Protocol 41304 Amendment 13.0

Approval date	02/10/2025
Expiration date	12/30/2025

1. Basic Information

1. Elements ID

For existing protocols, enter the ID assigned to this protocol in Topaz Elements.

9785

2. eACUC Number (Automatically Assigned)

41304

3. Principal Investigator

Duan, Dongsheng

Job title
CURATORS DISTINGUISHED PROFESS
Department
Molec Microbio & Immunology
Division
Medicine
Business unit
University of MO-Columbia

4. Protocol Title

AAV Gene Therapy in a Canine Model of Muscular Dystrophin Deficient Disease (9785, 13120)

5. Triennial Re-write

Is this protocol a triennial re-write of a protocol that was previously approved at the University of Missouri?

● Yes ○ No

A. Historical Protocol Number

What is the ACUC number this protocol was previously approved under?

Protocol 13120

B. 3 Year Progress Report

Give a brief summary of the work completed on the historical protocol listed above.

In the last three years we have successfully amended our techniques to better serve the research, as well as the animal welfare.
1.) Immune suppression is often used to inhibit the immune system from destroying the injected vector

or overreacting in the injected area. We have refined our immune suppression methods to achieve successful immune suppression while maintaining the health and safety of the animals.
2.) With continued analysis of data and better genetic diversity within the colony, we have also modified several anesthetic techniques. To avoid potential anesthetic complications post-operatively, the Duan lab has amended anesthetic and sedation protocols to include more cardiac friendly drugs. With increased understanding of the model, we are adjusting all techniques and protocols to benefit the animal's wellbeing and the research focus.
3.) We continue to evaluate breeding success and genetic potential to increase genetic diversity while maintaining the affected model. Over the last three years puppy care and requirements have greatly improved, meaning the puppies require less intervention. This has led to stronger more resilient puppies from the beginning of life, resulting in more viable study dogs for long term studies.
4.) With increased sample sizing with various ages, the Duan lab has expanded the knowledge of how normal, carrier and affected dogs all play a crucial role in understanding Duchene Muscular Dystrophy in humans.
The Duan lab has had 9 additional publications, to date, in the years since the latest protocol submission. Looking forward, the Duan lab is in contact with collaborators to add more data to make Duchenne Muscular Dystrophy research as translatable as possible to humans.

2. Species Section

1. Please note, the total number of animals requested is the amount of animals you will need for a 3 year period. This number should include all experimental animals plus animals used for colony maintenance (breeders and offspring produced that are not used for experiments). These numbers should match the amounts in the Justify Animal Numbers section. If this is a triennial re-write these amounts should also include any animals on the previous protocol that will be transferred to the new protocol.

Species	Strain/ Stock/Breed	Age/Weight	Pain/ Distress Category	Authorized	Ordered	Received	Adjustment	Available
Dog	Any dog; golden retriever or mix	newborn to 13 years/ weight 50g to 150lb	USDA Category D	320		50		270
Total Dogs:				320	0	50	0	270

2. Phenotypic consequences

Describe any phenotypic consequences of the genetic changes to the animals and the outcome of these consequences (e.g. whether or not any change in animal welfare or husbandry is anticipated).

	Strain	Describe
1	DMD affected	The DMD model does result in increased saliva production, thickening of the tongue and potentially megaesophagus (although this does not often result in clinical presentation for our colony). Because of these changes, the food dishes may be raised and/or the food may be provided in the form of a slurry or thickened mash. All affected dog food is puppy kibble soaked in water to facility swallowing.

3. Wild Animals

Are WILD ANIMALS to be used or studied?

○ Yes ● No

4. Client-Owned Animals

Are CLIENT-OWNED animals to be used or studied?

● Yes ○ No

Consent for Client-Owned Animals

Attach the client consent form in the attached files section

3. Proposal Overview

1. Purpose

Purpose of the study:

Duchenne muscular dystrophy (DMD) is an inherited lethal muscular dystrophy disease that is commonly seen in childhood. Patients are wheelchair bound around the age of 10-12 years old; and most of them pass away due to respiratory or cardiac complications around 25-year-old. Currently, no effective treatment is available for this fatal disease.

DMD is caused by mutations in the dystrophin gene. Using a gene therapy approach to replace and/or repair the mutated dystrophin gene (such as 6 to 8 kb mini-dystrophin gene and 4 kb micro-dystrophin gene), or to express alternative disease modifying genes (such as SERCA2a, utrophin, integrin, etc.) holds great therapeutic promise. Recombinant adeno-associated virus (AAV) has been shown to be one of the most promising viral vectors for the delivery of therapeutic genes in the treatment of muscle diseases. In this study, we will explore AAV gene therapy in the canine model of human DMD. In addition, we will apply the CRISPR Cas9 technology for therapeutic gene editing in our DMD dog model. We will maintain a dog colony with dystrophin deficiency and use the DMD dog model to obtain critical preclinical data for evaluating gene therapy of DMD in humans.

2. Value

Please provide the information necessary to allow the ACUC to evaluate the objectives of the study against potential animal welfare concerns.

This large animal study will provide critical information for the development of a gene therapy protocol to treat Duchenne muscular dystrophy. The information obtained from the dog model bridges the knowledge from rodent study and lead to human trials. The outcome of this study will provide valuable information towards an etiology-based gene therapy for the most common childhood lethal muscle disease.

3. Lay Term Description of Experimental Design

To put something in layman's terms is to describe a complex or technical issue using words and terms that the average individual (someone without professional training in the subject area) can understand. This section should be written so that someone with a **10th grade science education can easily understand the project.**

Duchenne muscular dystrophy (DMD) is an inherited disease due to the lack of an important muscular structure protein called dystrophin. Patients carry mutations in their dystrophin gene. These mutations abort dystrophin production. This disease is fatal and there is no effective treatment currently. Gene therapy holds great promise to treat DMD. Adeno-associated virus (AAV) is the most promising muscle gene therapy vector. Many studies have shown that AAV gene therapy can effectively ameliorate muscle disease in mouse models of DMD. Before applying mouse results to human patients, it is critical to validate these findings in a large animal model of DMD. The overarching goals of our proposed study are: 1.) To maintain a dog colony

including Normal, Carrier, and Affected dogs. Animals will be bred via natural service or artificial insemination. This dog colony provides us the resource of experimental animals. 2.) To test AAV gene therapy in the affected dogs that are symptomatic and have a body size similar to affected boys. Specifically, we will (1) screen new AAV capsids and find out the most effective one or ones by local muscle injection using a reporter vector, (2) identify the most potent dystrophin gene configuration with local muscle injection or systemic delivery, (3) determine the transduction efficiency, vector distribution and possible adverse effects of intravascular (systemic) delivery of a reporter AAV vector, (4) determine the therapeutic efficacy and adverse reactions of AAV-mediated systemic dystrophin gene therapy, (5) assess the natural history of affected dogs, and (6) test immunity of Cas9, a bacterial protein used in the CRISPR Cas9 technology, it is a promising tool for gene editing, (7) test CRISPR therapy for DMD in dystrophic dogs, (8) Explore other emerging new treatments for DMD patients. Results from these studies will be critical to guide future clinical studies. Besides above goals, we will also collect tissues and cells from dogs for our canine tissue bank that will allow us to share the precious dog tissues with other investigators who do not have access to dogs.

4. Scientific Description of Experimental Design

In language a scientific colleague can understand, provide a step-by-step, general description of the animal experiments you will perform including experimental groups and timing of procedures and manipulations. For complicated experimental designs, including a flow chart, diagram, or table in the Attachments section is recommended to help the ACUC understand what is proposed. DO NOT describe details of the procedures here as such details are requested later in the form.

Introduction:

Duchenne muscular dystrophy (DMD) is the most devastating inherited muscle disease affecting 1:5000 live male births worldwide. Affected boys become progressively weaker due to muscle wasting, inflammation, and contractures, leading to wheelchair confinement by the age of 10 to 12 years. DMD is caused by the loss of dystrophin. Dystrophin has been identified as an important structural protein, that scaffolds a series of cytosolic and trans-membrane proteins into the dystrophin-associated glycoprotein complex (DGC). Together, this complex plays an important role in connecting the cytoskeleton with the extra cellular matrix through the sarcolemma. This link up structure preserves the integrity of the sarcolemma from mechanical damage during muscle contraction. In the absence of dystrophin, the cell membrane becomes fragile and susceptible to tear. This results in myofiber degeneration that is eventually replaced by fibrotic and fat cells, all of which compromise patient mobility. Unfortunately, treatment is currently limited to corticosteroids and symptom management with no cure.

Adeno-associated virus (AAV) mediated gene therapy holds a great promise in treating DMD. AAV is a harmless virus consisting of a protein shell (called “capsid”) and a single stranded DNA genome inside the shell. Both the capsid and the content genome are highly relevant to the development of an effective gene therapy. The capsid plays a role in delivering its cargo (gene) to the target cell, while the gene should express the missing protein effectively. Many variables can affect the efficiency of gene therapy. For example, which AAV serotype should be used? There are 12 classic AAV capsids and they are called AAV serotype-1 to 12. In addition, over 12 more serotype were recently identified from different species and/or engineering in test tubes in various laboratories. More serotypes are expected to emerge in the upcoming years. Furthermore, which promoter should be used to control gene expression? There are two different kinds of promoters: tissue-specific and the ubiquitous promoters. Tremendous amount of work has been done by different laboratories including our laboratory to eliminate these variables and achieve effective gene therapy. Most of these studies were performed using mdx mice, a mouse DMD model. Unfortunately, mouse results often translate poorly to DMD patients due to lack of the dystrophic phenotype in mdx mice and/or the failure to scale up from mice to large mammals. Dystrophin-deficient dogs are the best-described large animal model for DMD. They show characteristic symptoms of muscular dystrophy and dogs also have the scale up model advantage. Results from studies performed in the canine DMD model may better inform the design of clinical trials.

Here, we intend to test the efficacy of different AAV capsids in dogs. This can be performed by doing local or systemic injection. Once we find an optimal capsid, we then test the therapeutic efficacy of the cargo (gene) in ameliorating the DMD disease in the canine model. To achieve this goal, we use dystrophin dependent treatment strategies (such as replacing the defective dystrophin gene with a new copy of the gene), or dystrophin independent treatment strategies (such as gene editing or modulating other genes that play role in the DMD disease, ex: SERCA2a). We also plan to test combine therapies (e.g., SERCA2a and micro-Dystrophin).

In this protocol, we will validate what was achieved in the mouse model into the dog model. Furthermore, we will test different combinations of AAV capsid, promoter and transgenes to find the most potent strategy that has the highest therapeutic effect and minimal adverse effect in the dog model.

Experimental Group Description:

We will be using the canine as large animal model to develop an effective gene therapy for DMD. All experimental dogs used in this protocol will be from in-house breeding, purchased from a biomedical breeding research company, or transferred from another researcher/research facility. Animals will be bred via natural service, artificial insemination, or surgical artificial insemination. Normal, carrier, and affected pups will be whelped, raised, and housed to maintain and expand the colony as well. Normal or carrier puppies may be adopted. Normal puppies have historically competed with affected puppies for nursing time to the detriment of the affected puppies. Therefore, normal puppies may be removed from the mother where they will be hand-raised until adoption. This situation gives the affected puppies the greatest chance of success while still being able to adopt normal or carrier puppies. Dogs from in-house breeding will be genotyped once they are born to identify normal, carrier and affected dogs. After weaning, dogs will be assigned to following experimental groups.

Experimental groups:

Group 1. Local injection with a reporter gene AAV vector

In this group, we will test muscle transduction efficiency of different AAV vectors (such as different serotypes, different promoters, different synthetic and/or engineered capsids, and different configurations including single, dual or triple vectors). The vector will express a reporter gene (such as, but not limited to: LacZ, GFP, alkaline phosphatase, luciferase, etc.).

Study timeline: up to 12 years

Gender: Mix

Genotype: Normal, Carrier, and Affected

Age group: (pre-weanling 1 wk to < 2 mo, juvenile 2 mo to < 6 mo, young adult 6 mo to < 12 mo and adult >12 mo).

Experiment details:

1. Skin tattoo may be used to mark injection site before injection.

2. Local injection can be performed while the animal is sedated or conscious (Sedation method is explained below in the 'Non-surgical Procedures' section).

3. Injection may include injection to a single muscle or multiple muscles.

4. We will choose the muscle that can be easily accessed with or without ultrasound and cause minimal discomfort to the animals. (Muscles that can be used in local injection are explained below)

5. Following local injection, dog will be monitored for any adverse reaction following injection, which includes monitoring them for up to one hour for immediate reaction (swelling, redness, gait abnormalities, etc.), and then again for similar adverse effects approximately 24 hours later. If adverse effects are noted, a veterinarian will be consulted.

6. AAV transfection efficiency will be determined by expression level of the reporter gene. To monitor the reporter gene expression, muscle samples will be obtained via periodic muscle needle biopsy or open biopsy (see below for details of our muscle biopsy guidelines).

7. Additional experimental and diagnostic procedures may be completed on dogs in this group as needed. These experimental and diagnostic procedures are described in 'Description of Non-Surgical Procedures' section of this protocol.

8. At the end of the study, we may euthanize the animal and complete a full necropsy evaluation.

Immune suppression:

We and other investigators have previously shown that direct AAV injection to canine muscle may causes cellular immune response. This untoward cellular immune response may be prevented by transient immune suppression.

The details of the immune suppressive method are described in 'Non-surgical Procedures' sections of this ACUC protocol. We will carefully monitor dogs for their wellbeing and general condition during transient immune suppression. In the case of cyclosporine, we will also carefully monitor the blood level of cyclosporine and blood profile.

Group 2. Systemic injection with a reporter gene AAV vector

Muscular dystrophy affects all muscles in the body. Hence, there is a need to test systemic AAV transduction with reporter gene to determine the efficacy of gene transduction at the whole-body level.

In this group, we will test systemic muscle and internal organ transduction efficiency and safety of different AAV vectors (such as different serotypes, different promoters, different synthetic and/or engineered capsids, and different configurations including single, dual or triple vectors).

Study timeline: up to 12 years

Gender: Mix

Genotype: Normal, Carrier and Affected

Age group: (pre-weanling 1 wk to < 2 mo, juvenile 2 mo to < 6 mo, young adult 6 mo to < 12 mo and adult >12 mo).

Experiment details:

1. The AAV vector will be delivered via intravenous infusion. The details of the systemic injection method are described in 'Non-surgical Procedures' sections of this ACUC protocol.

2. Dog will be monitored continuously during infusion, evaluated closely for signs of anaphylaxis. If adverse reactions are noted, a veterinarian will be consulted.

3. Following systemic injection, dog will be monitored for any adverse reaction following injection, which includes monitoring them for up to one hour for immediate reaction, and then again for similar adverse effects approximately twice daily for the first two days. If adverse effects are noted, a veterinarian will be consulted.

4. AAV transfection efficiency will be determined by expression level of the reporter gene. To monitor the reporter gene expression, muscle samples will be obtained via periodic muscle needle biopsy or open biopsy (see below for details of our muscle biopsy guidelines).

5. Additional experimental and diagnostic procedures may be completed on dogs in this group as needed. These experimental and diagnostic procedures are described in 'Description of Non-Surgical Procedures' section of this protocol.

6. At the end of the study, we may euthanize the animal and complete a full necropsy evaluation.

Immune suppression:

We and other investigators have previously shown that direct AAV injection to canine muscle may causes cellular immune response. This untoward cellular immune response may be prevented by transient immune suppression.

The details of the immune suppressive method are described in 'Non-surgical Procedures' sections of this ACUC protocol. We will carefully monitor dogs for their wellbeing and general condition during transient immune suppression. In the case of cyclosporine, we will also carefully monitor the blood level of cyclosporine and blood profile.

Group 3. Local injection with a therapeutic AAV vector.

The objective is to test therapeutic efficacy and safety of a therapeutic AAV vector by local muscle injection, an essential step before infusing the AAV therapy gene systemically. The AAV vectors used in this group are selected based on the information obtained from Group 1 study. We only select vectors with highest transfection efficiency to carry the therapeutic genes.

The therapeutic AAV vectors includes:
Notes: The therapeutic AAV vector may derived from different AAV serotype, contain different promoters and have different conditions (such as single, but not limited to dual or triple vectors).

1. Vectors that express different isoforms of dystrophin genes (such as, but not limited to: microgene, minigene and full-length dystrophin gene).

2. Vectors that express different disease modifying genes (such as, but not limited to: utrophin, nNOS, etc).

3. Vectors that express proteins involved in the pathogenesis of muscular dystrophy (such as, but not limited to: SERCA gene, catalase etc).

4. Vectors that can be used for genome editing to test gene repair therapy with the CRSPR technology, and /

or other newly developed gene editing technologies (these vectors may express Cas9 and/or gRNA).

Study timeline: up to 12 years

Gender: Mix

Genotype: Normal, Carrier, and Affected

Age group: (Newborn to 1 wk, pre-weanling 1 wk to < 2 mo, juvenile 2 mo to < 6 mo, young adult 6 mo to < 12 mo and adult >12 mo).

Experiment details:

1. Skin tattoo may be used to mark injection site before injection.

2. Local injection can be performed while the animal is sedated or conscious (Sedation method is explained below in the 'Non-surgical Procedures' section).

3. Injection may include injection to a single muscle or multiple muscles.

4. We will choose the muscle that can be easily accessed with or without ultrasound and cause minimal discomfort to the animals. (Muscles that can be used in local injection are explained below)

5. Following local injection, dog will be monitored for any adverse reaction following injection, which includes monitoring them for up to one hour for immediate reaction (swelling, redness, gait abnormalities, etc.), and then again for similar adverse effects approximately 24 hours later. If adverse effects are noted, a veterinarian will be consulted.

6. AAV transfection efficiency will be determined by expression level of the reporter gene. To monitor the reporter gene expression, muscle samples will be obtained via periodic muscle needle biopsy or open biopsy (see below for details of our muscle biopsy guidelines).

7. Additional experimental and diagnostic procedures may be completed on dogs in this group as needed. These experimental and diagnostic procedures are described in 'Description of Non-Surgical Procedures' section of this protocol.

8. At the end of the study, we may euthanize the animal and complete a full necropsy evaluation.

Immune suppression:

We and other investigators have previously shown that direct AAV injection to canine muscle may causes cellular immune response. This untoward cellular immune response may be prevented by transient immune suppression.

The details of the immune suppressive method are described in 'Non-surgical Procedures' sections of this ACUC protocol. We will carefully monitor dogs for their wellbeing and general condition during transient immune suppression. In the case of cyclosporine, we will also carefully monitor the blood level of cyclosporine and blood profile.

Group 4. Systemic injection with a therapeutic AAV vector.

In this group, we will study the therapeutic efficacy and safety of whole-body gene therapy via systemic

delivery of the therapeutic AAV vector.

The therapeutic AAV vectors include:
Notes: The therapeutic AAV vector may derived from different AAV serotype, contain different promoters and have different conditions (such as single, but not limited to dual or triple vectors).

1. Vectors that express different isoforms of dystrophin genes (such as, but not limited to: microgene, minigene and full-length gene)

2. Vectors that express different disease modifying genes (such as, but not limited to: utrophin, nNOS, etc.)

3. Vectors that express proteins involved in the pathogenesis of muscular dystrophy (such as, but not limited to: SERCA gene, catalase etc.)

4. Vectors that can be used for genome editing to test gene repair therapy with the CRISPR technology, and/or other newly developed gene editing technologies (these vectors may express Cas9 and/or gRNA).

Study timeline: up to 12 years

Gender: Mix

Genotype: Normal, Carrier, and Affected

Age group: (pre-weanling 1 wk to < 2 mo, juvenile 2 mo to < 6 mo, young adult 6 mo to < 12 mo and adult >12 mo).

Experiment details:

1. The AAV vector will be delivered via intravenous infusion with or without an infusion pump. This will depend on the animal age and volume to be injected. The details of the systemic injection method are described in 'Non-surgical Procedures' sections of this ACUC protocol.

2. Dog will be monitored continuously during infusion, evaluated closely for signs of anaphylaxis. If adverse reactions are noted, a veterinarian will be consulted.

3. Following systemic injection, dog will be monitored for any adverse reaction following injection, which includes monitoring them for up to one hour for immediate reaction, and then again for similar adverse effects twice daily for the first two days. If adverse effects are noted, a veterinarian will be consulted.

4. AAV transfection efficiency will be determined by expression level of the reporter gene. To monitor the reporter gene expression, muscle samples will be obtained via periodic muscle needle biopsy or open biopsy (see below for details of our muscle biopsy guidelines).

5. Additional experimental and diagnostic procedures may be completed on dogs in this group as needed. These experimental and diagnostic procedures are described in 'Description of Non-Surgical Procedures' section of this protocol.

6. At the end of the study, we may euthanize the animal and complete a full necropsy evaluation.

Immune suppression:

We and other investigators have previously shown that direct AAV injection to canine muscle may causes cellular immune response. This untoward cellular immune response may be prevented by transient immune

suppression.

The details of the immune suppressive method are described in Non-surgical procedure sections of this ACUC protocol. We will carefully monitor dogs for their wellbeing and general condition during transient immune suppression. In the case of cyclosporine, we will also carefully monitor the blood level of cyclosporine and blood profile.

Group 5. Controls.

In this group, we will collect the baseline data for normal, affected and carrier dogs, and use this data to compare with dogs receiving gene therapy treatment.

Study timeline: up to 12 years

Gender: Mix

Genotype: Normal, Carrier, and Affected

Age group: (pre-weanling 1 wk to < 2 mo, juvenile 2 mo to < 6 mo, young adult 6 mo to < 12 mo and adult >12 mo).

Experiment details:

1. Dogs will not receive AAV vector injections, but may undergo injections of vehicle (excipient), either locally or systemically.

2. Dogs may undergo transient immune suppression. The details of the immune suppressive method are described in 'Non-surgical Procedures' sections of this ACUC protocol. We will carefully monitor dogs for their wellbeing and general condition during transient immune suppression. In the case of cyclosporine, we will also carefully monitor the blood level of cyclosporine and blood profile.

3. We may take muscle biopsy. Muscle samples will be obtained via periodic muscle needle biopsy or open biopsy (see below for details of our muscle biopsy guidelines).

4. Additional experimental and diagnostic procedures may be completed on dogs in this group as needed. These experimental and diagnostic procedures are described in 'Description of Non-Surgical Procedures' section of this protocol.

5. At the end of the study, we may euthanize the animal and complete a full necropsy evaluation.

Group 6. Impella Supported Stop-Flow Method of Viral Delivery to the Heart.

In this group we will inject the AAV virus directly in the heart using balloon occlusion to saturate the lumen of the cardiac vessels directly.

Study timeline: up to 6 months post-procedure

Gender: Mix

Genotype: Normal, Carrier, and Affected

Age group: over 12 months old.

Experiment details:

A.) Rationale: Duchenne muscular dystrophy (DMD) patients often exhibit cardiac damage as the disease progresses. Heart failure is a primary cause of mortality. The adeno-associated virus (AAV) vector is currently the best gene therapy vector for DMD. A strategy that can efficiently deliver the AAV vector to the heart will pave the way to treat DMD cardiomyopathy by gene therapy. The Ishikawa lab has focused on cardiac gene therapy in large animal models for many years (Sahoo et al., 2021). Recently, the Ishikawa lab developed a procedure called the Impella-supported stop-flow method to deliver AAV to the heart (Mavropoulos et al., 2022). In the pig model of heart infarct, the Ishikawa lab found that this procedure can increase myocardial AAV transduction efficiency by 100 to 1000-fold without increasing risks compared with the conventional methods (Ishikawa lab, unpublished). We plan to establish the success of direct cardiac AAV delivery in dogs utilizing a stop-flow technique with Impella pump support.

B.) Equipment: 1. Sterile surgical drapes, gowns, gloves, bowls, towels, gauze, scalpel, syringes, catheters 2. Echocardiograph/Electrocardiograph machine (Veterinary Teaching Hospital) 3. LOGIQ e ultrasound machine (GE Healthcare) 4. 18 ga 2.75in Angiography introducer needle 5. 0.014” coronary guidewire. 6. Over-the-wire balloon dilator catheter (CS), Balloon wedge catheter 5Fr Swan-Ganz (CS), 5-7Fr introducer sheath + dilator (femoral artery), 6-7Fr deflectable sheath + dilator (jugular vein) 7. Fluoroscopy at NextGen: Artis Q Fluoroscopy with Volcano IVUS (performed by interventional cardiologist and/or Jan Ivey) 8. 3 Infusion pumps: Propofol, Saline, Nitroglycerin 9. DRE Bonair Electrical Ventilator with 800-3000ml Bellows (Avante Health Solutions) 10. 20-gauge butterfly catheter 11. Heating pad 12. Activated Clotting Time point of care testing (iStat) 13. Impella pump with Automated Impella Controller: 10Fr introducer and dilator sheath, 0.018” guidewire, 0.035” guidewire, 9Fr Impella pump, Purge Cassette

C.) Drugs: 1. Angiogram contrast agent: Iopamidol (Isovue-370) or Iodixanol (Visipaque) approximately 20-50ml as needed for catheter placement. 2. Acepromazine: 0.02mg/kg 3. Butorphanol: 0.4mg/kg 4. Propofol: 6mg/kg induction; 0.1-0.4mg/kg/min (maximal dosage of 1.2mg/kg/min) CRI for maintenance 5. Heparin: 100-300U/kg; hourly maintenance of 100U/kg 6. Nitroglycerin: 1mcg/kg/min infused for 15 mins prior to vector injection, continues for duration of injection, end after 10 mins elapsed from injection completion. 7. Protamine: 1 mg/100U of heparin to be inactivated, Dose decreased by 50% for every 30-60mins lapsed since heparin administered, given slowly. 8. Carprofen: 4.4mg/kg SQ after procedure, continue for up to 3 days following procedure. 9. Antibiotics (+/- cut down): veterinary recommendation 10. 70% alcohol and Chlorhexidine 11. 0.9% NaCl: IV fluids 10ml/kg/hr 12. 5% Dextrose/water solution: used for Impella cartridge 13. Atropine: 0.02-0.04 mg/kg for cardiac emergency 14. Epinephrine: 0.05-0.5 mg for cardiac emergency 15. Calcium gluconate: 1-1.5ml/kg slowly for cardiac emergency 16. Lidocaine: 2-8mg/kg for cardiac emergency

D.) Personnel involved: 1. Arun Kumar: Interventional cardiologist; placing and guiding catheters, guidewires, Impella pump and balloon occlusions. 2. Jan Ivey: NextGen equipment operation 3. Zhenguo Liu: Director Division of Cardiovascular Medicine (co-investigator) 4. Matt Burke: computer and supply set-up 5. Yongping Yue: surgical cut-down and closure; assistance with procedure as needed 6. James Teixeira (co-investigator): anesthesia; surgical cut-down and closure; assistance with procedure as needed 7. Dongsheng Duan (co-investigator) 8. OAR Veterinary Staff/Surgeon: surgical cut-down and closure; assistance with procedure as needed.

