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July 13, 2022

Ms. Tracy Egan
Executive Director
New York State Thoroughbred Breeding and Development Fund
One Broadway Center, Suite 601
Schenectady, NY 12305

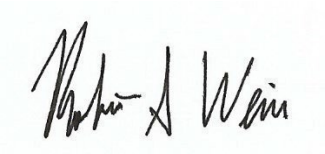
Dear Ms. Egan:

Enclosed is an electronic copy of the 2021 annual report for the Harry M. Zweig Memorial Fund for Equine Research, covering the award period of January 1, 2021 through December 31, 2021.

Included with the report are copies of the spring and fall issues of the Zweig News Capsule. Additional information about the Harry M. Zweig Memorial Fund for Equine Research can be found on the Zweig Memorial Fund public website at https://bit.ly/zweigfundcornell.

On behalf of Cornell University, we wish to extend our appreciation for your continued support of equine research.

Sincerely,



Robert S. Weiss, Ph.D.

Professor of Molecular Genetics
Associate Dean for Research & Graduate Education

Cc: Lorin D. Warnick, PhD, Austin O. Hooey Dean of Veterinary Medicine
Ms. Jill LaBoissiere, Comptroller, NYS Thoroughbred Breeding & Development Fund
Mr. Adam Lawrence, Registrar, NYS Thoroughbred Breeding & Development Fund

Cornell University
College of Veterinary Medicine

&

The Harry M. Zweig Memorial Fund for Equine Research

2021 Annual Report

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SUMMARY REPORT

The 2021 Annual Report covering the period of January 1, 2021, through December 31, 2021, is provided herein.

For this reporting period, the Harry M. Zweig Memorial Fund for Equine Research Committee awarded funding for three of the eight submitted projects. Six of the eight projects were new, first-time submissions, one was a renewal, and one was a revision. The total amount allocated for new awards for calendar year 2021 was $246,912. This report includes the “lay summaries” for the public website (Appendix A). There were also two continuation awards approved for second year funding in the amount of $146,645, allocated at the 2020 annual meeting. Two projects were granted a Year 1 no-cost extension due to significant challenges resulting from pandemic-related research restrictions.

The Zweig Family hosted the Annual Zweig Memorial Trot on August 28th, 2021 at the Vernon Downs Racetrack in New York. Additionally, on Wednesday, November 17, 2021, the Veterinary College hosted an in-person and Zoom seminar with scientific talks celebrating the collaboration between the Harry M. Zweig Memorial Fund for Equine Research and Cornell University College of Veterinary Medicine by showcasing faculty research to the College community and to the Zweig Committee.

The seminar can be viewed on the Zweig Virtual Presentations page.

RESEARCH AWARDS

CONTINUATIONS

Principal Investigator	Project Title	2021 Award
Reesink, Heidi	Unraveling lubricin signaling in equine joint injury	$89,440
Wagner, Bettina	Nasal immunity and its function in preventing transmission of EHV-1 in immune horses	$57,205
	SUBTOTAL:	**$146,645**

NEW AWARDS

Principal Investigator	Project Title	2021 Award
Delco, Michelle	Synovial fluid extracellular vesicles in equine joint disease and therapy	$64,463
Nixon, Alan/ Pigott, John	Multi-modal screening to identify Thoroughbred racehorses at increased risk for catastrophic injury of the metacarpophalangeal joint	$99,336
Wagner, Bettina	Intranasal biomarkers of EHV-1 susceptibility and protection	$83,113
	SUBTOTAL:	**$246,912**
		$393,557

PROGRESS IN 2021

PI	Project Title	Term Date	Report Type in Appendix B
Antczak, Douglas	2020 Horse Genome Project Workshop at Cornell	12/31/21	Final
Cheetham, Jonathan	Accelerating Recovery after Laryngeal Nerve Graft in Horses	12/31/22 With NCE*	Progress
Delco, Michelle	The role of mitochondrial Damage Associated Molecular Patterns (mDAMPs) in equine joint injury and disease	12/31/21	Final
Delco, Michelle	Synovial fluid extracellular vesicles in equine joint disease and therapy (Year 1)	12/31/21	Final
Diel de Amorim, Mariana	Can inflammatory markers in low-volume uterine lavage fluid be used to diagnose mares with endometrial fibrosis and with acute inflammation?	5/31/21	Final
Reesink, Heidi	Does Proximal Sesamoid Bone Mineral Loss Lead to Increased Fracture Risk?	12/31/21	Final
Perkins, Gillian	Equine gammaherpesviruses and equine gastric ulcer syndrome (EGUS) – is there a link?	6/30/22 With NCE*	Progress
Reesink, Heidi	Unraveling lubricin signaling in equine joint injury	12/31/22 with NCE*	Progress
VandeWalle, Gerlinde	The Mesenchymal Stem Cell Secretome Against Equine Herpesvirus Type I Infections	12/31/21	Final
VandeWalle, Gerlinde	Studying the replication kinetics of equine parvovirus hepatitis (EqPV-H)	12/31/21	Final
Wagner, Bettina	Nasal immunity and its function in preventing transmission of EHV-1 in immune horses	12/31/21	Final

*NCE = No Cost Extension

EXTERNAL FUNDING

The Incentive Program enables the Fund to leverage its investment in Zweig-sponsored research by encouraging Veterinary College faculty to seek either additional or supplementary monies from external sponsors that base their award decisions on a process that involves informed scientific review. The external grant must be closely related to a Zweig project. Eligible sponsors include, but are not limited to, the Grayson Foundation, the NIH, the NSF, and the USDA's National Research Initiative. Recipients provide an annual report on the use of these funds.

Heidi Reesink, December 2021 - $5000
Zweig Award: Unraveling lubricin signaling in equine joint injury ($147,061 1/1/20 – 12/21/22) Hong Kong Jockey Club Equine Welfare Research: Investigation of equine fetlock joint immunopathology and the immunomodulatory effects of intra-articular therapeutics ($186,055 1/1/2022 - 12/31/2023)

PUBLICATIONS

Cresswell EN, Ruspi BD, Wollman CW, Peal BT, Deng S, Toler AB, McDonough SP, **Palmer SE, Reesink HL.** Determination of correlation of proximal sesamoid bone osteoarthritis with high-speed furlong exercise and catastrophic sesamoid bone fracture in Thoroughbred racehorses. Am J Vet Res. 2021 Jun;82(6):467-477. doi: 10.2460/ajvr.82.6.467. PMID: 34032482.

Fasanello DC, Su J, Deng S, Yin R, Colville MJ, Berenson JM, Kelly CM, Freer H, Rollins A, **Wagner B**, Rivas F, Hall AR, Rahbar E, DeAngelis PL, Paszek MJ**, Reesink HL**. Hyaluronic acid synthesis, degradation, and crosslinking in equine osteoarthritis: TNF-α-TSG-6-mediated HC-HA formation. Arthritis Res Ther. 2021 Aug 20;23(1):218. doi: 10.1186/s13075-021-02588-7. PMID: 34416923; PMCID: PMC8377964.

Hammons V, Ribeiro L, Munyard K, Sadeghi R, Miller D, **Antczak D**, Brooks SA. GWAS Identifies a Region Containing the SALL1 Gene in Variation of Pigmentation Intensity Within the Chestnut Coat Color of Horses. J Hered. 2021 Aug 25;112(5):443-446. doi: 10.1093/jhered/esab037. PMID: 34343312; PMCID: PMC8386761.

Larson EM, **Babasyan S**, **Wagner B**. IgE-Binding Monocytes Have an Enhanced Ability to Produce IL-8 (CXCL8) in Animals with Naturally Occurring Allergy. J Immunol. 2021 May 15;206(10):2312-2321. doi: 10.4049/jimmunol.2001354. Epub 2021 May 5. PMID: 33952617; PMCID: PMC8185406.

Larson EM, **Wagner B**. Viral infection and allergy - What equine immune responses can tell us about disease severity and protection. Mol Immunol. 2021 Jul;135:329-341. doi: 10.1016/j.molimm.2021.04.013. Epub 2021 May 8. PMID: 33975251.

Marx C, Gardner S, Harman RM, **Wagner B, Van de Walle GR**. Mesenchymal stromal cell-secreted CCL2 promotes antibacterial defense mechanisms through increased antimicrobial peptide expression in keratinocytes. Stem Cells Transl Med. 2021 Dec;10(12):1666-1679. doi: 10.1002/sctm.21-0058. Epub 2021 Sep 16. PMID: 34528765; PMCID: PMC8641085.

Raza F, **Babasyan S**, Larson EM, Freer HS, Schnabel CL, **Wagner B.** Peripheral blood basophils are the main source for early interleukin-4 secretion upon in vitro stimulation with Culicoides allergen in allergic horses. PLoS One. 2021 May 26;16(5):e0252243. doi: 10.1371/journal.pone.0252243. PMID: 34038479; PMCID: PMC8153460.

Watkins A, Fasanello D, Stefanovski D, Schurer S, Caracappa K, D'Agostino A, Costello E, Freer H, Rollins A, Read C, Su J, Colville M, Paszek M, **Wagner B, Reesink H.** Investigation of synovial fluid lubricants and inflammatory cytokines in the horse: a comparison of recombinant equine interleukin 1 beta-induced synovitis and joint lavage models. BMC Vet Res. 2021 May 12;17(1):189. doi: 10.1186/s12917-021-02873-2. PMID: 33980227; PMCID: PMC8117281.

PATENTS

Wagner, Bettina. 2021**.** A Method to Induce Protection and Immune Altertness in Neonates. US Patent 11,167,026 file date May 12, 2016, 371 date November 9, 2021.

Heidi L. Reesink, VMD, PhD, DACVS-LA
Harry M. Zweig Assistant Professor in Equine Health 2019-2021

Heidi Reesink has been named the Harry M. Zweig Assistant Professor in Equine Health in honor of her ambitious research program to detect horses at risk for catastrophic injuries and to develop new treatments for arthritis.

The professorship is a three-year, endowed position for a junior faculty member who shows great promise for advancing equine research. It can be instrumental in helping junior faculty secure funding and develop high-level publications necessary for long-term success. Reesink has received grants previously from the Zweig Memorial Fund to support individual research projects. She has also received support from the Grayson-Jockey Club Research Foundation, the Cornell Center for Advanced Technology, the Cornell Center for Materials Research and the National Institutes of Health through a Mentored Clinical Scientist Development Award, a highly competitive grant to advance the careers of promising researchers.

"Dr. Reesink is recognized as a rising star among junior faculty and an important contributor to our college community," says Robert Weiss, Associate Dean for Research and Graduate Education. "One of the main concerns of the Harry M. Zweig Memorial Fund is catastrophic racehorse injury. Solving this problem is a critical need in the racing industry and she's doing some exciting work in that area."

Catastrophic musculoskeletal injuries – mainly broken legs – are the main cause of death for racehorses. "We would like to understand how these fractures occur and to develop better methods to screen for racehorses at risk of fracture," says Reesink.

She is working with Dr. Scott Palmer, the equine medical director for the New York State Gaming Commission, in addition to epidemiologists and pathologists to examine horses that died after sustaining fractures to their proximal sesamoid bones (PSBs) – two knobby, triangular bones at the back of the fetlock joint. Often, racehorses with this injury have no telltale signs of lameness during pre-race examinations or X-rays. Reesink is comparing the PSBs from the uninjured leg of those horses to PSBs from horses that died of other causes, using advanced CT scans. She hopes to develop better screening procedures to identify horses susceptible to fractures.

Reesink is also looking into new treatments and early detection methods for arthritis. "Joint disease and osteoarthritis are the leading cause of lameness in horses, but there are limited options for treating arthritis in horses and in humans," says Reesink. "A long term goal is to develop better therapies, that will both provide longer and better pain relief and that, ideally, will prevent or delay the development of arthritis."

After an injury, many – but not all – horses develop arthritis, so Reesink is examining the synovial fluid that bathes the joints to identify biomarkers that would indicate which horses are at risk and might benefit from preventive therapies. She is also investigating lubricin, a sugar-coated protein in the synovial fluid that provides lubrication, to see if an injection of lubricin can treat lameness. Additionally, along with pharmaceutical industry colleagues, Reesink is testing whether horses benefit from human arthritis medicines that are not available on the veterinary market.

Reesink is passionate about "one medicine," the concept that human and veterinary biomedical research can each inform the other. She believes that while providing treatment for animals, she can also offer insights to advance human health, and hopes her work will translate into clinical applications that benefit both horses and people.

As a former athlete herself, Reesink has fractured bones and injured joints while playing volleyball, competing in tae kwon do events, snowboarding and running, and so she understands the challenges and potential for developing better treatments for sports injuries. "I saw equine orthopedic surgery as a way to combine my love of the horse as well as my desire to advance the science of sports medicine."

Waldron, Patricia. "Reesink to be next Harry M. Zweig Assistant Professor in Equine Health". CVM News. 28 Feb. 2019, https://www.vet.cornell.edu/news/20190228/reesink-be-next-harry-m-zweig-assistant-professor-equine-health

CORNELL CLINICAL FELLOW IN EQUINE HEALTH

At the 2007 annual meeting, the Harry M. Zweig Committee approved the allocation of funds to help support a Cornell Clinical Fellow in Equine Health. Dr. Sophy Jesty was selected as Cornell's first Clinical Fellow, followed by Dr. Sarah Pownder, and more recently Dr. Joy Thomlinson. Supported in part by Zweig funds, all have been highly successful. Cornell's College of Veterinary Medicine's two- year Clinical Fellows Program is the first in the country to address a growing shortage of academic veterinarians who conduct research on animal diseases and basic biology.

The program is designed to help students meet the financial and time demands of qualifying for a position in veterinary academic medicine, which has traditionally required students to complete an M.S. or Ph.D. after they finish their doctorate in veterinary medicine (DVM). The two-year program, available to veterinarians who have completed a three-year residency, offers an annual salary of $65,000 plus benefits and an additional $15,000 per year to fund a research project.

There was no Clinical Fellow appointed for 2021.

APPENDIX A
Lay Summaries for New Awards

Delco, Michelle	Synovial fluid extracellular vesicles in equine joint disease and therapy
Nixon, Alan	Multi-modal screening to identify Thoroughbred racehorses at increased risk for catastrophic injury of the metacarpophalangeal joint
Wagner, Bettina	Intranasal biomarkers of EHV-1 susceptibility and protection

Principal Investigator:	Dr. Michelle L. Delco
Title:	Synovial fluid extracellular vesicles in equine joint disease and therapy
Project Period:	1/1/21 – 12/31/21

LAY SUMMARY

Background:

Cartilage provides near-frictionless joint surfaces and cushioning to protect underlying bone.

Even mild cartilage damage can impair its ability to dissipate loads, exposing the underlying bone to repeated micro-trauma, which can ultimately lead to fracture. Further, cartilage has little or no ability to heal after injury. Therefore, "regenerative therapies", including mesenchymal stromal cells (also known as stem cells or MSCs) are commonly used to promote cartilage healing after joint trauma in equine and human athletes. However, there are important drawback to using cells treatments, including the time and expense required to grow stem cells in the lab, adverse reactions, and the lack of uniformity within a pool of cells, which may lead to unpredictable results. Therefore, cell-free regenerative therapies, including extracellular vesicles (EVs) are gaining attention in human orthopedic medicine, but have not yet been investigated for horses.

EVs are small, spherical structures that are released from many cell types. EVs are wrapped in cell membrane, and contain diverse cargoes, which are loaded for export by their cells of origin. These cargos including proteins, DNA, and even whole mitochondria (so-called 'mitovesicles'). EV are messengers; they play a critical role in cell-cell communication. Cargos are delivered to, then taken up by target cells, affecting a wide array of functions in the recipient cell. The role of EVs in disease processes has long been recognized. More recently, stem cell-derived EVs have been investigated for their anti-inflammatory and pro-healing properties in human medicine. However little information is available regarding EVs in veterinary medicine. Furthermore, mitovesicles have not been investigated as possible regenerative orthobiologics.

Recent work by our group revealed that mitochondrial dysfunction is one of the very earliest responses of cartilage to overloading. Mitochondria are best known as the "powerhouses" of cells, because these organelles produce the energy required for normal tissue function and repair. Targeted therapies that improve mitochondrial function may be the key to stimulating repair mechanisms in poorly-healing tissues like cartilage. Our group also made the exciting discovery that MSCs can rescue injured cartilage cells by donating healthy mitochondria. Our findings also indicate that stem cells may deliver these life-saving mitochondria inside mitovesicles (mtEVs).

Our goal is to is to perform foundational investigations of **mitochondria-containing extracellular vesicles (mitovesicles, mtEVs)** as a new potential regenerative joint therapy in horses. We are also interested in examining EVs released into joint fluid after joint injury.

Hypothesis: Our overarching **hypotheses** are that EVs containing healthy mitochondria, such as those derived from stem cells, can rescue injured cells and promote tissue repair. Furthermore, EVs containing dysfunctional mitochondria are released into synovial fluid after joint injury, representing a potential early markers of disease. Finally, unhealthy mitovesicles can enhance cartilage repair mechanisms by stimulating the release of healthy mitovesicles by MSCs.

Strategy: In **Aim 1,** we will determine if mtEVs isolated from MSCs can improve mitochondrial function, prevent cell death and stimulate pro-healing mechanisms in injured chondrocytes. In **Aim 2**, we will characterize EVs from joint fluid of horses with and without injury, to determine if there is a difference in function of mitochondrial cargo within mtEVs between healthy and injured joints.

Finally, we will investigate if there is crosstalk between these two processes; we will determine if dysfunctional mtEVs can enhance healthy mtEV release by MSCs.

Relevance to equine health and racing: Understanding the role of mtEVs in the events after joint injury may lead to new diagnostic tests, and help identify horses requiring targeted therapy or modified training programs. These studies will also provide a foundation for developing MSC mtEVs as a new cell-free regenerative therapy in horses. These studies will provide data in direct support of a larger NIH grant proposal.

Principal Investigator:	Dr. Alan Nixon
Title:	Multi-modal screening to identify Thoroughbred racehorses at increased risk for catastrophic injury of the metacarpophalangeal joint
Project Period:	1/1/21-12/31/22

LAY SUMMARY

THE RESEARCH PROBLEM

Catastrophic fetlock injury exacts a major toll on Thoroughbred racing as a major sporting event and represents a significant mortality risk for individual horses. In the past year an unusual cluster of catastrophic injuries at Santa Anita Racetrack pushed Thoroughbred racing to the forefront of public scrutiny and threatened the future of the entire racing industry. Predisposition to fracture/ dislocation of the bones forming the fetlock articulation have resulted in extensive studies which define which bones break, and also hinted at why, but little progress has been made in accurate detection of stress fracture, trabeculae irregularity, bone bruising, or soft tissue injury which may set the stage for catastrophic failure during intense exercise. Certainly, pre-existing bone pathology is well recognized by autopsy studies to predispose Thoroughbred racehorses to catastrophic injury. Indeed, catastrophic injury of the fetlock joint is the most common cause of fatal musculoskeletal injury in racehorses world-wide. What this grant seeks is an early warning of impending fracture based on non-invasive epidemiologic, blood test, and imaging modalities.

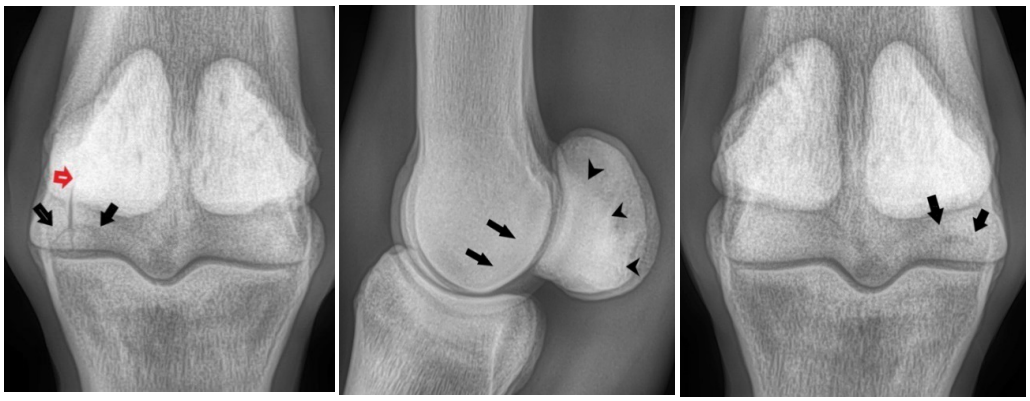

Fig 1. Stress accumulated palmar osteochondral disease (POD) fracture in both forelimbs of a TB 3-yr-old. The left fore (left image) has a condylar fracture (red arrow) propagating through the chronic osteochondral stress fracture (black arrows). The chronic density changes are evident on the lateral xray (center) in both the metacarpal condyles (arrows) and sesamoid bones (arrowheads). Chronic POD fragmentation is evident in the other fetlock of the same horse (right image; black arrows). This grant explores more sensitive imaging modalities to identify and categorize bone changes in the fetlock long before these types of fracture syndromes develop.

Previous studies[1] verify we can jump from digital radiographs (such as Fig 1), to standing MRI (Fig 2), to establish the extent of bone and soft tissue injury, but a complete MRI study is time consuming, requires multiple rounds of sedation, and costs several thousand dollars. A significant unmet need exists for a high throughput screening protocol, encompassing horse sensor data, rapid radiomic densitometry, and an abbreviated and economical MRI if ultimately indicated by the less complex indices.

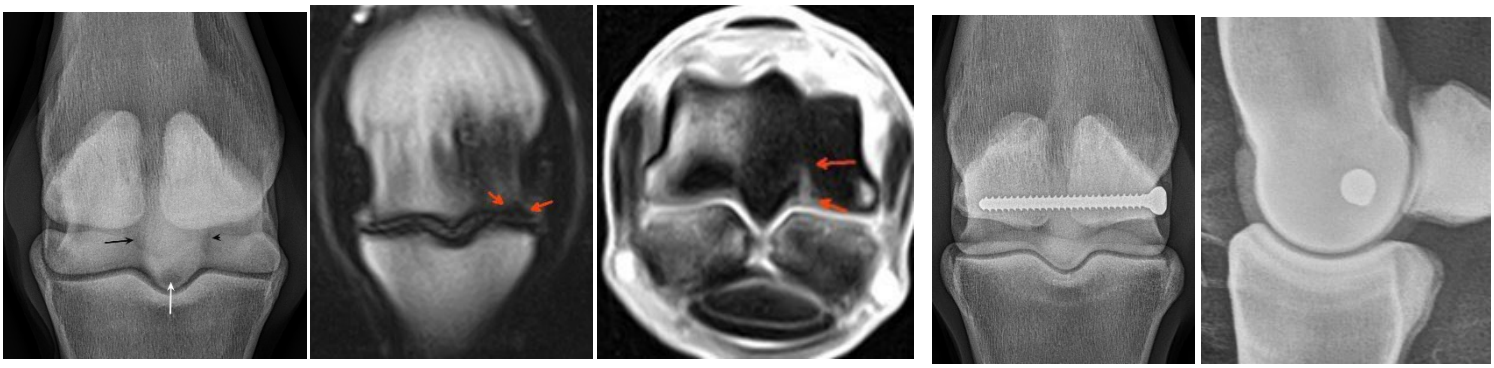

Fig 2. Referral radiograph (left) of left hind fetlock of a 3-yr-old TB persistently lame with training. Xrays show bone loss on the inside of the fetlock (arrow), an old OCD (white arrow) of unknown significance, and bone loss on the outside of the fetlock (arrow head). None of these findings help in sorting out how serious these bone loss regions are, or whether they represent fracture. Standing MRI (middle 2 images) indicate massive stress fracture induced bone accumulation and an incomplete condylar fracture of the back half of the cannon bone in the fetlock (red arrows). Treatment was by a single bone screw (right), placed low and toward the back of the cannon bone, and the horse returned to training after 4 months.

What can Digital Tomography and Image Biomarkers (Radiomics) add to improve fetlock imaging Amidst a desire for better multi-imaging studies, digital tomography has been added to quickly evaluate bone structure in fine detail, and define bone density build up, microfracture, and even early overt fractures. While it doesn't replace MRI for soft tissue imaging and the detection of bone edema, we have found in a limited series of cases that digital tomography may provide better information on the changes in fine bone structure at the heart of the bone disease leading up to condylar and sesamoid fractures, or at the end of the spectrum, untreatable catastrophic breakdown. This proposal will use digital tomography, and an abbreviated standing MRI, to complement the biosensor and serum bone marker information being derived. This adds to our combinatorial approach to assess horses with undiagnosed lameness involving the fetlock region as well as those failing a pre-race vet check due to a suspicion of fetlock injury

In summary, this proposal aims to critically assess multiple noninvasive screening methodologies to identify horses at increased risk for catastrophic injury and to provide evidence-based guidance for trainers to make informed decisions concerning ongoing training and racing. Recent incidents provide clear evidence that clinical examination and particularly the veterinary examination of horses prior to racing alone is not adequate to ensure the safety of horses and jockeys. Preliminary analysis of exercise characteristics, serum assay of circulating markers of excessive bone turnover, standing digital radiography tomosynthesis, and standing MRI, have revealed vital information about bone reaction to exercise and stress, and facilitated the development of potential screening protocols to identify horses with multiple risk factors. Standing MRI has also allowed us to develop a spectrum of disease from mild to dramatic that represents stages of failure of bone to adapt to training and racing; in short, a susceptibility index. But it would be wildly impractical to suggest an MRI for all horses entered to race. We propose to use GPS tracking, and biometric data capture that includes stride length and duration, the number of strides in a workout or race, and forces on each limb, to calculate measures of athletic efficiency. The system can then automatically detect and flag anomalies in stride data that can signal the need for additional studies, such as assay of blood biomarkers of abnormal bone degradation, digital radiography tomosynthesis, and abbreviated MRI screening. This sequential screening protocol will help to separate horses into at risk and normal conditioning homeostatic profiles. The *hypothesis* for this study is that multi-modal high- sensitivity screening, followed by confirmatory 2 and three-dimensional imaging, can reduce the incidence of catastrophic fetlock injuries.

Specific Aims:
1. To assess the ability of GPS tracking and automated biometric sensor analysis to identify abnormal gait characteristics of horses at increased risk for catastrophic fetlock injury.
2. Validate bone serum biomarkers as an adjunctive routine blood test for screening horses in race training
3. Assess image biomarkers (radiomics) of standing digital tomosynthesis as a means to quantify bone density that can be combined with epidemiologic variables to create an equine fracture susceptibility index.
4. Develop an abbreviated standing MRI protocol to confirm horses needing reduced training or surgical repair.

Although the prevalence of Thoroughbred racing fatalities has declined by more than 40% at New York racetracks over the past 6 years with implementation of a broad range of interventions, catastrophic fetlock injuries continue to represent nearly 50% of those fatalities. We hypothesize that an effective widespread screening protocol must include multiple layers of testing. Exercise analytics combined with serial serum bone biomarker assay will help to identify horses at increased risk for catastrophic injury. High throughput screening using digital radiography tomosynthesis may then allow formulation of a semiquantitative susceptibility index, similar to the FRAX index currently used to identify people at increased risk for hip fracture**.** The possibly of adding PET imaging would follow if funding can be secured, but the ultimate component of the index will include abbreviated MRI, to identify specific bone pathology recognized to increase the risk of catastrophic fetlock injury.

Impact of this Research

This study holds significant promise of being able to develop and verify an array of diagnostic imaging modalities capable of allowing the early detection of stress fracture and occult fracture that could culminate in catastrophic fetlock injuries. X-rays and bone scan are very limited in their ability to precisely define the susceptibility of the cannon bone, sesamoids, or proximal phalanx region to breakdown. Use of MRI has been helpful to define bone density increase and bone edema, but is time consuming, expensive, and does not provide the fine detail in bone structural change required to better define the susceptibility of bone to fracture. Radiomics of standing digital radiographic tomographic images will add to the diagnostic information, and will better allow the Cornell Ruffian Center to screen horses and potentially define high risk lesions in racehorses that may predispose to catastrophic breakdown. Ultimately, it is anticipated that the incidence of these injuries can be reduced by better screening.

Principal Investigator:	Dr. Bettina Wagner
Title:	Intranasal biomarkers of EHV-1 susceptibility and protection
Project Period:	1/1/21-12/31/22

LAY SUMMARY

Equine herpesvirus type 1 (EHV-1) continues to cause outbreaks in horse populations (https://www.equinediseasecc.org/alerts). Several of them include horses suffering from the neurological manifestation of EHV-1, called equine herpesvirus myeloencephalopathy (EHM) [Kydd et al. 2006, Lunn et al 2009, Perkins et al 2009]. The increased morbidity and mortality due to the neurologic manifestation of EHV-1 has prompted increased biosecurity [Henninger et al 2007, Kohn et al 2006, Perkins et al 2009]. During EHM outbreaks, horses are typically quarantined for 21-28 days. The medical and economic impact of EHV-1 outbreaks is often substantial through lost training and competing time, costs related to quarantine, treatment, and loss of horses due to death of severely neurologic horses [Goehring et al 2006, Lunn et al 2009].

EHV-1 outbreaks are confirmed by PCR detecting pathogen DNA in the nasal secretion of the first horse with clinical respiratory or neurologic signs. PCR is a sensitive technique but does not take into account the stage of EHV-1 infection or existing host immunity against EHV-1. Consequently, all horses on the grounds are quarantined after the positive PCR result for several weeks independent of infection stage and immune status. The existing PCR assays confirm EHV-1 infection in nasal secretion samples (nasopharyngeal swabs) by detecting EHV-1 DNA. In infected horses, the PCR is positive as long as viral DNA is present in the sample [Elia et al. 2006]. However, viral DNA is detectable for much longer than infectious virus is shed (sometimes for several weeks). For example, after the onset of immunity neutralized virus is taken up by cells residing in the respiratory tract, is no longer infectious but will still result in a positive PCR result. Methods and markers that give additional information on the immune status of EHV-1 PCR positive horses are still missing. Quarantine is consequently extensive and driven by pre-caution. A better understanding of when a PCR positive horse is still transmitting virus and can or cannot infect other horses, or when it developed immunity and viral DNA processed by immune cells is no longer a risk factor for other horses, will improve EHV-1 quarantine management and reduce costs associated with EHM outbreaks.

Our previous research funded by the Harry M. Zweig Memorial Fund for Equine Research and USDA/NIFA has shown that fully immune, protected horses are not shedding virus or developing clinical disease (Figure 1). The conclusions from our EHV-1 host immune and protection studies are: (1) a protected horse will not transmit the virus to another horse even if it was exposed to EHV-1; and (2) viremia is not happening in fully protected horses [Perkins et al. 2019, Schnabel et al. 2019]. Viremia is also the pre-requisite for developing neurologic disease [Edington et al. 1986, Borchers et al. 2006, Pusterla et al. 2009]. Thus, fully protected horses are at no risk of developing EHM.

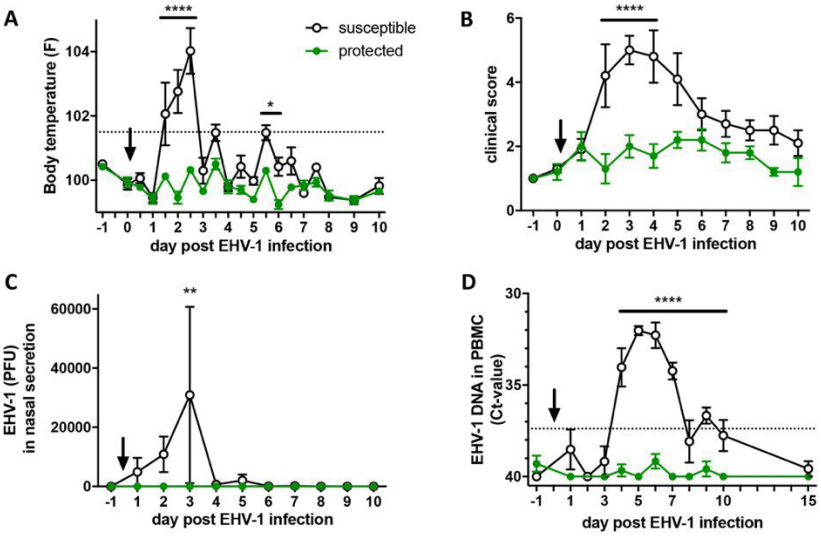

Figure 1. Susceptible and protected horse after experimental EHV-1 infection. (A) Body temperature, (B) clinical signs, (C) nasal viral shedding and (D) viremia in EHV-1 susceptible and protected horses. Susceptible and protected horses were infected (arrow) with $1x10^7$ Pfu of the neurogenic EHV-1 strain Ab4. Significant differences between the two groups are indicates by asterisks: * $p<0.05$, ** $p<0.01$,

Our recent findings initiated the development of a novel diagnostic Immune Biomarker assays for EHV-1 that aims to distinguish protected horses from those that are susceptible. The latter group will develop disease and spread the infection, the former will not. The assays will also support the identification of the infection stage in horses that are susceptible and get clinically ill during an EHV-1 outbreak (Figure 2). The biomarker identification in susceptible and protected horses during the course of infection resulted from projects funded by the Zweig Fund [Wagner et al. 2017, Wimer et al. 2018, Schnabel et al. 2018 & 2019, Perkins et al. 2019].

