OMB Number: 4040-0001
Expiration Date: 12/31/2022

APPLICATION FOR FEDERAL ASSISTANCE
SF 424 (R&R)

3. DATE RECEIVED BY STATE	**State Application Identifier**

1. TYPE OF SUBMISSION*

❍ Pre-application ● Application ❍ Changed/Corrected Application

4.a. Federal Identifier

b. Agency Routing Number

c. Previous Grants.gov Tracking Number

2. DATE SUBMITTED | **Application Identifier**

5. APPLICANT INFORMATION **UEI*:** JZWJLWEF1KR9

Legal Name*: SPAULDING REHABILITATION HOSPITAL
Department:
Division:
Street1*: SPAULDING REHABILITATION HOSPITAL
Street2: 300 1st Avenue
City*: CHARLESTOWN
County:
State*: MA: Massachusetts
Province:
Country*: USA: UNITED STATES
ZIP / Postal Code*: 021293109

Person to be contacted on matters involving this application
Prefix: First Name*: Steven Middle Name: Last Name*: Quinn Suffix:
Position/Title: Research Finance Manager
Street1*: 399 Revolution Drive
Street2:
City*: Somerville
County: Massachusetts
State*: MA: Massachusetts
Province:
Country*: USA: UNITED STATES
ZIP / Postal Code*: 02145-0000
Phone Number*: 8572821769 Fax Number: Email: STQUINN@partners.org

6. EMPLOYER IDENTIFICATION NUMBER *(EIN) or (TIN)** 1042551124A1

7. TYPE OF APPLICANT* M: Nonprofit with 501C3 IRS Status (Other than Institution of Higher Education)

Other (Specify):
Small Business Organization Type ❍ Women Owned ❍ Socially and Economically Disadvantaged

8. TYPE OF APPLICATION*

● New ❍ Resubmission
❍ Renewal ❍ Continuation ❍ Revision

If Revision, mark appropriate box(es).

❍ A. Increase Award ❍ B. Decrease Award ❍ C. Increase Duration
❍ D. Decrease Duration ❍ E. Other *(specify)* :

Is this application being submitted to other agencies?* ❍Yes ●No What other Agencies?

9. NAME OF FEDERAL AGENCY*
National Institutes of Health

10. CATALOG OF FEDERAL DOMESTIC ASSISTANCE NUMBER
TITLE:

11. DESCRIPTIVE TITLE OF APPLICANT'S PROJECT*
Menopausal Knee-ds: Elucidating mechanisms and treatments for knee osteoarthritis

12. PROPOSED PROJECT
Start Date* Ending Date*
07/01/2023 06/30/2027

13. CONGRESSIONAL DISTRICTS OF APPLICANT
MA-007

SF 424 (R&R) APPLICATION FOR FEDERAL ASSISTANCE

14. PROJECT DIRECTOR/PRINCIPAL INVESTIGATOR CONTACT INFORMATION

Prefix: First Name*: Gabrielle Middle Name: Last Name*: Gilmer Suffix:
Position/Title: MD/PhD Trainee
Organization Name*: Spaulding Rehabilitation Hospital
Department:
Division:
Street1*: 300 1st Ave
Street2:
City*: Charlestown
County:
State*: MA: Massachusetts
Province:
Country*: USA: UNITED STATES
ZIP / Postal Code*: 02129-3109
Phone Number*: 2057899607 Fax Number: Email*: ggilmer@mgh.harvard.edu

15. ESTIMATED PROJECT FUNDING

a. Total Federal Funds Requested*	$207,008.00
b. Total Non-Federal Funds*	$0.00
c. Total Federal & Non-Federal Funds*	$207,008.00
d. Estimated Program Income*	$0.00

16.IS APPLICATION SUBJECT TO REVIEW BY STATE EXECUTIVE ORDER 12372 PROCESS?*

a. YES ○ THIS PREAPPLICATION/APPLICATION WAS MADE AVAILABLE TO THE STATE EXECUTIVE ORDER 12372 PROCESS FOR REVIEW ON:
DATE:

b. NO ● PROGRAM IS NOT COVERED BY E.O. 12372; OR
○ PROGRAM HAS NOT BEEN SELECTED BY STATE FOR REVIEW

17. By signing this application, I certify (1) to the statements contained in the list of certifications* and (2) that the statements herein are true, complete and accurate to the best of my knowledge. I also provide the required assurances * and agree to comply with any resulting terms if I accept an award. I am aware that any false, fictitious, or fraudulent statements or claims may subject me to criminal, civil, or administrative penalties. (U.S. Code, Title 18, Section 1001)

● I agree*

** The list of certifications and assurances, or an Internet site where you may obtain this list, is contained in the announcement or agency specific instructions.*

18. SFLLL or OTHER EXPLANATORY DOCUMENTATION File Name:

19. AUTHORIZED REPRESENTATIVE

Prefix: First Name*: Maureen Middle Name: Elaine Last Name*: Kelly Suffix:
Position/Title*: Senior Grant Administrator/AOR
Organization Name*: Spaulding Rehabilitation Hospital
Department:
Division:
Street1*: 300 1st Ave
Street2:
City*: Charlestown
County:
State*: MA: Massachusetts
Province:
Country*: USA: UNITED STATES
ZIP / Postal Code*: 02129-3109
Phone Number*: 617-952-6179 Fax Number: Email*: mkelly37@partners.org

Signature of Authorized Representative*
Maureen Kelly

Date Signed*
12/06/2022

20. PRE-APPLICATION File Name:

21. COVER LETTER ATTACHMENT File Name:gilmerCoverLetter_final.pdf

424 R&R and PHS-398 Specific
Table Of Contents

OMB Number: 4040-0010
Expiration Date: 12/31/2022

Project/Performance Site Location(s)

Project/Performance Site Primary Location

❍ I am submitting an application as an individual, and not on behalf of a company, state, local or tribal government, academia, or other type of organization.

Organization Name:	SPAULDING REHABILITATION HOSPITAL
UEI:	JZWJLWEF1KR9
Street1*:	SPAULDING REHABILITATION HOSPITAL
Street2:	300 1st Avenue
City*:	CHARLESTOWN
County:	
State*:	MA: Massachusetts
Province:	
Country*:	USA: UNITED STATES
Zip / Postal Code*:	021293109
Project/Performance Site Congressional District*:	MA-007

Additional Location(s) File Name:

OMB Number: 4040-0010
Expiration Date: 12/31/2022

RESEARCH & RELATED Other Project Information

1. Are Human Subjects Involved?* ❍ **Yes** ● **No**

1.a. If YES to Human Subjects

Is the Project Exempt from Federal regulations? ❍ Yes ❍ No

If YES, check appropriate exemption number: _ 1 _ 2 _ 3 _ 4 _ 5 _ 6 _ 7 _ 8

If NO, is the IRB review Pending? ❍ **Yes** ❍ **No**

IRB Approval Date:

Human Subject Assurance Number

2. Are Vertebrate Animals Used?* ● **Yes** ❍ **No**

2.a. If YES to Vertebrate Animals

Is the IACUC review Pending? ❍ Yes ● No

IACUC Approval Date: 10-31-2022

Animal Welfare Assurance Number D16-00361

3. Is proprietary/privileged information included in the application?* ❍ **Yes** ● **No**

4.a. Does this project have an actual or potential impact - positive or negative - on the environment?* ❍ **Yes** ● **No**

4.b. If yes, please explain:

4.c. If this project has an actual or potential impact on the environment, has an exemption been authorized or an environmental assessment (EA) or environmental impact statement (EIS) been performed? ❍ Yes ❍ No

4.d. If yes, please explain:

5. Is the research performance site designated, or eligible to be designated, as a historic place?* ❍ **Yes** ● **No**

5.a. If yes, please explain:

6. Does this project involve activities outside the United States or partnership with international collaborators?* ❍ **Yes** ● **No**

6.a. If yes, identify countries:

6.b. Optional Explanation:

	Filename
7. Project Summary/Abstract*	gggProjectSummaryAbstract_Final.pdf
8. Project Narrative*	gilmerProjectNarrative_Final.pdf
9. Bibliography & References Cited	gilmerBibliographyRefCited_final.pdf
10.Facilities & Other Resources	gilmer_facilitiesOtherResources_Final.pdf
11.Equipment	gilmer_majorEquipment_Final.pdf
12. Other Attachments	gilmerIACUCapprovalLetter_final.pdf gilmerIACUCProtocol_final_removed_dec6_2022.pdf

PROJECT SUMMARY/ABSTRACT

Background: As of 2020, an estimated 654.1 million adults live with knee osteoarthritis (KOA), and women who are post-menopausal are nearly twice as likely to develop KOA compared to men. Despite this, most animal studies on KOA include only males, and, of the few studies utilizing females, menopause is typically not included, as it is not a naturally occurring process in non-primates. As such, there is a paucity of literature aimed at understanding mechanisms of menopause associated KOA and a corresponding absence of treatment interventions specifically for post-menopausal people with KOA. As a part of my PhD work thus far, I have developed a chemically-induced menopause model by injecting middle-aged female mice with 4-vinylcyclohexene diepoxide (VCD). I have confirmed this model displays a menopausal phenotype, including perimenopause, and that these mice display more severe KOA than age-matched, non-menopausal mice.

The research goals of this fellowship are to interrogate mechanisms of menopause-induced KOA using our VCD model (*Specific Aim 1*) and employ synthetic biology techniques to lay the groundwork for new treatment modalities for menopause-indued KOA and (*Specific Aim 2*).

Specific Aim 1: A component of E3 ubiquitin ligase was recently identified in a GWAS meta-analysis as a unique contributor to OA in women. As such, I will interrogate the role of estradiol-regulated, ubiquitin proteolysis in mediating menopause-induced KOA using our VCD menopause model. Changes in ubiquitin proteolysis signaling across perimenopause, menopause, and with estradiol treatment will be quantified both *in vivo* and *in vitro*. **We hypothesize that (1) menopause induction will disrupt ubiquitin proteolysis activity and (2) estradiol treatment started early in menopause will restore ubiquitin proteolysis signaling and ultimately quinch menopausal KOA.**

Specific Aim 2: I will design an estradiol-regulated controls circuit to modulate a gene of interest (GOI) *in vitro*. A significant drop in estradiol is a principal physiological change associated with menopause, and an estradiol-repressed promoter will be designed to turn the circuit on. GOI candidates will be generated from previous studies and will be systematically tested to determine ideality for mediating chondrogenicity. **We hypothesize that a genetic controls circuit modulated by estradiol will attenuate chondrocyte health *in vitro.***

Impact: To support my fellowship, I have assembled a multidisciplinary team with expertise in KOA, menopause, aging, and synthetic biology. I have strategized with my mentoring team to design a rigorous training plan that will take advantage of the extraordinary research environments offered at my sponsor's new institution (Spaulding Rehabilitation, Harvard Medical School) and the University of Pittsburgh, where I remain a full-time student. This fellowship will propel me towards my long-term goals of being a physician-scientist who practices orthopedic surgery and research leader in joint diseases and injuries presenting in women.

PROJECT NARRATIVE

Post-menopausal women are twice as likely to develop knee osteoarthritis than men; yet there are currently no animal studies aimed at understanding how natural menopause propagates knee osteoarthritis and, thus, no specific treatments for post-menopausal people with knee osteoarthritis. This proposal interrogates the role of pathologic protein turnover in menopause-induced knee osteoarthritis and employs synthetic biology techniques to develop an estradiol-regulated genetic controls circuit. Completion of these studies will provide insight into the underlying mechanisms of menopause-induced knee osteoarthritis and provide the first steppingstone for gene-therapy treatments for this disease.

BIBLIOGRAPHY & REFERENCES CITED

1. Cui A, Li H, Wang D, Zhong J, Chen Y, Lu H. Global, regional prevalence, incidence and risk factors of knee osteoarthritis in population-based studies. *EClinicalMedicine.* 2020;29-30:100587.
2. Liu Q, Niu J, Li H, et al. Knee Symptomatic Osteoarthritis, Walking Disability, NSAIDs Use and All-cause Mortality: Population-based Wuchuan Osteoarthritis Study. *Sci Rep.* 2017;7(1):3309.
3. Felson DT, Neogi T. Emerging Treatment Models in Rheumatology: Challenges for Osteoarthritis Trials. *Arthritis Rheumatol.* 2018;70(8):1175-1181.
4. Blagojevic M, Jinks C, Jeffery A, Jordan KP. Risk factors for onset of osteoarthritis of the knee in older adults: a systematic review and meta-analysis. *Osteoarthritis Cartilage.* 2010;18(1):24-33.
5. Kuyinu EL, Narayanan G, Nair LS, Laurencin CT. Animal models of osteoarthritis: classification, update, and measurement of outcomes. *J Orthop Surg Res.* 2016;11:19.
6. Diaz Brinton R. Minireview: translational animal models of human menopause: challenges and emerging opportunities. *Endocrinology.* 2012;153(8):3571-3578.
7. Iijima H, Gilmer G, Wang K, et al. Age-related increase in matrix stiffness downregulates α-Klotho in chondrocytes and induces cartilage degeneration. *bioRxiv.* 2021:2021.2003.2013.434679.
8. Brooks HL, Pollow DP, Hoyer PB. The VCD Mouse Model of Menopause and Perimenopause for the Study of Sex Differences in Cardiovascular Disease and the Metabolic Syndrome. *Physiology (Bethesda).* 2016;31(4):250-257.
9. Ibanez L, Alcaraz MJ, Maicas N, et al. Downregulation of the inflammatory response by CORM-3 results in protective effects in a model of postmenopausal arthritis. *Calcif Tissue Int.* 2012;91(1):69-80.
10. Engdahl C, Jochems C, Windahl SH, et al. Amelioration of collagen-induced arthritis and immune-associated bone loss through signaling via estrogen receptor alpha, and not estrogen receptor beta or G protein-coupled receptor 30. *Arthritis Rheum.* 2010;62(2):524-533.
11. Hoegh-Andersen P, Tanko LB, Andersen TL, et al. Ovariectomized rats as a model of postmenopausal osteoarthritis: validation and application. *Arthritis Res Ther.* 2004;6(2):R169-180.
12. Gilmer G BA, Iijima H, Jackson N, Thurston R, Ambrosio F. Uncovering the “riddle of femininity” in osteoarthritis: a systematic review and meta-analysis of menopausal animal models and mathematical modeling of estrogen treatment *Under Review at Osteoarthritis and Cartilage.*
13. The 2017 hormone therapy position statement of The North American Menopause Society. *Menopause.* 2018;25(11):1362-1387.
14. Boer CG, Hatzikotoulas K, Southam L, et al. Deciphering osteoarthritis genetics across 826,690 individuals from 9 populations. *Cell.* 2021;184(18):4784-4818 e4717.
15. Yuan G, Yang S, Yang S. Macrophage RGS12 contributes to osteoarthritis pathogenesis through enhancing the ubiquitination. *Genes Dis.* 2022;9(5):1357-1367.
16. Radwan M, Wilkinson DJ, Hui W, et al. Protection against murine osteoarthritis by inhibition of the 26S proteasome and lysine-48 linked ubiquitination. *Ann Rheum Dis.* 2015;74(8):1580-1587.
17. Kobak KA, Batushansky A, Borowik AK, et al. An In Vivo Stable Isotope Labeling Method to Investigate Individual Matrix Protein Synthesis, Ribosomal Biogenesis, and Cellular Proliferation in Murine Articular Cartilage. *Function (Oxf).* 2022;3(2):zqac008.
18. Shashova EE, Astakhova TM, Plekhanova AS, et al. Changes in proteasome chymotrypsin-like activity during the development of human mammary and thyroid carcinomas. *Bull Exp Biol Med.* 2013;156(2):242-244.
19. Kondakova IV, Spirina LV, Shashova EE, et al. [Proteasome activity in tumors of female reproductive system]. *Bioorg Khim.* 2012;38(1):106-110.
20. Kondakova IV, Spirina LV, Koval VD, et al. [Chymotripsin-like activity and subunit composition of proteasomes in human cancers]. *Mol Biol (Mosk).* 2014;48(3):444-451.
21. Kondakova IV, Shashova EE, Sidenko EA, Astakhova TM, Zakharova LA, Sharova NP. Estrogen Receptors and Ubiquitin Proteasome System: Mutual Regulation. *Biomolecules.* 2020;10(4).
22. Paciuc J. Hormone Therapy in Menopause. *Adv Exp Med Biol.* 2020;1242:89-120.
23. The Hormone Therapy Position Statement of The North American Menopause Society" Advisory P. The 2022 hormone therapy position statement of The North American Menopause Society. *Menopause.* 2022;29(7):767-794.
24. Tavani A, La Vecchia C. The adverse effects of hormone replacement therapy. *Drugs Aging.* 1999;14(5):347-357.

25. Ruder WC, Lu T, Collins JJ. Synthetic biology moving into the clinic. *Science.* 2011;333(6047):1248-1252.
26. Tan X, Letendre JH, Collins JJ, Wong WW. Synthetic biology in the clinic: engineering vaccines, diagnostics, and therapeutics. *Cell.* 2021;184(4):881-898.
27. Krzyszton R, Wan Y, Petreczky J, Balazsi G. Gene-circuit therapy on the horizon: synthetic biology tools for engineered therapeutics. *Acta Biochim Pol.* 2021;68(3):377-383.
28. Saxena P, Charpin-El Hamri G, Folcher M, Zulewski H, Fussenegger M. Synthetic gene network restoring endogenous pituitary-thyroid feedback control in experimental Graves' disease. *Proc Natl Acad Sci U S A.* 2016;113(5):1244-1249.
29. Humphreys IR, Sebastian S. Novel viral vectors in infectious diseases. *Immunology.* 2018;153(1):1-9.
30. McNerney MP, Zhang Y, Steppe P, Silverman AD, Jewett MC, Styczynski MP. Point-of-care biomarker quantification enabled by sample-specific calibration. *Sci Adv.* 2019;5(9):eaax4473.
31. Nims RJ, Pferdehirt L, Ho NB, et al. A synthetic mechanogenetic gene circuit for autonomous drug delivery in engineered tissues. *Sci Adv.* 2021;7(5).
32. Shah K, McCormack CE, Bradbury NA. Do you know the sex of your cells? *Am J Physiol Cell Physiol.* 2014;306(1):C3-18.
33. McLean AC, Valenzuela N, Fai S, Bennett SA. Performing vaginal lavage, crystal violet staining, and vaginal cytological evaluation for mouse estrous cycle staging identification. *J Vis Exp.* 2012(67):e4389.
34. Poole AR. Osteoarthritis as a whole joint disease. *HSS J.* 2012;8(1):4-6.
35. Waldstein W, Perino G, Gilbert SL, Maher SA, Windhager R, Boettner F. OARSI osteoarthritis cartilage histopathology assessment system: A biomechanical evaluation in the human knee. *J Orthop Res.* 2016;34(1):135-140.
36. Kraus VB, Huebner JL, DeGroot J, Bendele A. The OARSI histopathology initiative - recommendations for histological assessments of osteoarthritis in the guinea pig. *Osteoarthritis Cartilage.* 2010;18 Suppl 3:S35-52.
37. Nagira K, Ikuta Y, Shinohara M, et al. Histological scoring system for subchondral bone changes in murine models of joint aging and osteoarthritis. *Sci Rep.* 2020;10(1):10077.
38. Kurashina Y, Imashiro C, Hirano M, et al. Enzyme-free release of adhered cells from standard culture dishes using intermittent ultrasonic traveling waves. *Commun Biol.* 2019;2:393.
39. Rizzardi AE, Johnson AT, Vogel RI, et al. Quantitative comparison of immunohistochemical staining measured by digital image analysis versus pathologist visual scoring. *Diagn Pathol.* 2012;7:42.
40. Ingberg E, Theodorsson A, Theodorsson E, Strom JO. Methods for long-term 17beta-estradiol administration to mice. *Gen Comp Endocrinol.* 2012;175(1):188-193.
41. Sahu A, Clemens ZJ, Shinde SN, et al. Regulation of aged skeletal muscle regeneration by circulating extracellular vesicles. *Nat Aging.* 2021;1(12):1148-1161.
42. Faupel-Badger JM, Fuhrman BJ, Xu X, et al. Comparison of liquid chromatography-tandem mass spectrometry, RIA, and ELISA methods for measurement of urinary estrogens. *Cancer Epidemiol Biomarkers Prev.* 2010;19(1):292-300.
43. Wooding KM, Hankin JA, Johnson CA, et al. Measurement of estradiol, estrone, and testosterone in postmenopausal human serum by isotope dilution liquid chromatography tandem mass spectrometry without derivatization. *Steroids.* 2015;96:89-94.
44. Tu C, Fiandalo MV, Pop E, et al. Proteomic Analysis of Charcoal-Stripped Fetal Bovine Serum Reveals Changes in the Insulin-like Growth Factor Signaling Pathway. *J Proteome Res.* 2018;17(9):2963-2977.
45. Berthois Y, Katzenellenbogen JA, Katzenellenbogen BS. Phenol red in tissue culture media is a weak estrogen: implications concerning the study of estrogen-responsive cells in culture. *Proc Natl Acad Sci U S A.* 1986;83(8):2496-2500.
46. Handelsman DJ, Gibson E, Davis S, Golebiowski B, Walters KA, Desai R. Ultrasensitive Serum Estradiol Measurement by Liquid Chromatography-Mass Spectrometry in Postmenopausal Women and Mice. *J Endocr Soc.* 2020;4(9):bvaa086.
47. Carpenter AE, Jones TR, Lamprecht MR, et al. CellProfiler: image analysis software for identifying and quantifying cell phenotypes. *Genome Biol.* 2006;7(10):R100.
48. Chinn S. A simple method for converting an odds ratio to effect size for use in meta-analysis. *Stat Med.* 2000;19(22):3127-3131.

49. Serdar CC, Cihan M, Yucel D, Serdar MA. Sample size, power and effect size revisited: simplified and practical approaches in pre-clinical, clinical and laboratory studies. *Biochem Med (Zagreb).* 2021;31(1):010502.
50. Metzger MB, Pruneda JN, Klevit RE, Weissman AM. RING-type E3 ligases: master manipulators of E2 ubiquitin-conjugating enzymes and ubiquitination. *Biochim Biophys Acta.* 2014;1843(1):47-60.
51. Khatab S, van Buul GM, Kops N, et al. Intra-articular Injections of Platelet-Rich Plasma Releasate Reduce Pain and Synovial Inflammation in a Mouse Model of Osteoarthritis. *Am J Sports Med.* 2018;46(4):977-986.
52. Lekgabe ED, Royce SG, Hewitson TD, et al. The effects of relaxin and estrogen deficiency on collagen deposition and hypertrophy of nonreproductive organs. *Endocrinology.* 2006;147(12):5575-5583.
53. Zhang H, Li ZL, Su XZ, Ding L, Li J, Zhu H. Subchondral bone derived mesenchymal stem cells display enhanced osteo-chondrogenic differentiation, self-renewal and proliferation potentials. *Exp Anim.* 2018;67(3):349-359.
54. Zhao J, Ouyang Q, Hu Z, et al. A protocol for the culture and isolation of murine synovial fibroblasts. *Biomed Rep.* 2016;5(2):171-175.
55. Chevalier M, Gomez-Schiavon M, Ng AH, El-Samad H. Design and Analysis of a Proportional-Integral-Derivative Controller with Biological Molecules. *Cell Syst.* 2019;9(4):338-353 e310.
56. Vigneswaran K, Hamoda H. Hormone replacement therapy - Current recommendations. *Best Pract Res Clin Obstet Gynaecol.* 2021.
57. Treloar AE, Boynton RE, Behn BG, Brown BW. Variation of the human menstrual cycle through reproductive life. *Int J Fertil.* 1967;12(1 Pt 2):77-126.
58. Richardson H, Ho V, Pasquet R, et al. Baseline estrogen levels in postmenopausal women participating in the MAP.3 breast cancer chemoprevention trial. *Menopause.* 2020;27(6):693-700.
59. Li Z, Yates M, Lee A, Oesterreich S. EstroGene Browser. https://estrogene.org/. Published 2022. Accessed.
60. Wend K, Wend P, Krum SA. Tissue-Specific Effects of Loss of Estrogen during Menopause and Aging. *Front Endocrinol (Lausanne).* 2012;3:19.
61. Donahue PS, Draut JW, Muldoon JJ, Edelstein HI, Bagheri N, Leonard JN. The COMET toolkit for composing customizable genetic programs in mammalian cells. *Nat Commun.* 2020;11(1):779.
62. Muldoon JJ, Kandula V, Hong M, et al. Model-guided design of mammalian genetic programs. *Sci Adv.* 2021;7(8).
63. Leclerc GM, Boockfor FR, Faught WJ, Frawley LS. Development of a destabilized firefly luciferase enzyme for measurement of gene expression. *Biotechniques.* 2000;29(3):590-591, 594-596, 598 passim.
64. Li H, An J, Wu M, et al. LncRNA HOTAIR promotes human liver cancer stem cell malignant growth through downregulation of SETD2. *Oncotarget.* 2015;6(29):27847-27864.
65. Fuentes N, Silveyra P. Estrogen receptor signaling mechanisms. *Adv Protein Chem Struct Biol.* 2019;116:135-170.
66. Vrtacnik P, Ostanek B, Mencej-Bedrac S, Marc J. The many faces of estrogen signaling. *Biochem Med (Zagreb).* 2014;24(3):329-342.
67. Shang Y, Hu X, DiRenzo J, Lazar MA, Brown M. Cofactor dynamics and sufficiency in estrogen receptor-regulated transcription. *Cell.* 2000;103(6):843-852.
68. Morrey ME, Anderson PA, Chambers G, Paul R. Optimizing nonviral-mediated transfection of human intervertebral disc chondrocytes. *Spine J.* 2008;8(5):796-803.
69. Gresch O, Engel FB, Nesic D, et al. New non-viral method for gene transfer into primary cells. *Methods.* 2004;33(2):151-163.
70. Yang J, Tang Y, Chen W, et al. Establishment and characterization of an immortalized human chondrocyte cell line. *Biotechnol Lett.* 2020;42(5):707-716.
71. Sarpeshkar R. Analog synthetic biology. *Philos Trans A Math Phys Eng Sci.* 2014;372(2012):20130110.
72. Lefebvre V, Dvir-Ginzberg M. SOX9 and the many facets of its regulation in the chondrocyte lineage. *Connect Tissue Res.* 2017;58(1):2-14.
73. Eyre D. Collagen of articular cartilage. *Arthritis Res.* 2002;4(1):30-35.
74. Roughley PJ, Mort JS. The role of aggrecan in normal and osteoarthritic cartilage. *J Exp Orthop.* 2014;1(1):8.

75. Zuba-Surma EK, Kucia M, Abdel-Latif A, Lillard JW, Jr., Ratajczak MZ. The ImageStream System: a key step to a new era in imaging. *Folia Histochem Cytobiol.* 2007;45(4):279-290.
76. Grabner R, Till U, Heller R. Flow cytometric determination of E-selectin, vascular cell adhesion molecule-1, and intercellular cell adhesion molecule-1 in formaldehyde-fixed endothelial cell monolayers. *Cytometry.* 2000;40(3):238-244.
77. Hirai H, Umegaki R, Kino-Oka M, Taya M. Characterization of cellular motions through direct observation of individual cells at early stage in anchorage-dependent culture. *J Biosci Bioeng.* 2002;94(4):351-356.
78. Kino-Oka M, Chowdhury SR, Muneyuki Y, et al. Automating the expansion process of human skeletal muscle myoblasts with suppression of myotube formation. *Tissue Eng Part C Methods.* 2009;15(4):717-728.
79. Kramer BP, Viretta AU, Daoud-El-Baba M, Aubel D, Weber W, Fussenegger M. An engineered epigenetic transgene switch in mammalian cells. *Nat Biotechnol.* 2004;22(7):867-870.

FACILITIES AND OTHER RESOURCES

Laboratory. The Ambrosio lab is located on the 5th floor of CNY 149 as a part of Massachusetts General Hospital (MGH) Navy Yard campus, which is a three-minute walk from Spaulding Rehabilitation Hospital, where Dr. Ambrosio's primary appointment is held. Our space was renovated in the fall of 2022 and includes 1614 ft^2 of dedicated wet and dry laboratory space for the Ambrosio lab. This includes a wet lab with adequate bench space for immunofluorescence staining (833 ft^2), two chemical hoods, a 128 ft^2 room dedicated to molecular biology assays, a 231 ft^2 room for histological preparation of knee joints, and a SpectraMax plate reader (described further in the Equipment document). Additionally, we have a 312 ft^2 cell culture room equipped with two BSL2 tissue culture hoods, two incubators, and two brightfield microscopes. A 110 ft^2 imaging suite is outfitted with a confocal microscope. Shared facilities include a glass/autoclave room, cold room, conference rooms, shared equipment room, and a library.

Animals. Mice are housed in the animal facilities on the 8th floor of CNY 149 MGH Center for Comparative Medicine (CCM), just three floors above our laboratory space. Animal facilities at MGH CCM are directed by full-time veterinarians and have over 9,500 staff members. MGH CCM is accredited by the Association for Assessment and Accreditation of Laboratory Animal Care, has assurance with the Office of Laboratory Animal Welfare, and is registered with the US Department of Agriculture. MGH CCM complies with the NIH policy on Animal Welfare act. Mice are housed 3-5 per cage, and monitored daily by veterinarians, CCM staff, and the investigators. Veterinary consult is available 24 hours a day for any emergency animal issues.

Computers. In the Ambrosio lab, each employee (technician, lab manager) has a dedicated desktop computer and access to several other equipment specific computers in the lab. For improved computing powers, doctoral students and post-doctoral fellows have work-horse grade computers to increase computational speed. Specifically, each workhorse station is equipped with a Windows 10 64-bit processor, 4 Core Intel Xeon W-2123 3.6 GHz, 32 GB of RAM, and AI RTX 4000 graphics with 14" screens for data analysis. Each of these computers is equipped to access the computational resources available through Harvard, MGH, and Spaulding. The lab has a 2 TB server for data storage as well as unlimited data storage through OneDrive. Our confocal microscope is equipped with a workstation computer for microscope control and image processing. The lab is also equipped with a color laser jet printer and 9800 Microtek scanner. Dr. Ambrosio has her own computer with full access to the computational resources offered through Harvard, MGH, and Spaulding. VPN and remote desktop are also available for both equipment specific computers as well as personal desktop computers. All resources outlined here are available for use in this project.

Imaging. Within the Ambrosio lab, we have a Nikon A1R HD Confocal available for use on this project. Specifically, in our designated microscope room, we have a Nikon AXR point scanning confocal with four solid-state lasers at 405, 488, 561, and 640 nm and brightfield capabilities. This includes the largest field of view for any confocal on the market and deconvolution to resolve objects as small as 120 nm, and the highest numerical aperture available for our objectives (2, 10, 20, and 60X). Our microscope is also equipped with ultrafast point scanning (740 fps @ 2048 X 16) and AI driven image analysis using NIS-elements. NIS-elements also offers the ability to customize specific image analyses to a project's needs. Through our Nikon package, we have access to unlimited technical support from Nikon, and our microscope is fully upgradable should different microscopy needs arise.

We also have a SpectraMax i3x Multi-Mode Microplate Reader in the lab, that can record absorbance, luminescence, and fluorescence (top/bottom reads, time-resolved, or polarized). Excitation ranges for fluorescence range from 250-830 nm and emission from 270-850 nm. Lastly, we have access to several imaging facilities, including both the University of Pittsburgh's Center for Biological Imaging (CBI), Massachusetts General Brigham (MGB) Molecular Imaging Center, and the Ragon Institute. Details on facilities and equipment are available below and in the Major Equipment Section.

Software. Since Gabrielle remains a full-time student at the University of Pittsburgh, she maintains access to Pitt's Software Download Services (SDS). SDS provide students with free download access to a range of software, from MATLAB to GraphPad to SPSS. Software needed for the completion of this fellowship, including Aperio ImageScope 12.4.6, IDEAS, CellProfiler, SPSS, GraphPad, EndNote, Microsoft Office Applications, MATLAB, and Python Jupyter Notebook, are available either through SDS or free download from the internet.

Office. Desks are located within the lab and include the aforementioned desktop computers, 49 ft^2 of desk space per person, and 63 ft^2 of storage space person. Dr. Ambrosio's 144 ft^2 office is located in between the main lab space and cell culture/imaging area.

Classes/Training. Didactic research and training activities will occur through participation in both Pitt's and Harvard's monthly MSTP sessions, including Clinical Case Problem Solving and monthly workshops. Weekly seminar series through the University of Pittsburgh includes Women's Health Seminar, Pepper Seminar Research Series, Pathology Seminar, and Orthopaedic Surgery Department Grand Rounds. At MGH and Harvard, the Harvard Medical School Genome Engineering Series and Massachusetts Institute for Technology Bioinformatics Series are two biweekly seminars that Gabrielle will attend. Relevant courses in synthetic biology and cellular signaling are available at the University of Pittsburgh and have either already been completed or are in the process of being completed. Any synthetic biology specific laboratory training that is necessary will be completed in Dr. Pamela Silver's lab, and Gabrielle will continue to be involved with the Synthetic Biology Hive at Harvard Medical School. Lastly, for any statistical related concerns, we will consult the University of Pittsburgh's Clinical and Translational Science Institute (CTSI, details listed below).

Other facilities and resources available for this project.

- *Wyss Institute*. Dr. Silver serves as one of the core faculty members of the Wyss Institute and a member on Gabrielle's mentoring team. The Wyss Institute offers a variety of resources to expand biologically informed engineering, including access to equipment to perform qPCR for *Aim 2a* as well as collaboration and discussion groups on experiment design and technology development.
- *MGB Molecular Imaging Center*. The MGB Molecular Imaging Center is located on the 4th floor of CNY149, just one floor below our lab space. These facilities offer four different Nikon Confocal microscopes, including a Nikon Yokagawa CSU-W spinning disc/ SoRa super-resolution system with live cell imaging capacities. This microscope will be used for the live cell imaging experiments outlined in *Aim 1c*, is available for reserved experiment time, and has extensive technical support available for troubleshooting experiments.
- *Ragon Institute of MGH, MIT, and Harvard*. The Ragon Institute offers a number of different resources and services for scientific researchers, including a flow cytometry core. Within this core, we have been trained on and have regular access to the ImageStreamX Mark II (Luminex) which will be used for *Aim 2b*. In addition, technicians and assisted ImageStream runs are available for our use.
- *Histopathological core at MGH CNY 149*. The histopathological core, which is located one floor above our lab, offers a microtome for sectioning of knee samples that is regularly available within a two-day notice. Additionally, the core offers pay per service sectioning, with a two-week turnaround time. If any issues arise with our own microtome, we will use this core service for completion of *Aims 1a-1b*.
- *University of Pittsburgh's Center for Biologic Imaging (CBI)*. CBI is one of the largest optical imaging centers in the country. Although the Ambrosio lab is now physically located in Boston, Dr. Claudette St. Croix and faculty within CBI remain Co-Is on several of Dr. Ambrosio's grants. Collaborations and imaging still occur across institutions, sans difficulty. Thus, for any imaging related issues that come up throughout this proposal that are not able to be resolved internally or with resources available in Boston, CBI will be consulted.
- *University of Pittsburgh's Clinical and Translational Science Institute (CTSI)*. CTSI has a number of resources available for students, ranging from statistician consultations to tools for developing recruitment modalities in human participant studies. For this project, we will consult the statisticians at CTSI for any statistical questions or issues that come up while performing analyses. Gabrielle has used this service for prior projects and found the assistance to be both informative and productive.
- *University of Pittsburgh's Small Molecule Biomarker Core (SMBC) Mass Spectrometry Facility.* The SMBC provides accurate and highly sensitive quantitative analyses in serum using state-of-the-art ultra-performance liquid chromatography tandem mass spectrometry (UPLC-MS/MS). For this project, we will use these facilities to quantify estradiol concentrations for *Aim 1b & 1c*.
- *Atlantic Charter Discovery Center for Musculoskeletal Recovery.* Dr. Ambrosio was recruited to Spaulding Rehabilitation Hospital to serve as the inaugural director of this new research center. Given Gabrielle's interest in ultimately building a center on women's joint injuries, observing this early development will provide unique exposure to the process of building an academic institution and the opportunity to collaborate with incoming researchers.