E.) Pre-Procedure: Patients will undergo cardiac evaluations including: Holter Electrocardiogram (performed by the Duan lab), Electrocardiogram and Echocardiogram (performed at the Veterinary Teaching Hospital) before the procedure to establish baseline information about the patient. Cardiac rhythm and size will be closely evaluated and consulted upon with the University of Missouri Veterinary Teaching Hospital Cardiology department. Full physical examination and bloodwork (complete blood count and serum maxi), including cardiac biomarkers (cardiac troponin and NT-proBNP), will be performed to establish a starting baseline and

overall health of the patient. Veterinary advice will be consulted if values are significantly abnormal for the DMD model. Blood will be collected in accordance with the ACUC Standard Operating Procedure (blood collected over a period of 3 weeks will not exceed 8.6ml/kg). Patient size and body weight will be monitored to ensure the patient is an appropriate size for the equipment used. The patient will be fasted overnight with free access to water prior to the procedure. The patient may received immune suppression in accordance to our approved protocols beginning 3 days prior to the procedure date and continuing for the duration of study.

F.) Procedure: The patient will have blood drawn to test complete blood count, serum maxi panel cardiac troponin and NT-proBNP the day of the procedure. They will then be premedicated using our approved pre-anesthesia protocol of Acepromazine (0.02mg/kg) and Butorphanol (0.4mg/kg) via intramuscular injection. An intravenous catheter will be placed in the cephalic vein to administer the induction dose of propofol (6mg/kg) and to maintain anesthesia using a constant rate infusion of propofol at a dosage of 0.1-0.4mg/kg/min (up to a maximal dosage of 1.2mg/kg/min). The contralateral cephalic vein will be catheterized for administration of the nitroglycerin via a syringe pump. A third catheter will be placed in one of the lateral saphenous veins for 0.9% NaCl fluid therapy. Nitroglycerin and fluid therapy will both be administered using syringe pumps. The patient will be intubated and mechanically ventilated with medical oxygen throughout the procedure. The patient will be placed in dorsal recumbency with hindlimbs secured to allow for femoral artery access and preparation. Vascular access locations will be shaved and pre-scrubbed using 70% alcohol and chlorhexidine following a surgical scrub pattern. The area will then again be scrubbed using sterile scrub technique using the same agents and pattern with sterile gloves. Percutaneous access locations may include the left and/or right femoral artery, the jugular vein, and the left common carotid artery (Holmberg and Pettifer, 1997;Goodman and Goodman, 2016;Komornik et al., 2020;Mavropoulos et al., 2022). Peripheral arteries will be accessed using the Seldinger method where a hollow needle is inserted then a guidewire is advanced through the needle. The left common carotid artery will be assessed via intravascular ultrasound and accessed either using the Seldinger method or a cut-down approach to easily visualize and cannulate the artery (Holmberg and Pettifer, 1997;Goodman and Goodman, 2016;Komornik et al., 2020). This decision will be made by the interventional cardiologist and/or surgeon. The arteries will be punctured using the appropriately sized introducer needle as determined by the interventional cardiologist. Once the needle is withdrawn, a sheath is slid over the guidewire. The patient will then receive heparin at a dosage of 300U/kg IV and nitroglycerin at a dosage of 1 mcg/kg/min. The nitroglycerin will begin 15 minutes before the start of the AAV vector injection and continue until 10 minutes past the completion of the AAV vector injection. The heparin will be administered at a rate of 100U/kg/hr to achieve an activated clotting time of 250-300 seconds for Impella insertion. After insertion, the ACT will be maintained at 160-180 seconds. Activated clotting time will be monitored using an iStat point of care device. A 10Fr introducer/dilator is placed in the left common carotid artery for 9Fr Impella delivery. Using the 0.018” guide wire, the Impella is placed in the left ventricle and positioned using fluoroscopy. The Impella pump is then activated to support the blood flow out of the left ventricle, maintaining hemodynamics to the rest of the body during the procedure. The Impella is a device implanted in the left ventricle temporarily to maintain blood flow out of the heart during cardiac procedures. Using the pump, systemic circulation is maintained consistent while the cardiac blood flow is temporarily altered for viral injection (https://www.abiomed.com/products-and-services/impella/impella-25) (Glazier and Kaki, 2019). The Automated Impella Controller controls the flow of blood through the device’s pump (https://www.abiomed.com/products-and-services/impella/impella-25). This device is used in human medicine for acute myocardial infarcts, high risk coronary angioplasty, and off-pump coronary bypass (Glazier and Kaki, 2019). Studies suggest a clear benefit to the use of an Impella device during coronary procedures. The jugular access will be used to cannulate the coronary sinus and advance an occlusion balloon into the great cardiac vein. The occlusion balloon is then inflated to confirm proper placement. The femoral artery is used to advance an angioplasty balloon into the coronary artery. An angiogram is performed, and the balloon is placed in the proximal aspect of the left anterior descending artery (LAD). The coronary balloon is then inflated 3 times for 15 seconds each to precondition the heart for ischemia. The purpose of the preconditioning is to prepare the heart for the ischemic conditions during vascular occlusion. This should not cause cardiac damage and will minimize the adverse effects possible during the viral injection. Kiyotake Ishikawa has reported this technique minimizes the incidence of ventricular infarcts and arrhythmias in pig

subjects when Impella device is used. After preconditioning, the coronary sinus balloon is then inflated followed immediately by the coronary artery balloon for the duration of AAV vector injection, approximately 1 minute. This is repeated 3 times with the balloon being deflated between each injection. After three injections in the LAD, the coronary artery balloon is repositioned into the circumflex artery. The same preconditioning, inflation and injection procedure is followed. Upon the completion of the injection, protamine will be administered slowly, intravenously following the recommended antidote dosage. Catheters are removed following completion of the viral injection. Hemostasis is achieved using manual pressure to the insertion site for several minutes in conjunction with the protamine. If necessary, sutures will be used to close the vessels to reduce bleeding before manual pressure. This will be under the guidance of the cardiologist(s) and/or surgeon present. The Impella device is weaned down over 15 minutes to prevent acute decompensation. The Impella device is removed, and hemostasis is achieved in a similar manner. The left common carotid artery may also be ligated to achieve hemostasis. Based on literary review and veterinary consultation (email with Dr. Jim Lattimer and Dr. Stacey Leach), this has been done successfully in canines with minimal observable adverse effects (Holmberg and Pettifer, 1997;Goodman and Goodman, 2016;Komornik et al., 2020). If the left common carotid artery was accessed using a cut-down method, the surgical site will be sutured closed.

G.) Monitoring and Post-operative care: Throughout the procedure the dog will be monitored using the monitoring devices used for all surgical procedures. Heart rate, rhythm and electrical activity will be closely monitored prior to, throughout the experiment and following the procedure. Echocardiography and electrocardiography completed by the University of Missouri Veterinary Teaching Hospital Cardiology department will be performed and reviewed prior to the experiment date and prior to the termination date. During the procedure, the rate, rhythm, and peripheral blood pressure will be monitored and documented every 15 minutes. Immediately post-operatively, a Holter ECG will be placed on the dog for continuous monitoring for the first 24 hours. In the event of a cardiovascular incident, occlusion balloons will be deflated and the use of approved emergency drugs may be utilized in accordance with our approved dosages and routes. Emergency drug decisions will be made based on the situation and the opinions of the interventional cardiologist and/or veterinary consult. Bloodwork (CBC, Maxi, cTn1, NT-proBNP) will be collected the day following the procedure and periodically throughout the study duration. Pain and discomfort will be assessed according to the same guidelines used for muscle biopsies. Carprofen will be administered immediately post-operatively and use of analgesics will be used based on veterinary guidance. Dogs will be closely monitored for at least 7 days following the procedure for signs of pain, cardiac abnormalities, and neurologic abnormalities. Veterinary staff will be consulted if any concerns arise during or following the procedure. The dog will be evaluated for changes to the cardiac function and anatomy using non-invasive approaches (ECG/Echo/Blood work). Dogs will be kept alive up to 6 months after the procedure to evaluate changes to the cardiac parameters.

H.) Experts consulted during the protocol development Dr. Kiyotake Ishikawa: an Associate Professor at the Icahn School of Medicine at Mount Sinai. Dr. Ishikawa has successfully conducted Impella-based “stop-flow” AAV delivery to the porcine heart. Dr. Keita Saku: an investigator in the National Cerebral and Cardiovascular Center Research Institute, Suita, Japan. Dr. Saku has extensive experience using Impella in dogs. Dr. Esther Kim: a Scientist of Academic Research at Abiomed. Dr. Jerald Curran: the Associate Director of Academic Research at Abiomed Dr. Stacey Leach: an Associate Professor at the University of Missouri College of Veterinary Medical School. Dr. Leach is a board-certified veterinary physician specializing in canine cardiology. Dr. Jim Lattimer: a Professor at the University of Missouri College of Veterinary Medical School. Dr. Lattimer is a board-certified veterinary physician specializing in veterinary radiology. Dr. Arun Kumar: an Associate Professor in clinical medicine at the University of Missouri. Dr. Kumar is certified by the American Board of Internal Medicine in cardiovascular disease and interventional cardiology. Dr. Kumar has used Impella in human patients for many years. Dr. Zhenguo Liu: a Professor in clinical medicine at the University of Missouri. Dr. Liu is the Division Director of the Cardiovascular Medicine. Dr. Kumar is certified by the American Board of Internal Medicine in cardiovascular disease and clinical cardiac electrophysiology.

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Group 7. Subcutaneous Injection of Antisense oligonucleotides (ASO) weekly in affected dogs.

In this group we will inject the antisense oligonucleotides subcutaneously weekly to evaluate therapeutic efficiency.

Study timeline: 12 months of age

Gender: Mix

Genotype: Affected

Age group: Initial injection in dogs ≥1 month with end point based on the results of the treatment.

Experiment Details:

MicroRNA miR-128-1 has been shown to be elevated in plasma and muscle of DMD animal models and humans. Antisense oligonucleotide mediated miR-128-1 inhibition reduces the dystrophic phenotype in murine and zebrafish models. miR-128-1 is a master regulator that acts to promote energy storage by repressing the energy expenditure genes. It has been established that mitochondria dysfunction contributes to the DMD model. Interestingly, it has been shown that miR-128-1 expression is significantly increased in the muscle of the mouse and zebrafish DMD models as well as in human patients. Plasma concentrations are also elevated in the murine and canine models for DMD. Reduction in miR-128-1 has been shown to promote cardiomyocyte proliferation and regeneration while preventing ischemia-induced heart failure. Antisense oligonucleotide mediated miR-128-1 inhibition is achieved using weekly subcutaneous injections. Using the murine model of DMD, weekly injections significantly: improved mitochondrial function, reduced muscle fibrosis and serum creatine kinase, and decreased skeletal muscle abnormalities. Given these benefits, we propose testing this same delivery in the canine DMD model.

Beginning at ≥1 month of age, we will inject ASO subcutaneously in the scruff (between the scapulae). Injections will be once per week at a dosage range of 0.5-20 mg/kg. Given this dosage and frequency, there should be no concern with volume to be administered. The injection may be administered using a needle and syringe or a winged infusion set. The area will be clipped and cleaned with 70% isopropyl alcohol before puncture. Puncture locations will be rotated from slightly cranial to slightly caudal between the scapulae to minimize damage. The injection volume will be warmed to room temperature before administration and given over a period of time based on volume to be injected. The weekly injections may continue until approximately 12 months of age at which time the animal may be terminated, or treatment monitored for the remainder of the animal's life.

Several ASO drugs have been approved by the FDA for treatment of DMD, thus they have been extensively

tested in animal models and human patients. Of relevance to our study, ASO therapy has been successfully used in the canine model to establish exon-skipping therapy. Blood will be collected weekly during the first month, biweekly during the second month and then monthly until termination. In addition to the standard complete blood count, serum maxi panel and cardiac troponin, we will also measure NT-proBNP. Focus will be applied to the AST, ALT, serum CK, GLDH, GGT, cardiac troponin and NT-proBNP values to assess treatment and safety. Blood will be collected in accordance with the ACUC Standard Operating Procedure (blood collected over a period of 3 weeks will not exceed 6ml/kg in puppies and 8.6ml/kg for weaned dogs). Body weight and overall health will be documented before each injection. After each injection, the dog will be monitored for at least 10 minutes to ensure there is not adverse reaction. Veterinary staff will be consulted if any adverse reactions occur or if there are any concerns.

Local muscle injection guidelines:

The following guidelines apply to group 1, 3 and 5.

1. Up to a maximum of 10 muscles/muscle groups per side may be injected.

2. Muscles/muscles groups which may be injected percutaneously (with or without ultrasound guidance) includes:

A. Hindlimb:
a. Gastrocnemius
b. Cranial tibialis
c. Extensor digitorum longus
d. Biceps femoris
e. Semitendinosus
f. Semimembranosus
g. Vastus Lateralis
h. Rectus femoris
i. Cranial and Caudal Sartorius
j. Gracilis
B. Shoulder/Upper forelimb
a. Trapezius
b. Supraspinatus
c. Infraspinatus
d. Deltoid
e. Lateral head of triceps
C. Lower forelimb
a. Extensor group
b. Flexor group

Muscle Biopsy Guidelines:

The following guidelines apply to all experimental groups.

1. The first biopsy will be 1 week to 1 month post injection of AAV vectors/vehicle.

2. Biopsy procedures will be separated by at least a 1-week time period, alternating sides for each subsequent biopsy.

3. Biopsy of a single muscle (i.e., left Biceps femoris) will be separated by at least a 2-week time period.

4. Each biopsy will be limited to 2 muscles, with 1 skin incision per muscle.

5. For needle biopsies, 1-5 samples may be obtained from each site.

6. For open biopsies, a single sample will be taken, and open biopsy procedures will be limited to 6 times per dog over its lifetime.

7. Dogs less than 1 year of age may have up to 6 biopsies during that year. Dogs over 1 year of age may have up to 8 biopsies in a single year.

8. The maximal number of biopsies a single animal will undergo is limited to 18 biopsy events in a lifetime.

9. Number of times a single muscle/muscle group will be biopsied over a lifetime and whether or not it is accessible via needle or open biopsy is based on multiple parameters, including location, size, and proximity to vascular/neural components.

Muscles/muscle groups which may be used for biopsy includes:

A. Hindlimb:
a. Gastrocnemius – once (needle or open)
b. Cranial tibialis – once (needle or open)
c. Biceps femoris – eight (needle or open)
d. Semitendinosus – six (needle or open)
e. Semimembranosus – eight (needle)
f. Vastus Lateralis – four (needle)
g. Rectus femoris – two (needle)
h. Cranial and Caudal Sartorius – two (open)
i. Gracilis – two (needle or open)
B. Shoulder/Upper forelimb:
a. Trapezius – once (open)
b. Supraspinatus – once (needle)
c. Infraspinatus – once (needle)
d. Deltoid – once (needle)
e. Lateral head of triceps – two (needle)
C. Additional muscle groups that may be biopsied in systemically injected animals include:
a. Adductor - once (open biopsy)

Group 8. Oral administration of SAT-3153 daily in affected dogs to evaluate muscle function recovery.

In this group we will administer the drug SAT-3153 daily to 2 affected dogs and evaluate muscle function recovery. If the pilot study shows improvement, then we will consider a larger study using the drug.

Study timeline: approximately 12 months of age

Sample size: n=2

Genotype: Affected

Age group: Initial injection in dogs ~ 6 month with end point around 6 months post-initiation of treatment

Rationale:

Failure to regenerate is a major disease mechanism in Duchenne muscular dystrophy (DMD). Muscle regeneration is accomplished by the asymmetric division of muscle stem cells. In asymmetric division, one progeny cell retains the stem cell property while the other is committed to the progenitor cells that give rise to regenerated muscle. The asymmetric division depends on the polarized expression of dystrophin in muscle stem cells. The absence of dystrophin promotes symmetric division which yields two progeny cells with the stem cell property. As a result, the committed progenitor cells are not produced in the DMD muscle. The lack of committed progenitor cells underlies regeneration failure in DMD. Strategies that promote asymmetric muscle stem cell division would enhance regeneration and prevent muscle wasting in DMD.

SAT-3153 is an experimental drug that was found to promote asymmetric division in the mdx mouse model. Intraperitoneal administration of SAT-3153 at the dose of 10 mg/kg significantly enhanced muscle force. SAT-3153 has also been tested in normal dogs at a dosage of 10mg/kg PO without causing side effects. It is worth mentioning that an experimental drug that targets the same signaling pathway is currently in phase 3 clinical trials for treating other diseases. No toxicity concerns were raised for the clinical trial drug. Based on the above, we expect SAT-3153 to be safe in affected dogs

In this amendment, we would like to test the therapeutic potential of SAT-3153 in the canine DMD model. We will first perform a pilot study. If the pilot study yields positive results, we will amend the protocol for a full-scale study (n=6 dogs).

Experiment Details:
Daily oral administration of SAT-3153 at the dose of 10mg/kg for 6 months.
Weekly blood biochemistry and hematology in the first month and monthly thereafter.
Needle biopsy of BF muscle prior to drug administration
Needle biopsy of BF and/or Semimembranosus muscles bi-monthly or monthly
Overnight activity at the beginning (before dosing), 3 and 6 months after dosing.
Non-invasive hindlimb and forelimb force at the beginning (before dosing), 3 and 6 months after dosing.
Gait analysis at the beginning (before dosing), 3 and 6 months after dosing.
Terminal ECU force assay at 6 months (the end of therapy)
Full necropsy approximately 6 months at the end of the therapy.

Safety Assessment:
If blood values begin to change outside of the expected values for affected dogs, we will consult with veterinary staff to assess the health of the dog. During daily administration of the treatment the dogs will be evaluated for adverse effects and/or any changes. We will follow our approved guidelines for muscle biopsies and blood collection. In the event vomiting occurs during drug administration we may give Maropitant SQ and reassess for further vomiting. If vomiting continues, we will consult with veterinary staff and decide if the treatment route needs to be altered.

Amendment 10/24/2023: Change from SAT-3153 to SAT-3247.
After further discussion with the company, we had decided to utilize SAT-3247 for the purposes of the canine. This compound has the same mechanism of action and safety as SAT-3153 but better bioavailability and pharmacokinetics. All other approved aspects of the study will remain unchanged.

Group 9. A pilot study to evaluate the safety of systemic AAV delivery in adult dogs.

We have previously achieved successful systemic AAV delivery in neonatal and ≤ 3-month-old normal and affected dogs. However, gene delivery at this age will not treat older patients. Hence, we have included dogs that are ≥ 12 months in groups 2 and 4 of our approved protocol. Recently, a 27-year-old DMD patient was treated with systemic AAV CRSIRP therapy (Lek et al 2023 NEJM https://pubmed.ncbi.nlm.nih.gov/37754285/). Unlike our previous systemic AAV CRISPR study in 1-m-old affected dogs (Hakim et al 2021 NatComm https://pubmed.ncbi.nlm.nih.gov/34819506/), the CRISPR-treated human patient developed respiratory acidosis on day 3 after dosing, pericardial effusion on day 5 after dosing, and characteristic features of acute respiratory

distress syndrome (ARDS) on day 6 after dosing. The patient died on day 8 from multi-organ failure. In order for AAV DMD gene therapies to move forward safely in older and more advanced DMD patients, it will be critically important to determine why this one DMD patient experienced this toxicity and which characteristics of the vector or of the host may have triggered this.

Although we have been previously approved to test systemic AAV gene delivery in older dogs (≥ 12 months), given the findings in the human patient, we would like to amend our protocol to include more comprehensive pre- and post-dosing monitoring and evaluation. The goal of this study is to (1) replicate observations in the human patient and (2) identify the mechanisms underlying the acute toxicity seen in the human patient. We would also like to mention that our study will be performed in close collaboration with Dr. Terry Flotte (University of Massachusetts, Medical School), who directed the human study.

Study timeline: To study acute toxicity, we currently plan to conclude the study 10 days after injection or when animal quality of life and welfare is compromised. However, based on the data collected during the first 10 days, we may extend the study to 90 days or longer.

Gender: Mixed

Genotype: Normal, Carrier, and Affected

Age group: >12mo

Sample size: We will first use 1 affected dog to evaluate the reaction. Based on the results and observations, we will inject 2 to 3 more affected dogs at a later time with appropriate modifications. We will also include 1 to 2 normal dogs and 1 to 2 retired carrier dogs in the study to better understand the contribution of muscle disease to acute toxicity.

AAV vector: We will start with the same vector used in the human patient. To test whether the acute toxicity in the human patient is due to the transgene, we will also include a reporter gene AAV vector.

Experiment details:

1. Pre-injection evaluation: Dogs will undergo cardiac evaluation (ECG and echocardiography) at the Veterinary Hospital, radiographic evaluation of chest and abdomen, and have bloodwork submitted twice prior to injection. Bloodwork consisting of CBC w/ manual differential and serum biochemistry. We will pay special attention to cardiac biomarkers (cardiac troponin and NT-proBNP), liver function biomarkers (ALT, AST, ALP, GGT, GLDH, bilirubin), and kidney function biomarkers (creatinine and BUN). Since systemic AAV delivery in human patients has resulted in platelet drop and complement activation, we will also pay attention to the platelet count and complement activation will be measured by the serum C5b-9 complex level. Other immunological assays may include anti-AAV and anti-Cas9 antibodies, AAV and Cas9 interferon-gamma ELISpot, and serum cytokines. A full physical exam will be conducted and recorded prior to injection. Physical exam parameters that will be recorded are temperature, heart rate/rhythm/sound, respiratory rate/rhythm/sound, weight, CRT and mucous membrane color, general overall mentation, and BCS.

2. The injection will be performed under transient immune suppression as described in the 'Non-surgical Procedures' sections of this ACUC protocol. We will carefully monitor dogs for their wellbeing and general condition during transient immune suppression.

3. The AAV vector (up to 5e14 vg/kg) will be delivered via intravenous infusion with or without an infusion pump (1 mL/min). This will depend on the animal age and volume to be injected. The details of the systemic injection method are described in 'Non-surgical Procedures' sections of this ACUC protocol.

4. Dogs will be monitored continuously during infusion, evaluated closely for signs of anaphylaxis or adverse

reactions. If adverse reactions are noted, a veterinarian will be consulted.

5. Following injection, dogs will be monitored every hour for the first 6 hours. A full physical exam will be conducted every 1 to 2 hours and recorded. After the first 6 hours, blood will again be submitted to the Veterinary Hospital to identify changes in values.

6. Dogs will then be monitored (physical exam) every 4 to 8 hours in the first week of the study period. In the second week, the dog will be carefully monitored for eating, drinking, activity, body weight, body temperature, and other vital signs daily.

7. If during the study period the dogs present severe adverse effects, we will seek veterinary advice and/or complete emergency imaging (radiographs, ECG/ECHO) as needed.

8. At the end of the study or when deemed medically necessary the dogs will be euthanized and a full necropsy completed.

Safety Assessment:

The goal of this study is to assess safety and understand the mechanisms by which adverse effects occur. Prior to beginning the study, the dogs will undergo cardiac evaluation and radiographic evaluation including electrocardiogram, echocardiogram, and radiographic imaging of the thorax and abdomen. These evaluations will be used to confirm there are no abnormalities outside of the DMD model expectations. Bloodwork consisting of complete blood count with manual differential, serum biochemistry, cardiac troponin, NT-proBNP, and PBMC will be collected twice prior to beginning the study to evaluate organ function and cytology status. A full physical exam will be conducted and recorded prior to injection. Physical exam parameters that will be recorded are temperature, heart rate/rhythm/sound, respiratory rate/rhythm/sound, weight, CRT and mucous membrane color, general overall mentation and body condition score (BCS). *The radiographs would initially be evaluated by the OAR veterinarian. If something is noted or questionable then we would refer to radiology at the VHC (Dr. Jimmie Lattimer).

After beginning the study, dogs will be closely monitored every 1 to 2 hours for the first 6 hours post-injection. A full physical exam will be conducted every 1 to 2 hours and recorded. Blood will be resubmitted 6 hours post-injection to evaluate immediate changes to values. Beyond the first 6 hours, dogs will be examined every 4 to 8 hours for the 1st week of the study. If severe adverse events occur, monitoring frequency may be increased according to veterinary advice and/or James's discretion. Cardiac and radiograph evaluations may be conducted during the study period to identify progression of adverse effects or on an emergency basis.

To study acute toxicity, we currently plan to conclude the study 10 days after injection or when animal quality of life and welfare is compromised. However, based on the data collected during the first 10 days, we may extend the study to 90 days or longer. All blood collection volume, frequency, and technique will follow our ACUC approved values and procedures.

4. Justify

1. Justify Use of Animals in your Research

Justify the use of animals for your experimental goals. **DO NOT** describe details of the experimental design or justify animal numbers here.

Duchenne muscular dystrophy (DMD) is the most devastating inherited muscle disease affecting 1:5000 live male births worldwide. Dystrophin, the deficient protein in DMD, is an important structural protein in muscle

cells. It stabilizes muscle cell membrane and protects the membrane from contraction-induced damage. In the absence of dystrophin, patients become progressively weaker due to muscle degeneration, inflammation and wasting. Affected boys end up in a wheelchair by the age of 10 to 12-years-old. Gene therapy has the potential to restore dystrophin expression and/or ameliorate pathogenic process. Gene therapy has been extensively tested in rodent models. However, rodent models often do not develop the severe muscle disease as seen in human patients, rodent muscles have inherent properties different from that of larger mammals (for example, rodent muscles have a higher regenerative capacity), further the body size of the rodents is hundreds-fold smaller than human patients. For these reasons, results from rodent models often fail to translate to human patients during clinical trials. On the other side, the canine model does not have these drawbacks. The ultimate goal of our studies is to thoroughly test AAV gene therapy in the canine model with the hope of eventual application to human patients. The results obtained from in vitro cell culture study and/or computer-based analysis cannot be directly translated to treatment of DMD patients, so a large animal model is needed. There are no alternatives to replace the canine studies proposed in our studies.

2. Justify Animal Species

Justify the choice of species for your study.

Dogs:

Most DMD gene therapy research has been conducted in the mouse model of DMD. In contrast to the mouse model, the dog model has dystrophic clinical symptoms similar to human patients. In addition, the dog model has a body size closer to an affected child compared to the mouse model. Dogs also show a similar immune response as human patients. Thus, the dog model provides us a perfect animal model to bridge between mouse studies and clinical trials. Collectively, studies performed in dogs can better predict what will happen in human patients. We will use the following strains of dogs in our study.

