $p=0.02$, $n=5/\text{group}$), at 24h, plasma myoglobin had declined in both
19 groups (7.5 ± 1.2 vs. 6.2 ± 1.9 ng/mL in control and iMegKO respectively, $p=0.6$, $n=4-5/\text{gr}$). **Thus**
20 **myoglobin clearance was more than 11x greater than that of controls in mice with proximal**
21 **tubule-specific megalin interference (47.8 ± 10.9 vs. 4.2 ± 2.1 ng/h, $p=0.03$, figure 5C). Similarly,**
22 **cilastatin greatly increased urine myoglobin excretion (16.6 ± 4.23 ng/24h in vehicle vs.**
23 **127.7 ± 30.4 ng/24h in cilastatin-treated mice, $p=0.038$, figure 5D), resulting in reduced plasma**
24 **myoglobin (5.2 ± 2.0 in cilastatin-treated mice and 11.3 ± 1.0 ng/mL in vehicle-treated mice,**
25 **$p=0.04$, figure 5E), and a 16-fold increase in myoglobin clearance (0.06 ± 0.02 ng/h in vehicle vs**
26 **0.98 ± 0.24 ng/h in cilastatin-treated mice, $p=0.031$, figure 5F). Myoglobin clearance was similar**
27 **in cilastatin-treated mice and iMegKO mice ($p=0.38$). Renal myoglobin retention at 24h after**
28 **glycerol injection was not mediated by megalin interference (supplemental figure 7). Overall,**
29 **these data demonstrate that megalin interference and cilastatin administration similarly increase**
30 **urinary clearance of myoglobin in experimental rhabdomyolysis.**
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Cilastatin and megalin deletion have similar effects on renal function

To investigate the effects of cilastatin on renal function, we administered cilastatin or vehicle (identically to previous experiments) to surgically-naïve, wild-type mice and assessed renal function 24h later (figure 6A). Cilastatin administration did not alter urine output, or GFR (figure 6B and C) Urine electrophoresis revealed a similar pattern in cilastatin-treated mice to that of iMeggKO mice, with similar novel bands (figure 6D), although urine albumin was not significantly increased (figure 6E). Therefore to determine whether cilastatin inhibited megalin function, we quantified urine retinol binding protein 4 (RBP4), a small plasma protein and megalin ligand (20) which is specifically increased in the urine of megalin-deleted mice (21), and performed immunoblotting for megalin and cubilin in kidney lysate. In a striking similarity to iMeggKO mice, cilastatin administration to wild-type mice greatly increased urinary excretion of retinol binding protein 4 (RBP4, 18.3 ± 4.7 in vehicle, 32.4 ± 0.7 ng/24h in cilastatin-treated mice, $n=5/\text{group}$, $p=0.02$, figure 6F), strongly suggesting that cilastatin inhibits renal uptake of this megalin ligand. Cilastatin administration did not alter renal expression of megalin or cubilin (6G-I). Taken together these data indicate that cilastatin does not by itself alter urine output or GFR: rather, cilastatin increases the urinary excretion of megalin ligands, likely by acting directly on megalin. Next, to determine whether the protective effect of cilastatin administration was megalin-dependent, we administered cilastatin or vehicle to male and female iMeggKO mice subjected to experimental rhabdomyolysis (figure 6J). 24h after glycerol injection, GFR was similar in both groups to that of iMeggKo mice after glycerol injection; the mean GFR of vehicle-treated iMeggKO mice (859.3 ± 301.3) was not different than that of cilastatin-treated iMeggKO mice (813.7 ± 113.4 , $p=0.89$, figure 6K), indicating that cilastatin-mediated renoprotection from AKI due to experimental rhabdomyolysis is megalin-dependent.

Cilastatin reduces myoglobin endocytosis *in vivo*.

To evaluate a mechanism by which cilastatin might increase myoglobin clearance, we characterized and compared uptake of injected FITC-myoglobin *in vivo*. 15 minutes after injection to healthy wild-type mice FITC-myoglobin punctae were abundant at the apical brush border and within the cytoplasm of proximal tubular epithelial cells, but by 30 minutes, punctae were absent, suggesting rapid clearance (not shown). Therefore, to characterize the role of megalin in formation of myoglobin endocytic punctae, we administered FITC-myoglobin to iMegKO mice and controls. Histologic sections from iMegKO mice demonstrated considerable attenuation of puncta signal compared with controls figure (7A, B). 15 minutes after injection, the number of endocytic puncta was greater in control mice than that in iMegKO mice (0.33 ± 0.01 vs. 0.19 ± 0.01 puncta/ μm^2 , $n=3-5/\text{group}$, $p=0.00019$, this result was identical when not normalized to tissue area). Comparing puncta, puncta from control mice ($n=291330$) were larger (0.97 ± 1.12 vs. 0.88 ± 0.95 μm^2 , $p < 2.2 \times 10^{-16}$) and demonstrated greater FITC-fluorescence (19611 ± 3385 vs. 12603 ± 4481 arbitrary fluorescence units, $p < 2.2 \times 10^{-16}$, figure 7C) than those from iMegKO mice ($n=79826$). Thus, megalin deletion is characterized by reduction in myoglobin endocytic puncta number, size, and area.

To characterize alterations in endocytic punctae induced by cilastatin administration, we administered cilastatin or vehicle to wild-type mice, followed 10 minutes later by FITC-myoglobin. 15 minutes later, histologic sections demonstrated attenuated fluorescence in cilastatin-treated mice (example images, figure 7D, E). Although the number of puncta was not significantly different in cilastatin - and vehicle-treated mice (cilastatin: 0.11 ± 0.011 , vehicle: 0.14 ± 0.04 $p=0.33$), puncta from cilastatin-treated mice were smaller than those from vehicle-treated mice (0.92 ± 0.98 vs. 0.95 ± 0.99 μm^2 , $p < 2.2 \times 10^{-16}$, not shown), and demonstrated reduced

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3 FITC fluorescence (22603 ± 3775 vs. 24480 ± 4066 arbitrary fluorescence units, $p < 2.2 \times 10^{-16}$ figure
4 7F). Therefore cilastatin reduced the area and FITC-myoglobin content of endocytic puncta.

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8 Lastly, to confirm that overall renal myoglobin uptake is altered by cilastatin
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10 administration, we administered cilastatin or vehicle at the time of glycerol intramuscular
11 injection to wild-type mice, and assessed renal myoglobin immunofluorescence using unbiased
12 stereology, 2h after glycerol administration. Endocytic punctae were widely present in the
13 kidneys of mice in both groups (Figure 7G, H), and myoglobin-positive puncta were not different
14 between groups (0.18 ± 0.07 in vehicle vs. 0.11 ± 0.14 in cilastatin, puncta/ μm^2 , $n=3-5/\text{group}$,
15 $p=0.41$). However, unbiased stereology demonstrated that overall renal myoglobin
16 immunofluorescence was reduced by cilastatin treatment (0.216 ± 0.007 vs. 0.241 ± 0.003 ,
17 $V_{\text{myoglobin}}/V_{\text{kidney}}$, $p=0.032$, figure 7I). Taken together, these data demonstrate alterations in
18 myoglobin endocytic puncta by cilastatin, and further implicate altered endocytosis of myoglobin
19 as a mechanism of cilastatin's action in glycerol-induced rhabdomyolysis.
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33 Discussion

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35 This study has two important findings. The first is that the renal endocytic transporter
36 megalin plays a critical role in AKI due to rhabdomyolysis. The second important finding is that
37 cilastatin, a pharmacologic inhibitor of megalin, is renoprotective in rhabdomyolysis-induced
38 AKI in a megalin-dependent fashion.
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44 Proximal tubule-specific deletion of megalin prevented rhabdomyolysis-induced GFR
45 loss almost completely, and reduced other measures of renal injury including histopathologic
46 injury score, renal apoptosis, and renal KIM-1. Myoglobin clearance was greatly increased in
47 the absence of proximal tubule megalin. We conclude this dramatic increase in myoglobin
48 clearance preserved renal function. Increased myoglobin clearance may underlie three
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3 renoprotective mechanisms. First, myoglobin is present in the kidney for less time, resulting in
4 reduced renal exposure to a toxic molecule. Second, plasma myoglobin is more rapidly depleted,
5 resulting in more rapid reduction of tubular concentration of myoglobin, also potentially
6 reducing renal injury. Third, preservation of renal function, including urine output, due to the
7 first two mechanisms, likely further reduces plasma and urine concentrations, reducing renal
8 exposure to myoglobin.
9

10 Myoglobin was first identified as a megalin ligand in 2003 by Gburek et al, in mice with
11 incomplete but renal-specific megalin deletion. Investigators in this seminal study speculated
12 that megalin interference might have a beneficial effect in rhabdomyolytic renal failure (9).
13 Megalin functions as part of an endocytic complex including cubilin, amnionless (23), and
14 possibly other components (24). Together, the complex is promiscuous, with >40 known
15 ligands. Extensive and elegant investigations have elucidated the contributions of specific
16 components of the complex to ligand uptake and disposition in tubular epithelium; some ligands
17 (e.g. albumin) may have multiple paths to endocytosis. Based on molecular weight and
18 similarity to hemoglobin, which undergoes both megalin- and cubilin-mediated uptake (25),
19 myoglobin uptake could also be mediated by cubilin. In fact myoglobin binds both megalin and
20 cubilin, with lower K_D for megalin than for cubilin (9). Further, receptor specificity for tubular
21 endocytic complex ligands is concentration-dependent; for example, albumin uptake is megalin-
22 dependent in diabetic mice(11), but less so at nephrotic-range concentrations (26); recent study
23 suggests megalin and cubilin provide complementary pathways with differential affinities for
24 albumin uptake (27). This concentration-dependent pathway might be invoked when tubular
25 concentration of myoglobin is high in rhabdomyolysis. Since the model we employed
26 specifically depleted proximal tubule megalin, but not cubilin, we cannot eliminate the
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3 possibility that myoglobin clearance could be further increased by interference with cubilin.
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5 Together, the present data and the increasing understanding of this critical proximal tubule
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7 endocytosis complex provide tantalizing potential for specific therapy in rhabdomyolysis-
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9 induced AKI.
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12 We also found that megalin interference prevents progressive proteinuria and GFR loss
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14 due to rhabdomyolysis. Our findings in control mice, which progressively developed ~20%
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16 GFR loss and increased protein/albuminuria are in accordance with those of Belliere et al, which
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18 identified rhabdomyolysis as an initiator of AKI-CKD transition (4). Our study is distinct from
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20 this prior study in evaluating a lower dose of glycerol (6.5 mL/kg vs 7.5 mL/kg). We observed
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22 discordance between functional measures (GFR and albuminuria, which demonstrated
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24 impairment in controls) and structural evaluation (histology and α SMA quantification, which
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26 were similar and mild). We suggest these observations are consistent with early CKD, in which
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28 mild proteinuria and GFR loss occurs but structural injury may not be evident (28). Our finding
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30 may be contrasted with studies suggesting that partial megalin deletion does not alter
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32 tubulointerstitial fibrosis in severe glomerulonephritis (29, 30). Our distinct result may stem
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34 from the acute nature of rhabdomyolysis, contrasted to the unremitting injury of
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36 glomerulonephritis, or from more complete reduction in megalin function in our mouse model,
37
38 or both. Clinical literature supports that rhabdomyolysis-induced AKI may lead to long-term
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40 kidney disease, although the relationship appears complex and has only been studied in the
41
42 context of armed conflict. Rhabdomyolysis occurs in 31% of US combat casualties admitted to
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44 intensive care (2); these patients have elevated risk of developing CKD and hypertension, and
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46 AKI in this context independently quadruples the risk of subsequent CKD (5). However, a
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48 subsequent study by the same investigators in a similar population did not support a strong
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3 independent relationship between rhabdomyolysis alone and subsequent CKD (31). We found
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5 progressive decline in GFR after rhabdomyolysis accompanied by persistent proteinuria, both
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7 mediated by megalin. These findings further support AKI-CKD transition due to
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9 rhabdomyolysis, and provide data we hope may assist in future clinical studies. For example, we
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11 speculate that megalin polymorphisms may modify risk of CKD after rhabdomyolysis as they do
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13 other disease (32, 33); exploring such polymorphisms in clinical cohorts may help elucidate the
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15 relationship between rhabdomyolysis, AKI and subsequent CKD. Since rhabdomyolysis
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17 commonly occurs in young people (athletes and soldiers, for example) such studies would be
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19 impactful due to the decades-long burden of early-onset CKD.
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24 Finally, an important finding in this study is that the megalin inhibitor cilastatin preserves
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26 renal function in rhabdomyolysis in megalin-dependent fashion. As with megalin deletion,
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28 cilastatin administration preserved GFR. Cilastatin administration to healthy mice did not alter
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30 GFR or urine output, but caused selective proteinuria similar to that of iMegKO mice, including
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32 that of a specific marker of megalin function, RBP4. These findings further support the megalin-
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34 inhibiting role of cilastatin. In wild-type mice, cilastatin increased myoglobin clearance after
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36 glycerol injection, decreased the size and fluorescence of renal endocytic puncta when
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38 administered with FITC-myoglobin, and reduced overall renal myoglobin signal 2h after glycerol
39
40 injection (although not 24h after glycerol injection). In cultured proximal tubular epithelial cells,
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42 low concentrations of cilastatin modestly inhibited uptake of myoglobin. Therefore, although
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44 reduced myoglobin uptake is an action of cilastatin, this mechanism may not account for the
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46 entirety of the increased myoglobin clearance induced by cilastatin or all of the protective effect
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48 observed. Further investigation should include evaluation of other possible protective
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50 mechanisms induced by cilastatin.
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3 Cilastatin was originally identified as a renal dipeptidase inhibitor in the 1970s and
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5 received FDA approval for coadministration with the antibiotic imipenem, which is metabolized
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7 rapidly by renal dipeptidase. Therefore there is considerable clinical experience with
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9 administration of cilastatin, which has low toxicity – cilastatin LD50 estimates are > 30x the
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11 dose we employed in this study (34). Recent investigation identified that cilastatin also inhibits
12
13 megalin (22). Since rhabdomyolysis is commonly lethal in austere environments such as natural
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15 disasters and armed conflict, specific, effective therapy with low burden of administration is
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17 highly desirable and has great potential to transform care. Further study in clinically relevant
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19 models, and preparation for clinical study, is therefore imperative.
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24 Our study has limitations. We performed experiments entirely in a mouse model and
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26 results may not extend to humans. **We did not evaluate the role of myoglobin casts in injury.**
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28 Myoglobin is not the only cause of renal injury in rhabdomyolysis. Others have demonstrated
29
30 that hemolysis, and renal injury due to free hemoglobin also occurs and is ameliorated by heme
31
32 oxygenase (13, 14, 35, 36); we did not evaluate the role of hemoglobin or heme oxygenase in our
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34 studies. The proximal tubule may not be the only site of renal injury in rhabdomyolysis. We
35
36 note that glycerol-treated iMegKO controls developed lasting albuminuria, possibly a sign of a
37
38 damaged glomerular filtration barrier, but we did not rigorously evaluate for signs of glomerular
39
40 damage. Lastly, cilastatin has known off-target effects; we did not exclude a beneficial effect of
41
42 dipeptidase inhibition in rhabdomyolysis, and we cannot exclude other, unknown off target
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44 effects. Given the translational importance of the finding that cilastatin is renoprotective in
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46 rhabdomyolysis-induced AKI, we are pursuing further studies which will clarify these questions.
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3 In conclusion, the renal proximal tubule endocytic receptor megalin plays a critical role in the
4 development of AKI due to rhabdomyolysis. Administration of the megalin inhibitor cilastatin
5 recapitulates effects of megalin interference, and ameliorates AKI due to rhabdomyolysis.
6
7