MAJOR EQUIPMENT

For preparation of knees for histological assessment. In addition to the lab space and resources described in the Facilities and Other Resources section, we have equipment available to prepare knees for histological processing. Specifically, for embedding knees in paraffin, we have an embedding workstation, paraffin wax dispenser, and compact oven. For sectioning of the knees, we have a microtome in our laboratory that will be used. All of these pieces of equipment were bought this fall (2022) and are in outstanding condition. In case of any equipment issues or technical assistance needed, we also have access to the Histopathology Core at Harvard University (described in the Facilities and Other Resources document).

- In Ambrosio Lab
  - Epredia™ HistoStar™ Embedding Workstation (Cat. No. A81000002)
  - Boekel Scientific™ Paraffin Wax Dispenser (Cat. No. 22-046-917)
  - Thermo Scientific™ Precision™ Compact Ovens (Cat. No. 11-475-153)
  - Epredia™ HM 340E Electronic Rotary Microtome (Cat. No. 90-519-0ER)

For imaging. Imaging will primarily occur via our Nikon Confocal microscope, SpectraMax plate reader, the Nikon Yokagawa CSU-W spinning disc/ SoRa Microscope (Nikon SoRa) located in Massachusetts General Brigham (MGB) Molecular Imaging Center, and the ImageStreamX available through the Ragon Institute. Specifically, imaging of knee sections from *Aims 1a-b* and immunofluorescence imaging for *Aim 2b* will take place predominantly on our Nikon confocal microscope. If any unexpected issues arise, the Nikon SoRa at MGB Molecular Imaging Center is also available for this use. Imaging of proteasome activity via AnaSpec SUC-LLVY-AMC fluorogenic oligopeptide for *Aim 1c* and luciferase expression in *Aim 2a* will take place on our SpectraMax plate reader. The live cell imaging component of *Aim 1c* will be completed on MGB Molecular Imaging Center's Nikon SoRa. Lastly, for the imaging flow cytometry experiments outlined in *Aim 2b*, we will use the ImageStreamX Mark II that is available at the Ragon Institute.

- In Ambrosio Lab
  - Nikon AXR point focal scanning confocal (see detailed specs in Facilities and Other Resources)
  - SpectraMax® i3x Multi-Mode Microplate Reader (Cat. No. i3x)
- In the MGB Molecular Imaging Center
  - Nikon Yokagawa CSU-W spinning disc/ SoRa super-resolution system. Imaging capacities include Bright Field, Phase Contrast, DIC, Epi-Fluorescence, Confocal with super resolution capacities. Lasers include 405, 488, 561, 640 nm. Stage top is fit with a Nikon motorized stage with perfect focus, Piezo Z stage (200µm travel range) and Tokai HIT stage incubator (Temperature and CO_2 control) for high throughput live cell imaging
- In the Ragon Institute
  - ImageStreamX Mark II (Luminex) - 12-channel, 2-camera, 4-laser imaging flow cytometer for quantifying fluorescence signal and localization at the single cell level for cells in suspension

For cell culture. Within our designated cell culture area, we have two BSL2 grade hoods that can be used for performing experiments. There are also two 240 L incubators available for culturing the cells. We have a Zeiss microscope available for checking cells while they are in culture, which is particularly critical when culturing chondrocytes which tend to grow slower and easily shift into a fibroblast phenotype. For reagent storage, we have three 4°C refrigerators available, two -20°C freezers, and two -80°C freezers. Within our lab, we also have five centrifuges available for reagent prep and isolating cells.

- In Ambrosio Lab
  - Heracell™ 150i & 240i CO2 Incubator, 150L & 240L, Medical Device (Cat. No. 50116050)
  - Thermo Scientific™ TSX Series High-Performance Lab Refrigerators (Cat. No. TSX5005GD)
  - Zeiss Microscope Primovert HDcam (Cat. No. 491207-0003-000)
  - Eppendorf Centrifuge 5702 (Cat. No. 5702000010)
  - Thermo Scientific™ TSX Series High-Performance -20°C Manual Defrost Freezers (Cat. No. TSX2320FA)
  - Thermo Scientific™ Revco™ RDE Series Ultra-Low Temperature Freezer Package with Racks, Boxes, and LN2 Back-up System (Cat. No. RDE686FDRLN)
- In the Wyss Institute/Silver Lab
  - AriaMx Real-time PCR System with 6 filters (Cat. No. G8830A)

Institutional Animal Care and Use Committee
Massachusetts General Hospital
55 Fruit Street
Boston, MA 02114
Tel: 617-726-2000

Notification of IACUC Initial Review Approval

Protocol #: 2022N000171

Date:	10/31/2022
To:	Fabrisia Ambrosio, Ph.D MGH Mass General Brigham > MGH > Rehab Medicine
From:	Institutional Animal Care and Use Committee
Title of Protocol:	Investigating the effects of menopause on the musculoskeletal system
Sponsor Funding Support:	ADVANCE: The Anti-Aging Role of Klotho in Skeletal Muscle Regeneration NIH-National Institutes of Health 5R01AG052978-04
Species:	Mice (Mus)
Triennial Due Date:	10/31/2025

This protocol has been reviewed and approved by the MGH IACUC (OLAW Assurance D16-00361). The protocol is approved for a three-year period.

As Principal Investigator you are responsible for the following:

1. Conduct of procedures in compliance with the approved protocol.
2. Compliance with MGH IACUC/CCM Policies governing the care and use of animals.
3. Submission of project changes for IACUC review and approval prior to initiation of the change.
4. Submission of triennial review applications to the IACUC.
5. Adherence to safety requirements for the use of any hazardous agents approved on this protocol.
6. Informing the IACUC of unanticipated pain and distress, adverse events or unexpected deaths following the conduct of research procedures.
7. Ensuring only authorized personnel identified on an approved protocol or approved in advance by the CCM Director are permitted in the animal facilities.

Please direct questions and correspondence to **Rosemary Foster**, IACUC Administrator, Tel: 724-9619

CC: **Fabrisia Ambrosio, Rehab Medicine, Principal Investigator**
Gabrielle Gilmer, Mass General Brigham, PhD Student
Kai Wang, Dept of Physical Med and Rehab, Research Fellow

Title: Investigating the effects of menopause on the musculoskeletal system
Sponsor Name: NIH-National Institutes of Health
PI Name: Ambrosio, Fabrisia **Protocol #:** 2022N000171 **Type:** Current View
Species: MICE **# Of Animals:** 428 **Period Start Date:** October 31, 2022

Study Staff

Name	Role	Degree	Organization
Ambrosio, Fabrisia	Principal Investigator	Ph.D	MGH > Rehab Medicine
Gilmer, Gabrielle	PhD Student		Mass General Brigham
Hettinger, Zachary	Research Fellow		Mass General Brigham
Kopchak, Rylee	Research Technician		Mass General Brigham
Wang, Kai	Research Fellow	Ph.D	SRH > Dept of Physical Med and Rehab

Linked Agreements

Record #	Fund	Sponsor
2022A012789	500624	NIH-National Institutes of Health

Protocol Overview

Please answer the following questions using language a non-scientist will understand.

1. Study Goals

How would you explain the long term or overall scientific goals of the proposed work to a non-scientist? **[Please limit to 200 words.]**

Historically, women and diseases that disproportionately affect women have been excluded from medical research. From 1990-2000, 80% of pharmaceuticals pulled off the market by the FDA were due to adverse events in women. Within the preclinical world, male-only animal studies out number female-only studies 5 to 1. As such, there is an urgent need within the scientific community to study how diseases specifically present in women and understand the underlying mechanisms causing these presentations.

An important aspect of aging in women is menopause. Onset of menopause is associated with a number of different symptoms including vaginal dryness, hot flashes, and weight gain. Approximately 71% of menopausal women experience joint and muscle pain. Additionally, post-menopausal women are twice as likely to present with knee osteoarthritis than men, and the prevalence of sarcopenia rapidly increases with menopause onset. Yet, the underlying mechanisms driving these disease patterns remain unknown.

Thus, the purpose of this study is to investigate how menopause affects the musculoskeletal system (muscle, cartilage) in mice. Interestingly, rodents spontaneously rejuvenate their ovarian follicles in middle-age and thus do not present with a menopausal phenotype. As such, menopause will be chemically induced using the well-established vinyl-cyclohexene diepoxide (VCD) menopause model.

2. Benefit to be Gained by Animal Research

How would you explain to a non-scientist that the potential benefits of the study, in terms of biomedical advancement, justify the proposed animal use? **[Please limit to 200 words.]**

In humans, all women with intact ovaries will undergo menopause between the ages of 40-50 years old. As such, there is a lack of non-menopausal, age-matched controls for the purpose of understanding the underlying mechanism of disease. Differentiating age-related versus menopause-related effects is critical for developing effective therapeutics for said diseases. Additionally, collecting human cartilage samples is only possible at the time of joint replacement surgery or death, making time course and control samples challenging to obtain. In addition, muscle samples can only be collected via biopsy and the size of these samples is often small, making reproducible and robust studies challenging.

Research Objective: Research Objective 1

INSTRUCTIONS:
Complete a Research Objective form for each discrete aim of the protocol. See **Instructions** in the right pane for additional information.

To add an additional Research Objective, please click the **add New Research Objective** button at the bottom of this form.

Limit the discussion to activities involving animals. Do not describe *in vitro* procedures beyond collection of tissues, blood, or other biological products.

A. Rationale: [**Please limit to 200 words**]

The objective of this project is to characterize how menopause affects the musculoskeletal system. Specifically, we will induce menopause via 10 days of intraperitoneal (IP) injections of vinylcyclohexene diepoxide (VCD) (160 mg/kg/day) in sesame oil. Control mice will receive 10 days of IP injections of sesame oil. To characterize the trajectory of menopause, vaginal lavage and cytology will be performed daily to document this trajectory. Our previous studies have demonstrated that mice typically enter the perimenopausal transition 25 days after the first injection and menopausal 115 days after the first injection. To characterize the hormonal changes associated with menopause, blood will be collected via cardiac puncture immediately prior to euthanasia. Lastly, to document changes in muscle functional use, physiological testing will be performed to measure muscle contractile strength. Upon euthanasia, knee joints and muscle will be collected for various tissue processing and in vitro assays.

B. Experimental Design: For this research objective, outline the time-course indicating each activity. Describe each step and how it relates to an animal enrolled in this study. It should be clear what each animal will experience during the full course of this Research Objective.

- Include the length of time an animal is enrolled in an experiment
- Describe experimental endpoints
- Do not include descriptions of surgical and non-surgical procedures in the **Experimental Design**. Include this information in the specific **Procedure** forms.

See **Instructions** in the right pane for additional information.

Middle-aged (14-16 month) female C57/BL6 mice will be enrolled in this study. Mice will receive IP injections for 10 days of either VCD or sesame oil. After completion of injections, vaginal cytology will be performed daily to track the phase of the menstrual cycle, with menopause being defined as 10 days in a row of diestrus. Rectal temperatures and weights will also be tracked weekly to characterize the menopausal phenotype. Groups of mice will be euthanized at the following time points: immediately after injections for off target evaluation (day 11), start of perimenopause (day 25), mid perimenopause (day 70), end of perimenopause/start of menopause (day 115), mid-menopause (day 155), and late menopause (day 195).

When mice reach the appropriate end point, they will be anesthetized with isoflurane. One the animal is verified to be anesthetized, physiological testing to quantify muscle contraction will be performed. After completion, cardiac puncture will be performed. After the maximum amount of blood is collected, animals will be euthanized via cervical dislocations. Mice will then be removed from CCM facility and taken to our lab for tissue and cell collection.

C. Flow Chart: For this research objective, a schema or flow chart diagramming the overall picture of the study design and treatment groups must be included. The flow chart should include:

- all experimental groups
- the number of animals per group
- the procedures performed on the animal
- the length of time an animal is enrolled in the experiment

The IACUC must be able to understand the experience of each animal on the protocol.
See Instructions in right pane for additional institution-specific flow chart guidelines.

For Triennial submissions, please remove any outdated flow charts and replace with a new flow chart that describes the work to be conducted on this research objective over the next 3 years.

D. Health Status:

1. Describe the health status of the animals during this research objective. Include:

- Expected development and progression of clinical signs, including severity and time course
- The frequency of monitoring and what will be observed and documented
- Potential adverse events caused by the research model and/or experimental manipulations
- If a scoring system will be used to monitor animal health, please attach it to the protocol below

Please note: Describe longer-term health status monitoring here. Immediate post-procedural monitoring should be included in the relevant **Procedure** form.

IP injections are not expected to cause any significant clinical signs and symptoms, but details of adverse responses are addressed in the procedure form.

The phenotype of menopause is not expected to cause significant changes in risk of disease or distress. Menopausal mice due gain slightly more weight than age than non-menopausal mice and have slight variations in core body temperature, which may lead to some behavioral changes.

Weekly rectal temperature is a low-risk procedure, but it is possible for rectal prolapse to occur. In this case, the investigator will use sterile lube and a sterile q-tip to gently manipulate the rectum prolapse back into the anus. If the prolapse continues to displace for several days in a row, the mouse will be euthanized.

Weekly weight monitoring is not expected to cause any adverse effects outside of those associated with handling.

Vaginal lavage and cytology is another low-risk procedure. With daily collections, it is possible for trauma to the vagina and associated external genitalia. If this occurs, veterinary staff will be contacted for recommendations and vaginal lavage on that particular animal will pause until cleared by veterinary staff.

For physiological testing and cardiac puncture, the investigator will assure the mouse is fully anesthetized by squeezing the foot to ensure no pain is experienced during these procedures. If a mouse shows signs of losing anesthetic effects during procedure, the dosage of isoflurane will be slightly increased and the investigator will pause the procedure until confirming the animal is fully anesthetized again.

2. What action will be taken should clinical signs manifest?

If clinical signs manifest that are not outlined above, the investigators will consult the veterinary staff for review of the animal, and animals will be euthanized per the criteria listed in the Humane Endpoint, Disposition and Euthanasia form.

Attachments

Name	Mode
flowChartMenopauseProtocol_oct22_2022 (Flowchart)	Electronic

Mass General Brigham

Research Objective: Research Objective 2

INSTRUCTIONS:
Complete a Research Objective form for each discrete aim of the protocol. See **Instructions** in the right pane for additional information.

To add an additional Research Objective, please click the **add New Research Objective** button at the bottom of this form.

Limit the discussion to activities involving animals. Do not describe *in vitro* procedures beyond collection of tissues, blood, or other biological products.

A. Rationale: [**Please limit to 200 words**]

The most common treatment for menopause related diseases and symptoms is hormone replacement therapy (HT). However, the role of HT is treating musculoskeletal related diseases, such as sarcopenia and osteoarthritis, remains unknown. Additionally, in humans, there have been observations of the timing of treatment initiation modulating beneficial effects, a phenomenon described as the timing hypothesis. The timing hypothesis postulates that treatment given early in menopause results in therapeutic benefits, while treatment given late in menopause results in pathological effects. The timing hypothesis has been implicated in cardiovascular disease and dementia, but the effects of timing of initiation remain unclear in musculoskeletal related diseases. Thus, our goals are to study the effects of estradiol on modulating menopause-related musculoskeletal diseases in a time dependent manner.

B. Experimental Design: For this research objective, outline the time-course indicating each activity. Describe each step and how it relates to an animal enrolled in this study. It should be clear what each animal will experience during the full course of this Research Objective.

- Include the length of time an animal is enrolled in an experiment
- Describe experimental endpoints
- Do not include descriptions of surgical and non-surgical procedures in the **Experimental Design**. Include this information in the specific **Procedure** forms.

See **Instructions** in the right pane for additional information.

Middle-aged (14-16 month) female C57/BL6 mice will be enrolled in this study. All mice will be allowed to acclimate to the animal facility for at least 3 days prior to any procedures. Animals will be handled for five days, and then all animals will receive IP injections of VCD in sesame oil as described in Research Objective 1 and the IP injection procedure. Mice will then be randomized to one of three treatment periods (perimenopause, early menopause, late menopause) and one of two groups (estradiol treatment, control treatment). The perimenopause treatment group will receive daily treatment from days 75-115 and be euthanized on day 115. The early menopause group will receive daily treatment from days 115-155 and be euthanized on day 155. The late menopause group will receive daily treatment from days 195-235 and be euthanized on day 235.

When mice reach the appropriate end point, they will be anesthetized with isoflurane. One the animal is verified to be anesthetized, physiological testing to quantify muscle contraction will be performed. After completion, cardiac puncture will be performed. After the maximum amount of blood is collected, animals will be euthanized via cervical dislocations. Mice will then be removed from CCM facility and taken to our lab for tissue and cell collection.

C. Flow Chart: For this research objective, a schema or flow chart diagramming the overall picture of the study design and treatment groups must be included. The flow chart should include:

- all experimental groups
- the number of animals per group
- the procedures performed on the animal

- the length of time an animal is enrolled in the experiment

The IACUC must be able to understand the experience of each animal on the protocol.
See Instructions in right pane for additional institution-specific flow chart guidelines.

For Triennial submissions, please remove any outdated flow charts and replace with a new flow chart that describes the work to be conducted on this research objective over the next 3 years.

D. Health Status:

1. Describe the health status of the animals during this research objective. Include:

- Expected development and progression of clinical signs, including severity and time course
- The frequency of monitoring and what will be observed and documented
- Potential adverse events caused by the research model and/or experimental manipulations
- If a scoring system will be used to monitor animal health, please attach it to the protocol below

Please note: Describe longer-term health status monitoring here. Immediate post-procedural monitoring should be included in the relevant **Procedure** form.

IP injections are not expected to cause any significant clinical signs and symptoms, but details of adverse responses are addressed in the procedure form.

The phenotype of menopause is not expected to cause significant changes in risk of disease or distress. Menopausal mice due gain slightly more weight than age than non-menopausal mice and have slight variations in core body temperature, which may lead to some behavioral changes.

In previous studies, consuming Nutella daily was noted to be a minimally invasive way to provide mice estradiol treatment that does not have any side effects, including no changes in weight. In humans, estradiol treatment given late in menopause is thought to cause increased risk of a number of different diseases, including cardiovascular disease and stroke. However, given the paucity of age-related studies in estradiol treatment in mice, it is unclear whether we will see these patterns. If mice are noted to develop other health morbidities such as those listed, we will consult and work with veterinarians to identify the best solution for the mouse.

For physiological testing and cardiac puncture, the investigator will assure the mouse is fully anesthetized by squeezing the foot to ensure no pain is experienced during these procedures. If a mouse shows signs of losing anesthetic effects during procedure, the dosage of isoflurane will be slightly increased and the investigator will pause the procedure until confirming the animal is fully anesthetized again.

2. What action will be taken should clinical signs manifest?

If clinical signs manifest that are not outlined above, the investigators will consult the veterinary staff for review of the animal, and animals will be euthanized per the criteria listed in the Humane Endpoint, Disposition and Euthanasia form.

Attachments

Name	Mode
flowChartMenopauseProtocolE2Experiments_nov1_2022 (Flowchart)	Electronic

Mass General Brigham

Animals

The IACUC restricts protocols to a single species only. If the protocol will require xenografts, identify the donor species, and the applicable protocol number, in the appropriate **Research Objective** section of the protocol.

1. Select a species from the drop down list:
   Mice (Mus)

1.a Select breed(s), or strain(s), or specific type(s).

C57BL/6

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2. Do any of the animals have a genetic alteration and/or phenotype that is expected to have any impact on animal health and/or requirements for animal care?

◉ No

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3. Animal Source
   See **Instructions** in the right pane for transfer, order, and import policies/SOPs.

Select all that apply:

☑ Animals will be acquired from an outside institution

a. Have any of the animals being acquired from an outside institution undergone research procedures, excluding genotyping or identification procedures?

◉ No

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4. Sex

◉ Female

If you will be using one sex only, please explain:

We are studying menopause, which is only relevant in female animals

See Consideration of Sex as a Biological Variable in NIH-funded Research for more information.

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5. See Consideration of Sex as a Biological Variable in NIH-funded Research for more information. Indicate the method(s) of identification that will be used to track these animals *(select all that apply)*: See **Instructions** in the right pane for more information.

☑ Ear tag or notch

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6. The species selected is appropriate because *(select all that apply)*:

☑ Species lower on the phylogenetic scale cannot be used
☑ Tissues and/or substances needed are best/uniquely provided by this species
☑ Prior research has been conducted in this species

Potential Pain and Distress

1. Total number of animals requested for this three-year approval

Enter the number of animals in each pain and distress category. Each animal must be assigned to a category based on the most invasive procedure or the procedure that has the greatest potential to cause pain or distress. See **Instructions** in the right pane for definitions and examples.

- If an animal will be used in more than one **Research Objective**, count it only once, in the highest pain category that it will experience.
- If animals are bred in-house, include the progeny that may be culled. Progeny used for experiments should be counted in the specific **Research Objectives**. All other animals should be counted in **Other**. See **Instructions** in the right pane for more information.

TOTAL NUMBER OF ANIMALS REQUESTED					
USDA Pain & Distress Category (See Instructions for information)					
Animals	**B**	**C**	**D**	**E**	**Total**
Research Objective 1	0	0	252	0	252
Research Objective 2	0	0	126	0	126
Other (e.g. breeding, training):	0	0	50	0	50
Total requested	0	0	428	0	428
Animals currently in house					
Total approved for purchase, breeding, or other acquisition	0	0	428	0	428

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2. Justification for the number of animals requested (select all that apply):
   - ☑ Power analyses indicated that the proposed sample size, number of groups and/or number of experiments is the lowest required for statistically valid tests of the hypothesis (i.e., 80% power with 0.05 type I error).
   - ☑ Based on previous and/or published data, the numbers of animals requested are the minimum needed to achieve sufficient statistical power.

The following tools can be used to determine minimum sample size:

- Sample Size Calculations in Animal Research (W. W. LaMorte, BUMC)
- ClinCalc Sample Size Calculator
- Jackson Laboratories Breeding Colony Size Planning Worksheet

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3. Does the number of animals requested include extra animals to cover anticipated failures or to train or familiarize the staff with the procedures described?

◉ Yes

a. Discuss the anticipated failure rate and steps taken to minimize this rate. If some animals will be used to train staff on the procedures used in this protocol, describe that process here.

Given that the last endpoint is 195 days after the initial injections and the mice will be 21 months at this point, it is possible for some mice to pass or need euthanasia prior to desired outcomes. In our experience, 6% of sesame oil mice and 8% of VCD mice die or need to be euthanized unexpectedly. To minimize unnecessary animal usage, all personnel performing experiments (including tissue collections) will be extensively trained in procedures prior to experimentation.

The leader of the project (Gabrielle) will primarily perform all procedures and has extensive experience performing all procedures. New technicians and lab members will be trained by Gabrielle. First, Gabrielle will show the trainee the procedure, then will observe them performing the procedure in a step wise manner (e.g., if training for IP injections and has never handled mice perform, training will start with just holding mice and becoming comfortable there. Then, the trainee will become comfortable placing mice in restrainer and securing them there). Once Gabrielle has determined the trainee can perform the procedure independently, they will be allowed to do so on their own without supervision.

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Replacement, Reduction, and Refinement

The 3 Rs – replacement, reduction, and refinement – represent a practical strategy for researchers to apply when considering the use of animals in research and in designing humane animal research studies. Government policy and regulatory agencies require the IACUC to assure that researchers consider the 3 Rs when preparing research protocols.

- The Guide for the Care and Use of Laboratory Animals
- U.S. Government Principles for the Utilization and Care of Vertebrate Animals Used in Testing, Research, and Training
- USDA Policies 11 and 12

1. Alternatives to Animal Models

☑ Mathematical models are not a suitable alternative to live animals
☑ Computer simulations (in silico models) are not a suitable alternative to live animals
☑ In vitro biological systems are not a suitable alternative to live animals

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2. Duplication of Research

Unnecessarily duplicative research should be avoided for scientific and ethical reasons. Have the results fulfilling the experimental goals of this study been published in medical, scientific, or veterinary journals?

◉ No

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3. Search for Alternatives to Painful and/or Distressful Procedures

A literature search for alternative procedures must be performed for each procedure that has the potential to cause pain or distress, including prolonged use of restraint devices. Along with the literature search, consultation with experts in the field and attendance at scientific or professional meetings can be used to identify alternatives to painful and/or distressful procedures.

a. Indicate resources used to search for alternatives to painful and/or distressful procedures.
In addition to the selections below, other useful resources can be found at IACUC Central , the NIH Office of Laboratory Animal Welfare, and the USDA Animal Welfare Information Center.

☑ Pubmed (http://library.massgeneral.org/)
☑ Animal Welfare Institute (https://awionline.org/)

b. Indicate the date the literature search was completed. The search must be conducted within the last 6 months.

Click here to enter a date.
10/05/2022

c. Indicate the time period surveyed in the literature search:

1984-2022

d. Indicate the procedure(s) and keyword(s) searched for each potentially painful or distressful procedure or condition described in this protocol.

In the PROCEDURE column, list each individual Category D or E procedure or resulting condition e.g., immunosuppression, anything that has the potential to induce pain, stress, or distress to an animal.

In the KEYWORD column, on the corresponding row, please list all applicable keywords used in the search. These should include species, strain (if pertinent), agent, surgical approach, procedure etc. Please see instructions for more information/examples.

Procedure	Keywords
perfusion-fixation	thoracotomy, mice, non-survival

Procedure	Keywords
Muscle contractility testing	mice, peroneal nerve isolation, non-survival, tetanic force

e. Results of the Search for Alternatives to Painful and/or Distressful Procedures

☑ The literature search conducted indicates that there are no alternative procedures that would involve less pain or distress.

If there are any relevant citations or other documents that are needed to support this search for alternatives, please attach them to this form.

Humane Endpoint Disposition and Euthanasia

A. Humane Endpoints

See **Instructions** in the right pane for guidance on completing this form.

Mammals:

- Persistent recumbence; inability to rise; loss of righting reflex
- Pain or distress that cannot be alleviated by analgesics
- Difficulty with ambulation (paralysis, fractures, trauma, etc.)
- Severe central nervous system signs (e.g., circling, rolling, persistent seizures or convulsions)
- Abnormal breathing (dyspnea) and cyanosis
- Body condition score of 2 (out of 5) or less See **Instructions** in the right pane for more information.
- Excessive weight loss See **Instructions** in the right pane for more information.
- Vomiting/diarrhea resulting in severe dehydration
- Tumor production specific endpoints See **Instructions** in the right pane for more information.
- Model-specific endpoints (please describe and see **Instructions** in the right pane for more information.)

Please choose:

◉ Animals will be removed from the study and euthanized if any of the above clinical signs/conditions are found

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B. Moribundity and Mortality

The IACUC acknowledges that some studies may require moribundity (a clinically irreversible condition leading inevitably to death) or mortality (a fatal outcome) as an endpoint. The committee recommends that consideration be given to surrogate markers that can be utilized for a more humane endpoint, such as serial imaging or biomarkers that may permit the detection of experimental endpoints that precede the development of significant clinical signs, rather than allowing the animal to proceed to moribundity or mortality.

The use of moribundity or death as an endpoint is strongly discouraged and requires scientific justification.

Rationale

1. Will this protocol include models with severe clinical signs that require moribundity as an endpoint?

◉ No

2. Will this protocol use death as an endpoint?

◉ No

C. Animal Transfer and Disposition

Select all that apply. See **Instructions** in the right pane for more information.

☑ Euthanasia or Terminal Procedure
☑ Transfer to another protocol at this institution See Instructions in the right pane for institution-specific guidelines.

*Animal transfers may require other institutional reviews (e.g., grants, MTAs). Contact the appropriate administrator.

D. Euthanasia Method

Euthanasia methods must be consistent with the AVMA Guidelines for the Euthanasia of Animals. See **Instructions** in right pane for institution-specific guidelines/SOPs.

- A method must be indicated even if the protocol procedures are not terminal, for use in the event of an emergency.
- **MGH, SERI, and MEE protocols only:** A secondary physical method to confirm euthanasia by carbon dioxide overdose or Isoflurane anesthesia overdose is recommended, but not required.

Species:

Mice (Mus)

☑ Carbon dioxide overdose (with secondary physical method)

Death by Carbon Dioxide Overdose will be confirmed by:

☑ Cervical dislocation

☑ General anesthesia, followed by non-survival surgery or exsanguination. *Please complete a procedure form to cover this method of euthanasia.

☑ Isoflurane anesthetic overdose (5% isoflurane with secondary physical method)

Death by Isoflurane overdose will be confirmed by:

☑ Cervical dislocation

Will a sedative, tranquilizer, or anesthetic be administered prior to euthanasia?

◉ Yes

Provide the requested information for each agent (**Agent, Dose, Route**).

Agent	Dose	Route
Isoflurane	1-4%	Vaporize

Housing MGH

I. HOUSING LOCATIONS

A. CCM Centralized Facilities

Select all applicable housing areas.

149 CNY-8 (rodent)

B. Investigator-Managed Facilities or Satellite/Laboratory Housing Areas

- Please note that permission to house animals in investigator-managed centralized facilities must be obtained from the appropriate satellite facility manager. See **Instructions** in right pane for more information.
- All new satellite/laboratory housing areas must be inspected and approved by the IACUC and the Center for Comparative Medicine. Research cannot be conducted until the area has been inspected and notification of approval has been received.

Select applicable housing areas.

C. Offsite Housing

All offsite housing locations must be inspected and approved by the IACUC and the Director, Center for Comparative Medicine. Animals may not be housed in a new location until it has been inspected and notification of approval has been received.

II. SPECIAL HANDLING, HUSBANDRY, OR HOUSING REQUIREMENTS

Will the animals on this protocol require any special handling, husbandry, or housing requirements? This includes anything outside of normal routine husbandry/handling services utilized by CCM, as defined in the species specific SOPs (e.g., alterations in bedding types, cage change frequencies, housing densities, special diets/fluids, deviations from currently approved IACUC policies, etc). See **Instructions** in right pane for more information.

Exemptions from the Environmental Enhancement Program that are defined and approved by the IACUC Policy on Environmental Enrichment, Social Housing and Exercise of Laboratory Animals do not need to be described in the protocol.

Please discuss all special handling, husbandry, or housing requirements with CCM facility managers and/or veterinarians.

☑ Specialized diet or fluid (e.g., investigator provided diet, medication in drinking water)

Describe the requirements for specialized food and/or fluids.

Daily during the treatment period (see flow chart) in addition to the standard diets the mice receive, mice will be fed the following:

In the control group:

60 mg of Nutella will be administered daily for 40 days

In the experimental group:

60 mg of Nutella + 1.12 µg of 17β-estradiol + 0.312 µL of sesame oil will be administered daily for 40 days

Previous studies (https://pubmed.ncbi.nlm.nih.gov/22137913/) using this method have noted "Once fully habituated to the Nutella mixture, all animals consumed the entire portion within 2 min." Given that mice are social creatures and the treatment period is quite long, to avoid housing the mice individually permanently, we will separate the mice into small boxes to feed them the Nutella mixture, observe that they consume all proportions, and then place them back into their cage with the other mice.

If there are any relevant citations or other documents that are needed to support these special housing, husbandry, or handling requirements, please attach them to this form.

Anesthesia Regimen: Anesthesia Regimen: Isoflurane via Precision Vaporizer for Mice

Please assign a label for this anesthesia regimen (e.g. Isoflurane Option, Surgical – Minor Procedure, Imaging Sedation, etc.). This label will be used in dropdown lists for other forms in this protocol.

Anesthesia Regimen: Isoflurane via Precision Vaporizer for Mice

1. Enter the agents that will be used for this anesthesia regimen. Include sedatives, paralytic agents, and anesthetic reversal agents. Do not include local anesthetics or other drugs used for analgesia.

See **Instructions** in right pane for a link to the preferred formulary.

Agent	Dose	Frequency	Route
Isoflurane via precision vaporizer	5%	To effect	Induction in chamber
Isoflurane via precision vaporizer	1-3%	Continuous	Intubation or nosecone

2. Are any of the agents listed paralytics?

- ◉ No

3. The IACUC requires that all anesthetics administered to any animal species be of pharmaceutical grade, if that agent is available in pharmaceutical grade. Are all agents in this anesthetic regimen of pharmaceutical grade?

See **Instructions** in right pane for definition of pharmaceutical grade.

- ◉ Yes

Species:

Mice (Mus)

4. The adequacy or depth of anesthesia will be monitored by (select all that apply):

- ☑ Respiratory rate
- ☑ Toe pinch
- ☑ Corneal or palpebral (blink) reflex

5. How frequently will the depth of anesthesia be assessed?

See **Instructions** in right pane for more information.

Animal will be monitored continuously with depth assessed at least every 15 minutes.

Procedures: Administration of estradiol via the diet

Complete this form for each procedure/surgery to be performed.

A procedure is any manipulation of an animal for an experimental application, for examination purposes or for treatment of an induced or spontaneous disease or condition. For clarity of definition the IACUC uses the terms **Surgical Procedure** or **Non-Surgical Procedure** to describe all manipulations performed.

Surgical Procedure usually involves an incision and exposure of a tissue for an operative method or the operative manipulation of physiologic or physical parameters to create a model of a clinical disease process or condition and/or treatment of a disease or condition.

Non-Surgical Procedure is used to describe injections, bandaging or casting, imaging, antibody production, collection of blood and other clinical samples, non-invasive physiological monitoring, breeding, behavior observations, euthanasia, etc.

Enter a title for this procedure:

Administration of estradiol via the diet

Mass General Brigham

A. Procedure Type

1. What is the type of procedure?

- Non-Surgical Procedure

a. This procedure is:

- Survival

2. Please select the procedure from the list.
(Select the item that best represents the procedure or approach used.)

If you cannot find your procedure on the list, please enter the name below:

administration of estradiol via the diet

B. Location

Indicate the building where the surgery or procedure will be performed:

CNY-149

Indicate the room number(s):

8-rodent facility

D. Procedure

1. Will anesthesia be used for this procedure?

- No

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a. Why will anesthesia not be used for this procedure?

- Not painful/not required

---

2. Will pre-operative/pre-emptive analgesics be used?

- No

---

4. Description of procedure

Provide a complete description of the procedure. For surgical procedures, include the surgical approach used, the method(s) of wound closure, and intra-operative supportive care (e.g., IV fluids, mechanical ventilation)

In addition to the mice's normal diet, during the treatment periods outlined on the flow chart, the mice will receive daily administration of either Nutella or Nutella + sesame oil + estradiol. Specifically,

In the control group:

60 mg of Nutella will be administered daily for 40 days

In the experimental group:

60 mg of Nutella + 1.12 µg of 17β-estradiol + 0.312 µL of sesame oil will be administered daily for 40 days

a. Is this a tumor production procedure?

- ◉ No

E. Post-operative/Post-procedural Care

CCM provides routine veterinary oversight, but the investigators are responsible for all monitoring and care of the research animals, unless a specific service has been pre-arranged with CCM by contract. See **Instructions** in the right pane for more information.