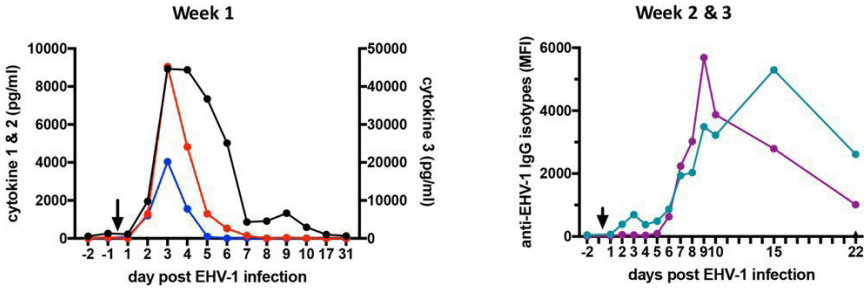

Figure 2. Intranasal immune responses and biomarkers identify the infection stage with EHV-1. Different intranasal biomarkers increase and decline at different times after experimental infection with $1x10^7$ Pfu EHV-1 in the nasal secretion. The biomarker changes are used in the novel EHV-1 Immune Biomarker assay to support the management of EHV-1 outbreaks in the US. **Left panel**: During week 1 three cytokine markers are detectable in nasal secretions of infected, susceptible horses. The cytokine biomarkers peak on day 3 post infection (pi), decline afterwards and become undetectable again by day 5-7 pi, with the exception of cytokine 3 that is maintained at low levels for another week. **Right panel**: IgG isotypes start rising in the nasal secretion in week 2. One of the IgG isotypes peaks early around day 9 pi and declines afterwards (purple line, IgG1). Another IgG isotype increases more slowly, peaks at the end of week 2 pi and then slowly declines (blue line, IgG4/7). In a clinical sample, the IgG isotype ratio of these two isotypes allows to distinguish horses that are beyond week 2 pi. These horses are immune, protected against disease induced by EHV-1, and past the infectious viral shedding phase.

Clinical EHM cases typically occur around day 6-12 pi. At this time, the inflammatory response in the nose weaned and antibodies just start to develop (Figure 2). Thus, most horses in an EHV-1 outbreak will have antibodies at that time which either originate from the current outbreak (susceptible horse) or existed prior to it (protected horse). The current Immune Biomarker assay lacks additional markers that help to more precisely detect this mid infection stage in horses on the premise that have been susceptible and thus, are a risk for transmitting the virus or developing EHM case themselves. To fill this gap we have performed an RNA expression analysis (RNAseq) during the first two years of this project. We used banked intranasal samples from our previous EHV-1 challenge and protection studies to comprehensively analyze gene expression in EHV-1 susceptible and protected horses. For each sample, clinical and immunological study outcomes were documented (Figure 1 & 2), representing well characterized samples to improve our understanding of immunity and viral transmission. We identified **nine novel EHV-1 immune biomarkers** in the upper respiratory tract that distinguish protected from susceptible horses in the mid or late stages of EHV-1 infection.

During this renewal application, we will further investigate these nine novel biomarkers and their roles during immunity against EHV-1. In Aim 1, we will develop assays for two newly identified secreted biomarkers of EHV-1 infection and add them to the existing EHV-1 Immune Biomarker assay. In Aim 2, the additional seven newly identified biomarkers will be further analyzed. All are expressed on T-cells, suggesting that T-cells play a major role in immunity and local protection against EHV-1. Thus, we will evaluate mucosal T-cell functions and their roles during infection and immunity. Mucosal T-cells have not yet been studied during EHV-1 infection. Based on the RNAseq results, they likely provide a very important first line of defense against EHV-1.

We plan to make the new EHV-1 Immune Biomarker assay available through the Animal Health Diagnostic Center at Cornell University to improve the management of EHV-1 outbreaks in the US. The current assay has been used at the AHDC during the past 1.5 years to measure field samples from horses in EHV-1 outbreaks and already provides valuable information on the immune status of the horses under quarantine. After adding the two new secreted mid infection biomarkers, the assay will be completed to measure biomarkers in nasal secretion of horses that are indicators for the stage of EHV-1 infection and protective immunity. The assay can be used to identify and distinguish (i) susceptible horses (will develop disease during an outbreak) from those that are in (ii) the early infection stage (high shedders of virulent pathogen), (iii) the later infection stage (developing immunity, low or no shedding), and (iv) immune horses that will not develop disease or shed virus. This information can support the separation of horse groups in an outbreak situation, will help to reduce new infections and the overall time of quarantine by improving management of groups. It will allow to release immune horses earlier from quarantine, and gives veterinarians a better tool to evaluate risk and prognosis for each horse. Importantly, shorter quarantine will significantly decrease costs during EHV-1 outbreaks.

The **outcome of this project** will be the characterization and evaluation of nine novel intranasal host protection biomarkers during EHV-1 infection to improve our understanding of protective immunity and risk of transmission. A new detection tools will be finalized to result in an advanced EHV-1 Immune Biomarker assay. The findings and the new diagnostic assay tool will directly support the management of EHV-1 outbreaks and reduce medical and economic losses for the horse industry.

APPENDIX B
Final & Progress Reports from 2021

PI	Project Title	Report Type
Antczak, Douglas	2020 Horse Genome Project Workshop at Cornell	Final
Cheetham, Jonathan	Accelerating Recovery after Laryngeal Nerve Graft in Horses	Progress
Delco, Michelle	The role of mitochondrial Damage Associated Molecular Patterns (mDAMPs) in equine joint injury and disease	Final
Delco, Michelle	Synovial fluid extracellular vesicles in equine joint disease and therapy (Year 1)	Final
DieldeAmorim, Mariana	Can inflammatory markers in low-volume uterine lavage fluid be used to diagnose mares with endometrial fibrosis and with acute inflammation?	Final
Perkins, Gillian	Equine gammaherpesviruses and equine gastric ulcer syndrome (EGUS) – is there a link?	Progress
Reesink, Heidi	Does Proximal Sesamoid Bone (PSB) Mineral Loss Lead to Increased Fracture Risk?	Final
Reesink, Heidi	Unraveling lubricin signaling in equine joint injury	Progress
VandeWalle, Gerlinde	The Mesenchymal Stem Cell Secretome Against Equine Herpesvirus Type I Infections	Final
VandeWalle, Gerlinde	Studying the replication kinetics of equine parvovirus hepatitis (EqPV-H)	Final
Wagner, Bettina	Nasal immunity and its function in preventing transmission of EHV-1 in immune horses	Final

Principal Investigator:	Dr. Douglas Antczak
Title:	2020 Horse Genome Project Workshop at Cornell
Project Period:	1/1/20 – 12/31/21
Reporting Period:	1/1/20 – 12/31/21

Project Title: **2020 Horse Genome Project Workshop at Cornell**

Principal Investigators: Doug Antczak

A. Specific Aims of the Study and Modifications

Specific Aims: If the aims have not been modified, state so. If they have been modified, provide the revised aims and the reason for the modification.

Original aims:

The specific aim of this proposal is to foster interactions between Cornell faculty and trainees who work in equine medicine and surgery and the international community of equine geneticists of the Horse Genome Project. Those interactions will take place in the context of a scientific workshop in the Havemeyer Foundation series.

Aim 1. To introduce all interested Cornell equine clinicians, scientists, and trainees to the member scientists of the Horse Genome Project.

Aim 2. To make member scientists of the Horse Genome Project aware of the varied types of equine research projects underway at Cornell.

Modifications:

The original aim was meant to be accomplished during the in-person Havemeyer Foundation Horse Genome Workshop that was scheduled for summer 2020 at Cornell. The Workshop was postponed in 2020 and 2021 because of the pandemic. The in-person workshop is now re-scheduled for July 2022, and we hope to achieve our original goal. However, it was not possible to carry over the Zweig award for two years. Instead, with permission of the CVM Research Office, we used the funds in 2021 to cover some of the costs of organizing and running a series of five virtual sessions that brought together participants of the Horse Genome community from around the world.

B. Summary of Scientific Findings

Describe the studies directed toward the specific aims and the positive and negative results obtained. If applicable, address any changes to the innovative potential of the project. If technical problems were encountered in carrying out this project, describe how your approach was modified.

The organizing committee (Doug Antczak and Don Miller from Cornell, Ernie Bailey and Jamie MacLeod from Kentucky, and Annette McCoy from the University of Illinois) developed a plan to hold a series of five short virtual (Zoom) workshop sessions throughout the 2021 year (see Table 1).

Table 1. Dates and Topics for the Horse Genome Project Virtual Sessions held in 2021

Session	Dates (all2021)	General Topic	# Participants
1	10 February	The Future of Large-scale Phenotyping and Lessons from Evolution	78
2	20 April	From Single Cells to Functional Networks	65
3	29 June	Finding and Using Transformative Technologies	68
4	31 August	Clinical & Commercial Genetic Testing and Gene Doping	53
5	27 October	For Trainees: Navigating a Path to a Career in Equine Genomics	57

The Organizing Committee selected the topics for each session based on scientific abstracts that had been submitted for the in-person Workshop and then recruited the presenters. Several creative elements were included in the sessions. All presenters prepared 15-to-20-minute pre-recorded talks that were viewed on-line prior to the 2-hour Workshops sessions. These recordings could be viewed multiple times to allow full understanding of the contents. During the actual sessions, the presenters gave 5-minute summaries of their recordings and then took questions from the audience. Strong moderators were recruited who kept the discussions flowing. Lists of questions were generated by the Workshop organizers before each meeting to make sure that good discussions took place. Written summaries of the sessions were prepared and made available to all attendees. The Organizing Committee held post-mortems after each session and made small improvements with each successive session.

The presenters for each of the sessions are shown in Table 2.

Table 2. Presenters at the virtual Horse Genome Project Workshop sessions.

Virtual session #1 2/10/2021
The Future of Large-scale Phenotyping and Lessons from Evolution

Speaker	*Affiliation*
Dr. Sian Durward-Akhurst	UMinn
Dr. Evelyn Todd	U of Toulouse
Dr. Samantha Brooks	U of Florida
Dr. Barbara Wallner	Vienna

Virtual session #2 4/20/2021
Topic: Looking at Both the Forest and the Trees: From Single Cells to Functional Networks

Speaker	*Affiliation*
Dr. Rebecca Harman	Cornell University
Dr. Alix Berglund	NC State
Dr. Felipe Avila	UC Davis
Dr. Jessica Petersen	U of Nebraska - Lincoln

Virtual session #3 6/29/2021
Topic: Beyond the Booth: Finding and Using Transformative Technologies

Speaker	*Affiliation*
Dr. Ted Kalbfleisch	U of Kentucky
Dr. Annik Gmel	U of Bern
Sichong Peng	UC Davis
Kevin Batcher	UC Davis

Virtual session #4 8/29/2021 Topic: Genetic Testing in the Horse

Speaker	*Affiliation*
Dr. Rebecca Bellone	UC Davis
Dr. Leanne van de Goor	VHL Genetics
Dr. Natasha Hamilton	Racing Australia
Dr. Sofia Mikko	Swedish University of Agriculture

Virtual session #5 10/27/2021
Topic: Navigating the Path to a Career in Genomics

Tentative Speaker	*Affiliation*
Dr. Ernie Bailey	U of Kentucky
Dr. Doug Antczak	Cornell University
Dr. Molly McCue	UMinn
Dr. Tosso Leeb	University of Bern
Dr. Gabriella Lindgren	Swedish University of Agriculture
Dr. Mandi de Mestre	Royal Vet College

C. Significance

Emphasize the significance of the findings and their potential impact.

The virtual sessions were very well attended and deemed to be very successful by the participants. The timing of the sessions was chosen to allow scientists from across the world to attend, and strong international participation was achieved. These virtual workshops helped to keep the international Horse Genome community of researchers engaged with one another during the pandemic. Partly as a result of these virtual workshop sessions, enthusiasm remains high for the in-person Horse Genome Workshop to be held at Cornell in July.

The call for abstracts for the 2022 workshop has just closed. We received 65 abstracts from equine geneticists from 15 countries and 11 US universities, including several from Cornell. We are optimistic that we will be able to host a successful meeting this coming summer and to achieve the original goals of the project.

D. Publications and Other Grant Submissions

If applicable, report publications resulting from the study, including manuscripts submitted or accepted for publication, and submissions and/or external grants resulting from the award.

Although we do not have a grant from the Zweig Fund to support the 2020 in-person workshop, we have partial funding in hand from the Havemeyer Foundation and the USDA NRSP-8 program. In addition, we have submitted a proposal to the USDA for a Conference Grant. That proposal is currently under review. A copy is attached to this report (redacted to achieve brevity by removing form pages and other non-essential information).

Principal Investigator:	Dr. Jonathan Cheetham
Title:	Accelerating Recovery after Laryngeal Nerve Graft in Horses
Project Period:	1/1/19 – 12/31/22
Reporting Period:	1/1/21 – 12/31/21

This project aimed to evaluate recovery of dorsal cricoarytenoid muscle (CAD) function after graft of the Recurrent Laryngeal nerve (RLn) with a donor nerve, with immunomodulation by addition of interleukimn-10 (IL-10) at the site of nerve anastomosis with the intent of modulating macrophage migration and type during the early phase of repair. The left RLn was transected and grafted with a branch of the first cervical nerve (C1); after completion of the anastomosis, a 0.7% agarose hydrogel loaded with recombinant equine IL-10 at 3ug/mL was injected sub-epineurally.

During the first year, we evaluated RLn reinnervation using the first cervical nerve (C1) as donor graft. Transection of the RLn induced complete loss of function of the left CAD muscle right after surgery. Signs of progressive reinnervation through C1 were detected starting from 10-16 weeks post-op, in the form of arytenoid abduction induced by electrical stimulation of C1, spontaneous twitching of the arytenoid, and improved laryngeal function during incremental exercise. Despite the promising outset, the arytenoid function during exercise decreased after 22 weeks, indicating an insufficient recruitment of C1 during exercise.

Thereafter we identified the ventral branch of the accessory nerve (XI cranial nerve, exclusively motor), as possible alternative nerve donor, and conducted investigations to support its use. After an initial anatomical cadaver study to verify the nerve pathway and surgical approach, we identified a site to perform transcutaneous stimulation of the proximal portion of the accessory nerve to monitor reinnervation overtime. Blunt simulating probes were used to stimulate (1Hz, 100µsec duration) the accessory nerve under the cranio-ventral border of the left wing of the atlas, while confirming activation of the sterno-cephalic muscle through electromyography.

After the long pause due to COVID-19 restrictions, we resumed training on high-speed treadmill on the last two horses recruited for the study. Once the horses were fit to sustain an incremental strenuous exercise, pre-operative assessment of their laryngeal function and CAD muscle structure were performed. One horse had a laryngeal function grade III.1-A, with minimal CAD asymmetry while the other one had a grade III.3-C laryngeal function with marked left CAD atrophy.

We encountered several further delays before being able to perform surgery due to restrictive surgery and anesthesia schedules in the large animal hospital. Both horses underwent surgery on early May 2021.
Considering the complete loss of function after the RLn transection required to perform an end-to-end nerve graft, an alternative surgical approach was done in these two horses. We used an end-to-side (ETS) anastomosis. Grafting the donor nerve through an epineurial or perneurial window allows maintenance of the recipient nerve structure and hopefully function. This surgical approach would be a more suitable option for client owned clinical cases presenting with recurrent laryngeal neuropathy with still some remaining RLn function. We selected this approach as it moves us closer to clinical application of these techniques in client owned animals with ‘Roaring’.

One horse (L) had a left C1- RLn epineural ETS, without IL-10 injection, while the horse with the most advanced disease (D) had a left accessory-RLn epineural ETS followed by IL-10 injection. Both surgeries were performed uneventfully with the horses standing under deep sedation. Despite the less invasive surgery on the RLn, both horses showed loss of left laryngeal function after surgery, but horse L showed again some spontaneous twitches of the left arytenoid starting 3 weeks after surgery. Endoscopy at rest and during exercise and laryngeal ultrasound have been performed on a monthly basis to assess recovery of laryngeal function. Horse L showed progressive improvement in left laryngeal function returning to full function within 4 months, while horse D laryngeal function never recovered. Horse L is not showing arytenoid response after electrical stimulation of the proximal C1, possibly indicating that RLn reinnervation derived from the proximal RLn and

not the C1-graft. The horses will continue to be monitored up to 10 months.

In the past year, the MyoSAT (Myofiber Segmentation and Analysis Tool) image-processing for evaluation of fiber size in full muscle cross-sections has been implemented and a protocol for osmium tetroxide staining for myelin staining in nerve cross section specimen has been tested and optimized on rodent nerve samples in collaboration with the Cornell Center for Material Research (CCMR). Currently we are testing the osmium tetroxide staining also in demo equine nerves to optimize the current protocol for use in lager specimen.
The plan is to process and stain all the muscles and nerves samples collected post-mortem at the same time to minimize variability in staining and optimize objective evaluation.

I. Explain the significance of findings.

Overall, the preliminary results of this study show that agarose loaded with recombinant Equine IL-10 is well tolerated and does not prevent reinnervation. C1 axons can reinnervate the RLn and the CAD muscle, providing adequate trophic support, but the spontaneous recruitment of C1 during intense exercise or the new morphologic characteristics of the reinnervated fibers are not satisfactory to induce a muscle contraction strong enough to sustain the inspiratory load during intense exercise.

Based on poor reinnervation in the 2 horses with the most advanced disease, grafted with 2 distinct nerves (C1 and accessory), we found that the distal portion of RLn affected by natural occurring recurrent neuropathy in advanced stage of disease may not represent an adequate environment for reinnervation.

We plan in the future to apply immunomodulation to direct nerve to muscle graft.

II. Outline goals for the remainder of the funding period.

The goals for the remainder funding period are to complete the clinical monitoring of the two remaining horses and then process and evaluate histologically the nerves and muscles collected throughout the study period.

III. List anticipated grant applications and publications resulting from Zweig funding.

Publication currently in preparation

Principal Investigator:	Dr. Michelle Delco
Title:	The role of mitochondrial Damage Associated Molecular Patterns (mDAMPs) in equine joint injury and disease
Project Period:	1/1/20 – 12/31/21
Reporting Period:	1/1/20 – 12/31/21

Project Title: The role of mitochondrial Damage Associated Molecular Patterns (mDAMPs) in equine joint injury and disease

Principal Investigators: Michelle L. Delco

A. Specific Aims of the Study and Modifications

The broad goal of **Aim 1** was to investigate the types of injury that lead to extracellular mDAMP release by cartilage. Specifically, **Aim 1a** tested the hypothesis that mitochondrial dysfunction is a specific trigger for mDAMP release**. Aim 1b** was to investigate if mDAMP release from cartilage occurs after mechanical overloading. ***These aims were successfully carried out.***
Specific **Aim 2** was to analyze mDAMPs in equine joint fluid, in order to determine if mDAMPs might be a useful indicator of early cartilage/bone injury. Specifically, we planned to investigate this in horses that had experimental injury to their articular cartilage, and 2) horses with naturally occurring joint injuries. ***These aims were successfully carried out.***

B. Summary of Scientific Findings

Early intervention, prior to the development of degenerative joint changes, has the potential for improved success in treating posttraumatic osteoarthritis but requires early detection of joint injury. In other tissue types, trauma is associated with the extracellular release of mitochondrial DNA (mtDNA), which can both serve as a biomarker of tissue injury and perpetuate inflammation as a mitochondria-specific Damage Associated Molecular Pattern (mDAMP). The goal of this study was to evaluate mtDNA release from injured chondrocytes and investigate the utility of synovial fluid mtDNA concentration in early detection of posttraumatic osteoarthritis. We measured mtDNA release using four models of osteoarthritis: *in vitro* IL-1b stimulation, *ex vivo* mechanical impact, *in vivo* mechanical impact, and naturally occurring equine intraarticular fracture.
We demonstrated that chondrocytes release mtDNA following cellular stress and that mtDNA is increased in equine synovial fluid following experimental and naturally occurring injury to the joint surface. In naturally occurring posttraumatic osteoarthritis, we found a strong correlation between the degree of cartilage damage and mtDNA concentration. Finally, impact-induced mtDNA release was mitigated by mitoprotective treatment. Our findings indicate that Changes in synovial fluid mtDNA occur following joint injury. Further investigation of mtDNA as a potentially sensitive marker of early articular injury is warranted.
Please see attached draft manuscript submitted for publication in Osteoarthritis and Cartilage

C. Significance

This work represents the first investigation of extracellular mtDNA in association with PTOA. Our findings indicate the mtDNA may be a valuable biomarker of early cartilage injury. Furthermore, our data suggest that mitoprotective drugs are capable of preventing mtDNA release and cell death, representing a promising new therapeutic strategy for the prevention and treatment of PTOA. Measurement of synovial fluid mtDNA concentration could provide a non-invasive method for detecting mitochondrial dysfunction and cartilage damage associated with early PTOA prior to the appearance of radiographic changes.

D. Publications and Other Grant Submissions

Submitted Manuscripts:

Seewald LA, Sabino IG, Montney KL, **Delco ML**. Synovial Fluid Mitochondrial DNA Concentration Reflects the Degree of Cartilage Damage After Naturally Occurring Articular Injury. August 2021 BioRxiv: doi.org/10.1101/2021.08.26.45757

Abstracts:

Sabino IG, Seewald LA, Jacobs CA, Lattermann C, **Delco ML** (2022). Elevated Synovial Fluid Mitochondrial DNA Is Associated with Inflammation After ACL Injury and Reconstruction. *Cornell Undergraduate Research Board (CURB) Annual Symposium *Selected for CURBx talk*

Sabino IG, Seewald LA, Jacobs CA, Chen J, Leifer CA, Lattermann C, **Delco ML** (2022). Elevated Synovial Fluid Mitochondrial DNA Is Associated With Inflammation After ACL Injury And Reconstruction. *ORS Annual Meeting*

Seewald LA, Sabino GS, Bohner A, **Delco ML** (2021). Synovial fluid mitochondrial DNA as a biomarker after naturally occurring intra-articular fracture. *ORS Annual Meeting *ORS Preclinical Models Section Podium Award Winner*

Seewald LA, Sabino GS, Bohner A, **Delco ML** (2021). Synovial fluid mitochondrial DNA as a biomarker after naturally occurring intra-articular fracture. *Osteoarthritis Research Society International OARSI World Congress**

Seewald LA, Sabino GS, Bohner A, **Delco ML** (2021). Synovial Fluid Mitochondrial DNA as a Biomarker of Articular Cartilage Injury. *American College Veterinary Surgeons Annual Symposium**

Seewald LA, Keller LE, Bennett MP, Casey JW, **Delco ML** (2020). Synovial fluid mitochondrial DNA concentration increases following cartilage injury. *ORS Annual Meeting*, Phoenix, AZ*

Seewald LA, Keller LE, Thomas M, Casey JW, **Delco ML** (2020). Mitoprotection prevents increased synovial fluid mitochondrial DNA concentrations after articular injury. *Osteoarthritis Research Society International World Congress (OARSI), Vienna, Austria.* Osteoarth Cart. Vol 28, Supl 1, S96, April 01, 2020 DOI: 10.1016/j.joca.2020.02.148

Seewald LA, et al., (2019). Synovial fluid mitochondrial DNA concentration increases following cartilage injury. Orthopedic Research Society Annual Meeting, Phoenix, AZ

Seewald LA, et al., (2019). Mitochondrial DNA is Released by Viable Chondrocytes After Induction of Mitochondrial Dysfunction. Osteoarthritis Research Society International World Congress, Toronto

Submitted Grants:

Delco (PI)

Sponsor/Mechanism: NIH Directors New Innovator Award (DP2)	2022 - 2027
Title: Next-Generation Mitochondria-targeted Therapies to Tackle the Most Common Causes of Pain and Disability	$2,355,000
Role: Principal Investigator	

We are also happy to report that this project has produced results above and beyond the original Aims; It will lead to at least 2 additional manuscripts, including: 1) investigation of mDAMPs in several different forms of articular pathology (OCD, chronic OA, intra-articular fracture, acute inflammation and sepsis) in the talocrural joint of horses, 2) the relationship of synovial fluid and plasma mtDNA to post-operative inflammation and outcome in human patients who had surgical repair of ACL tears.

*Our entire research group sincerely thanks the **Harry M. Zweig Memorial Fund for Equine Research** for supporting this work!*

Synovial Fluid Mitochondrial DNA Concentration Reflects the Degree of Cartilage Damage After Naturally Occurring Articular Injury

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Key Words: posttraumatic osteoarthritis, mitochondrial DNA, articular cartilage injury, mitoprotection, biomarker

Running Title: Synovial Fluid mtDNA and Cartilage Injury

Abstract:

Objective: Early intervention, prior to the development of degenerative joint changes, has the potential for improved success in treating posttraumatic osteoarthritis but requires early detection of joint injury. In other tissue types, trauma is associated with the extracellular release of mitochondrial DNA (mtDNA), which can both serve as a biomarker of tissue injury and perpetuate inflammation as a mitochondria-specific Damage Associated Molecular Pattern (mDAMP). The goal of this study was to evaluate mtDNA release from injured chondrocytes and investigate the utility of synovial fluid mtDNA concentration in early detection of posttraumatic osteoarthritis.

Design: We measured mtDNA release using four models of osteoarthritis: *in vitro* IL-1b stimulation, *ex vivo* mechanical impact, *in vivo* mechanical impact, and naturally occurring equine intraarticular fracture.

Results: We demonstrated that chondrocytes release mtDNA following cellular stress and that mtDNA is increased in equine synovial fluid following experimental and naturally occurring injury to the joint surface. In naturally occurring posttraumatic osteoarthritis, we found a strong correlation between the degree of cartilage damage and mtDNA concentration. Finally, impact-induced mtDNA release was mitigated by mitoprotective treatment.

Conclusions: Changes in synovial fluid mtDNA occur following joint injury. Further investigation of mtDNA as a potentially sensitive marker of early articular injury is warranted.

Introduction

Mounting evidence suggests that targeting very early pathomechanisms after articular injury may be the key to preventing osteoarthritis (OA) *(1)*; however, clinical signs of joint pain and immobility often appear years or decades after the inciting trauma, making early detection and intervention difficult or impossible *(2)*. Current diagnostics, including radiographs and MRI, often are not sensitive enough to detect disease prior to the onset of structural joint changes *(3)*. Therefore, an understanding of the response of cartilage to mechanical injury and events that perpetuate damage signals throughout the joint is essential to the development of more sensitive diagnostic tests and effective therapies for PTOA *(3, 4)*.

Recent work has demonstrated that mitochondrial dysfunction occurs as an acute response of chondrocytes to mechanical injury, resulting in several processes that lead to the development of PTOA, including the production of proinflammatory molecules *(5)*; a reduction in proteoglycan and collagen synthesis *(6, 7)*; glycosaminoglycan (GAG) release *(6, 8)*; and cartilage calcification *(7)*. Thus, mitochondrial dysfunction could serve as an indicator of cartilage damage in the peracute time frame after injury, and the preservation of mitochondrial structure and function, termed mitoprotection, may represent a valuable new strategy for preventing PTOA progression. Szeto-Schiller (SS) peptides are a class of mitoprotective agents that repair cristae structure and restore mitochondrial function, thereby improving cellular bioenergetics and preventing cell death *(9–11)*. In an *ex vivo* cartilage impact model, treatment with the mitoprotective peptide, SS-31 (elamipretide), shortly after injury prevented chondrocyte death and cartilage matrix degradation *(8)*. While mitoprotective agents are a promising new treatment strategy, their clinical application would require a practical and sensitive diagnostic test to identify patients experiencing mitochondrial dysfunction and to monitor response to therapy. No such disease markers have yet been identified.

One class of molecules, mitochondria-specific Damage Associated Molecular Patterns (mDAMPS), may prove to be useful indicators of early joint injury and mitochondrial dysfunction. mDAMPs are small molecules that are contained exclusively within the mitochondria in healthy cells *(12)*. When released into the cytosol or extracellular space, mDAMPs drive pro-inflammatory responses by a variety of pathways *(13)*. One mDAMP, mtDNA, has been validated as a biomarker of cellular stress in several tissue types and disease states *(14–16)*. Of particular relevance to orthopedic tissues, synovial fluid mtDNA was positively correlated with the degree of inflammation in rheumatoid arthritis patients *(17, 18)* and elevated serum mtDNA has been associated with femoral fractures *(19)*. As extracellular mtDNA has been associated with traumatic injury to cells *(14, 19, 20)*, synovial fluid mtDNA may be a promising candidate for early detection of orthopedic conditions precipitated by mechanical injury, such as PTOA.

Several potential mechanisms for extracellular mtDNA release have been proposed, including opening of the mitochondrial permeability transition pore *(21, 22)*, altered mitophagy *(23)*, and release of exosomes *(24, 25)*. These mechanisms occur concurrently with reactive oxygen species (ROS) formation and mitochondrial dysfunction *(23, 26, 27)*, suggesting a link between mtDNA release and mitochondrial dysfunction within cells. In the context of PTOA, synovial fluid extracellular mtDNA may provide a practical method for early diagnosis of cartilage injury and facilitate monitoring of response to mitoprotective treatment. Here, we use a robust series of translational models, from *in vitro* cell culture to clinical cases of equine intra-articular fracture, to investigate extracellular mtDNA release and the effects of mitoprotective therapy following acute cartilage injury.

The objectives of this study were to: 1) determine whether injured chondrocytes release mtDNA, 2) measure synovial fluid mtDNA in *in vivo* and naturally occurring models of PTOA, and 3) investigate the effect of mitoprotection on mtDNA release.

Methods

In vitro chondrocyte stimulation

Second passage chondrocytes previously harvested from normal femoropatellar joints of healthy adult horses (n = 4, 2-5 years old) and cultured under physoxic conditions (37°C, 5% O2, 5% CO2) were stimulated with interleukin-1b (IL-1b, 1 ng/mL) and compared to unstimulated cells. Conditioned media and trypsinized cells were collected 12 hours after stimulation. A second replicate was cultured for an additional 12 hours after stimulation media removal. Conditioned media and cells were collected at 24 hours.

Ex vivo cartilage impact injury

An explant model of articular injury was used to evaluate mtDNA release following mechanical overload, as previously described *(8)*. Briefly, cartilage explants were obtained from the medial femoral condyles of healthy bovids (n = 3, 1-3 days of age) within 48 hours of euthanasia using an 8 mm biopsy punch. Samples were rinsed with PBS and cultured in cartilage explant media under standard culture conditions (37°C, 21% O2, 5% CO2). One group of explants were impacted using a spring-loaded impacting device designed to deliver a rapid compression injury (24.0 ± 1.4MPa peak stress; 53.8 ± 5.3 GPa/s peak stress rate) and instrumented with a load cell to measure impact force. Explants were cultured as above and conditioned media was collected 1, 3, and 5 days after impact.

In vivo cartilage impact injury

An equine talocrural model of impact-induced articular injury was used to evaluate mtDNA content in synovial fluid following mechanical injury, as previously described *(28)*. Briefly, adult horses (n = 12, 2-5 years) with clinically and radiographically normal talocrural joints were placed under general anesthesia. Three focal cartilage injuries were created arthroscopically on the medial trochlea of both tali using a hand-held impacting device *(29)*. Either the mitoprotective peptide SS-31 (elamipretide, 1 uM, n = 6), or saline (control, n = 6) was administered intra-articularly to both joints 1 hour after injury. Synovial fluid was collected from each joint before injury, and 7, 14, 28, and 42 days after injury.

Microrespirometry

Real-time microscale respirometry was used to measure mitochondrial respiratory function and control in chondrocytes cultured with or without the addition of SS-31. Extracellular acidification rate (ECAR) and oxygen consumption rate (OCR) were measured over the course of a mitochondrial stress test using an XFe96 Analyzer (Seahorse Biosciences, North Billerica, MA). See supplemental methods for details. Microrespirometry data was normalized to cell number by dividing oxygen consumption rates by the number of cells per well. Mitochondrial functional indices were calculated as previously described *(30)*: non-mitochondrial respiration (NMR) = rotenone/antimycin A-stimulated OCR; basal OCR (bOCR) = initial OCR – NMR; maximal OCR (mOCR) = FCCP-stimulated OCR – NMR; spare respiratory capacity (SRC) = mOCR – bOCR; proton leak = (oligomycin-stimulated OCR - NMR)/bOCR; ATP production = (initial OCR – oligomycin-stimulated OCR)/bOCR.

Equine naturally occurring intra-articular fracture cases

Clinical cases were selected from equine patients with intra-articular carpal fractures presenting to either Cornell Hospital for Animals in Ithaca, NY, or Cornell Ruffian Equine Specialists in Elmont, NY, and from historic samples available through the Cornell Veterinary Biobank. Inclusion criteria

included any horse presenting for arthroscopic removal of osteochondral fragmentation of the carpal bones or distal radius (i.e. carpal chip fracture). Synovial fluid was obtained from patients during standard clinical care, such as during routine arthrocentesis or prior to joint distension for arthroscopy. A standard set of radiographs (5 views) obtained at presentation or by the referring veterinarian were blindly scored for changes associated with degenerative joint disease (table S3) by one author (M.L.D.). Arthroscopic video footage was blindly consensus scored for lesions associated with the cartilage, subchondral bone, and synovium (table S5) by two authors (L.A.S., M.L.D.). Cartilage lesion depth was scored at four sites: immediately surrounding the fracture, on the same bone but distant to the fracture site, on the opposing articular surface, and in remote locations within the same joint, using a modified ICRS scoring system *(31)* (table S5, fig. S6). Post-operative athletic performance was evaluated for all racing thoroughbreds. Race records were obtained through Equibase.com and used to evaluate return to racing and number of race starts following surgery. For comparison, synovial fluid was obtained from the carpal joints of healthy adult horses (n = 7) without clinical evidence of joint disease euthanized for reasons unrelated to this study.