8 **Author Contributions**

9
10 Katsuyuki Matsushita designed and performed experiments, analyzed data, and authored and
11 edited the manuscript. Kiyoshi Mori provided critical materials, critiqued experimental design,
12 analyzed results, and edited the manuscript. Turgay Saritas analyzed data, authored, and edited
13 the manuscript. Mahaba Eiwaz performed experiments and edited the manuscript. Yoshio
14 Funahashi designed and performed cell culture experiments, authored the manuscript, and
15 critiqued the manuscript. Jessica Hebert developed critical methods, performed immunoblot
16 experiments, authored the manuscript, and critiqued the manuscript. Megan Nickerson designed
17 experiments, contributed figures, performed critical experiments, and critiqued the manuscript.
18 Adam Munhall designed experiments, contributed figures, performed critical experiments, and
19 critiqued the manuscript. James McCormick critiqued and designed experiments, and edited the
20 manuscript. Motoko Yanagita provided critical materials, critiqued experimental design,
21 analyzed results, and edited the manuscript. Michael Hutchens conceived, designed, and
22 performed experiments, analyzed data, authored and edited the manuscript, and assembled and
23 led the research team.
24
25

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23 **Supplemental material Table of Contents**

24 Supplemental Table S-1

25 Supplemental Methods

26 Supplemental Figures 1-9
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Figure Legends

Figure 1: Animal and experimental models.

A-G: Results of tamoxifen treatment in $Lrp2^{fl/fl}; Ndr1^{CreERT2+}$ (iMegKO) and cre- littermates (control). A. High-power micrographs obtained 14 days after the first of 5 daily tamoxifen injections demonstrate that control mice exhibit robust immunostaining for megalin at proximal tubule brush borders, while iMegKO mice demonstrate near-complete absence of megalin. B: Megalin is absent in immunoblots performed on renal homogenate of iMegKO mice, while cubilin is not affected by iMegKO status. C: The urine of iMegKO mice contains low molecular-weight proteins (arrowheads) which are not present in the urine of controls as demonstrated by Coomassie-stained electrophoresis of equal volumes of urine obtained from 24h collection. D: Urine retinol binding protein 4 (RBP4), a megalin ligand, is greatly upregulated by iMegKO. E: Body weight and 24h urine output are not altered by megalin deletion. F: Clearance of fluorescein isothiocyanate (FITC)-conjugated sinistrin (FITC-sinistrin) is reduced by megalin deletion, indicating a reduction in glomerular filtration rate (GFR). G: Urine protein and urine albumin excretion are greatly increased by megalin deletion. Scale bars: 100 μ m. Statistical test employed for all comparisons: t-test.

Figure 2: Deletion of proximal tubular megalin mediates rhabdomyolysis-induced acute kidney injury (AKI) 24 hours after glycerol injection.

A: Experimental design. Tamoxifen induction started 15 days before glycerol injection; mice with inducible proximal tubule-specific megalin deletion (iMegKO) and littermate control mice received identical tamoxifen regimens. B-G: 24h after glycerol injection, control mice

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3 demonstrated AKI, which was attenuated in iMegKO mice. B: Control mice were oliguric,
4 while iMegKO mice demonstrated greater-than baseline urine output. C: Control mice
5 demonstrate severe loss of glomerular filtration rate (GFR), while iMegKO, GFR is not different
6 from baseline value. D: Serum urea nitrogen is much greater in control mice than iMegKO
7 mice. E: Photomicrographs of periodic acid-Schiff stained sections in control and iMegKO
8 mice are distinguished by extensive proteinaceous material in distal tubule and collecting ducts
9 (black arrowheads) and cell swelling and luminal effacement (white arrowheads) in control, with
10 more normal architecture in iMegKO. Composite injury score, right, is greater in control mice.
11 F: KIM-1 stain is greatly attenuated in iMegKO mice, and accordingly, apoptosis, indicated by
12 cleaved caspase-3 staining (G), is also reduced by megalin interference. Scale bars are 100 μ M.
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Statistical test employed for all comparisons: t-test.

Figure 3: Megalin mediates progressive kidney disease due to rhabdomyolysis.

A: Experimental design. B: Survival plot. One control mouse died during the 60 day
experiment. C: Body weight after glycerol injection was not mediated by induced proximal
tubule-specific megalin deletion (iMegKO). D: Control mice developed relative oliguria at 60
days when compared with 2 days after glycerol injection, while iMegKO mice demonstrated
unchanged urine output throughout the experiment. E: Glomerular filtration rate (GFR)
progressively declined in control mice, while in iMegKO mice, GFR did not change from
baseline. F and G: Because megalin interference causes proteinuria (see figure 1), urine protein
(F) and urine albumin (G) are displayed as change from baseline. Control mice demonstrated
increased proteinuria and albuminuria, compared with baseline, which persisted through the full
60 day experimental course, while in iMegKO mice, both proteinuria and albuminuria were
reduced at 60 days compared with 2 days after glycerol injection. iMegKO mice did not have

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3 significantly increased proteinuria at day 60, while controls did. H: Serum urea nitrogen,
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5 pathologic injury score (I), and α smooth muscle actin deposition (α SMA, an indicator of renal
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7 fibrosis) were not mediated by proximal tubule megalin status 60 days after glycerol injection.
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10 Statistical analysis presented is Mantel-Cox logrank test (B), repeated measures ANOVA (C-G),
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12 and t-test (H-J).
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15 Figure 4: Pharmacologic treatment with megalin inhibitor cilastatin recapitulates AKI
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17 amelioration by proximal tubule-specific megalin deletion.
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19 A. Experimental design. Cilastatin was administered immediately after glycerol. B-D: In
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21 accordance with findings in iMegKO mice, cilastatin administration at the time of
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23 rhabdomyolysis induction resulted in increased urine output (B), increased acute proteinuria (C),
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25 and increased urine albumin (D), compared with vehicle. Cilastatin administration also
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27 prevented severe loss of GFR observed in vehicle-treated mice (E), and prevented the highly
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29 elevated serum urea nitrogen seen in vehicle-treated mice (F). Periodic acid-Schiff stained
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31 sections were also generally in accordance with findings in control and iMegKO mice, although
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33 histopathologic injury scoring was not different between cilastatin and vehicle treated mice.
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35 KIM-1 was elevated in both groups (H), but significantly reduced in cilastatin-treated mice
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37 compared with vehicle-treated mice. Scale bars are 100 μ m. Statistical analysis presented is
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39 derived from the t-test.
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45 Figure 5: Myoglobin clearance is similarly altered by proximal tubule megalin interference and
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47 cilastatin administration.
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49 A: Induced proximal tubule-specific megalin deletion (iMegKO) status conferred much greater
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51 excretion of myoglobin in the urine. B: Plasma myoglobin concentration was elevated in
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53 controls relative to iMegKO mice with 6h, but not 24h, after glycerol injection, with rapid
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3 decline in plasma myoglobin occurring in both groups. C: Myoglobin clearance was
4 significantly greater in iMegKO mice than controls. D-F: Treatment with the pharmacologic
5 inhibitor of renal megalin, cilastatin, had similar actions to proximal tubule-specific deletion of
6 megalin. D: Urine myoglobin was increased by cilastatin treatment. E: 24h plasma myoglobin
7 was reduced by cilastatin treatment. F: Myoglobin clearance was increased ~16x by cilastatin
8 treatment. Statistical analysis presented is derived from repeated measures ANOVA (A, B) and
9 t-test (C-F).
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19 Figure 6: Cilastatin and megalin deletion have similar effects on renal function

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21 A: Experimental design for results B-I. Wild type mice received cilastatin (200 mg/kg) or
22 vehicle injection as in prior experiments, without injection of glycerol. 24h later, outcomes were
23 assessed. B and C: Urine output and glomerular filtration rate were not altered by cilastatin
24 administration. D: Coomassie-stained urine electrophoresis (loaded with identical volumes of
25 urine from each animal). Bands at ~23kD and ~40 kD marked by arrowheads appear
26 differentially expressed in cilastatin vs. vehicle samples. The same bands may be seen in the
27 urine of iMegKO mice in figure 1. Unaltered gel image shown in psuedo-color to better
28 visualize peak protein density. E: Urine albumin was not significantly increased. F: Urine
29 retinol binding protein 4 (RBP4), a plasma protein which is a known megalin ligand, and is
30 greatly increased in the urine of megalin-deleted mice, was nearly doubled by cilastatin
31 administration. G-I: Immunoblots performed on kidney lysate 24 hour after vehicle or cilastatin
32 injection. Megalin expression was reduced after cilastatin administration, while cubilin was not
33 significantly altered. J: Experimental design. iMegKO mice received cilastatin or vehicle with
34 injection of glycerol. 24h later, mean GFR was identical between groups (K), indicating that
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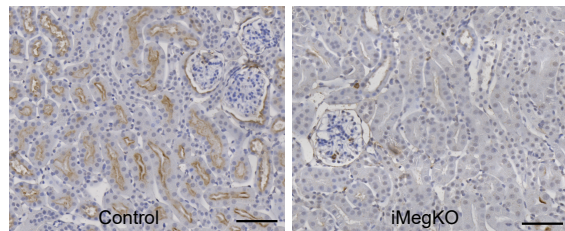
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3 cilastatin-dependent protection from rhabdomyolysis-induced AKI is absent in mice without
4 proximal tubule megalin. Statistical analysis presented is derived from the t-test.
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8 Figure 7: Similar interference in myoglobin uptake in iMegKO and cilastatin-treated mice.
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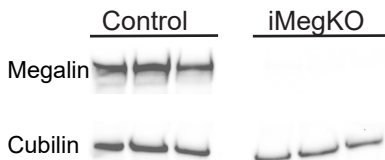
10 To characterize the role of megalin in myoglobin endocytosis, iMegKO mice and controls were
11 injected with FITC-myoglobin (0.5 mg). 15 minutes later, mice were killed and kidneys
12 prepared for histologic examination. A: Controls demonstrated abundant tubular epithelial
13 FITC signal, primarily organized in punctae within the apical brush border and the adjacent
14 cytoplasm, while (B), iMegKO mice demonstrated attenuated FITC-myoglobin signal, and many
15 fewer punctae. C: Mean fluorescence of all puncta (n=371,156) quantified by strain
16 demonstrates that puncta from iMegKO mice exhibit reduced fluorescence compared to those
17 from controls. Horizontal lines depict the mean. D-F: To characterize the effect of cilastatin on
18 myoglobin endocytosis, wild-type mice were injected with FITC-myoglobin (0.5 mg). 15
19 minutes later, mice were killed and kidneys prepared for histologic examination. Vehicle-treated
20 mice exhibited abundant FITC-myoglobin punctae, while (D) cilastatin-treated mice exhibited
21 fewer punctae and overall reduced FITC-fluorescence. H: Mean fluorescence of all puncta
22 (n=2,298,499) compared by drug treatment demonstrates that puncta from cilastatin-treated mice
23 exhibit reduced fluorescence compared to those from vehicle-treated mice. Horizontal lines
24 depict the mean. G-I: To determine whether cilastatin interfered with overall renal myoglobin
25 uptake, wild-type mice received vehicle or cilastatin injection 1h prior to glycerol intramuscular
26 injection. G, H: 2h after glycerol injection, myoglobin-directed immunofluorescence
27 demonstrates abundant myoglobin-positive punctae at the brush border and within the cytoplasm
28 of proximal tubules of vehicle injected mice, this signal appeared attenuated in tubules from
29 cilastatin-injected mice. I: Unbiased stereology demonstrates reduced total intrarenal myoglobin
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3 content in cilastatin-treated mice. Scale bars are 20 μ m. Statistical analysis presented is derived
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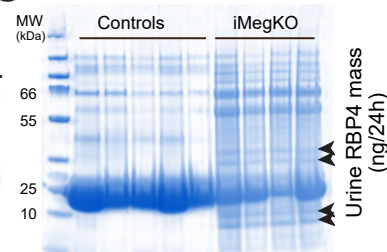
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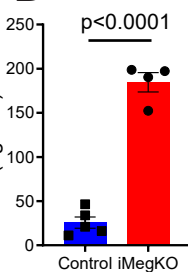
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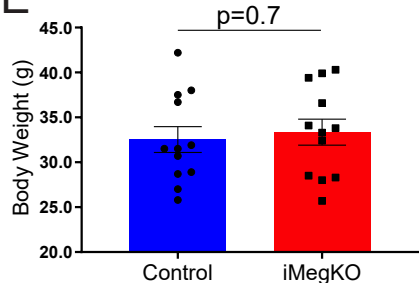
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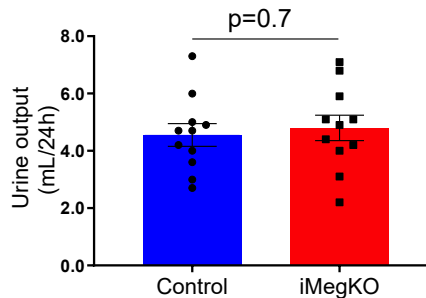
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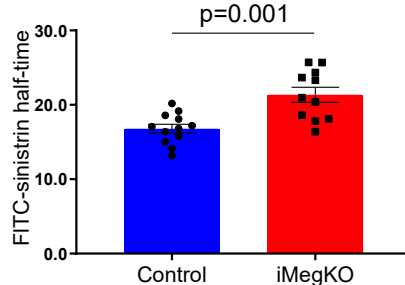
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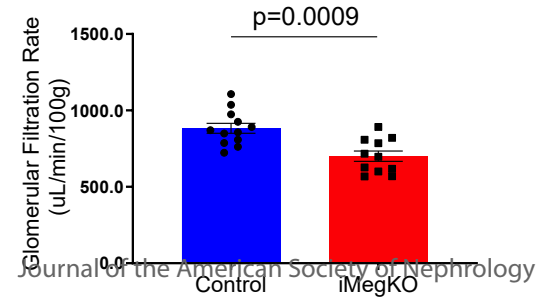
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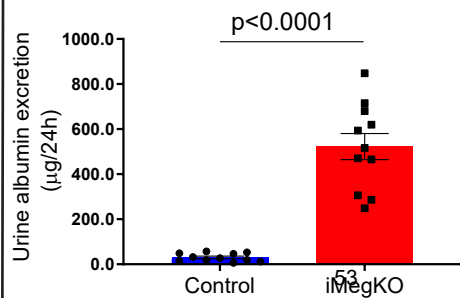
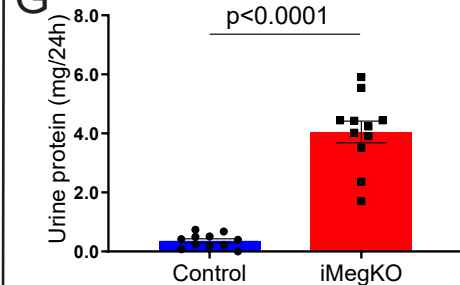
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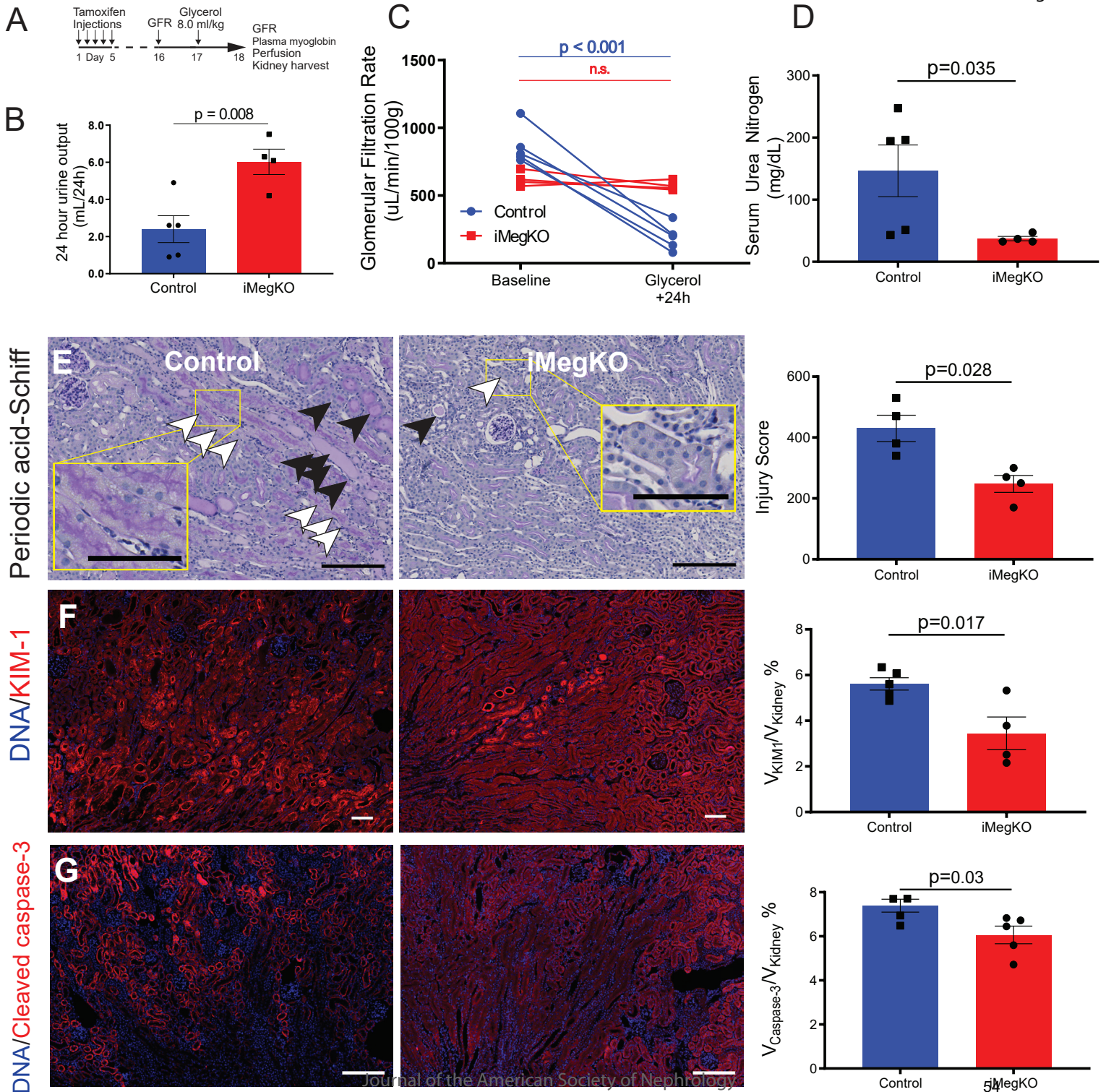