Dystrophin-deficient dogs:

The first reported dystrophin-null dog (1987) is on the golden retriever background with a point mutation in intron 6. This dog is called golden retriever muscular dystrophy dog (GRMD). Several different dystrophin-deficient dogs have been identified since then. Among these are (1) Corgi background dystrophin intron 13 LINE element insertion and (2) Labrador background dystrophin intron 19 LINE element insertion. In both cases, insertion introduces a new exon and stop codon. Dystrophin expression is thus aborted. Importantly, similar mutations have been reported in DMD patients. We currently have dogs with all three mutations. Specifically, affected males carry one of these three mutations and affected females carry one or two of these three mutations. Carrier females carry one of the three mutations. To avoid inbreeding associated problems and also based on the recommendation of Drs. Bruce Smith (Auburn University, an expert in DMD dogs) and Dietrich Volkmann (previously MU, an expert in canine breeding), we have been crossbreeding semen from different breeds to carriers of different breeds. The purpose of the mixed breeding is to prevent untoward genetic consequences of inbreeding (e.g., the lose the fertility, other mutations). For this reason, all our dogs are currently in the mixed genetic background. Since DMD can happen anywhere in the world, we believe that studying mix-breed dogs will be as informative (if not more informative) than studying pure breed dogs.

Please note, we are maintaining a dystrophin-deficient dog colony, not a GRMD colony. In addition to the dystrophic dogs, we will also use normal and carrier dogs in our study to find the most effective AAV gene therapy to treat DMD.

3. Justify Animal Numbers

Justify numbers of animals to be used (attach timeline or flow chart and power analysis, if possible, to describe study groups). This section should include a description of animals used for colony maintenance (breeders and all offspring produced) as well as a description of experimental animal numbers. Total

numbers should match the requested numbers in the species section.

- Animal Numbers Justification
- The Logical Determination of "N" in Animal Experimentation
- Non-Statistical Approach for Calculating the Optimum Number of Animals Needed in Research
- Statistics and the Issue of Animal Numbers in Research
- JUSTIFY ANIMAL NUMBERS EXAMPLE

Based on our records over the past several years (in house generation from breeding and the acquisition of dogs from other sources), we request to have a total of 300 dogs over the next three years. In addition, we currently have 20 dogs in our colony. Thus, the total number of dogs requested in this three-year renewal is 320 (300+20) dogs. These dogs are expected to be a mix of normal, affected and carrier dogs. Please note, this number could vary depending on the success of each breeding, the litter size, and the availability of dogs from other sources. If we are close to this requested number, we will make an amendment accordingly. We also like to point out that a maximum of 50 carrier females out of 320 dogs may be used for breeding to maintain different mutations in our colony. The need for breeding will be managed according to our experimental requirements. If these carrier dogs are no longer needed for breeding, we may use them for experiments.

Explanation of experimental animal number:

Once the dogs are born or acquired from other sources, they will be assigned right away to group 5. Thus, the total number of dogs requested in group 5 is up to 300 dogs (the current 20 dogs in our colony have been already assigned to a group).

According to the outcome of each breeding and needs of ongoing experiments in the laboratory, the principal investigator will later assign each dog accordingly to one of the following groups.

Group 1. Local injection with a reporter gene AAV vector.

We plan to test up to 15 different combinations of AAV serotype and reporter genes using local injection. Ideally, we would like to test up to 15 dogs for each vector combination. Thus, the total number of dogs requested in this group is 225 dogs (15 x 15 = 225 dogs).

Group 2. Systemic injection with a reporter gene AAV vector.

We plan to test up to 15 different combinations of AAV serotype and reporter genes using local injection. Ideally, we would like to test up to 15 dogs for each vector combination. Thus, the total number of dogs requested in this group is 225 dogs (15 x 15 = 225 dogs).

Group 3. Local injection with a therapeutic AAV vector.

We plan to test up to 15 different combinations of AAV serotype and reporter genes using local injection. Ideally, we would like to test up to 15 dogs for each vector combination. Thus, the total number of dogs requested in this group is 225 dogs (15 x 15 = 225 dogs).

Group 4. Systemic injection with a therapeutic AAV vector.

We plan to test up to 15 different combinations of AAV serotype and reporter genes using local injection.

Ideally, we would to like test up to 15 dogs for each vector combination. Thus, the total number of dogs requested in this group is 225 dogs (15 x 15 = 225 dogs).

Group 5. Control Animals

Once the dogs are born or acquired from other sources, they will be assigned right away to group 5. Thus, the total number of dogs requested in group 5 is up to 300 dogs (the current 20 dogs in our colony has been already assigned to a group).

Group 6. Impella Supported Stop-Flow Method of Viral Delivery to the Heart

We plan to test the effectiveness of viral delivery in the heart using a reporter gene in 3 normal and 1 affected dog first. If there is evidence that the virus successfully entered the heart, we plan to test 2 therapeutic viruses. We will use two different AAV vectors each containing 6 affected dogs. Thus, the total number of dogs requested in this group is 16 dogs.

Reporter: 3 normal 1 affected dog

Micro-dys: 6 affected dogs

SERCA: 6 affected dogs

Group 7. Subcutaneous Injection of Antisense oligonucleotides (ASO) weekly in affected dogs.

We plan to test the effectiveness of subcutaneously injected antisense oligonucleotides in 2 affected dogs first. We may use 2 untreated dogs as a control for evaluating effectiveness.

Group 9. A pilot study to evaluate the safety of systemic AAV delivery in adult dogs.

We plan to test the adverse reaction in 1 affected dog first to understand the possible effects. Based on the results from this first dog, we will modify our care and study plan to best maintain quality of life and welfare. We will then, at a later time, inject another 2-3 affected dogs, 1-2 normal, and 1-2 carrier dogs in the entire study.

5. Animal Husbandry

1. Facilities

In which animal facility will animals be housed?

	Facility
1	[redacted]

2. Housing Outside of Facility

Will animals be housed anywhere other than a designated animal housing facility for more than 12 hours (e.g., a laboratory)?

○ Yes ● No

3. Transportation Between Animal Housing/Use Facilities

Will animals be transported with a private vehicle between animal housing/use facilities?

● Yes ○ No

A. Description of Transportation

Describe how animals will be moved, including caging/transport carriers used (covered microisolator cages, dog/cat carrier, etc.), type of transport used (i.e. personal car or van), and projected transport time. (Note: Animals should be transported in a temperature controlled environment.)

Dogs will be transported in a temperature-controlled vehicle primarily by lab personnel vehicle listed in this protocol and occasionally by OAR technician. Normal and carrier animal may be placed in dog carriers for their safety during transportation. Affected animals will be accompanied by a lab personnel included in this protocol and monitored closely during transportation. Affected animals will not be placed in dog carriers.

4. Non-Standard Husbandry

A. Does this protocol contain any Prolonged Physical Restraint?

See: ACUC Physical Restraint policy

○ Yes ● No

B. Does this protocol contain any Food/Fluid Regulation?

See: ACUC Food and Fluid Restriction policy

○ Yes
○ No
● Overnight only

C. Does this protocol contain Multiple Survival Surgical Procedures?

See: ACUC Multiple Survival Surgical Procedures policy

○ Yes ● No

D. Does this protocol contain any of the following Non Standard Husbandry?

- ☑ Single housing of social species
- ☐ Wire-bottom cages
- ☑ Special diet/water
- ☐ Extended time to weaning
- ☐ Extended time between cage changes
- ☐ Alternative light cycles
- ☐ Out of range temperatures
- ☐ Cage-size exceptions
- ☐ Other

i. Explain non-standard husbandry and list the length of time the animal will undergo non-standard husbandry.

Dogs may be singly housed following sedation from muscle biopsies for up to 72 hours to allow for recovery from sedation and to monitor healing. To obtain video recordings overnight a dog may be housed singly for 24 hours while activity is monitored. An acclimation period is required when initially moved into the overnight recording run thus the dog may be housed in the run for up to 24 hours. On occasions where specialty diets or treatments are required per veterinary recommendation, dogs may be housed singly for the duration of treatment.

Animals will be singly housed following muscle biopsies to allow healing, when being video recorded in the dog run for gait analysis, and in cases when special diets are needed as recommended by veterinary staff. Otherwise attempts will be made to pair house dogs based on compatibility.

1. Affected puppies- These pups will be fed moistened food and may be supplemented with canned food as needed.

2. Affected dogs- Affected adult dogs will be fed moistened food and may be supplemented with canned food as needed.

Affected dogs are fed in elevated feeders due to difficulties swallowing and moving food down the esophagus into the stomach.

6. Description of Non-Surgical Procedures

1. Sample Collection

Will samples, such as blood or tissues, be collected from live animals? (Include sampling for genotyping.)

● Yes ○ No

A. Sample Type

Type of sample(s):

Umbilical cord, blood, muscle tissues, semen, vaginal swab, urine

B. Sample Volume

Volume of sample(s):

Umbilical cord: less than 5 mm; this is used for genotyping of the dog after birth.

Muscle biopsy:

For open biopsies on animals less than 2 months of age, we will take approximately 0.5cm x 0.3cm x 0.3 cm tissue; for animals >2 months of age, we will take approximately 1 cm x 0.5 cm x 0.5 cm muscle tissue.

For needle biopsies, we will use a 14 ga needle (2.2 mm OD) on animals less than 2 months of age, and a 10 ga (3.4 mm OD) on animals over 2 months of age. However, we may also decide to use a 10 ga needle on animals older than 1 month of age depending on their body weight, muscle size and accessibility of the muscle. The need of using a 10 ga over 14 ga is that we can acquire one or two sample biopsies with minimal muscle damage from the acquisition procedure. This determination of which needle to use will

be on individualized basis, and thus if we decide to use the 10ga on a 1–2-month-old dog, we will limit our sampling to two biopsies from each muscle.

Blood Draw:

Blood drawing will be performed according to the attached ACUC Standard Operating Procedure. Briefly, blood volume collected from dogs equal to or more than 1 month old will not exceed 8.6ml/kg over a period of three weeks. If our experiment needs exceed this limit, we will consult with the veterinarians.

For puppies (less than 1 month old), we will limit it to less than 6.4ml/kg over a three-week period.

We have been approved to collect blood from normal dogs at the University of Missouri outside of the DMD colony. We will collect approximately 2ml of serum and 2 ml of plasma per dog for as many dogs as the respective lab approves as a one-time collection. Blood will be collected from dogs free from any studies which may interfere with blood parameters needed for our research. In coordination with the PI of the study and associated lab personnel, we will arrange for collection of blood to not exceed approved quantities per body weight and to minimize undue stress to the animals. Blood will be collected by a member of the research lab who owns the dogs or by James Teixeira in coordination with the lab.

Semen Samples (normal ejaculate): Semen will be collected by extruding the penis and using manual stimulation. Fresh semen will be collected in a conical tube and immediately inseminated in the recipient female. Semen may also be collected by the University of Missouri Veterinary Health Center's Theriogenology service for immediate insemination or storage.

Vaginal swab: self-explanatory.

Urine: 0.5 - 10.0 ml per cystocentesis

C. Sampling Frequency and Duration

Frequency of collection and for how long:

Umbilical cord: once for every dog.

Muscle Biopsies:

Needle muscle biopsies using the VAB system cause minimal discomfort to the dogs, and the sedation/ anesthetic procedures cause minimal risk to the dogs. We will adhere to the following guidelines to acquire needle biopsies:

1. The first biopsy will be 1 week to 1 month post injection of AAV vectors/vehicle.
2. Biopsy procedures will be separated by at least a 1-week time period, alternating sides for each subsequent biopsy.
3. Biopsy of a single muscle (i.e., left Biceps femoris) will be separated by at least a 2-week time period.
4. Each biopsy will be limited to 2 muscles, with 1 skin incision per muscle.
5. 1-5 samples may be obtained from each muscle.
6. Dogs less than 1 year of age may have up to 6 biopsies during that year. Dogs over 1 year of age may have up to 8 biopsies in a single year.
7. The maximal number of biopsies a single animal will undergo is limited to 18 biopsy events in a

lifetime.
8. Number of times a single muscle/muscle group will be biopsied over a lifetime and whether or not it is accessible via needle or open biopsy is based on multiple parameters, including location, size, and proximity to vascular/neural components.

Open muscle biopsy will be completed under anesthesia. Since anesthesia may pose some risks to the animals, we will limit the need for open needle biopsies. Open muscle biopsies may be performed when: (1) in the event needle biopsy is not achievable due to equipment issues or supply shortages, (2) based on the specific study needs for a larger sample. We will adhere to the following guidelines to acquire open needle biopsies:

1. A single sample will be taken, and open biopsy procedures will be limited to 6 times per dog over its lifetime.
2. Biopsy procedures will be separated by at least a 1-week time period, alternating sides for each subsequent biopsy.

Muscles/muscles groups which may be used for biopsy includes (apply to needle and open biopsy):

A. Hindlimb:
a. Gastrocnemius – once (needle or open)
b. Cranial tibialis – once (needle or open)
c. Biceps femoris – eight (needle or open)
d. Semitendinosus – six (needle or open)
e. Semimembranosus – eight (needle)
f. Vastus Lateralis – four (needle)
g. Rectus femoris – two (needle)
h. Cranial and Caudal Sartorius – two (open)
i. Gracilis – two (needle or open)

B. Shoulder/Upper forelimb:
a. Trapezius – once (open)
b. Supraspinatus – once (needle)
c. Infraspinatus – once (needle)
d. Deltoid – once (needle)
e. Lateral head of triceps – two (needle)

Additional muscle groups that may be biopsied in systemically injected animals include: Adductor - once (open biopsy)

Blood:

We will seek veterinary consultation on blood draws if needed. For immune suppression monitoring see section 7 'Description of Non-Surgical Procedures' for detailed description of collection frequency. We may collect daily blood samples (up to 5 mL) when heart failure occurs in order to determine potential cardiac biomarkers (cardiac troponin, cardiac CK, NT-proBNP) and CBC+Maxi over the natural course of disease development in dystrophic dogs. For animals that undergo frequent blood collection, blood draws will be done from alternating blood vessels to prevent development of phlebitis and other associated problems. The veterinary staff will be consulted regarding monitoring the dog's condition. All collections will follow the ACUC standards for blood collections.

Semen: Up to 3x per week when a female is determined to be in estrus.

Vaginal Swab: When needed for estrus cycle determination.

Urine: We will follow the following guidelines for urine collection:

1. May be collected over the natural course of disease development in normal, carrier, and dystrophic animals.
2. Urine collection via cystocentesis will occur no more than once every four months unless required for clinical evaluation by OAR veterinary staff.

D. Sampling Method

Method of collection:

Umbilical cord:
We will cut with a set of sterilized surgical scissors following birth.

Muscle biopsy: (procedure detailed and explained in the surgical procedure section)

Needle muscle biopsy:
This method is routinely used in our lab now due a very short anesthetic period, quickly recovery, and a less traumatic procedure. With a tiny skin incision (less than 5 mm in length) a muscle biopsy needle (10 G or 14 G VAB needle) is inserted into the specific muscle. The device completes cutting and vacuuming and a muscle sample is collected. The device may be redirected into a separate part of the muscle, and a second sample obtained. Up to 5 samples may be obtained via a single skin incision (based on muscle and dog size). The skin incision is closed with surgical glue. During the procedure the dog will be monitored for discomfort and pain. The heart rate, respiration rate, SPO2, CO2 and body temperature will also be monitored.

Open muscle biopsy:
Dog will be anesthetized, and a skin incision (1.5-2 cm) will be made over the appropriate surface of the skin to visualize the specific muscle to be biopsied. A 1 cm x 0.5 cm x 0.5 cm section (for adult) or 0.5 x 0.3 x 0.3 cm (for less than 2-m-old) of the proposed muscle(s) will be removed surgically using a scalpel blade. The fascia is closed in a simple continuous suture pattern and then the skin incision will be closed with staples or in a continuous intradermal suture pattern. During the procedure the dog will be monitored for discomfort and pain. The heart rate, respiration rate, SPO2, CO2 and body temperature will also be monitored.

Blood:
Blood may be drawn from jugular, cephalic, femoral and saphenous vein using needle and syringe or butterfly needle. The needle gauge will be determined based on the site of collection, vein size and dog size. For Group 6, Stop-Flow cardiac injection of reporter or therapeutic AAV vector, blood will continue to be collected in the abovementioned locations and using the approved ACUC guidelines. Blood will be collected immediately before the procedure and again immediately following the procedure.

Semen Collection:
Manual stimulation.

Vaginal Swab:
Use of sterile swab to collect cells from vaginal wall.

Urine Collection (Cystocentesis):
The animal is briefly and gently restrained in dorsal recumbency. The bladder is located and isolated through abdominal palpation and/or transabdominal ultrasound. The skin overlying the bladder is prepared with alcohol or chlorhexidine. A sterile needle is passed through the abdominal wall into the bladder and urine (0.5 - 10.0 ml) is removed directly into a sterile syringe.

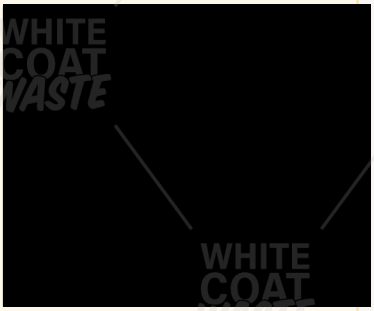
2. Induced or Spontaneous Neoplasia

Will induced or spontaneous neoplasia occur in live animals?

○ Yes ● No

3. Non-Surgical Procedures

1	Breeding procedures:	All breeding will be organized to attempt to meet the needs of the study this protocol supplies. Bitches will be evaluated for behavioral/physical signs of estrus (bloody vaginal discharge) 3x per week once they reach 6 months of age. This will be accomplished by a combination of OAR care staff and the Duan lab personnel. Any bitches that are found to be in heat and need to be bred will be reported to the Theriogenology Service of the VMTH for the optimal management of their breeding. When managing bitches for breeding the Theriogenology Service will consist of at least one board certified theriogenologist and senior veterinary students under his/her direct supervision. Procedures will be performed by the veterinary students under immediate supervision of the theriogenologist or by the latter him- or herself. None of the procedures described here will be performed by non-experienced persons. The Theriogenology Service will use conventional means of determining the time of the ovulatory luteinizing hormone (LH) surge in each bitch. Breeding reflexes, vaginal cytology, vaginoscopy and serum progesterone concentrations will be assessed every two to three days until the day of the LH surge has been identified. Blood samples (3-5 mL) for the determination of serum progesterone concentration will be aspirated out of the jugular or cephalic vein. It will be assumed that bitches ovulate 2 days after the LH surge and that the oocytes will be fertilizable another 2 days later. As oocytes remain fertilizable for 4 days, artificial insemination will be arranged to happen twice in each bitch if needed, between the 4th and 7th days after the LH surge. All males used for either AI or natural mating will undergo a semen quality evaluation (motility, morphology, etc.) to limit the number of sub-fertile males in the breeding population. Either affected or normal males belonging to UMC will be naturally mated to carrier females. Should two animals not be behaviorally or biosecurity compatible, semen will be collected from the male and the female will be bred by using AI. Should a normal male from outside of the UMC be needed to maintain an outbred colony, semen will be collected, and carrier females bred via AI. Natural mating will be performed every other day through the first day of diestrus, for an average of 5 matings per bitch per estrus. For natural mating, the male and female will be allowed to interact in a closely monitored, controlled environment in order to ensure behavior compatibility. If the animals are behaviorally compatible, they will be allowed to mate naturally under supervision. Whenever a genetically suitable male is available, his semen will be collected by manual stimulation on the day when it is needed for the insemination of a particular bitch. The semen will be extended in a commercially available semen extender (INRA 96, IMV), evaluated to confirm sperm motility and number and then used for insemination. All inseminations with fresh semen will be performed vaginally, using either a conventional insemination pipette or a MAVIC catheter (Minitube of America). Bitches will remain standing and fully conscious for insemination but may be sedated if deemed necessary



	Procedure	Description of procedure	Building name	Room number or area
		by the board certified theriogenolgist in rare circumstances (all attempts will be made to limit/avoid the use of sedation). As the males used for breeding in this population are mostly affected, their life expectancy will be reduced. In order to utilize males for breeding beyond their death, semen will be collected and frozen so that it can be used at any time after their death to optimize genetic outcome of all breeding attempts. Semen for freezing will also be collected manually and will be frozen using routine procedures for canine sperm preservation. Frozen semen inseminations will be performed in the same manner as fresh semen inseminations, but they will all be performed using a MAVIC catheter. The use of this catheter allows additional fluid to be injected behind the semen, placing the semen in the vagina under pressure, thus facilitating its movement through the cervix and up the uterine horns. Using this approach, the sperm dose can be reduced and less of the frozen semen is required to achieve satisfactory pregnancy rates. The additional fluid used in such inseminations may be filtered (sterile and sperm-free) autologous prostatic fluid or the same semen extender that is used for fresh semen inseminations. Amendment 11/04/2024: Addition for use of prophylactic antibiotics at the recommendation of Theriogenology service. Based on the recommendation of the Board Certified Veterinary Theriogenologist, we would like at add the ability for prophylactic antibiotics to be used to prevent complications due to endometritis in the aged female dog (>5y). We have had some failures in the older carriers potentially due to endometritis. In the rare instance it is advised by the Theriogenology service at the Veterinary Health Center at MU, we would like to incorporate this as an option.		
2	Breeding Gestation	Gestation: All bred females may have blood drawn for a relaxin test and ultrasounded to evaluate for pregnancy 30 days following diestrus. Following confirmation of pregnancy, bitches will be ultrasounded as needed to monitor the progression of the pregnancy. Whelping is anticipated approximately 57 days following diestrus. Pregnant females will be switched to puppy food approximately 3 weeks prior to whelping and stay on puppy food until weaning is complete. Pregnant females will be moved to room 110 into pens with whelping boxes approximately one week prior to the expected whelping date. Following this move, the female's temperature will be taken twice daily for several days prior to whelping to monitor for impending parturition. This will be performed by OAR care staff and/or Duan lab personnel. Animals will be closely monitored following temperature drop, which suggests impending parturition, and the OAR veterinarians contacted.	[redacted]	

	Procedure	Description of procedure	Building name	Room number or area
3	Breeding Parturition	Parturition: Most females are expected to whelp naturally. Based on the expected parturition day, Duan lab personnel will be present and prepared for the puppies' arrival. Should a dystocia be noted, appropriate veterinary care, including caesarian-section, will be provided as deemed necessary by the OAR veterinary staff. Dr. Baldrighi, UMC-VMTH theriogenology professor, will be consulted and utilized during the breeding/ gestation/parturition process. The temperature will be monitored by the facility technician from OAR as well as Duan lab personnel. In the past, the temperature has been provided by OAR technicians in the facility.	[redacted]	all rooms

4	Neonatal Care/ Weaning	Neonatal Care/Weaning: Whole blood and umbilical cord will be collected by Duan lab personnel from each puppy to determine serum creatine kinase concentrations and for PCR screening to determine normal/carrier/affected status, respectively. Pups will then be allowed to nurse normally until weaning at ~8weeks of age. Pups will be evaluated and weighed at least twice daily up to 2 weeks of age, then at least once daily until 4 weeks of age, and then three times per week until weaning. This will be performed by Duan lab personnel trained in puppy care in coordination with our colony manager and OAR veterinarians. Affected animals are usually weighed once weekly following weaning, whereas unaffected animals may be weighed monthly. Puppies that are not gaining weight, or that do not appear to be doing as well physically as their littermates, will be evaluated by an OAR veterinarian. These animals may be supplemented with a canine milk/colostrum substitute via tube feeding or other intensive care measures as determined by the OAR veterinarian. For puppies that are not doing well physically, we will have the following options. (1) We will euthanize the puppy and collect tissues for the tissue bank project; (2) if we do not need for tissues samples, we will consult with OAR doctors to see if the puppy is too weak to rescue. If the puppy is too weak, we will euthanize the puppy. If the OAR doctors think the puppy can be rescued with the care, we will try our best to care for the puppy. We will follow instructions from the OAR doctors periodically (at least on daily basis) to guide for the care of the weak puppy. We would like to point out that the reported neonatal death rate for affected puppies is 28-32%. With this information in mind, to prevent unnecessarily pain and suffering, we will actively seek advice from OAR veterinarians to timely euthanize the puppy that is too weak to rescue. We have talked with Dr. Jeff Henegar, to further improve the quality of care, we will emphasize on the training and re-training on puppy feeding for every new litter. All the involved lab members will be required to go through the puppy feeding protocol again before they begin to provide supportive feeding for each new litter. Training will review all basic puppy handling and care needs as well as hands on training whenever a puppy requires assistance of special needs. Training will be provided by James Teixeira and/or OAR veterinarians. All recordings will be documented by the attending lab member during their shift making notes of anything observed during the shift. The information will be emailed out at least daily to all lab personnel involved with puppy care as well as the attending OAR veterinarians and residents. Extra normal and carrier puppies: From our breeding, we expect that there will be more normal and carrier puppies produced than we can use in the study. After we have enough number of experimental normal/carrier puppies, those puppies may be adopted. We will euthanize few normal and carrier puppies to collect tissues if control tissue is required, but we do not have an immediate research need after we have confirmed their genotype.	[redacted]

	Procedure	Description of procedure	Building name	Room number or area
5	Thoracic radiograph image, ECG and Echocardiography for puppies.	Multiple puppies have died acutely without clinical signs. On necropsy, we noticed that these puppies all have enlarged and fibrotic hearts. We would like to screen all puppies for cardiomegaly shortly after birth to determine if they are at risk. The puppies will range from newborn to weaning. We will use thoracic radiography followed by ECG and echocardiography to screen the puppies. All procedures will be performed in the ARC and puppies will be transported from the whelping room to the veterinarian room under the veterinarian care to perform the procedure. Animals will be gently restrained in left lateral, right lateral, dorsal, or sternal recumbency for each image during radiography. All personnel will wear the appropriate PPE.	[redacted]	
6	Fortiflora, subcutaneous serum puppy care	Subcutaneous serum and Fortiflora to puppy care: Puppies that are born with muscular dystrophy tend to be weak sucklers and have a hard time ingesting enough colostrum to receive protective immunoglobulins. Serum will be collected from healthy adults in the colony following the previously established blood draw guidelines to collect 16cc of serum for each affected puppy. If multiple donors are required to reach this number, the samples will be pooled before administering. Each affected puppy may receive 16cc of serum subcutaneously within the first week of life. Serum will be handled sterilely at all times. This amount will be split over multiple doses and not exceed 60 ml/kg/day to prevent over hydration. To help prevent GI upset from supplementation with milk replacer, Fortiflora, a canine probiotic, may be added as needed to the milk replacer at a dose of 1 package to 60 mls of milk replacer. Reconstituted milk will not be kept longer than 24 hours after making. Fortiflora may also be added to the puppy food during weaning to help prevent diarrhea and subsequent weight loss.	[redacted]	all rooms