DNA quantification

mtDNA and nDNA were quantified by Taqman qPCR on a ViiA 7 real-time PCR system (Applied Biosystems, Foster City, CA, USA) using equine- and bovine-specific primers and probes.
Primers were validated *in silico* and *in situ* as per Bustin and Huggett *(32)*. Samples were analyzed in triplicate and the mean was used in subsequent calculations. A standard curve was created using isolated amplicons for each gene target and used to calculate DNA concentration (copies/µL) in unknown samples.

Statistical analysis

In vitro groups were compared using students' (paired) t test. Intracellular DNA was compared across individual and passage by two-way ANOVA. *Ex vivo* groups were compared within time points using students' t test, and across time points using a linear mixed effects model with joint and individual as random variables. For comparison between treated and untreated groups, data were normalized to baseline values and compared using a linear mixed effect model with joint as a random variable. For mixed effects models and ANOVAs, Tukey's post hoc test for multiple comparisons was used. Data from clinical cases were compared using students' t test or one-way ANOVA. Fisher's exact test was
used to determine associations between categorical variables. Pearson correlation analysis was used for associations between DNA concentration and radiographic, arthroscopic, and performance data. Soft tissue swelling scores were binned into < 2 (none/mild) and ≥2 (moderate/severe) and compared using students' t test. Of note, this method of stratification was not decided *a priori*. For each model, residuals were evaluated for normality and homogeneity. Data were log transformed as necessary. Two-sided tests were used for all comparisons and significance was set at $p < 0.05$. Statistical analyses were performed using JMP Pro 15 software (SAS, Cary, NC).

Results

Chondrocytes release mtDNA following inflammatory stress

To evaluate chondrocytes mtDNA release following cellular stress, we used an established *in vitro* model of osteoarthritis *(33)*. Chondrocytes from healthy, young adult horses were cultured under physiologic conditions (5% O2, 5 µM glucose) and stimulated with a sublethal dose of interleukin 1 beta (IL-1b, 1 ng/µL) (Fig. 1A). Total cell counts during stimulation (12 hours) and after removal of the

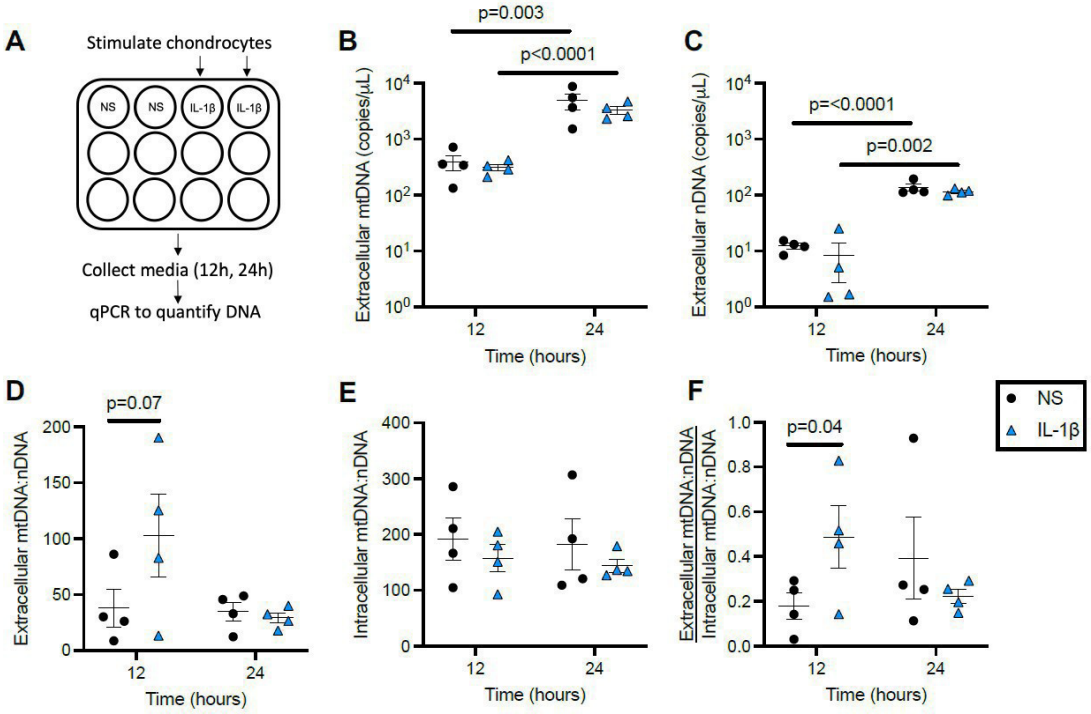

Fig. 1. Cultured chondrocytes selectively release mtDNA in response to an inflammatory stimulus. (**A**) Equinechondrocytes were cultured either with (IL-1b) or without (NS) a sublethal dose of IL-1b for 12 hours. Media was collected at 12 hours and 24 hours for mtDNA and nDNA quantification. (**B-C**) Extracellular mtDNA and nDNA concentration in chondrocyte-conditioned media. (**D**) Extracellular mtDNA concentration normalized to extracellular nDNA concentration (extracellular mtDNA:nDNA ratio). (**E**) Intracellular mtDNA concentration normalized to intracellular nDNA concentration (intracellular mtDNA:nDNA ratio). (**F**) Extracellular mtDNA:nDNA ratio normalized to intracellular mtDNA:nDNA ratio. All data are means ± SEM, n = 4, statistics by students' (paired) t test. Line connects groups different with p-values listed.

inflammatory stimulus (24 hours) were not different between control and stimulated groups (Fig. S1), confirming that IL-1b did not result in chondrocyte death. In both groups, extracellular mtDNA and nDNA isolated from chondrocyte-conditioned media increased from the 12- to 24-hour time point,
suggesting that cell death occurred over the course of chondrocyte culture regardless of treatment group. Neither extracellular mtDNA (Fig. 1B), nDNA (Fig. 1C), nor extracellular mtDNA:nDNA ratio (Fig.
1D) were different between IL-1b-stimulated and controls. Intracellular mtDNA:nDNA ratio did not
vary between groups or time points (Fig. 1E) but did vary between individuals and chondrocyte passage (fig. S2). To account for this biologic variability, we normalized extracellular mtDNA:nDNA ratio to intracellular mtDNA:nDNA ratio for each individual. This normalized extracellular mtDNA:nDNA ratio was elevated during IL-1b stimulation (12 hours; Fig 1F, p = 0.04) compared to controls, but returned to control levels after removal of the inflammatory stimulus (24 hours; Fig. 1F).

Chondrocytes release mtDNA as an acute-phase response following mechanical injury

We next investigated whether injurious mechanical loading of cartilage would result in mtDNA release from cartilage explants after a single, rapid compression (impact) injury (Fig. 2A). Extracellular mtDNA from cartilage conditioned media was elevated for injured explants (IN, n = 9) compared to uninjured explants (C, n = 6) at 1 day post-injury but not at later time points (Fig. 2B). Extracellular nDNA was increased 5 days after injury but not at earlier time points (Fig. 2C), suggesting that extracellular mtDNA release precedes cell death in this model of mechanical chondrocyte injury.

Mitoprotection mitigates mtDNA release following impact-injury in vivo

To evaluate mtDNA release *in vivo*, focal impact injuries of defined magnitude were delivered to
the articular surface of the medial trochlear ridge of adult horses under arthroscopic guidance (Fig. 3A-

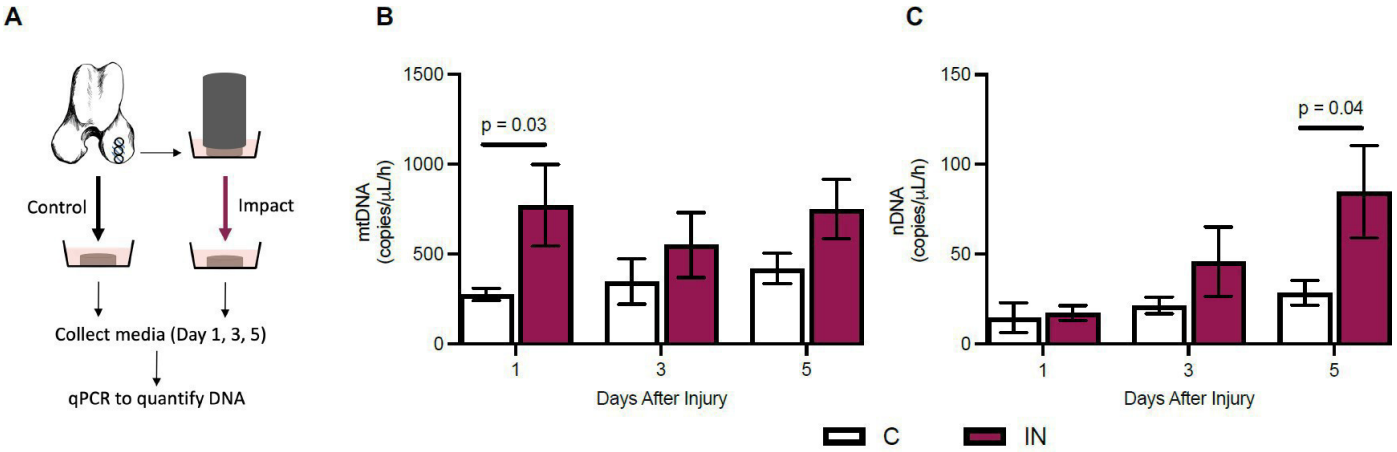

Fig. 2. Chondrocytes release mtDNA as an acute phase response to mechanical injury prior to cell death. (**A**) Cartilage explants were harvested from the medial femoral condyle of neonatal bovids. Explants were injured by delivering a single, rapid mechanical overload (IN, n = 9) and compared to unimpacted controls (C, n = 6). Cartilage conditioned media was collected serially for qPCR quantification of extracellular mtDNA and nDNA. (**B-C**) Conditioned media mtDNA and nDNA concentration for control (C) and injured (IN) explants. Groups compared by students' t test within time points. Line connects groups significantly different with p-values listed.

C, movie S1). The location of this experimental injury is analogous to the most common site of lesions on the human talus leading to talocrural joint OA (34, 40) (Fig. 3B). Experimental articular impacts produced an elevation in synovial fluid mtDNA at 7 days post-impact, but not at later time points (Fig. 3D). Further, elevated nDNA concentrations and decreased mtDNA:nDNA ratios occurred at all time points post-impact (Fig. 3E-F), suggesting this model of articular injury induces ongoing cell death within the joint. Mitoprotective therapy reduced concentrations of synovial fluid mtDNA compared to untreated joints at 14 and 28 days after injury (Fig. 3G). Cell death was also reduced in SS-31 treated joints at all time points (Fig. 3H). No differences in mtDNA:nDNA ratio were observed between treated and untreated joints (Fig. 3I).

Mitoprotective peptide SS-31 increases chondrocyte respiratory capacity

After observing the *in vivo* cytoprotective effects of SS-31, we investigated the mitoprotective effects on cultured chondrocytes by performing a microrespirometry assay and mitochondrial stress test, with and without SS-31 treatment. Treated chondrocytes had a higher maximum oxygen consumption rate (mOCR) and spare respiratory capacity (SRC) than controls (Fig. 4A-B, $p<0.05$). There was no difference in the relative contributions of proton leak and ATP turnover to basal oxygen consumption rate (bOCR) (Fig. 4C, $p < 0.05$).

Synovial fluid mtDNA correlates with cartilage damage in naturally occurring joint injury

Finally, we investigated synovial fluid mtDNA in cases of naturally occurring articular injury. The case population consisted of equine patients presenting for removal of intra-articular osteochondral carpal fragments, the most common arthroscopic procedure performed in athletic horses. Synovial fluid samples were analyzed from a total of 19 horses. Patients were predominantly male (84%), 3–5-year-old (79%) Thoroughbred (79%) racehorses (74%) presenting for chip fractures involving either the radiocarpal (n = 6, 32%) or middle carpal joint (n = 13, 68%). For comparison, synovial fluid was analyzed from the carpal joints of clinically normal horses (n = 7 horses, n = 12 joints). There was no

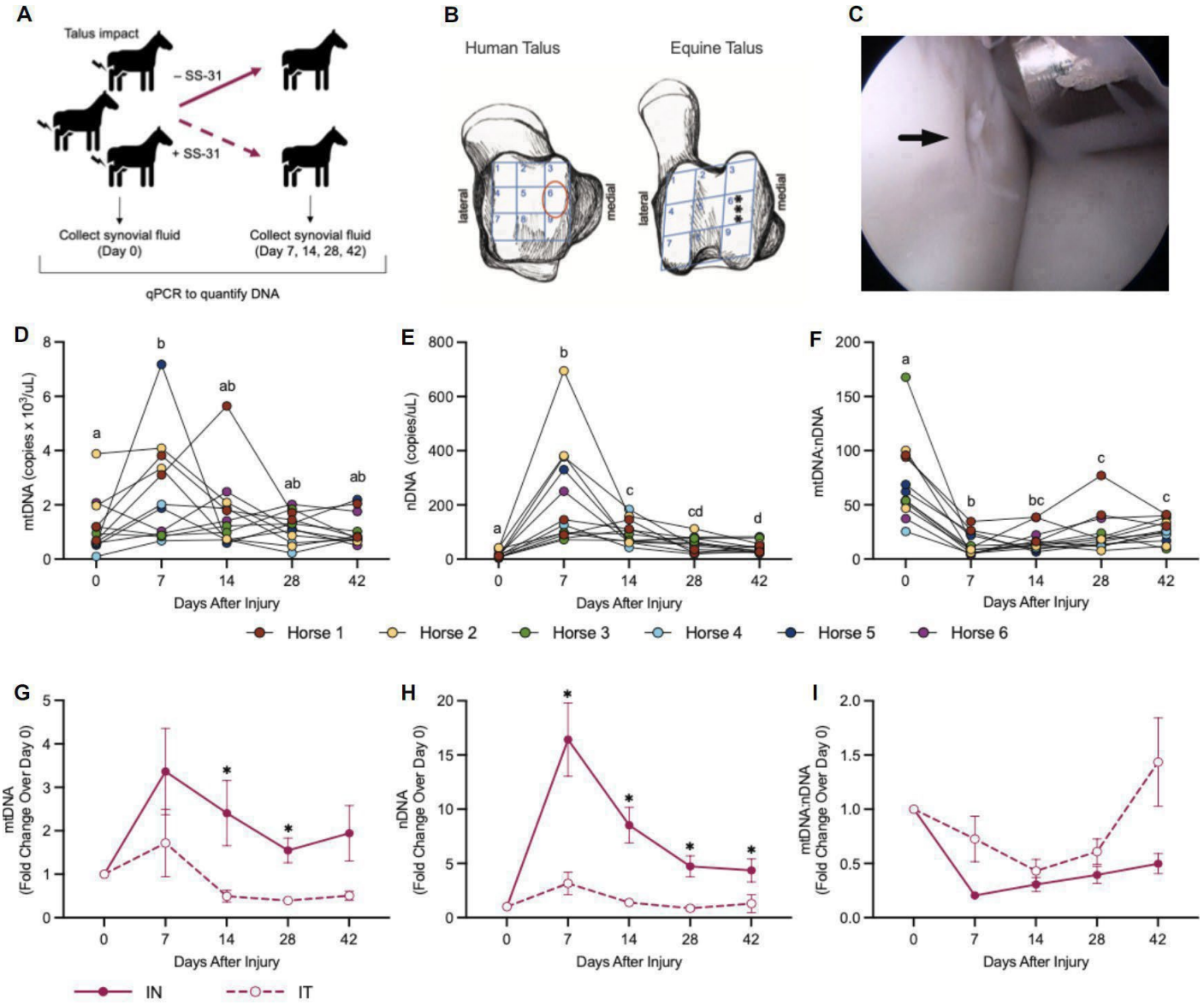

Fig. 3. Mitoprotection mitigates mtDNA release *in vivo* following mechanical articular cartilage injury. (**A**) Adult horses were placed under general anesthesia and three focal cartilage injuries were delivered to the talus in each talocrural joint. One group was treated with intra-articular injection of the mitoprotective peptide SS-31 one hour after injury. Synovial fluid was collected serially after injury and compared to pre-injury synovial fluid samples. (**B**) Schematic comparing the anatomy of the human and equine talus. Asterisks indicate sites of cartilage impact. Red circle identifies analogous region on the human talus *(from Delco, et al., AJSM 2020; permission pending).* (**C**) Arthroscopic image of the impacting device positioned on the medial trochlear ridge of the talus after impact. Arrow indicates cartilage defect caused by impact. (**D-F)** Synovial fluid mtDNA, nDNA, and mtDNA:nDNA ratio in injured, untreated joints (IN, n = 12). Time points not sharing a letter are significantly different ($p < 0.05$), statistics by linear mixed effects model. (**G-I)** Fold change over baseline (day 0, preinjury) of synovial fluid mtDNA, nDNA, and mtDNA:nDNA ratio in untreated (IN, n = 12) versus treated (IT, n = 10) joints. Data are means ± SEM, statistics by linear mixed effects model. *P<0.05 compared to IT within time points.

difference in age or sex distribution between the normal and fracture groups; however, differences in breed did exist between groups, with the fracture group consisting of a higher proportion of thoroughbreds ($p = 0.0002$) than the control group. See table S1 for details.

We found synovial fluid mtDNA and nDNA concentrations were elevated in injured compared to normal joints (Fig. 5A and 5B). Among injured joints, mtDNA but not nDNA concentrations were higher for middle carpal versus radiocarpal joint fragments (Fig. 5C and 5D). There were no differences in mtDNA:nDNA ratio between injured and non-injured or between middle carpal and radiocarpal

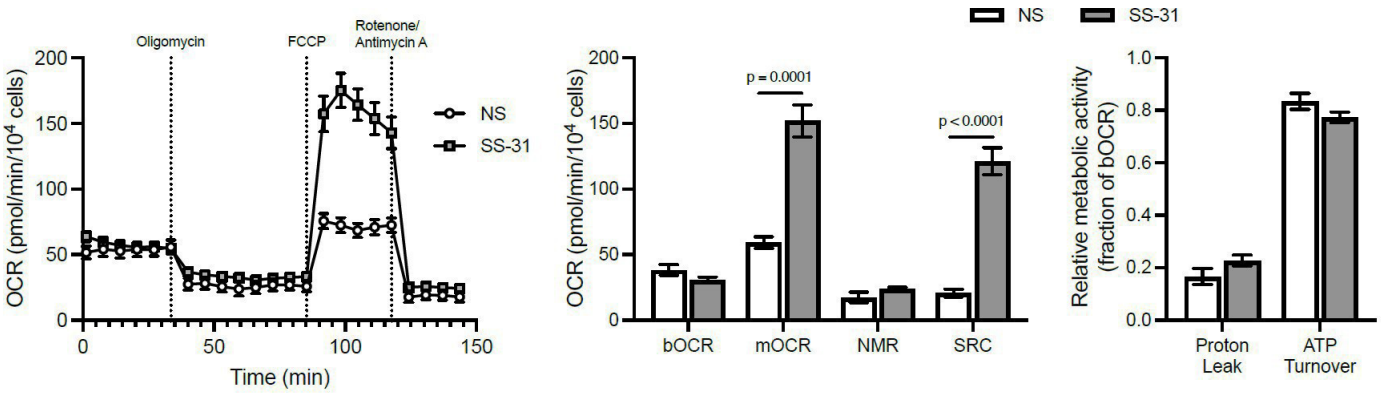

Fig. 4. SS-31 increases chondrocyte maximal oxygen consumption rate and spare respiratory capacity. (**A**) Chondrocyte oxygen consumption rate (OCR) during mitochondrial stress test with (SS-31) and without (NS) mitoprotective treatment (n = 7). (**B**) Basal OCR (bOCR), maximal OCR (mOCR), non-mitochondrial respiration (NMR), and spare respiratory capacity (SRC) for chondrocytes with and without treatment. (**C**) Proton leak and ATP turnover (as a fraction of bOCR) for chondrocytes with and without treatment. All data are means ± SEM, n = 7. Statistics by students' t test. Line connects groups different with p-values listed.

fragments (fig. S4). No significant differences existed between patient variables (age, breed, sex, side of injury) and mtDNA, nDNA, or mtDNA:nDNA ratio (table S2).

Radiographs were available for 17 of the 19 injured joints. The mean total radiograph score for joints with intra-articular fractures was 5.1 (95% CI, 3.4-6.8) out of a maximum score of 15, indicating that individuals in this study had little radiographic evidence of joint disease. No strong correlations were found between any scoring category and mtDNA concentration, nDNA concentration, or mtDNA:nDNA, except for moderate negative correlations between mtDNA:nDNA and fracture severity ($r = -0.61$, $p = 0.008$) and mtDNA:nDNA ratio and total radiograph score ($r = -0.49$, $p = 0.046$) (table S4). Joints with moderate to severe soft tissue swelling had significantly higher mtDNA (but not nDNA or mtDNA:nDNA ratio), than joints with no or mild soft tissue swelling (Fig. 5E-H, fig. S4).

Intraoperative video footage was available for 17 of the 19 injured joints. Total score, calculated as the sum of five component-scores, showed a strong positive correlation ($r = 0.76$, $p = 0.0004$) with synovial fluid mtDNA and nDNA concentration (Fig. 5I, fig S5). Mean cartilage lesion depth showed the strongest correlation ($r = 0.80$, $P = 0.0001$) with mtDNA of any of the component criteria (Fig. 5I-L). Several other component scores had moderate correlations with mtDNA or nDNA (Table 1, table S6). Race records were available for 13/14 thoroughbreds, and all (13/13) returned to racing after surgery. Synovial fluid mtDNA showed a moderate negative correlation ($r = -0.56$, $p = 0.046$) with the number of race starts in the first year after surgery (Fig. 5M).

Discussion

Extracellular mtDNA has been investigated as a biomarker in several tissue types and disease states *(14, 15, 18– 20, 24, 34, 35)*, and this work represents the first investigation of mtDNA as a potential biomarker in PTOA. We demonstrated that chondrocytes release mtDNA in response to mechanical and inflammatory stress. While cell death can result in passive release of mtDNA *(13, 36)*, our *in vitro* data suggest that viable, IL-1b-stressed chondrocytes actively release mtDNA. While mechanisms underlying this active release in cartilage have not been investigated, mDAMP release in other tissues is closely associated with mitochondrial dysfunction *(13, 22)*. Prior work has demonstrated that IL-1b induces chondrocyte mitochondrial dysfunction, characterized by increased NO production, decreased mitochondrial respiratory chain complex I activity, and membrane depolarization *(6, 37)*. Mitochondrial dysfunction increases cellular ROS production, which has been associated with mtDNA

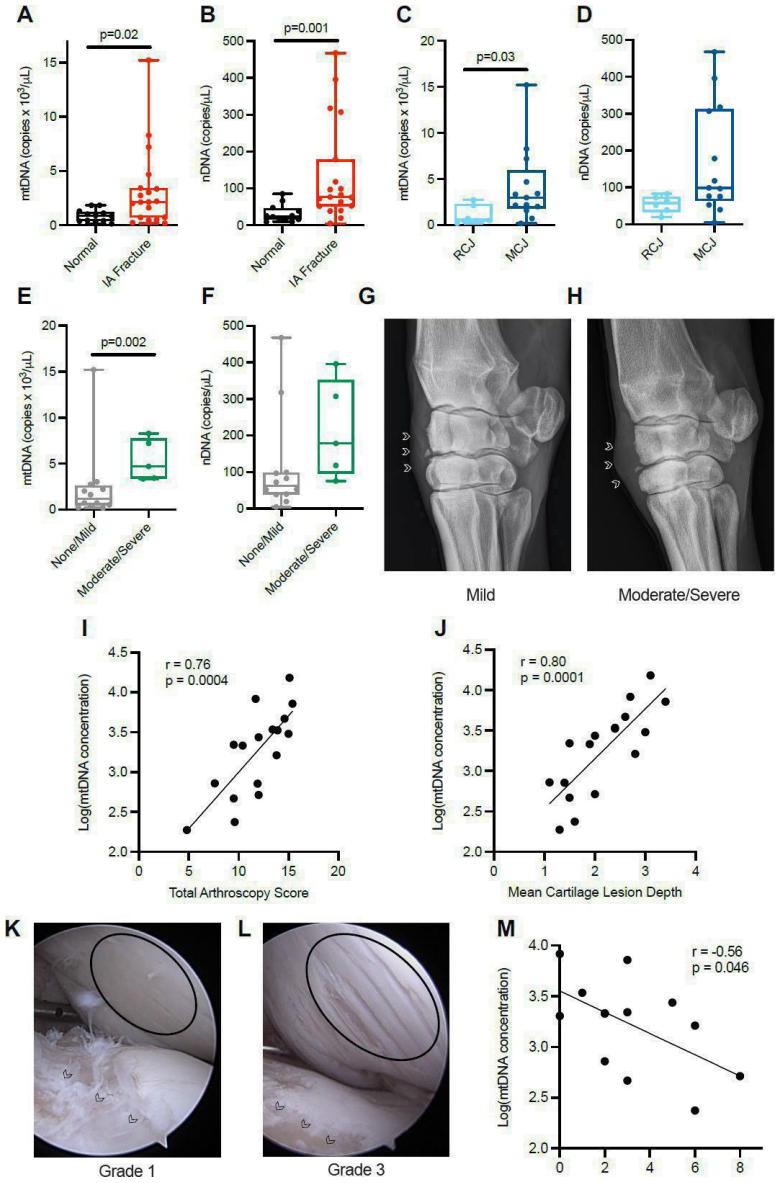

Fig. 5. Synovial fluid mtDNA concentration correlates with the severity of cartilage injury. (**A-B**) Synovial fluid mtDNA and nDNA concentration in joints with intra-articular (IA) carpal fractures compared to normal joints. (**C-D**) Comparison of mtDNA and nDNA between radiocarpal joint (RCJ) and middle carpal joint (MCJ) fractures. (**E-F**) Synovial fluid mtDNA and nDNA in joints with intra-articular carpal fractures and no or mild soft tissue swelling/joint effusion compared to those with fractures and moderate to severe soft tissue swelling/joint effusion. (**G-H**) Representative radiographs of joints with mild and moderate to severe soft tissue swelling/joint effusion. (**I**) Correlation of total arthroscopy score with mtDNA concentration in joints with IA fractures. (**J**) Correlation of mean cartilage lesion depth with mtDNA concentration in joints with IA fractures. (**K-L**) Representative arthroscopic images of joints with grade 1 and grade 3 cartilage lesion depth. Circle indicates area of cartilage damage on articulating surface. Arrow heads indicate fracture bed. (**M**) Correlation of number of race starts in the first year after surgery with mtDNA concentration. (**A-F**) Statistics by students' t test. Line connects groups that are significantly different with p-values listed. (**I, J, M**) Statistics by Pearson correlation analysis.

release via activation of the MPTP, alterations in mitophagy, or production of mitochondria-derived extracellular vesicles *(21–25)*. Any of these mechanisms may play a role in active release of mtDNA from chondrocytes and further studies would be necessary to identify which processes occur in the context of cartilage injury and early joint disease.

	mtDNA log(concentration)		nDNA log(concentration)	
	R	**P**	**R**	**P**
Cartilage Lesion Depth	**0.80**	**<0.001**	**0.75**	**<0.001**
Cartilage Lesion Size	0.59	0.012	0.69	<0.001
Subchondral Bone	0.30	0.237	0.19	0.456
Active Synovitis	0.64	0.006	0.61	0.010
Chronic Synovial Change	0.46	0.062	0.68	0.003
Total	**0.76**	**<0.001**	**0.79**	**<0.001**

Table 1. Arthroscopic scoring correlation data. Values with R > 0.7 and P < 0.001 are bolded. Statistics by Pearson's correlation analysis.

Teasing out active versus passive mtDNA release is complicated by variability in intracellular mtDNA concentration. Many factors affect the number of mtDNA copies per cell, including tissue type,energy balance, and disease state. For example, studies in several species indicate that mitochondrial content decreases with age *(39)*. Further, OA chondrocytes contain less mtDNA than healthy chondrocytes, reflected by a lower intracellular mtDNA:nDNA ratio, which can be attributed to mitochondrial dysfunction and impaired mitochondrial biogenesis *(38)*. This variability in intracellular mtDNA confounds interpretation of extracellular mtDNA:nDNA ratio when non- specific cell death causes the entire cellular DNA content to be released into the extracellular environment. As such, we normalized *in vitro* extracellular mtDNA:nDNA to intracellular mtDNA:nDNA for each chondrocyte population to better differentiate between active and passive mtDNA release. To the authors' knowledge, this method has not been reported previously, but our data suggest it is the most appropriate for *in vitro* detection of selective mtDNA release from primary cultured cells, as it accounts for differences we observed in intracellular mtDNA content during cell passage, as well as between individuals. Beyond *in vitro* studies, however, mtDNA:nDNA ratio diminishes in value. Our previous work suggests that *in situ*, chondrocytes experience differential levels of mitochondrial dysfunction in response to mechanical injury, *(40)* which is likely mirrored by a spectrum of mtDNA turnover (i.e. mitophagy and biogenesis), and release *(41)*. As such, mtDNA:nDNA ratio is not expected to accurately reflect changes in mtDNA release at the individual cell level. Furthermore, at the whole joint level, multiple cell and tissue types likely contribute to synovial fluid DNA concentrations, making mtDNA:nDNA ratios even more complicated to interpret. These factors likely account for the lack of difference between mtDNA:nDNA ratios *in vivo* . Thus, we believe absolute mtDNA concentration is likely the most practical and useful measure for synovial fluid samples, whereas mtDNA:nDNA ratio is best for investigating mechanisms of mtDNA release *in vitro*.

When translating our *ex vivo* findings to an *in vivo* model, we found that increases in synovial fluid mtDNA are detectable after articular injury, likely reflecting mitochondrial dysfunction within the joint environment. Importantly, evidence suggests that in addition to serving as a possible a marker of injury-induced mitochondrial dysfunction, extracellular mtDNA may also act as a signaling molecule that perpetuates joint inflammation in early OA. Due to its bacterial ancestry, mtDNA contains nonmethylated CpG islands that can bind and stimulate toll-like receptor 9 in immune cells, triggering proinflammatory signaling cascades *(42–45)*. Intra-articular injection of mtDNA produced inflammatory arthritis in mice, whereas injection with nDNA did not *(46)*. Thus, early release of mtDNA by chondrocytes following mechanical stress may serve to initiate a pro-inflammatory response throughout the joint via activation of immune cells such as synovial macrophages. Interestingly, in our *in vivo* model we found that synovial fluid mtDNA was elevated shortly after injury, then returned to baseline

levels, whereas elevated nDNA persisted for weeks. These data suggest that mtDNA elevations reflect acute joint injury and are consistent with our *in vitro* and *ex vivo* models in which mtDNA release occurred within 24 hours of injury. Moreover, the pro-inflammatory potential of mtDNA and, as our data indicate, the large number of mtDNA copies per cell and in synovial fluid compared to nDNA make it a relevant molecule to quantify. Further studies are warranted to investigate the link between extracellular mtDNA and persistent low-grade joint inflammation, which is an important contributing factor to the development of PTOA *(3)*.

We found that mitoprotective treatment with SS-31 increased spare respiratory capacity in chondrocytes. SS-31 is known to prevent mitochondrial dysfunction, ROS production, and cell death by stabilizing the inner mitochondrial membrane-specific phospholipid, cardiolipin *(11)*. Recently, we found SS-31 mitigated peracute mitochondrial dysfunction, preserved chondrocyte viability, and prevented cartilage matrix degeneration in a cartilage explant injury model *(40)*. In the current study, intraarticular SS-31 reduced cell death in our *in vivo* talar impact model. Mitochondrial dysfunction is a significant contributor to apoptotic and necrotic cell death in the early time course after articular injury, and these findings suggest mitoprotection may be a valuable therapeutic strategy to prevent PTOA. We also found that SS-31mitigated synovial fluid mtDNA elevations in injured joints. Although the mechanisms are not clear, evidence in other tissues suggest cells actively release mtDNA in response to mitochondrial dysfunction and increased ROS production *(23, 26, 27)*. Injured chondrocytes are known to experience strain-dependent mitochondrial dysfunction *(40, 47)* and ROS release *(48)*. Further, one study found that fibroblasts released mtDNA in response to changes in tissue stiffness, suggesting mechanotransduction may play an important role in selective mtDNA release *(16)*. Together, these findings suggest that mitochondrial dysfunction and extracellular mtDNA release occur concurrently in chondrocytes experiencing mechanical stress, and may represent a novel role for mitochondria-mediated mechanotransduction in OA. Extracellular mtDNA may represent a practical means for detecting mitochondrial dysfunction and for evaluating response to mitoprotective therapy following articular injury.