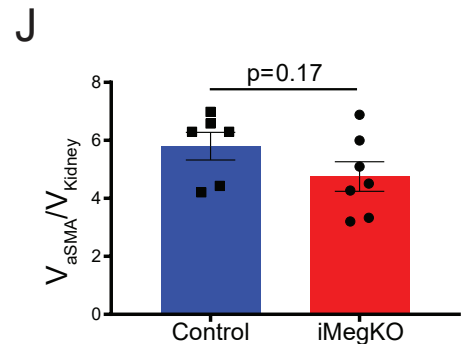
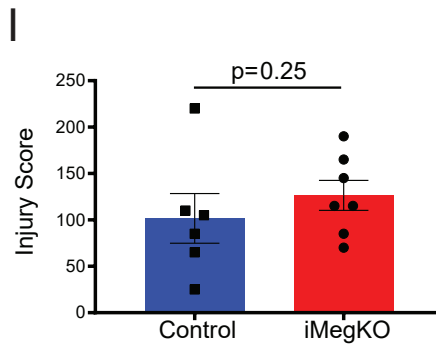
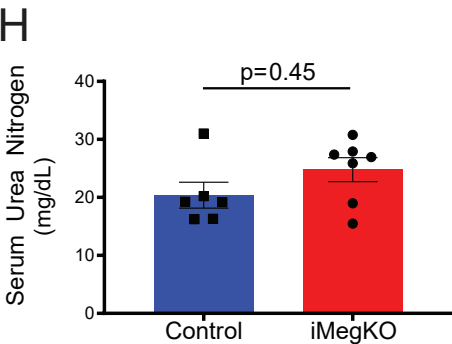
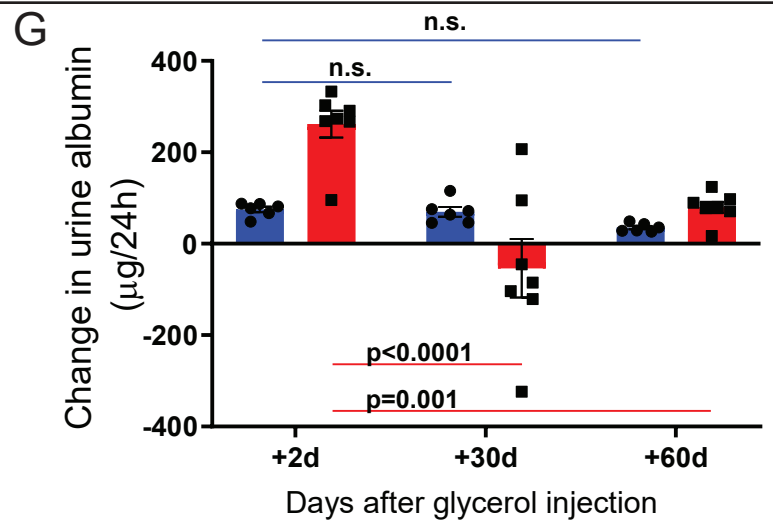
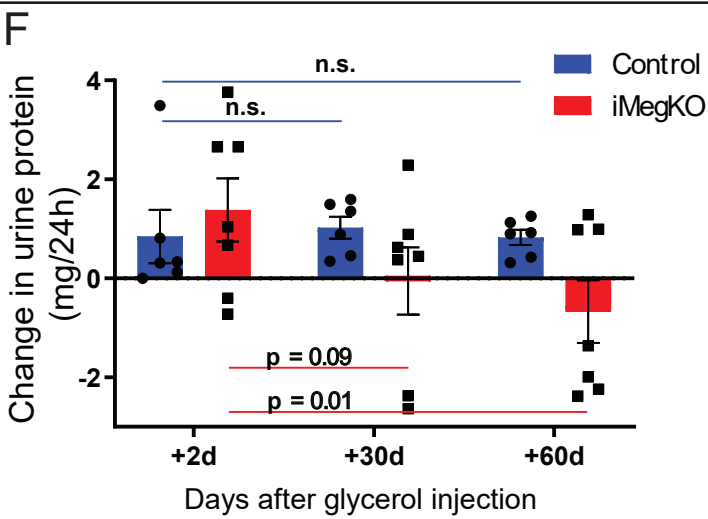
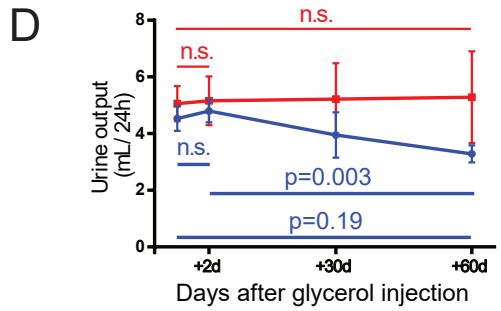
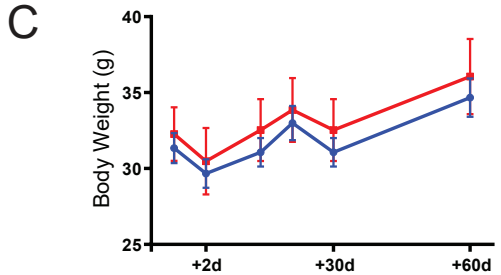
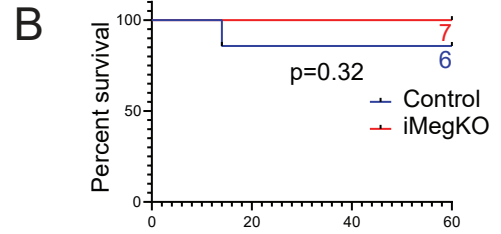
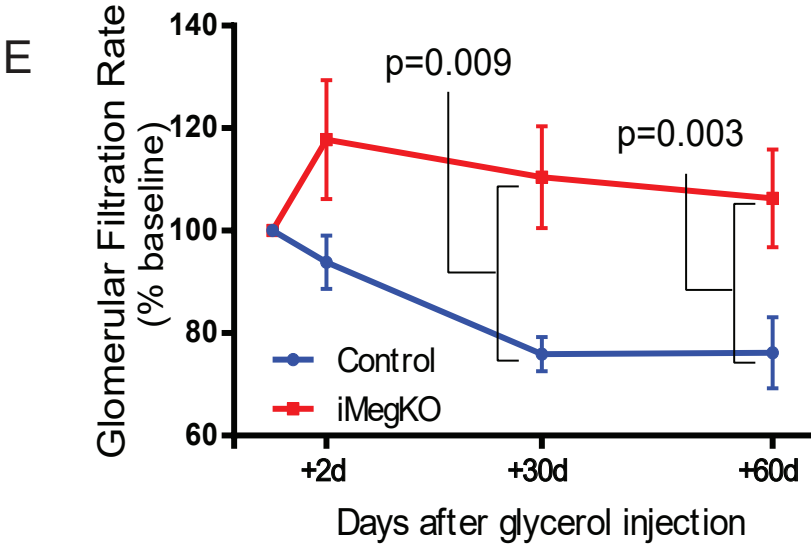
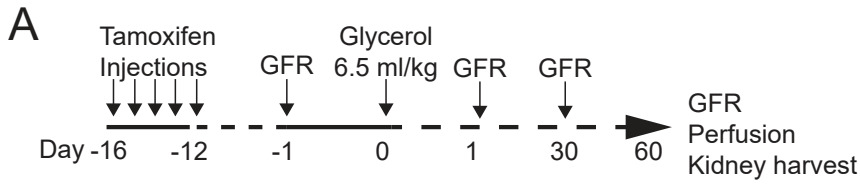
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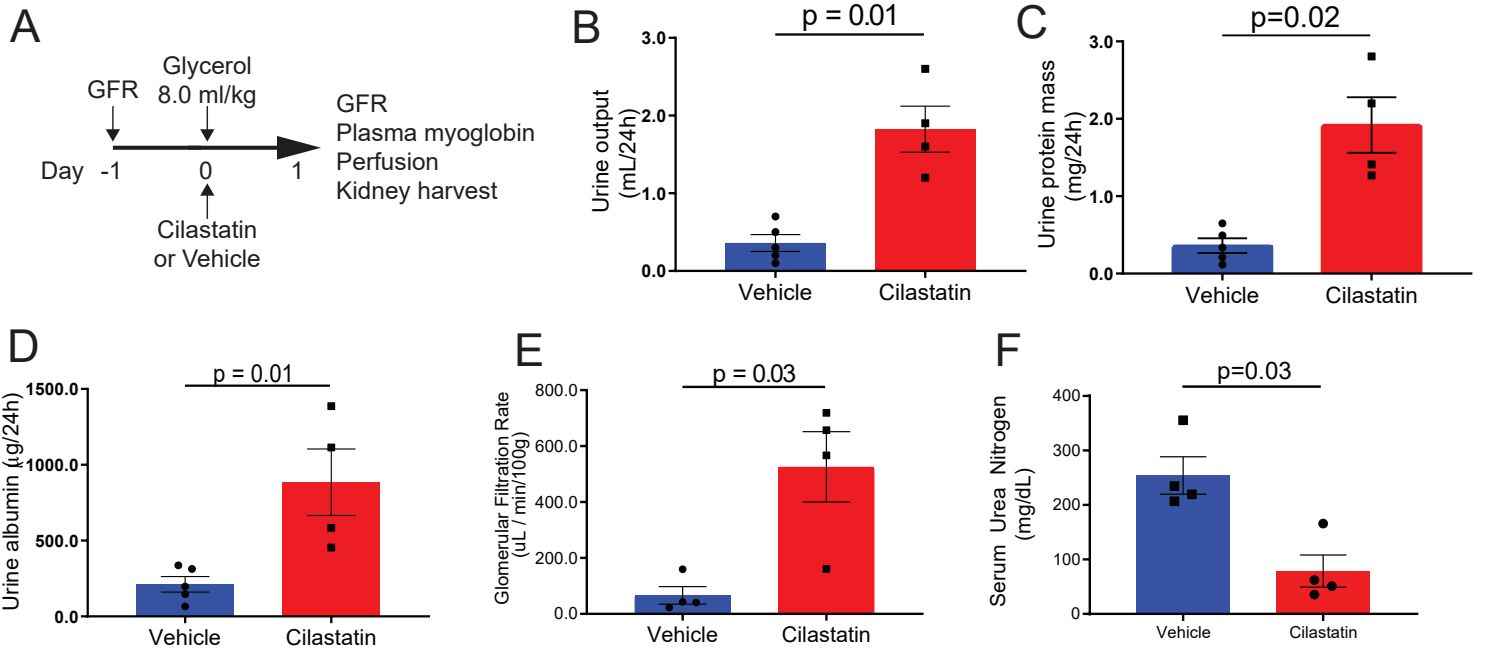