1. Indicate the frequency of post-procedural observations

Daily

2. Will post-operative/post-procedural analgesics be administered?

- ◉ No

a. Why will post-operative/post-procedural analgesics not be used for this procedure?

- ◉ Not painful/not required

If signs of pain persist past administration of the last dose of the analgesic regimen, contact a CCM veterinarian.

3. Will post-operative/post-procedural antibiotics be administered?

- ◉ No

4. Will other miscellaneous post-operative/post-procedural medications be administered?

- ◉ No

F. Non-Pharmaceutical Grade Substances

The IACUC requires that all substances administered to any animal species be of pharmaceutical grade, if that substance is available in pharmaceutical grade. See **Instructions** for more information.

1. Will all analgesics, antibiotics, or other medications administered during the course of this procedure be of pharmaceutical grade?

- ◉ No

Please include all non-pharmaceutical grade agents on the **Controlled** and **Non-Pharmaceutical Grade Substances** form.

Procedures: Cardiac puncture and exsanguination

Complete this form for each procedure/surgery to be performed.

A procedure is any manipulation of an animal for an experimental application, for examination purposes or for treatment of an induced or spontaneous disease or condition. For clarity of definition the IACUC uses the

terms **Surgical Procedure** or **Non-Surgical Procedure** to describe all manipulations performed.

Surgical Procedure usually involves an incision and exposure of a tissue for an operative method or the operative manipulation of physiologic or physical parameters to create a model of a clinical disease process or condition and/or treatment of a disease or condition.

Non-Surgical Procedure is used to describe injections, bandaging or casting, imaging, antibody production, collection of blood and other clinical samples, non-invasive physiological monitoring, breeding, behavior observations, euthanasia, etc.

Enter a title for this procedure:

Cardiac puncture and exsanguination

A. Procedure Type

1. What is the type of procedure?

◉ Non-Surgical Procedure

a. This procedure is:

◉ Non-Survival

2. Please select the procedure from the list.
(Select the item that best represents the procedure or approach used.)
Cardiac Puncture blood collection

B. Location

Indicate the building where the surgery or procedure will be performed:

CNY-149

Indicate the room number(s):

CCM procedure rooms

D. Procedure

1. Will anesthesia be used for this procedure?

◉ Yes

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a. Select the anesthesia regimen that will be used for this procedure, including induction and maintenance regimens. Please select a regimen that is appropriate for the duration of the procedure.

Anesthesia Regimen: Isoflurane via Precision Vaporizer for Mice

b. Indicate the duration of anesthesia

15-30 minutes

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4. Description of procedure

Provide a complete description of the procedure. For surgical procedures, include the surgical approach used, the method(s) of wound closure, and intra-operative supportive care (e.g., IV fluids, mechanical ventilation)

Once the mice have been anesthetized, blood will be withdrawn from the heart via syringe before euthanasia. Blood samples are taken from the ventricle of the heart, which is accessed either via the left side of the chest or by performing a thoracotomy. Blind puncture through the left side of the chest is the preferred method of blood collection. Thoracotomy will be performed if blind puncture is unsuccessful. A 23–25-gauge needle is used for blood collection.

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a. Is this a tumor production procedure?

- ◉ No

If signs of pain persist past administration of the last dose of the analgesic regimen, contact a CCM veterinarian.

F. Non-Pharmaceutical Grade Substances

The IACUC requires that all substances administered to any animal species be of pharmaceutical grade, if that substance is available in pharmaceutical grade. See **Instructions** for more information.

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1. Will all analgesics, antibiotics, or other medications administered during the course of this procedure be of pharmaceutical grade?

- ◉ Yes

Procedures: Ear Notch or Punch for Mice

Complete this form for each procedure/surgery to be performed.

A procedure is any manipulation of an animal for an experimental application, for examination purposes or for treatment of an induced or spontaneous disease or condition. For clarity of definition the IACUC uses the terms **Surgical Procedure** or **Non-Surgical Procedure** to describe all manipulations performed.

Surgical Procedure usually involves an incision and exposure of a tissue for an operative method or the operative manipulation of physiologic or physical parameters to create a model of a clinical disease process or condition and/or treatment of a disease or condition.

Non-Surgical Procedure is used to describe injections, bandaging or casting, imaging, antibody production, collection of blood and other clinical samples, non-invasive physiological monitoring, breeding, behavior observations, euthanasia, etc.

Enter a title for this procedure:

Ear Notch or Punch for Mice

A. Procedure Type

1. What is the type of procedure?

- ◉ Non-Surgical Procedure

a. This procedure is:

- ◉ Survival

2. Please select the procedure from the list.
(Select the item that best represents the procedure or approach used.)
Ear Punch

Mass General Brigham

B. Location

Indicate the building where the surgery or procedure will be performed:

CNY-149

Indicate the room number(s):

CCM-8th Floor Procedure room

D. Procedure

1. Will anesthesia be used for this procedure?

- ◉ No

---

a. Why will anesthesia not be used for this procedure?

- ◉ Not painful/not required

---

2. Will pre-operative/pre-emptive analgesics be used?

- ◉ No

---

4. Description of procedure

Provide a complete description of the procedure. For surgical procedures, include the surgical approach used, the method(s) of wound closure, and intra-operative supportive care (e.g., IV fluids, mechanical ventilation)

a. Animals ≥ 2 weeks of age are manually restrained by scruffing

b. The ear(s) is notched by cutting the minimum amount of tissue needed (approx. 2-3 mm) using a clean sharp scissors or punched using a commercial rodent ear punch device

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a. Is this a tumor production procedure?

- ◉ No

E. Post-operative/Post-procedural Care

CCM provides routine veterinary oversight, but the investigators are responsible for all monitoring and care of the research animals, unless a specific service has been pre-arranged with CCM by contract. See **Instructions** in the right pane for more information.

---

1. Indicate the frequency of post-procedural observations

Animals will be monitored immediately after the procedure to ensure no adverse effects.

---

2. Will post-operative/post-procedural analgesics be administered?

- ◉ No

---

a. Why will post-operative/post-procedural analgesics not be used for this procedure?

- ◉ Not painful/not required

If signs of pain persist past administration of the last dose of the analgesic regimen, contact a CCM veterinarian.

3. Will post-operative/post-procedural antibiotics be administered?

- No

4. Will other miscellaneous post-operative/post-procedural medications be administered?

- No

F. Non-Pharmaceutical Grade Substances

The IACUC requires that all substances administered to any animal species be of pharmaceutical grade, if that substance is available in pharmaceutical grade. See **Instructions** for more information.

1. Will all analgesics, antibiotics, or other medications administered during the course of this procedure be of pharmaceutical grade?

- Not applicable

Procedures: Intraperitoneal Injection for Mice

Complete this form for each procedure/surgery to be performed.

A procedure is any manipulation of an animal for an experimental application, for examination purposes or for treatment of an induced or spontaneous disease or condition. For clarity of definition the IACUC uses the terms **Surgical Procedure** or **Non-Surgical Procedure** to describe all manipulations performed.

Surgical Procedure usually involves an incision and exposure of a tissue for an operative method or the operative manipulation of physiologic or physical parameters to create a model of a clinical disease process or condition and/or treatment of a disease or condition.

Non-Surgical Procedure is used to describe injections, bandaging or casting, imaging, antibody production, collection of blood and other clinical samples, non-invasive physiological monitoring, breeding, behavior observations, euthanasia, etc.

Enter a title for this procedure:

Intraperitoneal Injection for Mice

A. Procedure Type

1. What is the type of procedure?

- Non-Surgical Procedure

a. This procedure is:

- Survival

2. Please select the procedure from the list.
(Select the item that best represents the procedure or approach used.)
Injection, IP

Mass General Brigham

B. Location

Indicate the building where the surgery or procedure will be performed:

CNY-149

Indicate the room number(s):

CCM-8th Floor Procedure room

D. Procedure

1. Will anesthesia be used for this procedure?

- ◉ No

---

a. Why will anesthesia not be used for this procedure?

- ◉ Not painful/not required

---

2. Will pre-operative/pre-emptive analgesics be used?

- ◉ No

---

4. Description of procedure

Provide a complete description of the procedure. For surgical procedures, include the surgical approach used, the method(s) of wound closure, and intra-operative supportive care (e.g., IV fluids, mechanical ventilation)

Agents to be injected: Sterile Vinylcyclohexene diepoxide (VCD), sterile sesame oil

Site: Lower right or left quadrant of the abdomen.

Volume: ≤ 100 microL using 25 gauge needle

Dose for each agent: VCD (160 mg/ml), sesame oil (because VCD is oily, sesame oil is the saline equivalent and there isn't an associated dose per say). Injection volume will be less than 100 microL

Dosing schedule for each agent: daily for 10 days

Method:

1. Animal will be restrained by via IP injection specific mechanical restrainer. Specifically, mouse will be grabbed by the tail, front paws will be placed in the restrainer as shown in the link, their rears will gently pushed into the restrainer, and the closer will be placed. (link that includes a general description: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3490254/ (Manual restraint: number 7)_)

2. The animal will be lifted such that its head is facing down and tail up. Injection site will be cleaned with an alcohol wipe

3. The needle will be inserted through the abdominal wall in the right or left caudal quadrant of the animal; the plunger will be pulled back to make sure no fluid is in the hub If there is fluid in the hub of the needle, the needle is removed, animal monitored and the procedure attempted again only if animal appears stable. If the hub is clean, material is injected.

4. The site of injection will be alternated between the lower quadrants of the animal for repeated injections.

Notes for consideration

1. Anesthesia is not necessary for performing this simple procedure, although exceptions may include the injection of hazardous agents. If instruction is needed, please contact CCM for training in handling and injection procedures.

2. Agents to be injected: (1) If multiple agents are to be injected IP, include all the required information for each agent. (2) If any agent to be administered is non-pharmaceutical grade, please indicate so in question F.

---

a. Is this a tumor production procedure?

- ◉ No

E. Post-operative/Post-procedural Care

CCM provides routine veterinary oversight, but the investigators are responsible for all monitoring and care of the research animals, unless a specific service has been pre-arranged with CCM by contract. See **Instructions** in the right pane for more information.

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1. Indicate the frequency of post-procedural observations

Animals will be observed immediately after injection for signs suggestive of abdominal discomfort such as hunched posture or unwillingness to ambulate, or signs consistent with aggressive restraint, such as labored breathing. If such signs are noted, a CCM veterinarian will be consulted.

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2. Will post-operative/post-procedural analgesics be administered?

- ◉ No

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a. Why will post-operative/post-procedural analgesics not be used for this procedure?

- ◉ Not painful/not required

If signs of pain persist past administration of the last dose of the analgesic regimen, contact a CCM veterinarian.

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3. Will post-operative/post-procedural antibiotics be administered?

- ◉ No

---

4. Will other miscellaneous post-operative/post-procedural medications be administered?

- ◉ No

F. Non-Pharmaceutical Grade Substances

The IACUC requires that all substances administered to any animal species be of pharmaceutical grade, if that substance is available in pharmaceutical grade. See **Instructions** for more information.

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1. Will all analgesics, antibiotics, or other medications administered during the course of this procedure be of pharmaceutical grade?

- ◉ No

Please include all non-pharmaceutical grade agents on the **Controlled** and **Non-Pharmaceutical Grade Substances** form.

Procedures: Muscle Contractility Testing

Complete this form for each procedure/surgery to be performed.

A procedure is any manipulation of an animal for an experimental application, for examination purposes or for treatment of an induced or spontaneous disease or condition. For clarity of definition the IACUC uses the terms **Surgical Procedure** or **Non-Surgical Procedure** to describe all manipulations performed.

Surgical Procedure usually involves an incision and exposure of a tissue for an operative method or the operative manipulation of physiologic or physical parameters to create a model of a clinical disease process or condition and/or treatment of a disease or condition.

Non-Surgical Procedure is used to describe injections, bandaging or casting, imaging, antibody production, collection of blood and other clinical samples, non-invasive physiological monitoring, breeding, behavior observations, euthanasia, etc.

Enter a title for this procedure:

Muscle Contractility Testing

A. Procedure Type

1. What is the type of procedure?

◉ Surgical Procedure

---

a. Select the type of surgical procedure:

◉ Non-survival: euthanasia is performed while the animal is under general anesthesia. The animal never awakens or regains consciousness

2. Please select the procedure from the list.
(Select the item that best represents the procedure or approach used.)
EMG and muscle contraction testing

B. Location

Indicate the building where the surgery or procedure will be performed:

CNY-149

Indicate the room number(s):

CCM-8th Floor Procedure room

C. Preoperative procedures

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1. Aseptic technique

For non-survival surgery, strict adherence to aseptic technique is not required; however, at a minimum, the surgical site should be clipped, the surgeon should wear gloves, and the instruments and surrounding area should be clean. For non-survival procedures of extended length, attention to aseptic technique may be more important in order to ensure stability of the model and a successful outcome.

Aseptic technique will be maintained by:

a. Preparation of the Animal
- ☑ Clipping/shaving fur around incision site
- ☑ Surgical soap scrub and alternating alcohol rinse, repeated three times

b. Preparation of the Surgeon
- ☑ Sterile gown, hat/cap, shoe covers, mask, sterile surgical gloves (for USDA regulated species)

c. Instruments and consumables (e.g., gauze, suture, etc.)
- ☑ Autoclave

2. Indicate other preoperative preparation:
- ☑ Other

Describe

none

D. Procedure

1. Will anesthesia be used for this procedure?
- ◉ Yes

a. Select the anesthesia regimen that will be used for this procedure, including induction and maintenance regimens. Please select a regimen that is appropriate for the duration of the procedure.

Anesthesia Regimen: Isoflurane via Precision Vaporizer for Mice

b. Indicate the duration of anesthesia

20 minutes

3. Will other medications (e.g., antibiotics, sedatives) be administered prior to the induction of anesthesia or the start of the procedure?
- ◉ No

4. Description of procedure

Provide a complete description of the procedure. For surgical procedures, include the surgical approach used, the method(s) of wound closure, and intra-operative supportive care (e.g., IV fluids, mechanical ventilation)

The animal will be anesthetized, and the peroneal nerve will be isolated by following the sciatic nerve to its branch point (the point at which the peroneal nerve starts) under sterile conditions. Superficial muscles will be carefully separated so that the electrode hooks are able to stimulate the peroneal nerve. The Achilles-tendon complex will be severed at the ankle in order to achieve isolated dorsi flexion of the hindfoot.

Once the nerve is isolated, the mouse hindfoot will be taped to a foot plate. The peroneal nerve will be stimulated once to confirm correct placement of the electrode. Once the test stimulation occurred, the

contractile computer program will be tuned for measuring contraction of the tibialis anterior (TA) and adjusted to its optimal length (i.e., the length at which maximal force production is achieved).

Frequency-force curves will be obtained by stimulating the TA at frequencies of 10-400 Hz (increments of 20-30 Hz for the tetanic protocol) and 100 Hz (fatigue protocol, 7 minutes long, stimulation every 4 seconds at 100Hz). Isometric contractile properties, including peak twitch, fatigue resistance, tetanic force, time to peak twitch force, and ½ relaxation time will be measured.

Following completion of the contractile testing, mice will be euthanized.

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a. Is this a tumor production procedure?

◉ No

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6. Indicate method of euthanasia

☑ Other (please describe)

Describe

Cervical dislocation

If signs of pain persist past administration of the last dose of the analgesic regimen, contact a CCM veterinarian.

F. Non-Pharmaceutical Grade Substances

The IACUC requires that all substances administered to any animal species be of pharmaceutical grade, if that substance is available in pharmaceutical grade. See **Instructions** for more information.

---

1. Will all analgesics, antibiotics, or other medications administered during the course of this procedure be of pharmaceutical grade?

◉ No

Please include all non-pharmaceutical grade agents on the **Controlled** and **Non-Pharmaceutical Grade Substances** form.

Procedures: Rectal Temperature Monitoring

Complete this form for each procedure/surgery to be performed.

A procedure is any manipulation of an animal for an experimental application, for examination purposes or for treatment of an induced or spontaneous disease or condition. For clarity of definition the IACUC uses the terms **Surgical Procedure** or **Non-Surgical Procedure** to describe all manipulations performed.

Surgical Procedure usually involves an incision and exposure of a tissue for an operative method or the operative manipulation of physiologic or physical parameters to create a model of a clinical disease process or condition and/or treatment of a disease or condition.

Non-Surgical Procedure is used to describe injections, bandaging or casting, imaging, antibody production, collection of blood and other clinical samples, non-invasive physiological monitoring, breeding, behavior observations, euthanasia, etc.

Enter a title for this procedure:

Rectal Temperature Monitoring

A. Procedure Type

1. What is the type of procedure?

- ◉ Non-Surgical Procedure

a. This procedure is:

- ◉ Survival

2. Please select the procedure from the list.
(Select the item that best represents the procedure or approach used.)
Rectal temperature measurement

B. Location

Indicate the building where the surgery or procedure will be performed:

CNY-149

Indicate the room number(s):

CNY-149 8th floor procedure room

D. Procedure

1. Will anesthesia be used for this procedure?

- ◉ No

---

a. Why will anesthesia not be used for this procedure?

- ◉ Not painful/not required

---

2. Will pre-operative/pre-emptive analgesics be used?

- ◉ No

---

4. Description of procedure

Provide a complete description of the procedure. For surgical procedures, include the surgical approach used, the method(s) of wound closure, and intra-operative supportive care (e.g., IV fluids, mechanical ventilation)

1. The mouse will be placed in a mechanical restrainer (https://www.braintreesci.com/restraint-containment-handling/restraint/rat-restrainers/flat-bottom/). Specifically, the tail will be grabbed at the base and the mouse will be encouraged to slide into the tube. Once the mouse is securely in the tube, the cap will be placed to hold the mouse in the tube.

2. Prior to use, the rectal probe will be cleaned with an alcohol swab.

3. The rectal probe will be placed in the rectum, no deeper than 5 cm.

4. The probe will be held in the rectum until the temperature reading is stable for 5 seconds.

5. The probe will be removed.

6. The mouse will be returned to her cage.

7. The probe will be cleaned with an alcohol wipe prior to use on another animal

a. Is this a tumor production procedure?

- ◉ No

E. Post-operative/Post-procedural Care

CCM provides routine veterinary oversight, but the investigators are responsible for all monitoring and care of the research animals, unless a specific service has been pre-arranged with CCM by contract. See **Instructions** in the right pane for more information.

1. Indicate the frequency of post-procedural observations

Daily

2. Will post-operative/post-procedural analgesics be administered?

- ◉ No

a. Why will post-operative/post-procedural analgesics not be used for this procedure?

- ◉ Not painful/not required

If signs of pain persist past administration of the last dose of the analgesic regimen, contact a CCM veterinarian.

3. Will post-operative/post-procedural antibiotics be administered?

- ◉ No

4. Will other miscellaneous post-operative/post-procedural medications be administered?

- ◉ No

F. Non-Pharmaceutical Grade Substances

The IACUC requires that all substances administered to any animal species be of pharmaceutical grade, if that substance is available in pharmaceutical grade. See **Instructions** for more information.

1. Will all analgesics, antibiotics, or other medications administered during the course of this procedure be of pharmaceutical grade?

- ◉ Not applicable

Procedures: Vaginal cytology

Complete this form for each procedure/surgery to be performed.

A procedure is any manipulation of an animal for an experimental application, for examination purposes or

for treatment of an induced or spontaneous disease or condition. For clarity of definition the IACUC uses the terms **Surgical Procedure** or **Non-Surgical Procedure** to describe all manipulations performed.

Surgical Procedure usually involves an incision and exposure of a tissue for an operative method or the operative manipulation of physiologic or physical parameters to create a model of a clinical disease process or condition and/or treatment of a disease or condition.

Non-Surgical Procedure is used to describe injections, bandaging or casting, imaging, antibody production, collection of blood and other clinical samples, non-invasive physiological monitoring, breeding, behavior observations, euthanasia, etc.

Enter a title for this procedure:

Vaginal cytology

A. Procedure Type

1. What is the type of procedure?

◉ Non-Surgical Procedure

a. This procedure is:

◉ Survival

2. Please select the procedure from the list.
(Select the item that best represents the procedure or approach used.)
vaginal swab

B. Location

Indicate the building where the surgery or procedure will be performed:

CNY-149

Indicate the room number(s):

CCM 8 procedure space

D. Procedure

1. Will anesthesia be used for this procedure?

◉ No

---

a. Why will anesthesia not be used for this procedure?

◉ Not painful/not required

---

2. Will pre-operative/pre-emptive analgesics be used?

◉ No

---

4. Description of procedure

Provide a complete description of the procedure. For surgical procedures, include the surgical approach used, the method(s) of wound closure, and intra-operative supportive care (e.g., IV fluids, mechanical ventilation)

1. A latex bulb will be placed on the end of a sterile 200 µl tip and approximately 100 µl of sterile ddH2O will be drawn using the gradations on the tip as a volume guideline.

2. The mouse will be placed in a mechanical restrainer.

3. Firmly grasp the tail and elevate the rear end. At this point the mouse may urinate. If so, investigator wiwll wait until urination stops. Should there be urine left at the entrance to the vaginal canal, the opening will be rinsed with excess ddH2O using a separate tip (i.e., not your sample collection tip).

4. The end of the ddH2O-filled tip will be placed at the opening of the vaginal canal taking care to not penetrate the orifice. Care will be taken to minimize the degree of invasiveness.

5. The bulb will be gently depressed to expel a quarter to half of the volume of water (~25-50 µl) at the opening of vaginal canal. The liquid will spontaneously aspirate into the canal without tip insertion. The pressure exerted on the bulb will be slowly released. The fluid will withdraw back into the tip. Investigator will avoid releasing pressure too quickly to prevent aspiration of fluid into the bulb. A filtered tip may be useful for this purpose.

6. The previous steps will be repeated 4-5 times using the same tip, bulb, and fluid to obtain a sufficient number of cells in a single sample.

7. TThe fluid will be placed on a glass slide, and the smear will be allowed to completely dry at room temperature. Once dry, these estrous smears can be stained immediately or stored and stained at a later date.

---

a. Is this a tumor production procedure?

- ◉ No

E. Post-operative/Post-procedural Care

CCM provides routine veterinary oversight, but the investigators are responsible for all monitoring and care of the research animals, unless a specific service has been pre-arranged with CCM by contract. See **Instructions** in the right pane for more information.

---

1. Indicate the frequency of post-procedural observations

Although extremely unlikely, vaginal trauma is possible with daily lavage. Since we will be performing this procedure daily, we will also check genital area to assure no trauma prior to procedure. If any trauma or abnormalities are noted, we will contact veterinary staff immediately

---

2. Will post-operative/post-procedural analgesics be administered?

- ◉ No

---

a. Why will post-operative/post-procedural analgesics not be used for this procedure?

- ◉ Not painful/not required

If signs of pain persist past administration of the last dose of the analgesic regimen, contact a CCM veterinarian.

---

3. Will post-operative/post-procedural antibiotics be administered?

- ◉ No

4. Will other miscellaneous post-operative/post-procedural medications be administered?

- ◉ No

F. Non-Pharmaceutical Grade Substances

The IACUC requires that all substances administered to any animal species be of pharmaceutical grade, if that substance is available in pharmaceutical grade. See **Instructions** for more information.

1. Will all analgesics, antibiotics, or other medications administered during the course of this procedure be of pharmaceutical grade?

- ◉ No

Please include all non-pharmaceutical grade agents on the **Controlled** and **Non-Pharmaceutical Grade Substances** form.

Hazardous Agent Administration and Use: vinylcyclohexene diepoxide (VCD)

All projects involving the use of any biological, chemical, or radiological hazard must be performed in accordance with Institutional Safety and Biosafety Policies for hazardous materials. See Instructions in right pane for more information. Principal Investigators are responsible for informing employees of any potential risks associated with hazardous agents they will be expected to use.

Capture information related to all hazardous agents used in the protocol using this form. Contact your institutional Safety Office if you have questions about which agents should be captured on this form.

Please provide the emergency study contact(s).

Contact Name	Phone Beeper Number	Email (Mass General Brigham Email only)
Gabrielle Gilmer	2057899607	ggilmer@mgh.harvard.edu

A. Hazard Type

Indicate the type of hazard to be used. See **Instructions** in right pane for more information on hazard types.

- ◉ Chemical

1. Please select the chemical hazard to be used.

If the chemical is not listed, enter the name:

vinylcyclohexene diepoxide (VCD)

In order to ensure chemical safety in the workplace, information about the identities and hazards of the chemicals must be available and understandable to workers. All employers with hazardous chemicals in their workplaces must have labels and safety data sheets (MSDSs) for their exposed workers, and train them to handle the chemicals appropriately. See **Instructions** in right pane for information on accessing MSDS Source.

2. Do you have a Chemical Hygiene Plan?

- ◉ Yes

Mass General Brigham

B. Hazard Use Location

Please indicate the building where this agent will be administered to an animal

CNY-149

Indicate the room number(s):

TBD

- The rooms indicated on this form must be consistent with the rooms indicated on your Procedure form(s). Biological hazards and radiological hazards may be used only in the rooms approved on your relevant IBC registration or radioisotope permit.
- If the hazard will be used within animal facilities, please contact the appropriate facility manager **before** use to ensure that any special requirements for animal housing, husbandry, and handling, and personnel safety are addressed.
- Please consult with your institutions Safety Office to discuss whether additional engineering controls are required for this agent. See **Instructions** in right pane for institution-specific contacts.

C. Agent Administration

1. What is the expected dose (range) per animal?

   160 mg/kg

2. What is the total number of doses an individual animal may receive?

   10

3. How frequently will an individual animal be dosed with this agent?

   daily

4. Indicate the duration of time between administration of the hazardous agent and planned euthanasia of the animals.

   24 hours

5. Will animals be returned to animal facilities after exposure to this agent?

   ◉ Yes

Please note, if animals will be housed in animal facilities after exposure to this agent or if the agent will be administered within animal facilities:

- Please contact the appropriate facility manager **before** use to ensure that any special requirements for animal housing, husbandry, and handling, and personnel safety are addressed. See **Instructions** in right pane for contact information.
- Include all special requirements for animal housing, husbandry, and handling on the Housing form.

Controlled Substance and Non-Pharmaceutical Grade Substance

A. Controlled Substances

◉ No

B. Non-Pharmaceutical Grade Substances

Both OLAW and AAALAC provide guidance regarding the use of non-pharmaceutical grade compounds in laboratory animals.

Pharmaceutical-grade substances, when available, must be used to avoid toxicity or side effects that may threaten the health and welfare of vertebrate animals and/or interfere with the interpretation of research results. However, it is frequently necessary to use non-pharmaceutical-grade substances such as investigational substances, veterinarian- or pharmacy-compounded substances, and/or Schedule I controlled substances to meet scientific and research goals.

A listing of pharmaceutical-grade drugs and biologics is available through the FDA database.
· The Orange Book is the reference for FDA-approved human drugs.
· The Green Book is the reference for FDA-approved veterinary drugs.

1. Are all substances to be administered to animals of pharmaceutical grade? See **Instructions** in right pane for definitions.

This includes:

- Anesthetics and analgesics (e.g., Avertin)
- Euthanasia compounds (e.g., pentobarbital *Note: Fatal-Plus is not an FDA-approved drug and is not considered pharmaceutical grade. See instructions for more information.*)
- Diluents and/or vehicles (e.g., DMSO, methyl cellulose)
- Test compounds

◉ No

2. List the non-pharmaceutical grade substance(s) that will be used.

Please address the use of these non-pharmaceutical grade substance(s) in the appropriate section(s) of the protocol (**Research Plan**, **Procedure** forms, etc.)..

Agent	Dose	Duration	Frequency	Route
4-vinylcycohexene diepoxide	160 mg/kg/day	10 days	daily	IP injection
Sesame oil	70 microL/day	10 days	Daily	IP injection
Nutella	60 mg/day	40 days	daily	Peroral
Sesame oil	0.312 µL/day	40 days	daily	peroral
17β-estradiol	1.12 µg/day	40 days	daily	peroral

3. Non-pharmaceutical grade substances must be the highest grade available and must be formulated using biocompatible solutions appropriate for the route of administration; departures from these guidelines must be described and justified below. In addition, non-pharmaceutical grade substances administered parenterally (e.g., IV, IP, IM, SC) will be sterilized according to the guidelines. See **Instructions** in right pane for institutional guidelines.

Describe and justify departures from the guidelines.

We will follow guidelines as stated above

4. Justification for use on non-pharmaceutical grade compounds (select all that apply):

☑ Pharmaceutical grade is not available in the appropriate concentration or formulation, or the appropriate vehicle control is unavailable.

☑ The Principal Investigator attests that he/she has read the institutional policy and will ensure that all protocol study staff will follow the policy.

Restraint and Device Acclimation

The Guide for the Care and Use of Laboratory Animals defines physical restraint as the use of manual or mechanical means to limit some or all of an animal's movement for the purpose of examination or experimental manipulation. Sedatives or anesthetics may be used to immobilize animals for the performance of non-painful procedures that might otherwise be painful or distressful to the animal.

Restraint

◉ Animals will undergo restraint as part of this research

1. Provide justification for the use and duration of restraint.

   Animals must be restrained to perform vaginal cytology

2. Will the animals be conscious or sedated during the restraint?

   ◉ Conscious

3. Indicate the type of restraint that will be used.
   ☑ Mechanical

   Select all that apply.
   ☑ Rodent plexiglass, metal, or Bowman style restrainer

   a. Indicate the duration of mechanical restraint (select all that apply).
   ☑ Routine mechanical restraint for less than 15 minutes

   b. Indicate the frequency of mechanical restraint.

   During vaginal cytology

   c. Describe the methods used to train and acclimate animals to the mechanical restraint device.

   The mouse will be restrained by the scruff and it's head will be directed into the opening of the restrainer. The securing device will be affixed to prevent the mouse from exiting the apparatus.

   d. Describe the plans for monitoring and care of the animals during the periods of mechanical restraint.

   The animal will be released if any change in breathing rate or change in coloration is observed during the vaginal cytology procedure.

The Principal Investigator is responsible for assuring that:
- Veterinary care will be provided if lesions or illnesses are observed
- The purpose and duration of restraint will be communicated to all personnel involved in the study.

Device Acclimation

Initial Survey

INSTITUTIONAL ANIMAL CARE AND USE COMMITTEE

In accordance with federal regulations and hospital policies, all animal research conducted at or funded through the Massachusetts General Hospital (MGH), the Shriners Hospitals for Children-Boston (SHC), Brigham and Women's Hospital (BWH), McLean Hospital (MCL), Massachusetts Eye and Ear (MEE) or Schepens Eye Research Institute (SERI) must be reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) prior to initiation of the study. This policy applies to any vertebrate animal used for any type of research, teaching, or testing. The IACUC has the sole authority to approve, require

modifications (in order to secure approval), or withhold approval of research protocols involving the use of animals at the selected Institution. Protocols can be approved for a maximum of three years, subject to satisfactory annual reviews where required. The IACUC also must review and approve **in advance** any changes or modifications to previously approved protocols.

Principal Investigator Eligibility: Please note that you must meet the eligibility requirements set by your institution's IACUC in order to serve as the principal investigator (PI) for an animal research protocol. See FAQ for links to institution-specific guidelines.

The questions below will help to identify if an IACUC protocol must be submitted to your institution's IACUC for your research project.

Please enter the full title of the study.

Investigating the effects of menopause on the musculoskeletal system

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A. At which Institution will the research be conducted?

- ◉ MGH or SHC

B. The proposed research project will involve the following:

- ◉ The entire animal research protocol will be conducted at the selected institution.

If the IACUC grants approval, it will oversee only the research component that is performed at your institution. Any research component(s) conducted at an outside institution will be conducted under the auspices of that institution's IACUC.

For the protocol to remain active, the investigator must submit satisfactory IACUC annual progress reports (if required), as well as provide annual documentation of the relevant outside IACUC approvals.

For more information, please refer to the IACUC website for your institution:

- MGH/SHC: http://is.partners.org/aniweb//Policies/Protocol%20Review%20Policy.pdf
- BWH: http://animal.bwh.harvard.edu/iacuc-review-process/
- MCL: https://partnershealthcare.sharepoint.com/sites/phrmApply/aieipa/iacuc/mi/Pages/IACUC-Home-Page.aspx
- MEE: http://thesource.meei.harvard.edu/index.php/animal-care/
- SERI : http://thesource.meei.harvard.edu/index.php/animal-care/

C. The proposed study involves the use of:

- ◉ Any live animal (ie. mouse, rat, rabbit, dog, cat, swine, sheep, nonhuman primate, etc)

Attachments

Name	Mode
Study Staff certification (Staff certification)	Electronic
Study Staff certification (Staff certification)	Electronic
Study Staff certification (Staff certification)	Electronic
Study Staff certification (Staff certification)	Electronic
Study Staff certification (Staff certification)	Electronic

Mass General Brigham

Title: **Investigating the effects of menopause on the musculoskeletal system**
PI Name: **Ambrosio, Fabrisia**
Protocol #: **2022N000171**

PRINCIPAL INVESTIGATOR CERTIFICATION

Please check all of the boxes and sign off at the bottom right.

As Principal Investigator on this protocol, I certify the following

✓ I am familiar with the MGH policy regarding PI eligibility for IACUC protocols and agree I meet the requirements and expectations of this policy. (Please access the Policy on Principal Investigator Eligibility on the IACUC website: https://mghresearch.partners.org/wp-content/uploads/Policy-on-Principal-Investigator-Eligibility.pdf)

✓ I am familiar with proper handling, experimental, and restraint techniques required for the species used, or will seek advice and assistance from the staff of the Center for Comparative Medicine.

✓ I certify that only staff proficient on the procedures approved in the species proposed will be allowed to independently perform these procedures. I will ensure all staff will be trained and supervised until proficient, as needed.

✓ I certify that I, and my staff, will adhere to the procedures as described in the approved protocol; I will ensure that animals will be provided with anesthetic, analgesic and supportive care, and will be monitored at the frequency as detailed in the protocol. I understand that failure to do this may result in a suspension of this research by the IACUC.

✓ I certify that all study staff have been informed of the hazards related to all procedures on this protocol. I will ensure that all study staff are trained in the safe use of hazardous agents and will follow all practices and procedures to ensure safe use of hazards on this protocol.

✓ I have read and agree to abide by the policies governing the care and use of animals at the Institution. It is understood that these polices include applicable local, State, and Federal policies and regulations.

✓ I recognize that failure to adhere to MGH policies relating to animal care and use may result in suspension or revocation of permission to perform animal research in Hospital facilities.

✓ I understand that IACUC approval is valid for three years only; every third year the IACUC must perform a new review of my protocol.

✓ I understand that any proposed changes to the approved protocol must be reviewed and approved by the IACUC prior to implementation. I will ensure the research team are aware of the changes made to the protocol on approval.

✓ If I use controlled substances in non-human research activities, I have the proper research specific (clinical is not sufficient) registrations for use of these substances in animal research.

Authenticated: **Ambrosio, Fabrisia**
Authenticated On: **September 30, 2022**

Title: **Investigating the effects of menopause on the musculoskeletal system**
PI Name: **Ambrosio, Fabrisia**
Protocol #: **2022N000171**

Study Staff Review and Certification

Please check all of the boxes and sign off at the bottom right.

As Study Staff on this protocol, I certify the following

✓ I am familiar with proper handling, experimental and restraint techniques required for the species used or will seek advice and assistance from the staff of the Center for Comparative Medicine/Animal Care Facility.