Finally, this is the first study to evaluate synovial fluid mtDNA following naturally occurring articular injury and we found that mtDNA concentration is elevated in cases of intra-articular fracture. Equine athletes represent a valuable model to study naturally occurring OA, as equine and human joints are similar in terms of cartilage thickness, cell structure, biochemical properties, and mechanical loading *(58, 59)*. Equine carpal fragments are a useful model because these injuries are common in racehorses *(50)*, are traumatic in origin resulting from either repetitive loading or a single acute overload, and frequently lead to OA *(60)*. Thus, our findings are particularly relevant to the study of PTOA, and analysis of synovial fluid from human patients to determine the association between joint injury, extracellular mtDNA, and OA is warranted. In one study investigating the association between mtDNA and immune-mediated arthritis, human synovial fluid mtDNA concentration ranged from 101 to 104 copies/mL for patients with chronic OA compared to 102 to 107 copies/mL for patients with rheumatoid arthritis *(18)*. Unfortunately, this study did not include healthy controls. In our equine intra-articular fracture patients, mtDNA concentrations were on the magnitude of 105 to 106 copies/mL, much higher than those reported for patients with chronic OA, and compared to ~101 copies/mL in our controls. Chronicity, type of joint pathology, species variation, and differences in mtDNA quantification method could contribute to these differences.

We found no association between mtDNA and radiographic bone changes, but a strong correlation with the degree of cartilage damage on arthroscopic joint evaluation. This is noteworthy, because racehorses with carpal fragments and severe concurrent cartilage damage return to racing at a significantly lower rates than those with mild cartilage injury *(49, 50)*. Radiographic and arthroscopic

findings are often disparate in early joint disease, with arthroscopic findings tending to be more severe and of higher prognostic value *(50–53)*. As such, synovial fluid mtDNA be a less invasive means to gain prognostic information and inform therapeutic decisions. Indeed, synovial fluid mtDNA in this subpopulation of elite equine athletes (flat-racing Thoroughbreds) was negatively correlated with the number of race starts after surgery. Given debate over the most appropriate post-operative performance measures in racehorses *(54)*, this association should not be over-interpreted; nevertheless, few studies have found associations between biomarkers and performance outcomes, so this finding in a small number of cases warrants further investigation to determine the prognostic value of mtDNA in a larger cohort. In addition, we found that patients with fractures in the middle carpal joint had higher synovial fluid mtDNA than those involving the radiocarpal joint, which is intriguing because middle carpal joint fractures carry a worse prognosis for return to racing than radiocarpal joint fractures *(55–57)*. Due to the difficulty in obtaining synovial fluid samples from healthy racehorses, we were unable to determine if mtDNA concentrations vary between normal middle and radiocarpal joints in this patient population, or this finding reflects subtle differences in the severity of early pathology not detectable using other diagnostic modalities.

Although this study provides preliminary evidence for the utility of mtDNA in the diagnosis and treatment of early joint disease, it has several limitations. First, while our *in vitro* experiments demonstrated that chondrocytes release mDAMPs following inflammatory stress, our methods did not allow us to identify the cell/tissue source(s) of mtDNA and nDNA in synovial fluid *in vivo*. As such, synovial fluid mtDNA cannot be considered specific to mitochondrial dysfunction in cartilage, and other joint tissues such as synovium, subchondral bone, or infiltrating immune cells likely contribute to synovial fluid mtDNA. Moreover, this study did not determine whether mtDNA is merely a marker of joint injury or actively contributes to low-grade inflammation characteristic of OA progression *(3)*. Further studies are necessary to investigate the role of mtDNA/TLR9 signaling pathway in crosstalk between joint tissues and in the inflammatory response following articular injury, as reported in other disease processes *(42–45)*.

Conclusions

This study represents the first investigation of extracellular mtDNA in association with PTOA. We demonstrated that chondrocytes release mtDNA following inflammatory and mechanical injury *ex vivo*. In a large animal *in vivo* model, elevations in synovial fluid mtDNA were detected following articular injury and mitoprotective therapy prevented this increase, suggesting mtDNA may be a sensitive indicator of both early articular injury and mitochondrial (dys)function within the joint environment. Finally, in a naturally occurring animal model of early PTOA, synovial fluid mtDNA had a strong correlation with severity of cartilage damage. Therefore, further studies to characterize synovial fluid mtDNA changes in human clinical cohorts and determine its diagnostic and prognostic utility are warranted.

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M.L.D performed *ex vivo* and *in vivo* experiments. All authors contributed to data collection, analysis, and interpretation. L.A.S. drafted the manuscript, with contributions from I.G.S and K.L.M., and revision by M.L.D. All authors contributed to editing the manuscript and approve the final submission.
Competing interests: No financial support or other benefits have been obtained from any commercial sources for this study and the authors declare that they have no competing financial interests. **Data and materials availability**: All data associated with this study are present in the paper or the Supplementary Materials.

Supplementary Materials

Materials and Methods

Intracellular DNA quantification for chondrocytes of increasing passage

Primary chondrocytes previously harvested and banked from normal femoropatellar joints of healthy adult horses (n = 3, 2-5 years old) were cultured in standard chondrocyte media (Ham's F12 containing 10% FBS, HEPES 0.025 mL/mL, penicillin 100 U/mL, streptomycin 100 U/mL, glucose 10mM) under physoxic conditions (37°C, 5% O2, 5% CO2). Chondrocytes were trypsinized when they reached 90% confluency, at which time an aliquot of cells was removed for lysis and intracellular DNA quantification. The remaining cells were replated. This process was repeated through passage 3 and for a total of four replicates per horse.

Specific culture conditions

For *in vitro* experiments, primary chondrocytes previously harvested and banked from normal femoropatellar joints of healthy adult horses were cultured under physoxic conditions in low-glucose chondrocyte media (1:1 Ham's F12: DMEM containing 10% FBS, HEPES 0.025 mL/mL, penicillin 100 U/mL, streptomycin 100 U/mL, glucose 5mM) for two passages. Second passage chondrocytes were plated on a 24-well plate at a density of 0.2 x 106 cells/well. Twelve hours after plating, chondrocytes were rinsed with phosphate buffered saline (PBS) before the addition of serum free stimulation media (1:1 Ham's F12: DMEM containing HEPES 0.025 mL/mL, penicillin 100 U/mL, streptomycin 100

U/mL, glucose 5mM) with or without the addition of interleukin-1b (1ng/mL). Each combination of horse and stimulant was plated in duplicate. Twelve hours after the addition of stimulation media, conditioned media was collected. For one replicate of each condition, the chondrocytes were trypsinized, rinsed with PBS, and centrifuged at 800xg for 5 min. An aliquot of cells from each well (20% of the cells) was removed for cell lysis and intracellular DNA quantification. For the second replicate of each condition, low- glucose chondrocyte media was added, and the cells were incubated for another twelve hours, after which the media and cells were collected in the same manner as the 12-hour time point.

For *ex vivo* experiments, cartilage explants were cultured in cartilage explant media (phenol free DMEM containing 1% FBS, HEPES 0.025 mL/mL, penicillin 100 U/mL, streptomycin 100 U/mL, glucose 2.25 mM). For microrespirometry experiments, fourth passage murine chondrocytes previously

harvested and banked from the coxofemoral joints of neonatal UBC mCherry mice (Jax stock 017614, n = 7, 5 days old) were cultured in low-glucose murine chondrocyte media (DMEM containing 10% FBS, L-glutamine 2mM, penicillin 50 U/mL, streptomycin 50 U/mL, glucose 5mM). At approximately 80% confluency, media was changed to serum-free low-glucose murine chondrocyte media (DMEM containing 2mM L-glutamine 2mM, penicillin 50 U/mL, streptomycin 50 U/mL, glucose 5mM) with or without the addition of SS-31 (1uM). Chondrocytes were cryopreserved in murine chondrocyte freeze media (80% low-glucose murine chondrocyte media, 10% FBS, 10% DMSO) until microrespirometry was performed. Prior to the respirometry assay, chondrocytes were thawed and plated onto a 96 well microplate (Seahorse Biosciences, North Billerica, MA) at a density of 20,000 cells per well.
Chondrocytes were incubated under standard culture conditions (37°C, 21% O2, 5% CO2) in lowglucose murine chondrocyte culture media for 24 hours. After 24 hours, media was changed to assay media (DMEM containing glucose 10mM, pyruvate 1mM, L-glutamine 2mM; Seahorse Biosciences, North Billerica, MA) and the mitochondrial stress test was performed.

Microrespirometry

Extracellular acidification rate (ECAR) and oxygen consumption rate (OCR) were measured for each well approximately every 7 minutes for ~150 minutes total. After baseline respiration was measured 6 times, a mitochondrial stress test was performed using the XF Cell Mito Stress Test Kit (Seahorse Biosciences, North Billerica, MA) according to standard protocols *(30)*; OCR was measured in response to the automated addition of the following i) oligomycin (1.5 µM; 8 measurements), an ATP synthase inhibitor, ii) carbonyl cyanide-4-(trifluoromethoxy) phenylhydrazone (FCCP; 2.0 µM; 5 measurements), a proton circuit uncoupler, and iii) rotenone (0.5 µM) + antimycin A combination (0.5µM; 4 measurements), inhibitors of mitochondrial complexes I and III, respectively. Following mitochondrial stress test, chondrocytes were washed with PBS and stained with calcein acetoxymethyl (4 µM) for 30 min at 37°C in the dark. Each well was fluorescently imaged with an Olympus IX73 inverted microscope, and viable cell count per well was determined using a custom ImageJ macro.

Sample processing and storage

Following collection, all media samples were centrifuged at 800xg for 5 min to remove cells and stored at -80°C until further analysis. Synovial fluid samples were centrifuged at 1800 xg for 15 min to remove cells and the resulting supernatant was stored at -80°C until further analysis.

Taqman qPCR

DNA was prepared using the QIAamp DNA Mini Kit (Qiagen, Germantown, Maryland, USA) according to the manufacturer's instructions. The following custom primers and probes were designed and validated: equine NADH dehydrogenase subunit 1 (mtDNA): forward 5'-

CCCATCATGACCCTTAGCCA-3', reverse 5'-ATGGAGCTCGGTTGGTTTCG-3', probe 5'-AATGTGATTCATCTCAACATTAG-3'; equine glyceraldehyde-3-phosphate dehydrogenase (nDNA): forward 5'-AGGCTCTTTGCTGCCTTTCT-3', reverse 5'-GCAGGATCAGCACCACTTCT-3', probe 5'-CCCTGGGGATCTGGGGAACCAG-3'; bovine NADH dehydrogenase subunit 1 (mtDNA): forward 5'-GCCTACTTCAACCCATCGCC-3'; reverse 5'-GAGGCTGAAGATGTAGCGGG-3'; probe 5'-TCATTAAAGAACCACTACG-3'; bovine glyceraldehyde-3-phosphate dehydrogenase (nDNA): forward 5'-TCGGTAAACAGCCCTTCACACT-3'; reverse 5'-CACCCTGTTGCTGTAGCCGAA-3';
probe 5'-TCCTTCCAGGTACGACAATG-3'. Quantitative PCR was conducted with denaturation at 95°C for 10 min, followed by 40 cycles of 15 s at 95°C and 1 min at 56°C. Samples were analyzed in triplicate and replicates that deviated more than 0.5 CT from the mean were discarded. The mean of sample triplicates was used in all subsequent calculations. A standard curve was created through amplification of the target sequence for each gene followed by isolation of the amplicon using the QIAquick Gel Extraction kit (Qiagen, Germantown, Maryland, USA) according to the manufacturer's instructions. Amplicon concentration was determined spectrophotometrically (NanoDrop 1000, Thermo Fisher, Wilmington, Delaware, USA) and used to create a standard curve through serial dilution. Unknown samples were compared to the standard curve to determine DNA concentration (CN/µL).

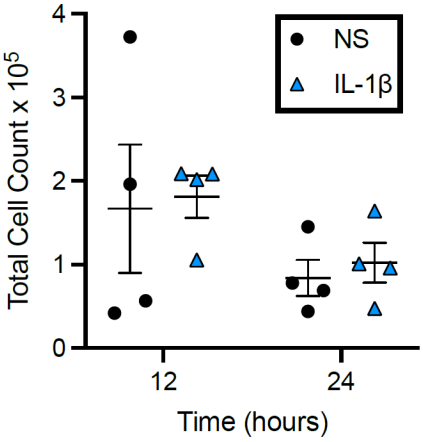

Fig. S1. Chondrocyte cell counts with *in vitro* IL-1β stimulation. Cell counts were not significantly different between treatment group or time point. All data are means ± SEM, n = 4, statistics by students' (paired) t test, $p < 0.05$.

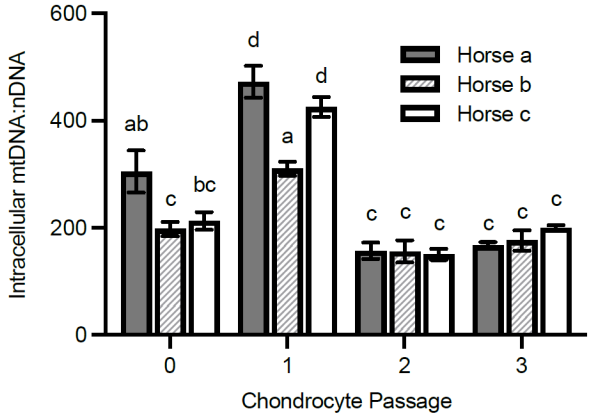

Fig. S2. Intracellular mtDNA:nDNA ratio varies by individual and cell passage. Intracellular mtDNA:nDNA ratio measured in primary (passage 0) through third passage chondrocytes of three healthy adult horses. Data are means ± SEM, n = 4, statistics by two-way ANOVA. Groups not sharing a letter are significantly different ($p < 0.05$).

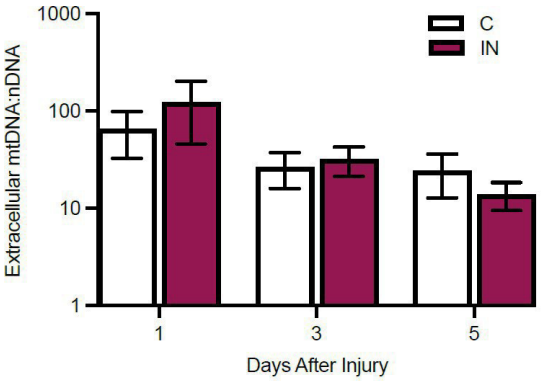

Fig. S3. mtDNA:nDNA ratio in cartilage explant conditioned media after impact injury. Cartilage conditioned media mtDNA:nDNA ratio from explants delivered a rapid impact injury (IN, n = 9) compared to uninjured controls (C, n = 6). Data are means ± SEM, statistics by students' t test within time points, $p < 0.05$.

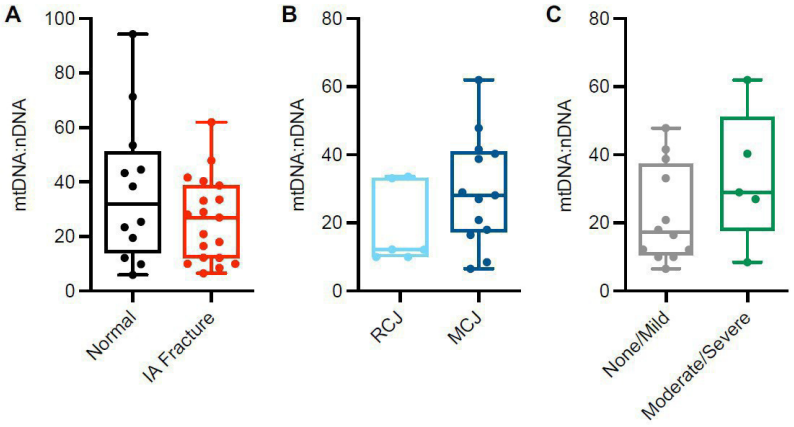

Fig. S4. mtDNA:nDNA ratio in synovial fluid of equine patients with intra-articular carpal fractures. (**A**) Synovial fluid mtDNA:nDNA ratio in equine carpal joints with intra-articular (IA) fractures (n = 19) compared to normal equine carpal joints (n = 12). (**B**) Comparison of synovial fluid mtDNA:nDNA ratio from radiocarpal (RCJ, n = 6) and middle carpal (MCJ, n = 13) fractures. (**C**) Comparison of synovial fluid mtDNA:nDNA ratio in joints with no or mild soft tissue swelling (n = 12) to those with moderate/severe soft tissue swelling (n = 5). Statistics by students' t test ($p < 0.05$).

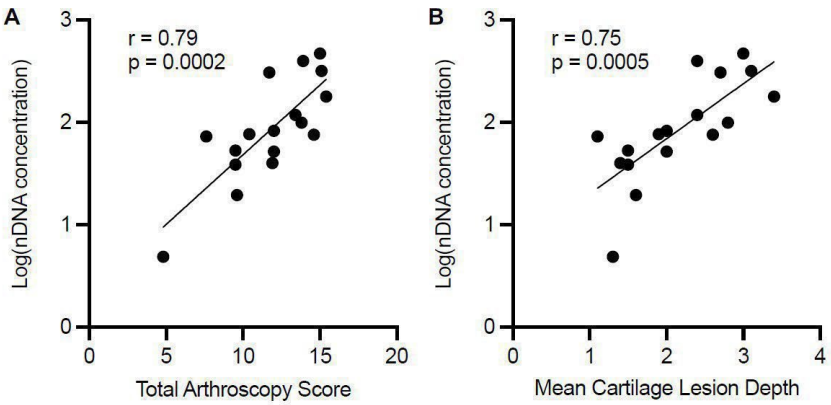

Fig. S5. Total arthroscopy score and cartilage lesion depth correlate with synovial fluid nDNA concentration in joints with intra-articular carpal fractures. (**A-B**) Correlation of total arthroscopy score and mean cartilage lesion depth with synovial fluid nDNA concentration in joints with IA carpal fractures. Statistics by Pearson's correlation analysis.

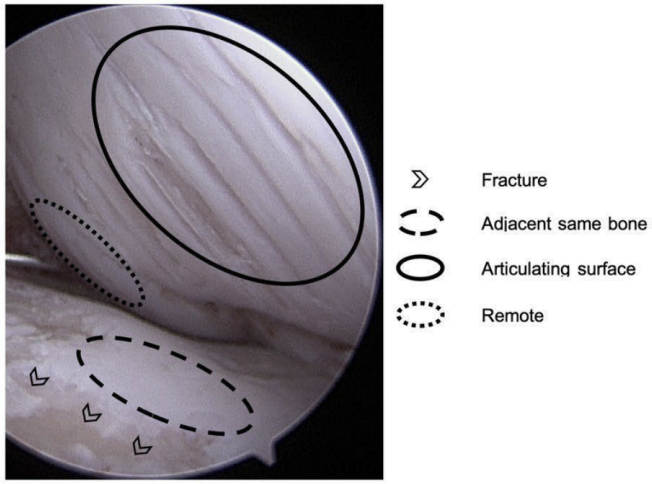

Fig. S6. Cartilage lesion depth scoring sites. Cartilage lesion depth was scored at four sites throughout the injured joint: immediately surrounding the fracture, adjacent to and on the same bone as the fracture, on the articulating joint surface to the fracture, and at remote locations within the same joint. These four scores were averaged to obtain a mean cartilage lesion depth score.

Table S1. Intra-articular carpal fracture patient demographics and sample information.

NA: information not available, TB: thoroughbred, SB: standardbred, QH: quarter horse, WB: warmblood, RC: radiocarpal joint, MC: middle carpal joint

Sample	Horse	Group	Sex	Breed	Age (yrs)	Racehorse	Joint	Side
1	A	Fracture	Male, castrated	TB	4	Yes	RC	Left
2	B	Fracture	Male, castrated	TB	4	Yes	RC	Left
3	C	Fracture	Male, intact	TB	3	Yes	RC	Left
4	D	Fracture	Male, castrated	TB	5	Yes	RC	Right
5	E	Fracture	Male, castrated	TB	4	Yes	RC	Right
6	F	Fracture	Male, castrated	TB	3	Yes	RC	Right
7	G	Fracture	Male, castrated	TB	4	Yes	MC	Left
8	H	Fracture	Male, castrated	TB	4	Yes	MC	Left
9	I	Fracture	Male, castrated	TB	3	Yes	MC	Left
10	J	Fracture	Male, castrated	TB	3	Yes	MC	Left
11	K	Fracture	Male, castrated	SB	5	Yes	MC	Left
12	L	Fracture	Male, castrated	QH	20	No	MC	Left
13	M	Fracture	Male, castrated	QH	4	No	MC	Left
14	N	Fracture	Male, intact	TB	3	Yes	MC	Right
15	O	Fracture	Male, intact	TB	2	Yes	MC	Right
16	P	Fracture	Female	TB	5	Yes	MC	Right
17	Q	Fracture	Female	TB	3	Yes	MC	Right
18	R	Fracture	Male, castrated	TB	6	No	MC	Right
19	S	Fracture	Female	SB	2	Yes	MC	Right
20	T	Normal	Female	NA	15	No	MC	Left
21	T	Normal	Female	NA	15	No	MC	Right
22	U	Normal	Female	NA	8	No	NA	Left
23	U	Normal	Female	NA	8	No	NA	Right
24	V	Normal	Male, castrated	NA	3	No	NA	Left
25	V	Normal	Male, castrated	NA	3	No	NA	Right
26	W	Normal	NA	NA	3	No	NA	Left
27	X	Normal	Male, castrated	WB	21	No	NA	Left
28	X	Normal	Male, castrated	WB	21	No	NA	Right
29	Y	Normal	Male, intact	Paint	1	No	NA	Left
30	Y	Normal	Male, intact	Paint	1	No	NA	Right
31	Z	Normal	Female	TB	1	No	NA	NA

Table S2. mtDNA, nDNA, and mtDNA:nDNA ratio in injured joints by sex, breed, age, and side of patient. Data are mean ± SD.

	mtDNA (copies/μL)	**nDNA** (copies/μL)	**mtDNA:nDNA**
Sex			
Stallion (male, intact)	1763 ± 1414	195 ± 240	13 ± 7
Gelding (male, castrated)	3542 ± 4122	128 ± 126	29 ± 16
Mare (female)	3182 ± 3510	106 ± 70	25 ± 13
Breed			
Thoroughbred	3463 ± 3980	136 ± 128	25 ± 13
Standardbred	2702 ± 2806	58 ± 25	40 ± 31
Quarter Horse	1767 ± 2233	201 ± 277	24 ± 21
Age			
<3 years	1873 ± 1634	254 ± 303	12 ± 8
3-5 years	2573 ± 2451	89 ± 73	28 ± 15
>5 years	9275 ± 8384	357 ± 55	28 ± 28
Side			
Left	2937 ± 2325	120 ± 128	31 ± 15
Right	3501 ± 4875	151 ± 148	20 ± 14

Table S3. Radiographic scoring rubric.

Criteria	0	1	2	3
Periarticular osteophytes	None	Mild (1, <2mm)	Moderate (1-2, 2-4mm)	Severe (>2, >4mm)
Subchondral bone sclerosis	None	Mild	Moderate	Severe
Subchondral bone lysis	None	Mild (focal shallow lysis)	Moderate (diffuse shallow lysis)	Severe (deep lysis)
Joint space narrowing	None	Mild (<25% narrowing)	Moderate (25-75% narrowing)	Severe (>75% narrowing)
Fracture	None	Mild (complete fracture, no comminution, involves one articular surface). Overall impression of acute based on appearance of fragment(s).	Moderate (complete fracture, mild to moderate fragmentation, opposing articular surface mildly affected).	Severe (complete fracture, severe fragmentation, opposing articular surface moderately to severely affected). Overall impression of chronicity based on fragments.
Soft tissue swelling/joint effusion	None	Mild	Moderate	Severe

Table S4. Radiographic scoring correlation data. Statistics by Pearson's correlation analysis.

	mtDNA log(concentration)		**nDNA** log(concentration)		**mtDNA:nDNA**	
	R	**P-value**	**R**	**P-value**	**R**	**P-value**
Periarticular Osteophytes	-0.36	0.151	-0.16	0.528	-0.37	0.144
Subchondral Bone Sclerosis	-0.05	0.852	0.10	0.715	-0.29	0.255
Subchondral Bone Lysis	-0.13	0.622	0.09	0.733	-0.39	0.124
Joint Space Narrowing	-0.32	0.209	-0.16	0.533	-0.34	0.186
Fracture Severity	-0.30	0.240	0.01	0.971	-0.61	0.008
Total	-0.27	0.293	-0.02	0.954	-0.49	0.046

Table S5. Arthroscopic scoring rubric.

Criteria	0	1	2	3	4
Lesion depth	No significant abnormalities	Nearly normal (soft indentations, mild fibrillation and/or superficial fissures)	Mild-moderately abnormal (lesion extending down to <50% cartilage depth)	Moderate-severely abnormal (lesions extending down to >50% cartilage depth but not through subchondral bone)	Severely abnormal (lesions extending down through subchondral bone)
Lesion area	No significant abnormalities	Focal lesion, <5% of articular cartilage examined	Lesions comprise 5-25% of articular cartilage	Lesions comprise 25-50% of articular cartilage	Lesions comprise >50% of articular cartilage
Subchondral bone	No significant abnormalities	Abnormal (avascular, yellow-tan discolored, brittle) SC bone closely associated with fracture fragment only, minimal bone loss	Focal area (<10% articular surface) abnormal SC bone not directly associated with fragment, mild bone loss	Multifocal or regional area (10-30% of articular surface) of abnormal SC bone, moderate bone loss	Widespread (>30% of articular surface) and severely abnormal SC bone, extensive bone loss
Active synovitis	No significant abnormalities.	Mildly abnormal; few hyperemic fronds (<5%, adjacent to fracture site).	Mild-moderately abnormal; <25% hyperemic fronds.	Moderately abnormal; 25-75% hyperemic fronds.	Severely abnormal: > 75% visible fronds hyperemic.
Chronic synovial change	No significant abnormalities	Mildly abnormal; rare (<5%) thickened/fibrotic fronds.	Mild-moderately abnormal; few (<25%) thickened/fibrotic fronds. Mildly increased volume.	Moderately abnormal; 25-75% visible fronds thick and fibrotic. Moderately increased volume.	Severely abnormal; >75% visible fronds thick and fibrotic. Severely increased volume and/or nodular proliferation.

Table S6. Correlation of arthroscopic scoring with mtDNA:nDNA ratio. Statistics by Pearson's correlation analysis.

	mtDNA:nDNA	
	R	**P**
Cartilage Lesion Depth	0.27	0.303
Cartilage Lesion Size	0.05	0.862
Subchondral Bone	0.18	0.484
Active Synovitis	0.19	0.467
Chronic Synovial Change	-0.16	0.545
Total	0.14	0.580

Principal Investigator:	Dr. Michelle Delco
Title:	Synovial fluid extracellular vesicles in equine joint disease and therapy (Year 1)
Project Period:	1/1/21 – 12/31/21
Reporting Period:	1/1/21 – 12/31/21

Project Title: Synovial fluid extracellular vesicles in equine joint disease and therapy (Year 1)
Principal Investigator: Michelle L. Delco

A. Specific Aims of the Study and Modifications

The Aims have not been modified substantially from the original proposal. Note that some of the foundational experiments were accomplished using a variety of non-equine cells due to availability of reagents, etc.

SPECIFIC AIMS

Aim 1: Investigate MSC-derived mitovesicles as potential orthopedic regenerative therapies in horses First, EVs derived from equine bone marrow MSCs will be isolated. Dynamic light scattering will be used to quantify and determine size distribution of EVs from healthy and stressed chondrocytes. High resolution flow cytometry will be used to determine EV mitochondrial content and function. Next, purified mitovesicles will be added to chondrocyte cultures after induction of mitochondrial dysfunction. Respirometry and confocal imaging will be used to determine if uptake of +mtEVs containing functional mitochondria can improve mitochondrial function, prevent cell death, and increase synthesis of type II collagen and glycosaminoglycan in chondrocytes. *We expect this aim to provide proof-of-concept for further investigation of MSC mtEVs to treat joint injury in equine athletes.*

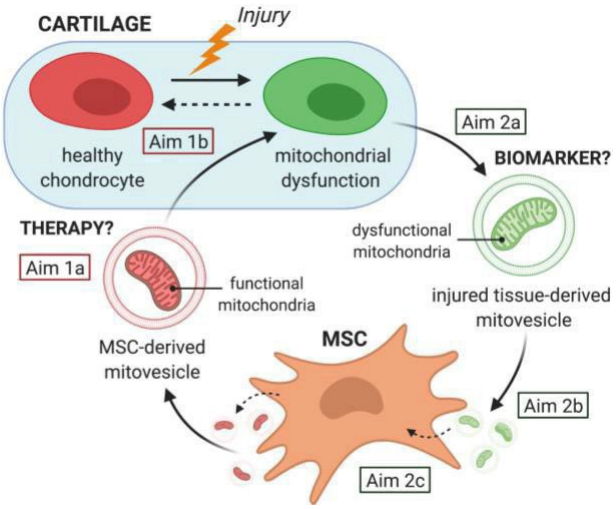

Fig 1. Conceptual overview & main question for each aim
Aim 1a: Are MSC-derived +mtEVs taken up by injured chondrocytes? Aim 1b: Can +mtEVs reverse MT dysfunction in chondrocytes?
Aim 2a: Are dysfunctional -mtEVs released into joint fluid after injury? Aim 2b: Are dysfunctional -mtEVs taken up by MSCs?
Aim 2c: Do -mtEVs stimulate MSCs to release functional +mtEVs?

Aim 2: Investigate mitovesicles derived from injured cells as potential biomarkers
First, EVs will be isolated from synovial fluid of racehorses with and without joint injury and characterized as described above to determine if injured joints are enriched in -mtEVs containing dysfunctional mitochondria. Next, chondrocytes and synoviocytes grown in culture will be stressed with inflammatory stimuli (IL-1β) or a mitochondrial inhibitor (rotenone). EVs will be isolated from the culture media and characterized as described above. Cultured MSCs will be stimulated with -mtEVs, to determine if these damage signals enhance MSCs
+mtEV release. *We expect this aim to provide rationale for further investigation of mtEVs as potential biomarkers of joint injury and mitochondrial dysfunction in racehorses. It may also identify a signal that recruits MSC mitochondrial donation.*

B. Summary of Scientific Findings

We isolated exosome, microvesicle, and vesicle-free fractions from MSC-conditioned media. Using a combination of dynamic light scattering and nanoparticle tracking, we determined that MSC-EV populations fall within the three size categories typically used to classify EVs (exosomes, microvesicles, apoptotic bodies).

Fluorescent nanoparticle tracking, immunoblotting, and flow cytometry revealed that mitochondrial cargoes are abundant across all EV size populations, and mitoEVs are nearly ubiquitous among the largest EVs.

Polarization staining indicated a subset of mitoEVs contain functional mitochondria. Finally, we used flow cytometry and fluorescent imaging to document uptake of mitoEVs by chondrocytes undergoing rotenone/antimycin-induced mitochondrial dysfunction. These data confirm that MSCs package intact, functional mitochondria into EVs, which can be transferred to chondrocytes in the absence of direct cell-cell interactions. This work suggests intercellular transfer of healthy MT to chondrocytes could represent a new, acellular approach to augment mitochondrial content and function in poorly-healing avascular skeletal soft tissues. ***Please see attached draft manuscript in final revisions for publication*** in *Frontiers in*

Bioengineering and Biotechnology; Emerging Technologies for Musculoskeletal Disease Modeling and Regenerative Medicine

C. Significance

This novel area of investigation combines our contemporary understanding of the importance of mitochondrial dysfunction in the pathogenesis of orthopedic injury, as well as the concept of optimizing mitochondrial function to promote healing. The evolving paradigm that MSCs can donate mitochondria to injured cells to rescue their viability, function and synthetic capacity represents an exciting opportunity for therapeutic development. The long-term goal is to develop the next generation of regenerative therapies that deliver healthy mitochondria to injured tissues to promote healing, and prevent career- and life-threatening orthopedic injuries in Thoroughbred racehorses.

D. Publications and Other Grant Submissions

Manuscripts

Thomas MA, Fahey MJ, Pugliese BP, Irwin RM, Antonyak MA, **Delco ML**. Human Mesenchymal Stromal Cells Release Functional Mitochondria in Extracellular Vesicles. *In Revision, Frontiers in Bioengineering and Biotechnology; Emerging Technologies for Musculoskeletal Disease Modeling and Regenerative Medicine,* March 2022 ***Please see attached PDF of revised submission***

Grants - Pending

Delco (PI)

Sponsor/Mechanism: NIH Directors New Innovator Award (DP2)	2022 - 2027
Title: Next-Generation Mitochondria-targeted Therapies to Tackle the Most Common Causes of Pain and Disability	$2,355,000
Role: Principal Investigator	

Grants - Funded

Delco (PI)
Sponsor/Mechanism: ACVS Foundation Surgeon-in-Training Research Grant — 2022 - 2024
Title: Investigating Mitovesicles in a Model of Equine Osteoarthritis — $15,000
Role: Role: Principal Investigator/Mentor (Dr. Brenna Pugliese)

Delco (PI)
Sponsor/Mechanism: Cornell Resident Research Grant — 2022 - 2023
Title: Investigation of Mitovesicles as a New Equine Orthobiologic — $10,000
Role: Principal Investigator/Mentor (Dr. Brenna Pugliese)

Abstracts

Pugliese BR, Fahey MJ, Thomas MA, **Delco ML** (2021). Equine Mesenchymal Stromal Cell- and Plasma-derived Extracellular Vesicles Contain Mitochondria, *American College of Veterinary Surgeons Annual Symposium.* *Winner; Large Animal Resident's Forum

Thomas MA, Miller JL, **Delco ML** (2021). Human Mesenchymal Stem Cells Release Functional Mitochondria in Extracellular Vesicles. *Osteoarthritis Research Society International (OARSI) World Congress.* *Podium Presentation.