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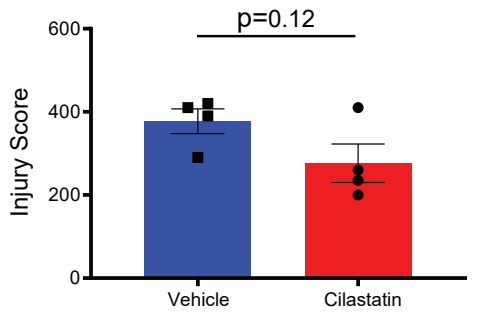
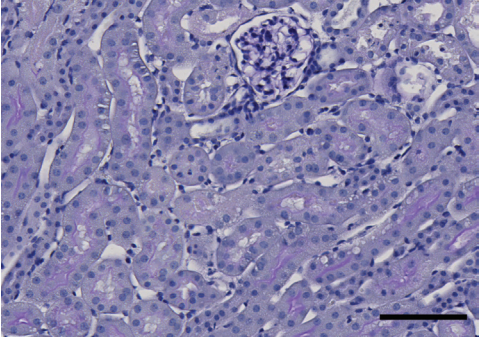
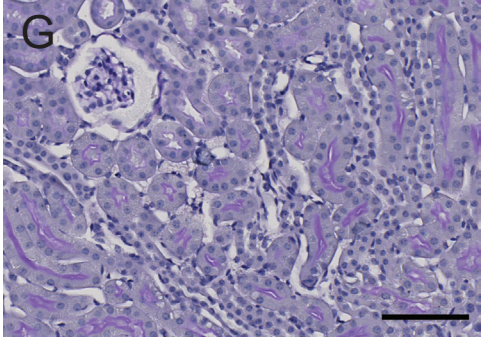




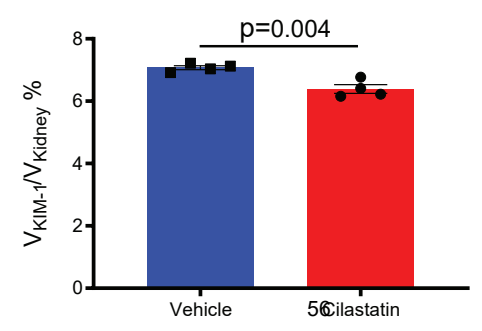
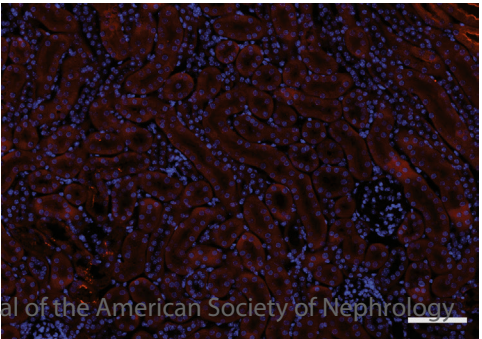
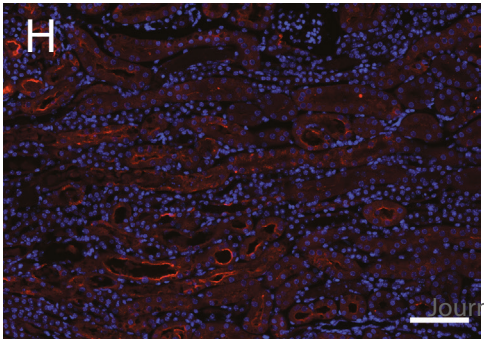
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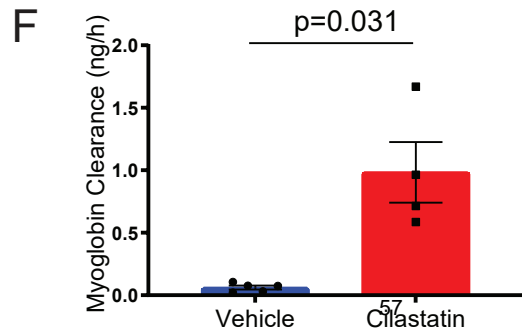
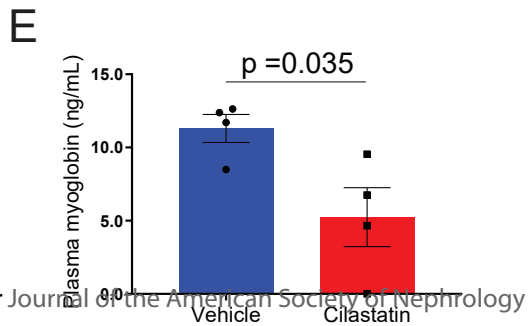
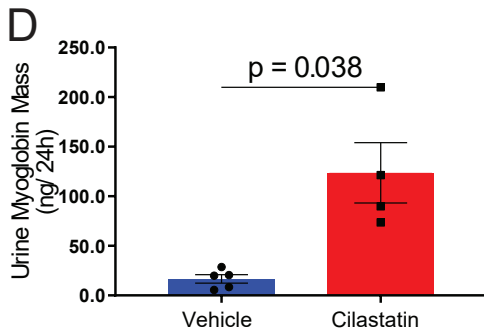
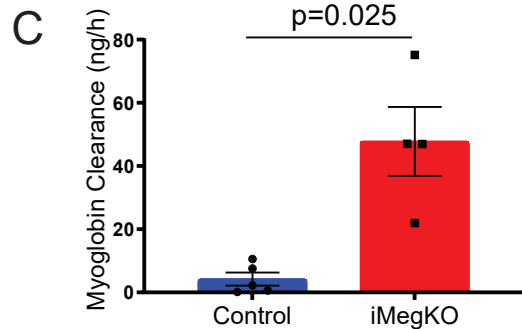
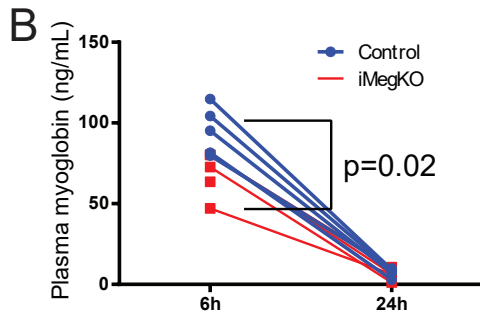
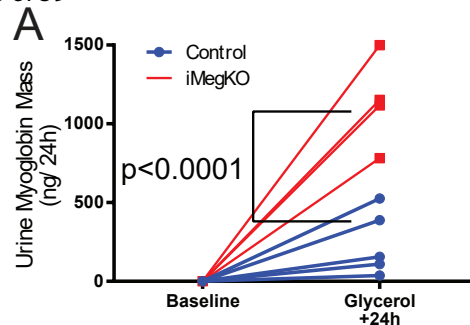
Cilastatin

Periodic acid-Schiff

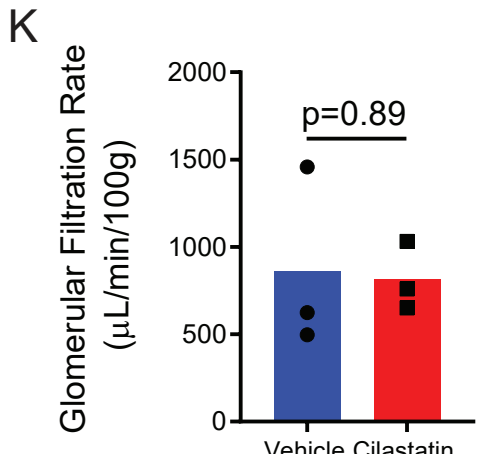
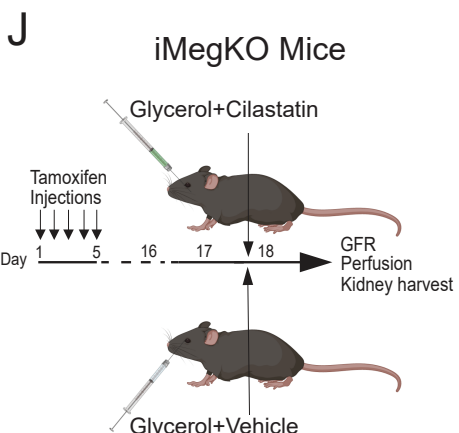
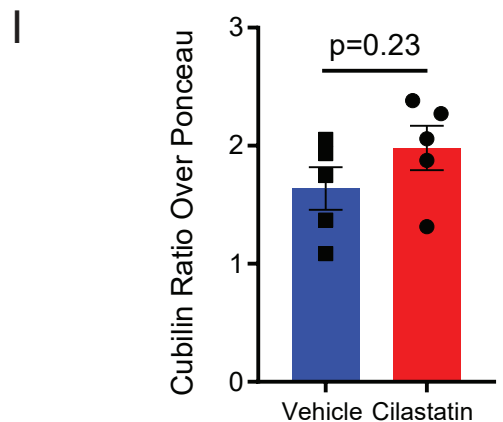
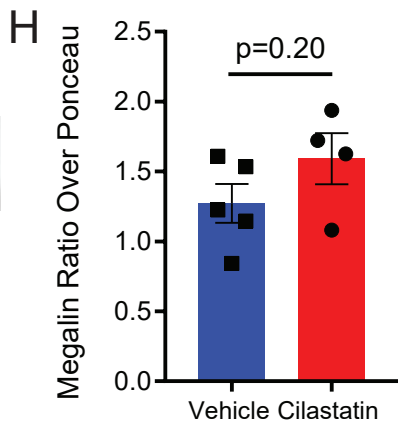
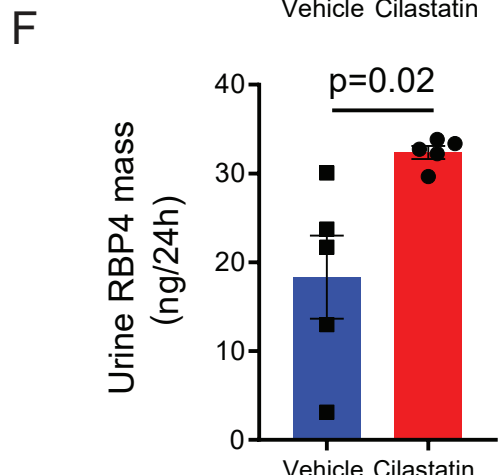
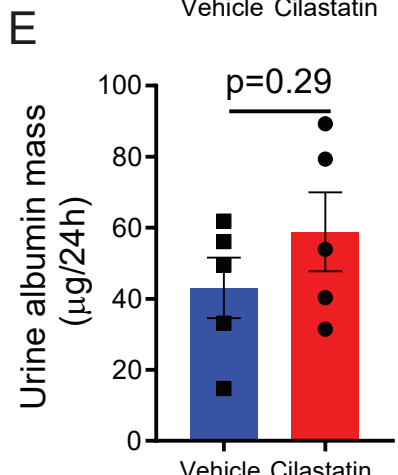
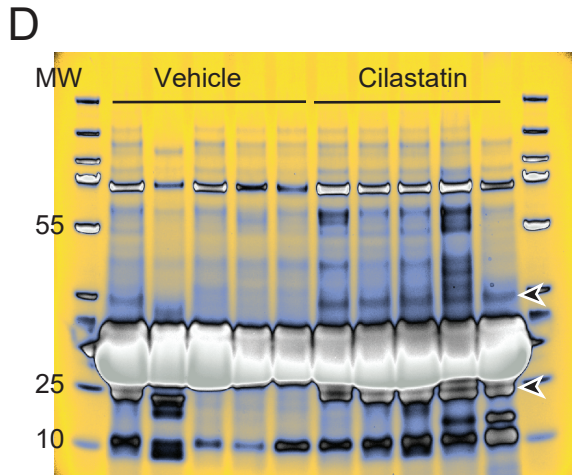
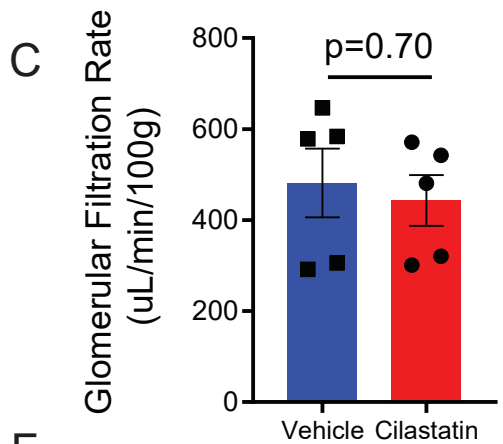
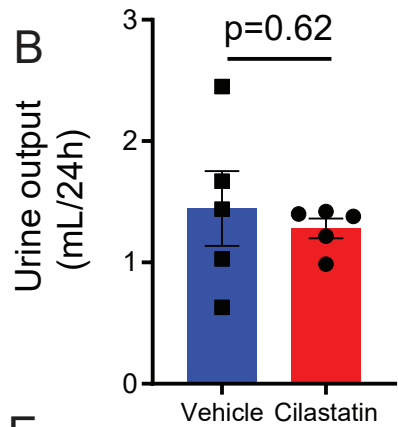
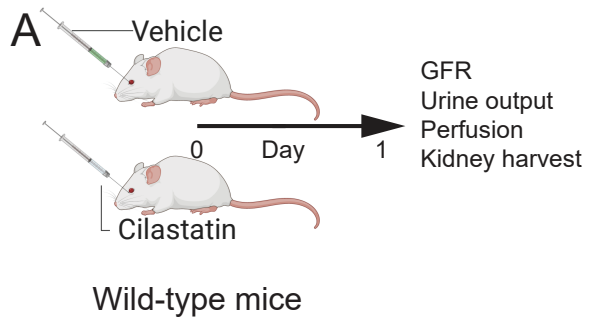