	Procedure	Description of procedure	Building name	Room number or area
7	Procedure 1 Heart function assessments: 1.1 Electrocardiography	Electrocardiograms are performed on conscious or lightly sedated animals that are gently restrained in right lateral recumbency with the limbs maintained perpendicular to the long axis of the body. The reason for light sedation is we are afraid that some dogs may not calm down during the procedure, for the three years, we do not apply any sedation, and can get the procedure done successfully. Dr. Leach will use a Philips Page writer Xli ECG machine, model #M1700A (Royal Philips Electronics, Eindhoven Area, Netherlands). ECG leads will be positioned using the hexaxial and precordial lead systems. The ECG electrodes consist of small flat metal plates that are attached to the distal limbs by flexible bands, and chest lead electrodes that are attached to the hemi-thorax by adhesive patches. Medical coupling gel is used to facilitate contact between the electrode and the skin. The ECG will be recorded at a paper speed of 50 mm/sec for measurements of electrical intervals and amplitudes and determination of the mean electrical axis. An additional recording will be made at 25 mm/sec for evaluation of arrhythmias. Leads I, II, III, aVR, aVL, aVF, V1, V2, V3, and V10 will be recorded in all experimental and control dogs. Standard measurements of intervals and amplitudes of the P, QRS, and T waves will be performed using lead II at a recording speed of 50 mm/second, and a calibration of 1 cm/mV. The Q, R, and S amplitudes and the Q/R and R/S ratios will be calculated in each lead. The mean electrical axis will be calculated as the net deflections of leads I and III. Evaluation of heart rate, and rhythm disturbances will be made from recordings obtained at 25 mm/second.	[redacted]	

	Procedure	Description of procedure	Building name	Room number or area
8	Procedure 1 Heart Function Assessment: 1.2 Echocardiography	Echocardiograms are performed in conscious or lightly sedated animals that are gently restrained in right and left lateral recumbence (gentle restraint refers to verbal soothing and gentle petting to keep animal quiet - duration is 10-60 minutes depending on conditions of the dog and number of images needed to provide definitive data). For dogs that are difficult to calm down, we may use a light sedaiton protocol at the discretion of the board-certified cardiologist. A small amount of hair is clipped from over the left and right precordia to facilitate image acquisition. The imaging is performed using a GE Vivid 7 ultrasound system or Toshiba Artida ultrasound machine (Toshiba America Healthcare, Rolling Meadows, Il). Probe will be selected according to image optimization from 3 to 7 mHz. We will simultaneously monitor ECG by Dr. Leach. The guidelines of the American Society of Echocardiography are used for measurement of the left ventricular (LV) end-diastolic and end-systolic diameters, and septal and posterior wall thickness. Fractional shortening, an index of contractility is calculated from the measurements of LV chamber dimensions. Two-dimensional imaging is used to guide pulsed-wave and continuous-wave Doppler evaluation of mitral inflow velocities, and aortic outflow velocities from an apical 4-chamber orientation. M-mode echocardiography is performed using the parasternal short-axis view of the left ventricle. Images are captured digitally and a series of 3 to 6 consecutive cardiac cycles are measured and averaged for each individual measurement.	[redacted]	

9	Procedure 1 Heart Function Assessment: 1.3 Dobutamine stress assay	This may be a terminal study when combined with cardiac catheterization procedure before necropsy, or it is a procedure will be used occasionally for screening. Rational: More than 90% of Duchenne muscular dystrophy (DMD) patients develop cardiomyopathy at the end of their life. Development of a gene therapy for DMD cardiomyopathy is thus an important goal in our study. We have previously performed dog ECG and echocardiography at rest. We would now like to include dobutamine stress test in our protocol. Exercise stress testing is routinely used in human beings to increase cardiac workload and assess heart function. However, this approach will not be applicable to dystrophic dogs that are restricted in movements due to muscle disease. Dobutamine stress has been recognized as an alternative to exercise testing. In comparison with electrocardiogram (ECG)/echocardiography at rest, dobutamine stress offers a better evaluation and early identification of cardiac dysfunction. Dobutamine is a synthetic positive inotropic agent developed for short-term intravenous infusion. During the subclinical (early) phase, the baseline values of cardiac performance in healthy and affected dogs overlap, therefore making the diagnosis difficult prior to the development of overt echocardiographic abnormalities. The inotropic challenge with dobutamine helps to disclose ECG/ echocardiographic abnormalities attributable to heart disease at the early stages of heart failure. Dobutamine stress assay has been widely used in dogs (Haidet et al., 1989; McEntee et al., 1998; McEntee et al., 1999). Actually, dobutamine stress assay has been used by Townsend et al to evaluate drug therapy in golden retriever muscular dystrophy dogs (Townsend et al., 2010). We believe that including this assay method in our protocol will enhance our study of DMD heart disease in the dog model. Method: A 20 G catheter (the size of the catheter may vary depending on the size of the dog. 20 G is the most commonly used size. The actual size of the catheter will be determined by Dr. Leach for the particular dog and will be inserted into the left cephalic vein for administration of dobutamine. The dog will be individually placed in right lateral recumbence and baseline ECG and echocardiography will be obtained at rest. Pharmacological grade dobutamine will be obtained from the MU VMTH hospital. Incremental dose will be administrated using a volumetric pump (such as the model Genie Plus from Kent Scientific). Dobutamine will be diluted in 5% dextrose solution (obtained from VMTH) to provide a concentration of 500 microgram/ml for iv infusion. The onset of action for dobutamine occurs within 2 minutes, and the peak of effects is within 10 minutes. McEntee et al used the dose of 7.5 µg/kg/ min (starting dose) and 42.4µg/kg/min (maximal dose) with the increment of 10 µg/kg/min every 15 min (McEntee et al., 1998; McEntee et al., 1999). Schmidt et al started dobutamine at the dose of 5 µg/kg/min and increased dose every 3 min up to 10, 20, 30 and 40 µg/ kg/min. We have consulted with Dr. Deb Fine at the VMTH. Dr. Fine suggests that we start out at 5 µg/kg/min since we don't know how these hearts are going to

respond to the potential arrhythmogenic effects of dobutamine and increase at 10 µg steps up to a maximum of 35 µg/kg/min holding each stage for 10 to 15 minutes. Five minutes after the start of each incremental administration, we will record ECG and echocardiography. All measurements will be performed again 10 minutes after cessation of dobutamine infusion. Arrhythmogenic effects of dobutamine: Dobutamine has been shown to precipitate arrhythmia in human patients (Hanson et al., 1997). Throughout the entire assay process (including during dobutamine infusion), we will continually monitor ECG. We will stop the test if one of the following conditions appears during the assay: decrease in ST value (ST value < (baseline ST value - 0.2 mY]), hypotension (SBP < {baseline SBP - 20 mm Hg]), hypertension (SBP > 240 mm Hg), sinus tachycardia (heart rate> 240 beats/min), ventricular tachycardia, or uncontrollable excitement of the dog (McEntee et al., 1998). In our protocol, we have proposed to start dobutamine stress at 5 ug/kg/min. We will increase the dose at the dose of 10 ug/kg/min until we reach 35 ug/kg/min. The holding time at each stage is 10-15 min. For four stages (5, 15, 25, 35 ug/kg/min), it will be a total of 40 to 60 min. In addition, we will need 10 min to record the baseline (before dobutamine stress) and we will also record echo and ECG at 10 min after the cessation of dobutamine infusion. So, in total, we will need 60 to 80 min to finish the experiment. We have tested the dobutamine in Normal and Carrier dogs with dose of 5 ug/kg/min, and induced tachycardia in those dogs and all dog tolerated well for the treatment. Total 24 dogs will used for dobutamine stress test (Normal = 6, Carrier control = 6, Affected control = 6, Affected-treated = 6). Provisions if the dog does not handle the stress assay well (unable to lay down for the entire time, gets agitated or excited, etc): The normal procedure for an animal that is overly excited or agitated during an echocardiogram is to attempt to sooth it by petting gently and speaking in a calm and quiet voice. This works 90% of the time in adult dogs to calm them down. In 9% of the time, giving them a break from the echocardiogram and allowing them to sit sternally on the echo table for 3 – 5 minutes calms them down enough to complete the examination. In 1% of cases, the dog does not calm down without sedation. We have always been able to complete our examination on the dogs with the combination of the first two techniques. We have never needed sedation. However, for purposes of the dobutamine study, I would add that any animal that becomes stressed to the point where sedation would be necessary to complete the study, the dobutamine infusion would be discontinued and the Echo/ECG study on that animal terminated. Dogs will not be restrained to perform this test. Dog age: Dr. Leach has successfully performed ECG and echo in 3-m-old normal, carrier and dystrophic dogs. However, at this age, we cannot detect statistically significant difference among three groups. Dobutamine stress may help us identify the difference. For this reason, the youngest dogs that we plan to test are 3-m-old. However, we also

understand that young dogs may more easily get agitated. If we find that it is too challenging to hold a 3-m-old dog on the examination bed for a period of 60 to 80 min, we will try 6-m-old dogs. If this is still too challenging, we will try one-year-old dogs. Based on the literature, there should be no problem to perform the assay in one-year-old dogs (For example, McEntee et al 1998 publication used 1-year-old dogs). Frequency of the assay per dog: We currently plan to do only once per dog. However, we will reserve the option to test one dog in a systemic AAV therapeutic gene treated group for multiple dobutamine assay (all other dogs will be euthanized at the end of dobutamine stress except one dog which we will test it again at 6 months later, we will do the assay again every 6 months until a total of five assays are done in this dog. This test will open the option for our future studies so that we can test a single dog with multiple dobutamine stress. This reduces the total number of dogs needed for future studies. Multiple dobutamine assay has been performed by others previously. McEntee et al 1998 stated that "To test repeatability of the technique, the full protocol was repeated within 24 hours". This is to say that the authors were able to conduct dobutamine stress assay twice within 24 hours in the same dog. In other words, the stress assay itself is well tolerated by the dog and does not cause apparent heart damage. In our study, we will not repeat the study in the same dog within a 24 hour-period. However, we may opt to test the stress response of the same dog at the different time points. In this case, we will give at least 6 months break between two tests. Further, we will limit the test to five times per dog in its lifespan. We will not do the stress test for every dog, we plan to have Normal control (n=6), Carrier control (n=6), and Affected control (n=6) as well as Affected treated (n=6) in the future, also we will use sedation/anesthesia if a dog appears to prevent the dog over-excited for the repeated test. References Haidet, G.C., Musch, T.I., Friedman, D.B., and Ordway, G.A. (1989). Cardiovascular effects of dobutamine during exercise in dogs. Am J Physiol 257, H954-960. Hanson, M.W., Morris, E.I., Borges-Neto, S., and DeLong, D.M. (1997). Analysis of cardiac arrhythmias during dobutamine pharmacologic stress testing in nuclear cardiology as related to the presence or absence of baseline arrhythmias. Journal of nuclear cardiology: official publication of the American Society of Nuclear Cardiology 4, 372-378. McEntee, K., Amory, H., Clercx, C., Soyeur, D., Michaux, C., Vanhaeverbeek, O., Jacqmot, O., and Henroteaux, M. (1998). Physiologic response to dobutamine infusion during cardiac stress testing of dogs. American journal of veterinary research 59, 1160-1165. McEntee, K., Clercx, C., Amory, H., Michaux, C., Dardenne, J.J., Soyeur, D., and Henroteaux, M. (1999). Doppler echocardiographic study of left and right ventricular function during dobutamine stress testing in conscious healthy dogs. American journal of veterinary research 60, 865-871. Townsend, D., Turner, I., Yasuda, S., Martindale, J., Davis, J., Shillingford, M., Kornegay, J.N., and Metzger, J.M. (2010). Chronic administration of membrane sealant prevents severe

	Procedure	Description of procedure	Building name	Room number or area
		cardiac injury and ventricular dilatation in dystrophic dogs. J Clin Invest 120, 1140-1150.		
10	Procedure 2: MRI	MRI assay has been used clinically to evaluate disease progression in DMD patients. This technology also has been used in animal models of DMD. Therefore, we want to use MRI to monitor muscular response to gene therapy. These assays will be performed by an expert in animal (especially dog) MRI at MU Vet School. Anesthesia for the MRI will be provided by qualified, and trained personnel who work for the hospital/clinic will be performing anesthesia during MRI as a hospital/clinic veterinary service. MRI Protocol: The animal will be placed in dorsal recumbence with the thoracic limbs extended forward. The proximal aspect of each antebrachium will be imaged with a general-purpose flex coil. The following MRI sequences will be performed sequentially. Transverse and sagittal T2 weighted Transverse Proton-density with fat saturationT1 weighted transverse images pre and post-contrast intravenous administration of 0.2 mmol/kg of gadolinium-DTPA. After the MRI, the animal will be brought back to the anesthesia service for recovering after which the animal will be ready for discharge and picked up. We plan to do MRI procedures once before gene transfer as a control, then approximately 1, 3, 6, and 12 months after gene transfer then once per year until the dog is euthanized. More times may be added but protocol will be modified to add these time points if needed. Only systemically injected animals will have MRI performed. MRI Image with new 3T MRI system: We will use this new system for MRI assay. Anesthesia will be performed as stated in above. As standard MRI, Magnetic Resonance imaging is conducted through excitation and relaxation of the protons within the tissues of investigation. The response of the protons in response to the variation to stimulation is characteristic of tissue or indicative of pathology. Both cardiac and skeletal muscle is investigated. Cardiac muscle is examined using ECG gating. For details procedure protocol, please refer to the attached MRI files. Contrast Enhanced MRI: Animals will be managed as above. Gadolinium-diethylenetriamine penta-acetic acid (Gd-DTPA), other Gadolinium chelates or magnetic agents suitable for MRI imaging is administered intravenously and several sequences are acquired. As above, 2D or 3D Gradient EchorRR interval 1.9488144 X 1925 mm13; Sequence TRTEB and width Matrix Slice Thickness Slice Gap Flip Angle will be used to delineate the post contrast studies performed on cardiac, and T1 3D7.2 ms 3.4 ms 279208 x 2801 mmNone9 will be applied to skeletal muscle.	[redacted]	

	Procedure	Description of procedure	Building name	Room number or area
11	Procedure 3 Respiratory Function Assay: 3.1 Fluoroscopy study	DMD patients mainly die from respiratory failure, so we want to study respiratory function in our dog model of DMD. These assays will be performed once as a control prior to gene transfer and then at approximately 3, 6, and 12 months after gene transfer then once per year until the dog is euthanized. More times may be added but protocol will be modified to add these time points if needed. Only systemically AAV vector delivered dog will have the respiratory function assay performed (Normal control dogs and Affected dogs with body-wide therapeutic gene delivery dogs). Overview: A total of four respiratory function assays protocols are listed. Procedures 3.1-3.3 are currently in clinical use by Drs John Dodam and Carol Reinero. Dr. Dodam will conduct the procedure. Procedure 3.4 will use a commercially available device. 3.1 Fluoroscopy study: Awake, spontaneously breathing dogs will be allowed to stand for fluoroscopic imaging. Gentle physical restraint will be used to allow imaging with fluoroscopy. Animal will be restrained and evaluated for 10 to 15 min. Regular respiration will be observed and recorded on video. Data will be analyzed offline.	[redacted]	
12	Procedure 3 Respiratory Function Assay: 3.2 Tidal breathing flow-volume loops (TBFVL)	Awake, spontaneously breathing dogs will be matched to a tight-fitting face mask to ensure minimal air leakage around the muzzle; gentle manual physical restraint will be used. The face mask will be attached to the breathing circuit in-line with the Engstrom Carestation Ventilator. A series of tidal breathing flow volume loops will be collected once the dog is acclimated to the face mask (i.e., no struggling or panting). A series of at least 10 inspiratory and expiratory loops will be collected; each should meet within 5%. Depending on patient compliance this is anticipated to take ~10 minutes. As needed to minimize stress, the dog will be allowed to take “breaks” by removing the face mask before trying to collect additional TBFVLs.	[redacted]	

	Procedure	Description of procedure	Building name	Room number or area
13	Procedure 3 Respiratory Function Assay: 3.3 TBFVL and compliance measurements in anesthetized dogs	Initial measurements of pulmonary mechanics will be made during spontaneous breathing in anesthetized dogs. Ventilation will be initiated until stable end-tidal carbon dioxide values are obtained (34-40 mm Hg), spontaneous ventilation not observed, and heart rate and blood pressure are stable. After equilibration and before collecting data, a short-acting neuromuscular blocking drug will be administered to prevent variability induced by alterations in chest wall muscle tone (atracuriium, 0.2 mg/kg). Pulmonary mechanical measurements will be obtained using software programs integral to the critical care ventilator. Ventilation will be continued until a train-of four response approaches normal (3/4). At this time, reversal of neuromuscular blockade may be initiated with administration of edrophonium (0.5 mg/kg, IV). When train-of-four response has returned to normal (4/4), the respiratory rate will be decreased to 2/min and end tidal carbon dioxide allowed to increase to 50-60 mm Hg. It will be maintained at this level until the dog is able to breath effectively (based upon peak CO2 values of < 50 mm Hg and pulse oximetry readings of >92%).	[redacted]	
14	Procedure 3 Respiratory Function Assay: 3.4 Non-invasive telemetry evaluation of dog pulmonary function	This will be conducted with the EMKA PACK system (www.emkatech.com). Basically, a respiratory belt will be put on awake spontaneously breathing dogs around the middle chest level. Signals generated during spontaneous breathing will be transmitted to a receiver through telemetry. This device can be used to generate & analyze (in real-time, on a breath-by-breath basis) flow/volume loops, as well as the standard respiratory flow parameters like breathing frequency, tidal volume, minute volume, & other related parameters. The EMKA device will allow monitoring respiration in 3 levels of capabilities: (1). A single RIP band; for breathing rate detection & relative volume change (non-calibrated approach). (2). Double RIP bands, for breathing rate, relative volume change, RIP summation signal (both bands summed as 1 band & provided as its own signal), and phase angle change (non-calibrated approach). (3). Double RIP bands, for all of the above, using a calibrated approach. This enables accurate calibrated Tidal Volume measurement. Calibration will be performed with a linear pneumotach (EMKA). The animals will be acclimated to wearing the monitoring equipment one day before measurements are taken. For each assay session, animal will be wearing the respiratory belt for approximately 30 min. Dr. Dodam will serve as consultants if we encounter any question.	[redacted]	

15	Procedure 4 Electric Impedance Myography (EIM):	This is a non-invasive assay to study muscle diseases. Since DMD is a muscle disease, we will use this non-invasive technique to assess muscle function. Electric impedance myography (EIM) is a safe, non-invasive bioimpedance-based technique assay for evaluation of muscle composition and function (reviewed in (Rutkove, 2009)) [This paper is attached for ACUC review]. This approach has been widely used in rodents and humans (Esper et al., 2006; Nie et al., 2006; Rutkove et al., 2012a; Rutkove et al., 2012b; Rutkove et al., 2007; Tarulli et al., 2005). We will use the EIM assay to non-invasively monitor disease progression in dystrophic dogs and control dogs. In EIM, low-intensity electrical alternating current (AC) is applied to a muscle using noninvasive surface electrodes. As current flows through tissue, voltages result on the surface of skin with values that depend on tissue structure and composition. Resulting voltages are measured using a second set of electrodes. EIM is strongly associated with localized muscle condition. EIM is already being used in neuromuscular disease evaluation in which muscle composition is also abnormal (Ogunnika et al., 2010; Rutkove et al., 2012b). EIM has also been used as a biomarker of muscle status in several clinical trials (Rutkove et al., 2012a; Rutkove et al., 2007; Tarulli et al., 2005). We will conduct EIM measurement using commercially available FDA-approved bioimpedance device connected to a transducer array (Convergence Medical Devices, Inc). Since the device use a low power DC current supplied by a rechargeable Li-ion battery, it is very safe and represents no risk of electrical shock to the animal. Two types of transducers will be tested separately to obtain optimal conductivity, the handheld array and the adhesive multiple electrodes array. These transducers are placed superficial and do not penetrate the animal skin, thus it will represent no harm to the animal. We will test the EIM approach on healthy, affected and carrier dogs at different ages and gender, in which several groups of muscles will be evaluated. This will include the sternohyoideus, sternocephalideus from the neck muscles; the deltoideus muscle from the shoulder; biceps femoris, gracilis, cranial sartorius, tibialis cranial and the medial/lateral gastrocnemius muscles from the hindlimb; the triceps brachii, extensor carpi radialis, extensor carpi ulnaris, extensor digitorum lateralis, flexor carpi radialis, and flexor carpi ulnaris from the forelimb. Based on experiment needs, we may measure some of the above listed muscles or all the muscles. Muscles may be evaluated all at once in one experiment or in multiple experiments. The EIM measurement Protocol: Awake dog will lie on its side and will be gently restrained for the duration of the measurement (5 to 10 min). The hair on top of the muscle of interest will be shaved to achieve optimal skin conductance. The skin will be cleaned with 70% ethanol and then dampened with water. The transducer will be placed on the skin. Data will be collected using the bio-impedance device and analyzed with a standard computer. We expect the duration of data collection to be ~ 30 seconds/ measurement and will be repeated 10-20 times for each	[redacted]

Procedure	Description of procedure	Building name	Room number or area
	muscle tested. References for the EIM assay Esper, G.J., Shiffman, C.A., Aaron, R., Lee, K.S., and Rutkove, S.B. (2006). Assessing neuromuscular disease with multifrequency electrical impedance myography. Muscle Nerve 34, 595-602. Nie, R., Sunmonu, N.A., Chin, A.B., Lee, K.S., and Rutkove, S.B. (2006). Electrical impedance myography: transitioning from human to animal studies. Clinical neurophysiology: official journal of the International Federation of Clinical Neurophysiology 117, 1844-1849. Ogunnika, O.T., Rutkove, S.B., Ma, H., Fogerson, P.M., Scharfstein, M., Cooper, R.C., and Dawson, J.L. (2010). A portable system for the assessment of neuromuscular diseases with electrical impedance myography. Journal of medical engineering & technology 34, 377-385. Rutkove, S.B. (2009). Electrical impedance myography: Background, current state, and future directions. Muscle Nerve 40,936-946. Rutkove, S.B., Caress, J.B., Cartwright, M.S., Burns, T.M., Warder, J., David, W.S., Goyal, N., Maragakis, N.J., Clawson,L., Benatar, M., et al. (2012a). Electrical impedance myography as a biomarker to assess ALS progression. Amyotrophic lateral sclerosis: official publication of the World Federation of Neurology Research Group on Motor Neuron Diseases 13, 439-445. Rutkove, S.B., Gregas, M.C., and Darras, B.T. (2012b). Electrical impedance myography in spinal muscular atrophy: a longitudinal study. Muscle Nerve 45, 642-647. Rutkove, S.B., Zhang, H., Schoenfeld, D.A., Raynor, E.M., Shefner, J.M., Cudkowicz, M.E., Chin, A.B., Aaron, R., and Shiffman, C.A. (2007). Electrical impedance myography to assess outcome in amyotrophic lateral sclerosis clinical trials. Clinical neurophysiology: official journal of the International Federation of Clinical Neurophysiology 118, 2413-2418. Tarulli, A., Esper, G.J., Lee, K.S., Aaron, R., Shiffman, C.A., and Rutkove, S.B. (2005). Electrical impedance myography in the bedside assessment of inflammatory myopathy. Neurology 65, 451-452.		