Human Mesenchymal Stromal Cells Release Functional Mitochondria in Extracellular Vesicles

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Keywords: MSCs, mesenchymal stem cells, mitovesicles, mitoEVs, secretome, mitochondrial transfer, regenerative orthobiologic

Abstract

Cartilage and other skeletal soft tissues heal poorly after injury, in part due to their lack of vascularity and low metabolic rate. No pharmacologic approaches have proven effective in preventing chronic degenerative disease after joint injury. Mesenchymal stromal cells (MSCs) have been investigated for their ability to treat pain associated with osteoarthritis (OA) and preserve articular cartilage. Limitations of MSCs include variability in cell phenotype, low engraftment and retention rates, and inconsistent clinical outcomes. Therefore, acellular biologic therapies such as extracellular vesicles (EVs) are currently being investigated. MSC-derived EVs have been found to replicate many of the therapeutic effects of their cells of origin, but the mechanisms driving this remain unclear. Recent evidence in non-orthopedic tissues suggests MSCs can rescue injured cells by donating mitochondria, restoring mitochondrial function in recipient cells, preserving cell viability, and promoting tissue repair. Our group hypothesized that MSCs package mitochondria for export into EVs, and that these so-called 'mitoEVs' could provide a delivery strategy for cell-free mitochondria-targeted therapy. Therefore, the goals of this study were to: 1) characterize the vesicle fractions of the MSCs secretome with respect to mitochondrial cargoes, 2) determine if MSC-EVs contain functional mitochondria, and 3) determine if chondrocytes can take up MSC-derived mitoEVs. We isolated exosome, microvesicle, and vesicle-free fractions from MSC-conditioned media. Using a combination of dynamic light scattering and nanoparticle tracking, we determined that MSC-EV populations fall within the three size categories typically used to classify EVs (exosomes, microvesicles, apoptotic bodies). Fluorescent nanoparticle tracking, immunoblotting, and flow cytometry revealed that mitochondrial cargoes are abundant across all EV size populations, and mitoEVs are nearly ubiquitous among the largest EVs. Polarization staining indicated a subset of mitoEVs contain functional mitochondria. Finally, we used flow cytometry and fluorescent imaging to document uptake of mitoEVs by chondrocytes undergoing rotenone/antimycin-induced mitochondrial dysfunction. These data confirm that MSCs package intact, functional mitochondria into EVs, which can be transferred to chondrocytes in the absence of direct cell-cell interactions. This work suggests intercellular transfer of healthy MT to chondrocytes could represent a new, acellular approach to augment mitochondrial content and function in poorly-healing avascular skeletal soft tissues.

1 Introduction

Cartilage is responsible for dissipating mechanical forces and providing a near frictionless articular surface for locomotion (Sophia Fox et al., 2009). Injury to the articular surface leads to chondrocyte death and cartilage matrix degradation. This cellular and structural damage initiates cycles of inflammation and irreversible tissue degeneration, culminating in end-stage osteoarthritis (OA)(Madry et al., 2011), a leading cause of chronic pain and disability worldwide (Economic Burden of Osteoarthritis; RC et al., 2008). Cartilage and other skeletal soft tissues such as meniscus, ligament and intervertebral disc have a low metabolic rate, poor vascularization and as such, possess limited healing capacity (Bianchi and Sinigaglia, 2012). Further, no current therapeutics can slow progression of OA after joint injury (Sarzi-Puttini et al., 2005).

Recent studies have shown that mitochondrial dysfunction is one of the earliest responses of chondrocytes to mechanical injury, implicating mitochondria (MT) as an important mediator of injury-induced OA (Blanco et al., 2011; Goetz et al., 2017; Bonnevie et al., 2018; Delco et al., 2018; Liu et al., 2019). We have found that the mitoprotective peptide SS-31 can improve MT function, preserve chondrocyte viability, and prevent tissue loss following cartilage injury (Delco et al., 2018; Bartell et al., 2020). These findings support the concept of protecting MT function after acute injury

to prevent cartilage loss, however MT-targeted strategies to improve healing of degenerated articular cartilage have not been investigated.

Tissue healing is an energy intensive process, and MT dysfunction mediates many degenerative diseases, including Parkinson's (Winklhofer and Haass, 2010), cardiomyopathy (Pacak et al., 2015), and pulmonary fibrosis (Watson et al., 2016). MT dysfunction has been less well studied in cartilage, in part because chondrocytes are largely glycolytic and do not heavily rely on MT for energy production during homeostasis (Bianchi and Sinigaglia, 2012). However, MT also act as signaling hubs in cellular processes such as apoptosis and autophagy, and are critical organelles for maintenance of cartilage homeostasis (Loeser, 2011). Inflammation that occurs in OA is associated with a shift towards glycolysis for energy production in chondrocytes (Zheng et al., 2021). This metabolic shift may represent an attempt to restore energy capacity after MT dysfunction reduces an already limited supply (Mobasheri et al., 2017). Therefore, replacing dysfunctional MT that have been lost to injury and/or disease may promote healing in avascular, metabolically quiescent tissues such as articular cartilage.

Intercellular MT transfer, a process whereby injured and dysfunctional cells recruit MSCs to donate functional MT, has been shown to restore MT function in energy-expensive tissues such as neurons (Berridge et al., 2016), cardiomyocytes (Pacak et al., 2015) and renal tubular epithelia (Konari et al., 2019), as well as more quiescent, poorly vascularized tissues such as the corneal epithelia(Jiang et al., 2016). *In vivo* murine models of acute lung injury revealed that MSC-mediated MT transfer protects against tissue degeneration after pulmonary injury (Islam et al., 2012; Jackson et al., 2016). Though the mechanisms mediating intercellular MT transfer have not been fully elucidated, evidence from a variety of cell and tissue types implicate processes requiring direct cell-cell contact, including filapodial extensions, tunneling nanotubules, gap junction formation, and cell-cell fusion events(Chinnery et al., 2008; Larsson et al., 2008; Murray and Krasnodembskaya, 2019).

In addition to direct cell-cell MT transfer, our group and others have documented apparent non-contact MT transfer via extracellular vesicles (EVs). EVs are small, membrane bound structures released by all cell types. EVs serve as vehicles for cell-cell signaling and orchestrate many biologic processes including inflammation, immunomodulation, homeostasis, metastasis, and tissue repair (Lai et al., 2010; AK and B, 2012; Y et al., 2013; Kogure et al., 2020). While EV populations are heterogenous and remain largely uncharacterized, they are most commonly classified into 3 categories based on size and mode of biogenesis: exosomes, microvesicles (MVs), and apoptotic bodies (Gould and Raposo, 2013). Recent work has identified mitochondria-specific cargoes, including DNA, RNA, and proteins in EVs originating from many cell types, including fibroblasts (Sansone et al., 2017), neurons (Peruzzotti-Jametti et al., 2021), and MSCs (Phinney et al., 2015). Furthermore, intact MT have been identified in larger vesicles derived from adipocytes (Crewe et al., 2021) and MSCs (Phinney et al., 2015; Morrison et al., 2017). In one study, at least 60% of human plasma-derived EVs contained MT, and MT cargoes were ubiquitous in the very largest vesicles (Zhang et al., 2020). Further, EV-mediated MT transfer has been documented between MSCs and professional phagocytic cells; in vitro, macrophages were found to take up MSC-derived EVs containing MT and incorporate them into their mitochondrial networks (Phinney et al., 2015; Morrison et al., 2017). This process of stem cell to phage mitoEV-mediated transfer has also been documented from neural stem cells (NSCs) to mononuclear phagocytes, with transfer restoring ATP capacity, upregulating oxidative phosphorylation, and reducing inflammation (Peruzzotti-Jametti et al., 2021). However, MSC-derived mitoEV transfer has not been demonstrated to chondrocytes. Therefore, the goals of this study were to characterize EVs produced by murine and human bone

marrow derived-MSCs (including mitochondrial cargoes), determine if MSC-mitoEVs contain functional mitochondria, and investigate whether chondrocytes are capable of taking up MSC-derived mitoEVs. While live MSCs have shown promise as an orthobiologic therapy (Kingery et al., 2019), the therapeutic use of live cells presents significant clinical challenges(Musiał-Wysocka et al., 2019). Harvesting autologous MSCs from sources such as bone marrow or cartilage requires painful and/or invasive procedures, while the use of non-autologous cells raises concerns of immunogenicity and tumorigenesis (Hall and Watt, 1989; Charron, 2013). Additionally, culturing, expanding, transporting, and storing live MSCs requires a large investment of time, resources, and expertise (Asghar et al., 2014). These obstacles, alongside the high regulatory burden associated with the clinical use of MSCs, have greatly limited their widespread use and caused clinicians to seek more practical and readily available orthobiologics.

2 Materials and Methods

2.1 Cell Sources and Culture

Bone marrow-derived MSCs were isolated from 5 week old PHaM mitoDendra2 (Jax stock 018385) mice, as previously described (Huang et al., 2015). Chondrocytes were isolated from 5 day old UBC mCherry mice (Jax stock 017614), as previously reported (Gosset et al., 2008). Cells were cryopreserved in liquid nitrogen then thawed and cultured for use as needed. Protocols were approved by the Institutional Animal Care and Use Committee at Cornell University. Murine cells expressing endogenous fluorophores were used for characterizing the size of the whole EV population using dynamic light scattering (DLS), and for assessing mitoEV uptake by chondrocytes with confocal imaging and flow cytometry. Human bone marrow-derived MSCs were purchased at passage two (Millipore Sigma: SCC034) and used for nanoparticle tracking, western blot, and identification of mitoEVs with flow cytometry. For all experiments using human MSCs, cells were passaged no more than 3 times, cultured to 85% confluence, rinsed thrice with PBS and serum starved for 24 hours before EV isolation.

2.2 Extracellular Vesicle Isolation

For all experiments, cell-conditioned media was removed and placed on ice immediately following 24-hour serum starvation. To characterize the size range of the entire MSC-derived EV population using (DLS), murine MSC cell-conditioned media (CCM) was centrifuged at 3,000 x g for 15 minutes. The supernatant was then processed using the Exoquick-TC ULTRA kit (System Bio: EQULTRA-20TC-1), according to manufacturer's instructions. For nanoparticle tracking, western blot, flow cytometry, and confocal imaging studies, a validated filtration-ultracentrifugation technique was used to isolate EV fractions of specific size ranges, as previously described (Wang et al., 2021). These fractions consisted of vesicle free media, (VFM, no EVs) exosomes, (EX, 30-100 nm) and microvesicles (MVs, 100-1000nm). Briefly, following CCM collection, adhered MSCs were lysed and placed on ice. The CCM was clarified of cellular debris with two consecutive centrifugation steps (1,000 x g for 5 minutes). To isolate MVs, CCM was vacuum filtered through a Steriflip filter (0.22 µm; Millipore: SE1M179M6). MVs were collected on top of the filter and were immediately placed on ice or lysed for protein analysis. The filtrate was subjected to 100,000 x g centrifugation at 4°C for 4 hours in a Beckman Coulter Optima XE Ultracentrifuge. The supernatant (vesicle-free media; VFM) was removed and concentrated using an Amicon centrifugal concentrator with a 10kDa pore size. The pellet (EX fraction) was lysed and immediately placed on ice. Total whole cell lysate (WCL), MV, EX, and VFM protein content was quantified by Bradford Assay. Validation of EV isolation was performed by western blot for EV-specific markers and calcein staining/flow cytometry(Gray et al., 2015).

2.3 EV Size Characterization

The relative size distribution of EVs from MT stressed and MT protected cells was characterized by DLS (Malvern Nano ZS Zetasizer). Murine MSCs were passaged 2-3 times, cultured to 85% confluence in standard growth media at 21% O_2 and 5% CO_2; then rinsed thrice with phosphate buffered saline (PBS) and stimulated with the mitochondrial inhibitor rotenone/antimycin (Sigma: R8875 ,0.5µM/Sigma: A8674, 0.5µM), treated with the mitoprotective peptide SS-31 (1uM), or left untreated during a 24-hour serum starvation prior to EV isolation. Three measurements were obtained per sample at 25 °C, a detection angle of 173°, and a refractive index of 1.334 (PBS), then averaged. The experiment was repeated twice, for an n=3. Percent area under the curve, maximum intensity, and mean particle size were calculated for major peaks. To obtain a more accurate quantification of sub-micron sized EVs, human MSCs were cultured as described above, then isolated using filtration/ultracentrifugation. Nanoparticle tracking was performed using a Malvern NanoSight NS300 configured with a 488 nm laser and 565 nm long pass filter for fluorescent detection. Five, 60 second videos were taken, individual particles were tracked, and hydrodynamic size was calculated via the Stokes-Einstein equation. To validate our isolation technique EX, MV, vesicle-free media, and PBS (control) were analyzed to confirm that each fraction fell within the expected size range based on previous work (Zhang et al., 2020). Samples were diluted in double filtered (.22um) PBS to a concentration between 10^7–10^9 per mL and analyzed at room temperature. Size distribution of EVs in each fraction was measured using the 488 nm laser. The size distribution of mitoEVs specifically was determined by staining microvesicle fractions with the mitochondrial probe Mitotracker Red (100 nM; Thermofisher: M22425) and using the 565 nm, long pass filter. Unstained and vesicle-free samples served as negative controls.

2.4 Characterization of MSC Secretome Fractions by Western Blot

A total of 25 µg protein from human MSC-EV fractions isolated by filtration/ultracentrifugation was resolved by SDS-PAGE under reducing conditions (4 to 20%; Invitrogen Novex Wedge Well, 1.0 mM; Tris-Glycine, Mini Protein Gel, 10-well; XCell SureLock Mini). The gel was transferred to a polyvinylidene difluoride membrane (0.45 µm; 10x10 cm, Thermofisher), which was blocked in tris-buffered saline with 5% bovine serum albumin, then cut based on molecular weight. Sections were incubated with respective primary antibodies overnight: HSP90 (Cell Signaling: 4877S) was used as a positive loading control; Flotillin (Cell Signaling: 3436S) was used as a positive EV marker expected to be detected in all lysates (WCL, MV, EX); IκBα (Cell Signaling: 4814S) was used as cellular marker to screen for cellular contaminants in conditioned media fractions; ATP5A1 (Thermofisher:459240) and COXIV (Cell Signaling: 4850S) were used to probe for mitochondrial proteins. The membrane sections were rinsed three times with tris-buffered saline and incubated with horseradish peroxidase-conjugated secondary antibody for 1 hour at room temperature with gentle rocking. All primary and secondary antibodies were used at 1:1000 dilutions. Proteins were detected using enhanced chemiluminescence reagents and a chemiluminescent imaging device (Bio-Rad Chemidoc XRS).

2.5 MitoEV Identification and Size Characterization by Flow Cytometry

To investigate the presence of mitoEVs, human MSC-MVs were cultured and isolated as described above, then stained with Calcein AM (10uM; Thermofisher: C3099) to identify intact vesicles (Gray

et al., 2015), and Mitotracker Deep Red (100nM; Thermofisher: M22426) to label intact mitochondria. MVs were resuspended in double filtered PBS and immediately analyzed by flow cytometry (FACSAria Fusion, BD Biosciences). Unstained control samples and EV fractions stained with individual fluorophores were used to set positive fluorescence thresholds. Events positive for both calcein and mitotracker were considered mitoEVs. Events positive for malcein only were considered EVs lacking whole mitochondria cargoes. Events positive for mitotracker alone were considered free mitochondria (i.e. not contained within EVs). Data analysis was conducted using FlowJo (version 10.8; FlowJo LLC) software. To investigate the relative size distribution of mitoEVs within the isolated MV population, the backgating tool in FlowJo was used. MitoEV events were plotted based on vesicle size (FSC-H vs. FSC-A). Gates were created to encompass progressively larger size percentiles (1^{st}, 50^{th}, 80^{th}, 90^{th}, 95^{th}) so that, for example, events falling within the 95^{th} percentile represented the largest 5% of all EVs. The fraction of mitoEVs within each size percentile was recorded.

2.6 MitoEV Polarity Assessment

In order to investigate the presence of functional (polarized) mitochondrial cargos, human MSC-MVs were stained with calcein AM and tetramethylrhodamine, methyl ester (TMRM; 200nM; Thermofisher: 2668), which fluoresces red-orange only when taken up across a polarized mitochondrial membrane. Single color and unstained controls were used to set thresholds for each fluorophore, as above. Events double positive for calcein and TMRM were considered mitoEVs containing functional mitochondria.

2.7 MitoEV Uptake by Articular Chondrocytes

2.7.1 Flow Cytometry of Chondrocytes Cultured with MSC-MVs

To gather quantitative data on rates of mitoEV-mediated mitochondrial transfer, PHaM mitoDendra2 MSCs were cultured to 85% confluence, rinsed thrice with PBS, and serum starved for 12 hours. MSC- microvesicles were isolated via filtration as described above. Murine articular chondrocytes were harvested from 5-6 day old pups, as previously described (Gosset et al., 2008). Passage 2-3 chondrocytes were cultured to 80% confluence and incubated for 12 hours in serum free media with or without murine MSC-derived MVs. Cells were then fixed and analyzed using an Attune NxT cytometer. Single cells were gated for using forward and side scatter. A negative control fluorescent threshold for dendra2 was set using non-MV treated chondrocytes. Chondrocytes fluorescing above threshold for dendra2 were considered to have taken up mitoEVs.

2.7.2 Confocal Imaging of Chondrocytes Cultured with MSC-MVs

PHaM mitoDendra2 MSCs were cultured, rinsed thrice with PBS and serum starved for 24 hours. Murine chondrocytes were cultured to 80% confluence on a chamber slide system (Lab-Tek: 177429), then stained with Mitotracker Red (100nM; Thermofisher: M7512) and incubated with MSC-derived MVs for 12 hours under serum starvation. Chondrocytes were then fixed on the chambered slide with 4% PFA, coverslipped with fluoromount containing DAPI nuclear stain. Chondrocyte cultures were imaged on a Zeiss LSM880 inverted i880 multiphoton microscope with a 63x oil immersion objective using sequential, 3-channel scans; 359/457nm (DAPI), 490/507nm (Dendra2) and 581/644 nm (Mitotracker red) excitation/emission, respectively. Z stacks were performed (14-20 slices at a step size of .38 μm) on a subset of co-fluorescent cells to investigate if MSC-derived fluorescent mitochondria were localized to the MT networks within recipient chondrocytes.

2.8 Statistical Analysis

For DLS parameters (percent area under the curve, maximum intensity, and mean particle size), a one-way ANOVA was used, followed by Dunnett's multiple comparison's test to assess differences between groups (NS, RA, SS31) at each peak. To validate mitoEV quantification and mitochondrial polarity data, one-way ANOVA followed by Dunnett's multiple comparisons tests were used to assess differences between the experimental groups (MitoTracker + calcein and TMRM + calcein, respectively), single color controls (MitoTracker, calcein and MitoTracker, TMRM, respectively), and unstained controls. Polarity data was log transformed for statistical analysis. To assess differences between MV-treated (+MV) and non-treated (-MV) chondrocytes, data was log transformed and an unpaired t-test was used to compare groups. All statistical analyses were performed using Graphpad Prism version 8.3, with significance set at $p \leq 0.05$.

3 Results

3.1 MSC-EV Isolation Validation and Basic Characterization

To characterize the entire MSC-EV population, a size distribution was obtained by DLS. Similar to previous reports (Zhang et al., 2020), EVs clustered into three size ranges: small vesicles (5-10 nm in diameter), medium (100-1000 nm), and a subgroup of much larger vesicles (5000-10000 nm) (Fig. 1A) likely representing exosomes, microvesicles (MVs), and apoptotic bodes, respectively. The non-stimulated group had a significantly higher percent area in the first size peak than either the rotenone/antimycin or the SS-31 stimulated group. Both stimulated groups showed a significantly higher percent area in the second (medium) peak than the non-stimulated group ($p < 0.05$). Since DLS is not well suited to quantifying heterogenous populations of particles, we also performed nanoparticle tracking analysis (NTA) of EV fractions. NTA data validated our isolation protocol; particles clustered in size ranges consistent with exosomes (10-150 nm) and MVs (100-1000 nm). The negative control diluent (double filtered PBS) was negative for EV-sized particles (Fig. 1B). Next, we used Western Blot to qualitatively characterize our separated MV, EX, and vesicle free media (VFM) fractions. To confirm that the fractions were devoid of cells and cellular debris, we probed for the cellular marker, IκBα, which was detected in our whole cell lysate (WCL) but not MV or EX. The ubiquitous marker for cells and conditioned media, HSP90, was detected in WCL, EX, and VFM, as expected. Flotillin, an EV marker, was detected in WCL, MV, and EX but not VFM. Mitochondrial proteins were detected in WCL (ATP5A1, COXIV) and MV (COXIV) (Fig 1C), indicating the presence of mitochondrial cargoes in the microvesicle fraction of the MSC secretome.

3.2 Characterization of MitoEVs

Quantification of mitoEVs was performed on flow cytometry. Events exceeding the fluorescence thresholds for both MitoTracker Deep Red and calcein (i.e. red-green double-positive) were considered mitoEVs (Fig. 2A). An average of 71% (range 58.6 - 97%) of intact EVs contained MT (Fig. 2B). The double stained group contained ~7-fold higher percentage of double positive events compared to controls, indicating that the mitoEV signal was not a result of autofluorescence or artifact (Fig. 2B). Backgating analysis revealed that mitoEVs were present throughout the entire size distribution of particles, however EVs lacking MT tended to be below the 50th percentile for size (Fig. 2C). Further, the vast majority (~90%) of the largest EVs (95th size percentile) contained MT. (Fig. 2D). Because size data gathered from backgating analysis is qualitative rather than quantitative, we also performed fluorescent nanoparticle tracking analysis on MVs stained with and without

MitoTracker Deep Red (Fig. 2E). Fluorescent nanoparticle tracking supported our flow cytometry data; mitoEVs were found to be distributed throughout the EV size spectrum but tended to be larger than the general population. Further, this more sensitive analysis revealed that mitoEVs clustered around 3-400nm, 600nm, and 1μm, suggesting the presence of distinct sub-populations of mitoEVs (Fig. 2E). Unstained samples were undetectable when analyzed using the 565 nm fluorescent laser, and groups stained with MitoTracker, but analyzed with brightfield tracking did not show a shift in size distribution, validating our method for detecting mitoEVs (Fig. 2E).

3.3 A Subset of MitoEVs Contain Functional Mitochondria

To characterize the functionality of mitochondrial cargoes within MSC-derived mitoEVs, we analyzed MT polarity via TMRM staining on flow cytometry, with events staining above threshold for calcein and TMR considered mitoEVs containing polarized MT (Fig. 3A). An average of 31% (range 5.38 - 69.16%) of EVs contained functional MT, suggesting that MT polarity in mitoEVs is highly variable (Fig. 3B). The double-stained experimental group contained significantly more double-positive events than unstained and calcein control groups. Although the experimental group contained a mean of 6-fold more double-positive events than the TMRM control, the difference did not reach statistical significance ($p = 0.0735$) due to high variability in the experimental group between trials (n=3).

3.4 MitoEVs Are Taken Up by Chondrocytes and Incorporated into Chondrocyte Mitochondrial Networks

To interrogate whether articular chondrocytes can take up MSC-derived mitoEVs from the extracellular environment, we stained murine chondrocytes with MitoTracker Red to label the MT networks, tthen incubated them with MVs isolated from MSCs expressing endogenous, MT-specific green fluorescence (Fig 4A). Confocal imaging revealed mitoEVs located in both the extracellular space and within chondrocytes (Fig 4B). Z-stacked imaging confirmed that MSC-derived MT localized to the MT networks within chondrocytes (see Supplemental Materials for representative 3D reconstructions). To confirm the phenomenon could be replicated, imaging experiments were performed twice on two separate days. As an orthogonal approach, we also cultured unstained chondrocytes with MSC-derived MVs and performed flow cytometry (Fig 5A). This method provided quantitative data on the rate of mitoEV-mediated MT transfer; an average of 0.4% of chondrocytes took up mitoEVs in culture (Fig. 5B). Although the rate of mitoEV uptake was low and variable in our experimental system, there was a significant difference between MV-treated and untreated groups (Fig 5B, $p < 0.05$).

4 Discussion

This study demonstrated that human MSCs release intact, functional MT in EVs. To the best of our knowledge, our work represents the first evidence of MSC to chondrocyte, EV-mediated MT transfer. Further, we confirmed that MSC-derived mitoEVs can serve as a vehicle for MT transfer in the absence of direct cell-cell interactions between MSCs and chondrocytes. Our findings are consistent with previous reports, where Phinney et al. found that MSCs undergoing oxidative stress produced vesicles containing dysfunctional, partially depolarized MT to outsource mitophagy. Macrophages engulfed these vesicles and repurposed functional components, improving bioenergetics for both the MSC and macrophage (Phinney et al., 2015). Morrison et al. used mitochondrial organelle staining of MSC-derived EVs and flow cytometry to show that mitoEVs released by MSCs can be incorporated by macrophages into their MT networks. MitoEV uptake by

macrophages enhanced phagocytosis by promoting oxidative phosphorylation, and improved their ability to treat lipopolysaccharide-induced lung injury *in vivo* (Morrison et al., 2017). Consistent with our findings, evidence that cells release both functional and non-functional MT in EVs suggests mitoEV transfer is a dynamic, context-driven process, likely involving crosstalk (i.e. bidirectional mitoEV transfer) between donor and recipient cells (Morris and Hollenbeck, 1993; Plotnikov et al., 2008). Studies of contact intercellular MT transfer, involving direct interactions between donor and recipient cells in other tissues indicate that calcium signaling and gap junction proteins play an important role (Islam et al., 2012; Hayakawa et al., 2016). However, the mechanisms of EV-mediated MT transfer are unclear, and further investigation is warranted. In our proof-of-concept experimental design, we found a low rate of mitoEV uptake by chondrocytes *in vitro*. However, it should be noted that several factors, including culture time and MV numbers were not optimized for these experiments. In similar work, where chondrocytes and MSCs were directly co-cultured, the frequency of MT transfer increased with a higher MSC: chondrocyte ratio. Therefore, we anticipate that future work to develop and optimize experimental models and understand the mechanisms mediating MSC-chondrocyte mitoEV transfer will allow us to identify strategies to promote mitoEV release by MSCs and uptake by chondrocytes.

To investigate functionality of MT packaged into mitoEVs we used TMRM staining, where fluorescent intensity is directly proportional to the inner MT membrane potential(Brand and Nicholls, 2011).Using this technique, we found that a subset (at least 5%, up to 70%) of mitoEVs contain functional MT, meaning that they have a competent electron transport chain and are capable of producing ATP. We suspect that the high variability in TMRM-staining between experiments is reflective of the exquisite sensitivity of mitochondria to depolarization, and subtle differences in MT stress induced by EV isolation and analysis techniques, especially flow cytometry, although further studies would be required to confirm this. Our preliminary experience suggests that while fluorescence activated cell sorting (FACS) of mitoEV subpopulations is possible, this approach is damaging to mitoEV polarity, and too time, effort, and cost-intensive to be practical at this time (Kormelink et al., 2016). However, our data suggest the possibility of more scalable, size-dependent methods of mitoEV isolation; We found that more than 70% of MSC-MVs are mitoEVs, a higher percentage than previously reported (Morrison et al., 2017). This is likely due to several experimental differences, such as cell of origin, EV isolation technique, and EV identification method. However, further size analysis of our mitoEV population indicated that nearly all of the MVs >500 nm contain mitochondria. Further studies to investigate methods of isolating mitoEVs based on a size will be necessary to investigate the functional impact of mitoEV uptake on injured chondrocytes. Based on work suggesting MSC donation of functional MT to injured cells can rescue cell viability and promote tissue healing, optimizing functionality of mitoEVs during isolation would be an important consideration for the investigation of mitoEVs as a regenerative orthobiologic therapy (Pacak et al., 2015; Berridge et al., 2016; Jiang et al., 2016; Konari et al., 2019).

Our data suggest mitoEVs themselves are not a homogenous population, but rather a diverse collection of vesicles containing distinct mitochondrial cargoes, with a range of functionality. Distinct mitoEV sub-populations may play biologically distinct roles, including cellular rescue through intercellular mitochondrial transfer, outsourcing of mitophagy and diverse signaling functions (Phinney et al., 2015; Morrison et al., 2017; Sansone et al., 2017; Zhang et al., 2020; Peruzzotti-Jametti et al., 2021). DLS size data further supports this. We found that agents known to affect mitochondrial dynamics and function shift the size profile of EV subgroups released by MSCs; When MSCs were stimulated with either a mitoprotective peptide or a MT specific stressor, we observed a significant shift towards a larger 100-1000 nm, ‘medium’ peak, presumably MVs.

Although our current experimental methods did not allow us to determine why both groups responded similarly, these data suggest mitoEV release is responsive to changes in cellular MT function. It is possible that following rotenone/antimycin-induced MT stress, MSCs increased packaging of dysfunctional MT into MVs, consistent with the previous work demonstrating stressed MSCs release unhealthy MT into EVs to outsource mitophagy (Phinney et al., 2015). Following mitoprotective treatment with SS-31, MSCs may similarly increase export of healthy, functional MT in mitoEVs. Further research into the origin, mechanisms of release, and function of mitoEV sub-populations is necessary to elucidate how MSC MT function is related to mitoEV biogenesis.

Measurement of particles smaller than 200 nm (i.e. exosomes) was limited to size and protein analysis. Neither of the MT proteins we probed for (COXIV an outer MT membrane protein and ATP5A1, an enzyme that catalyzes synthesis of ATP) was present in the exosome fraction. This may be due to the specific proteins we chose to investigate, rather than an indicator that MSC-exosomes do not transport MT components. Previous analysis has shown that exosomes may be more likely to contain miRNA and mtDNA, as opposed to the MT membrane proteins and intact MT seen in larger vesicles such as MVs (Phinney et al., 2015; Torralba et al., 2018; D'Acunzo et al., 2021). As techniques improve and high resolution, microparticle optimized flow cytometers become more widely available, characterization of individual mitoEV sub-populations will become more straightforward.

An underlying goal of this study was to characterize MSC-derived mitoEVs and investigate their potential suitability as vehicles for non-contact, acellular MT delivery in therapeutic applications. The properties that make EVs effective in long range endogenous communication, also make them excellent candidates for regenerative therapy. EV lipid bilayers are durable enough to retain structural integrity after freeze-thawing and remain intact following intraarticular injection(Li et al., 2019). EVs are non-replicating and have low levels of major histocompatibility complex proteins, posing minimal risk of immunogenicity or tumorigenicity (Yeo et al., 2013; Zhu et al., 2017; Tsiapalis and O'Driscoll, 2020). We confirmed the presence of functional MT in MSC-MVs, identified evidence of distinct sub-populations of mitoEVs, and found that, although MT are present in MVs of all sizes, large MVs are much more likely to contain MT. Further, we observed evidence that stressed chondrocytes will take up MT packaged in mitoEVs in the absence of physical interactions with the parent MSCs. The methods developed in this study will allow investigation into the effects of these mitoEVs after uptake and the mechanisms underlying this phenomenon, as well as those behind why distinct sub-populations such as functional and dysfunctional mitoEVs are released.