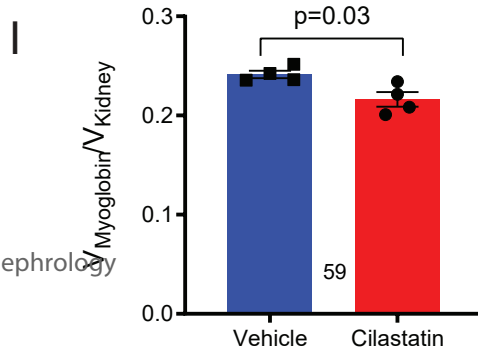
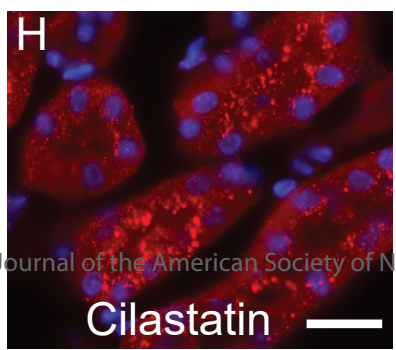
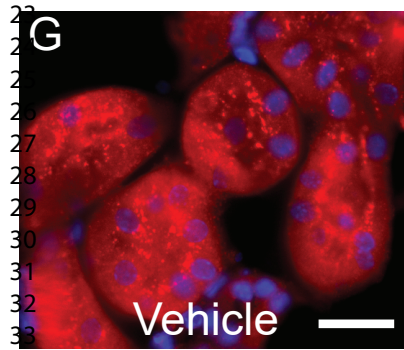
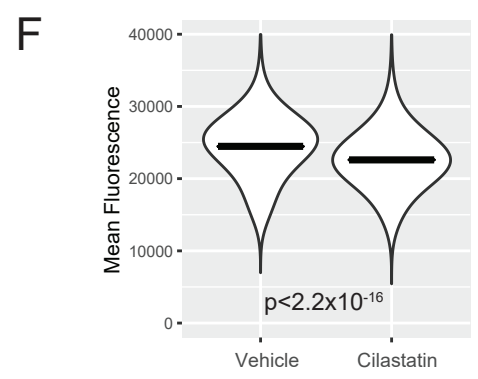
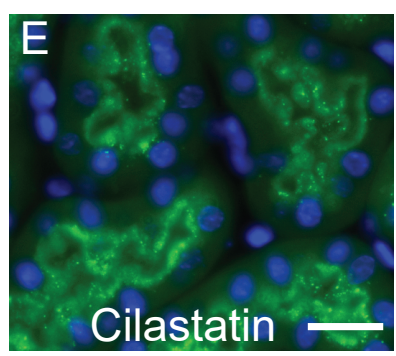
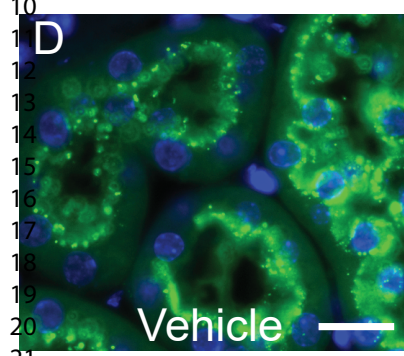
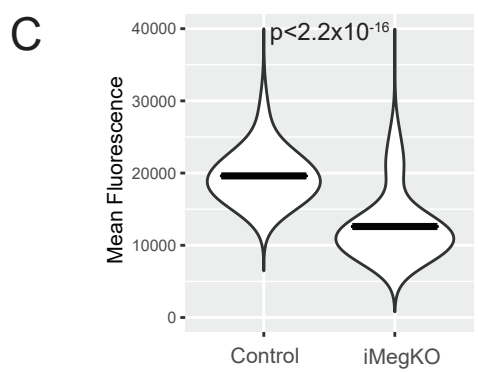
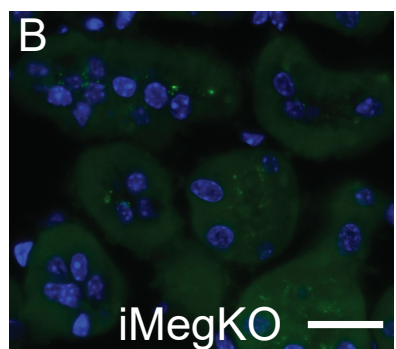
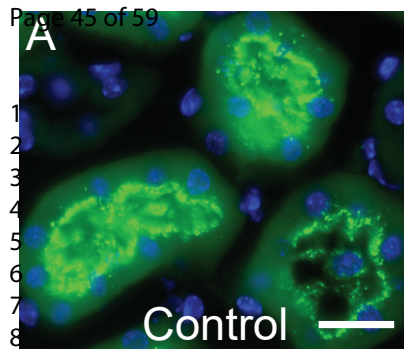
DNA/KIM-1





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Supplemental material Table of Contents

Supplemental Table S-1

Supplemental Methods

Supplemental Figures 1-9

Table S1. List of reagents and resources used in this study.

Reagent or Resource	Source, Catalog number	Dilution IF/IHC/W
Antibodies		
Goat anti-KIM-1 antibody	R&D, Cat#AF1817	1:180 (IF)
Rabbit anti-cleaved caspase-3	Cell signaling, Cat#9661S	1:500 (IF)
Mouse α -Smooth Muscle Actin- FITC	Sigma Aldrich, Cat#F3777	1:200 (IF)
Rabbit Anti-myoglobin antibody	Abcam # ab154292	1:50 (IF)
Mouse anti-megalin antibody	Santa Cruz Cat# sc-74525	1:100 (IHC)
Rabbit anti-megalin antibody	Invitrogen, Cat#PA5-67900	1:1000 (W)
Sheep anti-cubilin antibody	R&D, Cat#AF3700	1:1000 (W)
Mouse anti-myoglobin antibody	Santa Cruz Cat# sc-74525	1:200 (W)
Chemicals, Peptides, and Recombinant Proteins		
Radioimmunoassay (RIPA) buffer	50 mM Tris-HCl, 150 mM NaCl, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS, 2mM EDTA	
FITC-Sinistrin	Fresenius-Kabi Austria	

Antigen unmasking solution (citrate-based)	Vector Laboratories, Cat#H-3300	
DAPI mounting medium	Thermo Fisher Scientific, Cat#P36971	
Paraformaldehyde	Thermo Fisher Scientific, Cat#O4042-500	
Avidin/Biotin blocking kit	Vector Laboratories, Cat#SP-2001	
Peroxidase substrate kit	Vector Laboratories, Cat#SK-4100	
Vectastain ABC Kit	Vector Laboratories, Cat#PK-6100	
Hydrogen peroxide	Thermo Fisher Scientific, Cat#H323	
Methanol	Thermo Fisher Scientific, Cat#A412	
BSA	Sigma-Aldrich, Cat#A7906	
Glycerol	Sigma-Aldrich Cat# G9012	
Protein ladder (standard)	ThermoFisher #26620	
Protein ladder (high molecular weight)	ThermoFisher #LC5699	
Experimental Models: Organisms/Strains		
Mouse: C57BL/6J	The Jackson Laboratory, Stock No: 000664	

Supplemental Methods

Perfusion-fixation and histologic preparation

After exsanguination, the left ventricular apex was used to deliver 0.9% sodium chloride solution at a fixed pressure of 135 mmHg until both kidneys and the liver were blanched. The right renal pedicle was clamped, and 4% paraformaldehyde perfused through the left ventricular apex. Both kidneys were then removed; the right kidney (having been perfused only with saline) was immediately placed in liquid nitrogen and stored at -80°C for protein and messenger RNA assays. The left kidney was placed in formalin for 24h, followed by paraffin embedding.

1
2
3 Immunofluorescence, immunohistochemistry, and immunoblotting:
4

5 For immunofluorescence, sections were incubated for 2h at room temperature with primary
6 antibodies in 1% bovine serum albumin/phosphate buffered saline, followed by Cy2, Cy3 or
7
8 Cy5-conjugated secondary antibodies (all 1:500, Thermo Fisher Scientific, Carlsbad CA) for 1h
9
10 at room temperature, and stained with diaminopyridine (DAPI) in the mounting medium.
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14 Immunohistochemistry was performed using the Vectastain ABC kit, (Vector Labs, Burlingame
15 CA) according to manufacturer instructions. Antigens were unmasked in citrate-based
16
17 unmasking solution (Vector Labs, Burlingame CA), and sections were blocked with 10% normal
18
19 goat serum for 20 minutes, followed by incubation with primary antibody in 10% normal goat
20
21 serum for 1h. Then sections were incubated with biotinylated secondary antibody (Vector Labs,
22
23 1:200, 30 minutes), Vectastain ABC and DAB substrate, and mounted. For immunoblotting,
24
25 protein was extracted from renal homogenate in iced radioimmunoassay precipitation buffer and
26
27 treated with protease inhibitor (Complete, Roche Applied Science, Indianapolis IN). Lysate
28
29 protein concentration was measured using the bicinchoninic acid assay and 4-12% gradient
30
31 precast gels (Bis-tris, Invitrogen, Carlsbad, CA) were loaded with 80µg of protein,
32
33 electrophoresed, and transferred to polyvinyl difluoride membranes. After blocking with 5%
34
35 milk and incubation in primary anti-myoglobin, megalin, or cubilin and HRP-conjugated
36
37 secondary antibody (see supplementary table 1), blots were imaged using enhanced
38
39 chemiluminescence (Thermo Fisher Scientific). For quantification, total protein was optically
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41 semiquantified using the Ponceau stain image of the same membrane, and the specific protein
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43 quantity expressed as ratio of the specific band density to total Ponceau density.
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Pathologic scoring of kidney injury:

For each experimental replicate (one mouse) 100 periodic-acid Schiff-stained high-power fields were reviewed in a blinded, randomized fashion. Within each high-power field, the severity of three markers (loss of brush border, tubular atrophy, epithelial vacuolization) was assessed. A composite score for each replicate was computed with weighting for the severity of each finding.

Human proximal tubular epithelial cell culture

Human kidney-2 (HK-2) cells were obtained from American Tissue Type Culture and cultured as previously described (19). To test cilastatin inhibition of myoglobin uptake, fluorescein isothiocyanate (FITC)-myoglobin or vehicle were added to wells pretreated for 2h with cilastatin (0.1 mg/mL, 0.3 mM, equivalent to plasma concentration 4 half lives after injection in the animal model) or vehicle (dimethyl sulfoxide 0.1 mg/mL) in triplicate. Cells were washed after 2h, and fluorescence measured and that of treated cells was compared to that of vehicle control. Cell culture experiments were independently repeated 5 times.

Power analysis for iMegKO cilastatin vs vehicle experiment

A priori data (means and standard deviation) were taken from the wild type cilastatin vs vehicle experiment (depicted in figure 4 in the manuscript) and used to power the experiment testing the effect of cilastatin in iMegKO mice after glycerol injection (results depicted in figure 6 in the manuscript).