	Procedure	Description of procedure	Building name	Room number or area
16	Procedure 5 Dog Obedience	The purpose of this procedure is to train dogs to perform activities (walk, run, and jump) for data collection in other experimental procedures. For example, to train the dog to walk with limited leash restraining for gait study. This procedure will be performed on normal, affected, carrier and treated dogs. Training will begin at 6 weeks of age and may last for the life of the dog. Dogs with limited physical activity due to disease progression may be excluded from this procedure. We will consult the veterinary doctor to decide whether we should train these dogs or not. Training will occur in the housing facility. Frequency of test: We will work with the dogs on a daily, weekly and monthly basis. Dogs will be allowed to recover to full strength if they show soreness or fatigue following training. The dog training protocol includes: Basic training (obedience): sit, come, down, stay etc. Leash walking Walking 40 feet in a straight line with limited leash restraint Dog jump protocol a). A plastic adjustable wall is set up. This wall has the length sufficient that a dog cannot go around. Each step of barrier panel consists of 10 cm height. b). A dog is leashed and encouraged (may require dog treats) to jump over the wall. Dogs are not forced to perform when either unable or unwilling to proceed after the training period. For dogs those who cannot go through the training, we will stop the procedure. c). Evaluate dog performance once a month: the height of barrier starts with one step lower than the previous month's record. Dogs are rewarded after a round trip jump (jump over and jump back). Once it is accomplished, one more barrier panel is added, and the dog repeats jump. All trials (such as the height of the wall and number of times the dog can jump over a fixed height) will be recorded for each dog.	[redacted]	all rooms
17	Procedure 6 Overnight recording	The dog will be housed individually in a kennel with or without an elevated floor. The video camera is centered and secured to the ceiling on top of the kennel through a custom-made mounting bracket. The camera and light source are positioned at ~ 210cm above the kennel floor to ensure full view and even illumination of the cage floor. Spontaneous movement of the dog is recorded during the dark cycle from 6pm to 6am the next day. During this period, there is no interference from environmental cues or animal caregivers. Temperature and humidity are controlled and monitored during the recording period. References Shin JH, Greer B, Hakim CH, Zhou Z, Chung YC, Duan Y, He Z, Duan D (2013) Quantitative phenotyping of Duchenne muscular dystrophy dogs by comprehensive gait analysis and overnight activity monitoring. PLoS One 8: e59875. Hakim CH, Peters AA, Feng F, Yao G, Duan D (2015). Night Activity Reduction is a Signature Physiological Biomarker for Duchenne Muscular Dystrophy Dogs, J Neuromuscul Dis. 2015:2(4); 397-407	[redacted]	any

	Procedure	Description of procedure	Building name	Room number or area
18	Procedure 7 Gait: 7.1 Digital recording of the dog gait	Dog will be allowed to walk on the carpet back and forth (3 x 4 meters). Dog walking will be recorded with two digital cameras and image will be analyzed by computer software. Please note, for each dog the maximal walking time will be one hour. For dogs who cannot go through the basic obedience training (e.g., the dog is too weak and cannot walk or the dog suffers from any medical conditions that prevents him/her from training. We will consult the veterinary doctor to decide whether we should stop training), we will stop the procedure. The detailed protocol has been published. Shin JH, Greer B, Hakim CH, Zhou Z, Chung YC, Duan Y, He Z, Duan D (2013) Quantitative phenotyping of Duchenne muscular dystrophy dogs by comprehensive gait analysis and overnight activity monitoring. PLoS One 8: e59875.	[redacted]	any

19	Procedure 7 Gait: 7.2 Dog treadmill walking with motion sensor	Our previous studies have found that walking gaits are apparently different in the dystrophy dogs from those normal dogs. In order to better characterize dystrophic symptoms and signs and evaluate the effectiveness of whole body AAV gene therapy in muscular dystrophy dogs, we have collaborated with Dr. Gang (Gary) Yao in the Bioengineering Department to develop a more versatile dog gait analysis system. We expect to attach a motion sensor device to the dog and collect the data from the sensor while the dog walks at a constant speed on the treadmill. With the new gait analysis system, we should be able to monitor disease progression and effectiveness of AAV gene therapy non-invasively and repeatedly in the same dog over a period of time. Treadmill walking training: We will start the training after the dog is weaned. The dog will be familiarized with the treadmill walking by slowly introducing it to the treadmill. The detail training procedure as the follows: dog will be leashed and led to stand on the non-moving treadmill, then, let the dog gets used to the motor noise by turn on the treadmill while dog is not on the treadmill. After each dog gets used to the treadmill noise, we will set the treadmill to the slowest speed in the treadmill setting (≤1 miles/hr), dog will be led on the treadmill with both fore limbs first, if dog resisted to get on the treadmill, and let dog walk away. And using treats as positive award encourages dog walking on the treadmill. After the dog gets used to the slowest speed, we will gradually increase the speed at the rate of 0.5 mile/hr. The dog will be let to adjust to the new speed for at least 5 min before we increase the speed again. We will keep increasing the speed until the dog can walk comfortably on the treadmill without have to change its gait pattern from walk to trot or gallop. We define walk as more than two limbs always touching the treadmill ground and each limb moves one after another. Based on the internet search, we expect the maximum speed to be 3-4 miles/hour for walking. We may adjust the speed based on our training to get the steady walking speed. In general, we expect the training period to be 2 to 3 weeks until the dog can walk on the treadmill comfortably. At least two people will involve in the treadmill walking training. One person will be in charge of the treadmill on/off switch, and speed adjustment. Another person will hold the leash of the dog, to monitor the dog walking condition and make sure the dog walking comfortably and safely on the treadmill. We will perform the study on all the groups that have been planned for gait analysis. These include Normal, Affected untreated, and Affected with systemic AAV vector treated. The dog will be assessed at the ages that have been specified for the gait assay in above groups. Gait analysis: After the dog is able to walk steadily on constant speed, motion sensors will be put on dog's chest area and four limbs with elastic straps. The motion sensor uses Bluetooth wireless to communicate with the host computer, so there will be no ware to affect dog walking. The device provides 6 degrees of motion data (linear acceleration and rotational speed around x-, y-, and z-axis). The device is contained in a plastic box measured as 3.5 x 3.5		any

	Procedure	Description of procedure	Building name	Room number or area
		x 2 cm in size (length x width x height); about 20.5 grams in weight (please see attached photos). A small design will be used for limb movement analysis. We will collect the data from the sensors while the dog walks on a constant speed on the treadmill. We expect each period of data collection to be less than 10 min. The purpose of gait analysis is to obtain steady gait, as long as we get steady gait data, the test will be stopped. This usually needs 10 to 20 min walking duration. If the dog cannot walk on steady gait, the treadmill speed will be adjusted down accordingly. Alternatively, we will remove the dog from treadmill study if the dog cannot walk on the treadmill at the lowest speed setting. For actually tests, at least three people will be involved. One is in charge of treadmill, one watches the dong, and another one runs the gait data collection. During the training and gait analysis, extra caution will be taken to avoid dog injury. The front and back of the treadmill will be open allowing the dog to easily get on and off the treadmill. Only systemically injected dogs receive gait analysis and treadmill test, for comparison, Normal, Carrier, and Affected dogs will be use for the gait and treadmill analysis. The clinical point to stop dog gait testing is whenever the dogs developed health issues, that could affect walking gaits, or difficult to walk on the treadmill due to the muscular dystrophy disease, we will stop the treadmill test for the dog. The device is attached to dog's chest area and four limbs with elastic straps.		
20	Procedure 7 Gait: 7.3 Dog walking with motion sensor	1.) On the day of the testing, railways are set up along the hallway of the housing facility. The process will involve at least 2 individuals. 2.) Four IMU are connected to the computer running controller software and the sampling rate of IMU sensors was set on 100Hz and raw IMU data were collected under a Kalman filter. 3.) Dogs are put on a harness and leash walked from their kennels to the testing area. 4.) Four sensor mounts are attached to the dog's limbs and the dog is walked for 5 minutes to practice walking. 5.) The dog will then be led to the starting line of the railway and are allowed to walk freely using their unique gait for at least three passes. 6.) The sensors and mounts are removed immediately following data acquisition.	[redacted]	any

	Procedure	Description of procedure	Building name	Room number or area
21	Procedure 7 Gait: 7.4 Pressure sensitive walkway (GaitRite)	The GaitRite mat is a computerized mat that analyzes the pressure patterns and gait of the walking dog. The hallway of the housing facility is set up with the railways and the pressure sensing mat. The dogs are allowed to walk freely on the mat and investigate the area before data collection. The dogs are harnessed, and leash walked to the starting point of the mat and allowed to walk across the mat with their unique gait. The mat interprets the footsteps' pressures and records them into the computer software. The dogs are walked back and forth for the duration of time needed to gain the data with at least a 5-minute break every 10 minutes. If any dog shows signs of fatigue or soreness the test is stopped, and the dog is brought back to the kennel to rest. The process involves at least 2 people; one using the computer and the other walking the dog.	[redacted]	any

22	Procedure 8 Force function measurement of limb muscles	This is a non-invasive, non-terminal experiment. This procedure was developed by Dr. Kornegay group by using a footplate connected with Aurora level system to test a group of muscle function on the hind limb. (This protocol is included in surgery section too). This experiment allows us to assess the limb extensor or flexor muscle group function at different time points such as 1m, 3m, 6m, 12m etc. to follow-up the treatment effects. The Limb Muscle Force assay is another proven way to study muscle function. This assay is important because DMD is a muscle disease and alters muscle function. Groups to undergo this assay include Normal, Carrier and Affected animals. The hind limb procedure is conducted exactly as published by Kornegay et al (Childers et al, 2011; Kornegay et al, 1999; Kornegay et al, 2011; Tegeler et al, 2010). If the dog is unable to perform the work (due to disease stage), we will not do it for the dog. We also apply the same experiment procedure developed by Dr. Kornegay to measure the flexor muscle group on fore limb by modified the footplate and the stereotactic frame to support the front limb. Dogs will be pre-medicated using Acepromazine (0.02mg/kg), Butorphanol (0.4mg/kg) and Atropine (0.04mg/kg) all intramuscularly. If the total volume to be administered is over 2ml, then the Atropine will be administered subcutaneously. Dogs will be anesthetized using a propofol CRI at a dosage of 0.1-0.4 mg/kg/min up to a maximum of 1.2mg/kg/min. Anesthetized dogs will be positioned in dorsal recumbency, and the pelvic limbs will be alternately immobilized in a custom-made stereotactic frame (can be obtained commercially from Aurora), such that the tibia is parallel to the table and forms approximately a 90-degree angle with the femur. Isometric torque Measures: Anesthetized dogs are positioned in dorsal recumbency. One pelvic limb is immobilized in a muscle at L0 (muscle length at which tetanic torque is maximal) the tibiotarsal joint is positioned at 90 degrees. Adhesive wrap affixed the foot to a pedal (footplate) mounted on the shaft of a servomotor to measure torque (model 310LR, Aurora Scientific, Aurora, Ontario). Nerve stimulation activated hind limb muscles of the foot to push (extend) or pull (flex) against the pedal to generate torque. Percutaneous stimulation of the peroneal nerve induced tibiotarsal flexion, whereas tibial nerve stimulation induced tibiotarsal extension. Supramaximal 150 V, 100 ms pulses are applied (Model 701 stimulator, Aurora). Tetany is induced by a 1-s train of 50 HZ pulses. The limb is repositioned, and the sequence is repeated. Dynamic Muscle Control computer software (DMC, Aurora Scientific) controlled the servomotor, stimulation timing, and capture of torque responses. Tibiotarsal eccentric contraction (ECC) Protocol: Percutaneous peroneal nerve stimulation (100 ms square wave pulses, 50 HZ) activated tibiotarsal flexor muscles; stimulating electrodes are positioned until twitches (Pt) reached a maximum. The ECC protocol consisted of an initial isometric contraction followed by a forced stretch imposed by the servomotor. The servomotor rotated the lever arm 29_ opposite to contracting flexor muscles at a rate of approximately 0.7	

Procedure	Description of procedure	Building name	Room number or area
	muscle length/s followed by a rapid return to baseline position. During stimulation (100 ms square wave pulses over 1 s at 50 HZ) flexor muscles are subjected to 800 ms isometric and 200 ms eccentric contractions. This procedure is repeated every 5 s. To avoid fatigue, a 4-min rest followed every 10 contractions and a total of 30 contractions are performed in each animal. This assay will be performed once prior to gene transfer as a control, then approximately 3, 6, and 12 months after gene transfer. Based on our results, more time points may be needed. Amendments will be submitted to cover any additional time points. This assay has performed successfully by other groups multiple times in single animals. The same experiment protocol used in the hind limb will be used in the fore limb to evaluate the flexor function at different foot tarsal angles. ECC is determined at the optimal foot tarsal angles. References Childers MK, Grange RW, Kornegay JN (2011) In vivo canine muscle function assay. J Vis Exp: pii 2623 Kornegay JN, Bogan DJ, Bogan JR, Childers MK, Cundiff DD, Petroski GF, Schueler RO (1999) Contraction force generated by tarsal joint flexion and extension in dogs with golden retriever muscular dystrophy. J Neurol Sci 166: 115-121. Kornegay JN, Bogan JR, Bogan DJ, Childers MK, Grange RW (2011) Golden retriever muscular dystrophy (GRMD): Developing and maintaining a colony and physiological functional measurements. Methods Mol Biol 709: 105-123 Tegeler CJ, Grange RW, Bogan DJ, Markert CD, Case D, Kornegay JN, Childers MK (2010) Eccentric contractions induce rapid isometric torque drop in dystrophin-deficient dogs. Muscle Nerve 42: 130-132		

23	Procedure 9 Systemic injection	Since DMD affect all muscles in the body, so we need to test systemic gene delivery. Local muscle AAV vector injection will be used to test the potency of the AAV vector with a small volume delivery to individual muscle. Systemic AAV delivery into pre-weanling dogs (< 1 wk to 4 wk of age): The vector will be in HEPES buffered saline. Systemic AAV vector injection will be performed in conscious pre-weanlings. Injection can be performed either by a single injection into the jugular vein or other accessible veins on the limbs with a syringe, by jugular vein catheterization, or via infusion with a pump. Briefly, the region over the vein will be shaved and cleaned with a Betadine scrub. An 18–22-gauge venous catheter (depending upon the size of the dog) will be placed into one of the jugular veins (or other veins) to facilitate AAV injection. Once the vector is injected, the catheter will be removed, and pressure applied to the jugular puncture site for hemostasis. Based on our experience and pilot studies, a pre-weanling can tolerate up to 25 ul/g body weight volume and 1E+12 AAV particles/g body weight (Pan et al, 2013; Yue et al, 2008; Yue et al, 2011). In neonatal injection, injected pre-weanlings will be returned to the mother and continuously be monitored for a total of two hours. Dogs will be carefully monitored for the eating, drinking, activity, body weight, body temperature, nursing and other the vital signs (breathing, heart rate etc.) for a period of two days (twice a day). After two days, we will continue to monitor the dog daily. After two weeks, we will monitor them twice weekly. After one month, they will be monitored as other dogs in the colony. We will especially pay attention to potential distress responses and complications, including lethargy, unresponsiveness to feeding or other stimulations, lung murmurs likely due to fluid build-up, and cardiovascular changes. Post-injection, we will monitor animals for 1 month for signs of decreased nursing (suckling) response, lung function and cardiovascular function. Potential complications are managed by care and stabilized feeding, including tube-feeding and administration of subcutaneous fluids. We will also contact with the OAR veterinary doctors regarding the complications and the treatments. If the condition does not improve, we will euthanize the individual. We have already published this procedure in 7 pre-weanlings (n=4, Yue et al 2008; n=3, Pan et al 2013). So far, all dogs survived the procedure. We only noticed one incidence in one dog which became lethargic and less responsive after AAV injection. After bottle feeding and subcutaneous fluid supplementation, the condition of the dog was stabilized. At 2 weeks of age, this dog survived through anesthesia and surgery uneventfully during muscle biopsy (Yue et al 2008). Systemic AAV delivery in non-newborn dogs (=> 2 months of age): Systemic AAV vector injection will be performed in conscious dogs by a single injection through the jugular, cephalic, femoral or saphenous vein or via infusion pump. Briefly, the skin area will be shaved and cleaned with a Betadine scrub. An 18-22 butterfly catheter will be used for injection. Only personnel who are trained in this procedure will be allowed to place catheters. Once the		any

	Procedure	Description of procedure	Building name	Room number or area
		vector is injected, the catheter will be removed, and pressure applied to the jugular puncture site for hemostasis. Based on our experience and pilot studies, non-newborn dogs can tolerate 5ml/kg body weight volume. After systemic injection, dogs will be carefully monitored for the eating, drinking, activity, body weight, body temperature and other the vital signs for a period of two days (twice a day). Post-injection, we will monitor animals for 1 month for signs of respiratory and cardiovascular dysfunction. If any abnormalities are observed, we will consulate with OAR veterinary doctor immediately and solve the issue in a case-by-case manner. If the condition does not improve, we will euthanize the individual. References Pan X, Yue Y, Zhang K, Lostal W, Shin JH, Duan D (2013) Long-term robust myocardial transduction of the dog heart from a peripheral vein by adeno-associated virus serotype-8. Hum Gene Ther 24: 584-594 Yue Y, Ghosh A, Long C, Bostick B, Smith BF, Kornegay JN, Duan D (2008) A single intravenous injection of adenoassociated virus serotype-9 leads to whole body skeletal muscle transduction in dogs. Mol Ther 16: 1944-1952 Yue Y, Shin J-H, Duan D (2011) Whole body skeletal muscle transduction in neonatal dogs with AAV-9. Methods Mol Biol 709: 313-329		
24	Procedure 10 Local muscle injection (for AAV and plasmids)	This method allows us to get good muscle injections without the need of opening skin. We may do the muscle injection with or without ultrasound guidance. Some dogs may require light sedation during the injection procedure. We have been using Nalbuphine (0.5mg/kg) or Butorphanol (0.4mg/kg) combined with Dexmedetomidine (3-15 micrograms/kg) IM for these dogs. To reverse the sedation, Atipamezole (0.2mg/kg or an equivalent volume of Dexmedetomidine) IM or IV may be used. Alternatively, dogs may be sedated using Acepromazine (0.02mg/kg) and Butorphanol (0.4mg/kg) IM. 1.) The dog skin hair will be shaved and cleaned with ethanol 2.) If needed, topical anesthetic (EMLA cream) will be applied directly on the dog skin where we will do injection; alternatively, injectable Lidocaine (2%) may be injected intradermally at the projected injection site. 3.) The dog will be gently restrained for the procedure 4.) The skin will be sterilized with 70% ethanol 5.) Recombinant AAV vector (sterilized) or plasmid will be directly injected percutaneously into the target muscle. References Shin JH, Pan X, Hakim CH, Yang HT, Yue Y, Zhang K, Terjung RL, Duan D (2013) Microdystrophin ameliorates muscular dystrophy in the canine model of Duchenne muscular dystrophy. Mol Ther 21: 750-757 Shin JH, Yue Y, Srivastava A, Smith B, Lai Y, Duan D (2012) A simplified immune suppression scheme leads to persistent micro-dystrophin expression in Duchenne muscular dystrophy dogs. Hum Gene Ther 23: 202-209	[redacted]	

	Procedure	Description of procedure	Building name	Room number or area
25	Procedure 11 Antihistamine drug use	There has been reported acute allergic symptoms after AAV vector delivery at other research institutions. Although we have never encountered similar situations in our lab, we would like to always be prepared in the event it does occur. Diphenhydramine (2mg/kg) administered subcutaneously relieved the symptoms in the reported case, thus this is our primary choice for an acute allergic response.	[redacted]	any
26	Procedure 12 Skin tattoo marking	Skin tattoo may be used to mark injection sites. 1.) The dog skin hair will be shaved and cleaned with ethanol. 2.) If needed, topical anesthetic (EMLA cream) will be applied directly on the dog skin where the skin will be tattooed 30-45 minutes prior to tattooing; alternatively, injectable Lidocaine (2%) may be injected intradermally at the tattoo site. 3.) The dog will be gently restrained for the tattoo procedure 4.) The skin will be sterilized with 70% ethanol 5.) The skin will be tattooed (two parallel lines approximately 1 cm apart in order to delineate the injection site) using a sterilized needle and 30-50 uL sterilized Starbrite tattoo ink (Investus Inc., Point Roberts, WA) 6.) The dog will be checked the next day for any sign of inflammation that may happen at the tattoo site.	[redacted]	
27	Procedure 13 Muscle marking with Evans blue dye	This will be a backup option. We have not needed to use this technique to date. Evans blue (0.1%, sterilized by filtering through a 0.2-micron filter, 50-100 uL) may be used as an alternative to the skin tattoo. This will be performed at the time of muscle injection with the viral vector. In this case, there will be no need to use topical anesthetics. References Shin JH, Pan X, Hakim CH, Yang HT, Yue Y, Zhang K, Terjung RL, Duan D (2013) Microdystrophin ameliorates muscular dystrophy in the canine model of Duchenne muscular dystrophy. Mol Ther 21: 750-757 Shin JH, Yue Y, Srivastava A, Smith B, Lai Y, Duan D (2012) A simplified immune suppression scheme leads to persistent micro-dystrophin expression in Duchenne muscular dystrophy dogs. Hum Gene Ther 23: 202-209	[redacted]	

	Procedure	Description of procedure	Building name	Room number or area
28	Procedure 14 Ultrasonic evaluation of dog muscle	A variety of imaging modalities have been studied as a possible adjunct to physical examination or as a means for further exploration of the pathophysiology of DMD. Dr. Samuel Wickline, a Professor at the Washington University School of Medicine, has developed a sensitive ultrasound imaging method to study muscle disease in dystrophic muscles. Dr. Wickline and his colleagues have previous used the prototype of this ultrasound device to study the mdx model of DMD and the Syrian hamster model of limb-girdle muscular dystrophy (Davison et al., 1994; Lanza et al., 1997; Wallace et al., 2007). The FDA approved commercial version of the ultrasound device can now be purchase from Interson Corporation (http://www.interson.com/products/seemore-153-usb-probes). Dr. Wickline has trained us on use of this imaging modality, and we will conduct ultrasound measurement using commercially available FDA-approved device in healthy, affected and carrier dogs at different age and gender. The probe we will be using is a general-purpose USB interface ultrasound transducer operating at 2.5-5.0 MHz. Since the device use a low power DC current supplied by a rechargeable Li-ion battery, it is very safe and represents no risk of electrical shock to the animal. Muscles may be evaluated all at once in one experiment or in multiple experiments. The ultrasound measurement Protocol: 1.) Awake or sedated dog will lie on its side and will be gently restrained for the duration of the measurement (5 to 10 min). 2.) The hair on top of the muscle of interest will be shaved to achieve optimal skin conductance. 3.) The skin will be cleaned with 70% ethanol and the transducer will be placed on the skin. 4.) Data will be collected using the USB port and analyzed with a standard computer. We expect the duration of data collection to be ~ 30 seconds/measurement and will be repeated 10-20 times for each muscle tested.	[redacted]	any

	Procedure	Description of procedure	Building name	Room number or area
29	Procedure 15 Ultrasound guided AAV vector muscle injection	We will use the ultrasound to guide our muscle AAV vector injection. This will increase the accuracy of muscle injection, and more evenly delivery the vector within the muscle tissues. Some dogs may require light sedation during the injection procedure. We have been using Nalbuphine (0.5mg/kg) combined with Dexmedetomidine (3-15 micrograms/kg) IM for these dogs. To reverse the sedation, Atipamezole (0.2mg/kg or an equivalent volume of Dexmedetomidine) IM or IV may be used. The procedure will be as the follows: 1.) Awake or sedated dog will lie on its side and will be gently restrained for the duration of the measurement (5 to 10 min). 2.) The hair on top of the muscle of interest will be shaved to achieve optimal skin conductance. 3.) The skin will be cleaned with 70% ethanol and the transducer will be placed on the skin. 4.) Locate and isolate the muscle will be injected. 5.) Insert the needle into the muscle with the guide of Ultrasound image. 6.) Deliver the AAV vector (or carrier control) solution into the muscle until whole muscle is saturated. References: Davison, G., Hall, C.S., Miller, J.G., Scott, M., and Wickline, S.A. (1994). Cellular mechanisms of captopril-induced matrix remodeling in Syrian hamster cardiomyopathy. Circulation 90, 1334-1342. Lanza, G.M., Scott, M.J., Davison, G., Hall, C.S., Christy, D.H., Miller, J.G., and Wickline, S.A. (1997). Angiotensin II receptor blockade in Syrian hamster (T0-2) cardiomyopathy does not affect microscopic cardiac material properties: implications for mechanisms of tissue remodeling. Cardiovascular drugs and therapy / sponsored by the International Society of Cardiovascular Pharmacotherapy 11, 521-529. Wallace, K.D., Marsh, J.N., Baldwin, S.L., Connolly, A.M., Keeling, R., Lanza, G.M., Wickline, S.A., and Hughes, M.S. (2007). Sensitive ultrasonic delineation of steroid treatment in living dystrophic mice with energy-based and entropy-based radio frequency signal processing. IEEE transactions on ultrasonics, ferroelectrics, and frequency control 54, 2291-2299.	[redacted]	

30	Procedure 16 Immune suppression: 16.1 Oral Cyclosporine and MMF	Since viral vectors may induce an immune response, we will need to use immune suppression to make sure that our gene therapy vector will not be eliminated by the body. We will use the following approaches: these approaches also apply to Group 5. In dogs, Cyclosporin acts primarily through the inhibition of Calcineurin which subsequently blocks the transcription of cytokines such as Interleukin-2 and prevents the activation of T cells (Kobayashi 2007 JVM, Payne 1986 JCLI). MMF also inhibits T and B lymphocyte proliferation. Dosage for short-term study: This protocol is exactly the same as we have published (Shin et al, 2013; Shin et al, 2012). Animals will start with oral cyclosporine (Neoral Oral Solution, 100 mg/ml, Novartis or TEVA, Sellersville, PA) twice daily at 15 mg/kg per dose (up to 30 mg/kg/dose if initial dose did not yield the desired blood level) to achieve a whole blood level concentration of 400-600 ng/ml (Gregorevic et al, 2009; Yuasa et al, 2007). Concurrently, animals will receive oral mycophenolate mofetil (MMF, CellCept Oral Suspension, 200 mg/ml, Roche Laboratories, Nutley, New Jersey) twice daily at 20 mg/kg per dose. Dosing will start at one week before AAV gene transfer and for four more weeks after AAV injection. (Total of 5 weeks of immune suppression) Please note, if the drugs from the above listed companies are in back-order, we may use drugs provided by other FDA approved suppliers. Dosage for long-term study: The initial phase (1 week before injection to four weeks after injection) will be the same as described for short-term study. Oral cyclosporine twice daily at 15-30 mg/kg per dose to achieve a whole blood trough level concentration of 400-600 ng/ml and oral mycophenolate mofetil twice daily at 20 mg/kg per dose. Starting from the fifth week, we will reduce the cyclosporine dose to one-third or one-fifth of the starting dose to achieve a whole blood trough level concentration of 50-200 ng/ml (twice daily at 1 to 6 mg/kg per dose). We will also reduce mycophenolate mofetil to one-fourth of the starting dose (twice daily at 5 mg/kg per dose). We will continue dosing the animals for a total 24 to 52 weeks. The maintenance dose is similar to what have been published by Wang et al (Wang et al, 2012). Since DMD patients are often put on long-term immune suppressive drugs (especially steroid), we expect our maintenance dose to not result in dramatic increase in toxicity or side-effect. Alternative oral immune suppression drugs: Schultz et al have used azathioprine instead of MMF every two days at 5 mg/kg (Schultz et al 2008 American Society of Gene Therapy Annual Meeting). We would like to keep this option open in case our animals show any untoward responses such as fever, unable to eat and drink, dramatic weight reduction (a 10% loss of body weight from initiation of treatment) etc. We will consult with OAR DVM doctors and deal with the problem in a case-by-case manner. A previous study by Wang et al used coadministration of antithymocyte globulin (SangStat, Fremont, CA) at 1 mg/kg for four days (2 day before injection followed by 2-day post-injection) (Wang et al, 2007). They found this method could help deplete T-cells and further reduce immune reaction. We would like to keep this option open for our		all

study too. Drug formula and protection: The cyclosporine is packaged in gelatin capsules (please note this is not powder form) or in liquid form. MMF is in liquid form or coated capsules and no special protection is needed for administrating drugs. Flavoring cyclosporine for oral administration: Cyclosporine has a very distinctive bitter taste, and this often leads to the rejection of the drug by dogs. To overcome this problem, we request to flavor cyclosporine with a pharmaceutical grade natural flavor (chicken, beef or bacon). These flavors are commonly used for compounding basic canine medications. They are soluble in oil-based medications and have no known side effects on canines. We will purchase the flavor from Letco Medical, AL, USA (www.letcomedical.com). The catalog number for bacon, beef and chicken flavor are 685125, 685127 and 691606, respectively. Flavored cyclosporine will be prepared freshly by mixing the drug with the flavor at the ratio of 1:1. The mixture will then be administered to dogs orally. We are aware that adding flavor will reduce the bitterness of the drug and thus help oral administration, but the added flavor will not alter known side effects of the drug. MMF Reconstitution: Due to inconsistent availability of oral suspension formulation of MMF, we will make an oral suspension from commercially available MMF capsules. MMF can also be purchased as 250 mg capsules. On literature search, we found a method (attached) to make MMF oral suspension in house (Venkataramanan R et al Ann Pharmacother. 1998;32:755-757; PubMed 9681090). The protocol is straightforward and oral suspension made with this method is very stable. We would like to include this MMF oral suspension preparation method in our ACUC protocol. Step-by-step method for making MMF oral suspension (all procedures conducted in a vertical flow hood). 1.) Add six 250 mg MMF capsules into a mortar. 2.) Add 7.5 ml Ora-Plus and mix to a uniform paste. 3.) Mix while adding 15 ml of cherry/bacon flavored syrup in incremental proportions. 4.) Transfer to a calibrated bottle. 5.) Rinse the mortar with the flavored syrup. 6.) Add more flavored syrup to bring the final volume to 30 ml to reach the final concentration of 50 mg/ml. Label with "shake well before use" The MMF oral suspension prepared with this method is stable for 210 days at 5°C, for 28 days at 25°C to 37°C, and for 11 days at 45°C. Reagents: a.) Capsular MMF: Commercial pharmacy b.) Ora-Plus: NDC#0574-0303-16. www.perrigo.com/rx c.) Cherry/bacon flavored syrup: Commercial pharmacy Reference: Venkataramanan R, McCombs JR, Zuckerman S, et al. Stability of mycophenolate mofetil as an extemporaneous suspension. Ann Pharmacother. 1998;32(7-8):755-757.[PubMed 9681090] The use of ketoconazole to reduce cyclosporine dose: Based on our experience, we do not expect the above proposed cyclosporine dose to result in substantial toxicity as described in the "Safety" section. However, if it indeed happens, we will consult with the OAR doctor to care for the involved the dog. At the same time, we will look for alternatives. Co-administration of Ketoconazole has been used by some investigators to reduce cyclosporine dose

in healthy dogs (Dahlinger 1998) (paper attached). Dahlinger 1998 et al used oral ketoconazole (Janssen Pharmaceutica Inc, Titusville, NJ). This leads them to reduce the cyclosporine dose by 75% at a ketoconazole dose of 12.7 to 15 mg/kg/orally once daily. After the cyclosporine level reach a stable level, they reduced ketoconazole dose to 4.2 to 5.3 mg/kg/day once orally. This leads them to reduce the cyclosporine dose by 38%. If needed, we will cautiously test this approach in one normal and one dystrophic dog (since this approach may leads to weight loss and transient hypoalbuminemia of unknown clinical significance). If it works, we will adapt this protocol. If not, we will not pursue this further. We have not needed to test this approach yet in our research. Co-administration of Metoclopramide and flavored cyclosporine: We have been previously approved to administer oral immune suppressive medications, flavored cyclosporine and mycophenolate to dogs. However, some dogs lose their appetite and show symptoms of nausea, vomiting and diarrhea. A review of the literature suggests that all the symptoms are common side effects associated with oral cyclosporine administration [1-3]. To overcome this problem, we would like to co-administer Metoclopramide with our immune suppression regimen. Metoclopramide is an antiemetic and gastrointestinal prokinetic drug. It is commonly used by veterinary to relive nausea, vomiting and reflux disease in small animals [4]. Recent studies suggest that co-administration of Metoclopramide and cyclosporine effectively prevented nausea, vomiting and diarrhea in dogs for up to 16 weeks without causing any side effects [5, 6]. Importantly, there is no need to adjust the dose of cyclosporine when Metoclopramide is co-administrated [6]. To our knowledge, there is also no report showing drug interactions between Metoclopramide and pharmaceutical grade natural flavor (flavor is used to reduce the bitter taste of cyclosporine). In veterinary practice, Metoclopramide is usually administrated at the dose of 0.2-0.5 mg/kg orally every 6 to 8 hours (3 to 4 times a day) to dogs (see following links and attached reference "Kosecki 2003"). We would like to administer Metoclopramide orally at 0.5 mg/kg with every cyclosporine dose (2 to 3 times per day). Cyclosporine dose monitoring: Blood will be drawn from enrolled animals twice weekly for cyclosporine levels. Cyclosporine levels can be measured using the CSP Monoclonal Whole Blood Assay at the MU Hospital. The ideal cyclosporine concentration is 200 to 600 ng/mL. The laboratory results will be evaluated by the investigators. Veterinarians will also be consulted if needed. Abnormal values will be treated case-by-case according to the suggestion of the veterinarian. Safety: A blood sample will be drawn for CBC and Maxi twice a week or weekly. Animals will be carefully monitored daily by caregiver and/or laboratory personnel approved in this protocol for their wellbeing (such as body weight, food and water intake and general condition). If animals show any untoward responses such as fever, unable to eat and drink, dramatic weight reduction (a 10% loss of