5 Figures

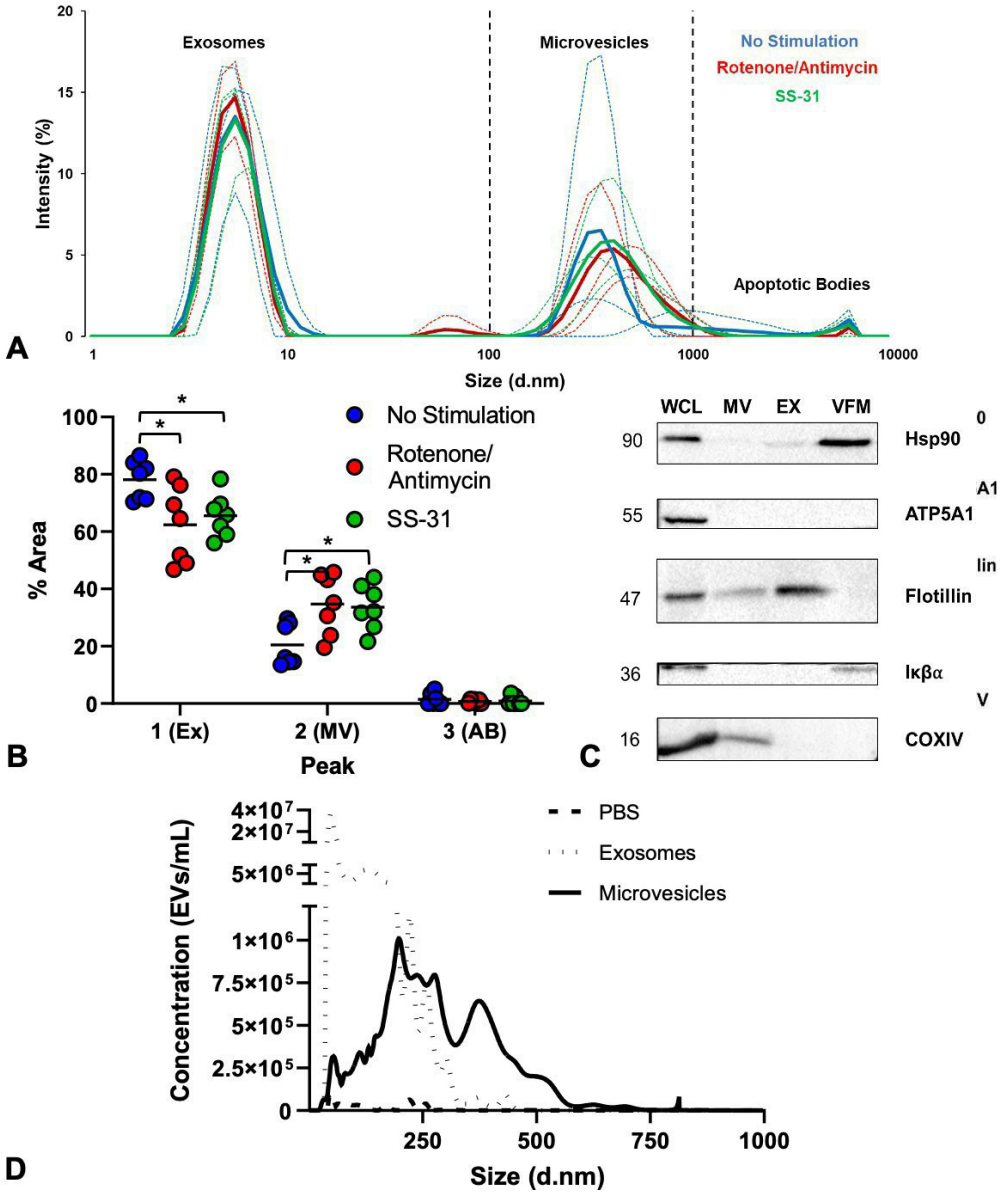
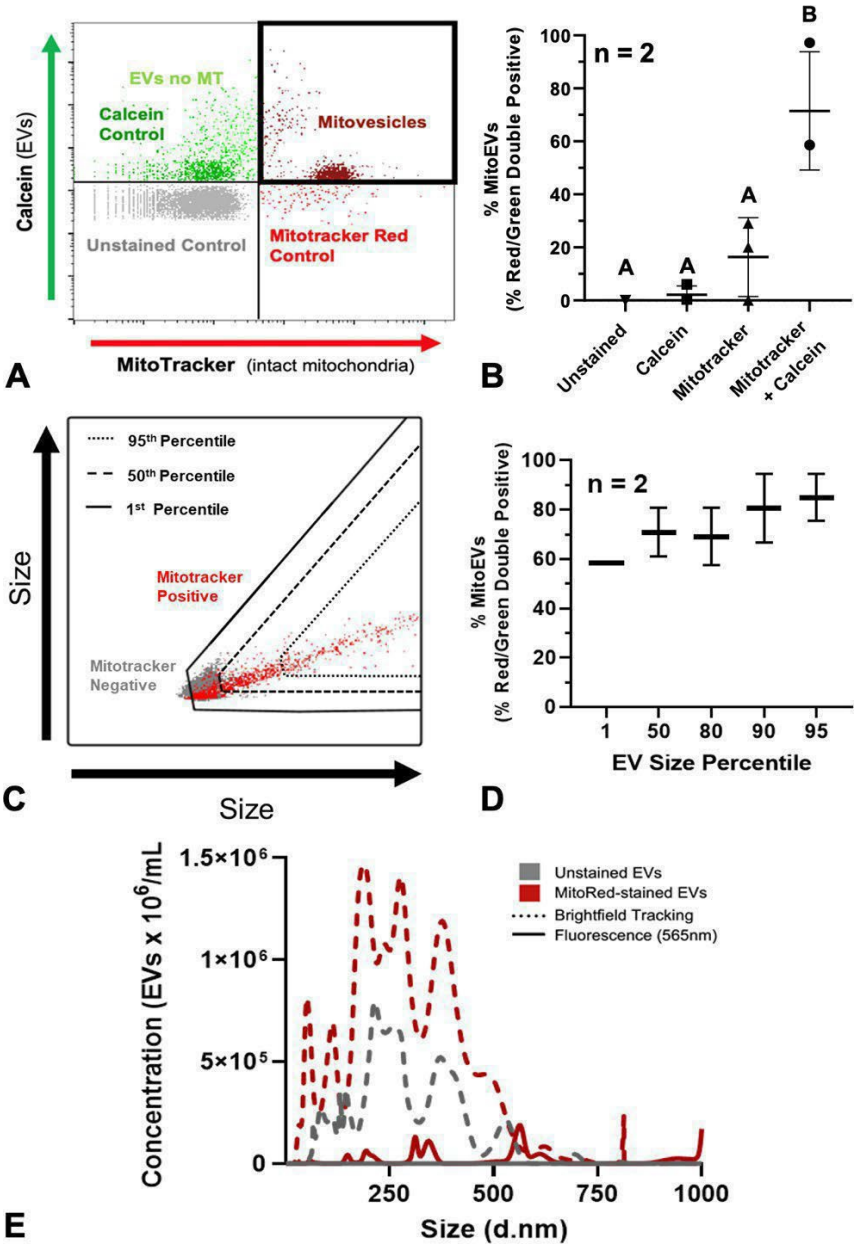


Figure 1. Basic characterization of human MSC-derived extracellular vesicles. A) Dynamic Light Scattering (DLS) of cell-conditioned media (CCM) from murine MSCs revealed three sub-populations of EVs based on size: small, (~5-10nm) medium, (~100-1000nm) and large (5,000-10,000nm), which likely represent exosomes, microvesicles, and apoptotic bodies, respectively. Thin lines represent individual trials (n=3), thick lines represent averaged curves. B) Analysis by one way ANOVA of average percent area under the curve on DLS data revealed a significantly smaller 1st (exosome) peak and a significantly larger 2nd (microvesicle) peak for both stimulated groups (* = $p \leq 0.05$). C) Immunoblotting of whole cell lysate (WCL) and microvesicle (MV), exosome (EX), and vesicle free media (VFM) fractions of CCM. Ubiquitous marker HSP90 is present in WCL, EX and VFM fractions. The EV marker flotillin is present in all fractions except VFM, and the cellular marker IκBα is absent from exosome (EX) and microvesicle (MV) fractions, as expected. MT protein ATP5A1 was found only in WCL, but COXIV (MT) was found in WCL and MVs. D) Nanoparticle tracking analysis (NTA) of PBS (negative control), EX, and MV fractions indicates that EXs outnumber MVs by several orders of magnitude amongst particles less than 1μm, the maximum detection size of NTA.



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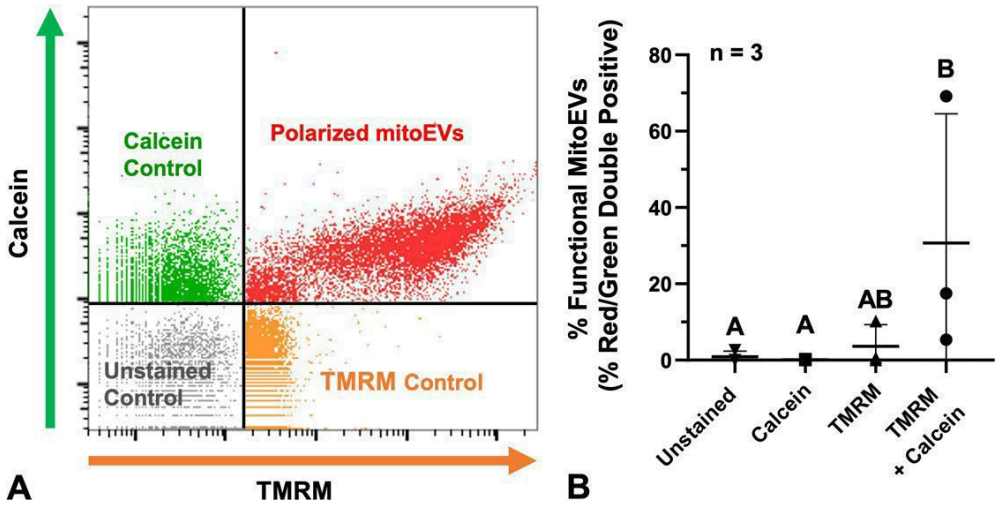
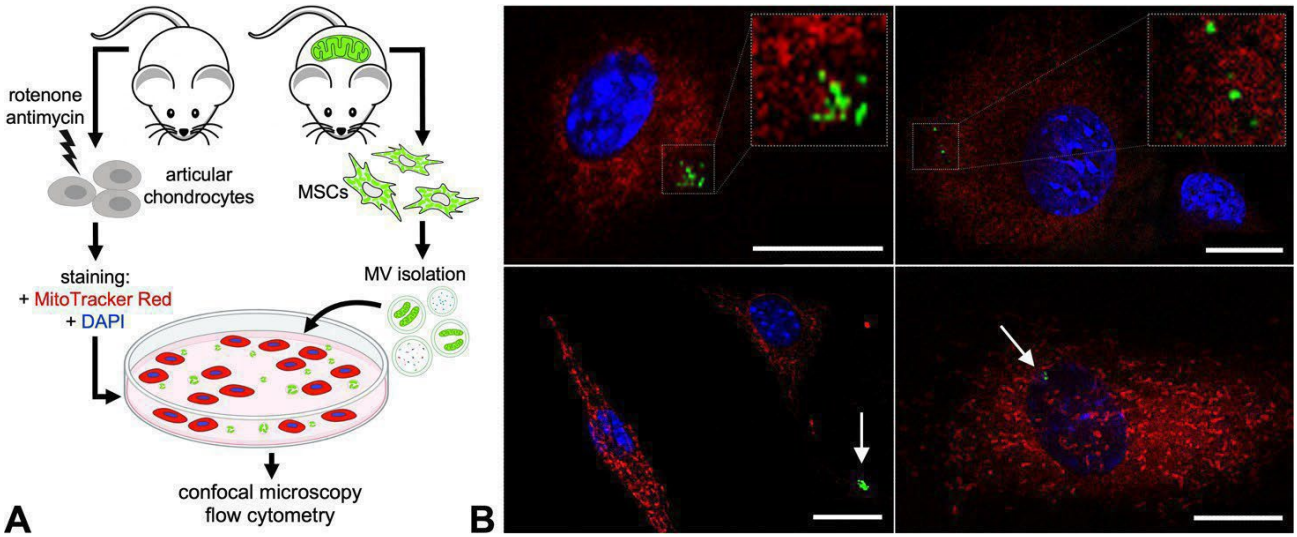
427 **Figure 2. MSC-derived EVs contain mitochondria (MT).** A) Events fluorescing above control
428 thresholds for Calcein AM (Green) and Mitotracker (Red) on flow cytometry were classified as EVs
429 containing intact MT (i.e., mitoEVs; dark red). B) Quantification of mitoEVs (red-green double
430 positive events) and three control groups; Mitotracker only (▲), calcein only (■), and unstained
431 control (▼), compared to EVs stained with calcein + Mitotracker (●). C) Representative flow plot
432 depicting backgating strategy for assessing relative sizes of mitoEVs (red) and MT-negative EVs
433 (gray). D) Backgating revealed that EVs of all sizes contain MT, but EVs lacking MT are generally
434 small, and the majority of the largest EVs contain MT. E) Fluorescent NTA revealed mitoEVs (solid
435 red line) trend larger than the general EV population (dotted lines) and cluster into distinct sub-
436 groups. Groups that do not share letters (Panel B) are significantly different ($p \leq 0.05$) by one way
437 ANOVA.



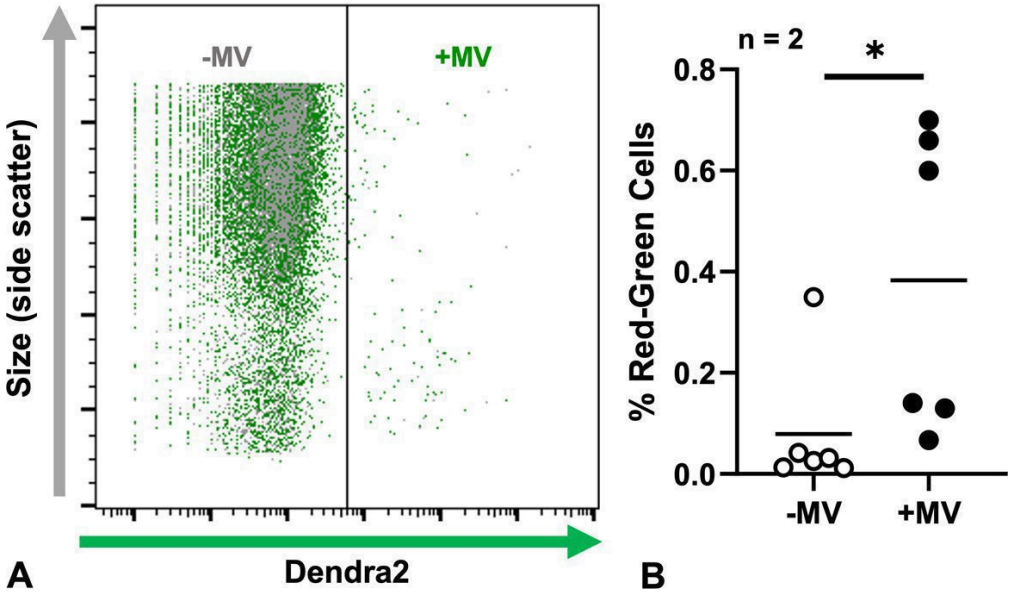
438438

439 **Figure 3. A subset of MitoEVs contain functional mitochondria.** A) Representative flow plot
440 identifying polarized mitoEVs; events fluorescing above control thresholds for calcein AM (green)
441 and TMRM (orange) were classified as mitoEVs containing functional MT (red). B). Quantification
442 of functional mitoEVs (red-green double positive events) and three control groups; TMRM only (▲),
443 calcein only (■), and unstained control (▼), compared to EVs stained with calcein + TMRM (●).
444 Data is expressed as the fraction of double-positive events in each group. of percent red/green double
445 positive showed statistically significant differences between the double stained experimental group
446 and single color/unstained controls Groups that do not share a letter are significantly different (n=3,
447 p≤0.05) by one-way ANOVA.



448448

449 **Figure 4. Murine Chondrocytes take up MT from MSC-derived MitoVesicles.** A) Schematic
450 depicting experimental methods for imaging non-contact intracellular MT transfer from MSCs to
451 chondrocytes. MV = microvesicles. B) Confocal images of chondrocytes (blue nuclei, red MT) that
452 were cultured for 12 hours with MSC-derived MVs. MSC MT (green, arrows) are incorporated into
453 chondrocyte MT networks (insets). Scale bars = 15μm.



454454

455 **Figure 5. Chondrocytes take up MSC-derived MT following incubation with MitoEVs** A)
456 Representative flow cytometry plots of chondrocytes incubated with (+MV) and without (-MV)
457 microvesicles isolated from MSCs expressing endogenous, MT-specific dendra2 fluorescence.
458 Dendra2 (green) threshold was set using control chondrocytes. Events fluorescing above threshold
459 were considered chondrocytes having taken up mitoEVs. B) Quantification of mitoEV uptake by
460 chondrocytes in culture (n=2) reveals the +MV group had a higher rate of transfer events than -MV.
461 Data are expressed as the fraction of chondrocytes above threshold for green fluorescence. * =
462 $p \leq 0.05$, by one-way ANOVA.

463

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7 Acknowledgements

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This study was funded by NIH NIAMS grants K08AR068470 and R03AR075929 and the Harry M. Zweig Fund for Equine Research.

8 Supplementary Figures

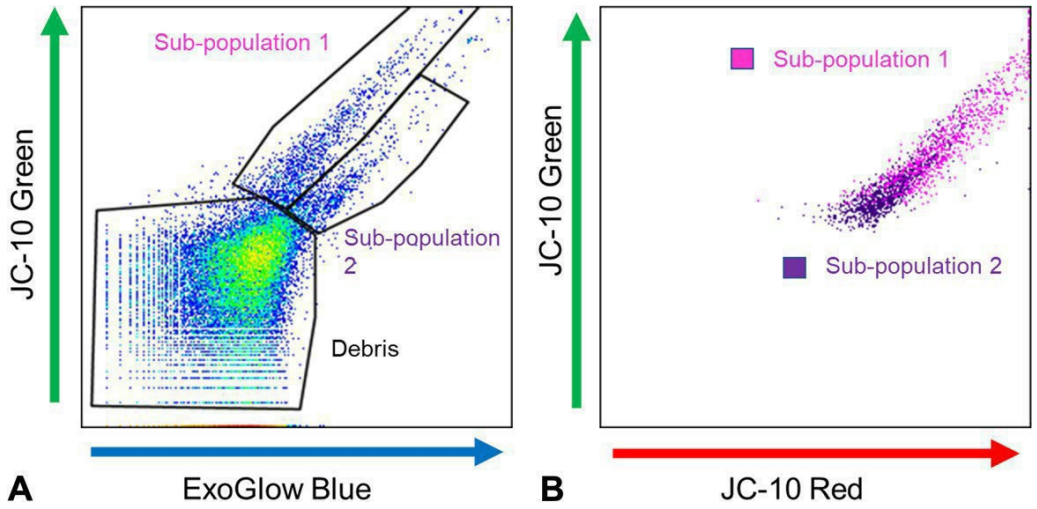
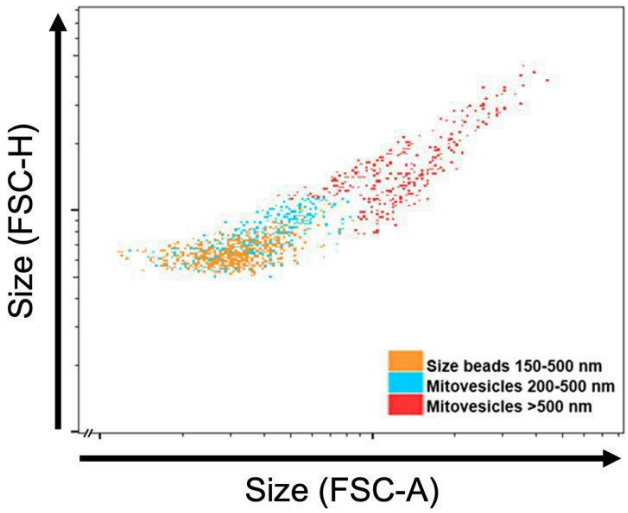


Figure S1. JC10 staining reveals that individual EVs contain mitochondrial cargo with a range of MT function. A) Exo-glow blue (EV) staining reveals two sub-populations of EVs. Events fluorescing above blue threshold were considered EVs, all events below that threshold were counted as debris. Blue positive events were gated into two sub-populations and plotted based on JC-10 red/green fluorescence. B) JC-10 fluoresces red in polarized MT. When the mitochondrial membrane becomes depolarized, JC-10 shifts to green fluorescence. Both sub-populations of EVs contained red and green fluorescence, indicating that MSCs release both polarized and depolarized MT in mitoEVs.



643

644 **Figure S2**. **Validation of mitoEV size ranges using calibration beads of known diameter.** MSC-
645 derived mitoEVs were identified with calcein/mitotrackerdeep red staining, and compared to beads of
646 known size on flow cytometry to provide an orthogonal, quantitative measurement of size to
647 complement backgating analysis data presented in Figure 2c,d. 47% of mitoEVs from the
648 microvesicle fraction are >500 nm. Calibration beads (160 -500nm; MegamixSSC, StagoDiagnostica:
649 01078) were used to set FSC/SSC thresholds and provide size references for flow cytometry
650 experiments. 53% of mitovesicles were within the 200-500 nm range, while 47% were >500 nm.

Principal Investigator:	Dr. Mariana Diel de Amorim
Title:	Can inflammatory markers in low-volume uterine lavage fluid be used to diagnose mares with endometrial fibrosis and with acute inflammation?
Project Period:	6/1/20 – 5/31/21
Reporting Period:	6/1/20 – 5/31/21

Project Title: 'Can inflammatory markers in low-volume uterine lavage fluid be used to diagnosemares with endometrial fibrosis and with acute inflammation?'

Principal Investigators: **Mariana Diel de Amorim**

A. Specific Aims of the Study and Modifications

Specific Aims: If the aims have not been modified, state so. If they have been modified, provide the revised aims and the reason for the modification.

Aim 1. Not modified

Aim 2. Not modified

B. Summary of Scientific Findings

Purpose.

Identify the presence of inflammatory markers in low-volume uterine lavage fluid and correlate those levels to mares with poor endometrial biopsy scores (fibrosis) or mares with acute inflammation (endometritis) based on cytology and histology to provide an efficient and less-invasive diagnostic technique for equine practitioners.

Methods.

Mares in estrus were be sampled in the following order: all mares had a low-volume uterine lavage (LVL) (250mL of 0.9% Sodium Chloride Solution) performed; followed by a single endometrial biopsy. After centrifugation of the LVL, the pellet was used for an endometrial cytology to grade acute inflammation. Endometrial biopsy was graded by Dr. Andrew Miller according to the standard Kenney-Doig scale, which is composed of 4 grades according to the amount of inflammation and fibrosis and carries a prognosis to carry a foal to term.

The low-volume uterine lavage supernatant was used in a commercially available ELISA kit for Horse CTGF and ANXA1 according to the manufacturer's instructions (MyBioSource, San Diego, CA, USA) and for the multiplex Assay to detect inflammatory markers (CCL2, CCL3, CCL5, and CCL11 along with TNF-α, IFN-γ, IL-1β, IL-10, IL- 17, and sCD14). This multiplex assay is established fluorescent-bead based technology that was performed in collaboration with Dr. Wagner at the AHDC.

Results.

Forty-seven samples were analyzed and consisted of healthy mares (n = 20), chronic endometritis (n = 17), and mares with acute endometritis (n = 10). Four out of 10 markers tested, IFN-γ, IL-17A, TNF-α, and CCL2 were significantly increased in CHRONIC mares. Furthermore, TNF-α was significantly increased in CHRONIC compared to ACUTE; and IL-17A was only increased in mares' uterine lavage fluid with CHRONIC inflammation compared to HEALTHY mares (Fig. 1).

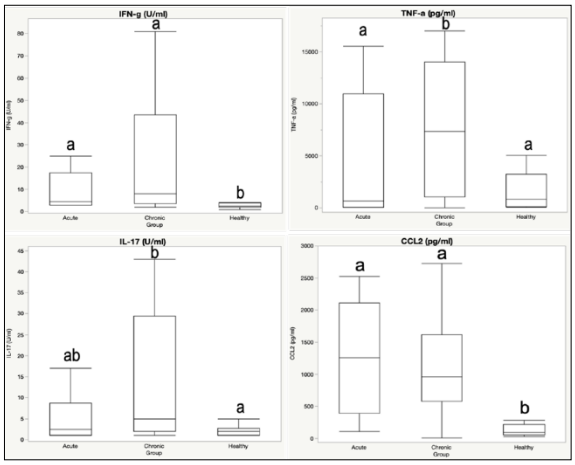


Fig. 1 Multiplex assay of inflammatory markers in low-volume lavage uterine fluid of mares with ACUTE and CHRONIC endometritis and HEALTHY mares. The different letter denotes statistical significance difference ($P < 0.05$)

No significant difference ($P>0.05$) was detected between the ACUTE, CHRONIC and HEALTHY mares groups on the CTGF and ANXA1 in the ELISA.

Conclusions.

We were able to demonstrate that bead-based multiplex assays provide a novel sensitive tool for detection of inflammatory markers in Low-volume lavage uterine fluid and that we were able to differentiate between ACUTE, CHRONIC and HEALTHY mares. A larger scale study is warranted so reference intervals can be established for those inflammatory markers in both the uterine lavage fluid but also in endometrial swabs and cytobrush, as those endometrial swabs and cytobrush are faster screening sample tools for the equine practitioner.

C. Significance

Emphasize the significance of the findings and their potential impact.

Our findings demonstrate that in the face of chronic endometritis, four out of the ten inflammatory markers investigated were significantly increased in mares with chronic inflammation compared with healthy mares; and we were also able to identify further differences between CHRONIC and ACUTE and CHRONIC and HEALTHY. These findings show promise for the use of an easy, fast, and less invasive test to detect chronic inflammation rather than only detecting through an endometrial biopsy (currently the gold standard) that is not always available to the equine practitioner. Even though, future research is warranted, far reaching implication in which the inflammatory markers might not only be used to screen and diagnose mares with endometritis, but also help practitioner tailor their therapies in response to the inflammatory markers levels.

D. Publications and other grant submissions

Report publications resulting from the study, including manuscripts submitted or accepted for publication, and submissions and/or external grants resulting from the award.

Lection JM, Wagner B, Miller A, Chenier T, Cheong SH, Diel de Amorim M. Investigation of inflammatory proteins as novel diagnostic biomarkers for endometritis in the mare. Clinical Theriogenology, 2020:12(3):365 (Society for Theriogenology (SFT/ACT), Pittsburg, PA, July 2020)[Abstract]

Lection JM, Wagner B, Miller A, Chenier T, Cheong SH, Diel de Amorim M. Inflammatory proteins biomarkers in the

low volume lavage of mares with endometritis and degenerative endometrial fibrosis, in Proceedings. American Association of Equine Practitioners (AAEP), 2020;66:151. [Abstract]

Lection J, Wagner B, Miller A, Byron M, Chenier T, Cheong SH, Diel de Amorim M. Novel Diagnostic Biomarkers for Differentiation of Endometritis in the Mare. Equine Veterinary Journal [In preparation]

E. Resident's Assessment

Research Development: Briefly describe your involvement in activities throughout the project to increase your research skills. Include, for example, formal course work, informal instruction in specific research skills, scientific seminars and meetings, training in the responsible conduct of research, or visits to other laboratories.

The Resident Research Grant program afforded me many opportunities to become more familiar with the scientific method, hypothesis-driven research and laboratory techniques. While the sample collection part of my study was already second nature to me because of my clinical duties, the laboratory component of my project was all new to me. I was taught by Dr. Diel de Amorim's laboratory technique to run the quantitative PCR for my study to support the multiplex assay results from Dr. Wagner's laboratory. I also gained exposure to other techniques such as immunohistochemistry and Western blotting, both of which I am now utilizing in my PhD program. The data I gathered from my resident research project was well-received at the two national conferences that I presented at (Society for Theriogenology Conference and American Association of Equine Practitioners Convention) along with being chosen as the abstract with the Best Scientific Content at Cornell's Clinical Investigator's Day. I also learned more regulatory side of research such as developing my own IACUC protocol for the mares enrolled in the study as well as creating an informed consent form for client horses.

Other Activities: Briefly describe your involvement in activities other than research and research training during the project. Describe activities such as teaching, clinical care, service on advisory groups or committees, and administrative activities.

During the project, I was mostly in the second year of my theriogenology residency, and therefore, I had greater and more independent clinical responsibilities both in the small animal clinic and equine clinic along with on-call hours for emergencies. Teaching is one of my passions, and so, I sought out extra opportunities to lecture to students both in the core curriculum and in elective courses. I also published two other first-author papers- a case report and a retrospective study. I also passed my boards to become a Diplomate of the American College of Theriogenologists.

F. Mentor's Report

Provide a mentor's statement assessing the Resident's progress and performance throughout the project, both in research productivity and in terms of research understanding.

Dr. Lection excelled in her research while in a busy clinical residency program. Dr. Lection was able to recruit mares for the equine endometritis project. She collected all the research samples, she interpreted all the endometrial cytologies and learned alongside Dr. Andrew Miller how to grade the endometrial biopsies, a great skill for an equine theriogenologist. Dr. Lection was self-motivated to learn how to do some statistical analysis and asked for initial help but was able to trouble shoot to do her data analysis and graphs. Besides the ELISA and learning the multiplex system, she also joined my lab to learn other molecular techniques and tried to trouble shoot immunohistochemistry for some of the cytokines that she had interest due to her promising data. She also learned to do some Western Blots and qPCR. The research findings were presented at the Society for Theriogenology conference in July of 2020 under a Resident competitive category, for which Dr. Jennine Lection won third place. Additional data was also presented in the American Association of Equine Practitioners (AAEP) Annual meeting in December of 2020 and she also won the first place in the best scientific content in the Clinical Investigators Day demonstrating that she

was highly motivated. She demonstrated to improve her research productivity and scientific knowledge rapidly during her program. I believe this research project along with her high motivation and mentorship was the key to her being successful candidate to get accepted in a prestigious PhD program, where she is exceeding her current mentor's expectation.

Dr. Lection had to take a break from writing her full manuscript this summer as she was studying for her specialty boards, which she was one of the two candidates out of 35 that passed, but since then she has started a PhD program at PennState and she has made significant progress with the manuscript uodate me weekly. She is expected to have a complete draft by the fall, which will be submitted to the Equine Veterinary Journal.

Principal Investigator:	Dr. Gillian Perkins
Title:	Equine gammaherpesviruses and equine gastric ulcer syndrome (EGUS) – is there a link?
Project Period:	1/1/20 – 6/30/22
Reporting Period:	1/1/21 – 12/31/21

The pandemic that started in March 2020 and closed the research labs has significantly delayed progress on the study. Additionally, initial results showed that almost all horses have some detectable EHV2 and/or EHV5 in their stomach samples, therefore the analysis method had to be adjusted to evaluate quantitative data of positive cells/area, instead of simply categorizing positive and negative tissues. This requires significantly more time and effort as all slides must be scanned for digital area measurement, and all samples must be manually counted. Because of this we decided to prioritize completing the ISH analysis of the glandular and normal samples only, since glandular disease remains the greater etiologic conundrum in equine medicine. We recruited a veterinary student and another large animal medicine resident to aid in this endeavor.

Full analysis by ISH for EHV2 and EHV5 has been completed for 9 horses with paired normal glandular mucosa and ulcerated glandular mucosa. This showed high prevalence of infection with all having EHV2 and all but one having EHV5 detected in the glandular mucosa. There was no significant difference in the number of infected cells per area between ulcerated and healthy tissue. The remaining samples from 28 horses have all been processed by ISH and cell counting is nearly complete. These represent 9 horses with no ulcers and 19 with glandular ulcers. Although the areas haven't been measured yet, both groups have very high prevalence of EHV2 and EHV5 infection, and there are many normal tissue samples from both healthy and ulcerated horses that have too numerous to count positive cells. Therefore, it is looking less likely that we will detect a significant difference between groups in the level of infection.

The remaining step is to perform DNase prior to ISH on positive samples from healthy and ulcerated horses to see if there is a different rate of active infection (i.e. RNA-positive) in ulcerated tissue.

I. Explain the significance of findings.

The overall hypothesis of the study is that equine gammaherpesviruses are associated with equine gastric ulcer syndrome. It is hypothesized that EHV-2 and EHV-5 are more prevalent in horses with ulcers compared to horses without ulcers. The preliminary results at this time suggest that might not be true. It is also hypothesized that horses with virus-positive ulcers will have EHV-2 and EHV-5 present in an active state at the ulcer site and a latent state in the normal stomach tissue. This is yet to be determined with further testing during the remainder of the study period.

II. Outline goals for the remainder of the funding period.

Due to the setbacks listed above, we will be requesting a no cost extension as there are 3 goals to be completed. First, finish measuring the tissue areas by digital scanning and imaging software to determine the number of positive cells per area of sample. Second, ISH will be repeated on positive samples using DNase pre-treatment to determine if the virus is in a latent or active state. The third goal during the remainder of the study period is to assimilate the data, present the findings at a scientific meeting in 2021 and complete the manuscript.

III. List anticipated grant applications and publications resulting from Zweig funding.

Upon successful completion of this project, we can use the data generated as strong rationale to further investigate the role of these 2 equine herpesviruses in equine ulcers, which are highly prevalent amongst horse populations. Such research proposals would be attractive to various equine-focused funding agencies, such as the Morris Animal Foundation, Grayson Jockey Club, American Quarter Horse Association, and others. The USDA could be interested in this research as well, based on previous funding of equine ulcer-related proposals by this funding agency.

Principal Investigator:	Dr. Heidi Reesink
Title:	Does Proximal Sesamoid Bone Mineral Loss Lead to Increased Fracture Risk?
Project Period:	1/1/19 – 12/31/21
Reporting Period:	1/1/19 – 12/31/21

PROJECT TITLE: Does Proximal Sesamoid Bone (PSB) Mineral Loss Lead to Increased Fracture Risk?

PRINCIPAL INVESTIGATOR: Dr. Heidi Reesink

A. Specific Aims of the Study and Modifications

The aims have not been modified.

Specific Aim 1. To determine whether proximal sesamoid bone volume fraction, bone mineral density and fetlock osteoarthritis (OA) differ between: 1) racehorses sustaining PSB fracture, 2) racehorses not sustaining PSB fracture and 3) non-racehorses.
Specific Aim 2. To determine how well PSB ash fraction measurements, the gold-standard for measuring bone mineral density, correlate with non-invasive measurements of bone mineral density, including: 1) clinical CT, 2) dual-energy x-ray absorptiometry (DXA), 3) Raman spectroscopy and 4) Raman and ash fraction measurements of the iliac crest.
Specific Aim 3. To determine whether PSB or iliac crest bone mineral density measurements or PSB bone volume fraction measurements increase the likelihood of appropriate classification of fractures or controls as compared to a previously reported mathematical model.