Text below captured from RStudio. Lines in blue beginning with '>' denote user input.

```
> mean1<-67
> mean2<-526
> meandif<-mean2-mean1
> std1<-48.9
> std2<-217.6
> stds<-c(std1,std2)
```

```
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3 > sstds<-stds^2
4 > pooled_std<-sqrt(mean(sstds))
5 > dee<-meandif/pooled_std
6 > pwr::pwr.t.test(d=dee, power=0.8, type="two.sample")
7
```

```
8       Two-sample t test power calculation
9
```

```
10             n = 3.180799
11             d = 2.897838
12     sig.level = 0.05
13         power = 0.8
14 alternative = two.sided
15
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16 NOTE: n is number in *each* group
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Control

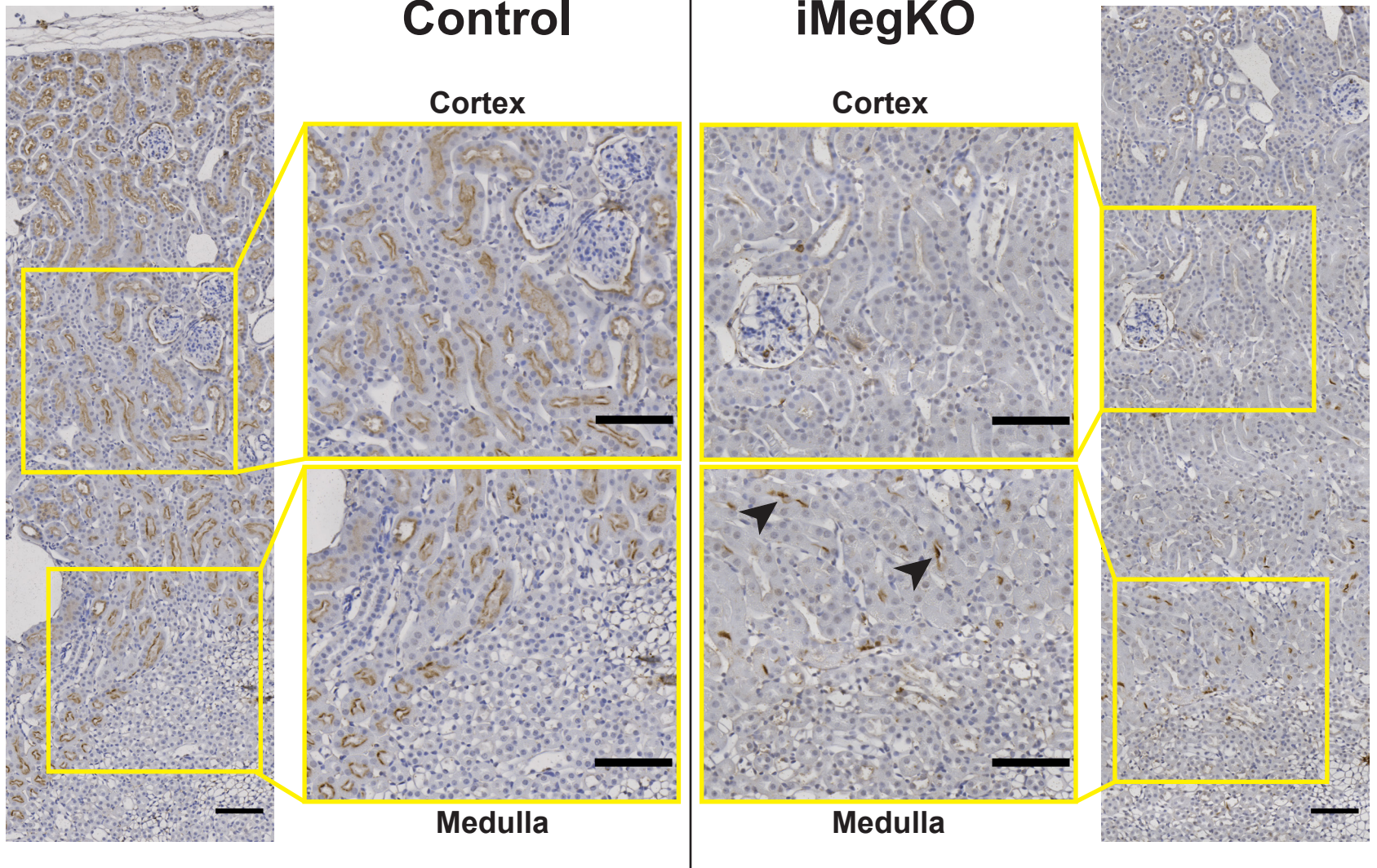
iMeggKO

Cortex

Cortex

Medulla

Medulla

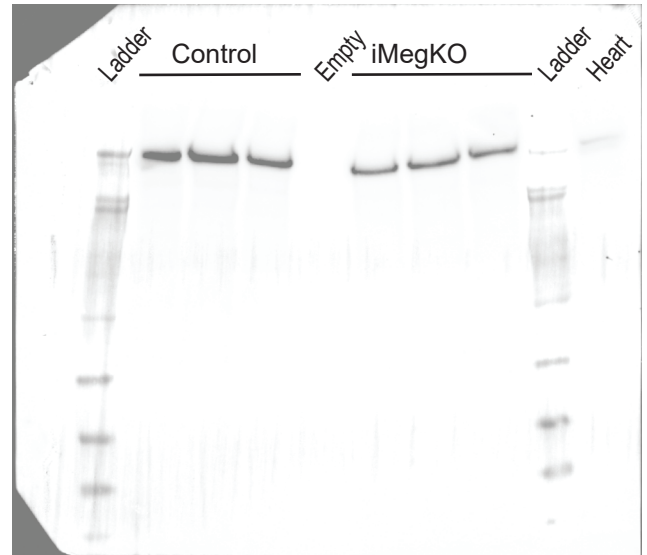
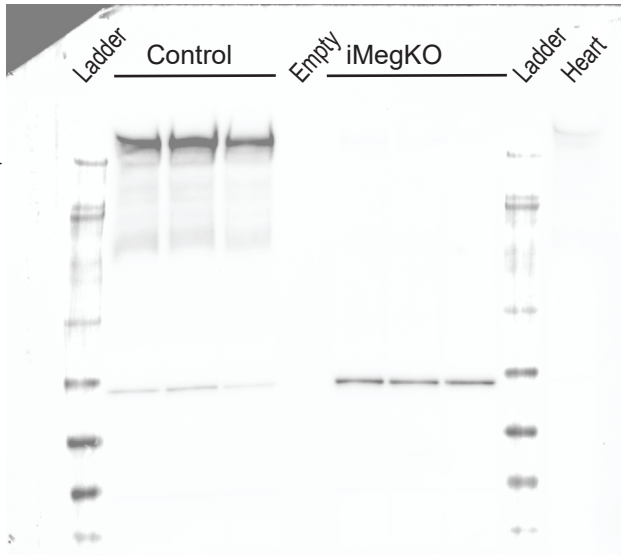


33 Supplemental Figure 1: Immunohistochemistry performed using antibody directed at megalin in kidneys of control and
 34 iMeggKO mice after induction of cre recombinase. Control mice demonstrate robust expression of megalin along the brush
 35 border of tubular epithelial cells in the cortex and medulla (i.e. in S1, S2, and S3 segments of the proximal tubule). After
 36 induction of cre recombinase, iMeggKO mice demonstrate absence of megalin from tubular brush border in the cortex with
 37 small areas of expression in tubular epithelial cells of the outer medulla (arrowheads). Scale bars are 100 μ m.
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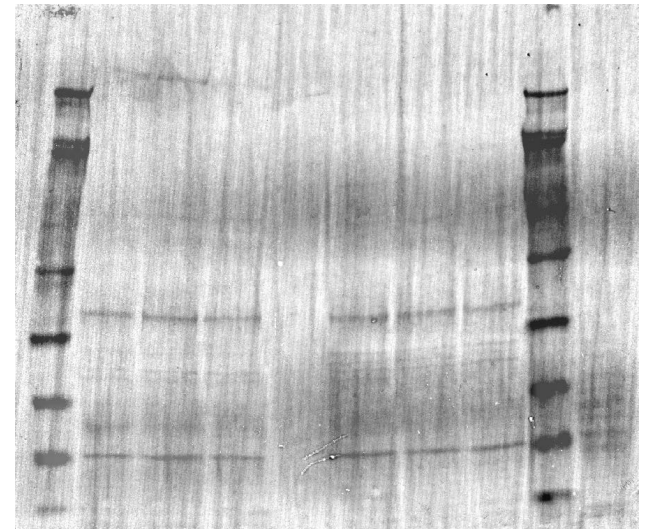
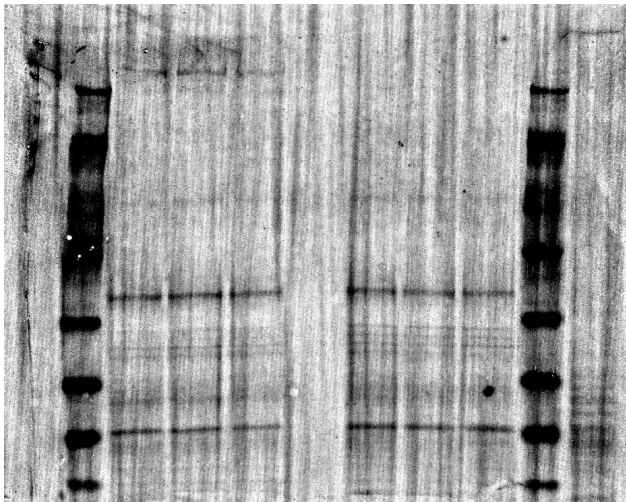
Megalin

Cubilin

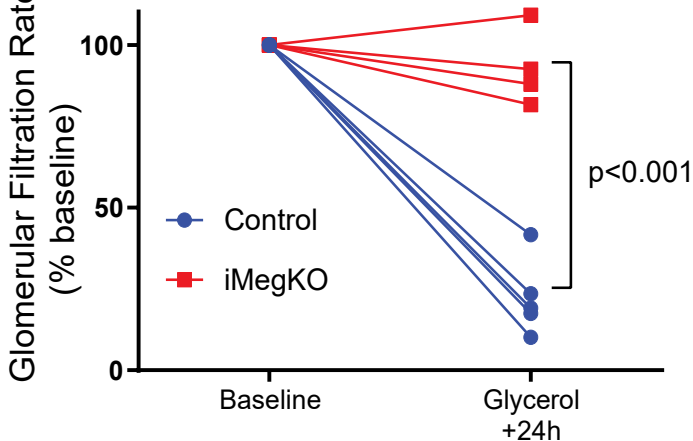


Ponceau

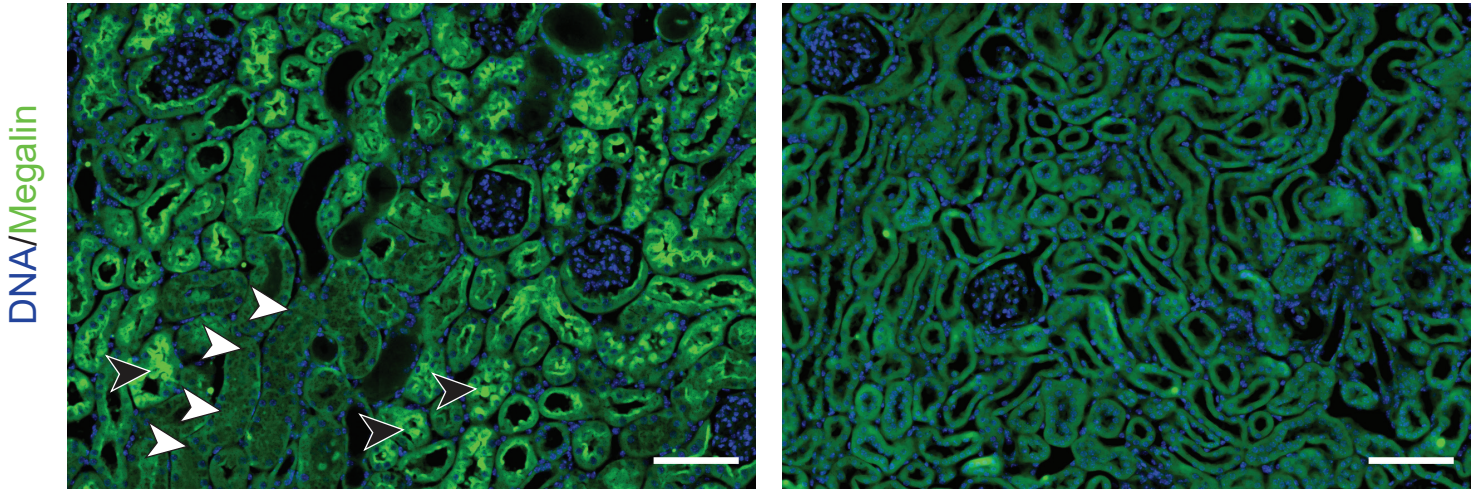
Ponceau



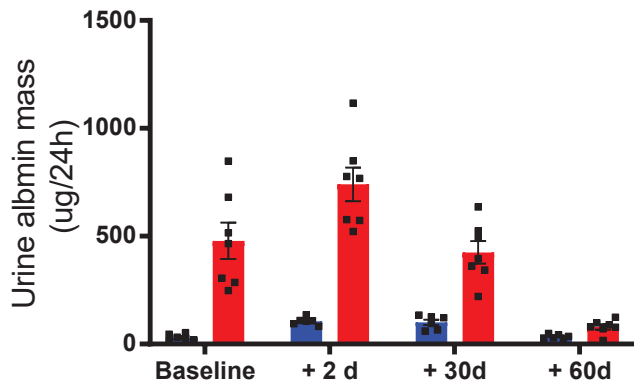
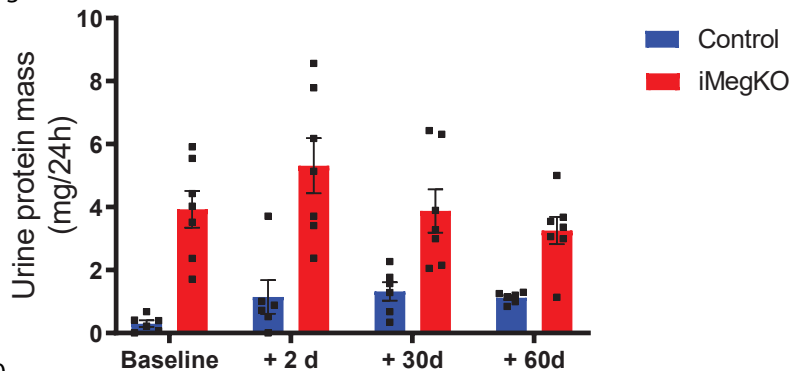
Supplemental Figure 2: iMegKO mice do not express megalin in the kidney. Full-length kidney lysate immunoblots directed at megalin (left) and cubilin (right) with respective Ponceau stains. Induction of cre recombinase after tamoxifen injection results in the deletion of megalin, without change in the abundance of cubilin.



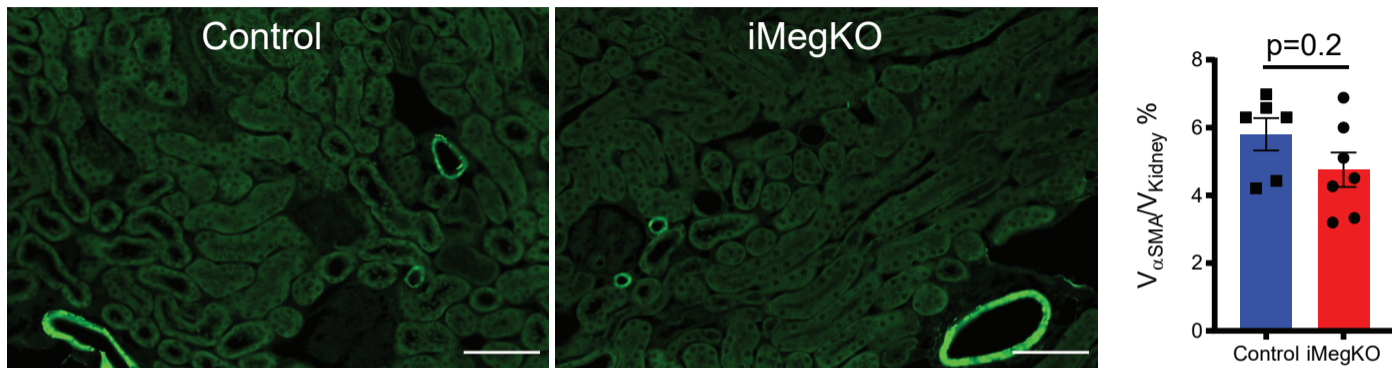
Supplemental Figure 3: Glomerular filtration rate (GFR) expressed as percent of baseline, measured before and 24h after glycerol injection. While control mice developed severe impairment of renal function (no control GFR was >50% of baseline and most were <30% of baseline), iMegKO mice demonstrated stable GFR.



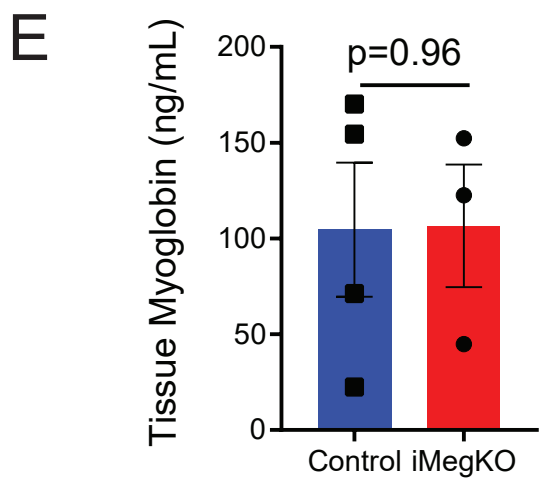
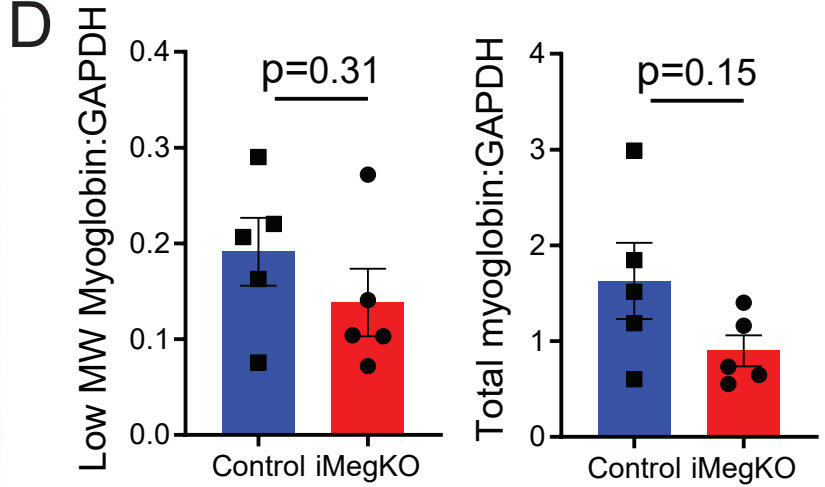
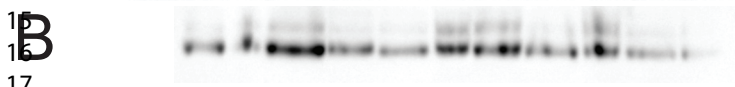
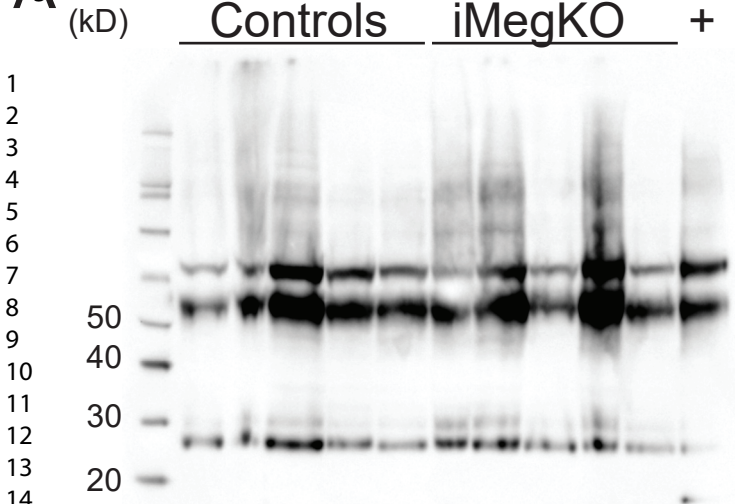
Supplemental Figure 4: Immunofluorescence with antibody directed at megalin 24 hours after glycerol injection in a control mouse (left) and one with inducible, proximal tubule-specific megalin deletion (iMegKO, right). 24h after AKI, megalin is distributed at the brush border of tubular epithelial cells in most tubules. However, tubules which exhibit cell swelling and luminal effacement consistent with injury also demonstrate absent or reduced megalin expression (white arrowheads). In some tubular lumens, megalin-positive material can be observed (black arrowheads). In iMegKO mouse, megalin is absent. Scale bars are 100 μ m.



Supplemental Figure 5: Quantitation of urine protein and albumin in control and iMegKO mice over 60 days following glycerol injection (relative change from baseline is presented in figure 3). iMegKO mice demonstrate increased urine protein and albumin all time points when compared to control mice. Both strains of mice demonstrate increased proteinuria and albuminuria after glycerol injection



Supplemental figure 6: Representative renal sections from control and iMegKO mice 60 days after glycerol injection, stained with fluorescent antibody to α -smooth muscle actin (α SMA). Interstitial α SMA is not observed; vascular smooth muscle in vessel walls serves as positive control. Quantification, shown at right, demonstrates no difference between strains.

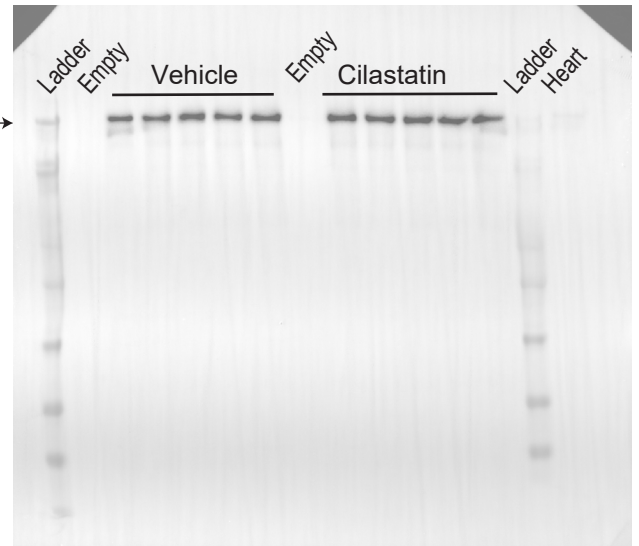
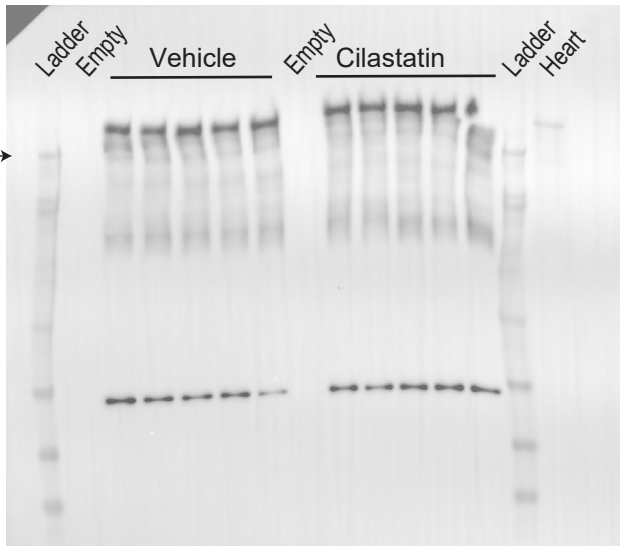


Supplemental Figure 7: Uncropped immunoblot performed on renal homogenate from control and iMeggKO mice 24 hours after glycerol injection. A. Visualization of blot after incubation with anti-myoglobin antibody demonstrates multiple bands, consistent with multimers of myoglobin. The positive control is dilute mouse cardiac lysate. B. Cropped, contrast-enhanced image of the low molecular-weight bands from image A as seen in figure 6. C. Visualization of blot after incubation with anti-GAPDH antibody. D. Quantification of the ratio of all myoglobin-positive bands ("total myoglobin") to GAPDH and that of the low molecular-weight myoglobin-positive band to GAPDH. There was no difference between groups in the quantity of myoglobin retained in the kidney. Results are representative of >4 repeated experiments. E. Confirmatory quantitative ELISA performed on the same renal homogenates demonstrates nearly-identical renal tissue concentration of myoglobin.

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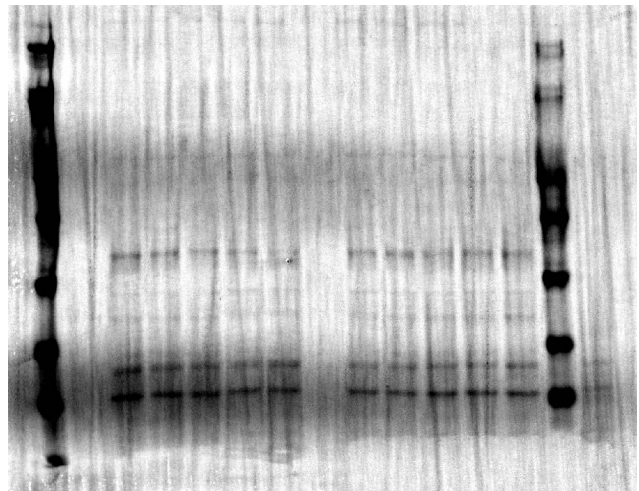
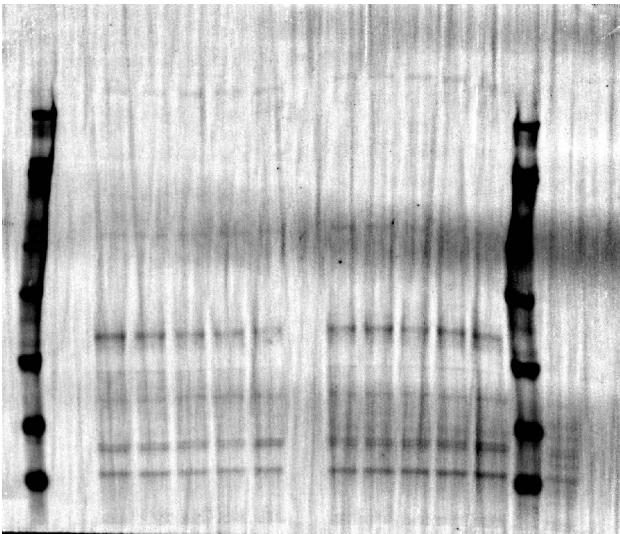
Megalin

Cubilin

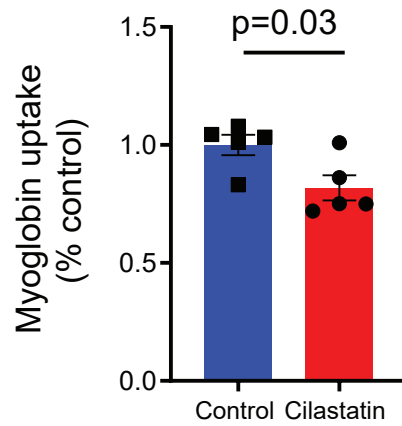


Ponceau

Ponceau



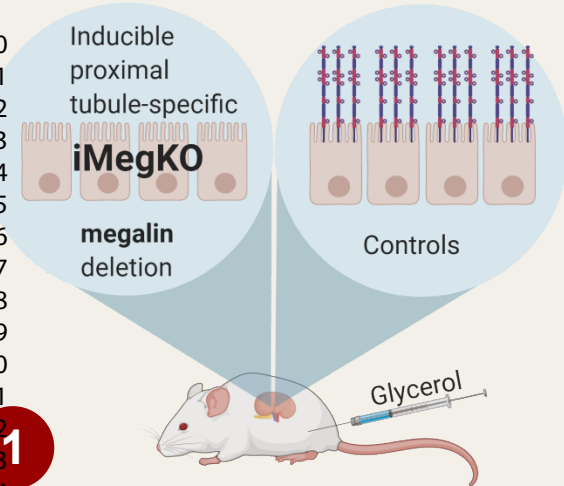