✓ I certify that I have been informed of the hazards related to all chemicals on this protocol. I have been trained in the safe use of chemicals and will follow all practices and procedures to ensure safe use of chemicals on this protocol.

✓ I have read and agree to abide by the policies governing the care and use of animals at the Institution. It is understood that these policies include applicable local, State, and Federal policies and regulations.

✓ I recognize that failure to adhere to the Institution's policies relating to animal care and use may result in suspension or revocation of permission to perform animal research in Hospital facilities.

Authenticated: **Wang, Kai**
Authenticated On: **October 11, 2022**

Title: **Investigating the effects of menopause on the musculoskeletal system**
PI Name: **Ambrosio, Fabrisia**
Protocol #: **2022N000171**

Study Staff Review and Certification

Please check all of the boxes and sign off at the bottom right.

As Study Staff on this protocol, I certify the following

✓ I am familiar with proper handling, experimental and restraint techniques required for the species used or will seek advice and assistance from the staff of the Center for Comparative Medicine/Animal Care Facility.

✓ I certify that I have been informed of the hazards related to all chemicals on this protocol. I have been trained in the safe use of chemicals and will follow all practices and procedures to ensure safe use of chemicals on this protocol.

✓ I have read and agree to abide by the policies governing the care and use of animals at the Institution. It is understood that these policies include applicable local, State, and Federal policies and regulations.

✓ I recognize that failure to adhere to the Institution's policies relating to animal care and use may result in suspension or revocation of permission to perform animal research in Hospital facilities.

Authenticated: **Kopchak, Rylee**
Authenticated On: **October 11, 2022**

Title: **Investigating the effects of menopause on the musculoskeletal system**
PI Name: **Ambrosio, Fabrisia**
Protocol #: **2022N000171**

Study Staff Review and Certification

Please check all of the boxes and sign off at the bottom right.

As Study Staff on this protocol, I certify the following

✓ I am familiar with proper handling, experimental and restraint techniques required for the species used or will seek advice and assistance from the staff of the Center for Comparative Medicine/Animal Care Facility.

✓ I certify that I have been informed of the hazards related to all chemicals on this protocol. I have been trained in the safe use of chemicals and will follow all practices and procedures to ensure safe use of chemicals on this protocol.

✓ I have read and agree to abide by the policies governing the care and use of animals at the Institution. It is understood that these policies include applicable local, State, and Federal policies and regulations.

✓ I recognize that failure to adhere to the Institution's policies relating to animal care and use may result in suspension or revocation of permission to perform animal research in Hospital facilities.

Authenticated: **Gilmer, Gabrielle G**
Authenticated On: **October 11, 2022**

Mass General Brigham

Title: **Investigating the effects of menopause on the musculoskeletal system**
PI Name: **Ambrosio, Fabrisia**
Protocol #: **2022N000171**

Study Staff Review and Certification

Please check all of the boxes and sign off at the bottom right.

As Study Staff on this protocol, I certify the following

✓ I am familiar with proper handling, experimental and restraint techniques required for the species used or will seek advice and assistance from the staff of the Center for Comparative Medicine/Animal Care Facility.

✓ I certify that I have been informed of the hazards related to all chemicals on this protocol. I have been trained in the safe use of chemicals and will follow all practices and procedures to ensure safe use of chemicals on this protocol.

✓ I have read and agree to abide by the policies governing the care and use of animals at the Institution. It is understood that these policies include applicable local, State, and Federal policies and regulations.

✓ I recognize that failure to adhere to the Institution's policies relating to animal care and use may result in suspension or revocation of permission to perform animal research in Hospital facilities.

Authenticated: **Hettinger, Zachary**
Authenticated On: **October 11, 2022**

OMB Number: 4040-0001
Expiration Date: 12/31/2022

RESEARCH & RELATED Senior/Key Person Profile (Expanded)

PROFILE - Project Director/Principal Investigator

Prefix: First Name*: Gabrielle Middle Name Last Name*: Gilmer Suffix:

Position/Title*: MD/PhD Trainee
Organization Name*: Spaulding Rehabilitation Hospital
Department:
Division:
Street1*: 300 1st Ave
Street2:
City*: Charlestown
County:
State*: MA: Massachusetts
Province:
Country*: USA: UNITED STATES
Zip / Postal Code*: 02129-3109

Phone Number*: 2057899607 Fax Number:

E-Mail*: ggilmer@mgh.harvard.edu

Credential, e.g., agency login: GABBYGILMER

Project Role*: PD/PI Other Project Role Category:

Degree Type: MD,PHD,BS Degree Year: 2027,2025,2018

Attach Biographical Sketch*: File Name: gilmerBiosketch_Final.pdf

Attach Current & Pending Support: File Name:

PROFILE - Senior/Key Person

Prefix: First Name*: Fabrisia Middle Name Last Name*: Ambrosio Suffix:

Position/Title*: Discovery Center Director
Organization Name*: Spaulding Rehabilitation Hospital
Department:
Division:
Street1*: Unit 8
Street2: 13th St #4002
City*: Boston
County:
State*: MA: Massachusetts
Province:
Country*: USA: UNITED STATES
Zip / Postal Code*: 021290000

Phone Number*: 412-657-1525 Fax Number:

E-Mail*: fambrosio@mgh.harvard.edu

Credential, e.g., agency login: AMBROSIOF

Project Role*: Other (Specify) Other Project Role Category: Sponsor

Degree Type: PHD Degree Year: 2005

Attach Biographical Sketch*: File Name: Ambrosio_biosktech_final.pdf

Attach Current & Pending Support: File Name:

OMB No. 0925-0001 and 0925-0002 (Rev. 10/2021 Approved Through 09/30/2024)

BIOGRAPHICAL SKETCH

Provide the following information for the Senior/key personnel and other significant contributors.
Follow this format for each person. DO NOT EXCEED FIVE PAGES.

NAME: Gilmer, Gabrielle

eRA COMMONS USER NAME (credential, e.g., agency login): gabbygilmer

POSITION TITLE: Trainee in the Integrated Clinical and Geroscience Research Training Program

EDUCATION/TRAINING *(Begin with baccalaureate or other initial professional education, such as nursing, include postdoctoral training and residency training if applicable. Add/delete rows as necessary.)*

INSTITUTION AND LOCATION	DEGREE (if applicable)	END DATE MM/YYYY	FIELD OF STUDY
Auburn University, Auburn, AL	BENG	05/2018	Chemical Engineering
University of Pittsburgh, Pittsburgh, PA	PHD	05/2025	Cellular and Molecular Pathology
University of Pittsburgh, Pittsburgh, PA	MD	05/2027	Medicine
NHLBI, NIH, Bethesda, MD	Other training	02/2019	Post-baccalaureate fellowship

A. Personal Statement

If you asked 17-year-old me what I would be when I grew up, I would have answered a professional soccer player. However, after signing a full athletic scholarship and contract for the Olympic Development Team, I tore my anterior cruciate ligament (ACL) for the third time, an injury that plagues female athletes ten times more frequently than male athletes. As I have moved through my early medical and research training, I have realized this sex-based discrepancy is not unique: knee and hand osteoarthritis, femoroacetabular impingement syndrome, and rotator cuff tears are all more common in women. More appalling is the paucity of data exploring *why* these trends exist and targeted solutions designed specifically for women. With this in mind, **my long-term research goals are**: *(1) to understand how sex and gender affect joint functionality and injury from the molecular to whole organism level, (2) to use this understanding to design creative and accessible clinical interventions for the treatment of joint injuries in women, and (3) to use these clinical interventions to empower women to get back to the movement patterns they love.*

To provide a framework for my goals, I will merge (1) Biomechanics and Rehabilitation, (2) Physiology Informed Tissue Engineering, and (3) Clinical Interventions. From this foundation, **my long-term career goals** are to build a comprehensive research and clinical care center that focuses on joint injuries in women. As a researcher, I intend to lead an NIH-funded, multi-disciplinary laboratory that brings together investigators with diverse backgrounds, including biomechanists, engineers, and biologists, to merge findings from these three fields and propel the next generation of therapeutics for treating joint injuries in women. As a clinician, I aim to provide patients with access to a diverse set of support, including physical therapists, orthopedic surgeons, and psychologists, to help balance all the needs that are associated with recovering from a joint injury. With this long-term vision in mind, my training focuses on developing expertise in the three aforementioned arenas.

My dedication to achieving my long-term goals is evidenced by my success in the research lab early in my career and my involvement in leadership positions. My work as an undergraduate resulted in 20 peer reviewed publications, eight of which are first-authored, and my productivity rate illustrates my ability to systematically test research questions in a highly rigorous and efficient manner. Moving into my MD/PhD training at the University of Pittsburgh, my focus during graduate school has progressed to developing a foundation in the Physiology Informed Tissue Engineering arm of my goal. To explore my training opportunities, I completed several research rotations between my first two years of medical school and realized my objectives most centrally aligned with Dr. Fabrisia Ambrosio's values due to her extensive expertise in regenerative rehabilitation. Together, we have designed this proposal which will provide me with the latitude to develop footing in knee osteoarthritis, menopause, and synthetic biology. My rotations and early work in graduate school have resulted in three first-authored manuscripts (one recently published [1] and two are under review) and three co-authored papers (two that were recently published [2,3] and one that was recently accepted at *Nature Communications* [4]). My multi-disciplinary background, unique combination of technical skills, and productive track record consisting of twenty-five peer-reviewed publications (ten of which are first-authored) equips me with the necessary skills to tackle the complex and neglected research questions that inform medical care for the millions of women with joint-related injuries and diseases.

1. **Gilmer G**, Jackson N, Koscumb S, Marroquin O, Sowa G. A Retrospective Analysis of Clinical Utilization between Patients who used Telemedicine and Office Visits in Outpatient Physical Medicine & Rehabilitation Clinics during the COVID-19 Pandemic. Am J Phys Med Rehabil. 2022 Mar 12; PubMed Central PMCID: PMC9464794.
2. Kwan JM, Noch E, Qiu Y, Toubat O, Christophers B, Azzopardi S, **Gilmer G**, Wiedmeier JE, Daye D. The Impact of COVID-19 on Physician-Scientist Trainees and Faculty in the United States: A National Survey. Acad Med. 2022 Oct 1;97(10):1536-1545. PubMed Central PMCID: PMC9547818.
3. Iijima H, **Gilmer G**, Wang K, Sivakumar S, Evans C, Matsui Y, Ambrosio F. Meta-analysis Integrated With Multi-omics Data Analysis to Elucidate Pathogenic Mechanisms of Age-Related Knee Osteoarthritis in Mice. J Gerontol A Biol Sci Med Sci. 2022 Jul 5;77(7):1321-1334. PubMed Central PMCID: PMC9255692.
4. Iijima H, **Gilmer G**, Wang K, Bean A, He Y, Lin H, Evans C, Ambrosio F. Age-related increase in matrix stiffness downregulates α-Klotho in chondrocytes and induces cartilage degeneration. [Preprint]. 2021 March 14. DOI: 10.1101/2021.03.13.434679. *Accepted at Nature Communications.*

B. Positions, Scientific Appointments and Honors

Positions and Scientific Appointments

2022 -	Member, Massachusetts General Hospital Martinos Center Women in Science, Policy Advocacy Sub-Committee
2021 -	Trainee in the Integrated Clinical and Geroscience Research Training Program, Department of Geriatrics, University of Pittsburgh
2021 - 2022	Co-Chair, Workshop Committee, University of Pittsburgh MSTP
2020 -	Member, Pediatric Research in Sports Medicine Female Athletes Research Interest Group
2020 -	Member, Point of Care Ultrasound Certification Program, University of Pittsburgh School of Medicine
2020 -	Member, Women's Health Area of Concentration, University of Pittsburgh School of Medicine
2020 - 2022	Founder & President, American Physician Scientist Association University of Pittsburgh Local Chapter
2020 - 2021	Chair, Advocacy Committee, American Physician Scientist Association University of Pittsburgh Local Chapter
2020 - 2021	Co-coordinator, Physical Medicine & Rehabilitation Interest Group, University of Pittsburgh
2020 - 2021	APSA MD-DO/PhD Member at Large, American Physician Scientist Association
2020 - 2020	Co-chair, Welcome Committee, University of Pittsburgh MSTP
2019 -	Trainee in Medical Scientist Training Program, University of Pittsburgh
2019 -	Mentor, Pitt Women in Healthcare Club, University of Pittsburgh
2019 - 2020	Student Interviewer, University of Pittsburgh School of Medicine
2018 - 2019	Writer for The Science Beat, NHLBI Fellows Newsletter
2018 - 2019	Postbaccalaureate Intramural Research Training Award Fellowship, NIH
2017 - 2018	Undergraduate Teaching Assistant for Principles of Chemical Engineering, Department of Chemical Engineering, Auburn University
2017 - 2018	Secretary, Omega Chi Epsilon Chemical Engineering Honor Society, Auburn University
2017 - 2018	Service Chair, Tau Beta Pi Engineering Honor Society, Auburn University
2017 - 2018	President, Undergraduate Research Ambassadors, Auburn University
2017 - 2017	National Heart, Lung, and Blood Institute Summer Intramural Research Training Award , NHLBI, NIH
2016 -	Member, American Physician Scientist Association
2016 - 2018	Physics Learning Assistant for General Physics II, Engineering Physics I, and Engineering Physics II, Physics Department, Auburn University
2015 - 2018	Director of Curriculum Support, Catapult Engineering Academy
2015 - 2018	Undergraduate Research Fellow, Sports Medicine & Movement Laboratory, Auburn University
2014 - 2018	Lead Teaching Assistant for Introduction to Solid Modeling and Engineering & Engineering Instrumentation and Analysis, Catapult Engineering Academy
2014 - 2018	Member, Honors College, Auburn University

Honors

2022	Travel Award Recipient, Regenerative Rehabilitation Symposium
2022	First Place Poster Presentation, Regenerative Rehabilitation Symposium
2021	Student of the Month, University of Pittsburgh School of Medicine Student Executive Committee
2021	Bevier Award, University of Pittsburgh Department of Bioengineering
2020	Travel Award Recipient, American Physician Scientist Association Annual Meeting
2020	Service Award, University of Pittsburgh MSTP
2018	First Place Undergraduate Student Research Award, Southeast Chapter of the American College of Sports Medicine
2018	100 Women Strong in Engineering Leadership Award, Auburn University
2018	First Place in Oral Presentations in STEM , Auburn University Student Symposium
2017	Travel Award Recipient, Southeastern Medical Scientist Symposium
2016	Member, Tau Beta Pi Engineering Honor Society
2016 - 2018	Cleburne A. Basore Endowed Scholarship, Auburn University
2016	Member, Omega Chi Epsilon Chemical Engineering Honor Society
2014 - 2018	Founder's Scholarship, Auburn University
2014 - 2018	100 Women Strong Scholarship, Auburn University
2014	Soccer Scholarship, Hoover High School

C. Contribution to Science

1. Undergraduate Research.

As an undergraduate in Dr. Gretchen Oliver's lab at Auburn University, I led two projects. The first of which was focused on the relationship between throwing mechanics and lumbopelvic hip complex (LPHC) stability in female athletes (a, b), and my second project focused on understanding the relationship between biomechanical predictors for anterior cruciate ligament (ACL) injury and relaxin (c, d). For both projects, I led the study design, participant recruitment, motion capture and analysis, statistical analyses, and scientific presenting and writing. For my projects, I also generated a code used to process the motion capture and identify the time points of interest for comparing athletes to each other. To this day, the lab uses this code, and it has sped up the post-processing of data from a few weeks to a few hours.

a. **Gilmer GG**, Gascon SS, Oliver GD. Classification of lumbopelvic-hip complex instability on kinematics amongst female team handball athletes. J Sci Med Sport. 2018 Aug;21(8):805-810. PubMed PMID: 29366828.

b. **Gilmer GG**, Washington JK, Dugas JR, Andrews JR, Oliver GD. The Role of Lumbopelvic-Hip Complex Stability in Softball Throwing Mechanics. J Sport Rehabil. 2019 Feb 1;28(2):196-204. PubMed PMID: 29140180.

c. **Gilmer GG**, Washington JK, Roberts MD, Oliver GD. Preliminary Evaluation of Dynamic Knee Valgus and Serum Relaxin Concentrations After ACL Reconstruction. JB JS Open Access. 2020 Jan-Mar;5(1):e0060. PubMed Central PMCID: PMC7147639.

d. **Gilmer GG**, Roberts MD, Oliver GD. The Relationship between Serum Relaxin Concentrations and Knee Valgus. Int J Sports Med. 2020 Mar;41(3):182-188. PubMed PMID: 31902127.

2. Postbaccalaureate Research.

As a summer student and then a post-bac in Dr. Mark Knepper's lab at the NIH, my research focused on (1) generating a mathematical model of fluid flow along the nephron and (2) elucidating the mechanisms of lithium-induced nephrogenic diabetes insipidus. For my first project, I created a mathematical model of fluid flow in the nephron that showed the highest points of resistance to flow are the thin limb of Henle and cortical collecting duct (a, b). Through the simulations I ran, we demonstrated that Bowman's capsule glomerular pressure is a high enough driving force for fluid flow. Additionally, as a part of our studies aimed at understanding how lithium causes nephrogenic diabetes insipidus, I led microdissection of cortical collecting ducts and isolation of proteins for mass spectrometry proteomics. Alongside a post-doc in the lab, I performed mass spectrometry proteomics to map out changes in protein expression at the single

tubule level. I also quantified the ratio of principal to intercalated cells and generated all figures that were included in the manuscript (c).

a. **Gilmer GG**, Deshpande VG, Chou CL, Knepper M. Flow resistance along the rat renal tubule. Am J Physiol Renal Physiol. 2018 Nov 1;315(5):F1398-F1405. PubMed Central PMCID: PMC6293284.

b. *Poster Presentation:* **Gilmer G**, Deshpande V, Schnermann J, and Knepper M. (2018) Thin Limbs of Henle and Inner Medullary Collecting Ducts are the Major Points of Flow Resistance Along the Renal Tubule. *Experimental Biology*. San Diego, CA.

c. Sung CC, Chen L, Limbutara K, Jung HJ, **Gilmer GG**, Yang CR, Lin SH, Khositseth S, Chou CL, Knepper MA. RNA-Seq and protein mass spectrometry in microdissected kidney tubules reveal signaling processes initiating lithium-induced nephrogenic diabetes insipidus. Kidney Int. 2019 Aug;96(2):363-377. PubMed Central PMCID: PMC6650374.

3. Graduate Research.

As a rotating student in Dr. Ambrosio's lab, I worked closely with Dr. Hirotaka Iijima, a post-doc in the lab, to elucidate the mechanisms of age-related knee osteoarthritis. We started by performing a systematic review, in which I served as a reviewer for articles and assisted significantly with the writing of the manuscript (a). In our own experiments, I suggested and led a mass spectrometry proteomics analysis of cartilage across the lifespan of mice. Additionally, I independently repeated Dr. Iijima's histological experiments in female mice (b). Our work revealed that male mice had more severe knee osteoarthritis than female mice. This finding led me to interrogate this discrepancy between mice and humans more and ultimately, I realized that female mice do not exhibit a menopausal phenotype. I have since then generated a chemically-induced menopause model and both confirmed a menopausal phenotype and that knee osteoarthritis is more severe in the menopause mice than the age-matched non-menopause mice. Additionally, I led my own systematic review on menopause and knee osteoarthritis. With the data generated from this review, I developed a mathematical model of estrogen treatment for cartilage. My simulations revealed that starting estrogen treatment early in menopause leads to more benefits in cartilage quality than starting treatment later (c, d).

a. Iijima H, **Gilmer G**, Wang K, Sivakumar S, Evans C, Matsui Y, Ambrosio F. Meta-analysis Integrated With Multi-omics Data Analysis to Elucidate Pathogenic Mechanisms of Age-Related Knee Osteoarthritis in Mice. J Gerontol A Biol Sci Med Sci. 2022 Jul 5;77(7):1321-1334. PubMed Central PMCID: PMC9255692.

b. Iijima H, **Gilmer G**, Wang K, Bean A, He Y, Lin H, Evans C, Ambrosio F. Age-related increase in matrix stiffness downregulates α-Klotho in chondrocytes and induces cartilage degeneration. [Preprint]. 2021 March 14. DOI: 10.1101/2021.03.13.434679. *Accepted at Nature Communications.*

c. **Gilmer G**, Bean A, Iijima H, Jackson N, Thurston R, Ambrosio F. Uncovering the "riddle of femininity" in osteoarthritis: a systematic review and meta-analysis of menopausal animal models and mathematical modeling of estrogen treatment. *Under revision at Osteoarthritis and Cartilage.*

d. *Poster Presentation:* **Gilmer G**, Jackson N, Bean A, Iijima H, Evans C, Thurston R, Ambrosio F. A mathematical model to evaluate the impact of estrogen treatment timing on cartilage degeneration. *Systems Aging Gordan Research Conference.* Newey, ME, May 2022.

Complete List of Published Work in My Bibliography:
https://www.ncbi.nlm.nih.gov/myncbi/gabrielle.gilmer.1/bibliography/public/

D. Scholastic Performance

Scholastic Performance

YEAR	COURSE TITLE	GRADE	YEAR	COURSE TITLE	GRADE
		AUBURN UNIVERSITY			
2014	Introduction to Computations - MATLAB	A	2016	Directed Reading in Physics: Learning Assistant Pedagogy	A
2014	Special Topics: Self Defense	A	2016	Chemical Engineering Progress Assessment I	P
2014	Freshmen Fit	A	2016	Organic Chemistry II	A
2014	World History I	A	2016	Organic Chemistry II Lab	A
2014	Honors Calculus I	A	2016	Phase and Reaction Equilibrium	A

YEAR	COURSE TITLE	GRADE	YEAR	COURSE TITLE	GRADE
2014	Engineering Physics I	A	2016	Computer Aided Chemical Engineering	A
2014	English Composition I*	S	2016	Biochemistry I	A
2014	Precalculus Trigonometry*	S	2016	Transport II	A
2014	Engineering Orientation	P	2016	Performance Activity: Yoga	A
2014	Honors Freshmen Exploration	P	2016	Honors Lyceum: Research at Auburn	A
2015	English Composition II	A	2017	Chemical Engineering Analysis	A
2015	Introduction to Mechanical Engineering	A	2017	Honors Ethics and Health Sciences	A
2015	World History II	A	2017	Chemical Engineering Separations	A
2015	Wellness	A	2017	Chemical Reaction Engineering	A
2015	Calculus II	A	2017	Chemical Engineering Lab I	A
2015	Honors Physics II	A	2017	Chemical Engineering Progress Assessment II	P
2015	Principles of Chemical Engineering	B	2017	Chemical Engineering Lab II	A
2015	Honors Biology	A	2017	Digital Process Controls	A
2015	Fundamentals Chemistry II	A	2017	Process Simulation, Synthesis, and Optimization	A
2015	Linear Differential Equations	A	2017	Process Economics and Safety	A
2015	Fundamentals of Chemistry II Lab	A	2017	Honors Undergraduate Research	A
2016	Honors Organismal Biology	A	2017	General Microbiology	A
2016	Organic Chemistry I	A	2018	Protein Engineering	A
2016	Organic Chemistry I Lab	A	2018	British Literature	A
2016	Transport I	B	2018	Honors Thesis	A
2016	Thermodynamics	B	2018	Process Design Practice	B
			UAB		
2015	Calculus III	A	2015	Music Appreciation	A
2015	General Chemistry I	A	2015	Introduction to Psychology	A
		UNIVERSITY OF PITTSBURGH – PHD COURSE WORK			
2021	Mathematical Modeling for Chemical Engineers	A	2021	Synthetic Biology	A-
2021	Introduction to Quantum Mechanics	A	2022	Biomedical Informatics Statistics	A
2022	Bioengineering Seminar Series	S			
		UNIVERSITY OF PITTSBURGH – MD COURSE WORK			
2019	Patient, Physician & Society 1	Satisfactory	2019	Foundations of Medicine 1	Satisfactory
2019	Introduction to Patient Care 1	Satisfactory	2019	Evidenced Based Medicine Fundamentals	Satisfactory
2019	Foundations of Medicine 2	Satisfactory	2020	Introduction to Patient Care 2	Satisfactory
2020	Patient, Physician, & Society 2 - Behavioral Medicine	Satisfactory	2020	Foundations of Medicine 3	Satisfactory
2020	Neuroscience/Psychiatry	Satisfactory	2020	Body Fluid Homeostasis	Satisfactory
2020	Introduction to Patient Care 3	Satisfactory	2021	Patient, Physician, & Society 3 - Population Health	Satisfactory
2021	GI/Endocrine/Hematology/Skin-Musculoskeletal/Repro & Dvlp	Satisfactory	2021	Introduction to Patient Care 4	Satisfactory
2021	Integrated Case Studies	Satisfactory	2021	Integrated Case Studies	Satisfactory
2021	Preclerkship Course	Satisfactory	2021	Pediatric Clerkship	High Satisfactory
2022	Longitudinal Clinical Clerkship 1	Honors			

Auburn: Grades are scored on a standard 4.0 system, with A = 4.0 and B = 3.0. Classes marked with P are pass/fail. * = tested out of with AP testing
Pitt MD courses: All preclinical courses (everything listed except the two clerkships) are scored Satisfactory or Unsatisfactory. Clerkship courses are graded as Honors, High Pass, Pass, or Fail.
Pitt Grad Courses: Grades are evaluated on a full scale, with A+ = 97-100, A = 93-97, and A- = 90-93. Courses listed with S were pass/fail.

OMB No. 0925-0001 and 0925-0002 (Rev. 12/2020 Approved Through 02/28/2023)

BIOGRAPHICAL SKETCH

Provide the following information for the Senior/key personnel and other significant contributors.
Follow this format for each person. **DO NOT EXCEED FIVE PAGES.**

NAME: Ambrosio, Fabrisia

eRA COMMONS USER NAME (credential, e.g., agency login): AMBROSIOF

POSITION TITLE: Director of the Atlantic Charter Discovery Center for Musculoskeletal Recovery, Spaulding Research Institute at Harvard Medical School

EDUCATION/TRAINING *(Begin with baccalaureate or other initial professional education, such as nursing, include postdoctoral training and residency training if applicable. Add/delete rows as necessary.)*

INSTITUTION AND LOCATION	DEGREE *(if applicable)*	Completion Date MM/YYYY	FIELD OF STUDY
Mansfield University, Mansfield, PA	BS	05/1996	Biology, minor Chemistry
Laval University, Québec, Canada	MSc	05/1998	Physiology-Endocrinology
Drexel University, Philadelphia, PA	MPT	06/1999	Physical Therapy
University of Pittsburgh, Pittsburgh PA	PhD	05/2005	Rehabilitation Science & Technology

A. Personal Statement

Dr. Ambrosio's research has the long-term goal of developing Regenerative Rehabilitation approaches to enhance skeletal muscle function with increasing age and in the setting of disease. Her laboratory uses murine and human models to investigate the underlying mechanisms by which targeted and specific mechanotransductive signals can be used to enhance donor and/or host stem cell functionality. Dr. Ambrosio's research has been supported by the NIH, the DOD, the Foundation for Physical Therapy, the Claude D.Pepper Older American's Independence Center, and the University of Pittsburgh Institute on Aging, among others. In 2017 and 2018, Dr. Ambrosio was the highest NIH-funded investigator of all Physical Medicine & Rehabilitation departments in the United States. She is currently the 2nd highest funded PM&R investigator, with the bulk of her grants awarded by the National Institute on Aging. Dr. Ambrosio also co-directs the NIH-funded Alliance for Regenerative Research & Training (AR3T), and she is the Founding Director of the International Consortium for Regenerative Rehabilitation, which includes 16 participating institutions representing North America, Europe, and Asia.

Zhang C; Ferrari RJ; Beezhold K; Stearns-Reider K; D'Amore A; Haschak M; Stolz D; Robbins PD; Barchowsky A; **Ambrosio F***. Arsenic promotes NF-kB-mediated fibroblast dysfunction and matrix remodeling to impair muscle stem cell function. *Stem Cells,* 2016 Mar 34(3):732-42 PMID: 26537186; PMCID: PMC4817845. ***NIEHS NIH Top Paper of 2016 (top 25 of ~2700 papers from NIEHS-funded research)***

Stearns-Reider K, D'Amore A; Beezhold K; Rothrauff B; Cavalli L; Wagner W; … Zhang C; Barchowsky A; Rando T; Tuan R; **Ambrosio F***. Aging of the skeletal muscle extracellular matrix drives a stem cell fibrogenic conversion. *Aging Cell,* 2017, Jun 16(3):518-528. ***Aging Cell's 'Best Paper of the Year'.***

Sahu A; Mamiya H; Shinde SN; Cheikhi A; Winter LL…St. Croix C; Sanders LH; Van Houten B; Barchowsky A; **Ambrosio F*.** Age-related declines in α-Klotho drive dysfunctional muscle progenitor cell bioenergetics and impaired skeletal muscle regeneration. *Nature Communications,* 2018, 19;9(1):4859.

Sahu A; Fitz N; Shinde SN; Clemens ZJ; Pius A…Lefterov I; Barchowsky A; Koldamova R; **Ambrosio F***. Regulation of cell and tissue aging by circulating extracellular vesicles, *Nature Aging,* 2021, 12/06

B. Positions, Scientific Appointments, and Honors

2001-2003 Research Assistant, Human Engineering Research Laboratories, Department of Rehabilitation Science and Technology, University of Pittsburgh, Pittsburgh, PA

2003-2005 National Science Foundation IGERT Fellow, Department of Rehabilitation Science and Technology, University of Pittsburgh, Pittsburgh, PA

2005-2006 Visiting Instructor, Stem Cell Research Center, University of Pittsburgh, Pittsburgh, PA

2006-2015 Assistant Professor, Department of Physical Medicine & Rehabilitation, University of Pittsburgh, Pittsburgh, PA

2007-2015 Assistant Professor, Department of Physical Therapy, secondary appointment, University of Pittsburgh, Pittsburgh, PA

2007-2015 Assistant Professor, Department of Orthopaedic Surgery, secondary appointment, University of Pittsburgh, Pittsburgh, PA

2009- 2022 Assistant Professor, Department of Microbiology & Molecular Genetics, secondary appointment, University of Pittsburgh, Pittsburgh, PA

2015- 2022 Associate Professor, Department of Physical Medicine & Rehabilitation, University of Pittsburgh, Pittsburgh, PA

2015-2022 Associate Professor, Department of Bioengineering, University of Pittsburgh, Pittsburgh, PA

2015-2022 Associate Professor, Department of Physical Therapy, secondary appointment, University of Pittsburgh, Pittsburgh, PA

2015-2022 Associate Professor, Department of Orthopaedic Surgery, secondary appointment, University of Pittsburgh, Pittsburgh, PA

2016-2022 Director of Rehabilitation, UPMC International

2017-2022 Associate Professor, Department of Environmental and Occupational Health, secondary appointment, University of Pittsburgh, Pittsburgh, PA

2022 - Director of the Discovery Center for Musculoskeletal Recovery, Schoen Adams Research Institute at Spaulding, Boston, MA

2022 - Associate Professor, Department of Physical Medicine & Rehabilitation, Harvard Medical School, Boston, MA

C. Contributions to Science

1. **Skeletal muscle physiology and function:** Beginning with my Master of Science at Laval University I have maintained an interest in functional skeletal contractile muscle testing in murine models using both *in vitro* and *in situ* systems. Most recently, I have used these systems to evaluate muscle strength, fatigue resistance, and recovery from a fatiguing protocol following stem cell transplantation into models of muscle injury and disease. We have also implemented these systems in other models of muscle pathology, such as following exposure to the environmental contaminant, arsenic. Our work has investigated the mechanosensitivity of muscle stem cells, as well as the impact of circulating longevity factors of skeletal muscle regenerative capacity.
    a. Stearns-Reider K, D'Amore A; Beezhold K; Rothrauff B; Cavalli L; Wagner W; Vorp D; Tsamis A; Shinde S; Zhang C; Barchowsky A; Rando T; Tuan R; **Ambrosio F**. Aging of the skeletal muscle extracellular matrix drives a stem cell fibrogenic conversion. *Aging Cell,* Jun 16(3):518-528. PMID: 28371268; PMCID: PMC5418187.
    b. Sahu A; Mamiya H; Shinde SN; Cheikhi A; Winter LL; Vo NV; Stolz D; Roginskaya V; Tang WY; St Croix C; Sanders LH; Van Houten B; Barchowsky A; **Ambrosio F*.** Age-related declines in α-Klotho drive dysfunctional muscle progenitor cell bioenergetics and impaired skeletal muscle regeneration. *Nature Communications,* 2018, 19;9(1):4859. PMID: 30451844
    c. Cheikhi A; Barchowsky A; Sahu A; Shinde SN; Pius A; Clemens ZJ; Li H; Kennedy CA; Hoeck JD; Franti M; **Ambrosio F***. Klotho: An elephant in aging research. *J Gerontol A Biol Sci Med Sci,* 2019,18;74(7):1031-1042. PMID: 308472365

d. Clemens Z; Sivakumar S; Pius A; Sahu A; Shinde S; Mamiya H; Luketich N; Cui J; Dixit P; Hoeck J; Kreuz S; Franti M; Barchowsky A; Ambrosio F. The biphasic and age-dependent impact of Klotho on hallmarks of aging and skeletal muscle function. *eLife,* April 20. 61138. PMID: 33876724

2. **Cellular and tissue engineering therapeutics to improve skeletal muscle healing after injury and with disease**: The host microenvironment, or niche, plays an essential role in determining transplanted cell fate, highlighting the importance of developing synergistic approaches to optimize the niche and maximize the efficacy of cellular therapies. We have used pharmacologic, genetic, and mechanical strategies as a means to modulate the skeletal muscle microenvironment and enhance the regenerative response of injured or diseased skeletal muscle.
    a. Deasy BM, Feduska J, **Ambrosio F**, Huard J. Role of VEGF-mediated angiogenesis in muscle stem cell transplantation to Dystrophic Muscle. *Molecular Therapy.* 2009. 17(10): 1788-1798; PMID: 19603004; PMCID: PMC2835014.
    b. **Ambrosio F**; Ferrari RJ; Plassmeyer J; Fitzgerald GK; Carvell GE; Distefano G; Boninger ML; Huard J. The synergistic effect of treadmill running and stem cell transplantation to heal injured skeletal muscle. *Tissue Eng Part A*. 2010. 16: 839-849. PMID: 19788347; PMCID: PMC3602431.
    c. Han N; Yabroudi MA; Stearns-Reider K; Helkowski W; Sicari BM; Rubin JP; Badylak SF; Boninger ML; **Ambrosio F**. Electrodiagnostic evaluation of individuals implanted with extracellular matrix for the treatment of volumetric muscle injury. *Physical Therapy Journal,* April 96(4):540-9. PMID: 26564252; PMCID: PMC4817212.
    d. Sicari BM; Rubin JP; Dearth CL; Wolf MT; **Ambrosio F**; Boninger M: Turner NJ; Weber DJ; Simpson TW; Wyse A; Brown EP; Dziki JL; Fisher LE; Brown S; Badylak SF. An acellular biologic scaffold promotes skeletal muscle formation in mice and humans with volumetric muscle loss. *Science Translational Medicine,* 2014, 30:6(234):234ra58. PMID: 24786326; PMCID: PMC5942588.