B. Summary of Scientific Findings

Aim 1 Findings (Published Abstracts)
Background: Proximal sesamoid bone (PSB) fractures are the most common fatal musculoskeletal injury in racehorses. Computed tomography has the potential to detect morphological changes in bone structure but can challenging to analyze efficiently, reliably, and quantitatively.
Objectives: To adopt a radiomics approach for analyzing image biomarkers and features. We hypothesized that image biomarker features from microCT images of equine PSBs from a previously published study can be reliably reproduced. We hypothesized that other undetected and imperceptible features are also revealed.
Study Design: Retrospective study of published data using a radiomics approach.
Methods: Radiomics features were computed on standardized CT datasets to benchmark the software. Features from microCT images of PSBs from 8 horses that sustained PSB fracture and 8 controls were computed using the contralateral, intact forelimb from horses sustaining PSB fracture (cases, n=16) and all available forelimbs for controls (n=30). Two-hundred and fifteen image features were calculated, and radiomic features were compared with features reported in a previous study.
Results: Morphologic features, such as volume, long axis dimensions and anisotropy were calculated and were highly correlated with previously published data. Several families of imperceptible features were also revealed. **Main limitations:** Only datasets obtained from cadaver specimens were included in the study.
Conclusions: Measurements and differences in PSB morphology were reproduced from previously published work using a radiomics approach. A number of imperceptible features were also revealed. These image biomarkers can be used in conjunction with other clinical data in the development of AI tools for predicting risk of PSB fractures.

OBJECTIVE

The objectives of this study were to examine whether proximal sesamoid bone (PSB) articular cartilage and bone osteoarthritic changes or palmar osteochondral disease (POD) scores were associated with exercise history and catastrophic PSB fracture in Thoroughbred racehorses. We hypothesized that horses that sustained a PSB fracture would have more advanced osteoarthritis as compared to controls and that osteoarthritis would correlate with total career high-speed exercise.

ANIMALS

Proximal sesamoid bones from 16 Thoroughbred racehorses (8 fracture, 8 control) euthanized on New York racetracks.

PROCEDURES

The Osteoarthritis Research Society International (OARSI) scoring system was used to evaluate sagittal PSB histological sections. Cartilage thickness and bone necrosis, or the proportion of empty to full osteocyte lacunae, were measured using blinded sagittal PSB sections. Osteophyte size was quantified using micro-CT, and limbs were assigned a macroscopic palmar osteochondral disease (POD) score by a board-certified pathologist at necropsy. Total accumulated high-speed furlongs was used as the measure of total exercise performed per horse. **RESULTS**

Total OARSI score, bone necrosis, osteophyte size and POD score were greater in horses with more accrued total furlongs. Total OARSI score, tidemark advancement, osteophyte size, and POD score were greater in the fracture group.

CONCLUSIONS AND CLINICAL REVELANCE

OARSI histologic PSB scoring revealed that more advanced cartilage arthritic changes correlated with both total furlongs and PSB fracture. Although OA changes correlated with fracture, the effect of exercise was dominant, suggesting that inclusion of exercise history will be important in future models that aim to assess catastrophic PSB fracture risk.

Aim 2 and 3 Findings (Unpublished Abstract) Summary

Background: Proximal sesamoid bone (PSB) fracture is the leading cause of fatal limb fracture in Hong Kong and the US. Efforts are underway to investigate diagnostic modalities that could help identify racehorses at increased risk of fracture; however, features associated with PSB fracture risk are still poorly understood.

Objectives: The objectives of this study were to: (1) investigate third metacarpal (MC3) and PSB density and mineral content using dual energy x-ray absorptiometry (DXA), computed tomography (CT), Raman spectroscopy and ash fraction measurements, and (2) investigate PSB quality and metacarpophalangeal joint (MCPJ) pathology using Raman spectroscopy and CT.

Study Design: Cross-sectional cadaver morphologic study.

Methods: Forelimbs were collected from 29 Thoroughbred racehorse cadavers (n=14 PSB fracture, n=15 control) and imaged using DXA and CT. An axial parasagittal section of one intact medial PSB was analysed by Raman spectroscopy, and a mid-sagittal section of the same PSB was sectioned into 9 sub-regions for ash fraction measurements.

Results: Bone mineral density (BMD) was greater in the third metacarpal (MC3) condyles and PSBs, and percent mineral by weight was greater in the subchondral region of PSBs in horses with more accrued furlongs. MCPJ pathology, including palmar osteochondral disease (POD) scores, MC3 condylar sclerosis, and MC3 subchondral lysis were greater in horses with more accrued total furlongs, and MC3 dorsal cavitation was greater in control cases. There were no differences in BMD or Raman parameters between fracture and control groups; however, Raman spectroscopy and ash fraction measurements revealed regional differences in PSB BMD and tissue composition.

Main limitations: Sample size was limited, and Raman spectroscopy was only performed on a subset of 16 PSBs. **Conclusions:** DXA, CT, and Raman revealed few differences between fractures and controls; however, many parameters, including MC3 and PSB bone mineral density, were strongly correlated with total accrued furlongs.

C. Significance

Aim 1 Significance

We demonstrated that a radiomics approach was able to identify differences in perceptible features, such as morphology, and other imperceptible features, such as entropy and complexity, between case and control PSBs. The significance of these findings is that image biomarkers may be able to be used in conjunction with other clinical data in the development of future artificial intelligence tools for predicting PSB fracture risk. We also demonstrated that PSB osteoarthritic changes correlated with both total accrued high-speed furlongs and PSB fracture; however, the effect of exercise was dominant. These data suggest that accounting for measures of exercise history and/or age will be essential in future models that aim to assess catastrophic PSB fracture risk.

Aim 2 and 3 Significance

Bone mineral density (BMD) was greater in the third metacarpal (MC3) condyles and PSBs in horses with more accrued total furlongs. Percent mineral by weight was greater in the subchondral region of PSBs in horses with more accrued furlongs. Metacarpophalangeal joint (MCPJ, fetlock) pathology, including palmar osteochondral disease (POD) scores, MC3 condylar sclerosis, and MC3 subchondral lysis were greater in horses with more accrued furlongs. There were no significant differences in BMD, Raman parameters, or MCPJ pathology between fracture and control groups; however, Raman spectroscopy and ash fraction measurements revealed regional differences in PSB BMD and tissue composition. The significance of these findings is that, while clinical CT, DXA and Raman spectroscopy have value in assessing fetlock (MC3 and PSB) morphology and composition, they are unable to differentiate between PSB fracture and control horses on the basis of bone density alone. Because of the strong correlation between BMD and total accrued high-speed furlongs, BMD may still be useful as a covariate in a fracture risk model or as a surrogate measure for exercise. The fact that BMD was so strongly correlated with accrued furlongs provides evidence to suggest that the pathophysiology underlying catastrophic PSB fracture differs between young horses and older, 'long campaigners'. Future studies may need to restrict focus to only one group or include much larger sample sizes to be sufficiently powered to differentiate between fracture and controls on the basis of imaging data because of these differences between young, exercise-naïve and older, more heavily exercised horses.

D. Publications and Other Grant Submissions

Publications

Basran P, Gao J, Palmer SE, Reesink HL. A radiomics platform for computing imaging features from μCT images of Thoroughbred racehorse proximal sesamoid bones: benchmark performance and evaluation. *Equine Vet J.*
2020 Mar;53(2):277-286. doi: 10.1111/evj.13321. Online ahead of print. PMID: 32654167.

Cresswell EN, Ruspi BD, Wollman CW, Peal BT, Deng S, Toler AB, McDonough SP, Palmer SE, Reesink HL. Determination of correlation of proximal sesamoid bone osteoarthritis with high-speed furlong exercise and catastrophic sesamoid bone fracture in Thoroughbred racehorses. *Am J Vet Res.* 2021 June;82(6):467-477. doi: 10.2460/ajvr.82.6.467. PMID: 34032482.

Noordwijk KJ, Chen L, Ruspi BD, Schurer S, Papa B, Fasanello DC, McDonough SP, Palmer SP, Porter IR, Donnelly EL, Basran P, Reesink HL. Exercise history predicts metacarpophalangeal joint pathology and proximal sesamoid and third metacarpal bone mineralization in Thoroughbred racehorses. In preparation for submission.

Grant Proposals Submitted and Funded

(Reesink, PI) 4/1/2020 - 03/31/2023
Grayson Jockey Club Research Foundation
Bisphosphonates and Fatal Musculoskeletal Injury

The goal of this project is to determine the prevalence of bisphosphonate use in racehorses and to determine whether bisphosphonates are associated with fatal musculoskeletal injury.
Role: Principal Investigator

Grant Proposals Submitted and Not Funded

(Reesink, PI) 1/1/2022 – 6/1/2024
Hong Kong Jockey Club Equine Welfare Research Foundation
Multi-regional investigation of risk factors and CT imaging parameters associated with catastrophic PSB fracture. The goal is to identify multi-regional risk factors for catastrophic proximal sesamoid bone (PSB) fracture in Thoroughbred racehorses and to validate a CT-based radiomics approach for PSB fracture risk assessment.
Role: Principal Investigator

Principal Investigator:	Dr. Heidi Reesink
Title:	Unraveling lubricin signaling in equine joint injury
Project Period:	1/1/20 – 12/31/22
Reporting Period:	1/1/21 – 12/31/21

PROJECT TITLE: Unraveling lubricin signaling in equine joint injury

PRINCIPAL INVESTIGATOR: Heidi Reesink

Progress was substantially delayed during the 3-4 months in which research labs were closed and subsequently re-activated due to the COVID-19 pandemic; however, we are now actively making progress on both Aims 2 and 3. We encountered some unexpected challenges as described in Aim 1 below; therefore, we have abandoned the genome editing portions of this Aim to focus instead on the effects of treatment with exogenous recombinant lubricin and experiments in Aims 2-3.

Aim 1. **To identify transcriptional networks regulated by proteoglycan 4 (*PRG4)* expression in equine synoviocytes, chondrocytes and mesenchymal stromal cells (MSCs).**

In last progress report, we shared the results of equine *PRG4* knockout (PRG4KO) experiments in equine chondrocytes using lentiviral vectors containing CRISPR-Cas9 cassettes. A total of 5 PRG4KO (with 5 different guide RNAs) chondrocyte cell lines were tested, and the KO4-transduced chondrocytes showed genome editing with DNA deletions and/or insertions in a subset of the transduced cells. However, due to an extremely slow growth rate, single cell-derived clones of the KO4-transduced chondrocytes were not generated.

Lentiviral-mediated genome editing of PRG4 in equine synoviocytes:
The same five equine PRG4KO lentiviral vectors used for chondrocyte cell lines were used to transduce equine primary synoviocytes. PCR analysis of the genomic DNA from the transduced synoviocytes indicated that KO1-, KO2- and KO4-transduced cells showed CRISPR-Cas9 induced indels. To detect specific genome editing, single cell cloning was performed using KO2-transduced synoviocytes. A total of 6 independent clones were generated. Four out of 6 clones showed genome editing with deletions (3-, 7-, 45- and 87-nucleotide deletion), and two clones with single nucleotide (A or T) "insertion". However, the cloned PRG4KO-synoviocytes also grew extremely slowly and/or resulted in lethality. Therefore, further studies on the PRG4KO clones could not be performed. Instead, we attempted an alternative CRISPR-Cas9-HDR strategy for genome editing as described below.

CRISPR-Cas9 induced genome editing of PRG4 in equine synoviocytes:
A "CRISPR-Cas9-HDR (Homology Directed Repair)" approach was employed using the KO1 and KO2 gRNAs in both equine synoviocytes and mesenchymal stromal cells (MSCs). We used 40-nt donor oligos around the KO1 and KO2 gRNA sites to reversely transfect equine synoviocytes and MSCs. Unfortunately, equine PRG4KO cells could not be obtained as detected by PCR analysis and restriction digestion, despite several attempts at protocol optimization. To our knowledge, these approaches have not been previously reported to be successful using equine primary cell lines. We hypothesize that the rate of reverse transfection and/or homologous recombination for the equine synoviocytes and MSCs was too low, resulting in undetectable homologous recombination events in the mixed (uncloned) cells. Therefore, positive PRG4KO clones could not be obtained using single cell cloning.

Because the CRISPR-Cas9-HDR approach was unsuccessful, and because equine PRG4KO synoviocytes transduced with lentiviral vectors demonstrated extremely low growth rates or lethality, we were not able to pursue further studies (e.g., qRT-PCR, RNAseq, etc).

Aim 2. To investigate how recombinant equine lubricin regulates cell growth and adhesion, gene expression, and biochemical parameters in equine primary cell lines and explant cultures.

Anti-adhesive effect of recombinant lubricin on equine synoviocytes and chondrocytes:

To verify the anti-adhesive properties of our recombinant lubricin (Lub), adhesion experiments were performed in three equine primary synoviocyte cell lines. Synoviocytes were plated on tissue culture plastic with or without lubricin (240, 120 and 60 µg/mL) in serum-free synoviocyte media. Equine synovial fluid (SF) was added to serum-free media at 3 concentrations (25%, 12.5% and 6.25%) as a positive anti-adhesion control. Synoviocytes were seeded in the Lub- or SF-treated wells, followed by crystal violet staining and quantitation (absorbance measurement at 570nm). As shown in **Fig. 1A-B**, both lubricin and SF resulted in dose-dependent decreases in **(A)** synoviocyte and **(B)** chondrocyte adhesion.

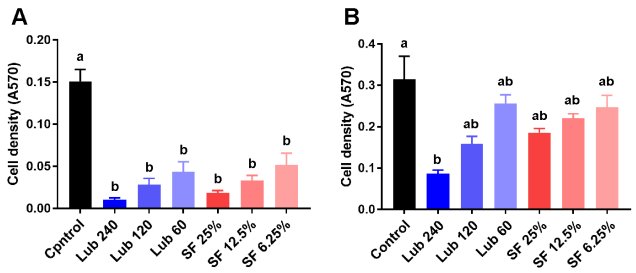


Fig 1. Lubricin inhibits equine synoviocyte and chondrocyte adhesion in a dose-dependent manner. Quantitation of **A)** synoviocyte (n=3) and **B)** chondrocyte (n=3) adhesion using crystal violet staining.

Effects of synovial fluid or recombinant lubricin on IL-1β-mediated GAG release from cartilage explants

To measure the amount of GAG released into culture media in response to IL-1β with or without synovial fluid (SF), a dimethylmethylene blue (DMMB) assay was performed using equine cartilage explants. The cartilage explants were cultured in media supplemented with either 1) IL-1β in a range of concentrations (0.01, 0.1, 1.0, 10.0, 25.0 ng/mL) with or without equine synovial fluid (0%, 6.25%, 12.5%) or recombinant lubricin (60, 120, 240 µg/mL).

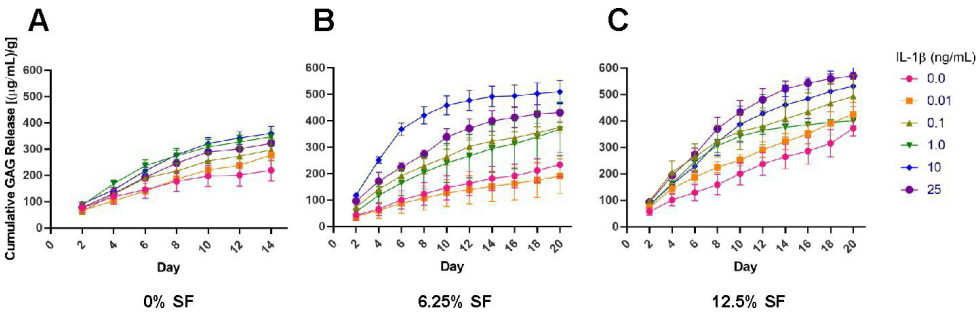


Fig 2. Effect of IL-1β concentration on GAG release into supernatant grouped by % SF composition. Increased IL-1β corresponded to increased GAG release in media with **(A)** 0% SF, **(B)** 6.25% SF, and **(C)** 12.5% SF.

Media supernatants were first digested with papain, then the papain-digested samples were quantified for GAG release spectrophotometrically at 525 nm. The results demonstrated that increasing IL-1β concentration from 0.01 to 25.0 ng/mL results in a dose-dependent increased GAG release into supernatant over time (2 to 20 days post-treatment, **Fig. 2)**. Media supplementation with moderate-dose (6.25%) SF may result in less GAG release than 0% SF at low IL-1β concentrations, whereas this effect was not observed with higher-dose (12.5%) SF supplementation **(Fig. 3).**

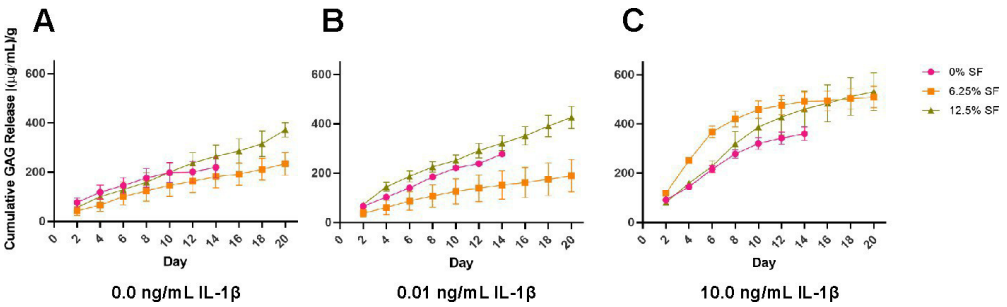


Fig 3. Effect of media supplementation with equine SF on GAG release, grouped by IL-1β treatment. Media supplemented with 6.25% SF resulted in lower GAG release in control media (0.0 ng/mL IL-1β) and low-dose (0.01 ng/mL) IL-1β conditions, but not in high-dose (10.0 ng/mL) IL-1β conditions. Media supplemented with 12.5% SF did not alter GAG release as compared to media without SF (0% SF).

Aim 3. To determine whether recombinant equine lubricin can modulate clinical, cytologic or biochemical parameters following intra-articular injection in IL-1}-induced carpal synovitis, and to demonstrate safety and absence of adverse effects following lubricin injections into naïve fetlock joints.

Animal experiments are anticipated to commence this winter due to delays associated with the COVID-19 pandemic.

II. Explain the significance of findings.

In addition to its lubricating functions, lubricin/*PRG4* has been shown to have anti-inflammatory effects in synovial joints. Evidence is accumulating for both pro-regenerative and anti-inflammatory roles; however, the precise mechanisms by which lubricin/*PRG4* performs these functions are largely unknown. In order to investigate these less understood roles for lubricin in joint homeostasis and repair, RNA-Seq will help us to identify transcriptional networks regulated by lubricin and cartilage explant experiments will allow us to assess the biochemical effects of lubricin in an *in vitro* joint inflammation model. This data will provide critical insight into the mechanisms by which lubricin functions as a chondroprotective agent and anti-inflammatory agent in the joint, leading to possible new applications for lubricin therapy. In addition, we will investigate the safety and short-term efficacy of our recombinantly-produced lubricin in the first-in-horse application of lubricin supplementation. Together, these experiments represent the first step towards bringing lubricin therapy closer to clinical application in equine practice.

III. Outline goals for the remainder of the funding period.

Goals for the remainder of the funding period include evaluating the effect of recombinant lubricin in the IL-1β-treated cartilage explant model in Aim 2 and performing animal experiments in Aim 3. Animal experiments are anticipated to commence this winter due to delays associated with the COVID-19 pandemic.

IV. List anticipated grant applications and publications resulting from Zweig funding.

Publications

Watkins A, Fasanello D, Stefanovski D, Schurer S, Caracappa K, D'Agostino A, Costello E, Freer H, Rollins A, Read C, Su J, Colville M, Paszek M, Wagner B, **Reesink HL**. Investigation of synovial fluid lubricants and inflammatory cytokines in the horse: a comparison of recombinant equine interleukin 1β-induced synovitis and joint lavage models. *BMC Vet. Res.* 2021 May 12;17(1)189. doi: 10.1186/s12917-021-02873-2. PMID: 33980227.

Fasanello DC, Su J, Deng S, Yin R, Colville MJ, Berenson J, Kelly C, Freer H, Rollins A, Wagner B, Rivas F, Hall AR, Paszek MJ, **Reesink HL**. Hyaluronic acid synthesis, degradation and crosslinking in equine osteoarthritis: TNF-α-TSG-6-mediated HC-HA formation. *Arthritis Res. Ther.*, 2021 Aug 20;23(1):218. doi: 10.1186/s13075-021-02588-7. PMID: 34416923.

Grant Proposals Submitted and Funded

Secor (Trainee) / Reesink (Mentor) 1/1/2022 - 12/31/2023 0.1 CM
Hong Kong Jockey Club Equine Welfare Research Foundation
Research Training Scholarship Application
Investigation of equine fetlock joint immunopathology and the immunomodulatory effects of intra-articular therapeutics
The goal is to illustrate the immune cell distribution in equine osteoarthritis, correlate lubricin glycosylation with immune cell populations, and evaluate the immunomodulatory effects of lubricin and common intra-articular therapies.
Role: PI (Supervisor)

Grant Proposals Submitted and Funding Pending

1R03AR078961-01A1 (Reesink) 4/1/2022 - 3/31/2024 0.6 CM
National Institutes of Health
Engineering recombinant lubricin to combat orthopedic infection
The goal of this project is to investigate how lubricin attenuates orthopedic biofilms and to determine whether distinct lubricin *O*-glycans mediate these anti-biofilm properties.
Role: Principal Investigator

Principal Investigator:	Dr. Gerlinde Van De Walle
Title:	The Mesenchymal Stem Cell Secretome Against Equine Herpesvirus Type I Infections
Project Period:	1/1/18 – 12/31/21
Reporting Period:	1/1/18 – 12/31/21

Project Title: The Mesenchymal Stem Cell Secretome Against Equine Herpesvirus Type I Infections.

Principal Investigators: Gerlinde Van de Walle

A. Specific Aims of the Study and Modifications

In this study, we proposed to evaluate the efficacy of factors secreted by equine mesenchymal stromal cells (MSC) against equine herpesvirus type 1 (EHV-1) *in vitro* (Aim 1) and *in vivo* (Aim 2). We successfully completed these 2 aims, and this work has resulted in a manuscript published in the peer-reviewed journal *Research in Veterinary Science* (see under D).

B. Summary of Scientific Findings

All our scientific findings from this study are published in a manuscript, which is included on the following pages of this Final Report in pdf format.
After publishing our work, we did begin to explore the identity of the secreted bioactive factors from equine MSC that could be responsible for the inhibition of EHV-1. One of the factors we explored was secretory leukocyte protease inhibitor (SLP-1), a multifunctional protein in host defense responses. Our collaborator, Dr. Bettina Wagner, recently identified this protein as being upregulated in nasal secretions of horses that are protected against EHV-1 infection and her graduate student developed an antibody against this equine protein. Based on their findings and the fact that SLP-1 is secreted by various different cell types, we proposed that the anti-EHV-1 effects from the equine MSC secretome could be mediated by secreted SLP-1. To this end, we collected MSC secretome from MSC from 3 different tissue sources (i.e., adipose tissue, peripheral blood, and bone marrow) and the graduate student performed her SLP- 1 ELISA to detect this protein in these secretomes. However, SLP-1 was not detected in any of the secretomes, indicating that the anti-EHV-1 effects are mediated by other, yet-to-be-determined, bioactive factors.

C. Significance

Our results with our *ex vivo* equine nasal mucosa explant system show that MSC secrete factors can reduce viral replication in a physiologically relevant 3D model. Moreover, the *in vivo* mouse study preliminary shows that factors secreted by MSC can protect lung tissue from damage associated with infection, potentially via modulating immune responses, and reduces infection in nasal cavities locally.

This is the first study that focused on the antiviral properties of equine MSC secreted factors against EHV-1. Our generated *in vitro* and *in vivo* data provide the scientific foundation for developing and implementing strategies to reduce the negative impacts of EHV-1 infections in horses. Pending successful *in vivo* studies in horses, the equine MSC secreted factors have potential as a cost-effective, off-the-shelf, biological alternative therapy or therapeutic adjunct to control EHV-1, and possibly also other infections important to the equine industry.

D. Publications and Other Grant Submissions

Harman R.M., Churchill K.A., Jager M.C., & **Van de Walle G.R.** (2021). The equine mesenchymal stromal cell secretome inhibits equine herpes virus type 1 in epithelial cells. *Research in Veterinary Science*, 141: 76-80.

Principal Investigator:	Dr. Gerlinde Van De Walle
Title:	Studying the replication kinetics of equine parvovirus hepatitis (EqPV-H)
Project Period:	1/1/20 – 12/31/21
Reporting Period:	1/1/20 – 12/31/21

Project Title: Studying the replication kinetics of equine parvovirus hepatitis (EqPV-H)

Principal Investigators: Gerlinde Van de Walle

A. Specific Aims of the Study and Modifications

The aims of this 1-year funded study have not been modified.

B. Summary of Scientific Findings

The overarching goal of this study was to better understand the replication dynamics and pathogenesis of the novel equine parvovirus, EqPV-H. This information will be critical for determining risk factors associated with severe disease, design a safe and efficacious vaccine, and identify potential antiviral or therapeutic drugs for EqPV-H- induced hepatitis. Completion of this study has provided significant new insights into the pathogenesis and replication kinetics of this virus, as outlined below.

To better understand the prevalence and significance of EqPV-H in New York State (NYS) racehorses, we have completed PCR and in situ hybridization (ISH) of 192 liver biopsies from NYS racehorses. We found a PCR prevalence of 22%, which is similar to the reported prevalence in the general population. We then performed ISH on these PCR- positive samples and 75% were ISH-positive. After evaluating these ISH-positive samples histologically, 35% showed signs of active liver pathology associated with viral infection of hepatocytes. This demonstrates that EqPV-H is both prevalent in NYS racehorses and can be associated with active liver pathology, warranting additional studies examining the effects of (sub)clinical EqPV-H-induced liver disease on racehorse performance.

We performed a retrospective analysis of EqPV-H-positive equine liver tissues with a range of clinical disease and identified an interesting relationship between the level of cellular division and viral replication level. Using a combination of immunohistochemistry (IHC), ISH, and advanced image analyses, we found that (i) EqPV-H infection of hepatocytes correlates with the severity of disease and (ii) cellular division is increased in cases of moderate hepatitis but not in severe, fatal cases. Importantly, the severity of EqPV-H infection does not correlate with cellular division status of hepatocytes, indicating that this virus is not reliant on dividing cells for its replication.

To determine whether EqPV-H, like the parvovirus Human bocavirus-1 (HuBoV-1), utilizes the nonstructural protein (NS) to initiate the DNA damage repair pathways, and as such bypass the need for host cell division, we developed several mammalian expression vectors of the NS protein. Western blot analysis and immunofluorescent imaging demonstrated that expression of the EqPV-H NS protein in mammalian 293T cells results in increased activation of the host DDR factor H2AX, but not RPA32. These findings identify a potential mechanism through which the virus may activate the DDR to facilitate viral genome replication and completion of the virus life cycle. Experiments examining the immediate upstream and downstream mediators of yH2AX and p-RPA32 are currently underway. Once these experiments are complete, we will submit a manuscript entitled, “The NS1 protein of Equine parvovirus- H activates the DNA damage repair pathways through activation of H2AX” for publication in a peer-reviewed journal.

Similar to the NS protein constructs, we also developed multiple expression systems for the viral capsid, known

as VP. Production of these capsids through an insect cell expression system resulted in complete auto-assembling viral-like particles that have been confirmed using traditional electron microscopy. High-definition cryogenic electron microscopy in collaboration with the Parrish laboratory are underway. In addition to providing the first detailed structural analysis of a *Copiparvovirus*, of which EqPV-H is a member, these capsids will provide a platform for the

development of an ELISA diagnostic test and a vaccine composed of these virus-like particles. Once these experiments are complete, we will submit a manuscript entitled, "Production and structural analysis of self-assembling equine parvovirus-hepatitis virus-like particles in an insect baculovirus cell system" for publication in a peer-reviewed journal. A reliable, robust *in vitro* culture system is necessary to study the replication and molecular characteristics of any virus. Our laboratory has developed multiple novel and renewable primary hepatocyte cultures systems, including 2D and 3D cultures. We have successfully maintained these cultures for at least 30 days and have developed a novel technique for collecting source material from minimally invasive percutaneous liver biopsies of adult hoses. Importantly, we have demonstrated that DDR activation can successfully occur in spheroids treated with UV radiation. We have also developed replication-competent liver organoids using a similar collection technique that involves embedding tissue fragments in Matrigel substrates. More recently, we found low levels of infection of 2D primary liver cell cultures using ISH and additional experiments are currently underway to repeat these findings and optimize a strategy for maintaining cultures and maximizing viral replication. Importantly, an optimized *in vitro* equine hepatocyte model can then also be used to study other equine hepatotropic viruses, such as equine hepacivirus.

Lastly, we have designed two infectious clones of EqPV-H using a combination of traditional cloning methods and commercial nucleotide synthesis. These infectious clones encode the entire EqPV-H genome and include the majority of the inverted terminal repeat regions that are critical for auto-replication of parvoviruses. Evaluation of these constructs for successful viral production and infectivity are currently underway and will be critical for *in vitro* assays and the much-needed pure viral inoculum for *in vivo* infection experiments.

C. Significance

Determining how EqPV-H replicates in the liver will provide a better understanding of the pathogenesis of this virus. This will be critical in the rational design of a safe and efficacious vaccine and in the identification of antiviral or therapeutic drugs for EqPV-H hepatitis, as well as to improve management strategies. Our studies are important to the racing industry because of the devastating economic impact of Theiler's disease in broodmares and because of the potential that EqPV-H hepatitis negatively impacts race performance given the high prevalence in racehorses. Our data demonstrate that EqPV-H is prevalent in the NY racehorse population and that infection is associated with pathology, supporting the need for additional studies examining associations between performance and EqPV-H infection. Lastly, our novel, renewable, and minimally invasive primary hepatocyte model may be useful for studying infectious and non-infectious diseases of the horse liver, including toxicologic studies.

D. Publications and Other Grant Submissions

Publications:

1. Jager MC, Tomlinson JE, Lopez-Astacio RA, Parrish CR, Van de Walle GR. (2021). Small but mighty: old and new parvoviruses of veterinary significance. *Virology Journal*, 18: 210.

2. Jager MC, Henry C, Fahey M, Tomlinson JE, Van de Walle GR. Prevalence and pathology of equine parvovirus- hepatitis in New York racehorses. In preparation.

3. Jager MC, Van de Walle GR. The NS1 protein of Equine parvovirus-H activates the DNA damage repair pathways through activation of H2AX. In preparation.

4. Jager MC, Wasik B, Weichert W, Parrish C, Van de Walle G. Production and structural analysis of self- assembling equine parvovirus-hepatitis virus-like particles in an insect baculovirus cell system. In preparation

Grant application:

NIH K08 application for Dr. Mason Jager (trainee; Van de Walle: mentor): primary submission was not selected for funding despite an excellent score, but was resubmitted in November 2021.

Principal Investigator:	Dr. Bettina Wagner
Title:	Nasal immunity and its function in preventing transmission of EHV-1 in immune horses
Project Period:	1/1/20 – 12/31/21
Reporting Period:	1/1/20 – 12/31/21

Project Title: Nasal immunity and its function in preventing transmission of EHV-1 in immune horses

Principal Investigators: Bettina Wagner (PMDS)

A. Specific Aims of the Study and Modifications

Specific Aims: If the aims have not been modified, state so. If they have been modified, provide the revised aims and the reason for the modification.

The specific aims of this project were to find out if immune horses that are exposed to EHV-1 can infect other horses (Aim 1) and to analyze the mechanistic roles of neutralizing IgG antibodies on EHV-1 binding to and/or entry into epithelial cells (Aim 2). The aims have not been modified and were performed as planned.

B. Summary of Scientific Findings

Describe the studies directed toward the specific aims and the positive and negative results obtained. If applicable, address any changes to the innovative potential of the project. If technical problems were encountered in carrying out this project, describe how your approach was modified.

For **Aim 1**, we performed the experiment to test if immune horses that are exposed to EHV-1 can infect other horses. Immune horses were experimentally infected with EHV-1. On the next day, each horse was naturally exposed to a group with EHV-1 susceptible and also EHV-1 immune horses by letting them mingle for 24 hours. Afterwards, all horses were stabled in individual box stalls to followed the horses body temperatures, clinical signs of disease, nasal virus shedding and cell-associated viremia daily to identify if infectious virus was transmitted or not by an immune horse. We also took blood and nasal secretion samples to analyze immune responses to EHV-1. Overall, we found that immune horses can still shed small amounts of virus without showing any signs of disease themselves. Only susceptible horses in the mingling groups picked up the virus and developed high fever and respiratory disease typical for EHV-1. In contrast, the immune horses in the mingling group were not infected by the experimentally infected immune horses. Immune horses had high pre-existing mucosal and serum antibodies against EHV-1 combined with some pre-existing T-cell immunity.

For **Aim 2**, we analyzed the mechanisms by which mucosal IgG antibodies can prevent EHV-1 infection at the respiratory entry site. In particular, we tested whether mucosal IgG inhibited EHV-1 binding to and/or entry into cells. We infected equine cells (PBMC) and cell lines (RK13) *in vitro* with fluorescent EHV-1 virus (EHV-1-GFP) and identified the time point and viral dose for optimal infection (maximal fluorescence/best viability) for each cell type. In addition, we performed neutralization assay with samples from horses with different EHV-1 immune status, such as susceptible and immune horses, and at different times pre- and post-experimental EHV-1 infection. This showed that immune horses are able to neutralize EHV-1 post-infection, while susceptible horses lack neutralizing mucosal antibodies during at least the first week post infection. Next, we infected RK13 cells with EHV-1-GFP in the presence or absence of neutralizing antibodies. This showed that EHV-1 can still bind to the cells when mucosal antibodies are present. However, neutralizing IgG antibodies prevented internalization of EHV-1 into the cells almost completely.