Supplemental Figure 8: Full blots for megalin and cubilin obtained from renal homogenate of wild-type mice treated with vehicle or cilastatin. 200 mg/kg cilastatin was injected retroorbitally and kidneys harvested 24h later. Presented are full-length kidney lysate immunoblots directed at megalin (left) and cubilin (right) with respective Ponceau stains for total protein (below). Quantification is in figure 9.



Supplemental Figure 9: Results of cell culture experiment testing uptake of FITC-myoglobin in human kidney cells (HK2 cell line). Cells were pretreated for 2h with vehicle or cilastatin; then FITC myoglobin was added to confluent cells for 2h, and rinsed off. Total fluorescence was measured; cilastatin-treated cells demonstrated reduced fluorescence, indicating that *in vitro*, cilastatin interferes with FITC-myoglobin uptake by proximal tubular epithelial cells.

Cilastatin ameliorates acute kidney injury due to rhabdomyolysis in mice

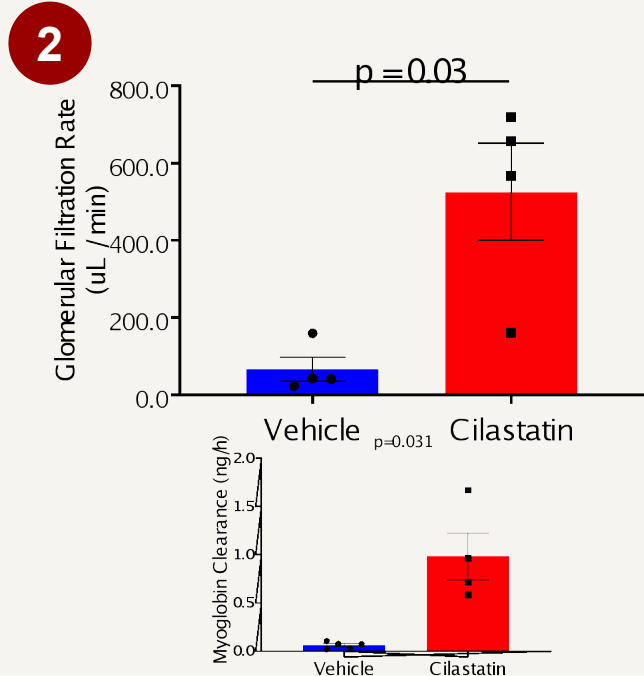
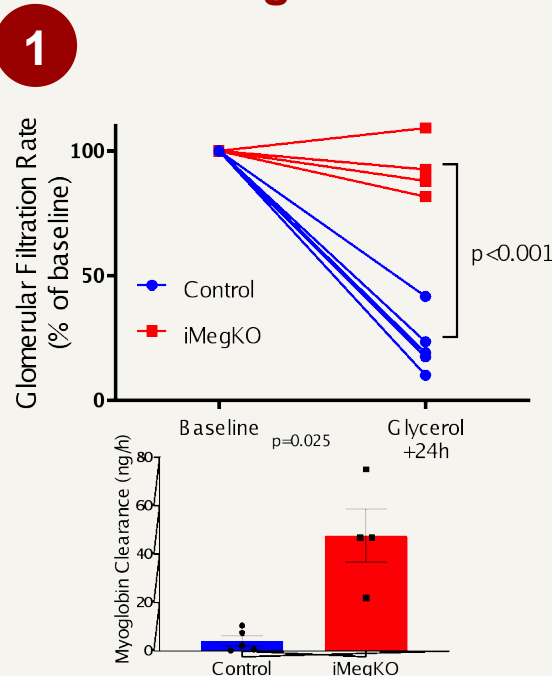
Mouse Rhabdomyolysis



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Megalín interference ameliorates AKI



CONCLUSION Megalín critically mediates rhabdomyolysis mediated AKI. Cilastatin ameliorates rhabdomyolysis-induced AKI.

doi: 10.1681/ASN.

Appendix 2:

Abstract #MHSRS-21-03948 Oral presentation. Military Health System Research Symposium (MHSRS).

23-26 August, 2021. Kissimmee, FL

Rapid interference with crush syndrome-induced myoglobin endocytosis in the kidney

Authors: Hebert, Matsushita, Nickerson, Munhall, Eiwaz, Groat, Funahashi, McCormick, Hutchens

Crush injury, causing rhabdomyolysis, induces acute kidney injury (AKI). There is no specific therapy. Muscle-derived myoglobin is a renal toxin via megalin-mediated endocytosis, but the kinetics of renal uptake and means to interfere with myoglobin endocytosis are poorly understood. We characterized myoglobin uptake *in vivo* and tested the hypothesis that a putative megalin inhibitor, cilastatin, recapitulates effects of megalin interference.

Methods: Mouse procedures in wild-type (WT) male C57BL/6 mice and inducible, proximal tubule-specific megalin-deleted mice (iMeggKO), were approved by OHSU or PVAMC IACUC. FITC-myoglobin (FMb) and cilastatin (200 mg/kg) were injected retroorbitally. Experimental rhabdomyolysis was induced via intramuscular injection of 50% glycerol (8 mL/kg). Glomerular filtration rate was measured 24h later, and renal immunoblotting and immunofluorescence were performed.

Results: FMb was visible in punctae within proximal tubular cells 15 minutes after injection, but largely absent 30 minutes after injection in control mice, while in iMeggKO mice, FMb puncta were nearly absent at all time points ($p < 0.0001$). In experimental rhabdomyolysis, iMeggKO prevented AKI, with 24h GFR 5x control, ($p < 0.001$). Injection of cilastatin to healthy wild-type mice did not alter GFR or megalin expression, but induced selective low molecular-weight proteinuria identical to that of iMeggKO and reduced endocytosis of FMb. Cilastatin injection ameliorated AKI in wild-type mice (GFR 8x vehicle, $p = 0.03$), but not in iMeggKO mice ($p = 0.89$), and in wild-type increased urine myoglobin clearance (8x vehicle, $p < 0.01$) while decreasing tubular uptake of endogenous myoglobin.

Conclusions: Renal uptake of myoglobin occurs within minutes of injection or experimental rhabdomyolysis. Megalin-mediated endocytosis of myoglobin is essential for development of AKI in crush syndrome. Cilastatin inhibits megalin-mediated uptake of small proteins without effect on megalin expression. Megalin interference prevents AKI due to experimental rhabdomyolysis by reducing myoglobin endocytosis; cilastatin recapitulates this effect in a megalin-dependent fashion. Cilastatin and related drugs are attractive potential therapeutics, but timing may be critical.

Appendix 3: Hutchens lab SOP: Pig Kidney Harvest and Perfusion:

1. Remove both kidneys with enough vascular pedicle to secure tubing
2. Cannulate renal artery and secure cannula. Cannula needs to have a Luer-lock fitting (i.e., a large-bore intravenous catheter, rapid infusion catheter, or IV tubing with end-Luer.)
3. Perfuse kidney via renal artery cannula with normal saline until effluent from renal vein runs clear.
4. Leave renal artery cannula in place.
5. Place kidneys in container for transport. Ensure container is labeled with pig# and date.
6. Transport ASAP to Nicole Andeen, Pathology. Cell: 503-360-7396/office: 503-418-1595

In OHSU Pathology:

7. Perfuse one kidney with 4% PFA
8. Bivalve
9. Embed in paraffin
10. Do not perfuse contralateral kidney with PFA
11. Cut ~ 1 cc from mid cortex and ~1 cc from medulla
12. Place cut sections in containers labeled with pig # and "med" or "cor"
13. Snap-freeze cut sections in dry ice/isopropanol or liquid nitrogen and transfer to -80°C storage.

Appendix 4a: Hutchens Pig Samples Collection Standard Operating Procedures (SOP) v.6.11.21

Intro:

1. This SOP refers to companion **Samples Timeline** and **Samples Table**, which give further details on sampling times, volumes, and recommended containers.
2. Keep all samples chilled (on ice or at 4C) as soon as possible. Temporary storage of all samples on ice is great. Move samples to 4C when time allows. Do not freeze any samples at any time.
3. Centrifugation of plasma should occur at RT for 10 min at 1500 RCF.
4. All sample containers should be completely labeled (see next section).

Labeling:

1. Each animal will have been assigned a unique identification number, incrementing with each surgery.