3. **Mechanisms for pathogenic skeletal muscle dysfunction and impaired regenerative response with environmental exposure:** Arsenic is an odorless and tasteless semi-metal that contaminates drinking water supplies from natural deposits in the earth. It is estimated that over 140 million individuals worldwide are exposed regularly to drinking water that exceed government arsenic standards. Our work addresses key questions regarding the health impacts of environmental exposures remain, including the underlying mechanisms by which exposures negatively impact the stem cell phenotype and, ultimately, skeletal muscle maintenance and healing capacity. An improved understanding of how such exposures contribute to the complexity of skeletal muscle health is an important first step toward developing targeted and specific interventions aimed at preventing or delaying skeletal muscle declines and, ultimately, health and quality of life for the millions of arsenic-exposed individuals worldwide.
    a. Garciafigueroa DY, Klei LR, **Ambrosio F**, Barchowsky A. Arsenic-stimulated lipolysis and adipose remodeling is mediated by G-protein-coupled receptors. *Toxicol Sci*. 2013 Aug;134(2):335-44. PMID: 23650128; PMCID: PMC3707436.
    b. **Ambrosio F***, Brown E, Stolz D, Ferrari R, Goodpaster B, Deasy B, Distefano G, Roperti A, Cheikhi A, Garciafigueroa Y, Barchowsky A. Arsenic induces sustained impairment of skeletal muscle and muscle progenitor cell ultrastructure and bioenergetics. *Free Radic Biol Med*. 2014 Sep;74:64-73. PMID: 24960579; PMCID: PMC4159748.
    c. Zhang C; Ferrari RJ; Beezhold K; Stearns-Reider K; D'Amore A; Haschak M; Stolz D; Robbins PD; Barchowsky A; **Ambrosio F***. Arsenic promotes NF-kB-mediated fibroblast dysfunction and matrix remodeling to impair muscle stem cell function. *Stem Cells,* 2016 Mar 34(3):732-42. PMID: 26537186; PMCID: PMC4817845.
    d. Cheikhi A; Wallace C; St Croix C; Cohen C; Tang W; Wipf P; **Ambrosio F***; Barchowsky A*. Mitochondria are a substrate of cellular memory. *Free Radical Medicine & Biology,* 2019. Jan.; 130: 528-541

4. **Regenerative Rehabilitation:** In 2010, our group first proposed a charge to the fields of regenerative medicine and rehabilitation, where we highlighted the need for an increased communication and interaction between the two fields so as to maximize the efficiency and efficacy of cellular and tissue engineering-based technologies. Since this time, the integration of rehabilitation protocols into the application of tissue

engineering technologies has been progressively appreciated, nationally and internationally, in large part because of our work.

a. Avin KG; Coen PM; Huang W; Stolz D; Sowa GA; Dube JJ; Goodpaster B; O'Doherty R; **Ambrosio F***. Skeletal muscle as a regulator of the longevity protein, klotho. *Frontiers in Physiology,* 2014, 17;5: 189. PMID: 24987372; PMCID: PMC4060456.
b. Gentile NE; Stearns KM; Brown EP, Rubin JP; Boninger ML; Dearth CL; **Ambrosio F**; Badylak SF. Targeted rehabilitation following extracellular matrix scaffold transplantation in patients with volumetric muscle loss. *American Journal of Physical Medicine & Rehabilitation,* 2014, 93(11 Suppl 3):S79-87. PMID: 25133624.
c. Dalise S; **Ambrosio F**; Modo M. Brain plasticity and recovery I preclinical models of stroke. Arch Ital Biol. 2015, 152(4):190-215.; PMID: 25987181
d. Rando TA* & **Ambrosio F***. Regenerative Rehabilitation: Applied biophysics meets stem cell therapeutics. *Cell Stem Cell,* 2018, 1;22(3): 306-309. PMID: 29499150; PMCID: PMC5931336.

5. **Biomechanics and Assistive Technology.** During my doctoral training, I conducted clinical research evaluating skeletal muscle function in relation to mobility in individuals with spinal cord injury and multiple sclerosis. This work was recognized in the form of three 'Best Paper' awards.

a. Souza AL, Boninger ML, Fitzgerald SG, Shimada SD, Cooper RA, **Ambrosio F**. Upper Limb Strength in individuals with spinal cord injury who use manual wheelchairs. 2005. *Journal of Spinal Cord Medicine.* 28(1):26-32. PMID: 15832901.
b. **Ambrosio, F**; Boninger, ML; Souza, AL; Fitzgerald, SG; Koontz, AM; Cooper, RA. Biomechanics and strength of manual wheelchair users. 2005. *Journal of Spinal Cord Medicine.* 28: 407-414; PMID: 16869087; PMCID: PMC1808266.
c. **Ambrosio F,** Boninger ML, Fitzgerald SG, Schwid SR, Cooper RA. A comparison of mobility device delivery within the Veterans Administration for individuals with multiple sclerosis versus individuals with a spinal cord injury. *Journal of Rehabilitation Research and Development.* 2007. 44(5): 693-702. PMID: 15832901.
d. Lai S; Panarese A; Lawrence R^{++}; Boninger ML; Micera S; **Ambrosio F**. A murine model of robotic training to evaluate skeletal muscle recovery after injury. *Medicine and Science in Sports and Exercise.* 2017. Apr 49(4):840-847. PMID: 27875498.

PHS Fellowship Supplemental Form

OMB Number: 0925-0001
Expiration Date: 09/30/2024

Introduction

1. Introduction to Application
(for Resubmission applications)

Fellowship Applicant Section

2. Applicant's Background and Goals for Fellowship Training*	gilmer_ApplicantBackGroundGoalsForFellowship_final.pdf

Research Training Plan Section

3. Specific Aims*	gilmerSpecificAims_final.pdf
4. Research Strategy*	gilmerResearchStrategy_final.pdf
5. Respective Contributions*	gilmerRespectiveContributions_Final.pdf
6. Selection of Sponsor and Institution*	gilmerSelectionOfSponsor_Final.pdf
7. Progress Report Publication List (for Renewal applications)	
8. Training in the Responsible Conduct of Research*	gilmerTrainingInResponsibleConductOfResearch_Final.pdf

Sponsor(s), Collaborator(s) and Consultant(s) Section

9. Sponsor and Co-Sponsor Statements	gilmerAmbrosio_SponsorStatement_final.pdf
10. Letters of Support from Collaborators, Contributors and Consultants	LettersOfSupport_gilmer_final.pdf

Institutional Environment and Commitment to Training Section

11. Description of Institutional Environment and Commitment to Training	gilmer_InstitutionalEnviornment_final.pdf
12. Description of Candidate's Contribution to Program Goals	

Other Research Training Plan Section

Vertebrate Animals

The following item is taken from the Research & Related Other Project Information form and repeated here for your reference. Any change to this item must be made on the Research & Related Other Project Information form.

Are Vertebrate Animals Used? ☑ Yes ☐ No

13. Are vertebrate animals euthanized?	☑ Yes	☐ No
If "Yes" to euthanasia Is method consistent with American Veterinary Medical Association (AVMA) guidelines?	☑ Yes	☐ No
If "No" to AVMA guidelines, describe method and provide scientific justification		
14. Vertebrate Animals	gilmerVertebrateAnimals_final.pdf	

PHS Fellowship Supplemental Form

Other Research Training Plan Information

15. Select Agent Research

16. Resource Sharing Plan: gilmerResourceSharingPlan_final.pdf

17. Authentication of Key Biological and/or Chemical Resources: gilmerAuthenticationBioChem_final.pdf

Additional Information Section

18. Human Embryonic Stem Cells

Does the proposed project involve human embryonic stem cells?* ☐ Yes ☑ No

If the proposed project involves human embryonic stem cells, list below the registration number of the specific cell line(s), using the registry information provided within the agency instructions. Or, if a specific stem cell line cannot be referenced at this time, please check the box indicating that one from the registry will be used:

☐ Specific stem cell line cannot be referenced at this time. One from the registry will be used.

Cell Line(s):

19. Alternate Phone Number:

20. Degree Sought During Proposed Award:

Degree:	If "other", indicate degree type:	Expected Completion Date (MM/YYYY):
OTH: Other	MD/PhD	05/2027

21. Field of Training for Current Proposal*: 198 Biology/Biomedical Sciences, General

22. Current or Prior Kirschstein-NRSA Support?* ☐ Yes ☑ No

If yes, identify current and prior Kirschstein-NRSA support below:

Level*	Type*	Start Date (if known)	End Date (if known)	Grant Number (if known)

23. Applications for Concurrent Support?* ☐ Yes ☑ No

If yes, describe in an attached file:

24. Citizenship*

U.S. Citizen — U.S. Citizen or Non-Citizen National? ☑ Yes ☐ No

Non-U.S. Citizen
☐ With a Permanent U.S. Resident Visa
☐ With a Temporary U.S. Visa

If you are a non-U.S. citizen with a temporary visa applying for an award that requires permanent residency status, and expect to be granted a permanent resident visa by the start date of the award, check here: ☐

25. ☐ Change of Sponsoring Institution — Name of Former Institution:*

PHS Fellowship Supplemental Form

Budget Section

All Fellowship Applicants:

26. Tuition and Fees*:

☐ None Requested ☑ Funds Requested

Year 1	$51,752.00
Year 2	$51,752.00
Year 3	$51,752.00
Year 4	$51,752.00
Year 5	
Year 6 (when applicable)	
Total Funds Requested:	$207,008.00

27. Childcare Costs*:

☑ None Requested ☐ Funds Requested

Year 1	
Year 2	
Year 3	
Year 4	
Year 5	
Year 6 (when applicable)	
Total Funds Requested:	$0.00

Senior Fellowship Applicants Only:

	Amount	Academic Period	Number of Months
28. Present Institutional Base Salary:			

29. Stipends/Salary During First Year of Proposed Fellowship:

a. Federal Stipend Requested:	Amount	Number of Months
b. Supplementation from Other Sources:	Amount	Number of Months
	Type (e.g.,sabbatical leave, salary)	
	Source	

Appendix

30. Appendix

GOALS FOR FELLOWSHIP TRAINING AND CAREER

If you asked 17-year-old me what I was going to be when I grew up, I would have answered a professional soccer player. However, three days after signing a full athletic scholarship and contract for the Olympic Development Team, I tore my anterior cruciate ligament (ACL) for the third time, an injury that plagues one out of ten female athletes. As I have moved through my early medical and research training, I have come across many other joint diseases that disproportionately affect women, including knee osteoarthritis, femoroacetabular impingement syndrome, and rotator cuff tears.

With this new perspective in mind, **my long-term research goals are**:

(1) to understand how sex and gender affect joint functionality from the molecular to whole organism level and understand how this functionality is disrupted with disease and injury
(2) to use this understanding to design creative and accessible clinical solutions for the treatment of joint diseases and injuries in women
(3) to use these clinical interventions to empower women to get back to the movements they love.

In envisioning a framework for my goals, I intend to merge (1) Biomechanics and Rehabilitation, (2) Physiology Informed Tissue Engineering, and (3) Clinical Interventions. As a physician-scientist, **my long-term career goal** is to independently lead a Women's Movement Center with an NIH-funded, multi-disciplinary lab and a multi-disciplinary clinic that merges these three arenas to holistically interrogate and treat joint diseases and injuries in women. Thus, my goals in training are to gain expertise in each of these fields.

Biomechanics and Rehabilitation. During my undergraduate, I worked in the Sports Medicine and Movement Lab at Auburn University, where I studied the mechanics of how athletes move. Specifically, I led two projects: the first examined how lumbopelvic-hip complex stability affects mechanics in female throwing athletes, and my second project focused on linking sex-hormones to biomechanical predictors of ACL injury. Pathomechanical movement patterns are a fundamental aspect of joint disease, and the expertise I built during this time will be paramount to designing rehabilitation protocols that couple well with novel therapeutic modalities. The culmination of my undergraduate work resulted in twenty publications, eight of which are first authored.

Physiology Informed Tissue Engineering*.** During my PhD under the mentorship of Dr. Fabrisia Ambrosio, my focus is on expanding my expertise to include regenerative rehabilitation strategies for the treatment of joint diseases. Since the Ambrosio laboratory has recently moved to Spaulding Rehabilitation Center at Harvard Medical School, I will complete my PhD training in Boston while remaining a full-time student at the University of Pittsburgh. As such, I have designed a training plan to utilize resources in both Boston and Pittsburgh. Specifically, I will build competency in interrogating molecular mechanisms of menopause-induced knee osteoarthritis by completing ***Specific Aim 1 under the guidance of Dr. Ambrosio. I will also build proficiency in designing and constructing genetic controls circuits in mammalian cells by completing ***Specific Aim 2*** with intensive complementary mentorship from Dr. Pamela Silver. My training in the Cellular and Molecular Pathology (CMP) graduate program at Pitt coupled with my chemical engineering background will combine to facilitate engineering design that is informed by fundamental biology. My PhD curriculum will be complemented with weekly seminars focused on aging, orthopedics, women's health, and engineering principles (see Seminar Attendance Plan). I will continue to hone and improve on my oral communication skills by presenting monthly at lab meetings, journal clubs, and mentor meetings, semesterly in Dr. Silver's lab, and annually at local seminars and retreats, as well as conferences focused on osteoarthritis, menopause, and regeneration principles (see Attendance and Presentations at Scientific Meetings). My scientific writing skills will be further developed by writing abstracts for each of the outlined conferences, publication of one first authored manuscript per year based off my Preliminary Data and ***Specific Aims 1 and 2***, 2-4 co-authored publications per year, and completion of my thesis during graduate school.

***Clinical Interventions*.** I have begun my clinical development via completion of one clinical clerkship in Pediatrics and one Longitudinal Clinical Clerkship (LCC) in orthopedic surgery. Since Dr. Ambrosio and our lab have moved from the University of Pittsburgh to Harvard Medical School, I will complete the remainder of my clinical training at both institutions. Specifically, while in Boston for the remainder of my graduate training, I will shadow Dr. Miho Tanaka, an orthopedic surgeon who is the Director of the Women's Sports Medicine Program at Massachusetts General Hospital (MGH). I will travel back to Pittsburgh during graduate school to complete

my second LCC under the guidance of Dr. John Fowler, a successful orthopedic surgeon who rigorously applies and expands evidence-based practices. When in Pittsburgh for my LCC, I will also continue to volunteer at the Women's Clinic and Shelter of Greater Pittsburgh. To continue my connection to the clinical world from a research perspective, I have been and will continue to collaborate with Dr. Gwendolyn Sowa, with whom I completed one of my research rotations and who now serves as my career advisor. I have elaborated on the work I completed in her lab by designing a survey study based on our findings from our retrospective chart review to examine perceptions of telemedicine in physical medicine and rehabilitation.

After completion of my graduate studies in Boston, I will return to Pittsburgh to finish my third and fourth years of medical school. The culmination of medical training offered during my third and fourth years of medical school at PittMed, LCCs through my MSTP, and my clinical research experience will ensure I develop the rigorous clinical skills I need to succeed in a research-based orthopedic surgery residency tract.

With support from this fellowship training, my mentors and I will have the tools necessary to facilitate my career development as a surgeon-scientist in regenerative rehabilitation, and I will have the tools necessary to springboard towards innovative solutions for treating joint injuries in women.

ACTIVITIES PLANNED UNDER THIS AWARD

Fellowship Years 1-2 (Graduate School Years 3-4):

Research: During the first two years of my fellowship, I will focus on the research plan and experiments outlined in my ***Specific Aims*** and **Research Strategy** (***Figure 1***). By completing ***Specific Aim 1***, I will investigate mechanisms of menopause-driven knee osteoarthritis. My training will focus on expanding my experience with animal procedures, immunohistochemistry/immunofluorescence, and cell culture as well as providing me the opportunity to develop new skills in microscopic imaging, including live cell imaging. With execution of ***Specific Aim 2***, I will train in the application of synthetic biology techniques to mammalian cells. This will entail extensive training in promoter design and generation, cellular transfection, and stable genetic engineering. Both of my aims will afford me the opportunity to generate hypotheses, design experiments to test said hypotheses in a rigorous manner, and communicate my scientific findings, both orally and via writing.

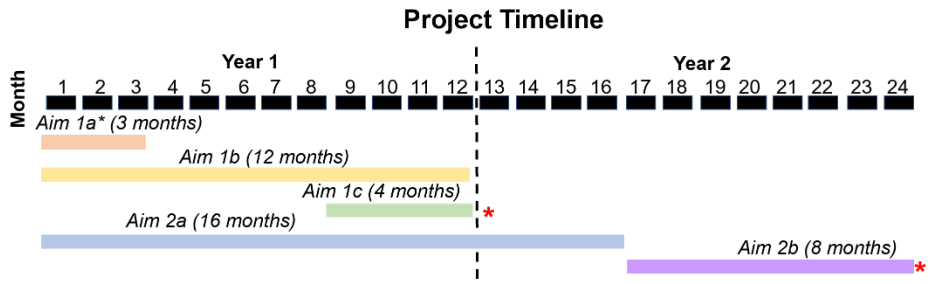

Figure 1*: Project timeline * = samples already collected. * = planned publication*

Mentoring: To complete this research training, I will continue to have weekly meetings with my primary mentor, Dr. Ambrosio. In addition, I will hold monthly meetings with my mentoring team (Drs. Ambrosio, Christopher Evans, Rebecca Thurston, Pamela Silver), and semesterly meetings with my career advisor (Dr. Sowa) and thesis committee. Mentor meetings will be focused on discussing my experimental designs, troubleshooting technical issues, and big picture feedback on manuscripts. Detailed feedback on manuscript drafts will be provided by my mentoring team via email. All mentors are available for more frequent communication via email or scheduled one-on-one meetings.

In addition to my formal mentoring team, I will also receive mentoring and support from members within the Ambrosio laboratory. Dr. Hirotaka Iijima is a former post-doctoral fellow in the Ambrosio lab who trained me in performing knee histology as well as culturing chondrocytes. He is available via email or scheduled meetings to troubleshoot any issues related to preparing knee sections or culturing chondrocytes. Within the physical lab, Dr. Kai Wang is a scientist with expertise in cellular transfections and genetic engineering and is available to help with training and troubleshooting in these related areas. Dr. Zachary Hettinger, a post-doctoral fellow in the lab, has extensive experience in microscopic imaging and quantification, and is also available to help with imaging and microscopy related issues. Our lab manager, Dr. Ekaterina Creed, is a veterinarian by training, has extensive experience with animal procedures and work, and is available to troubleshoot any animal related

procedures. We also have two lab technicians to assist with executing experiments and analyses. At our lab meetings, I will continue to regularly present progress reports on a monthly basis to receive peer feedback. For more specific synthetic biology techniques, I will train in Dr. Silver's lab under the mentorship of her senior lab members, as well as present at her lab meetings on a semesterly basis.

Clinical: I have already completed one longitudinal clinical clerkship (LCC) under the mentorship of Dr. Volker Musahl, who is a successful orthopedic surgeon-scientist. To continue my clinical development during graduate school, I will spend one day per month in the operating room with Dr. Tanaka during my third and fourth years of graduate school. I will complete my second LCC with Dr. Fowler by traveling back to Pittsburgh and working with him in the clinic and operating room for two weeks at the beginning of my third year of graduate school. I will also continue working with Dr. Sowa on examining the impact of telemedicine in physiatry clinics. These clinical experiences will allow me to maintain and expand my clinical skills throughout graduate school and will also provide further opportunity to integrate my research and clinical interests.

Courses: During my first and second years of graduate school as a student in CMP at Pitt, I completed course work covering synthetic biology principles, mathematical modeling, quantum mechanics, bioinformatic statistical analyses, and molecular mechanisms of tissue growth and differentiation. During my fellowship period, I will continue participating in CMP's weekly pathology journal club and our own Ambrosio lab journal club and attend the seminars outlined below. In Pitt's MSTP, I have completed all the required summer courses, including ethics, and will continue to attend our monthly workshop series. Additionally, with the Ambrosio lab move to Spaulding, I am connected with the Harvard MSTP and will continue to take their courses as supplements, including Clinical Problem Solving Cases.

Enrichment/Service: At PittMed, I founded our American Physician Scientist Association (APSA) chapter and served as President for three years (2019-2022). I will continue my involvement with APSA as an active member of the advocacy committee. I have also served as a volunteer at the Women's Center & Shelter of Greater Pittsburgh during early medical and graduate school, and I will continue to do so while in Pittsburgh for my LCC, I have joined the Martino Center Women in Science (WiS) group at Massachusetts General Hospital (MGH) and will work specifically with the policy sub-committee to generate trainings on how scientists can help lead evidence-based policy development. To enhance my synthetic biology training, I am involved with the "Synthetic Biology Hive" at Harvard Medical School and will continue attending biweekly discussion groups and tea times. Lastly, mentoring is an important skillset for my development as a future leader in orthopedics and rehabilitation. I am currently mentoring one technician, one undergraduate student, and one high school student in the lab and will continue to do so throughout my fellowship.

Seminar Attendance Plan: At the University of Pittsburgh (all weekly and virtual): Orthopedic Surgery Department Grand Rounds, Women's Health Seminar, Pepper Seminar Research Series, Pathology Seminar

At MGH (all biweekly and in person): Harvard Medical School Genome Engineering Series, Massachusetts Institute for Technology Bioinformatics Series

Attendance and Presentations at Scientific Meetings:

International Conferences: Osteoarthritis Research Society International (OARSI) World Congress on Osteoarthritis (yearly); Synthetic Biology Gordan Research Center (GRC) (biennially); Alliance on Regeneration Rehabilitation Research and Training (AR^3T) Regenerative Rehabilitation Symposium (yearly)

National Conferences: Synthetic Biology: Engineering, Evolution & Design (SEED) (biennially); North American Menopause Society Annual Meeting (biennially)

Local, Pittsburgh: All Yearly - Women's Health Seminar; Pitt MSTP Retreat; Pitt CMP Retreat; Pitt Biomedical Graduate Student Association (BGSA) Symposium; Pepper Seminar Research Series

Local, Boston: Martino Center WiS Symposium (yearly); Celebration of Synthetic Biology (yearly); Synthetic Biology Hive Discussion Groups (semesterly)

Mental Health and Wellbeing: While training as a professional athlete, one thing we discussed frequently was maintainability of our training. Given the enormous rates of burnout among physician-scientists, these same principles are necessary for my success, and I will be proactive in my mental care and wellbeing as a part of my training. As such, I will practice self-care by sleeping at least 7 hours every night, practicing yoga 5 times per

week, journaling and reading daily, going on a weeklong backpacking trip annually, and spending at least one hour outside daily, regardless of weather.

Fellowship Years 4-5 (Medical School Years 3-4):

Clinical: My third and fourth years of medical school will be focused on completing the core clinical clerkships through a variety of medical and surgical specialties at the University of Pittsburgh. My fourth year will focus on completing clinical electives through the Department of Orthopedic Surgery and away rotations.

Research: I will have three research elective months throughout the third and fourth years of medical school. My research months will focus on completing and submitting any manuscripts from my aforementioned graduate work and presenting the remainder of my thesis work at conferences.

Enrichment/Service: I will continue to attend monthly Pitt MSTP workshops and be an active member of our APSA chapter at PittMed.

Mental Health and Wellbeing: I will continue to practice the self-care plan outlined above.

DOCTORAL DISSERTATION AND OTHER RESEARCH EXPERINCE

***Undergraduate Research Fellow*,** Auburn University (Auburn, AL) 11/2015 to 05/2018

Advisor: Gretchen Oliver, Ph.D., Professor in Kinesiology Auburn University

Project Title 1: The role of lumbopelvic-hip complex stability in throwing mechanics in female athletes

- **Project Focus:** During my undergraduate training, one of my projects examined how energy transfer from the lumbopelvic hip complex (LPHC) correlates to measurable pathomechanics in the upper extremity during softball and handball throwing in female athletes.
- **My Contributions:** My role in this project was to design the study, recruit participants, lead motion capture and analysis, perform appropriate statistical analyses, and document our results through manuscript writing and scientific presentation. For my projects, I also generated a code used to process the motion capture and identify the time points of interest for comparing athletes to each other. This code is still used in the lab and has sped up post-processing of data from a few weeks to a few hours.
- **Deliverables:** Seven peer-reviewed publications, four of which are first authored. Five oral presentations and eight poster presentations at local, regional, and national conferences.

Project Title 2: The relationship between serum relaxin concentrations and biomechanical predictors for anterior cruciate ligament (ACL) injury

- **Project Focus:** My honors thesis revealed serum relaxin correlations are positively correlated with commonly used biomechanical metrics to determine risk for injury (e.g., knee valgus). Completion of this study highlighted the importance of mechanistic studies linking the interaction of sex hormones and mechanical properties of joint tissues.
- **My Contributions:** Within this project, I led the design of the study, participant recruitment, motion capture and analysis, statistical analyses, and documentation of findings via initially drafting manuscripts. In addition, I also began to gain wet laboratory skills by performing phlebotomy and ELISAs for hormone quantification.
- **Deliverables:** Three first authored peer-reviewed publications and three poster presentations at local, regional, and national conferences.

***National Heart, Lung, and Blood Institute Summer Intramural Research Training Award*,** NIH NHLBI (Bethesda, MD) 05/2017 to 08/2017

Advisor: Mark Knepper, M.D., Ph.D., Senior Investigator of Epithelial Systems Biology Laboratory

Title: Flow resistance along the rat renal tubule

- **Project Focus:** In classical mechanics, it is well-established that fluid flow is driven by a pressure gradient, and the rate of fluid movement is determined by physical characteristics of the tube (e.g., shape,

diameter) and the fluid (e.g., density, viscosity). During my first summer at the NIH, I created a mathematical model of fluid flow in the nephron based on the aforementioned parameters. We showed that the highest points of resistance to flow are the thin limb of Henle, due to the small diameter, and cortical collecting duct, due to the increased volume accumulating within each tube. Based on our models, we demonstrated that Bowman's capsule glomerular pressure is indeed a high enough driving force for fluid flow.

- **My Contributions:** I tabulated and compiled the dimensions of the different segments of the nephron in different species from previous studies, along with a fellow summer student. I used this data in our models to simulate the Hagen–Poiseuille equation and predict the pressure gradient at each point along the nephron. I then designed the figures and helped write and edit the associated manuscript resulting from this study.
- **Deliverables:** One co-first authored peer-reviewed publication in *Am J Physiol Renal Physiol* and one co-first authored poster presentation at *Experimental Biology*

***Postbaccalaureate Intramural Research Training Award Fellowship*,** NIH NHLBI (Bethesda, MD) 05/2018 to 02/2019

Advisor: Mark Knepper, M.D., Ph.D., Senior Investigator of Epithelial Systems Biology Laboratory

Title: RNA-Seq and protein mass spectrometry in microdissected kidney tubules reveal signaling processes initiating lithium-induced nephrogenic diabetes insipidus

- **Project Focus:** This project begins to elucidate the mechanism by which lithium, a treatment for bipolar disorder, alters water balance in the kidney. We characterized transcript-level, proteome-level, and morphological changes observed shortly after administration of lithium. Morphologically, we found that the number of principal cells increased, while the number of intercalated cells decreased. Combining our transcript and proteomic analyses, we found that lithium increases ERK activation, NF-κB signaling, and an inflammatory-like response that ultimately represses aquaporin-2 transcription and interferes with water balance.
- **My Contributions:** I led the collection of cortical collecting ducts via microdissection and isolation of proteins for mass spectrometry proteomics. Under the mentorship of a post-doc in the lab, I performed proteomic analyses to map out changes in protein expression at the single tubule level. I also quantified the ratio of principal to intercalated cells and generated all figures that were included in the manuscript.
- **Deliverables:** One co-authored peer reviewed publication in *Kidney International*

***MSTP Summer Rotation*,** University of Pittsburgh (Pittsburgh, PA) 06/2020 to 07/2020

Advisor: Gwendolyn Sowa, M.D., Ph.D., Co-Director of the Ferguson Laboratory for Orthopaedic and Spine Research and Chair of the Department of Physical Medicine and Rehabilitation

Title: A Retrospective Analysis of Clinical Utilization between Patients who used Telemedicine and Office Visits in Physical Medicine & Rehabilitation Clinics during the COVID-19 Pandemic.

- **Project Focus:** The COVID-19 pandemic presented a unique set of circumstances and large cohorts of patients were switched over to telemedicine. We used this as an opportunity to gain insight into who used telemedicine and how telemedicine affected clinical utilization within physiatry. We found that proportionally more patients of color, older patients, and patients with more comorbidities used telemedicine than in-person visits. We also found that clinical utilization was not different between patients who used telemedicine and those who were seen in person.
- **My Contributions:** I generated the questions and primary hypotheses for this study based on observations Dr. Sowa had in the clinic. I then performed a retrospective chart review and tabulated all data used for the study through the UPMC Clinical Analytics Application. Through consultation with Pitt's Clinical and Translational Science Institute (CTSI), I designed the appropriate statistical tests. I also wrote the manuscript and generated all associated figures.

- **Deliverables:** One oral presentation at *Physiatry '21* and a first authored peer-reviewed publication that I am the corresponding author of in *Am J Phys Med Rehabil*

***MSTP Summer Rotation*,** University of Pittsburgh (Pittsburgh, PA) 05/2020 to 06/2020

Advisor: Fabrisia Ambrosio, Ph.D., Director of Rehabilitation for UPMC International and an Associate Professor in the Department of Physical Medicine & Rehabilitation

Title: Aging and knee osteoarthritis in murine studies

- **Project Focus:** Knee osteoarthritis remains one of the most debilitating diseases in our aging population. We performed a systematic review and implicated AGE-RAGE signaling as a potential driver of age-related knee osteoarthritis that has not been previously studied. In addition, we performed mechanistic studies mapping the trajectory of knee osteoarthritis across the lifespan in male and female mice. We found that extracellular matrix stiffness regulates α-Klotho expression via epigenetic reprogramming in male, but not female, mice.
- **My Contributions:** I served as a reviewer to assess eligibility of articles in our systematic review. For our original research study, I performed histological sectioning and staining of the knees isolated from female mice. I also led a mass spectrometry proteomics study by micro-dissecting the cartilage and performing an associated pathway analysis. For both of these studies, I helped significantly with the writing of our manuscripts and figure generation.
- **Deliverables:** Two second-authored manuscripts (one published at *The Journal of Gerontology* and one accepted at *Nature Communications)* and Trainee Award (T32) under the University of Pittsburgh Integrated Clinical and Geroscience Research Training Program (5T32AG021885-18)

***Trainee under the University of Pittsburgh Integrated Clinical and Geroscience Research Training Program (T32)*,** University of Pittsburgh (Pittsburgh, PA) 07/2021 to Present

Advisor: Fabrisia Ambrosio, Ph.D., Director of Discovery Center for Musculoskeletal Recovery at the Schoen Adams Research Institute at Spaulding, Harvard Medical School

Title: The effects of menopause on knee osteoarthritis

- **Project Focus:** Post-menopausal women are nearly twice as likely to develop knee osteoarthritis than men. Interestingly, in our aforementioned studies, we found that male mice displayed significantly worse knee osteoarthritis than female mice, which is antithetical to observations in humans. We confirmed the aged female mice in our study were non-menopausal, thus we next aimed to investigate how menopause may modulate knee osteoarthritis. To do this, first we performed a systematic review of the literature aimed at understanding menopause and knee osteoarthritis. Meta-analyses revealed that cartilage degeneration is worse in ovariectomized animals compared to age-matched controls. Additionally, mathematical models revealed that estrogen treatment given early in menopause leads to more benefits to cartilage than treatment that is started late in menopause. For our web laboratory experiments, we developed a chemically induced menopause model that displays more severe knee osteoarthritis than controls, and we will use this model in ***Specific Aim 1.***
- **My Contributions:** I generated the primary hypotheses and research questions based on the work outlined from my research rotation. To test the hypothesis that menopause induces knee osteoarthritis, I developed a chemically-induced menopause model based on previous studies. I have since validated that these mice become menopausal, while control mice do not, and confirmed they have worse knee osteoarthritis than age-matched controls. Additionally, for the systematic review, I led the systematic search and review of manuscripts, performed meta-analyses, and designed and performed the mathematical models used in this study. I have also been the primary writer of the associated manuscript and generated the associated figures.
- **Deliverables:** One poster presentation at *Systems Aging Gordan Research Conference* and one first-authored, co-corresponding authored manuscript currently under revision at *Osteoarthritis & Cartilage*

SPECIFIC AIMS

As of 2020, an estimated 654.1 million adults live with knee osteoarthritis (KOA) worldwide. Notably, women who are post-menopausal have higher incidence and severity of KOA compared to men. Despite this disproportionate effect on women, most KOA animal models include only males. In the few studies examining sex-differences, female mice paradoxically develop less severe KOA than males. One reason for this discrepancy may be that rodents spontaneously rejuvenate their ovarian follicles in middle-age and do not demonstrate a menopausal phenotype. Indeed, ovariectomized (OVX) animals have significantly worse KOA compared to sham animals. However, despite OVX being the most common menopause model in KOA studies, the mechanistic insights offered here are restricted by the well-established disconnect in translatability between OVX and natural menopause in humans. To address these limitations, we validated the 4-vinylcyclohexene diepoxide (VCD) model in middle-aged female mice. The VCD model is commonly employed in young mice to study menopause-associated cardiovascular disease and is known to better replicate natural menopause than OVX. We confirmed that middle-aged, VCD-injected mice display more severe KOA than age-matched controls, thereby providing us with a reliable tool for translational mechanistic studies.

In searching for mechanistic insights into KOA, mutations in ubiquitin ligase E3 were recently identified as uniquely associated with OA in women, and previous studies have implicated maladaptive protein turnover as a mechanism of age-related KOA. In other tissues, the ubiquitin system and estradiol-signaling share complex, mutual regulation, though this relationship has not been explored in cartilage. However, based on our modeling work, estradiol treatment improves OVX-driven cartilage degeneration, with timing of initiation moderating efficacy. **Thus, in *Specific Aim 1*, we will employ our VCD model to test the hypothesis that time-dependent, estradiol signaling regulates ubiquitin proteolysis in menopause-induced KOA.**

Correlative to the lack of studies examining menopausal effects on KOA, there are no disease-modifying treatments for menopause-associated KOA. For other menopausal diseases, hormone therapy (HT) is the most effective treatment. However, the use of HT in women 10+ years beyond the onset of menopause is limited due to increased risk of adverse events, including thromboembolism. In cartilage, HT outcomes are inconsistent but appear to be mediated by the timing of treatment initiation. Thus, an alternative is to apply synthetic biology techniques to treat menopause-driven KOA. Synthetic biology as a field aims to engineer new biological systems or redesign existing ones to optimize performance. One tool often employed for therapeutics is the synthetic circuit, which act as a biological controls system. Feedback loops between artificially introduced promoter elements from synthetic circuits can enable modifiable control of pathway coupling, or even reverse the direction of effect. **Thus, in *Specific Aim 2,* we hypothesize that such a genetic circuit can re-frame how loss of estradiol affects genes of interest (GOI) and ultimately chondrocyte integrity *in vitro*.**

Taken together, the overarching goals of this project are to elucidate estradiol driven mechanisms (*Specific Aim 1*) and treatments (*Specific Aim 2)* for menopause-induced KOA.

Specific Aim 1. To interrogate how estradiol mediates ubiquitin proteolysis in KOA. *We hypothesize that (1) menopause induction will disrupt ubiquitin proteolysis activity and (2) estradiol treatment started early in menopause will restore ubiquitin proteolysis signaling and ultimately quench menopausal KOA.* *Aims 1a-1b*: We will quantify expression of functional components in ubiquitin signaling via immunohistochemistry in the following groups: menopausal, non-menopausal and estradiol treatment started during (1) perimenopause, (2) early menopause, or (3) late menopause. KOA pathology will also be quantified across groups using the Osteoarthritis Research Society International histopathology scoring. *Aim 1c:* Proteasome activity and protein ubiquitination will be quantified using live cell imaging in chondrocytes exposed to estradiol and non-estradiol conditions.