C. Significance

Emphasize the significance of the findings and their potential impact.

Our previous data leading to this project have shown the crucial role of mucosal immunity and, more specifically, intranasal EHV-1 specific IgG antibodies in protecting horses from infection and all disease outcomes (Perkins et al. 2019, Schnabel et al. 2019). The data supported that protective immunity in the upper respiratory tract is associated with pre-existing EHV-1-specific IgG4/7 antibodies, the absence of IFN-α and inflammatory marker secretion, and rapidly increasing IgG4/7 upon challenge infection. While pre-existing systemic EHV-1 specific IgG4/7 antibodies highly correlated with protection, T-cell immunity was overall low, even in fully protected horses (Larson and Wagner 2021). Overall, the findings suggested immediate virus neutralization at the local infection site by pre-existing antibodies in protected horses. It further suggested that immune and protected horses not only quickly inactivate EHV- 1 at the respiratory mucosa, they are likely also transmitting no or only small amounts of infectious virus.

During this current project, we tested the neutralizing capacity of mucosal antibodies in a more mechanistic way. We also determined whether immune horses with mucosal antibodies can still transmit EHV-1 to other horses. This provided the first direct evidence that nasal antibodies can quickly inactivate EHV-1 at the respiratory mucosa and thereby prevent viral entry into respiratory epithelial cells of immune horses. The experiments also showed that transmission of EHV-1 is interrupted in an immune population while susceptible horses can still develop all signs of disease even after exposure to minor amounts of infectious virus in their environment. Overall, the additional information and data resulting from this project can have a substantial impact on quarantine management and release procedures during EHV-1 outbreaks. The data also emphasize the importance of EHV-1 vaccination for all horses to effectively prevent outbreaks in the US.

D. Publications and Other Grant Submissions

If applicable, report publications resulting from the study, including manuscripts submitted or accepted for publication, and submissions and/or external grants resulting from the award.

Two manuscripts describing the outcomes of this project are in preparation. The findings on (i) the neutralizing capacity and mechanism of mucosal IgG antibodies, and (ii) that EHV-1 transmission is majorly inhibited by pre- existing immunity will be submitted for publication.

A USDA/NIFA application is planned for 2023 to evaluate the capacities of different commercial EHV vaccines on inducing IgG4/7 antibodies against EHV-1, with particular emphasis on mucosal antibodies.

Publications from Zweig funding related to this project:

Perkins G, Babasyan S, Stout AE, Freer H, Rollins A, Wimer CL, Wagner B. 2019. Intranasal IgG4/7 antibody responses protect horses against equid herpesvirus-1 (EHV-1) infection including nasal virus shedding and cell- associated viremia. *Virology*, 531: 219-232.

Schnabel CL, Babasyan S, Rollins A, Freer H, Wimer CL, Perkins GA, Raza F, Osterrieder N, Wagner B. 2019. An equine herpesvirus type 1 (EHV-1) Ab4 open reading frame (ORF)2 deletion mutant provides immunity and protection from EHV-1 infection and disease. *J Virol*, 93(22):e01011-19.

Larson EM, Wagner B. 2021. Viral infection and allergy – what equine immune responses can tell us about disease severity and protection. *Mol Immunol*, 135: 329-341.

APPENDIX C
Summary of 2021 Expenditures

2021 Research Awards	$393,557
2022 Public Relations and Administrative Budget	$29,600
2021 Incentive Awards	$5000
TOTAL EXPENDITURES:	**$428,157**

APPENDIX D
2021 Research Presentations

The Harry M. Zweig Memorial Fund for Equine Research

2021 Zweig Faculty Research Presentations
Wednesday, November 17, 2021, 3:00-4:30 PM

Everyone is welcome to attend

Lecture Hall 5

Zoom Link

Meeting ID: 918 3663 7415 - Passcode: 535098

3:00 pm **Opening Remarks by Associate Dean for Research & Graduate Education, Robert Weiss**

3:05 pm USE OF RADIOMICS AND BIOMETRIC WEARABLE SENSORS TO IDENTIFY HORSES AT RISK FOR CATASTROPHIC INJURY

Dr. Scott Palmer
New York State Equine Medical Director
Adjunct Professor, Dept of Population Medicine & Diagnostic Sciences

3:25 pm VETERINARY BIOBANKING: THE DARK HORSE OF EQUINE RESEARCH

Dr. Lara Mouttham
Laboratory Coordinator & Research Associate
Cornell Veterinary Biobank

3:45 pm INVESTIGATING EQUINE MITOVESICLES AS A NEXT-GENERATION ORTHOBIOLOGIC

Dr. Michelle Delco
Assistant Research Professor
Department of Clinical Sciences

4:05 pm GENOMICS OF THE EQUINE IMMUNE SYSTEM

Dr. Doug Antczak
Dorothy Havemeyer McConville Professor of Equine Medicine
Baker Institute for Animal Health

4:25 pm Closing remarks

APPENDIX E
2022 Research Awards

CONTINUATIONS

Principal Investigator	Project Title	2022 Award
Felippe, Julia*	Diagnostic markers in mares with placentitis	$79,352
Pigott, John	Multi-modal screening to identify Thoroughbred racehorses at increased risk for catastrophic injury of the metacarpophalangeal joint	$99,297
Todhunter, Rory*	Genomics of Autopsy–Negative Sudden Cardiac Death in Racing Thoroughbreds	$49,672
Wagner, Bettina	Intranasal biomarkers of EHV-1 susceptibility and protection	$86,451
	SUBTOTAL:	**$314,772**

NEW AWARDS

Principal Investigator	Project Title	2022 Award
Antczak, Douglas	Factors Affecting Durability in Standardbred Racehorses	$65,827
Delco, Michelle	Synovial fluid extracellular vesicles in equine joint disease and therapy	$83,229
DieldeAmorim, Mariana	Inflammatory markers from endometrial swab/cytobrush as a screening test for equine endometritis and endometrial fibrosis	$47,948
Reesink, Heidi	Equine joint sepsis and synovial fluid mucins	$67,973
Wagner, Bettina	Inflammatory biomarkers for prediction of breakdown injuries in horses	$91,661
	SUBTOTAL:	**$356,638**
		$671,410

*Received a 1 year no cost extension in 2020 to extend year 1 of the project.

APPENDIX F
Zweig News Capsules

Issue #71 (June 2021) and #72 (December 2021) are attached.

The full archive (1988 – present) is available online at https://ecommons.cornell.edu/handle/1813/22528.

Zweig

From the Harry M. Zweig Memorial Fund for Equine Research at the Cornell University College of Veterinary Medicine

No. 71 June 2021

Healing with horses

By Olivia Hall

When Teagan Manning first began working with Hero, she felt an immediate kinship with the horse — a five-year-old mustang gelding who was still unused to being handled. The veteran recognized the same kind of exhausting stress, anxiety and hypervigilance that had been plaguing her ever since a severe training accident had cut short a 14-year career with the United States Marine Corps. "I realized that if I was going to help Hero, I was going to have to find this calm centeredness within myself, in order to help him learn and achieve the things that he needs to achieve," Manning said. "And that calm centeredness I haven't felt in years."

The pair continued to train together under the auspices of Mission Mustang©, one of a diverse array of programs serving individuals with disabilities, at-risk youth, veterans and their families at EquiCenter in Honeoye Falls, New York. Housed at the 200-acre William and Mildred Levine Ranch, the nonprofit is a Professional Association of Therapeutic Horsemanship International Premier Accredited Center and offers therapeutic equestrian and related activities, including horseback riding and horsemanship, adaptive yoga, canine-assisted activities and farm-to-culinary classes. In 2018, in collaboration with Cornell Cooperative Extension and the Canandaigua VA Medical Center, EquiCenter secured an $850,000 contract from the U.S. Department of Veterans Affairs' Office of Rural Mental Health to teach therapeutic horticulture and farming to veterans on its three-acre organic farm.

Mission Mustang© brings captive mustangs together with veterans burdened with the emotional and physical scars of war. Photo: EquiCenter

When Manning joined Mission Mustang©, it was a pilot program between EquiCenter and the Bureau of Land Management (BLM) to bring captive mustangs together with veterans burdened with the emotional and physical scars of war. "Veterans are really given a purpose," said Jonathan Friedlander, EquiCenter's CEO and co-founder with his wife Stacy, who is an accomplished equestrian. The purpose: Gentle mustangs and prepare them for a new home.

The first four horses arrived at EquiCenter from Wyoming in 2018, joined later by six others purchased at a BLM auction at Cornell's Oxley Equestrian Center. All come from among some 50,000 wild mustangs and burros held by the BLM in off-range corrals and pastures to ease pressure off the nearly 100,000 free mustangs that crowd into 10 western states with insufficient space to roam and thrive. Being separated from their herd and brought into captivity only heightens the mustangs' natural self-protective instincts as prey animals. "The hypervigilance and anxiety and the desire to fit back in really mirror what many veterans with post-traumatic stress are experiencing," Friedlander explained. "They feel a sense of

common struggle and connection to the mustangs."

As the veterans learn — step-by-step and under the guidance of such experts as lead mustang trainer Steve Stevens — how to handle the wild animals, they themselves begin to change. "To watch the veteran have to dig deeply into his or herself, to be quiet, to be able to gentle the mustangs is a really powerful thing to see," said Pat Wehle, a retired standardbred breeder and Zweig committee member, who serves as an informal advisor to EquiCenter. Ann Dwyer, D.V.M. '83, also a Zweig committee member and EquiCenter advisor, is another shared connection.

A veteran named Mike no longer harbors thoughts of suicide, Friedlander reports, and another stopped wishing he'd died in Afghanistan. Manning has regained confidence and focus that extend into life outside the ring. In a letter to the future owners of Liberty, one of 10 mustangs who have been adopted to date, participant Brett Avery wrote, "I'm sure I could speak for everyone who worked with her when I say that she was the light and guidance we needed. We as veterans went to Mission Mustang© to save these horses, but in actuality they saved us!"

Having lost three military friends to suicide in the year after he retired from the U.S. Marine Corps, Avery hopes to bring such healing to many more of his fellow veterans. Last year he joined forces with Friedlander to organize Hoof-it for Heroes©. During the inaugural challenge, people from 19 states and six countries ran, walked, rode or otherwise covered a 2.2-mile distance — one pilot in South Korea flew his jet 2.2 miles in 30 seconds — to call awareness to the 22 veterans andactive military personnel who end their own lives every day. Funds raised support Mission Mustang©, which Friedlander intends to serve as a model and inspiration for other facilities around the country and world.

"[Veterans] feel a sense of common struggle and connection to the mustangs." - Jonathan Friedlander, EquiCenter president and CEO. Photo: EquiCenter

Friedlanderinvites anyone who wants to raise awareness for veteran and active military suicide and support Mission Mustang© to participate in this year's event — likely joined by members of Queen Elizabeth II's Household Cavalry, with whom EquiCenter has ongoing working exchanges to share best practices and programs to help wounded veterans and active military in both countries. The second annual Hoof-it for Heroes© will begin Nov. 7 and continue for 22 days. For more information, please visit: www.equicenterny.org/hoof-itforheroes •

Asthe veterans learn how to handle the wild anima[P]l[a]s[g], e[th]e[1]y[0] themselves begin to change. Photos: EquiCenter[5]

Harry M. Zweig Memorial Fund for Equine Research Awards

NEW

$99,336 to John Pigott and Alan Nixon for "Multi-modal Screening to Identify Thoroughbred Racehorses at Increased Risk for Catastrophic Injury of the Metacarpophalangeal Joint"

$83,113 to Bettina Wagner for "Intranasal Biomarkers of EHV-1 Susceptibility and Protection"

$64,463 to Michelle Delco for "Synovial Fluid Extracellular Vesicles in Equine Joint Disease and Therapy"

CONTINUED

$89,440 to Heidi Reesink for "Unraveling Lubricin Signaling in Equine Joint Injury"

$57,205 to Bettina Wagner for "Nasal Immunity and Its Function in Preventing Transmission of EHV-1 in Immune Horses"

Zweig Memorial Trot

Saturday, August 28, 2021

Vernon Downs, Vernon, New York

Post time: 6:10 p.m.

Information: 1-877-888-3766 | vernondowns.com/racing

Cornell bids farewell to leaders in equine medicine excellence

By Lauren Cahoon Roberts

In recent months, several equine luminaries have retired from their positions at the college. Their accomplishments have defined the cutting edge in clinical care, driven innovative discoveries and trained the next generation of world-class veterinarians. While saying goodbye to such living legends is not easy, it offers a chance to reflect on their impact.

Diving deep

Dr. Thomas Divers, the Rudolph J. and Katharine Steffen Professor Emeritus of Veterinary Medicine, has played a pivotal part in placing Cornell at the front of the pack when it comes to equine medicine. He and his colleagues have made foundational discoveries in equine motor neuron disease, Lyme disease, protozoal myelitis, equine hepatitis, equine liver disease, equine leptospirosis and more — many of his research projects funded by the Harry M. Zweig Foundation.

Dr. Thomas Divers, the Rudolph J. and Katharine Steffen Professor Emeritus of Veterinary Medicine. Photo: Cornell Ve

Divers was drawn to Cornell thanks to its mix of both dairy cattle and equine caseload, and for the opportunity to do collaborative research concurrent with his clinical work. "That's what drew me here, and it all worked out perfectly," he says. "My knowledge of the clinical questions allowed me to jump in on research projects and allowed our research teams to make a difference."

Divers has won three teaching awards at three universities, as well as the Educator of the Year Award from both the American Association of Equine Practitioners and the American College of Veterinary Emergency Critical Care and the Cornell University Hospital for Animals (CUHA) Distinguished Service Award for service to referring veterinarians. He also received the American Veterinary Medical Association's Samuel F. Scheidy Memorial Research Award for his collaborative foundational work on equine motor neuron disease. He has authored several textbooks, book chapters and over 190 peer-reviewed scientific publications. He says the best part of working at Cornell was without question the people whom he got to work with: "faculty, staff, students, residents and referring veterinarians!"

Respiratory inspiration

Dr. Dorothy Ainsworth, professor emerita of large animal medicine, came to Cornell in 1991, drawn by its reputation, students and residents, and the chance to collaborate with world-class veterinarians. She was eager to apply her expertise in equine respiratory disease and exercise physiology, and began doing clinical research on equine heaves, roaring, asthma, foalhood pneumonia and eventually the connection between environmental contaminants and dysphagia in foals. Her research has beenfunded by Zweig Memorial Fund, the USDA and the NIH.

Among her foundational discoveries in equine health were her 1990s Zweig-funded studies which evaluated the normal physiology of the diaphragm — its linkage to stride frequency (entrainment) during high-intensity exercise and the effect of upper airway obstruction on this synchronization. This work made it possible to evaluate

diaphragm function in the horse for the first time and to study under what circumstances the diaphragm fatigues. Her electromyography techniques were later adapted by Dr. Norm Ducharme for evaluation of upper airway muscle activities.

Another line of Ainsworth's Zweig-funded research uncovered the genomic origins of recurrent laryngeal neuropathy, or roaring — a common and economically important cause of exercise intolerance in horses — revealing that growth traits connected to height also dispose horses to the condition.

One of her morerecentNIH-funded studies focused on the correlation between dysphagia in newborn foals and ground water chemical contaminants associated with natural gas fracking.

Implementation of a well water filtration system reduced chemical concentrations and the dysphagia.

In addition to having received a Norden Teaching Award, she was also awarded the SUNY Chancellor's Award for Excellence in Faculty Service.

Dr. Dorothy Ainsworth, professor emerita of large animal medicine. Photo: Jonathan King/Cornell Vet

For Ainsworth, the best aspect of working at Cornell has been the diversity of cases she saw. "Nothing was ever boring — it was always fun," she says. "I did internal medicine because I like puzzles, and every case was a puzzle to solve."

Airway pioneer

James Law Professor Emeritus of Surgery Dr. Norm Ducharme is a world-renowned expert in the upper airways of the horse, with years of practice that have refined his surgical techniques, such as the standing tie back forward surgery — a procedure he pioneered and perfected. He also established efforts in reinnervating nerve tissue in the equine airway through nerve transplants, as well as the electrical stimulation of the muscles surrounding the larynx.

In addition to contributing to Cornell's expertise in the upper airways of the horse, Ducharme has also been a leader in equine arthritis, sports medicine, animal rescue and general surgical techniques. "I strongly believe that there are solutions to the pressing problems that exist and that we just have to find them and never settle for what's good enough," says Ducharme.

While at Cornell, Ducharme served as medical director at

Dr. Norm Ducharme, the James Law Professor Emeritus of Surgery. Photo: Rachel Philipson/Cornell Vet

CUHA from 1990-2014 and as chief medical officer at Cornell Ruffian Equine Specialists (CRES) since 2017. He acted as president and chair of the board of the American College of Veterinary Surgeons from 2005-2007 and was inducted in the University of Kentucky Hall of Fame for Equine Research in 2016.

His interest and excellence in the world of equine airway management wasn't something he always planned on. "I thought at first, okay, I'll try this for four years and then something else that's more exciting will come along. It just never happened — it has been fun the whole time."

Joints and genes

Dr. Alan Nixon, professor emeritus of large animal surgery, made his name as the director of Cornell's Comparative Orthopaedics Laboratory, where he was able to push the boundaries of knowledge and capabilities in orthopedic surgery, regenerative medicine and orthopedic research. He has also served as the Chief Medical Officer of CRES from 2014-2017 and served as chief of surgery at CUHA from 2002-2006.

His groundbreaking work in these areas has landed him on a 15 most influential veterinarians list and induction into the Equine Research Hall of Fame in 2009.

Nixon has served on the editorial review board for journals such as Veterinary Surgery, served as a consultant to the FDA panel on cell and gene therapy, written two textbooks on equine orthopedics and authored roughly 280 papers and book chapters, and served as a member of numerous veterinary organizations.

Dr. Alan Nixon, professor emeritus of large animal surgery. Photo: Cornell Vet

Among his achievements is the transplantation of cartilage cells and gene therapy to treat acute joint injuries — a cutting-edge treatment that has also benefitted human patients. In a culmination of many years of support from the Zweig Memorial Fund, he was awarded a $1.8 million grant from the NIH to investigate his innovative techniques in healing damaged joints.

Farewell but not goodbye

Although these luminaries in equine scholarship won't be walking the hallways at Cornell as often as they once did, their legacy lives on through the hundreds of students they taught, residents they guided and research teams they advised. Years of compassionate care for patients, combined with their expertise in the field of equine medicine, established a foundation on which others can build for years to come. •

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The Harry M. Zweig Memorial Fund for Equine Research honors the late Dr. Harry M. Zweig, a distinguished veterinarian, and his numerous contributions to the state's equine industry. In 1979, by amendment to the pari-mutuel revenue laws, the New York State Legislature created the fund to promote equine research at the College of Veterinary Medicine at Cornell University. The Harry M. Zweig Committee is established for the purpose of administering the fund and is composed of individuals in specified state agencies and equine industry positions and others who represent equine breeders, owners, trainers and veterinarians.

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Our site provides information on the projects and publications resulting from the Zweig Memorial Fund, and demonstrates the objectives of the Fund in promoting equine health in the racing industry. The Zweig News Capsule is published twice a year, and can be downloaded at bit.ly/ZweigNews. Please encourage other equine enthusiasts to visit the site.

Zweig

From the Harry M. Zweig Memorial Fund for Equine Research at the Cornell University College of Veterinary Medicine

No. 72 December 2021

Farewell, Fortier: Surgeon-scientist shifts from CVM to AVMA

By Lauren Cahoon Roberts

Lisa Fortier, Ph.D. '98, the James Law Professor of Surgery, has always had a passion for veterinary medicine. This love of the profession has carried her through her training — from her D.V.M. program at Colorado State University, to her Ph.D. and residency training at Cornell, and her current role as clinician-scientist at the Cornell University College of Veterinary Medicine. This year, Fortier's dedication leads her to a new chapter in her career: Taking on the role of editor-in-chief and communication division director of the American Veterinary Medical Association (AVMA).

"I think being a veterinarian is the coolest profession of all," says Fortier. "I've always loved being a veterinarian. And now, I have the ability, with the help of my staff, to impact 97,000 veterinarians with the work I am now doing."

This level of impact speaks strongly to Fortier, who has devoted her career to developing cutting-edge treatments that can dramatically improve both horses' and humans' lives. As a board-certified large animal surgeon, she dealt with numerous horses suffering from osteoarthritis — a condition that often arises after injury in equine athletes. These clinical cases drew her to study cartilage biology, and how to leverage the body's natural immune system to treat and even prevent arthritis from developing. Numerous studies funded by the Harry M. Zweig

Lisa Fortier, Ph.D. '98, the James Law Professor of Surgery, at the Cornell Equine Park. Photo: Platinum Performance, Inc./Cornell

Memorial Fund for Equine Research and led by Fortier have expanded the suite of clinical therapies available to equine patients — from stem cells, to bone marrow concentrate, to mitochondria-stabilizing peptides, to platelet-rich plasma. "The Zweig Fund is really what allowed me to establish myself as a researcher," says Fortier. "That funding was critical, and allowed me to produce the scientific manuscripts to get the word out about the work of my team at Cornell."

Fortier is proud of the fact that her body of research has not only made a difference for animal patients, but has also paved the way for human therapies as well. "We really have been able to fly the One Health flag with this work and showcase the role of veterinarians in advancing human health."

> *"The Zweig Fund is really what allowed me to establish myself as a researcher. That funding was critical, and allowed me to produce the scientific manuscripts to get the word out about the work of my team at Cornell. — Fortier*

In addition to pushing the boundaries of science and medicine, Fortier has also served in many other ways at the college, including being director of the Cornell Equine Park, associate chair for graduate education and research, serving two terms on the Institutional Animal Care and Use Committee, and working as a surgeon at the Cornell University Hospital for Animals and the Cornell Ruffian Equine Specialists clinic in Elmont, New York.

As she steps into her new role at the AVMA, Fortier will be doing a phased retirement at Cornell, still working on some key research projects and seeing some select clients at the hospital, concluding in the spring of 2023. At the same time, she is ramping up her work as editor-in-chief and is excited about the new horizons ahead. Her team just launched the AVMA's new journals website. "We've revamped the journals to be more author-friendly and increase their clinical and career relevance for our members," says Fortier. "We're really prioritizing the value of the manuscript for our readers, and getting them the information they need to enhance their profession in a timely manner."

Fortier is not new to this type of work, having launched two other journals during her career — Cartilage and The Journal of Cartilage and Joint Preservation. "I already knew I liked being an editor, I liked helping authors getting their work published and get their message out to the world," says Fortier. "I like to help them have their voice heard."

As she begins to step away from her duties at Cornell, Fortier reflects on the impact the college has had on her life. "The collaborative spirit at Cornell is truly unique," she says. "That spirit has always been here, and it has truly elevated the work I've done. I also can't stress enough how important all the administrative support has been — from the research office, to all of our administrative assistants, to all the support staff — everyone does so much to allow us to focus on teaching, research and clinics. The support is amazing at Cornell."

After three decades of discovery and service at Cornell, Fortier's new role with the AVMA is sure to be an exciting new chapter in her career. ●

Fortier prepares for surgery. Photo: Platinum Performance, Inc. /Cornell

Renowned equine hepatitis virus researcher receives AAEP research award

By the American Associaton of Equine Practitioners

The American Association of Equine Practitioners (AAEP) presented its 2021 AAEP Research Award to Joy Tomlinson '06, D.V.M. '10, whose groundbreaking research into four recently discovered equine hepatitis viruses has revolutionized the diagnosis, treatment and prevention of equine liver disease.

The AAEP Research Award recognizes an individual who has completed research that has or will make a significant impact on the diagnosis, treatment or prevention of equine disease. Tomlinson was recognized Dec. 7 during the AAEP's 2021 Annual Convention and Trade Show.

Tomlinson is a Ph.D. candidate, research associate and lecturer at the Cornell University College of Veterinary Medicine, from which she received her veterinary degree in 2010. Following graduation, she completed a large animal internal medicine residency at the University of Pennsylvania's New Bolton Center before returning to Cornell in 2014 as a clinician in the university's Equine Hospital and research associate at the Baker Institute for Animal Health.

Tomlinson's foundational research demonstrates equine parvovirus (EqPV-H) as the cause of Theiler's disease, a frequently fatal acute hepatitis disease of horses that has baffled the veterinary community for over a century. Her research shows that transmission is possible not only by serum or other biological products contaminated by the virus from infected horses, but also by natural means, such as direct contact and biting flies.

In addition, Tomlinson's research into the immune response of horses following equine hepacivirus (EqHV) infection has significantly advanced understanding of the equine immune system. Meanwhile, her landmark research into two recently discovered equine pegiviruses confirm that these viruses are not hepatropic and do not cause liver disease, but instead cause non-clinical persistent infection in the bone marrow of horses.

"Dr. Tomlinson's research focus is on the investigation of equine viral hepatitis. She has become the recognized world leader in this research area while also continuing her clinical work in internal medicine," said nominator Dr. Thomas Divers, professor of large animal medicine. "Dr. Tomlinson's 2.5 years of research on these four viruses has been extraordinary and answered many questions regarding the biology and clinical significance of these viral infections in horses." ●

Joy Tomlinson '06, D.V.M. '10. Photo: Chris Kitchen/Cornell

Notable publications

Antczak DF, Allen WRT. "Placentation in equids." Advances in Anatomy, Embryology and Cell Biology. 2021.

Cresswell EN, Ruspi BD, Wollman CW, Peal BT, Deng S, Toler AB, McDonough SP, Palmer SE, Reesink HL. "Determination of correlation of proximal sesamoid bone osteoarthritis with high-speed furlong exercise and catastrophic sesamoid bone fracture in Thoroughbred racehorses." American Journal of Veterinary Research. June 2021.

Harman RM, Theoret CL, Van de Walle GR. "The horse as a model for the study of cutaneous wound healing." Advanced Wound Care. July 2021.

Palmer S, Gomez AMM, Mohammed HO. "Attrition of Thoroughbred and Standardbred racehorses at New York racetracks due to exercise and non-exercise related fatalities during the 2016-2019 racing seasons." Journal of Equine Veterinary Science. Sept. 2021.

Raza F, Babasyan S, Larson EM, Freer HS, Schnabel CL, Wagner B. "Peripheral blood basophils are the main source for early interleukin-4 secretion upon in vitro stimulation with Culicoides allergen in allergic horses." PLoS One. May 2021.

Zoetis honors Reesink with research excellence award

Dr. Robert Weiss (left), and Reesink accepting the Zoetis Award for Veterinary Excellence from Lorin D. Warnick, D.V.M., Ph.D. '94, the Austin O. Hooey Dean of Veterinary Medicine. Photo: Darcy Rose/Cornell

By Melanie Greaver Cordova

Heidi Reesink, Ph.D. '16, the Harry M. Zweig Assistant Professor in Equine Health, is the 2021 winner of the Zoetis Award for Veterinary Research Excellence. This award recognizes outstanding research effort, productivity and the advancement of knowledge in areas relevant to veterinary medicine.

Each year, the recipient gives a presentation at the Zoetis Award Recognition Event, followed by a reception. Reesink presented "Horses, Hounds and Humans: A One-Health Approach to Osteoarthritis" Sept. 14 to virtual and in-person attendees at the Cornell University College of Veterinary Medicine. Her research covers pathophysiology, epidemiology and the treatment of orthopedic disease.

The award is intended for an outstanding faculty member at an early stage of his or her career. Nominations are restricted to individuals who, at the submission deadline, are not more than six years beyond their first faculty appointment at Cornell. With generous support from Zoetis, the college has highlighted and celebrated the exceptional research achievements of accomplished junior faculty for 37 years. ●

Cornell Ruffian Equine Specialists welcomes Morris to faculty

Tate Morris, D.V.M. '16. Photo provided.

By Melanie Greaver Cordova

Cornell Ruffian Equine Specialists (CRES) welcomed Tate Morris, D.V.M. '16, to its faculty July 26. "It is a pleasure to have a veterinarian as skilled as Dr. Morris join our team of clinicians," said Dr. John Pigott, medical director and associate clinical professor. "I look forward to seeing his enthusiasm and talents as a surgeon advance the health of our equine patients."

Morris grew up on the coast of Connecticut and spent time in the dairy barns of Vermont's Champlain Valley. Upon graduation from the University of Vermont, he worked in Wyoming on cattle and horse ranches, where he developed his passion for veterinary medicine. This prompted a return to the Northeast in 2012, when he enrolled at the Cornell College of Veterinary Medicine. At Cornell, Morris worked in the Equine and Nemo Farm Animal Hospital as a student technician, assisted clinical research in the Comparative Orthopedics Laboratory and treadmilled poor performance candidates in the Equine Performance Clinic.

After receiving his doctorate of veterinary medicine, he spent the next year as an intern with Randwick Equine Centre in Sydney, Australia, and with Rood and Riddle in Saratoga, working with a mixed population of Thoroughbred racehorses and sport horses. Following an internship with CRES in 2017, Morris joined the New Bolton Center, the large animal hospital and campus for the Veterinary School of the University of Pennsylvania. He now returns to CRES as an equine surgeon and emergency clinician, where he will continue to advance the elective, emergency and sports medicine case load. He brings with him an interest in airway and orthopedic surgery, novel therapy development and poor performance evaluation. ●

Touching a nerve in equine medicine

By Melanie Greaver Cordova

A passion for horses is foundational for the veterinarians at the Cornell Equine Hospital. It has led to groundbreaking procedures, state-of-the-art techniques and an impressive wealth of knowledge that has saved and improved the quality of life of many animals. Up-and-coming experts are already making waves in the fields that Cornell legends pioneered, fostering an equine surgery frontier that is exciting both for the outlook of horses and the veterinarians who care for them.

Cheetham at work in the Cornell Equine Hospital in 2018. Photo: Jonathan King/Cornell

Jonathan Cheetham, Ph.D. '08, associate professor in the large animal surgery section, is making important forays into equine airway research, with a clinical interest in upper airway surgery and equine sports medicine. After receiving his bachelor of veterinary medicine and master's degrees from Cambridge University, Cheetham worked in first-opinion and referral equine practices in the United Kingdom for several years. He then came to Cornell for a residency in large animal surgery and completed his Ph.D. in 2008 under the tutelage of equine airway pioneer Dr. Norm Ducharme, the James Law Professor of Surgery Emeritus. He joined the Department of Clinical Sciences as the Harry M. Zweig Research Scientist in 2012, followed by his appointment to associate professor in 2016. Cheetham has received several Zweig awards since 2011 to further his work.

"His research is really top-shelf," says Ducharme. "The work he's doing will have made an amazing difference even five, 10 years from now. It's promising and exciting."

Cheetham's concern is with peripheral nerve repair — specifically, understanding the relationship between the immune response to nerve injury and recovery, and modulating that immune response to improve functional outcome after an injury, as well as restoring laryngeal function using regenerative medicine techniques combined with reinnervation.

"I think of my process as a sort of wheel," says Cheetham. "The wheel can spin around, discovering at one point, modifying and translating at another and finally having the application from those findings spit out in unique ways."

The connective thread that runs throughout Cheetham's many projects — understanding the immune system's initial response to injury and leveraging it to improve patients' outcomes — is proving successful in the equine larynx, as well as the canine laryngeal process and human peripheral nerve injury, via collaborations with colleagues at Cornell's Companion Animal Hospital, the Cornell University Hospital for Animals and Weill Cornell Medicine respectively.

This work on nerve injuries in the lab is approaching the exciting point of application in clinics. "We're working on something that helps promote nerve growth after a nerve graft," Cheetham says. "There are a few promising candidates that have seen success in mice, rats and other

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species, so we're looking forward to bringing that to the clinic."

This project is building from Ducharme's work. "This would be an addition to the amazing work he has done to encourage those nerves we graft onto the muscle to regrow more quickly," says Cheetham.

From the operating room to work in the lab, Cheetham emphasizes that success relies on trusting team expertise. "Fellow surgeons will share their experience, the residents are great and the overall environment is excellent," he notes. "The technicians in the hospital have a sort of sixth sense about cases and have amazing relationships with clients. They're a core part of the team. And from a research perspective — we have a rich environment that allows and encourages innovation. A strength is knowing that our work is supported by the college."

Cheetham contributes to multiple services at the Cornell Equine Hospital, including the equine sports medicine and rehabilitation service; the soft tissue surgery service; the emergency and critical care service; the orthopedic service; and the regenerative therapies service. The Cornell Equine Hospital is a world leader in its field largely due to the expertise of the veterinarians and team members in each of these services and more.

Cheetham agrees it's a team attitude that creates success. "We all feel free to exchange ideas and support each other."

Whether it's bringing his drive, determination or collaborative attitude to the clinical space or the lab, Cheetham is upholding the Cornell Equine Hospital's commitment to the best possible care for its equine patients now and in the future. ●

Cheetham talks to a group touring the Cornell Equine Hospital in early 2020.
Photo: Darcy Rose/Cornell

Snapshots from the 2021 Zweig Memorial Trot

Photos provided

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