Procedure	Description of procedure	Building name	Room number or area
	body weight from initiation of treatment), abnormal liver and/or kidney function. We will consult with OAR veterinarians and deal with the problem in a case-by-case manner. Most likely, we will immediately stop the dosing or reduce the drug dose according to veterinarian suggestion. References: 1. Robson, D., Review of the pharmacokinetics, interactions and adverse reactions of cyclosporine in people, dogs and cats. Vet Rec, 2003.n152(24): p. 739-48. 2. Wilson, D.V., A.T. Evans, and W.A. Mauer, Influence of metoclopramide on gastroesophageal reflux in anesthetized dogs. Am J Vet Res, 2006. 67(1): p. 26-31. 3. Tinker, J. and A.G. Cox, Effect of metoclopramide on transport in the small intestine of the dog. Gut, 1969. 10(12): p. 986 -9. 4. Kretzing, S., et al., In vivo assessment of antiemetic drugs and mechanism of lycorine-induced nausea and emesis. Arch Toxicol, 2011. 85(12): p. 1565-73. 5. Cohen, L., S. Zabel, and R.A. Rosychuk, Relationship of body weight to maintenance cyclosporine a dose in canine atopic dermatitis. J Am Anim Hosp Assoc. 2014. 50(3): p. 174-80. 6. Radwanski, N.E., et al., Effects of powdered whole grapefruit and metoclopramide on the pharmacokinetics of cyclosporine in dogs. Am J Vet Res, 2011. 72(5): p. 687-93. 7. Wadhwa, N.K., et al., The effect of oral metoclopramide on the absorption of cyclosporine. Transplantation, 1987. 43(2): p. 211-3. http://www.petcarerx.com/medication-guides/using-metoclopramide-tablets-as-vomiting-treatment/1051?page=all http://www.1800petmeds.com/Metoclopramide-prod10210.html http://www.medi-vet.com/Metoclopramide-Oral-Solution-p/10446.htm http://ratguide.com/meds/miscellaneous/metoclopramide.php Plumb, D.C. (2008). Metoclopramide HCl. Plumb's veterinary drug handbook (6th ed. pp. 606-608).		

	Procedure	Description of procedure	Building name	Room number or area
31	Procedure 16 Immune suppression: 16.2 SQ Cyclosporine	This is an alternative approach for dogs that cannot take the immune suppressive drugs orally. However, we want to point out that injectable cyclosporine may not always be in stock. They can be backordered for substantial amount of time. Nevertheless, we will investigate an outside pharmacy, if the injectable is unavailable from the pharmaceutical company. Cyclosporine and mycophenolate mofetil can be purchased in SQ injection formula in pharmaceutical grade (commercially available). For example, SQ injectable cyclosporine can be purchased from Sandoz Pharma (Princeton, NJ). They will be administered twice daily. Since SQ drugs may have a better bioavailability, we will start the drug at a lower dose (cyclosporine from 5 mg/kg twice daily and MMF from 10 mg/kg twice daily). We will use the same blood levels as described for oral drugs (see above) as our guide to determine if we need to increase the dose. We will also monitor cyclosporine level in the blood as described above. Both drugs have been used in dogs via SQ injection by other investigators in the literature. We will take same precautions and safety monitoring as we described for oral immune suppression. References 1. Gregorevic P, Schultz BR, Allen JM, Halldorson JB, Blankinship MJ, Meznarich NA, Kuhr CS, Doremus C, Finn E, Liggitt D, Chamberlain JS (2009) Evaluation of vascular delivery methodologies to enhance rAAV6-mediated gene transfer to canine striated musculature. Mol Ther 17: 1427-1433 2. Shin JH, Pan X, Hakim CH, Yang HT, Yue Y, Zhang K, Terjung RL, Duan D (2013) Microdystrophin ameliorates muscular dystrophy in the canine model of Duchenne muscular dystrophy. Mol Ther 21: 750-757 3. Shin JH, Yue Y, Srivastava A, Smith B, Lai Y, Duan D (2012) A simplified immune suppression scheme leads to persistent micro-dystrophin expression in Duchenne muscular dystrophy dogs. Hum Gene Ther 23: 202-209 4. Wang Z, Kuhr CS, Allen JM, Blankinship M, Gregorevic P, Chamberlain JS, Tapscott SJ, Storb R (2007) Sustained AAV mediated Dystrophin Expression in a Canine Model of Duchenne Muscular Dystrophy with a Brief Course of Immunosuppression. Mol Ther 15: 1160-1166 5. Wang Z, Storb R, Halbert CL, Banks GB, Butts TM, Finn EE, Allen JM, Miller AD, Chamberlain JS, Tapscott SJ (2012) Successful regional delivery and long-term expression of a dystrophin gene in canine muscular dystrophy: a preclinical model for human therapies. Mol Ther 20: 1501-1507 6. Yuasa K, Yoshimura M, Urasawa N, Ohshima S, Howell JM, Nakamura A, Hijikata T, Miyagoe-Suzuki Y, Takeda S (2007) Injection of a recombinant AAV serotype 2 into canine skeletal muscles evokes strong immune responses against transgene products. Gene Ther 14: 1249-1260	[redacted]	all

32	Procedure 16 Immune Suppression: 16.3 Oral Prednisolone	Some dogs cannot tolerate cyclosporine. Dr. Jerry Mendell used Prednisolone at 1 mg/kg/day in a clinical trial of AAV gene therapy for spinal muscular dystrophy (SMA). According to the data Dr. Mendell presented in conferences, this immune suppression scheme worked great in SMA patients. According to a study by Dr. Joe Kornegay, his team achieved excellent AAV gene transfer in dystrophic dog under immune suppression with oral prednisone at 1mg/kg/day starting from 1 week before AAV injection and continuing until four weeks after AAV injection. Based on these observations, we have used corticosteroids as a potential immune suppression method in all of our AAV injection studies. Below are our 4 Prednisolone protocols in which we have successfully administered without adverse effects noted. 1.) Low dosage: Prednisolone or prednisone will be administered at 1 mg/kg PO SID 3 days before AAV treatment and then continued at the same dosage for 4 more weeks post injection. The dosage will not be tapered for the low dose. 2.) High dosage: Prednisolone or prednisone will be administered at 4 mg/kg PO SID for 3 days before AAV treatment and continued at this dosage for 1 week post injection. Then, the dosage will be reduced to 2 mg/kg PO SID for 1 week; then 1 mg/kg PO SID for 1 week; then 0.5 mg/kg PO SID for 1 week; and then 0.5 mg/kg PO EOD for 1 week. 3.) Extended High Dose: Prednisolone or prednisone will be administered at 4 mg/kg PO SID for 3 days before AAV treatment and continued at this dosage for 1 week post injection. Then, the dosage will be reduced to 2 mg/kg PO SID for 1 week, then 1 mg/kg PO SID for the duration of study, up to 365 days total. Justification: Currently, we taper down prednisolone to 0.5 mg/kg in our protocol. While using this protocol, we observed a clear immune response at the time of prednisolone tapering down. We believe tapering down to 0.5 mg/kg may have allowed the immune system to begin responding since the level of immune suppression is beginning to drop. Maintaining a dosage of 1 mg/kg would allow for a constant suppression of the immune system for a longer duration of time to avoid the immune response to gene therapy. The goal is to maintain a steady level of immune suppression for the entire duration of therapy (up to 365 days total). 4.) Super High Dose: Prednisolone or prednisone will be administered at 4 mg/kg PO SID for 3 days before AAV treatment and continued at this dosage for 1 week post injection. Then, the dosage will be reduced to 3 mg/kg PO SID for 2 weeks, then 2 mg/kg PO SID for 4 weeks, then 1 mg/kg PO SID for another 8 weeks. Justification: We believe that tapering down and stopping the immune suppression may have allowed the immune system to begin responding to the gene therapy vector. We hypothesize that (1) a gentler and slower tapering down protocol and (2) continued prednisolone dosing may prevent the immune response to gene therapy. On literature search, we find that clinicians are currently using the following dosing regimen in treating canine autoimmune diseases such as IMHA. Specifically, clinicians start with 4 mg/kg for three weeks, then tapering down to 3 mg/kg (a 25% decrease)		all

Procedure	Description of procedure	Building name	Room number or area
	for four weeks, then tapering down the dose to 2 mg/kg (50% decrease of the initial dose) for another 4 weeks (Archer T and Mackin A Today's Veterinary Practice 2014 March/April page 41-46). Hence, we designed our new tapering down protocol based on this reference paper. Formulation: A pharmaceutical grade powder of Prednisolone or prednisone will be purchased from the veterinary teaching hospital pharmacy. The powder will be formulated and flavored using the Flavor Rx, https://www.flavorx.com/veterinary/ (see attachment). Safety: We will adhere to the following guideline to ensure treatment safety. A blood sample will be drawn for CBC and Maxi before beginning immune suppression and at least once a week for the duration of the study. Animals will be carefully monitored daily by caregiver or/and laboratory personnel approved in this protocol for their wellbeing (such as body weight, food and water intake and general condition. If animals show any untoward responses such (ex: fever), unable to eat and drink, dramatic weight reduction (a 10% loss of body weight from initiation of treatment), abnormal liver and/or kidney function. We will consult with veterinary staff and deal with the problem on a case-by-case manner. Most likely, we will immediately stop the dosing or reduce the drug dose according to veterinary suggestion. If deemed necessary by veterinary advice, the animal may be terminated to maintain quality of life.		

33	Procedure 16 Immune Suppression: 16.4 Combine immune suppression of Prednisolone, Cyclosporine and MMF	We are currently using two different approaches for immune suppression in our dogs. In one approach, we co-administered flavored cyclosporine (CsA), MMF and Metoclopramide to dogs (normal, affected or carrier). We observed minimal side effects (see above, oral cyclosporine). In another approach, we have been using prednisolone or prednisone (low, high, and super high dosage) as an immune suppression regimen (see above, oral prednisolone). The dogs have also tolerated this very well. In dogs, CsA acts primarily through the inhibition of calcineurin, which subsequently blocks the transcription of cytokines such as Interleukin-2 and prevents the activation of T cells. MMF also inhibits T and B lymphocyte proliferation. Metoclopramide is an antiemetic and gastrointestinal prokinetic drug. It is commonly used by veterinary to relive nausea, vomiting and reflux disease in small animals. Co-administration of Metoclopramide and cyclosporine has effectively prevented nausea, vomiting and diarrhea in dogs without causing any side effects. Corticosteroids have two main immunosuppressive effects on the immune system including (1) the sequestration of CD4+ T-lymphocytes in the reticuloendothelial system (RES), and (2) inhibition of both proliferation and function of lymphocytes via inhibition of lymphokines and cytokines. Here, we will combine these two different immune suppression regimens together in the hopes of more effectively blocking the untoward immune response following AAV gene therapy in dogs. The rationale of combining immunosuppressive drugs is to achieve synergistic effects. Steroids will remain the backbone of immune therapy because of their potent anti-inflammatory and immunosuppressive effects. The addition of CsA will allow a reduction in the steroid dose to avoid and/or minimize adverse effects of high dose steroid use. Similarly, the combined regimen may allow us to achieve a more rapid tapering of the steroid dose, and thereby avoid and/or minimize adverse effects of high dose steroid use. The combined prednisolone (or prednisone) and cyclosporine regimen has been used in the dogs to treat atopic dermatitis (Dip, Carmichael et al., 2013). Specifically, prednisolone was given at the dose of 1 mg/kg once daily for 7 days, followed by alternate dosing for 14 days. Cyclosporine was given at the dose of 5 mg/kg orally, once daily for 28 days. Treated dogs tolerated this protocol well. The combined prednisolone (or prednisone) and cyclosporine regimen has been used in GRMD dogs to see if this protocol can be used to treat DMD in the canine model (Barthelemy, Uriarte et al., 2012). Specifically, prednisolone was given at the dose of 2 mg/kg/d. Cyclosporine was given at the starting dose of 20mg/kg/d to reach a trough level of 300-400 ng/ml. The authors treated affected dogs for 7 months. Treated dogs tolerated this protocol well. Dosage: The following drugs will be co-administered as described below for a max study duration of 90 days. Flavored Prednisolone or prednisone will be administered at 4mg/kg PO SID for 10 days, then will be reduced to 2mg/kg PO SID for 1 week, then 1mg/kg PO SID throughout the remaining study duration. Flavored		all

Procedure	Description of procedure	Building name	Room number or area
	cyclosporine will be administered at 15-30 mg/kg PO BID throughout the study duration. The dosage will be adjusted to achieve a whole blood level concentration of 200-600 ng/ml. Alternatively, we may perform SQ cyclosporine as described in detail in section 16.2. Mycophenolate mofetil (MMF) will be administered at 20 mg/kg PO BID throughout the study duration. Metoclopramide will be administered at 0.5 mg/kg PO BID with every cyclosporine dose throughout the study duration. Cyclosporine dose monitoring: Blood will be drawn from enrolled animals twice weekly for cyclosporine levels. Cyclosporine levels can be measured using the CSP Monoclonal Whole Blood Assay at the MU hospital. The target cyclosporine concentration is 200 to 600 ng/mL. The laboratory results will be evaluated by the investigators. Veterinary doctor will also be consulted. Safety: A blood sample will be drawn for CBC and Maxi twice a week or weekly. Animals will be carefully monitored daily by caregiver or/and laboratory personal approved in this protocol for their wellbeing (such as body weight, temperature, food and water intake and general condition. If animals show any untoward responses such (ex: fever), unable to eat and drink, dramatic weight reduction (a 10% loss of body weight from the initiation of treatment), abnormal liver and/or kidney function, we will consult with OAR veterinarians to determine appropriate approaches to reduce side-effects. For example, we may immediately stop the dosing or reduce the drug dose according to veterinary suggestion. Following the up to 90 days of immune suppression, dogs will continue to be monitored based on experimental plan and design on an independent basis. (ie. blood collection based on age time points in the animal's life.) Dogs will also be monitored for any changes or abnormalities related to immune suppression following the conclusion of treatment. References Barthelemy I, Uriarte A, Drougard C, Unterfinger Y, Thibaud JL, Blot S (2012) Effects of an immunosuppressive treatment in the GRMD dog model of Duchenne muscular dystrophy. PLoS One 7: e48478 Dip R, Carmichael J, Letellier I, Strehlau G, Roberts E, Bensignor E, Rosenkrantz W (2013) Concurrent short-term use of prednisolone with cyclosporine A accelerates		

	Procedure	Description of procedure	Building name	Room number or area
34	Procedure 16 Immune Suppression: 16.5 Tacrolimus	We would like to add Tacrolimus (injectable) as an immune suppression method to our protocol. Tacrolimus is commonly used in humans to prevent organ rejection by the host immune system. Dr. Akiko Ishii MD, Ph. D et al. recently showed that daily tacrolimus injection (0.06 mg/kg intramuscularly) in non-human primates can effectively suppress the immune response induced by adeno-associated virus (AAV) for at least 42 weeks without obvious toxicity (https://ars.elscdn. com/content/image/1-s2.0-S2329050120300991-mmc2.pdf). We would like to add Tacrolimus (Prograf) to our protocol as an immunosuppression option. We plan to administer tacrolimus using the same protocol published by Dr. Akiko Ishii et al (the dosage of 0.06mg/kg intramuscularly once daily). Dogs will be monitored for toxicity and overall health in accordance to our approved immune suppression safety guidelines stated below. We will adhere to the following guidelines to ensure treatment safety: A blood sample will be drawn for CBC and Maxi at least once a week for the duration of the study. Animals will be carefully monitored daily by caregiver or/and laboratory personnel approved in this protocol for their wellbeing such as body weight, food and water intake and general condition. If animals show any untoward responses such (ex: fever), unable to eat and drink, dramatic weight reduction (a 10% loss of body weight from initiation of treatment), abnormal liver and/or kidney function, we will consult with OAR veterinarians and deal with the problem in a case-by-case manner. 1.) How long are you planning on administering daily IM injections? a.) In Ishii et al study, the authors administered daily Tacrolimus for up to 42 weeks without obvious toxicity. Hence, we will limit our treatment duration to ≤ 42 weeks. 2..) Tacrolimus is only available in oral, topical or IV formulations. Can you have the oral form compounded into an oral suspension for administration? a.) In Ishii et al paper, the authors used IM injection using the drug that is for IV injection use. Since tacrolimus is supplied in both oral form and iv form. We will start with oral form. If animals cannot tolerate the oral form, we will switch to injection form as described by Dr. Ishii. See our communication with Dr. Ishii "Ishii 2020 MTM.FK506_Response from the authors". Please also see page 7 of "Ishii 2020 MTM.FK506_Astellas Pharma's Prograf Injection 5mg" Both are also attached in the Attachment Section.	[redacted]	all

35	Procedure 16 Immune Suppression: 16.6 Conduct a pilot study administering high dose Prednisolone with intramuscular Tacrolimus injection in one puppy	We are currently approved to administer oral Prednisolone at a dosing regimen of 4 mg/kg PO SID for 10 days. Then, the dosage will be reduced to 3 mg/kg PO SID for 2 weeks, then 2 mg/kg PO SID for 4 weeks, then 1 mg/kg PO SID for the remainder of the study up to 180 days total. This dosage was tolerated well during the pilot study. We also were approved to administer Tacrolimus intramuscularly at a dosage of 0.06mg/kg SID for up to 42 weeks (294 days). We have previously shown that high-dose Prednisolone successfully mitigated the immune response to systemic AAV micro-dystrophin gene therapy in affected puppies. Surprisingly, we just found that high-dose Prednisolone alone is insufficient to mitigate the immune response to systemic AAV CRISPR therapy in affected puppies. To overcome the immunological barrier to systemic AAV CRISPR therapy for DMD in puppies, we would like to experimentally test in one puppy (> 1 month old) whether combining Tacrolimus and high-dose Prednisolone can lead to more effective immune suppression. Since Tacrolimus and Prednisolone use different mechanisms, there is a high chance that we may see synergistic effect when two drugs are combined. The prednisolone will be administered based on our approved method of 4 mg/kg PO SID for 10 days; then 3 mg/kg PO SID for 2 weeks; then 2 mg/kg PO SID for 4 weeks, then 1 mg/kg PO SID for the remainder of the study up to 1 days. Tacrolimus would be administered at 0.06mg/kg IM SID for up to 180 days of total treatment. We will monitor this puppy closely for signs of toxicity following the below guidelines. A blood sample will be drawn for CBC and Maxi once a week for the duration of the study and consult with the veterinary doctors if we notice any abnormalities. We will strictly follow the instructions of the veterinary doctors. We will closely monitor kidney and liver values and adjust immune suppression dosages according to veterinary advice. · Animals will be carefully monitored daily by caregiver or/and laboratory personnel approved in this protocol for their wellbeing and general condition (including but not limited to mentation, activity level, appetite, urine/fecal quality, behavior and body condition score). · We will monitor food and water intake daily. If the dog shows reduction in food intake, we will supplement with canned food. If the dog continues to be inappetence we will immediately consult with the veterinary doctors. We will strictly follow the instructions of the veterinary doctors. If the condition of the experimental dogs does not improve after interventions recommended by the veterinary doctors, we will euthanize the dog. · Each dog will be assessed daily for activity level and overall mobility to monitor muscle health. · Each dog will be monitored daily for urine quality. If clinical signs present such as (licking at urogenital area, difficulty or strained urination, alterations in urine color, turbidity, odor or frequency) we will consult with veterinary staff for guidance and possible urinalysis. Urinalysis may consist of a free catch mid-stream or cystocentesis following veterinary recommendation.		all

	Procedure	Description of procedure	Building name	Room number or area
36	Procedure 17 Chest, Limb and Joint Radiographs	We add chest radiographs, to evaluate abnormality in heart growth during development (limited to once a month in the first six months of the dog age, following with once a year if abnormality is noted during growth) The chest radiographs will be applied for some selected affected and normal dogs. In addition, we also will take Joint radiographs, to evaluate joint contracture in affected dogs. We need to take a radiograph during the following times: 3, 6, 9, 12, 18, 24-month-old and then once annually for up to 12 years of age. We will limit to a radiograph every three months in the first year of life and then one every six months for the second year, and then once yearly, up to 12 years of age. This will be done with the X-ray machine in the ARC with assistant of a veterinarian. This procedure will be conducted in conscious animals, and the veterinarian will determine if the dog needs to be sedated.	[redacted]	

37	Procedure 16 Immune Suppression: 16.7 Triamcinolone acetonide as an immunosuppressive agent	Prednisolone is a widely used immunosuppressive agent to inhibit immune response to viral vector therapy. We have utilized several dosing strategies of prednisolone to attempt to achieve optimal AAV expression while limiting potential adverse effects of corticosteroid administration. A recent paper demonstrated the usefulness of triamcinolone acetonide as the immunosuppressive agent in mice for AAV therapy (Gan et al., 2025 https://www.cell.com/molecular-therapy-family/methods/fulltext/S2329-0501(24)00215-8). They found that mice had better expression of the therapeutic gene when triamcinolone acetonide was used. We would like to utilize a similar strategy in the canine model of DMD. Triamcinolone acetonide is estimated to be 6-8 times more potent compared to prednisolone while also having a longer duration of action. These two principles are ideal for minimizing the immune response to AAV therapies. There have been studies conducted to evaluate effective dosing of triamcinolone acetonide for clinical cases, comparing to prednisolone dosing (Ganz, 2012 https://pubmed.ncbi.nlm.nih.gov/22681547/; Mueller, 2021 https://onlinelibrary.wiley.com/doi/10.1111/vde.12933). It is generally estimated that 5mg of prednisolone is roughly equivalent to 4mg of triamcinolone acetonide. Triamcinolone acetonide is also commonly used in the veterinary field for localized and systemic immune dysfunctions, as well as in combination for topical dermatitis cases. Based on the estimated effective dosage ranges and our approved prednisolone dosage strategies we propose the following dosing strategies. 1.) High dosage: Triamcinolone acetonide will be administered at 3.2mg/kg PO SID for 3 days before AAV treatment and continued at this dosage for 1 week post injection. Then, the dosage will be reduced to 1.6mg/kg PO SID for 1 week; then 0.8mg/kg PO SID for 1 week; then 0.4mg/kg PO SID for 1 week; and then 0.4mg/kg PO EOD for 1 week. 2.) Extended High Dose: Triamcinolone acetonide will be administered at 3.2mg/kg PO SID for 3 days before AAV treatment and continued for 1-week post-injection. Then, the dosage will be reduced to 1.6mg/kg PO SID for 1 week, then 0.8mg/kg PO SID for the duration of study, up to 365 days total. Formulation: A pharmaceutical grade powder triamcinolone acetonide will be purchased from a medical supplier (MedChem Express). The powder will be formulated and flavored using the Flavor Rx, https://www.flavorx.com/veterinary (see attachment). Safety will be evaluated like our approved methodology for other immune suppression protocols. A blood sample will be drawn for CBC and Maxi before beginning immune suppression and at least once a week for the duration of the study. Animals will be carefully monitored daily by caregiver or/and laboratory personnel approved in this protocol for their wellbeing such as body weight, food and water intake and general condition. If animals show any untoward responses such as fever, unable to eat and drink, dramatic weight reduction (a 10% loss of body weight from initiation of treatment), abnormal liver and/or kidney function we will consult with veterinary staff and deal with the problem on a case-by-case manner.	 all

Procedure	Description of procedure	Building name	Room number or area
	Most likely, we will immediately stop the dosing or reduce the drug dose according to veterinary suggestion. If deemed necessary by veterinary advice, the animal may be terminated to maintain quality of life. It is anticipated that adverse effects will be similar to those seen with ongoing prednisolone administration.		