Specific Aim 2. To design an estradiol-repressed genetic controls circuit that modulates chondrocyte health. *We hypothesize that a genetic circuit modulated by estradiol will attenuate chondrocyte integrity in vitro.* *Aim 2a:* An estradiol promoter will be designed such that, when menopause is induced and estradiol is lost, the circuit will turn on. GOI candidates will be determined from a GWAS meta-analysis identifying mutations associated with KOA. Circuits will be generated with the estradiol promoter and GOI candidates, and candidate circuits will be transfected into chondrocytes. *Aim 2b:* To confirm therapeutic benefits, chondrogenic markers will be quantified via imaging flow cytometry and immunofluorescence. Responses to estradiol doses will be tested in transfected and non-transfected chondrocytes to confirm circuit controllability.

Impact Statement. By completing these aims, we will better understand estradiol-driven proteolysis as a mechanism of KOA and take the initial steps towards alternative treatment options for menopause-induced KOA. I will grow as a physician-scientist by gaining extensive training in the interrogation of molecular mechanisms and physiology-informed tissue engineering as they relate to menopause-induced KOA.

SIGNIFICANCE & BACKGROUND

Current murine models fail to capture menopause-specific mechanisms of knee osteoarthritis (KOA). KOA is a leading cause of disability in our aging population,[1,2] and there are currently no disease modifying treatments available.[3] One likely factor contributing to the limited treatment options is that our animal models do not recapitulate the disease observed in humans. Women who are post-menopausal present with the highest incidence and severity of KOA.[4] Yet, most animal studies for KOA include only males.[5] Of the few studies utilizing females, the impact of menopause is typically not investigated, as rodents do not present with a menopausal phenotype.[6] In fact, we found that non-menopausal female mice displayed a near total absence of KOA pathology.[7] The lack of menopause specific KOA studies becomes most apparent when searching the literature on PubMed: the Boolean search terms “knee osteoarthritis” results in 30,064 articles, while “knee osteoarthritis” AND “menopause” yields only 68. Of these few animal studies, ovariectomy (OVX) is the primary menopause model utilized.[8-11] However, these studies still have notable shortcomings. First, OVX lacks a perimenopausal phase, which is problematic since many menopause-related pathologies begin during perimenopause.[6] Additionally, OVX removes all ovarian tissue, resulting in collateral hormonal changes that do not occur in natural menopause, such as a drop in testosterone.[6] Finally, most studies perform OVX on young animals.[9-12] In humans, older women undergoing natural menopause and young women undergoing oophorectomy are considered distinct populations with separate clinical needs.[13] Thus, it is inappropriate to use young, OVX animals to model natural menopause. Here, we address these limitations by using a chemically-induced menopause model in middle-aged mice that presents with more severe KOA than controls.

Given the limited translatability of OVX-KOA studies, to identify possible mechanisms, we turned our attention to a recent meta-analysis that identified mutations in E3 ubiquitin ligase as uniquely associated with OA in women.[14] Ubiquitination is thought to play a critical role in mediating cartilage extracellular matrix (ECM) maintenance,[15,16] and a recent study concluded that a central issue in age-related OA is increased ECM protein half-life, suggesting maladaptive protein turnover and clearance.[17] In other tissues, there is evidence that estrogen-signaling modulates proteasome subunit composition and ultimately proteasome activity.[18-21] When examining estrogen signaling in the context of treatment for menopausal symptoms, timing of treatment initiation is a critical variable mediating effects, a phenomenon described as the “timing hypothesis”. The timing hypothesis suggests that if hormone therapy (HT) is given early in menopause, it exerts protective effects against sex-hormone sensitive diseases, while if started later in menopause, pathologic effects are observed.[22] Indeed, in our mathematical modeling work, we found estrogen treatment given “early” after OVX resulted in beneficial effects on cartilage while treatment started “late” resulted in no such effects (Gilmer et al., *Osteoarthritis & Cartilage*, *in revision*). Taking this with the aforementioned background, we intend to apply our menopause model to study a novel mechanism of time-dependent, estradiol-regulated protein ubiquitination within KOA. The studies proposed here will be the first to study age-related KOA in menopausal, female mice.

Therapeutics for sex hormone-sensitive diseases are limited by pathologic side effects. HT is the gold standard for treatment of many menopause-related diseases and symptoms.[13,23] However, for women 10+ years beyond the onset of menopause, HT is associated with increased risk of coronary heart disease and stroke.[23,24] Another approach to address menopause-related pathologies is to correct the signaling dysregulation caused by menopause induction within the tissue itself using synthetic biology techniques. The goal of synthetic biology is to engineer new biological systems or redesign existing ones for optimal performance.[25,26] One tool often employed is the synthetic genetic controls circuit (synthetic circuit),[27] which acts as a biological controls system. A predefined gene or protein serves as the metric to turn the switch ON or OFF, ultimately changing expression of some target gene of interest (GOI) to modify disease outcomes.[27] Over the last 20 years, synthetic circuits have been designed to address the pituitary-thyroid dysregulation seen in Graves’ disease,[28] develop SARs-CoV2 vaccines,[29] and diagnose communicable diseases, such as HIV.[30]

Synthetic biology has also been applied to diseases in cartilage[31] as well as in other diseases regulated by hormonal targets,[28] thereby confirming the feasibility of our studies. However, the application of synthetic biology in addressing sex-differences in disease progression and response to treatment has remained untouched.[32] We begin to address this gap by designing a treatment modality specific to menopausal cells. Although here we focus on KOA, these techniques could be employed to treat many other sex hormone-sensitive diseases, including cardiovascular disease.[23] More so, the genetic circuit we will develop with an estradiol-promoter could directly be used in other studies investigating menopause-related diseases.

APPROACH

Preliminary Data: Building upon previous studies,[8] we developed a chemically-induced menopause model. Briefly, middle-aged (14-16 months), female mice were randomly assigned to either menopause or non-menopause groups. The menopause group received 10 days of 4-vinylcyclohexene diepoxide (VCD, 160 mg/kg/day) in sesame oil intraperitoneal (IP) injections. VCD is a specific ovarian toxin that causes atresia in the primordial and primary ovarian follicles.[8] The non-menopause group received 10-days of sesame oil IP injections at the same volume (**Figure 1A**). Menopause onset was defined as 10 consecutive days of diestrus[8] as determined by vaginal cytology[33] and occurred 115±2 days after the first injection in the menopause group (**Figure 1B-C**). Conversely, the non-menopause group continued to have normal estrus cycles. In this injection schedule, perimenopause began at day 25 as determined by assessing estrus cyclicity and comparing time points to previous studies.[8] Internal organs were also collected at all time points to assess for off-target effects, and a blinded pathologist noted no signs of toxicity in any of the tissues evaluated.

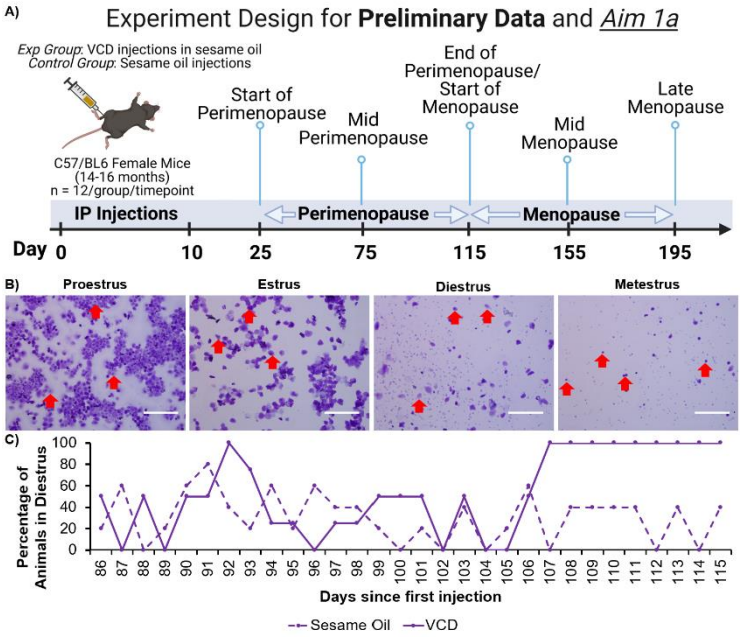

Figure 1: *(A) Experimental Design and injection schedule. Euthanasia and tissue collection occurred at the blue dots. Created in BioRender. (B) Representative images from each phase of the estrus cycle. Arrows point to cells used in determination of cycle phase. (C) Percentage of mice in the sesame oil and VCD groups in diestrus, evaluated under blinded conditions.*

We then collected knees from menopausal and non-menopausal mice at the start of perimenopause, mid-perimenopause, start of menopause, mid-menopause, and late menopause (**Figure 1A**). Late menopause is defined as menopause in older mice (21-24 months; 60–70-year human equivalent). We also collected knees on day 10 to assess for direct effects of VCD and verified there were no changes in any joint tissues. Decalcified knees were embedded in paraffin, sectioned, and stained using Safranin-O/Fast Green, as in our previous study.[7] Since KOA is considered a disease of the whole joint,[34] we quantified cartilage degeneration, synovium pathology, and subchondral bone pathology in a blinded manner using the well-established Osteoarthritis Research Society International (OARSI) scoring system, synovium grade, and subchondral bone grade, respectively.[35-37] The OARSI score quantifies the grade (degeneration depth progression) and the stage (extent of involvement within the joint) of cartilage degeneration. Interestingly, we found that menopausal aging results in progressive cartilage degeneration, while non-menopausal aging does not (**Figure 2A**). Non-menopausal aging led to increased synovium pathology, effects that were amplified with menopause induction (**Figure 2B**). Lastly, we did not observe any effects of aging or menopause on subchondral bone pathology (**Figure 2C**).

Research Plan

Specific Aim 1. To interrogate how estradiol mediates ubiquitin proteolysis in KOA.

<u>*Rationale:*</u> A component of E3 ubiquitin ligase was recently identified to be a unique contributor to OA in females.[14] In post-traumatic OA in males, ubiquitin proteolysis modifies expression of matrix metalloproteinase 13,[15,16] and in malignant tumors, estradiol signaling regulates ubiquitin proteasome subunit composition

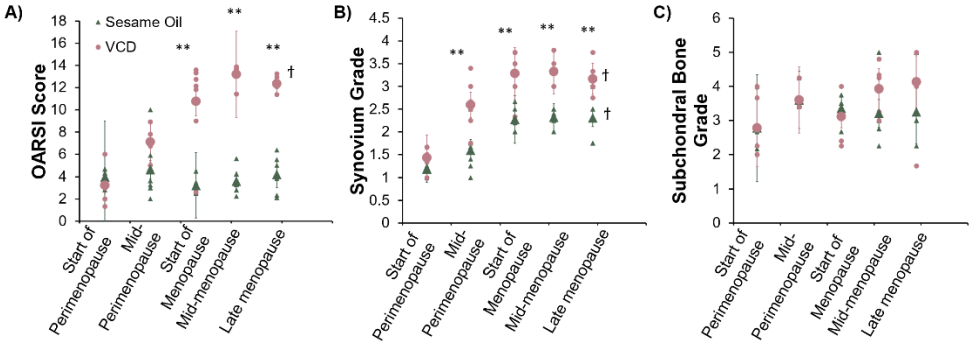

Figure 2: *Knees collected at the time points in Figure 1A were processed for histological analysis. OARSI Score (A), Synovium Grade (B), and Subchondral Bone Grade (C) were quantified under blinded conditions across perimenopause and menopause between VCD and sesame oil injected animals. OARSI Score is a metric of cartilage degeneration, and a higher score indicates worse pathology for all scoring systems presented. ** = significant difference between groups; † = significant difference across time.*

and activity.[18-21] Thus, a logical next step is to investigate the role of estradiol based ubiquitin proteolysis in KOA using a natural menopause model. Given estradiol signaling in menopause may be modulated by time within menopause, we also designed our studies to capture time-based effects (Gilmer et al., *Osteoarthritis & Cartilage*, *in revision*).[38]

Plan overview: First, we will outline changes in ubiquitin proteolysis component expression in the knee over the course of menopause. We will then directly test whether protein ubiquitination signaling is mediated by estradiol and if mediation is time dependent. Menopausal mice will be randomized into one of four groups: (1) no treatment versus estradiol treatment given in (2) perimenopause, (3) early menopause, or (4) late menopause. Expression of the ubiquitin proteolysis components and degree of cartilage, synovium, and subchondral bone pathology will be quantified in knee sections. Lastly, ubiquitination and proteasome activity will be quantified in chondrocytes under different estradiol exposures.

Aim 1a. To define how menopause induction modulates ubiquitin signaling within the knee. As a first step, we will quantify changes in ubiquitin signaling using knees from menopausal and non-menopausal mice across menopause that we currently have biobanked (12 mice per group, 8 slides per mouse, 4 knee sections per slide). Specifically, we will quantify (1) the time course (**Figure 1A**), (2) tissue specificity (cartilage, synovium, subchondral bone), and (3) which components of protein ubiquitination are affected. We will perform immunohistochemistry to quantify expression of E1, E2, E3, ubiquitin, and proteasome subunit components (α1α2α3α5α6α7, LMP7, Rpt6, LMP2, PA28β; subunits noted to change via estradiol signaling[20]). Immunohistochemistry will be analyzed by a blinded investigator using Aperio GENIE (Leica Biosystems).[39]

Aim 1b. To map the effects of estradiol treatment on ubiquitin signaling in KOA in vivo. Middle-aged female mice will receive 10 days of VCD IP injections. Mice will then be randomized to one of the following time periods: (1) perimenopause, (2) early menopause, and (3) late menopause. Within time periods, an experimental group will receive estradiol treatment, with an appropriate control group (**Figure 3**).

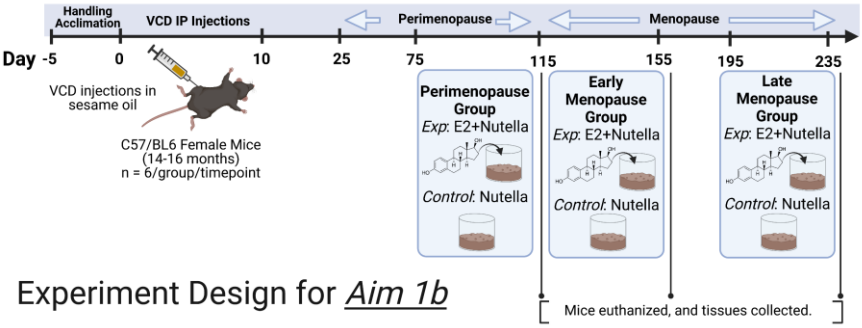

Figure 3: *Experiment design for Aim 1b. Briefly, all mice will receive VCD injections. Mice will then be split into three time periods (perimenopause, early menopause, late menopause) and two groups (experimental and control). Exp and control treatments are given daily. Euthanasia and tissue collection will occur at the end of treatment. E2 = estradiol. Created in BioRender.*

For the experimental groups, 17β-estradiol will be administered perorally in Nutella. In a previous study, peroral administration resulted in physiologically relevant concentrations in estradiol over 35 days while being minimally invasive.[40] Specifically, mice will be individually served 60 mg of Nutella containing 17β-estradiol daily (1.12 µg of 17β-estradiol in 0.312 µL of sesame oil).[40] Control animals will be given Nutella in the same amount and duration. Using methods well-established in our lab,[41] serum will be collected via cardiac puncture at the time of euthanasia to measure serum estradiol concentrations with mass spectrometry[42,43] for *Aim 1c*.

As before, knees will be collected, and expression of components along the ubiquitin signaling cascade will be quantified via immunohistochemistry. Here, we will focus on the components that are changing across perimenopause and menopause from *Aim 1a*. We will also quantify cartilage degeneration, synovium pathology, and subchondral bone pathology as described in the **Preliminary Data**.

Aim 1c. To quantify estradiol-driven changes in protein ubiquitination and proteasome activity in chondrocytes. We will isolate and expand chondrocytes from the cartilage in our *Aim 1b* mice, using techniques well-established in our lab.[7] All chondrocytes will set a baseline under culture conditions that mimic those of their *in vivo* environment. In other words, chondrocyte media will contain estradiol concentrations that match that of the serum from the mice they were isolated from, making up two groups: experimental and control chondrocytes. All culture experiments will use charcoal stripped FBS to remove sex steroids from the serum[44] and will be phenol red free, since phenol red is a weak estrogen.[45] Using a Ubiquitination Live-Cell BRET-Based Assay, we will perform live cell imaging to assess changes in protein ubiquitination with changes in estradiol conditions. A group of experimental and control chondrocytes will be exposed to estradiol concentrations reflective of receiving estradiol treatment for 24 hours (in mouse ~30 pg/mL[40]), and then exposed to estradiol concentrations reflective of no treatment conditions for 24 hours (in mouse ~5 pg/mL[46]). A separate group of experimental and control

chondrocytes will receive the same exposure in reverse order (no treatment conditions for 24 hours, then estradiol treated conditions for 24 hours). For the duration of the experiment, live cell imaging will be performed, and immunofluorescent intensity of ubiquitin will be quantified and compared between groups and over time. Lastly, we will quantify Proteasome Activity via AnaSpec SUC-LLVY-AMC fluorogenic oligopeptide. Fluorescent intensity will be quantified using a SpectraMax plate reader for the first hour after media changes and the last hour prior to media changes. Both live cell imaging and plate reader images will be analyzed by a blinded investigator using *CellProfiler*.[47] (**Figure 4**)

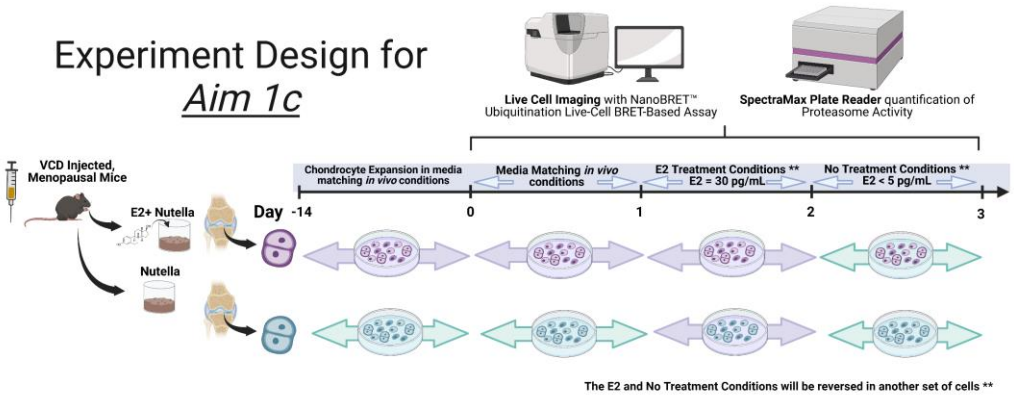

Figure 4: *Experiment design for Aim 1c. Briefly, chondrocytes will be isolated and expanded from the mice in Aim 1b in media that matches in vivo conditions (i.e., estradiol concentrations will reflect that of the mouse). Live cell imaging will then be performed for 72 hours on the following conditions: in vivo matched concentrations, estradiol (E2) concentrations from the experimental group (30 pg/mL), and E2 concentrations from the control group (<5 pg/mL). Proteasome activity will be quantified for the first hour after media changes and last hour prior to media changes on each day. Created in BioRender*

<u>*Statistical Analysis:*</u> For <u>*Aims 1a-b*</u>, an *a priori* power analysis was performed using the effect size calculated from the meta-analysis identifying E3 ubiquitin ligase mutations.[14,18,48] For a statistical power of 0.8 with an effect size of 0.377, 12 mice are needed per group per time point. For normally distributed data, we will perform a repeated-measures ANOVA. For non-normally distributed data, Kruskal Wallis tests will be used to assess differences across time, and Mann Whitney U-tests will be used to assess between group differences. For <u>*Aim 1c,*</u> we estimated the required sample size (i.e., number of unique culture wells) using guidelines generated for *in vitro* studies.[49] For an estimated effect size of 1.0, power of 0.8, and alpha level of 0.05, a sample size of 30 is needed.[49] Based on the normality of datasets, independent samples t-tests or Mann Whitney U-tests will be used to assess differences in proteasome activity, and repeated measures ANOVA or Kruskal Wallis tests will be used to assess changes in ubiquitination.

<u>*Expected Outcomes:*</u> Since E3 ubiquitin ligase strongly mediates E2 activity,[50] we hypothesize menopause will result in decreased co-localization of E3 and E2, decreased E3 expression, and abnormal proteasome structure. We hypothesize E3 expression, E2 and E3 co-localization, and proteasome structure will be restored with early, but not late, estradiol treatment. We also hypothesize proteasome activity and protein ubiquitination will be higher in estradiol culture conditions compared to no treatment conditions. (**Figure 5**)

<u>*Potential Pitfalls & Alternative Strategies:*</u> Throughout this aim, we may find there are no menopause-mediated effects on protein ubiquitination, despite previous literature.[21,16] In this case, we will investigate general changes in protein turnover and degradation using isotype labeling, as previously described.[17] Additionally, we intend to use various imaging software (GENIE, CellProfiler), but if analyses expand beyond the scope of these algorithms, we can design customized NiS-Elements analyses using our Nikon microscope and the associated packages.

In <u>*Aims 1b-1c*</u>, we are assuming that serum estradiol concentrations reflect that of the synovial fluid. If we do not observe the expected effects, we will first repeat <u>*Aim 1c*</u> with a wider range of estradiol titrations. If an ideal estradiol concentration is identified, we will then repeat <u>*Aim 1b*</u> with intra-articular injections of estradiol at the ideal concentration.[51] If we do not identify an ideal estradiol concentration, we will quantify other sex-hormones that

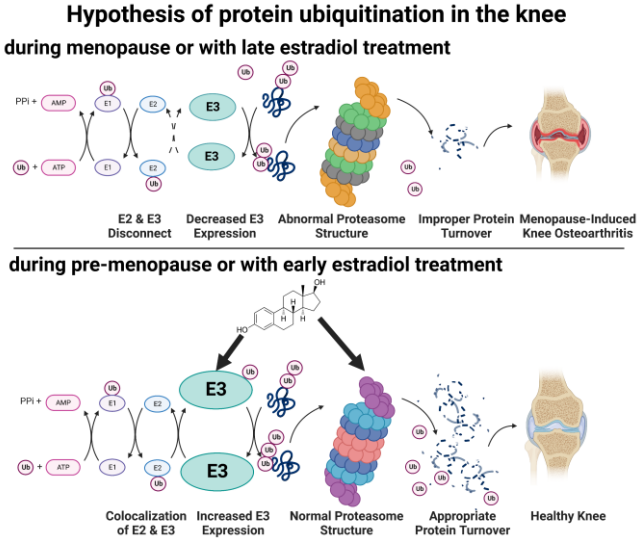

Figure 5: *Hypothesis schematic for Specific Aim 1. Ub = ubiquitin. Created in BioRender*

change in menopause[52,23] from the mice used in *Aim 1a*. We will correlate these hormone concentrations with changes observed in *Aim 1a*. If any sex hormones have a moderate correlation with *Aim 1a* outcomes, we will perform the experiments outlined in *Aim 1c* using that sex-hormone (e.g., relaxin, progesterone).

In *Aim 1c*, we assume that changes in protein ubiquitination will occur in cartilage, however, changes may predominantly be in the subchondral bone or synovium. There are techniques for isolating mesenchymal stem cells from the subchondral bone[53] and fibroblasts from the synovium,[54] and if we predominantly see changes here, we will implement these techniques in our lab and focus *Aim 1c* on these cell types.

Specific Aim 2. To design an estradiol-repressed genetic controls circuit that modulates chondrocyte health.

Rationale: In traditional controls engineering, a controller is used to monitor a parameter of interest (e.g., temperature) and modulate a controllable factor (e.g., heat applied to the system) to achieve the desired outcome (e.g., water bath maintained at 37°C).[55] Similarly, here, we can use the principal drop in systematic estradiol that is characteristic of menopause[56] to regulate a promoter that ultimately modulates expression of a gene of interest (GOI) and attenuates menopause-driven loss of chondrogenicity.

Brief Plan: We will design and optimize an estradiol-repressed promoter such that our circuit will turn ON when menopause is induced and remain OFF during non-menopausal conditions. GOI candidates will be generated using a previous GWAS meta-analysis which identified 18 unique genes associated with KOA, total knee replacement, early onset OA, and female sex.[14] Estradiol-repressed promoters and GOI candidates will be combined to generate a library of circuits. Circuits will then be transfected into chondrocytes, and we will test which circuit results in the greatest improvement in markers of chondrogenicity. Lastly, we will verify that our circuit can successfully switch from the ON and OFF position under varying estradiol concentrations.

Aim 2a. To assess viability of estradiol-repressed promotors & assemble estradiol-repressed genetic controls circuits. Since there are many native genetic systems regulated by estradiol signaling, we will start with identifying native sequences that may offer the kinetics we are seeking. Specifically, we are seeking promoter repression in the presence of cyclical fluctuations in estradiol, as occurs during the normal menstrual cycle (in humans 30-400 pg/mL[57]) but repression loss (i.e., activation) with a drop in estradiol resembling menopause (in humans < 30 pg/mL[58]). We will identify native estradiol-estrogen receptor promoter sequences via a data base with all studied estrogen receptors from breast cancer samples (5,000+ promoter sequences).[59] These promoters typically contain “estrogen receptor elements” (ERE) and begin with a 13 base pair sequence – GGTCANNNTGACC.[60] COMET Toolkit will be used to model promoter behavior and narrow down potential candidates.[61,62] We will start off by identifying the top 20 most likely promoters to offer our desired kinetics. Candidate promoters will then be synthesized onto a lentiviral plasmid expressing a short half-life firefly luciferase[63] using a Multiple Lentiviral Expression System Kit.[31,64]

All culture experiments will use charcoal stripped FBS[44] and will be phenol red free (as in *Aim 1c*).[45] Given that estradiol-promoter interactions rely on a complex set of cofactors and machinery that differs quite drastically between different tissues[65-67] and the documented challenges of transfecting primary chondrocytes,[68,69] all experiments will be performed in immortalized chondrocytes.[70] Chondrocytes will be plated in standard chondrocyte media. Our engineered lentivirus plasmids will be diluted to 10% in FBS with polybrene.[31] Standard chondrocyte media will be replaced with plasmid containing media. Cells will then be incubated in plasmid containing media for 24 hours, followed by five days of incubation in standard chondrocyte media prior to use in experiments. In previous reports, this method results in ~95% of chondrocytes being transfected with stable copy numbers,[31] and we will confirm this success rate using quantitative polymerase chain reaction (qPCR).

Cells will be placed in a 96-well plate at a constant concentration, and firefly luciferase expression will be measured using our SpectraMax plate reader at varying doses of estradiol (0-400 pg/mL) to quantify kinetics. Since biological promoter behavior tends to be “digital” and we are seeking “analog” behavior,[71] we will identify promoter sequences that are the closest to being completely ON during menopause and completely OFF during menstrual cycle conditions. Random mutations in individual nucleotides across the promoter will then be used to generate a library of candidates, and COMET Toolkit will again be used to model promoter behavior and gain a better understanding of how changes in the promoter sequence affects repressibility.[61,62] Once candidates are designed, engineered promoters will be placed on lentiviral plasmids for testing at varying doses of estradiol. This will be repeated until an ideal, “analog” candidate is identified.

Using our ideal estradiol-repressed promoter, we will generate a library of circuits by replacing the luciferase gene with the GOI candidates,[14] similar to previous studies.[31] Specifically, 18 GOI candidates will be generated from target genes associated with female sex, total knee replacement, and KOA from a previous study.[14]

Aim 2b. To test responsiveness of the estradiol-GOI synthetic circuit in chondrocytes. To determine which GOI provides the greatest potential for therapeutic benefits, we will quantify well-established markers of chondrogenicity (Sox9,[72] type II collagen,[73] aggrecan[74]) (**Figure 6**). Imaging flow cytometry will be used to quantify markers, as this method allows for high-throughput single cell protein quantification in 500-1000 cells.[75] Standard immunofluorescence of non-detached cells will also be performed to assure trypsinization of cells does not result in changes in expression.[38,76-78] The GOI that provides the largest increase in all three markers relative to non-transfected controls will be used in subsequent experiments (analyzed under blinded conditions).

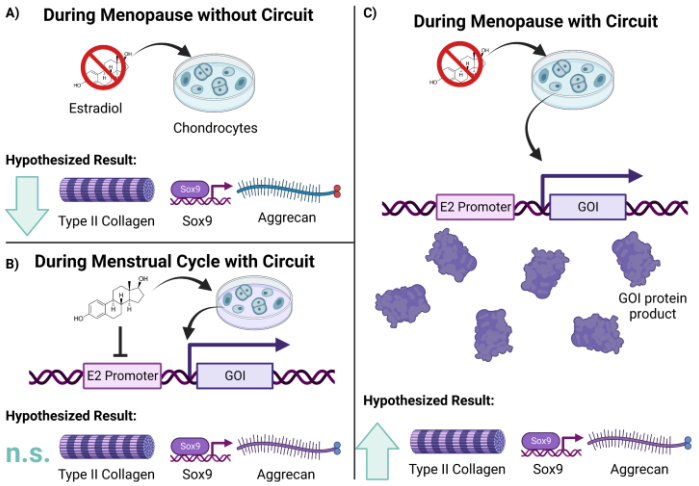

Figure 6: *Hypothesized results from Specific Aim 2: (A)* During menopause without circuit*. In comparison to menstrual cycle cells without the circuit, we expect to see a decrease in markers of chondrocyte health. (B)* During menstrual cycle with the circuit*. In comparison to menstrual cycle cells without the circuit, we expect to see no changes in makers of chondrogenicity. (C)* During menopause with the circuit*. In comparison to the group in A and B, we expect to see an increase in markers of chondrogenicity. Created in BioRender*

To assure the circuit is reversible, we will perform a time-course study in which cells will be cultured under menopausal conditions (E2<30 pg/mL) for four times the half-life of our GOI protein product. This same monolayer will then be cultured under menstrual cycle conditions (E2=30-400 pg/mL) for four times the half-life of our GOI. This will be repeated for three sets (menopausal conditions and then menstruation conditions) to assure adequate control of the circuit. Transfected chondrocytes under constant menstrual cycle conditions and non-transfected chondrocytes under this rotating schedule will be used as controls. GOI protein expression will be quantified using imaging flow cytometry and immunofluorescence at the end of each set.[75]

Statistical Analysis: To estimate the required sample size (i.e., number of unique culture wells), we again use guidelines for *in vitro* studies.[49] For an effect size of 1.0, power of 0.8, and alpha level of 0.05, a sample size of 30 is needed.[49] Two-way ANOVA or Kruskal Wallis tests will be used to assess group and time dependent effects for normally and non-normally distributed data, respectively.

Expected Outcomes. We expect to design an estradiol promoter that reversibly represses gene expression under menstrual cycle conditions (E2=30-400 pg/mL) while allowing for gene expression in menopausal conditions (E2<30 pg/mL). We hypothesize that by applying GOI candidates identified from a previous study,[14] we will recover chondrogenicity in menopause when compared to non-transfected cells. (**Figure 6**).

Potential Pitfalls & Alternative Strategies: Given the challenges in designing promoter repression,[61] in *Aim 2a*, we may be unable to generate a promoter that is consistently repressed with estradiol signaling. It is, however, much easier to design an activated promoter.[61] In this case, we will design a more complex circuit with an activated estradiol promoter, followed by a PIP-KRAB gene. PIP-KRAB expression will then repress a PIR promoter, which is a well-established genetic system in mammalian synthetic biology.[79] In our circuit, the PIR promoter will be followed by luciferase or a GOI, such that when estradiol activation is lost, PIP-KRAB expression will decline, the repression of the PIR promoter will dissipate, and luciferase/GOI expression will occur.

In *Aim 2b*, our GOI candidates may not modulate markers of chondrogenicity. In this case, we will perform mass spectrometry proteomics from cartilage samples from menopausal and non-menopausal mice to determine a more robust and menopause-specific set of GOI candidates (top 20 proteins that are down regulated between non-menopause and menopause groups will be used).

Another possibility is that our circuit may fail to turn ON and OFF with successive stimulation. In this case, we will interrogate samples that offer better relative control of the circuit and separate samples that offer worse relative control of the circuit. qPCR will be used to sequence the circuit of these sample sets to determine if individual mutations within the circuit are associated with controllability. If specific mutations that affect the circuit stability are identified, plasmid sequences will be modified to purposefully integrate these mutations.

RESPECTIVE CONTRIBUTIONS

This proposal was originally developed and drafted by the applicant, Gabrielle Gilmer, with the exception of the Sponsor Information, which was written by the primary sponsor, Dr. Fabrisia Ambrosio, and the Institutional Environment, which was written by Pitt's MSTP Director, Dr. Richard Steinman. Gabrielle developed the base of the project outlined in this proposal after a summer rotation in the Ambrosio laboratory and with extensive discussion with Dr. Ambrosio. Gabrielle and Dr. Ambrosio began discussing the concept of a menopause mouse model in application to knee osteoarthritis in January 2021, when she was applying for the University of Pittsburgh Integrated Clinical and Geroscience Research Training Program. Gabrielle has continued to receive extensive and valuable feedback from Dr. Ambrosio on this proposal, including discussion of preliminary data, technical difficulties, resource availability, scientific rigor, and effective communication. These discussions have largely taken place through weekly meetings and frequent email exchanges. In addition, feedback on this proposal was received from Gabrielle's primary mentoring team, Drs. Christopher Evans, Rebecca Thurston, and Pamela Silver, as well as from members of the Ambrosio laboratory and Gabrielle's comprehensive examination committee members, Drs. Nam Vo, Steffi Oesterreich, Mo Ebrahimkhani, and Hang Lin.

The proposal outlined here will be a closely mentored experience that encourages Gabrielle to develop into an independent physician scientist. Specifically, Gabrielle will perform the experiments and collect the data outlined in the **Research Strategy**. Any technical difficulties and all associated results will be shared with Dr. Ambrosio in weekly one-on-one meetings. Once a month, Gabrielle will present project updates to both the entire Ambrosio laboratory and to her mentoring team (Drs. Evans, Thurston, and Silver). Gabrielle will draft all manuscripts associated with this proposal. Manuscripts will then be edited and approved by Dr. Ambrosio, Gabrielle's mentoring team, and all co-authors prior to submission. Gabrielle will write annual updates to the funding agency on progress through this training plan, which will also be reviewed and edited by Dr. Ambrosio prior to submission. Dr. Ambrosio and the mentoring team will provide research, clinical, and professional development opportunities to Gabrielle, as outlined in the **Applicant Background & Goals for Fellowship Training**.

SELECTION OF SPONSOR AND INSTITUTION

Institution. When identifying an institution for my physician-scientist training, the University of Pittsburgh's Medical Scientist Training Program (MSTP) first stood out to me because of the director, Dr. Richard Steinman. Dr. Steinman invests in each student, taking the time to learn about our specific research and clinical interests and helps design customized training plans. At the program level, he has created a structure for us to grow as physician-scientists that includes workshops, longitudinal clinical experiences throughout graduate training, career coaches at transition points, and MSTP course work that fills in the gaps from our graduate and medical training, including ethics, statistics, and grant writing courses.