2. Many samples are taken at several timepoints during the 2-day surgery. A sequence number, should be included in the label (e.g. standard draws will be numbered 0 through 7 to match the timeline: pre-injury, 0, 2, 6, 12, 18, 24, and 48h). For iohexol plasma sequences, an addition “set number” should be added to identify set 1 from the first day, and set 2 from the second day.
3. Essentially, label each container with the following: pig number, date, sample type, sample number.

Urine:

1. All urine is to be taken from the inlet tube leading to the collection bag. Urine from the bottom of the Foley bag is averaged over time and not useable for this reason.
2. For standard lab draws, take 1-2 mL urine into a 2 mL screw cap vial.
3. For the proteomics urine draw, transfer 2-3 mL urine into the 10 mL screw cap vial—roughly one third the volume of the container. Then add protease inhibitor solution (ThermoFisher HALT) at a 1:100 dilution (e.g. 30uL for 3mL) and shake gently to mix.
4. Put all urine samples on ice or at 4C immediately.
5. Store all urines at 4C for later pick-up by Hutchens lab couriers.

Blood plasma:

General notes:

1. It is important to take steps to avoid hemolysis. Use vacutainers for blood collection. If using a needle, it should be 21 gauge.
2. Several vials of lithium heparin plasma are to be merged into a single storage container. Avoid merging any hemolyzed heparin samples. If possible, recollect the hemolyzed vial or store it in its own container.

Procedure:

1. After taking arterial whole blood into the specified vacutainers, invert the filled vacutainer 8-10 times gently to mix the anticoagulants.
2. Store vacutainers upright on ice until centrifugation. Samples can be centrifuged within 1 hour after collection if stored on ice in the meantime.
3. Centrifuge blood samples for 10 minutes at 1500 RCF at room temperature.
4. Transfer the plasma layer above the buffy coat to the designated storage container for each plasma type. Include any lipid floating near the top of the plasma layer.
5. Store upright at 4C for later pick-up by Hutchens lab couriers.

Kidney:

1. Following euthanasia of the pig, dissect one kidney (either). Place kidney in a clear plastic bag and store bag under ice.
2. A Hutchens lab courier (Adam Munhall or Tahnee Groat) should transport without delay to Nicole Andeen, RJH 1137 (office) 503-360-7396 (cell) 503-418-1595 (office).

iStat Chem8+ and CG4+:

1. Abbott iStat Chem8 and CG4 cartridges are run several times during a surgery.
2. Use the pig ID number at the iStat “patient ID” prompts.
3. The iStat will record wall-clock time but this will need to be translated into sample sequence number or time for the repeated samples of each surgery.
4. An online spreadsheet will be used to share the iStat results between Schreiber and Hutchens labs.

Stago PT/INR:

1. The Schreiber Lab Stago analyzer will be used to measure Prothrombin Time (PT).
2. Aliquots of the citrate plasma samples will be made in the Hutchens lab and brought back to the Schreiber lab for analysis.
3. The online spreadsheet for iStat data (see above) will also be used to share Stago results.

Iohexol aka. OmniPaque 300:

1. Iohexol solution (5 mL, OmniPaque 300, GE Healthcare) is injected IV twice, at 19 hours and 43 hours post injury, hereafter called iohexol #1 and #2 respectively. A series of 7 small plasma draws are made at specific times post injection, to be numbered 1 through 7 for each iohexol series.
2. It is especially important the first few closely spaced draws are drawn after the specified time after injection.

PlasmaLyte:

1. PlasmaLyte A (500mL, Baxter) will be infused IV post-injury over 15 minutes or less.
2. Start infusion at least 30 minutes after chest impacts.
3. Any treatment drugs will be predisolved in the plasmalyte in the Hutchens lab and Schreiber lab will be blind to the contents.
4. The PlasmaLyte bag will be labeled with the pig number and brought to the Schreiber lab before each surgery.

Sample type	Sample volume (mL)	Collection container	Collection containers per time point	Storage container (for plasmas)	Storage containers per time point	Total endpoint containers in 48h sx	Special handling notes: (See Samples Collection SOP for full details)
urine	1 - 2	2 mL screw cap tube	1	---	---	7	
heparin plasma	4	4 mL lithium heparin vacutainer	5	15mL conical tube (Falcon, blue cap)	1	8	Merge the plasma from the 5 collection tubes into 1 storage tube.
citrate plasma	2.7	2.7 mL sodium citrate vacutainer	1	2mL screw cap cryo tube (Corning, orange cap)	1	8	
-omics urine	2 – 3	10 mL screw cap tube	1	---	---	1	Collection vial will contain protease inhibitor cocktail for 3mL volume. Do not overfill.
Iohexol plasma	2	2 mL lithium heparin vacutainer	1	1.5 mL conical snap cap microtube	1	14	

Collection container part numbers:

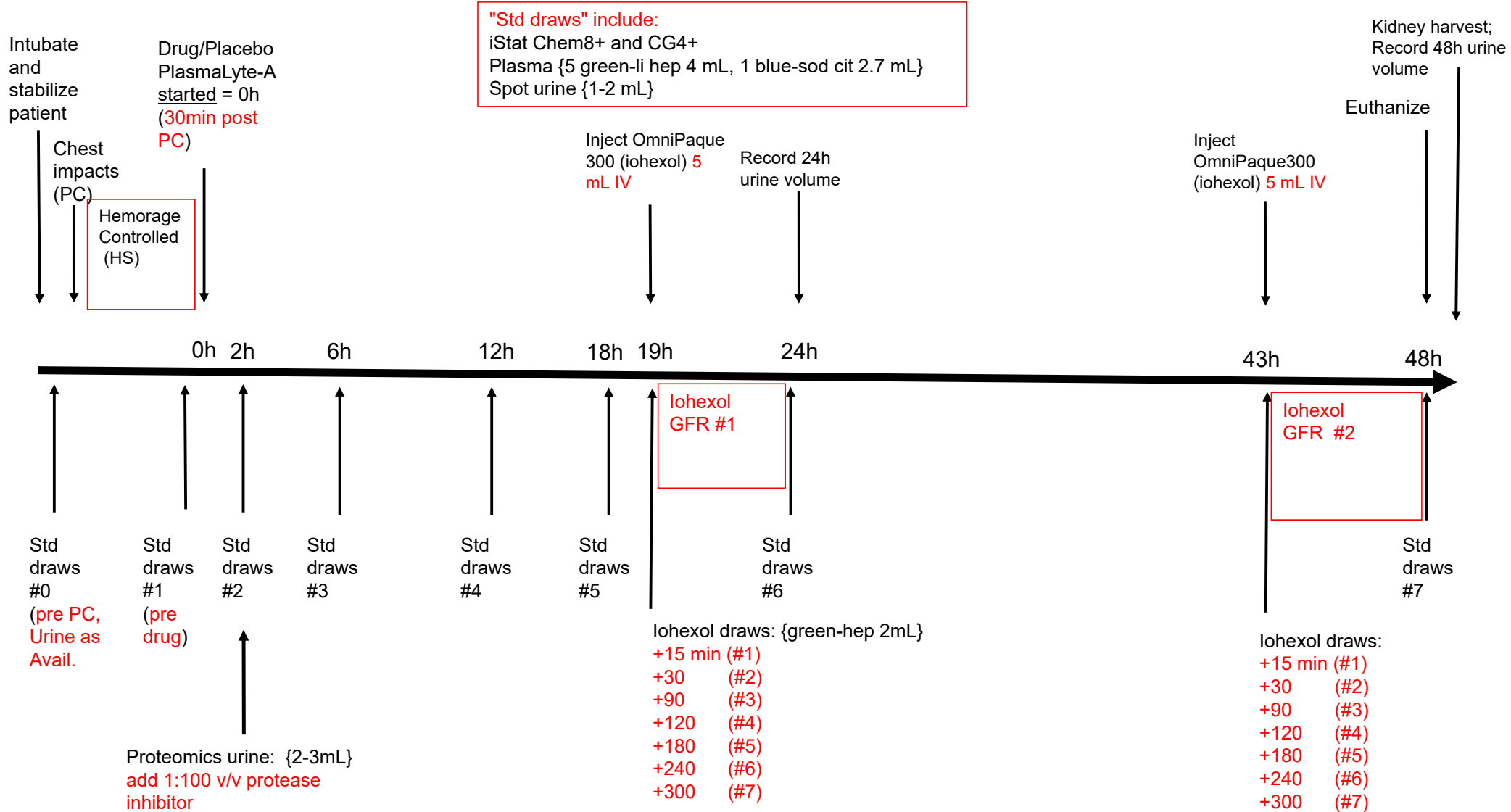
Urine:	Sarstedt 72.649.006	2 mL microtube, clear screw cap	Research Stores 142339
Omics urine:	Sarstedt 60.551	10 mL tube, clear screw cap	Research Stores 159031
Heparin plasma:	Becton Dickson 367884 124149	4 mL 13x75 lithium heparin green hemogard	Med Supplies
Citrate plasma:	Becton Dickson 369714 124838	2.7 mL 13x75 sodium citrate lt blue hemogard	Med Supplies
Iohexol plasma:	Becton Dickson 366664	2 mL 13x75 lithium heparin green hemogard	Fisher Sci 0265729

Storage container part numbers:

Heparin plasma:	Becton Dickson 352097	15 mL "Falcon" conical, blue screw cap	Research Stores 144916
Citrate plasma:	Corning 430052	2 mL cryo tube, orange screw cap	Research Stores 144917
Iohexol plasma:	Fisher 05408129 142340	1.5 mL microcentrifuge tube, snap cap	Research Stores

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Appendix 4c: Rhabdo pig samples timeline



v.6/11/21