7. Substances Used in Animals

1. Substances Used in Animals

   List the substances you will give the animals here (including vehicles given to controls, hazards, radiation, etc.):

	Substance	Amount/Dose/Volume	Route	Frequency/ Duration	Hazard	Pharmaceutical Grade
1	Cyclosporine	1-30 mg/kg/day	PO	BID	Yes	Yes
2	Cyclosporine	5 mg/kg/day; 0.1-0.5 ml/ kg/day	SQ	BID	Yes	Yes
3	Mycophenolate mofetil	5-20 mg/kg/day	PO	BID	No	Yes
4	Mycophenolate mofetil	10 mg/kg/day; 0.4ml/ kg/day	SQ	BID	No	Yes
5	Azathioprine (optional)	5 mg/kg	PO	EOD	No	Yes
6	Tattoo dye	30-50 ul	ID	prn	No	No
7	Dextrose solution	5% as solvent for dobutamine	IV	prn	No	Yes
8	Dobutamine	500 ug/ml; start at 5ug/ kg/min, increase at 10ug steps up to a max of 35ug/kg/min. Holding each stage for 10-15 mins.	IV, 500 ug/ml	N/A	No	Yes
9	Gadolinium-DTPA	0.2 mmol/kg IV once for each MRI procedure	IV	once per procedure	No	Yes
10	Antithymocyte globulin	1 mg/kg	PO	SID	No	Yes
11	Ketoconazole	4.2-15 mg/kg/day	PO	BID	No	Yes
12	Plasmid	vary depending on study	IM, IV	N/A	Yes	No
13	Diphenhydramine	2 mg/kg	SQ, IV	prn	No	Yes
14	Evans Blue Dye	0.1% in 50-100 ul	IM	prn	No	No
15	AAV Vector	vary depending on study	IM, IV	N/A	Yes	No
16	DNA	delivered in AAV	delivered in AAV	N/A	Yes	No
17	HEPES buffered saline	AAV carrier	IM, IV	N/A	No	No
18	Metoclopramide	0.5 mg/kg	PO	BID or TID	No	Yes
19	Physiological saline	prn	IM, IV, SQ	prn	No	Yes

	Substance	Amount/Dose/Volume	Route	Frequency/ Duration	Hazard	Pharmaceutical Grade
20	Prednisolone	vary depending on study	PO	N/A	No	Yes
21	Prednisone	vary depending on study	PO	N/A	No	Yes
22	Cas-9	delivered in vector	IM, IV	N/A	Yes	No
23	Fortiflora (probiotic)	1 pkg to 60ml milk replacer	PO	prn	No	Yes
24	Serum from healthy adult dog	Each affected puppy will receive 16cc (via multiple doses) over first week of life; not to exceed 60 ml	SQ	once per puppy over the course of 1 week	No	No
25	Tacrolimus	0.06 mg/kg	IM	SID	No	Yes
26	Tacrolimus	0.2 mg/kg	PO	BID	No	Yes
27	Iodixanol	20-50ml	IV	based on procedural needs determined by interventional cardiologist	No	Yes
28	Iopamidol	20-50ml	IV	based on procedural needs determined by interventional cardiologist	No	Yes
29	Antisense Oligonucleotides	2.5 ml/kg	SQ	SID/weekly	Yes	No
30	Norepinephrine	Study doses of 0.75ml, 1.5ml, 3ml, 6ml, 12.5ml, 25ml	IA	twice during terminal bloodflow function given over 30s	No	Yes
31	SAT-3247	10mg/kg	PO	daily	Yes	No
32	Triamcinolone acetonide	3.2mg/kg; 1.6mg/kg; 0.8mg/kg; 0.4mg/kg	PO	SID	No	Yes

2. Non-Pharmaceutical Grade Substances

For those substances that are marked "no" as pharmaceutical grade, list a justification in the space below. Also, include instructions for how they will be mixed to maintain sterility and adjust pH.

For those items listed as unavailable as pharmaceutical grade; Evan's blue agent, DNA, AAV, ASO and plasmid will be sterilized by filtering through different sized filter to remove pathogens. HEPES buffered saline is sterilized by autoclave. Serum will be collected following aseptic technique for blood collection into a sterile tube via vacutainer. Administration to puppies will follow same aseptic technique. Tattoo dye will be kept in

the sterilized bottle with small amounts being removed for administration when needed via a sterile needle.

3. Substances Used in Animals Personal Protective Equipment (PPE)

PPE is needed to safely handle most materials in the laboratory. In general, a minimum of gloves and lab coat should be used. Other substances would require more PPE such as eye protection, respiratory protection, fume hood, etc. Please notify laboratory members if there are any special precautions that need to be taken when working with the above substances.

Describe the PPE required to handle these substances. You may group substances (e.g., "All substances" or "non-hazardous substances") if all or some use the same PPE. Please list any substances needing alternative or additional PPE separately. You do not have to include additional PPE needed for work with hazards as that will be described in the Hazards section, however, you may include here as well if you wish.

	Substance	Gloves	Eye Protection	Lab Coat	Face Mask	Fume hood	Biosafety cabinet	Double-Gloves	Other	Other PPE
1	Non-hazardous substances	☑	☑	☑	☑	☐	☐	☐	☑	head cover

Hazardous Agent

If you marked "yes" under Hazard, please complete the "Hazardous Materials" Section that follows.

8. Hazardous Materials

1. Will you use any Biological Hazards?

● Yes ○ No

A. Biological Hazard

List all biological hazards that will be used in live animal work.

	Agent or type of hazard	Donor species	Receiving species	Dose	Route/ Volume of Admin.	Frequency of Admin.	Other
1	AAV Vector	X	dogs	Vary depending on study	IM, IV	Once per animal group	
2	DNA	Human	dog	Will be used to make AAV	Will be delivered in format of AAV vector	Once per animal	
3	DNA	Dog	Dog	Will be used to make AAV	Will be delivered in format of AAV vector	Once per animal	
4	Plasmid	Dog	Dog	200ug in 500ul	IM	1 plasmid per muscle, 10 plasmids per dog (in 10 different muscles)	
5	Cas-9	from Bacteria	dog	will be used to make vector	IM, IV	Once per animal	
6	Antisense Oligonucleotides	X	Canine	0.5-20mg/kg	SQ	weekly	
7	SAT-3247	X	Canine	10mg/kg	PO	daily	

B. IBC Protocol Number (if applicable for recombinant DNA or biological materials)

List your IBC Approval Number or attach your current IBC application. (Include attachments in the attached files section.)

15240

- ☐ Unsubmitted
- ☐ Submitted
- ☑ Approved

i. Expiration date

08/15/2027

C. Biological Hazard - Anticipated Effect(s)

List any anticipated effect(s) of biological hazards on animal.

AAV is considered BL1 by NIH. We don't anticipate any side effects from AAV itself.

D. Biological Hazard - Housing/Procedure Sites

Where do you anticipate housing/working with animals receiving hazardous or potentially hazardous biological agents? Coordinate with the facility manager then list building and room numbers below.

	Agent	Receiving species	Building	Room or Area	Housing	Procedure
1	All hazards	Dog	[redacted]	Housed in canine rooms/ Procedure to be completed in	☑	☑

E. Biological Hazard - Animal Identification

Explain how animals treated with a biological hazard will be identified (ex. cage card, ear tag, etc.)

- ☑ Cage Card
- ☑ Chip
- ☐ Door Sign
- ☑ Other

i. Please specify:

implanted electronic microchip number

F. Hazardous Agents or By-Products /Presence

The biological hazard or by-products may be present in which of the following?

- ☐ None
- ☐ Feces/Urine/Bedding
- ☐ Saliva
- ☐ Blood
- ☐ Aerosols
- ☐ Animal bite/scratch
- ☑ Animal carcasses/tissues
- ☐ Surgical site wound or sore
- ☐ Other

G. Biological Hazard - Personal Protection Equipment (PPE) and Engineering Controls

PPE to be worn when handling biological hazards. LIDR ABSL-3 includes protective suit, shoe covers, double gloves, full-face PAPR.

	Biological Hazard	Gloves	Eye Protection	Lab Coat	Double-Gloves	Face Mask	Biosafety cabinet	LIDR ABSL-3	Other	Other PPE
1	AAV Vector	☑	☐	☑	☐	☑	☑	☐	☑	head cover
2	DNA	☑	☐	☑	☐	☑	☑	☐	☑	head cover
3	Plasmid	☑	☐	☑	☐	☑	☑	☐	☑	head cover
4	Cas-9	☑	☐	☑	☐	☑	☐	☐	☑	head cover
5	Antisense Oligonucleotides	☑	☐	☑	☐	☑	☐	☐	☐	
6	SAT-3247	☑	☐	☑	☐	☐	☐	☐	☐	

H. Additional Information

List additional information, i.e., special precautions for pregnant women, immunocompromised individuals, special handling, or storage, etc.

N/A

2. Will you use any Chemical Hazards?

● Yes ○ No

A. Chemical Hazards

List all chemical hazards that will be used in live animal work.

	Agent or type of hazard	Receiving species	Dose	Route/Volume of Admin.	Frequency of Admin.	Other
1	Cyclosporine	dog	1-30 mg/kg/ day	PO	BID	

B. Is this an FDA Approved Drug?

● Yes ○ No

C. Chemical Hazard - Anticipated Effect(s)

List any anticipated effect(s) of hazardous chemical on animal.

Immune suppression, Gastrointestinal upset, Weight loss

D. Chemical Hazard Housing/Procedure Sites

	Agent	Receiving Species	Building	Room or area	Housing	Procedure
1	Cyclosporine	canine	[redacted]	all rooms	☑	☐

E. Chemical Hazard - Animal Identification

Explain how animals treated with a chemical hazard will be identified (ex. cage card, ear tag, etc.)

- [x] Cage Card
- [ ] Chip
- [ ] Door Sign
- [ ] Other

F. Chemical Hazard - By-Products/Presence

- [ ] None
- [ ] Feces/Urine/Bedding
- [ ] Saliva
- [x] Blood
- [ ] Aerosols
- [ ] Animal Bite/Scratch
- [x] Animal Carcasses/Tissues
- [ ] Surgical Site Wound or Sore
- [ ] Other

G. Chemical Hazard - Personal Protection Equipment (PPE)

PPE to be worn when handling chemical hazards include: (ex. safety glasses, surgical mask, etc.)

	Chemical Agent	Gloves	Eye Protection	Lab Coat	Double-Gloves	Face Mask	Fume hood	Other	Other PPE
1	Cyclosporine	☑	☐	☑	☐	☑	☐	☑	head cover

H. Additional Information

List additional information, i.e., special precautions for pregnant women, immunocompromised individuals, special handling, or storage, etc.

N/A

3. Will you use any Radiation Hazards?

○ Yes ● No

9. Anesthetic Procedures, Pain Control, Other Clinical Drugs

1. Anesthetics, Preanesthetics & Tranquilizers

Will any anesthetics, preanesthetics, or tranquilizers be used?

● Yes ○ No

2. Preanesthetic Agent(s)

List preanesthetic agents here

	Species	Agent	Dose/Volume	Route	Frequency of Admin.
1	Dog	Atropine sulfate	0.02-0.04 mg/kg	IM, SQ, IV	prn
2	Dog	Acepromazine maleate	0.02-0.12 mg/kg	IM,SQ	prn
3	Dog	Butorphanol tartrate	0.2-0.4 mg/kg	IM, SQ	prn
4	Dog	Nalbuphine	0.5-1 mg/kg	IM,SQ	prn
5	Dog	Dexmedetomidine	3-15 mcg/kg	IM, IV	prn
6	Dog	Hydromorphone	0.05-0.1 mg/kg	IM	prn
7	Dog	Buprenorphine	0.005-0.03mg/kg	IM	prn
8	Dog	Diazepam	0.2-0.5mg/kg	IV	prn to assist with induction

3. Anesthetic Agent(s)

List anesthetic agents here. **Do not list isoflurane here, it will be listed later in the form.**

	Species	Agent	Controlled Substance	Dose/Volume	Route	Frequency of Admin.
1	Dog	Propofol	No	Induction: 6-10 mg/kg, Maintenance CRI: 0.1-0.4 mg/kg/min (maximal 1.2 mg/kg/min)	IV	Once for induction, subsequent boluses may be used to increase anesthetic depth as required to maintain an appropriate plane of anesthesia for surgical procedures.
2	Dog	Ketamine	Yes	6-15 mg/kg	IV	prn
3	Dog	Diazepam	Yes	0.2-0.5 mg/kg	IV	prn
4	Dog	Xylazine	No	0.05-2 mg/kg	IM, IV, SQ	Induction prn
5	Dog	Alfaxalone	Yes	Induction: 2-3 mg/kg (no preanesthetic) OR 1-2 mg/kg (preanesthetic)	IV	Once
6	Dog	Etomidate	No	1 mg/kg	IV	Once
7	Dog	Ketamine	Yes	loading dose 0.5mg/kg; 10mcg/kg/min CRI	IV	Once for induction; CRI to maintain anesthetic depth
8	Dog	Lidocaine	No	loading dose 3mg/kg; 50mcg/kg/min CRI	IV	Once for induction; CRI to maintain anethetic depth and cardiac stability
9	Dog	Alfaxalone	Yes	Maintenance (CRI): 8-9 mg/kg/hr (no preanesthetic) OR 2-9 mg/kg/hr (preanesthetic)	IV	Prn for maintenance, boluses may be used to increase anesthetic depth as required to maintain an appropriate plane of anesthesia for surgical procedures

4. Isoflurane Use

A. Will you use isoflurane?

● Yes ○ No

B. What species?

Dog

C. How will it be administered?

- ☐ Vaporizer with nose cone
- ☑ Vaporizer with endotracheal tube
- ☐ Induction box connected to vaporizer
- ☐ Open drop method (bell jar, etc.)
- ☐ Other

5. Non-Pharmaceutical Grade Anesthesia

If non-pharmaceutical grade anesthesia must be used, strong scientific justification must be provided. In addition, describe how the solution will be made and stored. Also, describe how pH, osmolarity, and sterility

will be kept in acceptable physiological ranges. Please see ACUC policies for Anesthesia and Non-pharmaceutical Anesthesia and ACUC Guidelines for pentobarbital, avertin, inactin, and urethane/chloralose.

N/A

6. Monitoring and Life Support

Monitoring and life support systems to be utilized to ensure adequate depth of analgesia or anesthesia and to prevent overdose:

All animals undergoing anesthesia (including muscle biopsy and terminal procedure) will have blood drawn and sent to the VMDL for CBC, maxi and cardiac troponin before beginning the procedure. Animals will be monitored using an ECG, SpO2, ETCO2 monitor continuously. Non-invasive blood pressure is monitored approximately every 15 minutes only in affected animals. Appropriate anesthetic depth will be monitored by loss of the pedal withdrawal and palpebral reflexes, heart rate, respiratory rate and mentation every 5-15 min. Temperature support will be provided by a circulating water heating pad placed under the animal during the procedure or heating lamp.

For MRI study:
Crystalloid replacement fluids (LRS) will be given at 5-10ml/kg/h IV during anesthesia. Fluid rate and anesthetic drug doses may be adjusted as necessary to maintain appropriate cardiopulmonary function and anesthetic depth. Anesthesia will be induced using Nalbuphine and Dexmedetomidine sedation, followed by IV Propofol administration and anesthesia maintained using Isoflurane. Respiratory function (for MRI) will be assessed by monitoring mucous membranes, respiratory rate, exhaled carbon dioxide (capnography) and hemoglobin saturation (pulse oximetry) whenever possible. A mechanical ventilator may be used during the imaging procedure to treat or prevent hypoventilation. Cardiovascular function (for MRI) will be monitored using heart rate, pulse quality, and non-invasive blood pressure whenever possible. Cardiovascular parameters, respiratory parameters, ocular reflexes and jaw tone will be used to infer anesthetic depth (for MRI).

For pulmonary function assay protocol 3:
When paralytic agent is used, animals will be monitored by ECG, Pulse oximetry, and non-invasive blood pressure. Capnography will also be used when the animal is being weaned from mechanical ventilation. Dogs will be premedicated with acepromazine (0.02 mg/kg) and butorphanol (0.2 mg/kg) intramuscularly. After allowing for sedation, a 20g cephalic intravenous catheter will be placed. Dogs will be anesthetized with propofol (6 mg/kg IV, to effect) and maintained with an intravenous infusion of the same (0.1-0.4 mg/kg/min). Changes in heart rate and blood pressure will be assessed during anesthesia and neuromuscular blockade in order to assess depth of anesthesia after administration of the neuromuscular blocking drug. They will be orotracheally intubated with a cuffed endotracheal tube and connected to the ventilator. We estimate that the animal will be ventilated for less than 30 min. Animals will be monitored using capnography, pulse oximetry, ECG, and non-invasive blood pressure continuously throughout the procedure. Initial measurements of pulmonary mechanics will be made during spontaneous breathing. Ventilation will be initiated until stable end-tidal carbon dioxide values are obtained (34-40 mm Hg), spontaneous ventilation is observed, and heart rate and blood pressure are stable.

For needle muscle biopsy:
We use Dexmedetomidine 6-15 µg/kg combined with Nalbuphine 0.5-1 mg/kg or Butorphanol 0.2mg/kg as a short anesthesia method for dog muscle needle biopsy and found this dose can completely anesthetize the animal as suggested by Dr. John Dodam (anesthesiologist at MU Veterinary Hospital). He originally suggested that starting dose of dexmedetomidine at 6 mcg/kg + nalbuphine at 1 mg/kg. However, almost all of our dogs need a second dose of dexmedetomidine, even when we started at 9 mcg/kg of dexmedetomidine + 1 mg/kg of nalbuphine. Some larger dogs still cannot get satisfactory anesthesia; therefore, we increase the Dexmedetomidine to 15 ug/kg for large dogs. Also, we can use local lidocaine at 1 mg or bupivacaine at 2-3 mg/kg total dose in all sites and use an oxygen mask to help support the sedated dogs.

Recently we have appreciated the severe side effects of Dexmedetomidine on the cardiovascular system of the DMD model. After consulting with a board-certified cardiologist and veterinarians, we need to alter our sedation/anesthetic protocol for muscle biopsies. We currently are approved to administer propofol for induction at 6mg/kg and maintain with a CRI at 0.1-0.4 mg/kg/min (up to 1.2mg/kg/min) for forelimb and hindlimb function. The patients behave in a predictable manner and a satisfactory plane of anesthesia is achievable with this method. We have implemented this anesthetic protocol for needle muscle biopsies as well. Since there will be more appreciated pain, we will administer Buprenorphine intramuscularly prior to beginning anesthesia using our approved range of 0.005-0.03mg/kg. This will alleviate the negative cardiovascular effects of the dexmedetomidine, while providing a predictable and adequate plane of anesthesia to the patient.

For patients with known cardiac abnormalities, alternative anesthetic protocols may be used to minimize cardiovascular effects following consultation with a veterinary anesthesiologist. Etomidate and Alfaxalone may be used as alternatives for propofol for induction. Etomidate is an intravenous induction agent with minimal cardiopulmonary depression. Etomidate will only be used for induction and not for maintenance of anesthesia with boluses or constant rate infusion (CRI). Alfaxalone may also be employed as an induction agent and for maintenance of anesthesia through CRI due to its short and rapid duration of action with minimal side-effects. In comparison to propofol, Alfaxalone has little to no cardiovascular effects when given in the normal dosage. Etomidate and Alfaxalone do not possess adequate analgesic properties. The concurrent use of an appropriate and approved analgesic agent such as Nalbuphine or Buprenorphine is required for potentially painful procedures (i.e., needle muscle biopsy).

During the use of propofol, etomidate, or Alfaxalone, overdose may cause cardio-respiratory depression. Overdose is likely to cause apnea. In cases of respiratory depression, we stop drug administration, establish a patent airway and initiate assisted or controlled ventilation with pure oxygen. Cardiovascular depression should be treated with plasma expanders, vasopressor agents, and/or antiarrhythmic agents.

Cardiac emergency:
In the past we have encountered several cases of sudden death of the affected dogs during recovering from anesthesia. To increase the success of recovery, Dr. Korte has suggested to include the following procedures/ medications as a routine in our protocol. These are now added in the section of other agents. Calcium gluconate (10%) 0.5-1.5 ml/kg IV slowly (over 10-20 mins) if arrhythmia is noted. Calcium gluconate 0.25-0.5 ml/kg IV throughout procedure if history of hyperkalemia or signs consistent with hyperkalemia. $NaHCO_3$ 0.5 mEq/kg IV over course of procedure. 5% Dextrose in 0.9% NaCl or LRS IV 30-90 ml/kg/hr. Alternatively, we may also administer Lidocaine at a dosage of 2-8 mg/kg IV slowly to effect while monitoring ECG trace.

Preanesthetic Drugs:
Based on discussions with Dr. John Dodam and Dr. Scott Korte, we have added pre-anesthetic drugs before induction of anesthesia for the terminal muscle functions and whole limb functions. We previously had difficulty anesthetizing subjects to a plane of anesthesia suitable for surgical incision during muscle function. After seeking veterinary advice, we utilized pre-anesthetic agents as a means of achieving a surgical plane of anesthesia. The following recommendations will allow for a gradual progression into maintenance anesthesia.

The first recommendation is to administer Carprofen subcutaneously at a dosage of 2.2 mg/kg and Maropitant subcutaneously at a dosage of 1-5 mg/kg 2 hours before the procedure start. The Carprofen-Maropitant combination will help to decrease the amount of Isoflurane required to achieve a surgical plane of anesthesia. Approximately 10-15 minutes before induction with propofol we will premedicate with either Dexmedetomidine (2mcg/kg IM) or Acepromazine (0.01-0.04mg/kg IM), in combination with Hydromorphone (0.05-0.01 mg/kg IM) or Butorphanol (0.4mg/kg IM). This preanesthetic regimen will enable a smooth transition to a balanced anesthesia using Isoflurane. We may also administer an additional dose of Maropitant during the procedure as needed to maintain proper MAC control.

Based on previous experiments conducted by Dr. Joe Kornegay we may also use a preanesthetic combination of Acepromazine (0.02 mg/kg IM), Butorphanol (0.4 mg/kg IM), and Atropine (0.04 mg/kg IM or SQ). Utilizing this preanesthetic combination will allow for a smooth transition into induction using propofol and maintenance anesthesia using Isoflurane. This combination is already included in our approved protocol for Pulmonary Function Assay.

Alternatively, we may also use a constant rate infusion throughout the procedure in conjunction with propofol for induction and Isoflurane for maintenance of anesthesia. Carprofen and Maropitant will be administered 2 hours before the procedure as stated above. A loading dose of Ketamine (0.5 mg/kg IV) and Lidocaine (3 mg/kg IV) will be administered prior to propofol induction. Then a CRI will be used at the following rates: Ketamine (10 mcg/kg/min) and Lidocaine (50 mcg/kg/min) throughout the procedure along with Isoflurane to maintain anesthetic depth. The addition of this CRI regimen will help to maintain cardiac stability while also maintaining an adequate anesthetic plane with Isoflurane.

In general, a veterinarian will help with the anesthesia for high-risk dogs on a case-by-case basis. If a veterinarian is not available, James Teixeira may help with the anesthesia. James Teixeira has a BS in Veterinary Medicine and has assisted with anesthesia for over 13 years. He has performed anesthesia and sedation successfully for this specific DMD colony for over 4 years.

7. Post Anesthetic Recovery

Complete description of post-anesthetic recovery monitoring and care:

Isoflurane recovery: Dogs will remain intubated until they have regained the gag reflex and can successfully swallow. All animals will be recovered on a heating pad (water recirculating type) for temperature support.

The first dose of Carprofen will be given prior to recovery from anesthesia for painful procedures such as muscle biopsies. We may also give it twice a day for up to 3 days post-surgery as needed for painful procedures. Alternatively, Ketoprofen may be given immediately post-operatively and then SQ once daily.

Surgical sites are evaluated at least once daily for a minimum of 10 days until staples are removed or incision is completely healed. The sutures are intradermal and absorbable, so removal is not required. If staples are used, we will remove the staples 10 to 14 days later. For muscle biopsy, medical grade glue will be used to seal the tiny incision (<4 mm) and will be follow-up daily for a minimum of 7 days to check the healing of the wound. An OAR veterinarian will be contacted if signs of infection (pain, swelling, redness, heat, or discharge) are noted or if the wound begins to dehisce.

Emergency equipment and supplies will be readily available in the event of cardiac or respiratory complications. Our anesthesia machine is equipped with a ventilator should we need one. Supplies may include epinephrine (0.05-0.5 mg IV, sublingual, IT), Doxapram (5-10mg/kg IV, SQ, IM or sublingual), or other potential live saving measures as deemed necessary by the veterinarian present.

In case of no veterinarian present when an emergency happens, James Teixeira will make the decision to manage the treatment of the animals. James Teixeira will start to treat the animals, along with Duan lab personnel present, and emergency contact to on-duty veterinary staff will be initiated.