I was also drawn to Pitt given its excellent reputation for innovation in musculoskeletal research. While interviewing, I was introduced to Dr. Gwendolyn Sowa, Professor and Chair of the Department of Physical Medicine & Rehabilitation. Dr. Sowa's lab combines clinical, biomechanics, and basic science to study low back pain, and she is a prominent leader at Pitt and in her field. As a female physician-scientist in leadership positions, her career trajectory and lab set up provide a framework for what I envision for myself. She now serves as my career advisor and facilitated my introduction to Dr. Fabrisia Ambrosio, my primary mentor.

Sponsor. My long-term career goal is to be an orthopedic surgeon-scientist who studies and treats joint injuries and diseases in women, and my primary goal during this fellowship is to become competent in physiology-informed tissue engineering within the musculoskeletal system. As such, my first pass at identifying a mentor was finding an expert in this aforementioned niche, and Dr. Fabrisia Ambrosio stood out to me immediately. She has been credited with founding the fusion field, regenerative rehabilitation, which combines tissue engineering and physical therapy approaches in a truly bench-to-bedside paradigm. She directs the NIH-funded Alliance for Regenerative Rehabilitation Research and Training (AR^3T) as the lead Principal Investigator. I also sought to find a sponsor who understands the delicate balance associated with dual degree training. Dr. Ambrosio completed MPT and PhD training herself and has personal experience with the challenges associated with balancing clinical and basic training. Additionally, I aimed to be sponsored by a mentor who has ample funding and a strong publication record. Indeed, Dr. Ambrosio has three active R01s and publishes regularly, with trainees almost always as first authors on manuscripts.

While completing my research rotation, I confirmed that the lab environment and culture were an excellent fit for me. Early on, I noticed the energy in lab meetings was extremely collaborative; everyone was actively participating and unafraid to share critiques or dissenting thoughts. In addition to our scientific presentations, lab members present on "innovation heroes" from different fields and talk about how this person inspires them as a scientist. Dr. Ambrosio also scheduled training on the "art of observation" with a professional art curator for some of our lab meetings. Through these discussions, our lab group identified how to apply these observational skills back to our science. Dr. Ambrosio creates an environment where I can explain my wildest of experiment ideas and lowest of scientific failures. She meets them all the same with an open mind and willingness to pivot. Dr. Ambrosio also quickly picked up on my struggle with overcommitting, and she has become a central advocate for keeping me focused on my projects and tasks that support my training goals.

To complement the mentoring offered by Dr. Ambrosio, I have assembled a team with expertise in each component of my proposal: Dr. Rebecca Thurston in hormone replacement therapy applied to menopause and Dr. Christopher Evans in gene therapies for knee osteoarthritis, with whom I have worked with over the last year and a half. Dr. Pamela Silver will also serve as my mentor, as she is an expert in synthetic biology techniques applied to mammalian cells. Since adding Dr. Silver to my team, she has connected me to synthetic biology resources and peer groups in the Boston area and provided extensive feedback on this proposal.

Institutional Change. At the beginning of my PhD, Dr. Ambrosio informed me she would be moving our laboratory to the Spaulding Rehabilitation Institute at Harvard Medical School. After contemplation, I decided to move with the lab to Boston for completion of my PhD prior to returning to Pitt to finish medical school. I worked and continue to work extensively with Drs. Ambrosio, Sowa, and Steinman to design a framework for my success while in Boston. Specifically, for my clinical development, I will return to Pittsburgh to complete my second longitudinal clinical clerkship (LCC) with Dr. John Fowler. While in Boston, I will shadow Dr. Miho Tanaka, a colleague of Dr. Volker Musahl who is my mentor from my first LCC. I will continue to attend Pitt MSTP workshops virtually and attend both the Pitt MSTP and Cellular and Molecular Pathology (CMP) graduate retreats in person. Dr. Steinman connected me with Dr. Loren Walensky, the MSTP Director at Harvard, and I have since been participating in Harvard's MSTP events in person. I will complete the remainder of my course work, journal clubs, career advisor meetings, and thesis committee meetings virtually. I have also identified seminars both at Pitt and Harvard that will supplement my research training.

RESPONSIBLE CONDUCT OF RESEARCH

Responsible conduct of research (RCR) is a pillar to my training as a physician-scientist, and I am strongly committed to integrating RCR into the experiments outlined in this proposal. Within the University of Pittsburgh's Medical Scientist Training Program (MSTP) as well as the Ambrosio lab, RCR is emphasized as a critical part of our community and commitment to scientific research.

Within the MSTP, I took our ethics course in the summer of 2021, which was led by Dr. Steinman and consisted of four 2-hour sessions. The first session focused on challenges in clinical trials and specifically how this relates to the COVID-19 pandemic. Our second session dove into the intricacies of conflicts of interests and how to appropriately report our conflicts. We discussed the ethics of authorship, credit, and peer review in our third session through case discussions. I found this particular session so helpful and insightful that, with permission from Drs. Steinman and Ambrosio, I led two, one-hour workshops to review these cases in the Ambrosio lab. Our final session in ethics covered public communication and how physician-scientists interact with the larger society with a specific emphasis on social media presence. In addition to this course, each year, we have four hours of MSTP workshop time focused on ethics in one or two sessions. This past academic year, I led one workshop focused on the ethics of near-death conversations with patients and another on the ethics of the "hidden curriculum".

While in medical school, I took "Ethics, Law, and Professionalism" as a part of our "Patient, Physician, and Society" course block. This course included both lectures and small group discussions in two hours sessions per week over the course of 15 weeks. Lectures and discussions were focused on critical ethical issues including informed consent, genetic manipulation, and confidentiality. In addition to this course, I also completed several training modules related to RCR in humans that focused on HIPAA, informed consent, and bloodborne pathogens. Additionally, while working with Dr. Sowa on my project examining telemedicine use in physical medicine & rehabilitation, Dr. Sowa and I would actively discuss the importance of maintaining patient confidentiality as I performed my chart reviews. Lastly, during my 8-week Pediatrics clerkship, my attending physicians would actively discuss the principles I learned in my courses and training modules as I began navigating the Electronic Medical Record.

Within the Cellular and Molecular Pathology (CMP) graduate program, I completed modules on appropriate conduct of research. Specifically, these modules were assembled through the Collaborative Institutional Training Initiative (CITI), sponsored by the University of Miami. Thus far, I have completed modules on RCR, Biomedical Human Subjects Research, Conflicts of Interest, and Information Privacy & Security. As a part of my work in the Ambrosio lab and with the Institutional Animal Care and Use Committee (IACUC), I have completed several CITI modules pertaining specifically to working with animals, both at the University of Pittsburgh and Massachusetts General Hospital (MGH). Specifically, these have included Working with Mice in Research Settings, Working with Genetically Modified Mice in Research Settings, Working with Small Animals, Animal Care and Use – Working with the IACUC, Reducing Pain and Distress in Laboratory Mice and Rats, Aseptic Surgery, and Rodent Anesthesia. Lastly, to enhance my RCR training, I attend the RCR series offered by the University of Pittsburgh's Clinical and Translational Science Institute (CTSI). These training lectures are focused on considerations when submitting an Institutional Review Board (IRB) protocol, methods to enhance reproducibility and transparency in reporting, preprints, among other critical RCR topics.

Within the Ambrosio lab, as a reflection of our steadfast commitment to ensuring rigor and reproducibility in our work, we review the ARRIVE Guidelines and discuss the strategies (or lack thereof) taken to ensure rigor in our weekly journal club paper presentations. We also map out these guidelines in our own experiment designs and as we prepare manuscripts.

In addition to the extensive RCR training I have completed thus far, I will continue to maintain this level of awareness throughout my fellowship. To do so, I will renew and stay up-to-date on my CITI trainings. Within the Pitt MSTP, we will continue to have four hours of ethics-based workshops per year, which I will attend virtually, and I will continue to attend the CTSI RCR series. Within the Ambrosio lab, we will continue our extensive discussions of the ARRIVE guidelines in journal clubs, experiment designs, and in manuscript drafts. Through these trainings and active discussions within our lab, I am better equipped to address any conduct considerations related to animals that may come up during the time of my fellowship.

SPONSOR STATEMENT

A. Research Support Available

The Ambrosio laboratory is highly funded and offers abundant resources available to support Gabrielle's training. Dr. Ambrosio's grant support, which totaled $2.7M for 2022, has primarily been provided by the National Institutes of Health (NIH, and the Department of Defense (Abridged listing in **Table 1**). Additionally, Dr. Ambrosio received over $5.5M in recruitment/center funds from Spaulding Rehabilitation Center to support novel research directions such as the one proposed by Gabrielle in this fellowship application.

Table 1. Ambrosio current funding

Grant Number	Grant Title	Role	Years Inclusive	Amount
2P2CHD086843	*ALLIANCE FOR REGENERATIVE REHABILITATION RESEARCH AND TECHNOLOGY (AR^{3}T)*	Contact PI	7/2020-6/2025	$5,010,083
R01ES0255929	*DYSFUNCTIONAL MUSCLE REMODELING AND REGENERATION IN ENVIRONMENTAL DISEASE*	Contact PI	2/2016-1/2022	$2,420,732
R01AG052978	*THE ANTI-AGING ROLE OF KLOTHO IN SKELETAL MUSCLE REGENERATION*	PI	9/2017 - 5/2023	$2,036,643
R01AG061005	*ROLE OF EXTRACELLULAR MATRIX IN AGE-RELATED DECLINES OF MUSCLE REGENERATION*	Contact PI	8/2019-4/2024	$2,166,518
R01AG061005-03S1	*ROLE OF EXTRACELLULAR MATRIX IN AGE-RELATED DECLINES OF MUSCLE REGENERATION*	Contact-PI	8/2019-4/2024	$281,359
R01AG066198	*PHYSICAL EXERCISE AND BLOOD-BRAIN COMMUNICATION: EXOSOMES, KLOTHO AND CHOROID PLEXUS*	Co-PI	1/2020-12/2024	$3,841,686
W81XWH-21-1-0542	*OPTIMIZING MUSCLE AND BONE MECHANOADAPTATION TO PHYSICAL TRAINING: MECHANISTIC CONTROL PATHWAYS VIA MUSCLE BONE*	Co-I	8/2021-7/2024	$61,988
W911NF-21-2-0208	*ENHANCING SOLDIER PROTECTION AGAINST EVOLVING THREATS*	Sub-award PI	9/2021-8/2023	$1,780,668

B. Previous Trainees

As of September 2022, Dr. Ambrosio is a faculty member in the Department of Physical Medicine & Rehabilitation at Harvard Medical School. She is also the inaugural Atlantic Charter Director of the Discovery Center for Musculoskeletal Recovery at the Schoen Adams Research Institute at Spaulding. Dr. Ambrosio has supervised 7 doctoral students, 11 postdoctoral fellows, 8 international visiting scholars, and 25 undergraduates. Eight of Dr. Ambrosio's former postdoctoral fellows are currently in faculty positions at major research Universities, and many of her graduate students have gone on to post-doctoral fellowships (abridged listing in **Table 2**). Dr. Ambrosio is recognized internationally for her leadership in launching and expanding the field of Regenerative Rehabilitation. Regenerative Rehabilitation represents the integration of rehabilitation approaches together with regenerative medicine/regenerative biology technologies to promote functional recovery after injury or in the setting of disease. This interdisciplinary mindset has guided Dr. Ambrosio's mentorship style. For example, Dr. Ambrosio encourages her trainees to read articles from other disciplines, interact with colleagues and students in other programs, and attend a wide spectrum of seminars so that they may gain exposure to tools and techniques from other fields. This tendency to move outside disciplinary boundaries is facilitated by the fact that the trainees in the Ambrosio laboratory have highly diverse research backgrounds and interests, spanning rehabilitation, bioengineering, physics, women's health, and molecular biology. The Ambrosio lab group's diversity extends even beyond scientific backgrounds, and as a female researcher of Latina descent, Dr. Ambrosio is especially committed to promoting diversity, equity, and inclusion among her laboratory members. This commitment is evidenced by the array of races and ethnicities that are/have been represented.

Table 2. Abridged list of former trainees and their current positions

Position in Ambrosio Lab	Time of Supervision	Mentorship Role	Current employer	Current position/title

Ricardo Ferrari *PhD student*	*2011-2016*	Primary research supervisor	University of Pittsburgh	Instructor
Amrita Sahu *PhD student*	*2015-2019*	Primary research supervisor	University of Pittsburgh	Assistant Professor
William Conkright *PhD student*	*2019-2021*	Primary research supervisor	Madigan Army Medical Center	Research Director
Meghan Beckner *PhD student*	*2019-2021*	Primary research supervisor	US Army Research Institute of Environmental Medicine	Post-doctoral fellow
Keith Avin *Post-doctoral fellow*	*2012-2013*	Primary research advisor	University of Iowa	Assistant Professor
Kristen Stearns-Reider *Post-doctoral fellow*	*2012-2014*	Primary research advisor	UCLA	Assistant Professor
Kevin Beezhold *Post-doctoral fellow*	*2013-2016*	Co-research advisor	University of Pittsburgh	Assistant Professor
Prabaha Sikder *Post-doctoral fellow*	*2019-2020*	Primary research advisor	Cleveland State U.	Assistant Professor
Hirotaka Iijima *Post-doctoral fellow*	*2019-2022*	Primary research advisor	Nagoya University	Assistant Professor

C. Training Plan, Environment, Research Facilities

Gabrielle's primary career goal is to lead an NIH-funded multi-disciplinary center focused on merging biomechanics/rehabilitation, tissue engineering, and clinical interventions to holistically interrogate and treat joint diseases and injuries in women. As her mentor, Dr. Ambrosio is fully committed and extremely enthusiastic to help her achieve this goal and to provide the research environment and training necessary for Gabrielle to excel. Her long-term research goals are to interrogate molecular and macroscopic mechanisms driving joint injuries and diseases in women, use these mechanisms to inform clinical interventions, and use these clinical interventions to propel women back to movement. In this F30, Gabrielle is investigating molecular mechanisms driving menopause-induced knee osteoarthritis and exploring novel gene therapy options as a treatment modality. Her work is addressing the long-neglected facts that post-menopausal women present with more severe knee osteoarthritis than men and that rodents do not present with a menopausal phenotype. These fundamental questions will lay the groundwork for understanding and addressing osteoarthritis for half the population. This detailed training plan was written by Gabrielle with frequent input from Dr. Ambrosio to ensure that she has the structure and support necessary to achieve her research, training, and professional goals. Together, we are confident this plan will lay the foundation for Gabrielle's career as an orthopedic surgeon-scientist equipped to tackle research questions related to joint injuries in women from all levels.

C1. Intellectual training and environment: Even prior to joining the Ambrosio laboratory when at the University of Pittsburgh, Gabrielle has had extensive training in biomechanics and -omics analyses. However, to be optimally positioned to achieve her ambitious long-term career goal of directing an NIH-funded multidisciplinary center for orthopedic research, the formalized and comprehensive research and didactic training offered through the F30 training plan—as outlined throughout Gabrielle's application—is essential. In the Ambrosio lab at Harvard Medical School and in close collaboration with her mentoring team spanning Harvard University and the University of Pittsburgh, Gabrielle will receive rigorous scientific training in aging biology, osteoarthritis, menopause-induced pathogenesis, and synthetic biology. Harvard University and the University of Pittsburgh are an ideal training environment for Gabrielle that will provide unique research, mentorship, clinical, and educational opportunities. The Ambrosio lab is physically located within a major research building that is a part of Massachussets General Hospital (CNY149) and is just one block away from the Spaulding Rehabilitation Hospital, home of Dr. Ambrosio's faculty appointment. CNY149 also houses numerous resources available for this project, including a Molecular Imaging Center, Histopathological core, and animal facility. The Ambrosio laboratory is also located conveniently for other resources that are to be used for this project. The Silver laboratory, Wyss Institute, and Ragon Institute are all within a 10-minute bike ride.

Gabrielle has already started her comprehensive training in synthetic biology and molecular mechanisms through her completed coursework, seminars, workshops, retreats, and scientific conferences, and these activities will continue. Within the Ambrosio laboratory, Gabrielle presents work in progress at lab meetings and

leads article discussions in journal clubs both on a monthly basis. She will also present semesterly within the Silver laboratory and annually at local seminar series, such as the Women's Health Seminar, and retreats, such as the Pitt MSTP retreat. As a part of her graduate course work, Gabrielle attends and participates in weekly pathology journal clubs that are focused on discussion of rigorous experiment design for evaluating molecular mechanisms. She also regularly attends weekly and biweekly seminars on women's health, osteoarthritis, aging, and engineering principles. Lastly, Gabrielle has already presented her research in both poster and oral presentations at a variety of conferences and will continue to do so throughout her fellowship.

C2. Technical training and environment: Gabrielle's proposal combines a variety of methodologies and approaches to study menopause-induced knee osteoarthritis. This topic is well-aligned with the specific expertise available in the Ambrosio lab and with the rest of Gabrielle's mentoring team. Dr. Ambrosio will provide structured technical mentoring on experiment design, troubleshooting technical issues, result interpretation, and manuscript structure through weekly one-on-one meetings. Within the Ambrosio laboratory, a structured mentorship program is set up such that post-doctoral fellows and scientists mentor graduate students, and graduate students mentor undergraduates and technicians. Dr. Kai Wang, who is a scientist with expertise in genetic engineering and methodologies for cellular transfection, is available for troubleshooting of related experiments (***Specific Aim 2***). Dr. Zachary Hettinger, who is a post-doctoral fellow with extensive training in various microscopic imaging techniques, is available for trouble shooting imaging modalities (***Specific Aim 1*** and *Aim 2c*). Dr. Hirotaka Iijima, who is a former post-doctoral fellow that laid the foundation for knee-related studies in the Ambrosio laboratory, trained Gabrielle in processing mouse knees for histological analyses and chondrocyte culture techniques. He continues to be available via email or Zoom meetings to help with any issues that arise during this proposal (***Specific Aim 1*** and *Aim 2a-2b*). The Ambrosio lab manager, Dr. Ekaterina Creed, is a veterinarian by training who is available for troubleshooting animal-related experiments (*Aim 1b*). The Ambrosio laboratory keeps two lab technicians on staff to provide additional research support. One critical aspect of Gabrielle's training is in synthetic biology. Fortunately, the Ambrosio laboratory is located a brief 10-minute bike ride away from Dr. Pamela Silver's laboratory in Cambridge. Any synthetic biology related questions/training needed can and will be completed in this laboratory space.

C3. Mentoring to be a successful physician-scientist: Dr. Ambrosio is committed to providing focused mentoring to Gabrielle on developing high-impact research questions, testable hypotheses, and rigorous and reproducible research plans. To provide such an environment, Dr. Ambrosio and Gabrielle meet weekly for one hour to discuss these different aspects as they pertain to her project. Dr. Ambrosio is also available to meet more frequently and address quick issues via email. To completement the mentoring offered by Dr. Ambrosio, Gabrielle will have monthly meetings with her mentoring team, which includes Dr. Christopher Evans (an expert in knee osteoarthritis), Dr. Rebecca Thurston (an expert in menopause), and Dr. Pamela Silver (an expert in synthetic biology). Drs. Evans and Thurston have mentored Gabrielle since starting graduate school, and Dr. Silver, although relatively new to Gabrielle's mentoring team, has already provided ample support. For example, Dr. Silver connected Gabrielle to synthetic biology groups and seminars within Boston and helped develop the experiments outlined in this proposal. Detailed manuscript feedback will be provided by all members of her mentoring team via email, and all mentors are available to meet more frequently. Gabrielle will also have semesterly meetings with her career advisor, Dr. Gwendolyn Sowa, and her thesis committee.

In addition to research training, it is critical that Gabrielle receive training and exposure to other aspects of being a physician-scientist. One such aspect is mentoring students. Gabrielle currently mentors one technician, one undergraduate student, and one high school student within the Ambrosio laboratory and will continue to do so throughout this fellowship period. With the Ambrosio laboratory's move to Harvard University, Gabrielle has also received exposure to lab set up, project planning, lab management skills, and hiring processes, which will be critical for her as a future principal investigator. In addition, since Dr. Ambrosio is the Inaugural Director of the Atlantic Charter Discovery Center for Musculoskeletal Recovery, Gabrielle will receive exposure to the process of building an academic center, which will be helpful for her long-term goals of building and leading a women's movement center.

Lastly, it is highly important for Gabrielle to continue receiving clinical training throughout this fellowship. She has already completed one clinical clerkship in Pediatrics and one Longitudinal Clinical Clerkship (LCC) in orthopedic surgery under the mentorship of Dr. Volker Musahl, a highly successful orthopedic surgeon-scientist. To continue this training while in Boston, Dr. Musahl has connected Gabrielle to Dr. Miho Tanaka, who is the Director of the Women's Sports Medicine Program at MGH and a practicing orthopedic surgeon. Gabrielle will continue shadowing Dr. Tanaka monthly throughout this fellowship. She will complete her second LCC under the mentorship of Dr. John Fowler, another successful orthopedic surgeon-scientist at the University of

Pittsburgh, by traveling back to Pittsburgh for a 10-day period. While in Pittsburgh, she will also continue to volunteer at the Women's Clinic and Shelter of Greater Pittsburgh. She is also continuing her clinical research with Dr. Sowa. After completion of her PhD training in Boston, Gabrielle will return to Pittsburgh to finish the third and fourth years of medical school.

C4. Presentations: Gabrielle has already generated high-quality research within the first year of being a full-time student in the Ambrosio laboratory. She has had poster presentations at three international conferences and two local conferences, winning first place for the best poster award at the Regenerative Rehabilitation Symposium in 2022. Gabrielle has also given an oral presentation at Physiatry '21 to a national audience of experts in physical medicine and rehabilitation. Over the course of this training period, Gabrielle will continue to build on her oral presentation skills by presenting monthly at Ambrosio lab meetings and journal clubs as well as mentor meetings. Additionally, she will present semesterly to the Silver laboratory and annually at a variety of seminars, retreats and conferences focused on osteoarthritis, aging, menopause, and synthetic biology. These conferences are specifically outlined in the Training Plan but include Osteoarthritis Research Society International (OARSI) World Congress on Osteoarthritis, Synthetic Biology: Engineering, Evolution & Design (SEED), and the North American Menopause Society Annual Meeting. At these meetings, Gabrielle will submit an abstract for oral presentation and will also plan to present a poster. While oral presentations will provide valuable experience speaking to large, unfamiliar groups, presenting a poster will be valuable for helping Gabrielle gain experience in how to articulate her project succinctly and clearly.

C5. Course work and didactic training: Gabrielle has already completed the coursework required for students in the MSTP at the University of Pittsburgh, and she will complete the last of her graduate coursework through the Cellular and Molecular Pathology (CMP) graduate program in the spring of 2023. This course work entailed training in ethics, synthetic biology principles, mathematical modeling, quantum mechanics, bioinformatic statistical analyses, and molecular mechanisms of tissue growth and differentiation. Thus far in her graduate course work, she has maintained a 3.94 GPA. In addition to these required courses, during this fellowship period, she will continue to participate in the University of Pittsburgh's MSTP workshops and the weekly Ambrosio laboratory and CMP journal clubs. She will also attend a variety of seminars focused on orthopedics, women's health, engineering principles, and aging, both at Harvard University and the University of Pittsburgh. To maintain her clinical reasoning throughout graduate school, Gabrielle is also connected with Harvard University's MSTP, and will continue to attend the Clinical Problem-Solving Cases. This didactic training coupled with Gabrielle's undergraduate coursework in chemical engineering will provide her with the groundwork for successful completion of this proposal.

C6. Research ethics: Gabrielle has previously been trained in the responsible conduct of research through her medical school and MSTP coursework, online module training both for her clinical work and research, and through workshops hosted in the Ambrosio lab. Although Gabrielle has completed all her ethics related course work, she continues to educate herself on the subject matter by attending virtual monthly University of Pittsburgh Clinical and Translational Science Institute (CTSI) lectures on a variety of topics, ranging from preprints to informed consent. Within the Ambrosio laboratory and Pitt MSTP, workshops on responsible conduct of research will continue to be held on a regular basis, and Gabrielle will stay up to date on her online training modules. In addition, at the University of Pittsburgh, CTSI offers one-on-one meetings and training for specific questions related to research projects, and Gabrielle has already and will continue to utilize this resource.

C7. Assessment of training progress: A rigorous assessment plan has been defined for Gabrielle, which includes publications, presentations at national and international conferences, awards, and success in mentoring her own mentees, which currently include one high school student, one undergraduate student, and a laboratory technician. Consistent with Gabrielle's track record in the laboratory to date, we have agreed on a publication plan targeting one first author paper and 2-4 co-author publications per year. Though these benchmarks may seem ambitious, they are consistent with Gabrielle's performance to date as well as the manuscripts that are currently in various stages of submission. Dissemination of research findings is also a primary aspect of the evaluation of training progress. Our agreed upon plan for Gabrielle's oral presentation skill development is outlined above in the Presentations section.

Within the first year of her fellowship, Gabrielle will form her thesis committee. Per departmental guidelines, this committee will be composed of her primary mentor (Dr. Ambrosio), four departmental experts on an aspect of her project, and an external member, likely Dr. Rebecca Thurston. The committee will meet semesterly to ensure Gabrielle successfully completes her doctoral training and the goals outlined in this fellowship.

C8. Research facilities: Gabrielle will have access to all the needed equipment and facilities to complete the

aims as proposed. The Ambrosio Laboratory is located on the 5th floor of Building 149 at Mass General Hospital in the Navy Yard, Boston, MA (CNY149). This space occupies 1614 sq feet of wet and dry laboratory space including a cell culture room and four lab benches. The following is a breakdown of said space: General Lab and Cell culture (312sqft); General lab space (833sqft); Microscopy and Imaging room(110sqft); Molecular biology room (128sqft), Histology room (231sqft). *Shared facilities:* Refrigerator/Freezer room, glass/autoclave room, cold room, conference rooms, shared equipment room, and a library. All animal experiments will be conducted at the MGH animal facility. This facility is equipped with a complete surgical center, and the animal care space and is a satellite facility of and overseen by Mass General Hospital Animal Resources. *Computers:* Gabrielle and Dr. Ambrosio each have a computer that contains Windows 10 64-bit processor, 4 Core Intel Xeon W-2123 3.6 GHz, 32 GB of RAM, and AI RTX 4000 graphics with 14" screens for data analysis. Each large piece of equipment (e.g., Nikon confocal microscope) is equipped with its own desktop computer of similar specs as outlined above. Gabrielle has access to all the computational resources available at both the University of Pittsburgh and Harvard, including SPSS and EndNote. Our lab also has 2 TB of storage space and a color laser jet printer and 9800 Microtek scanner.

D. Number of Fellows/Trainees to be Supervised During the Fellowship

Faculty	Trainee Name	Training Period	Prior Degrees	Support
Ambrosio	Kai Wang	2019-present	PhD	2P2CHD086843
Ambrosio	Zachary Hettinger	2021-present	PhD	R01AG061005
Ambrosio	Post-doctoral fellow (TBN)	2023-	PhD	R01AG066198

E. Applicant's Qualifications and Potential for a Research Career

Gabrielle Gilmer (Gabby) is exactly the type of exceptional doctoral student the NRSA F30 predoctoral fellowship awards were designed to support. I have known Gabby for almost two years now, and I have found her to be among the most motivated, hard-working, bright, and creative students with whom I have ever worked (top 1% of all my trainees). Gabby is currently enrolled in the MD/PhD program at the University of Pittsburgh, where she began her research training with me in 2021. When I was recruited in September 2022 to join the Harvard faculty through the Department of Physical Medicine & Rehabilitation and to direct the Discovery Center for Musculoskeletal Recovery, I was thrilled when Gabby accepted my offer to join us in Boston. Gabby has already completed the first two years of medical school, and her plan is to finish her MD degree in Pittsburgh once she has successfully defended her PhD thesis in Boston with me. I have been working closely with her MSTP director, Dr. Richard Steinman, to ensure that Gabby's training plan is smooth and seamless. Now, two months following our move, our lab is already nearly fully functional, and Gabby has been advancing her proposed studies without delay.

Gabby initially reached out to me in the first year of her MD/PhD program to discuss the possibility of completing a rotation in my lab. What immediately struck me was Gabby's crystal-clear vision for her future career aspirations. Specifically, Gabby articulated to me her long-term goal of leading a research center focused on joint injuries and diseases in women that comprises musculoskeletal investigators whose studies span basic to translational science. She already had experience with biomechanical studies during her undergraduate training as well as reductionist research working at the NIH. This latter experience allowed Gabby to gain valuable experience in proteomics, which she has already extended to her doctoral studies.

Gabby scheduled a rotation in my laboratory for the summer of 2020. Before, during, and even after her lab rotation, Gabby consistently demonstrated herself to be a force of nature. She immediately jumped into the research projects she was assigned, working incredibly efficiently and already establishing herself as an extremely reliable and productive lab member. She officially started her PhD training last summer (2021) and has continued to progress at a meteoric pace. As a testament to her hard work, she is already 2nd co-author on two manuscripts. The first manuscript, published in the *Journal of Gerontology: Biological Sciences*, took a multi-scale omics approach to identify hallmarks of cartilage degeneration according to age and sex. For this work, Gabby's primary contributions consisted of performing a systematic review and bioinformatic analysis. She also did a considerable amount of the writing. The second paper, just accepted at *Nature Communications,* evaluated age- and sex-dependent molecular mechanisms underlying knee osteoarthritis. For this paper, Gabby was responsible for all aspects of the mass spectrometry analysis, including sample collection; designing, troubleshooting, and optimizing the protocol in collaboration with a core facility at CalTech; as well as data analysis and interpretation. Gabby also played a major role in other aspects of data collection, including repeating

histological analyses in female mice, as well as writing. These papers are an incredible accomplishment for a student who, for most of the study period, was juggling other responsibilities such as medical school classes, leadership roles in various societies, and even rotations in other labs.

Gabby also has another first author paper that was submitted to *Osteoarthritis & Cartilage* that presents a mathematical model aimed at evaluating the effects of timing of initiation of estrogen treatment on cartilage degeneration. The first round of reviews (received November 2, 2022) were exceedingly positive, with reviewers stating: (Reviewer 1) "*Overall, this review addresses important aspects and gaps that should be considered in future studies involving menopausal animal models in OA*," (Reviewer 2) "*This is a topic of importance to the osteoarthritis research field and highlights multiple key gaps between the preclinical model and the clinical situation. The analysis is very well done and highlights several key points in the rigor and reproducibility in the field*," (Reviewer 3) "*It is great to see a meta-analysis of animal studies, these are highly needed in the field I think. Submission of the code is also fantastic. Overall I think that this is a valuable study*." Because Gabby took the initiative to write this paper above and beyond the other projects she had been working on, she and I are co-corresponding authors. Only minor revisions were requested for this paper, and Gabby will be submitting the revised manuscript by early December 2022.

Gabby's current research project exemplifies her commitment to developing novel engineering technologies. Specifically, her project aims to design and create a menopause-induced genetic controls circuit. Menopause-associated diseases are understudied while at the same time becoming increasingly prevalent as our population ages. While the circuit Gabby is designing will have many applications, we are building off our previous work to focus on incorporating this control loop into chondrocytes to prevent, delay, and potentially even reverse menopause-induced knee osteoarthritis. Our design approach will be informed by the natural progression of knee osteoarthritis and incorporate systems biology bioinformatic and proteomic analyses for gene product selection. Synthetic circuit design is a new technique in our lab, and Gabby is committed to incorporating this new skillset into our group and building collaborations with her mentoring team.

Our group is highly committed to well-rounded training experiences that are designed to stimulate creativity and boldness in generating new ideas. In what we have called the Innovation Hero Series, one laboratory group member is invited to share their own 'Innovation Hero', highlighting unique traits about their chosen individual, such as background, tribulations, motivators, and responses to failure. Not only have these examples served as valuable lessons towards our personal growth, but we have also found these sessions to be a powerful team-building experience, as they often reveal personal interests of our group members.

With great ideas and findings comes an even greater need to effectively disseminate the ideas and findings to a larger audience. While the importance of spoken and written communication skills is indisputable, there are arguably too few opportunities for doctoral student and fellow training in these areas. To address this gap, we have created and launched the Craft of Writing session. These sessions are a series of conversations in which our group "explores the writing process through a lens of creativity, defines artistic elements in our work, and cultivates tools for evaluation and reflection that are rooted in artistic research and practice." Topics that have been a part of the Craft of Writing sessions have included: *Narrowing: musical elements in writing, defining, finding, and incorporating*; *Broadening: literary elements in music, including leitmotif and idee fixe and the narrative thread to cure abstraction*; *Self-reflection: finding growth in the folds of the spiral*; and more. While these series started before Gabby joined our lab, she has stepped up to help organize these and other group activities, including providing lectures to our group on topics that span rigor and reproducibility, authorship standards, and mindfulness. I feel incredibly lucky to have Gabby as a part of our group, and I am confident that, in the future, her own trainees will thrive under her leadership. Her exceptional mentoring skills have already been on full display as she has mentored students ranging from high school to post-bac. In fact, her passion for her work has already inspired her mentees to pursue research careers of their own.

In the long-term, this collective training experience offered through the F30 pre-doctoral fellowship will provide Gabby with a unique skillset that will potentiate an enduring pursuit of cutting-edge and transformative research. Gabby certainly has the makings of an outstanding physician-scientist. She is smart, hardworking, curious, and collaborative—all to the n^{th} degree! I am confident that under my mentorship, her mentoring team, and through the MSTP programs at the University of Pittsburgh and Harvard University, she will flourish. I look forward to watching Gabby grow, as she is on a sure path to becoming an international leader in her field.

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Director, Rehabilitation Medicine Research Center
Director, Musculoskeletal Gene Therapy Research Laboratory
Department of Physical Medicine & Rehabilitation

8th December 2022

RE: Gabrielle Gilmer Application for NRSA F30 Grant (PA-21-049)

Dear NRSA Review Committee:

I am very happy to write this letter confirming my unequivocal support for the F30 application of Gabrielle Gilmer ("Gabby"), entitled "*Menopausal Knee-ds: Elucidating mechanisms and treatments for knee osteoarthritis*". She is a brilliant MD-PhD student who, in my opinion, is certain to become a future national and international leader as a clinician-investigator in the field of aging, women's health and osteoarthritis.

I know Gabby well because I serve already as a member of her PhD thesis committee. This involves regular meetings by zoom with additional, *ad hoc* interactions via email. Once Gabby obtains F30 support, we plan to have mentoring meetings every month, and I will maintain my willingness to meet more frequently with her and exchange emails as needed.

Gabby's research sits at the intersection of aging, women's health and osteoarthritis; despite just beginning her research career, she has already made substantial contributions to our understanding of this complex interface. Although it has been appreciated for decades that osteoarthritis is a disease of aging, nearly all pre-clinical research on osteoarthritis has been conducted in young animals where osteoarthritis is induced by injury to the joint. This induces post-traumatic osteoarthritis, which is distinct from the idiopathic osteoarthritis that occurs as a result of aging and is the major form of the disease seen clinically. Gabby has made a major contribution to research showing that changes in gene expression occurring during post-traumatic and aging-related osteoarthritis overlap by only 3% (Iijima, H., Gilmer, G. *et al.* Aging and knee osteoarthritis in murine studies: integration of meta-analysis and bioinformatics to elucidate disease mechanisms. *Journal of Gerontology: Series A.* 77: 1321–1334, 2022*).* This insight directs attention towards the neglected area of aging, as opposed to injury, as the main driver of osteoarthritis and highlights the need for more research in this area. In conjunction with this, Gabby's research has also underlined the importance of aging-related changes in the mechanical properties of the cartilaginous matrix as an important component of the etiopathogenesis of osteoarthritis (Iijima, H., Gilmer, G. *et al.* Epigenetic regulation of α-Klotho by age-related matrix stiffening drives cartilage degeneration. *Nature Communications* Provisionally accepted for publication). Given my extensive expertise in knee osteoarthritis, I was a collaborator, co-author, and mentor for both Gabby and Hirotaka, the lead post-doctoral fellow, in both of these projects.

The project that forms the basis of this F30 application has its origins in another component of Gabby's studies, which address the effect of sex and age on the incidence of osteoarthritis. It is well established that the incidence of human knee osteoarthritis increases dramatically after menopause such that the incidence of osteoarthritis in post-menopausal women is approximately twice that of men of a similar age. However, in mice, the most commonly used species in pre-clinical studies of osteoarthritis, females get much milder osteoarthritis than males. Indeed, this is the reason why most murine studies of osteoarthritis use male mice despite the human clinical picture. (Another disconnect that Gabby's research has highlighted).

Gabby's research has provided a plausible mechanism why older, female mice are protected from osteoarthritis – they do not undergo the equivalent of human menopause. Instead, female mice rejuvenate their ovarian follicles which continue to produce estrogen in middle and old age. In view of this, Gabby has developed a very sophisticated new approach to induce the equivalent of human menopause in mice. It involves the injection of 4-vinylcyclohexene diepoxide to deplete estrogen without also producing the additional, confounding changes induced by the blunt instrument of ovariectomy. Gabby has shown that mice treated with 4-vinylcyclohexene diepoxide have more severe osteoarthritis than age-matched controls. She has also developed a mathematical model that implies estradiol protects against osteoarthritis in mice lacking estrogen. Her explanatory hypothesis, to be tested in Specific Aim 1, focuses on estradiol-regulated ubiquitin proteolysis. This hypothesis is novel and evinces great insight. Specific Aim 2 will develop a genetic circuit whereby a reduction in estradiol turns on the expression of therapeutic genes. As someone with considerable interest and expertise in the use of gene therapy to treat osteoarthritis, I find this aim intriguing and am excited to mentor Gabby specifically in the use of gene therapy for osteoarthritis, along side Dr. Pamela Silver, who has expertise in synthetic biology in application to mammalian cells. Although the combination of these aims may seem ambitious, the combination of Gabby's extensive track record and exceptional mentoring team set her up for success to meet the challenges associated with this proposal.

Inspection of Gabby's c.v. reveals that she is a student with outstanding potential. She graduated *Summa Cum Laude* in Chemical Engineering at Auburn University and was accepted into the highly competitive MD-PhD program at the University of Pittsburgh School of Medicine. She has won prizes for student research, and has availed herself of a wide range of research exposures at the University of Pittsburgh, NIH, and Auburn University. Remarkably for someone at her stage of career, she is an author on over 25 peer reviewed publications; this is phenominal. That she has achieved all of this while engaged in a multitude of volunteer and teaching activities speaks to her industry, commitment and drive.

From my experience as a member of Gabby's mentoring committee, I can confirm that she is highly intelligent and, importantly, able to get things done. Her enthusiasm is without question, and I would rank her within the top 1-2% of PhD and MD-PhD students I have mentored or co-mentored. She comes with my highest recommendation.

Yours Sincerely,

C. H. Evans Ph.D.
John and Posy Krehbiel Professor of Orthopedics

HARVARD MEDICAL SCHOOL | BLAVATNIK INSTITUTE SYSTEMS BIOLOGY

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December 8, 2022

Dear NRSA Review Committee,

It is with great enthusiasm that I provide my strongest support for Gabrielle "Gabby" Gilmer and her F30 fellowship submission on "Menopausal Knee-ds: Elucidating mechanisms and treatments for knee osteoarthritis". I am a Professor of Biochemistry and Systems Biology at Harvard University, a founding core faculty of the Wyss Institute at Harvard University, and Director of the Harvard University Graduate Program. Additionally, I run the Silver Lab, which is a grant funded laboratory focused on applying synthetic biology techniques to therapeutic design, cellular programming, and environment and pathogen sensing. As such, I very intimately understand the challenges associated with synthetic biology applications in mammalian cells, and I am uniquely positioned to mentor Gabby as she navigates these applications specifically to knee osteoarthritis and menopause.

In addition to my expertise in synthetic biology, I also have an extensive track record of mentoring graduate students and post-doctoral trainees, and mentoring is a pillar to my commitment to advancing science and synthetic biology. Specifically, of the nearly 90 trainees from my lab, 8 (6 female/2URM) PhD students and 24 (11 female/2 URM) post docs now hold faculty positions at academic research universities. All others are currently employed in science related careers in either private industry, government or NGOs.

Although Gabby and I have only recently come to know each other, I am already very committed to her training and development as an independent scientist. This is evidenced by my assistance in connecting her to the synthetic biology community here in Boston by inviting her to attend our Celebration of Synthetic Biology local conference and join our Hive community group. It is already evident to me that Gabby is a thoughtful scientist with an exceptional work ethic and an impressive amount of resilience. I am truly thrilled to have the opportunity to work closely with her through this fellowship.

As a member of Gabby's mentoring team throughout this fellowship, I will continue to connect Gabby to members of the synthetic biology community that may be helpful in navigating her project. Additionally, I will attend monthly mentoring meetings with her and the rest of the team to provide critical feedback on experimental design, technical issues, and developing results. Specifically, I will provide suggestions for considerations and programs for accurate promoter design, other methods for plasmid transfection in mammalian cells, and alternatives to achieving stable copy numbers as well as controllability of the circuit in chondrocytes. I am also more than happy to meet with Gabby more frequently as needed and exchange ideas and thoughts over email. In addition, when training in specific synthetic biology techniques may be helpful, I will welcome Gabby into my own laboratory to train under some of the senior members, which is located quite close to Fabrisia's lab (Gabby primarily bikes, so a short 10-minute ride for her). Lastly, I have invited Gabby to present semesterly at my own lab meetings as well as at the Hive Community Group discussions so that she can receive feedback from other synthetic biology peers.

Gabby and Fabrisia have designed an exciting fellowship project that has the potential to provide both an exceptional training environment for a physician-scientist and an impactful scientific contribution to applying synthetic biology to menopause-induced knee osteoarthritis. Although the experiments outlined here are expansive, my expertise in mammalian synthetic biology coupled with my track record of mentoring will enhance the phenomenal training Gabby will receive as an MD/PhD student here in Boston.

Please do not hesitate to reach out with any questions.

Sincerely,

Pamela Silver, PhD
Elliott T. and Onie H. Adams Professor of Biochemistry and Systems Biology
Harvard Medical School

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December 8, 2022

To the Review Committee:

I am writing in strong support of Gabrielle Gilmer's application, "Menopausal Knee-ds: Elucidating mechanisms and treatments for knee osteoarthritis" for an F30 (PA-21-049) Individual Predoctoral Fellowship. Gabrielle is an exceptionally promising physician-scientist who is well-positioned to undertake high-impact research in the area of sex, gender and joint injury and rehabilitation. I find her program of research and her F30 research well-designed, innovative, and high-impact. With this support, she is poised to become an exceptional future physician-scientist.

Ms. Gilmer has a remarkably strong background and research experience. She obtained her bachelor's degree in Chemical Engineering *Summa Cum Laude* at Auburn University, where she carried out an honors project entitled *Evaluation of the relationship between hormonal, biomechanical, and neuromuscular risk factors for anterior cruciate ligament injury*. Her undergraduate work resulted in a striking 20 publications, eight of which are first authored. She subsequently pursued post baccalaureate research at the National Institutes of Health and next entered the MD / PhD program at the University of Pittsburgh Medical School. At the University of Pittsburgh under the primary mentorship of Dr. Ambrosio, she has been investigating the mechanisms of sex differences in age-related knee osteoarthritis (KOA). This work has underscored the importance of the menopause transition and sex hormones in women's joint health. She has developed a novel chemically-induced menopause model that advances beyond traditional ovariectomy models that induce an abrupt change in sex hormones in the context of young animals. She instead implements a method that produces more gradual ovarian senescence among older mice that more closely mimics natural human menopause. This model represents a major advance in the basic science of female reproductive aging and its impact on health. She has gone on to next develop a mathematical model of estrogen treatment for cartilage which has underscored the importance of early estrogen treatment, a finding that parallels findings about hormone therapy in other physiologic systems. Ms. Gilmer has been exceptionally productive even at this early stage, authoring 25 publications, 10 of which are first-authored.

Her proposed F30 research builds upon this strong foundation of work. In her proposed research, she will utilize her chemically-induced menopause mouse model to investigate the role of menopause induction in ubiquitin proteolysis activity and test whether estradiol treatment started early in menopause will restore ubiquitin proteolysis signaling. She also plans to design a genetic controls circuit that compensates for menopause-driven molecular perturbations within chondrocytes; this research will inform initial steps towards gene-therapy treatments for menopause-associated KOA. Her work is programmatic, well-formulated, and high-impact. As a member of Ms. Gilmer's mentoring team, I will meet with her monthly during all years of the award period to provide oversight and training in the menopause transition, hormone therapy, and in the translation of her basic science research to menopause research and clinical care in women. I am also available to meet more frequently if needed and exchange ideas and manuscript edits over email.

My background and experience in the menopause transition serves me well in performing this role. I am a Principal Investigator (PI) of the NIH U19-funded Study of Women Across the Nation (SWAN), the largest multi-ethnic 25-year cohort study of women transitioning through the menopause; SWAN has provided seminal information about the menopause transition and its implications for women's health as they age. Further, I am a PI of the MsHeart (R01HL105647, PI: Thurston) and MsBrain studies (RF1AG053504/R01AG053504: mPIs: Thurston & Maki), two studies that investigate the menopause transition, its hormonal changes and symptoms, and the implications of these menopause-related changes for women's cardiovascular and brain health. I have published over 170 peer-reviewed publications on women's health and the menopause transition. I am Past President of the North American Menopause Society, the premier medical society devoted to menopause science and care, and I am recipient of the top award for distinguished contributions to the science of

menopause globally, the Henry Burger Prize from the International Menopause Society. Further, I have co-authored *The 2022 Hormone Therapy Position Statement of The North American Menopause Society*, the leading clinical guideline regarding the use of hormone therapy during the menopause. Finally, I am a fellow of both the Academy of Behavioral Medicine Research and American Psychosomatic Society. This background and experience will serve me well in helping to train Ms. Gilmer on menopause-related research.

I also have a strong track record of mentoring. To date, I have mentored 42 trainees across disciplines, including four who have recently obtained NIH funding (e.g., K23 and R01 grants). I serve as a Director of the NIH T32-supported (T32HL007560, PIs: Thurston & Gianaros) *Cardiovascular Behavioral Medicine Research Training Program*, a 40 year-long NHLBI T32 training program. Further, I am a member of 10 additional T32 / KL2 training programs. Finally, I am PI of a NIH K24 award focused on interdisciplinary training in women's health research. This award recognizes excellence in mentoring and provides me with protected time and resources to provide high-quality interdisciplinary mentoring to trainees in women's health.

In summary, Ms. Gilmer is an exceptionally strong physician-scientist trainee and scholar. She has a clear track record of productivity, ability to design and execute rigorous research, and a passion and commitment to improving the joint health of women. I hold her in the highest regard and have no doubt of her future success. With this F30 support, she is poised to become an exceptional physician-scientist who will make breakthroughs in the area of sex, gender and joint injury and rehabilitation.

Sincerely,

Rebecca C. Thurston, PhD, FABMR, FAPS
Pittsburgh Foundation Chair in Women's Health and Dementia
Professor of Psychiatry, Psychology, Epidemiology and Clinical and Translational Science
Director, Women's Biobehavioral Health Laboratory
Director, Cardiovascular Behavioral Medicine Research Training Program
University of Pittsburgh

DESCIPTION OF INSTITUTIONAL ENVIORNMENT AND COMMITMENT TO TRAINING

Written by Richard Steinman MD PhD, Director MSTP at University of Pittsburgh, Steinman@pitt.edu

Although Gabrielle will complete her PhD training with Dr. Fabrisia Ambrosio at Spaulding Rehabilitation Hospital/Harvard Medical School, she remains a full-time student at the University of Pittsburgh and member of Pitt's MSTP. As such, MSTP structure and curriculum remains an integral part of her training. Details on resources available during Gabrielle's PhD training are outlined in the Facilities and Other Resources section.

A. University of Pittsburgh-Carnegie Mellon University MSTP Structure. MSTP students in Pittsburgh complete a MSTP-specific enrichment curriculum beyond the standard courses in medical and graduate school. This consists of 3 summer research rotations, 3 summer professional development courses, a 3-semester weekly journal club featuring research papers consistent with the coincident SOM curriculum, a 4-week case-based ethics course, a monthly program-wide workshop, a 40-week longitudinal clinical clerkship (1/2 day/week) during the graduate years, a 11-day Junior Hospitalist service and yearly special events such as the two-day MSTP Scientific Retreat.

B. Laboratory Research Rotations. Research rotations begin the summer prior to the start of medical school. In addition to developing manuscripts and presenting at scientific meetings based on their rotation results, all students turn in a written scientific report that is reviewed by MSTP leadership and present their work at the annual MSTP Scientific Retreat. The choice of thesis laboratories by students is informed by their rotation history and by discussion with their individual Career Advisors (who follow them longitudinally in the program).

C. Professional Development. Students take three successive 10-week long Professional Development Courses during summers prior to starting graduate school. The first course (PD1) focuses on scientific writing and introduces students to biomedical software and to key methods used by different disciplines to approach scientific problems. The PD2 course focuses on scientific design and career development strategies, with particular emphasis on reproducibility and biostatistics. The PD3 course focuses on grant review and writing.

D. Training in Reproducibility in Science. The PD2 course focuses on optimizing reproducibility of findings, to power experiments, and analyze data with appropriate statistical testing. Topics for classes include problems arising from non-reproducible work, optimal experimental and reagent documentation, and handling, the ARRIVE guidelines for animal work, measurement validity and sources of error, robust hypothesis testing, and a series of sessions on biostatistics including customized problem solving tied to student data. In addition to this course, each thesis student is required to give a lab meeting on rigor and reproducibility as pertains to his or her project and the field broadly. The laboratory environment is highly collaborative in a program that strongly values student support and diversity. In keeping with his rigorous training, Gabrielle has already led an Ambrosio laboratory meeting focused on rigor and reproducibility as it applies to her research.

E. Biomedical and ethical expertise. During MS1 and 2 years, students build biomedical knowledge through a 3-semester MSTP literature review course in which students' present papers after formal consultation with local faculty experts in the field of that paper. During the G1 year of graduate school, MSTP students take a month-long, weekly, case-based research ethics course. Throughout both medical school and graduate school, all MSTP students meet monthly for student-arranged seminars that pose scientific, logistical, clinical and/or ethical dilemmas. Students and/or guest faculty experts present these workshops.

F. Clinical and Research Integration. This is a central focus to better model the physician scientist career.

F.1 Clinical Activities During the Research Years. Prior to starting graduate school, all MSTP students complete 8 weeks of required clinical core clerkships. This front-loads requirements once students re-enter medical school post thesis and enables research engagement in MS3 and 4. MSTP students are required to complete a (credited) minimum of two 20-week long Longitudinal Clinical Clerkships during graduate school. For each LCC, students spend a half day per week with a clinician scientist receive one-on-one clinical mentoring by a clinician scientist in an area of interest chosen by the student with guidance from the MSTP LCC director, Paul Monga, MD. Student objectives for the LCC and write-ups at the end are reviewed by MSTP leadership. In Gabrielle's case completing her PhD training in Boston, she will complete a "compact" LCC with a 10 workday / two week LCC in Pittsburgh. This situation has been arranged and discussed extensively with Paul, and Gabrielle has already identified that her second LCC will be completed with John Fowler, MD, who is an exceptional orthopaedic surgeon at the University of Pittsburgh. To maintain the more "longitudinal" aspect of clinical exposure while completing her PhD in Boston, Gabrielle has arranged to shadow Miho Tanaka, MD, who is a close colleague and fellow orthopaedic surgeon of her first LCC mentor, Dr. Volker Musahl.

F.2 Transition from Graduate to Clinical Years. After the student's thesis defense but prior to returning to medical school, students take the MSTP required Junior Hospitalist Service, also known as the LCC3. A master

clinician mentors the returning students for 11 days as they examine, discuss, diagnose and plan treatment for surrogate patients presenting with common outpatient or inpatient ailments.

F.3 Research During Clinical Years. Our students continue their research focus after re-entry to medical school generally in four ways: (1) MS3 and MS4 students continue to plan and execute MSTP Workshops that feature research topics and research challenges to be discussed with MSTP peers. (2) Students complete formal reflective and goal-oriented self-assessment evaluations during twice-yearly Career Advisor meetings. (3) Students average 2.8 new publications during the MS3 and MS4 years (at least one first authored), averaging 5-7 papers upon graduation. (4) Most students elect to take 1-2 Research Elective months during their MS4 year to extend findings of thesis work and/or to build skillsets in a translational area. Another novel feature of our MSTP, the Postdoctoral Fellowship, provides support for 5 months of postdoctoral research prior to residency for MSTP students graduating in December (25% of graduates in recent years). Applications address research hypotheses and aims, career development aims, planned deliverables, mentor fit and intellectual goals.

G. Monitoring and Evaluating Student Progress. Prior to matriculation, the Program Director assigns each new student a Career Advisor based on matching research interests who helps orient and guide the students throughout their careers. Most of a trainee's time in the graduate program is spent in research training under the guidance of their research mentors, program leadership, and eventually their doctoral dissertation committee. To customize advice and resource allocation, all MSTP students complete and share *individual development plans* with the Director and with their Career Advisor. The form allows students to identify specific skills that they want to develop, set technical, intellectual, and professional goals, and identify how goals will be achieved and measured. Resources to reach goals and obstacles that could compromise success are discussed. Progress toward goals is regularly reviewed with the Advisor and new goals are set.

H. Career Counseling. In addition to biannual meetings with their Career Advisor, each MSTP student benefits from individualized Executive Coaching sessions with professional Career Coaches. These meetings during G2 and MS4 focus on building resiliency, decision strategies aligned with students' strengths and interests, time management and conflict resolution skills.

I. Program Duration and Outcomes. Over the past 10 years, our time from enrollment to graduation has averaged 7.65 years. The Pittsburgh MSTP has 218 alumnae. 81% of graduates from the past 10 years are in the academic pipeline (either still in training or in academic positions). Senior MSTPs graduating over the past 3 years have averaged 9 total and 4 first-authored manuscripts from medical schoolwork (median 6.0 total, 4.0 first-authored).

J. Gabrielle Gilmer is a superb member of our MSTP who matriculated into the MSTP program in June 2019 and is in her G2 year as a graduate student in the Cellular and Molecular Pathology (CMP) Program. She is pursuing her doctorate in the laboratory of Dr. Fabrisia Ambrosio, an internationally recognized expert in the novel fusion field regenerative rehabilitation. Gabrielle's stellar performance in the MSTP to date is described in the Letter of Recommendation from the MSTP Director.

Gabrielle completed her MS1 and MS2 coursework and passed USLME Step 1 in April 2021. She completed the MSTP Professional Development courses, 3 laboratory rotations, 3 Research Basis of Medical Knowledge courses, and an 8-week clinical rotation in Pediatrics prior to beginning graduate training. Gabrielle formally entered the CMP graduate program in the fall of 2021. She has already distinguished herself with four peer reviewed publications, two first-authored papers under review, and an oral presentation at a national conference. The coursework for CMP program consists of a total of 5 courses including Molecular Mechanisms of Tissue Growth and Differentiation and Pathology Seminar as core courses and 4 elective courses.

The CMP has several program milestones with timelines adjusted for expeditious completion by MSTP students. Gabrielle completed her Comprehensive Exam in November 2022, where she prepared an F30-style grant and defended the proposal in front of pre-selected training faculty. Following this, Gabrielle is formulating her thesis committee and will propose the aims that she will complete to fulfill her PhD in June 2023 to her this committee. As a formal PhD candidate, Gabrielle's progress will be monitored at regular, biannual committee meetings and Career Advisor Meetings where her updated Individualized Development Plans will be reviewed.

Gabrielle has already completed one Longitudinal Clinical Clerkship (LCC) in orthopedic surgery and expects to complete her second LCC in summer 2023 during graduate school. She expects to defend her PhD and return to medical school in September 2025, putting her on track to complete her remaining medical school clerkships and graduate from our program in May 2027. For the terms of the fellowship proposed, Gabrielle plans to complete an additional 26 months of research, followed by clinical work for 20 months (of which 20 months would be under the purview of this F30)**.** Should Gabrielle elect the route in which she graduates medical school in December of 2026, she will then undertake the 5-month MSTP Postdoctoral Fellowship which is not included in the time of covered support requested in the current application.

PHS Human Subjects and Clinical Trials Information

OMB Number: 0925-0001

Expiration Date: 09/30/2024

Use of Human Specimens and/or Data

Does any of the proposed research in the application involve human specimens and/or data *	❍ Yes ● No
Provide an explanation for any use of human specimens and/or data not considered to be human subjects research.	
Are Human Subjects Involved	❍ Yes ● No
Is the Project Exempt from Federal regulations?	❍ Yes ❍ No
Exemption Number	❑ 1 ❑ 2 ❑ 3 ❑ 4 ❑ 5 ❑ 6 ❑ 7 ❑ 8
Other Requested Information	

VERTEBRATE ANIMALS

1. Procedures and Breakdown of Animals Required

The tissues needed for *Aim 1a* have previously been collected, thus, no new animals are needed for completion of this sub-aim. For the groups proposed in *Aim 1b*, a total of 72 mice will be utilized (12 mice per group per treatment period X 3 treatment periods X 2 groups). The Ambrosio laboratory receives up to 60 C57/BL6 mice per month free of charge from the NIA Rodent Colony for muscle related studies (20 mice per R01; age range 14-16 months and 21-24 months both male and female). In addition to collecting the muscle for the grants that provide these mice, we will use an aliquot of these mice's knees for the proposed studies. Mice will be housed at Massachusetts General Hospital (MGH) in the CNY 149 8th Floor Animal Facility. Cages will be kept in a temperature (22°-23°C) and humidity (55-65%) controlled environment with 12-hour light/dark cycle. Domes will be included in cages for enrichment, food and water access will be ad libitum, and 3-5 mice will be housed per cage. All procedures and euthanasia will be performed in accordance with MGH's Institutional Animal Care and Use Committee (IACUC).

4-Vinylcyclohexene Diepoxide (VCD) Model of Menopause

Mice will acclimate to the new environment for at least two days prior to any handling. All handling and intraperitoneal (IP) injections will take place between 4:30-6:30 am EST. For five days prior to the start of injections, investigators will place the mice in an IP injection specific mechanical restrainer and then place them back in their cage to acclimate the mice to the restrainer. Cage location within the animal facility will be randomized via a random number generator. VCD will be given at a dose of 160 mg/kg/day via IP injection using a well-established protocol. The total volume of all injections will range from 70-80 µL depending on the mouse's bodyweight.

On the morning of injections, sterile syringes and needles will be prepped using sterile technique, and one needle/syringe set will be used per mouse. Each mouse will be placed in the mechanical restrainer. Injection placement will alternate between the right and left quadrant due to the nature of consecutive daily injections. The injection site will be defined at the level of the hip or second set of nipples and cleaned with an alcohol pad. One investigator will hold the restrainer such that the mouse's tail is above her head. The other investigator will insert the needle, going no deeper than half of the needle inserted, bevel up, at a 30-40° angle. The plunger will be pulled back to ensure negative pressure prior to injection and then the material (VCD in sesame oil) will be delivered. Mice will be placed in a tall bucket temporarily for monitoring behavior and bleeding prior to being placed back in their cage. These steps will be repeated for 10 consecutive days.

Vaginal Lavage

Vaginal lavage will be performed based on established protocols to verify a menopausal phenotype in the mice used in *Aim 1b*. Briefly, double distilled water (ddH2O) will be autoclaved and stored at room temperature until collections. All vaginal cytology and lavage collections will take place between 4:30-6:30 am EST and will be performed daily starting at day 90 until the VCD injected mice became menopausal. Approximately 200 µL of ddH2O will be drawn up into two different plastic, sterile transfer pipets. Each mouse will be individually placed within the mechanical restrainer described above. Once the mouse is secure and ceases to urinate and defecate, one pipet will be used to wash the genital area. After cleaning, the other ddH2O filled pipet will be placed at the vaginal canal, with attention paid to not disturb the opening. The pipet will be gently depressed to dispense approximately 50-100 µL at the opening of the vaginal canal. In our experience as well as in previous reports, the vaginal canal spontaneously aspirates the contents. The pipet bulb will then be released, which pulls the ddH2O back into the pipet. This step will be repeated 15-20 times. After the final collection of the ddH2O back into the pipet, the contents will then be dispersed onto a clean, glass slide. Three slides will be collected per day per mouse. Each mouse will then be removed from the restrainer and placed back into their cage.

Estradiol Treatment

For these experiments, all mice will have menopause induced via VCD IP injections as detailed above. Mice will then be randomly split into one of three treatment periods (perimenopause, early menopause, late menopause) and two groups (control, experimental). With day 1 being the first day of IP injections, mice in the perimenopause group will receive daily treatment from day 75 to day 115 and be euthanized on day 115; the early menopause group will receive daily treatment from day 115 to day 155 and be euthanized on day 155; and the late menopause group will receive daily treatment day 195 to day 235 and be euthanized on day 235.

Prior to the treatment period, all mice will be given 60 mg of Nutella daily to habituate them to the treatment modality (five days total). For the experimental group, 17β-estradiol will be administered perorally in Nutella. Specifically, mice will be placed in a small bucket and served 60 mg of Nutella containing 17β-estradiol on a ceramic tile between 4:30-6:30 am EST daily during the designated treatment period. For successful dissolving, 1.12 µg of 17β-estradiol will be mixed in 0.312 µL of sesame oil, and this sesame oil mixture will be added to the Nutella. Control animals will be given Nutella in the same amount and quantity without the sesame oil/estradiol mixture. Previous studies using this method have reported physiologically relevant concentrations in estradiol over 35 days while being minimally invasive. There have also been reports that this method does not lead to significant changes in weight, but to confirm this, we will track daily weights of the mice by placing them on a scale immediately prior to consuming their daily treatment. Previous reports have also stated that the Nutella is entirely consumed within 2 minutes, and an investigator will observe the mice to confirm this is the case.

Cardiac Puncture

Mice will be placed under anesthesia with 1-4% isoflurane in oxygen in an enclosed container on our anesthesia workstation for 4-5 minutes. After the mouse appears to be anesthetized within the box (slowed breathing), the mouse will be placed supine on an operating table with an anesthesia tube over the mouth and nose. Prior to any interventions, the mouse will be confirmed to be adequately anesthetized via toe pinch and observing for any pain reactions or twitching. Once no resistance or twitching are observed, the upper and lower limbs will then be taped to the procedure table, and the thorax and abdomen will be sprayed with ethanol. Autoclaved tweezers and scissors will be used to cut the skin at the level of the xiphoid process along the border of the diaphragm. Once fully exposed, the left and right side of the ribs will be carefully cut, and the diaphragm will be removed to expose the heart. The chest cavity will be held open by autoclaved clamp scissors. A 25 5/8 gauge needle attached to a 1 mL syringe will be gently inserted to the apex of the heart into the left ventricle. Once a flash is observed, blood will be pulled from the heart into the needle and ultimately the syringe. After blood is collected, the mouse will immediately be euthanized via cervical dislocation.

2. Justifications

C57/BL6 mice were chosen due to the lack of reported adverse events with the VCD model (in comparison to rats who have reported 100% peritonitis in aged animals). Mice are also the lowest phylogenetic species that can mimic human menopause. Because mice do not display a menopausal phenotype naturally, we are able to have non-menopausal, age-matched controls. Since menopause results in changes to the entire organism, live organisms are necessary to fully model this phenotype.

3. Minimization of Pain and Distress

IP injections are a relatively low-risk procedure, but to minimize distress from the animals, we will handle the mice for 5 consecutive days prior to beginning injections so that they are used to being held. Prior to injection, the injection site will be cleaned with an alcohol swab. Since this protocol calls for multiple consecutive days of injections, we will alternate between the right and left lower quadrants for the injection site. The mice will also be carefully monitored for bleeding immediately following the injections. If bleeding is noticed, we will clean the area with an alcohol wipe. If any severe reactions or pain are noticed, including lethargy, poor grooming, 20% weight loss, or hunching, we will notify a vet immediately for discussion of a plan. If early euthanasia is deemed necessary by the vet, we will comply.

Vaginal lavage and cytology are also low risk procedures. With daily collections, it is possible for trauma to occur to the vagina and associated external genitalia. In our experience of performing this procedure on over 100+ animals, this has never occurred, so this is extremely unlikely. However, in the rare case that it does, veterinary staff will be contacted for recommendations and vaginal lavage on that particular animal will pause until cleared by veterinary staff.

In humans, estradiol treatment given late in menopause is thought to cause increased risk of a number of different diseases, including cardiovascular disease and stroke. However, given the paucity of age-related studies in estradiol treatment in mice, it is unclear whether we will see these patterns. If mice are noted to develop other health morbidities such as those listed, we will consult and work with veterinarians to identify the best solution for the mouse.

For cardiac punctures, the investigator will ensure the mouse is fully anesthetized by squeezing the foot to ensure no pain is experienced during these procedures. If a mouse shows signs of losing anesthetic effects during

procedure, the dosage of isoflurane will be slightly increased, and the investigator will pause the procedure until confirming the animal is fully anesthetized again.

4. Euthanasia

Euthanasia will be performed by isoflurane overdose followed by cervical dislocation and will be completed in compliance with the recommendations of the American Veterinary Medical Association (AVMA) Guidelines for the Euthanasia of Animals.

RESOURCE SHARING PLAN

We are committed to sharing our experimental design, methods, results, and resources with fellow scientists. Additionally, both the University of Pittsburgh and Harvard Medical School share this commitment and adhere to the NIH Grants Policies on Sharing of Unique Research Resources, Sharing Research Data, and Sharing Model Organisms.

Data Sharing Plan: We are willing to share data and associated materials with other scientists and researchers through established means. Specifically, data will be presented and discussed with collaborators and members of the field as soon as available via local seminars and talks as well as at local, regional, and national scientific meetings. Additionally, data collected through this fellowship will be published as peer-reviewed manuscripts and shared with a broader audience via press interviews through our universities.

Resource Sharing Plan: Resources generated through the fellowship, including the estradiol repressed promoter, will be dispersed broadly throughout the scientific community. Specifically, resources will be shared with collaborators and other scientists as soon as available if to be used for work that is distinct from our work. Upon publication of our findings, we will also share any resources we develop with the broader scientific community upon request. If transfer of resources requires physical movement of materials, we will facilitate this transaction through Materials Transfer Agreements with our universities. Both the University of Pittsburgh and Harvard Medical School have offices dedicated to technology transfer, commercialization of new ideas, and patent development of novel inventions. For for-profit organization requests, we will proceed using standard non-exclusive licenses.

AUTHENTICATION OF BIOLOGICAL AND CHEMICAL RESOURCES

In *Aim 1b*, we will use our 4-vinylcyclohexene diepoxide (VCD) menopause model to study the role of estradiol signaling in mediating protein ubiquitination and ultimately knee osteoarthritis (KOA). Although we and other groups have used this model before and verified a consistent and reproducible menopausal phenotype, to assure this is the case in the experiments outlined in this proposal, we will perform daily vaginal lavage and cytology using previously established methods from day 90 to menopause onset. As before, menopause will be defined as 10 days in a row of diestrus, and we expect this to occur at day 115.

One aspect of *Aims 1a-1b* is quantifying expression of components along the ubiquitination signaling pathways using immunohistochemical staining. To accomplish this, we will use the following primary antibodies:

- E1: ThermoFisher Cat. No. 15912-1-AP
- E2: Millipore Sigma Cat. No. ABE1407
- E3: ThermoFisher Cat. No. BS-9663R
- Ubiquitin: ThermoFisher Cat. No. 13-1600
- α1α2α3α5α6α7: Abcam Cat. No. ab22674
- LMP7: Abcam Cat. No. ab3329
- Rpt6: MyBioSource Cat. No. MBS9429032
- LMP2: Abcam Cat. No. ab3328
- PA28β: CellSignaling Cat. No. 2409S

Prior to beginning any experiments, we will perform western blots to verify specific binding to the target protein of interest. We will then perform titration tests (1:20, 1:50, 1:100, 1:200, 1:500, 1:1,000, and 1:2,000) on knee sections to identify the ideal concentration necessary to achieve consistent and reproducible stains with minimal batch effect.

A central component to *Aim 1b* is treating a subset of our VCD mice with estradiol. To both verify that estradiol concentrations are physiologically relevant and inform the concentrations used in *Aim 1c*, we will collect serum from these mice and quantify estradiol concentrations using mass spectrometry through the Small Molecule Biomarker Core (SMBC) at the University of Pittsburgh.

Camptothecin is known to stimulate protein ubiquitination, while Chlorofusin is known to be a protein ubiquitination inhibitor via disruption of E3 ubiquitin ligase and p53–HDM2 (MDM2) interactions. As such, we will use Camptothecin and Chlorofusin as positive and negative controls in our live cell imaging experiments for *Aim 1c*, respectively. Likewise, Fulvestrant is known to be a proteasome activator, while CEP-18770 (delanzomib) is known to be a proteasome inhibitor, and we will use these as positive and negative controls, respectively, in our plate reader experiment for quantifying proteasome activity in *Aim 1c*.

The experiments outlined in *Aim 1c* and the entirety of ***Specific Aim 2*** rely heavily on culturing chondrocytes. Given how sensitive chondrocytes are to cell-cell and cell-matrix interactions as well as the native low density of chondrocytes within cartilage, investigators will pay particularly close attention to cell density, assuring cells are not overly populated prior to experiments. To verify the purity of cells in each experiment, investigators will closely observe morphology of the cells over the expansion period, and we will have a control well that is used for immunofluorescence staining of type II collagen, Sox9, and aggrecan, as these are well-established markers of chondrogenicity.

In *Aim 2b*, we will use imaging flow cytometry and immunofluorescence to quantify type II collagen, aggrecan, and Sox9 expression. For our primary and secondary antibodies, we will use the following antibodies, which we have used in our lab previously and presented in our previous work:

- Type II collagen: AbCam, Cat. No. Ab34712 (1:200)
- Aggrecan: ThermoFisher, Cat. No. MA3-16888 (1:200)
- Sox9: Abcam, Cat. No. ab185230 (1:200)

- Alexa Fluor 488 anti-rabbit: ThermoFisher, Cat. No. A-11094 (1:100)
- Alexa Fluor 568 anti-mouse: ThermoFisher, Cat. No. A-11031 (1:100)

In both our imaging flow cytometry and immunofluorescence experiments, we will include one well of plates as a negative control which contains secondary antibody staining with no primary antibody staining. Imaging flow cytometry runs will also include compensation beads stained for each individual antibody as a control as well.

One we determine our ideal gene of interest (GOI) for our genetic controls circuit in *Aim 2b*, we will confirm circuit controllability by quantifying GOI protein expression using imaging flow cytometry and immunofluorescence. This will likely require the use of a new antibody for our lab, and as outlined above, we will start by performing western blotting to verify specific binding to the protein of interest and then titration tests (1:20, 1:50, 1:100, 1:200, 1:500, 1:1,000, and 1:2,000) on chondrocytes to identify the ideal concentration for reproducible staining.