8. Pharmaceutical Analgesia

● Yes ○ No

9. Pharmaceutical Analgesia

	Species	Agent/Non-pharm.	Dose/Volume	Route	Frequency of Admin.
1	Dog	Carprofen	2.2-4.4mg/kg	SQ	1 dose during surgery, then up to 3 days following surgery SID
2	Dog	Butorphanol	0.2-0.4mg/kg	IM	prn
3	Dog	Nalbuphine	0.5-1mg/kg	IM	prn
4	Dog	Hydromorphone	0.05-0.1mg/kg	IM	prn
5	Dog	Ketoprofen	2mg/kg	SQ	1 dose during surgery, then up to 3 days following surgery SID
6	Dog	Buprenorphine	0.005-0.03mg/kg	IM	prn

10. Non-pharmacologic control of pain

○ Yes ● No

11. Paralytic Agents

● Yes ○ No

12. Paralytic Agents

	Species	Agent	Dose/Volume	Route	Frequency of Admin.
1	Dog	Atracurium	0.1-0.2 mg/kg	IV	prn

13. Antibiotics and Other Agents

(Include any emergency drugs, fluids, etc. here)

● Yes ○ No

14. Antibiotics and Other Agents

List other agents such as antibiotics and other emergency drugs

	Species	Agent	Dose/Volume	Route	Frequency of Admin.
1	Dog	Lactated Ringer's Solution	5-10 ml/kg/hr	IV	prn
2	Dog	EMLA cream	as needed to cover skin over tattoo site	Topical	once 30-45 min prior to tattooing
3	Dog	2% Lidocaine	max dose 1 mg/kg	ID	Several minutes before tattoo
4	Dog	Epinephrine	0.05-0.5 mg (0.5-5ml of 1:10,000)	IV, sub-lingual for cardiac arrest	If needed during emergency
5	Dog	Doxapram	5-10 mg/kg	IM, IV, SQ and sub-lingual for respiratory arrest/puppies	If needed during emergency
6	Dog	Edrophonium	0.5 mg/kg	IV	Started prior to respiratory function test and maintained until test complete then reversed.
7	Dog	Cephazolin	22 mg/kg	IV	Once prior to procedure
8	Dog	Heparin	100-300 ul/kg	IV	Hourly maintenance doses (100u/kg) to prevent thrombosis during heart catheter assay. 3ml bolus given immediately prior to exsanguination during terminal assay(s).
9	Dog	Atipamezole	0.2 mg/kg or same volume as dexmedetomidine	IM, IV	Once to reverse Dexmedetomidine
10	Dog	Calcium gluconate	0.5-1.5 ml/kg	IV	slowly if arrythmia is noted
11	Dog	Calcium gluconate	0.25-0.5 ml/kg	IV	Throughout procedure if history of hyperkalemia or signs consistent with hyperkalemia.
12	Dog	NaHCO3	0.5 mEq/kg	IV	Over course of procedure
13	Dog	5% Dextrose in physiological saline (0.9% NaCl) or Lactated Ringer's solution (LRS)	30-90 ml/kg/hr	IV	Over course of procedure
14	Dog	Bupivacaine	2-3 mg/kg	ID	prn
15	Dog	Lidocaine	2-8 mg/kg slow bolus or 0.8mg/kg/min CRI	IV	as needed for cardiac emergency
16	Dog	Bupivacaine	0.67 mg/kg	at site of nerve for block	prn

	Species	Agent	Dose/Volume	Route	Frequency of Admin.
17	Dog	Maropitant	1-5 mg/kg	SQ	1 dose given 2 hours before procedure to decrease MAC of Isoflurane required during procedure.
18	Dog	Maropitant	1-2mg/kg	IV	perioperatively if needed
19	Dog	Nitroglycerin	1mcg/kg/min	IV pump infusion	Begins 15 mins before vector injection, continues for duration of vector delivery, ends after 10 mins have elapsed from injection.
20	Dog	Protamine	1mg/100IU of heparin; dose decreased by 50% for every 60 mins elapsed since heparin delivery	IV slowly	End of procedure to reverse heparin effects
21	Dog	Naloxone	0.04mg/kg	IV, IM	as needed for opioid reversal
22	Dog	Maropitant	1 mg/kg	SQ	prn for vomiting up to 5 days

10. Description of Surgical Procedures

1. Surgical Procedures

Will there be any surgical procedures?

● Yes ○ No

2. Surgery: Pre-surgical Prep

Describe pre-surgical preparation of the animals. Include information about fluid/food restriction, skin, and instrument prep.

Food is withheld for 4 hours for nursing puppies (puppies will be separated from the dam for 4 hours before the procedure to prevent nursing) and at least 12 hours for adult dogs. All dogs will be examined and then anesthetized with the injectable agents listed above and then intubated and maintained on isoflurane. Preanesthetic drugs, if administered, will be administered IM approximately 10 minutes before anesthetic induction using propofol. An IV catheter (20 to 22 gauge) may be placed in the jugular, cephalic, femoral, or saphenous vein and the dogs will be maintained on Lactated Ringer's solution (LRS) or 0.9% NaCl at a flow rate of 10 mL/kg/hr. Each incision site is clipped and then alternately scrubbed at least three times each with Chlorhexidine scrub and alcohol before being draped in preparation for aseptic surgery. Eyes will always be lubricated using eye lubrication ointment. Prophylactic antibiotic treatment (Cephazolin 22mg/kg) may be administered IV along with LRS for the duration of the procedure. Instruments will be steam sterilized, prior to use for survival surgery. Medical devices such as needles for muscle biopsies are sterilized using Ethylene oxide.

Preanesthetic Drugs:
Based on discussions with Dr. John Dodam and Dr. Scott Korte, we have added pre-anesthetic drugs before induction of anesthesia for the terminal muscle functions and whole limb functions. We previously had difficulty anesthetizing subjects to a plane of anesthesia suitable for surgical incision during muscle function.

After seeking veterinary advice, we utilized pre-anesthetic agents as a means of achieving a surgical plane of anesthesia. The following recommendations will allow for a gradual progression into maintenance anesthesia.

The first recommendation is to administer Carprofen subcutaneously at a dosage of 2.2 mg/kg and Maropitant subcutaneously at a dosage of 1-5 mg/kg 2 hours before the procedure start. The Carprofen-Maropitant combination will help to decrease the amount of Isoflurane required to achieve a surgical plane of anesthesia. Approximately 10-15 minutes before induction with propofol we will premedicate with either Dexmedetomidine (2mcg/kg IM) or Acepromazine (0.01-0.04mg/kg IM), in combination with Hydromorphone (0.05-0.01 mg/kg IM) or Butorphanol (0.4mg/kg IM). This preanesthetic regimen will enable a smooth transition to a balanced anesthesia using Isoflurane. We may also administer an additional dose of Maropitant during the procedure as needed to maintain proper MAC control.

Based on previous experiments conducted by Dr. Joe Kornegay we may also use a preanesthetic combination of Acepromazine (0.02 mg/kg IM), Butorphanol (0.4 mg/kg IM), and Atropine (0.04 mg/kg IM or SQ). Utilizing this preanesthetic combination will allow for a smooth transition into induction using propofol and maintenance anesthesia using Isoflurane. This combination is already included in our approved protocol for Pulmonary Function Assay.

Alternatively, we may also use a constant rate infusion throughout the procedure in conjunction with propofol for induction and Isoflurane for maintenance of anesthesia. Carprofen and Maropitant will be administered 2 hours before the procedure as stated above. A loading dose of Ketamine (0.5 mg/kg IV) and Lidocaine (3 mg/kg IV) will be administered prior to propofol induction. Then a CRI will be used at the following rates: Ketamine (10 mcg/kg/min) and Lidocaine (50 mcg/kg/min) throughout the procedure along with Isoflurane to maintain anesthetic depth. The addition of this CRI regimen will help to maintain cardiac stability while also maintaining an adequate anesthetic plane with Isoflurane.

Surgical AI: Once anesthetized, the animal will be placed in dorsal recumbency, and the eyes will be lubricated. The abdomen will be shaved from sternum to pubis and approximately 10 cm on each side of the midline. The shaved region will be aseptically prepared using a Betadine or Chlorhexidine surgical scrub, followed by a 70% alcohol scrub. This process will be repeated 3 times to ensure the appropriate amount of contact time. All instruments will be autoclaved prior to use. Eyes will be lubricated using an ointment.

Impella Supported Stop-Flow Method of Viral Delivery to the Heart:

Patients will undergo cardiac evaluations including: Holter Electrocardiogram (performed by the Duan lab), Electrocardiogram and Echocardiogram (performed at the Veterinary Teaching Hospital) before the procedure to establish baseline information about the patient. Cardiac rhythm and size will be closely evaluated and consulted upon with the University of Missouri Veterinary Teaching Hospital Cardiology department. Full physical examination and bloodwork (complete blood count and serum maxi), including cardiac biomarkers (cardiac troponin and NT-proBNP), will be performed to establish a starting baseline and overall health of the patient. Veterinary advice will be consulted if values are significantly abnormal for the DMD model. Blood will be collected in accordance with the ACUC Standard Operating Procedure (blood collected over a period of 3 weeks will not exceed 8.6ml/kg). Patient size and body weight will be monitored to ensure the patient is an appropriate size for the equipment used. The patient will be fasted overnight with free access to water prior to the procedure. The patient may receive immune suppression in accordance with our approved protocols beginning 3 days prior to the procedure date and continuing for the duration of study.

3. Surgical Procedures

List surgical procedures (include incision location and size, tissue(s) manipulated, and closure methods and materials).

	Procedure	Description of procedure	Survival or Terminal	Building Name	Room Number or area
1	Procedure 1 Surgical Artificial Insemination:	Under exceptional circumstances (when frozen semen of a particular dog is limited, or the outcome of the insemination is critical for the continuation of a bloodline in the colony) frozen semen inseminations may be performed surgically. No bitch will be subjected to more than one such insemination in her lifetime. For a surgical insemination the bitch will be anesthetized, her abdomen will be clipped and prepared for aseptic surgery. Using routine procedures, a 50 mm incision will be made through the linea alba into the peritoneal cavity, the uterine horns will be pulled through the incision and the semen will be injected through the uterine wall directly into the uterine lumen. Once injected, the semen will be held inside the uterus for 5 minutes by occluding the uterine horns proximal to the injection site. Hemorrhage at the injection site will be controlled by digital pressure. Abdominal closure will be the same as for any other abdominal surgery. The incision will be closed in 3 layers: 1) abdominal wall – 3-0 to 0 PDS or Vicryl depending upon the size of the dog in a simple interrupted pattern; 2) subcutaneous layer – 3-0 to 0 PDS or Vicryl depending upon the size of the dog in a simple continuous pattern; 3) skin - 3-0 or 2-0 PDS or Vicryl in an intradermal pattern or staples. Following cessation of anesthesia, the animals will be monitored continuously until they are able to place themselves in and maintain sternal recumbency. The endotracheal tube will be removed when the pharyngeal (gag) reflex returns along with swallowing. All animals will be recovered on a circulating water heating pad for temperature support. Once the animals gain sternal recumbency, they will be evaluated hourly until normal (presurgical) behavior returns. All animals undergoing surgery will be evaluated daily for 10-14 days or until staples/skin sutures (approximately 10 days) are removed. All animals will receive Carprofen (2.2 mg/kg SQ BID or 4.4 mg/kg SID) or ketoprofen (2 mg/kg SQ SID) postoperatively and for at least 2 days following the procedure. Should the animals appear to show discomfort or pain longer, pain management will continue, and a veterinarian will be contacted for further evaluation.	Survival	[redacted]	

	Procedure	Description of procedure	Survival or Terminal	Building Name	Room Number or area
2	Procedure 2 Surgical muscle injection in adult dog:	We currently do not plan to do surgical muscle injection; however, this is how we have done it in the past. We have information listed here as a backup to direct muscle injection. Muscle injection will be performed in operating/surgical room. We will strictly follow all sterile rules and techniques. Anesthesia will be carried out using isoflurane. For direct muscle injection, we will only use this technique on muscle/muscle groups which are extremely small or difficult to access. If surgical muscle injection is to be used, after the muscles are identified, we will make 1 to 2 cm skin incision over the appropriate surface of the crus to access the target muscle. AAV vector will be injected at the volume of 500 ul to 1.6 ml/muscle into the muscle belly through one to five insertion points. The volume is based on our published studies and pilot studies (Shin et al, 2013; Shin et al, 2012). The fascia is closed in a simple continuous pattern with 4-0 Vicryl and then the skin incision is closed in a continuous pattern also with 4-0 Vicryl (or equivalent material from a different supplier) or staples. We expect to inject a maximal of five muscles per side (two on the forelimb and three on the hindlimb) and a total of 10 muscles for both the left and right sides for each dog. For each muscle, we will be performed 1 to 5 injections to saturate the muscle. All the injection will be performed at one time point. References Miller ME, Evans HE (1993) Miller's Anatomy of the dog, 3rd ed. Philadelphia: Saunders. Nghiem PP, Hoffman EP, Mittal P, Brown KJ, Schatzberg SJ, Ghimbovschi S, Wang Z, Kornegay JN (2013) Sparing of the Dystrophin-Deficient Cranial Sartorius Muscle Is Associated with Classical and Novel Hypertrophy Pathways in GRMD Dogs. Am J Pathol 183: 1411-1424	Survival	[redacted]	

	Procedure	Description of procedure	Survival or Terminal	Building Name	Room Number or area
3	Procedure 3 Surgical muscle biopsy:	This procedure now is only used as backup for muscle needle biopsy. This procedure only applies to those muscles that can be accessed by needle biopsy, and can be over 6 times/dog, and only limited to those muscles listed under the muscle needle biopsy section (see below). This will be a sterile procedure. Skin incisions will be made over the appropriate surface of the crus to visualize the muscles injected (see end of this section for the full list of muscle names). For dogs younger than 2-m-old, we will take 0.5cm x 0.3cm x 0.3cm sample. For dogs older than 2-months, a 1 cm x 0.5 cm x 0.5 cm (or smaller) block of the proposed muscle(s) will be removed surgically using a scalpel blade. The fascia is closed in a simple continuous pattern with 4-0 Vicryl and then the skin incision is closed in a continuous intradermal pattern also with 4-0 Vicryl (or equivalent material from a different supplier) or staples.	Survival	[redacted]	
4	Procedure 4 Needle muscle biopsy with the VAB system:	The Vacora vacuum assisted biopsy system was initially developed for breast biopsy. It was found that the VAB system also worked for muscle biopsy (Akarolo-Anthony et al 2012 and Drobnic et al 2014). The VAB system causes minimal discomfort and has not been associated with major complications when used in human patients. A small incision (~0.5 cm) will be made on the surface of the target muscle (See section on surgical biopsy). A 10ga or 14ga VAB needle will be inserted to the muscle at an angle of ~ 45 degree. The VAB system will be turned on to collect muscle tissue (~ 0.5 cm long and 0.3 cm diameter). The skin will be closed with surgical glue or suture. Needle biopsy can be performed under regular anesthesia protocol as we normally do for surgical biopsy or using one of the sedation/anesthesia methods described above. Reference: Akarolo-Anthony et al., Office based muscle biopsy using Vacora vacuum assisted biopsy system. Afr. J. Med. Sci (2012) 41:313-316.	Survival	[redacted]	

5	Procedure 5 In situ ECU muscle force measurement +/- blood flow:	This is a terminal procedure, and the dog will be euthanized after the procedure. Dr. Yang and Dr. Hakim are experts in muscle force measurement in animal models. We have published this protocol before (Shin et al, 2013; Yang et al, 2012). Yongping Yue has been trained by Dr. Hakim on several dogs and has successfully performed this procedure over the past year. Method description: Fur in the surgical area is shaved. The right carotid artery is surgically exposed. A catheter is inserted into the carotid artery and advanced to the thoracic aorta for blood pressure monitoring. Another catheter is inserted into the saphenous vein for intravenously saline infusion. Body temperature (rectal temperature), central blood pressure, electrocardiography, heart rate, respiratory rate, SPO2 and ETCO2 are monitored throughout the experiment. The dog is placed in a supine position on a force transducer plate that is specially designed for in situ muscle function assay. A 3 cm incision is made at the medial side of the upper forelimb. The brachial artery is exposed and a 3P transonic flow probe is put around the brachial artery for blood flow measurement (Module TS 420, Transonic Systems, Ithaca, NY). A catheter is placed in a lower branch of the brachial artery to administer the norepinephrine for the blood flow experiment. Another incision is made on the lateral side of the forearm to expose the entire ECU muscle. The length of the entire ECU preparation (muscle plus tendon) is measured as the distance from the proximal tendon insertion at the medial epicondyle of the humerus to the distal tendon insertion at the carpus. The length of the tendons (proximal and distal) accounts for 16% of the length of a complete ECU preparation (muscle plus tendon) (Yang et al, 2012). The length of the experimental ECU muscle is calculated by subtracting the tendon length (16% of the total length) from the total measured length. The distal ECU tendon is cut at the insertion on the carpus. The free end of the distal tendon of ECU muscle is then sewn on a metal chain with #2 surgical silk. The chain is tightly connected to a level system (310CLR Dual Mode Level System, Aurora Scientific Inc) for muscle force recording. The forearm is subsequently fixed with two bone pins to allow the ECU muscle in line with the muscle force transducer. One stainless steel bone pin is placed on the olecranon and the other is placed on the radius about 3 cm away from its distal end. The radial nerve is located at the lateral side of the distal humerus bone. To expose the radial nerve for electric stimulation, the forearm incision is slightly extended proximately until the nerve is clearly visible. The radial nerve is carefully dissected and tied. The nerve is then cut, and its distal end is mounted on a bipolar electrode for triggering muscle contraction. Before the nerve is cut, bupivacaine (0.67mg/kg) may be injected	Terminal	

around the nerve to alleviate sensation. The Radial nerve stimulation with a 701B Aurora constant current/voltage field stimulator resulted in contraction of the whole extensor muscle groups. However, since the force transducer is only connected to the ECU muscle tendon, only the force produced by the ECU muscle is recorded. It should be pointed out that since the forelimb is tightly held by two strong bone pins (one on the olecranon and the other on the radius), radial nerve stimulation did not cause any movement of the forelimb except for foot kicking due to extensor muscle contraction. To determine whether foot kicking affected tension measurement, we compared the results with or without foot fixation. We did not see any difference in the recorded tension. The exposed ECU muscle and tendon were moistened with warm (37 °C) saline gauze. The temperature of the ECU muscle surface is maintained at 37 °C with a surgical lamp. After the preparation is done, the subject is allowed to stabilize for 10 minutes before force measurement. In some subjects, the same experiment is conducted on both sides of the forelimb. At the end of study, the subject is euthanized and necropsied. Force measurement: For all experiments, electric stimulation is set at 10 Amp and 0.2 ms pulse duration (Hi-power, Biphase stimulator, Cat# 701C, Aurora Scientific, Inc., Aurora, ON, Canada). Muscle force, brachial arterial blood flow and aorta blood pressure are recorded with Powerlab (AD Instruments, Castle Hill, Australia) interfaced via an Aurora 601 Signal Interface with a Mac power PC computer. The optimal muscle length is determined from the optimal isometric force method. Briefly, a 60Hz stimulation frequency for 200 ms is applied while the muscle is held at different lengths. The muscle length which yields the highest isometric force is defined as the optimal muscle length (Lo). At the optimal muscle length, the force-frequency relationship is determined by applying 200 ms tetanic stimulation at various stimulation frequencies. The frequency that yielded the highest force (usually 90 to 120 Hz) is defined as the optimal stimulation frequency. The optimal muscle length (Lo) and optimal stimulation frequency are used in all subsequent force measurements. The peak tetanic force is determined as the highest force produced during the 200 ms tetanic stimulation. Muscle force data were recorded using dynamic muscle control (DMC, version 5.30) and analyzed using dynamic muscle analysis (DMA, version 5.010) window-based software (Aurora Scientific, Inc). For eccentric contraction, the ECU muscle was stimulated for a total of 1,200 msec. In the last 1,100 msec, the ECU muscle was stretched by ~ 5% of its original length. After a 2 min rest, a second cycle of eccentric contraction was applied. A sequence of eccentric contraction was conducted

Procedure	Description of procedure	Survival or Terminal	Building Name	Room Number or area
	in each ECU muscle. The percentage of force drop after each cycle of eccentric contraction was calculated. At the end of study, the subject was euthanized with Euthasol (Virbac Animal Health, Fort Worth, TX) or other approved euthanization methods. References Kornegay JN, Bogan DJ, Bogan JR, Childers MK, Cundiff DD, Petroski GF, Schueler RO (1999) Contraction force generated by tarsal joint flexion and extension in dogs with golden retriever muscular dystrophy. J Neurol Sci 166: 115-121. Shin JH, Pan X, Hakim CH, Yang HT, Yue Y, Zhang K, Terjung RL, Duan D (2013) Microdystrophin ameliorates muscular dystrophy in the canine model of Duchenne muscular dystrophy. Mol Ther 21: 750-757 Shin JH, Yue Y, Srivastava A, Smith B, Lai Y, Duan D (2012) A simplified immune suppression scheme leads to persistent micro-dystrophin expression in Duchenne muscular dystrophy dogs. Hum Gene Ther 23: 202-209 Yang HT, Shin JH, Hakim CH, Pan X, Terjung RL, Duan D (2012) Dystrophin deficiency compromises force production of the extensor carpi ulnaris muscle in the canine model of Duchenne muscular dystrophy. PLoS ONE 7: e44438			

	Procedure	Description of procedure	Survival or Terminal	Building Name	Room Number or area
6	Procedure 6 Dog heart catheter assay (pressure-volume loop assay, PV loop):	Heart function will be assessed by PV loop in dogs between the age of 3-m-old to 5 year-old. This terminal surgery will measure heart pressure, volume and flow characteristics. The measurement will be taken by inserting a pressure-volume catheter into one of the heart chambers. Following heart function assessment, the dog will be euthanized and tissue collected for further analysis. Femoral--an incision (± 4-6 cm) will be made in the right inguinal region. Using blunt dissection, the deep femoral artery and vein will be isolated. The vein will be cannulated with a balloon occlusion catheter. Via angiography, the deflated balloon is advanced to the caudal vena cava at the level of the apex of the heart. The femoral artery is cannulated with a 6F sheath and introducer. At this time, the animal will be giving a loading dose of 300u/kg heparin and will then receive hourly maintenance doses (100u/kg) to prevent thrombosis. A standard 6F-guiding catheter will be attached to a manifold assembly to allow continuous central pressure monitoring. Sternotomy--an incision (± 15 cm) will be made from the manubrium to sternum and the rib retracted. Using a 14–16-gauge needle, a small puncture within the apex of the heart will be created to allow for insertion of the PV loop catheter into the left ventricle. With catheters in place, cascading pressure-volume loops can be generated under condition of reduced preload reflecting the contractile state of the myocardium. Reduction in preload will be achieved by inflating the balloon catheter using an angioplasty inflation syringe filled with a 50:50 mix of saline/visipaque contrast media. Balloon inflation will prevent return of blood flow to the left ventricle (approximately 30 sec). After which, the balloon will be rapidly deflated and blood flow allowed to resume to normal within 20-30 seconds. Approximately 3-5 trials will be attempted during the procedure.	Terminal	[redacted]	

7	Procedure 7 Force function measurement of limb muscles:	This is a non-invasive procedure that is not a terminal experiment but may be performed prior to a terminal function described above in In situ ECU muscle force measurement. This procedure developed by Dr. Kornegay's group by using a footplate connected with Aurora level system to test a group of muscle function on the hind limb. (This protocol is included in surgery section too). This experiment allows us to assess the limb extensor or flexor muscle group function at different time points such as 1m, 3m, 6m, 12m etc. to follow-up the treatment effects. The Limb Muscle Force assay is another proven way to study muscle function. This assay is important because DMD is a muscle disease and alters muscle function. Groups to undergo this assay include normal, carrier and affected animals. For hind limb, the procedure is conducted exactly as published by Kornegay et al (Childers et al, 2011; Kornegay et al, 1999; Kornegay et al, 2011; Tegeler et al, 2010). If the dog is unable to perform the work (due to disease stage), we will not do it for the dog. We also apply the same experiment procedure developed by Dr. Kornegay to measure the flexor muscle group on fore limb by modified the footplate and the stereotactic frame to support the front limb. Dogs will be premedicated using Acepromazine (0.02mg/kg), Butorphanol (0.4mg/kg) and Atropine (0.04mg/kg) combine IM if the total volume is less than 2ml; or Acepromazine+Butorphanol IM and Atropine SQ if over 2 ml. Then the animal is anesthetized using a propofol CRI at a dosage of 0.1-0.4 mg/kg/min up to a maximum of 1.2mg/kg/min. Anesthetized dogs will be positioned in dorsal recumbency, and the pelvic limbs will be alternately immobilized in a custom-made stereotactic frame (can be obtained commercially from Aurora), such that the tibia is parallel to the table and forms approximately a 90-degree angle with the femur. Isometric torque Measures: Anesthetized dogs are positioned in dorsal recumbency. One pelvic limb is immobilized in a muscle at L0 (muscle length at which tetanic torque is maximal) the tibiotarsal joint is positioned at 90 degree. Adhesive wrap affixed the foot to a pedal (footplate) mounted on the shaft of a servomotor to measure torque (model 310LR, Aurora Scientific, Aurora, Ontario). Nerve stimulation activated hind limb muscles of the foot to push (extend) or pull (flex) against the pedal to generate torque. Percutaneous stimulation of the peroneal nerve induced tibiotarsal flexion, whereas tibial nerve stimulation induced tibiotarsal extension. Supramaximal 150 V, 100 ms pulses are applied (Model 701 stimulator, Aurora). Tetany is induced by a 1-s train of 50 HZ pulses. The limb is repositioned and the sequence is repeated. Dynamic Muscle Control computer software (DMC, Aurora Scientific) controlled the servomotor, stimulation timing, and capture of torque	Survival	

Procedure	Description of procedure	Survival or Terminal	Building Name	Room Number or area
	responses. Tibiotarsal eccentric contraction (ECC) Protocol: Percutaneous peroneal nerve stimulation (100 ms square wave pulses, 50 HZ) activated tibiotarsal flexor muscles; stimulating electrodes are positioned until twitches (Pt) reached a maximum. The ECC protocol consisted of an initial isometric contraction followed by a forced stretch imposed by the servomotor. The servomotor rotated the lever arm 29_ opposite to contracting flexor muscles at a rate of approximately 0.7 muscle length/s followed by a rapid return to baseline position. During stimulation (100 ms square wave pulses over 1 s at 50 HZ) flexor muscles are subjected to 800 ms isometric and 200 ms eccentric contractions. This procedure is repeated every 5 s. To avoid fatigue, a 4-min rest followed every 10 contractions and a total of 30 contractions are performed in each animal. This assay will be performed once prior to gene transfer as a control, then approximately 3, 6, and 12 months after gene transfer. Based on our results, more time points may be needed. Amendments will be submitted to cover any additional time points. This assay has performed successfully by other groups multiple times in single animals. The same experiment protocol used in the hind limb will be used in the fore limb to evaluate the flexor function at different foot tarsal angles. ECC is determined at the optimal foot tarsal angles. References Childers MK, Grange RW, Kornegay JN (2011) In vivo canine muscle function assay. J Vis Exp: pii 2623 Kornegay JN, Bogan DJ, Bogan JR, Childers MK, Cundiff DD, Petroski GF, Schueler RO (1999) Contraction force generated by tarsal joint flexion and extension in dogs with golden retriever muscular dystrophy. J Neurol Sci 166: 115-121. Kornegay JN, Bogan JR, Bogan DJ, Childers MK, Grange RW (2011) Golden retriever muscular dystrophy (GRMD): Developing and maintaining a colony and physiological functional measurements. Methods Mol Biol 709: 105-123 Tegeler CJ, Grange RW, Bogan DJ, Markert CD, Case D, Kornegay JN, Childers MK (2010) Eccentric contractions induce rapid isometric torque drop in dystrophin-deficient dogs. Muscle Nerve 42: 130-132			

8	Procedure 8 Impella Supported Stop-Flow Method of Viral Delivery to the Heart	Rationale: Duchenne muscular dystrophy (DMD) patients often exhibit cardiac damage as the disease progresses. Heart failure is a primary cause of mortality. The adeno-associated virus (AAV) vector is currently the best gene therapy vector for DMD. A strategy that can efficiently deliver the AAV vector to the heart will pave the way to treat DMD cardiomyopathy by gene therapy. The Ishikawa lab has focused on cardiac gene therapy in large animal models for many years (Sahoo et al., 2021). Recently, the Ishikawa lab developed a procedure called the Impella-supported stop-flow method to deliver AAV to the heart (Mavropoulos et al., 2022). In the pig model of heart infarct, the Ishikawa lab found that this procedure can increase myocardial AAV transduction efficiency by 100 to 1000-fold without increasing risks compared with the conventional methods (Ishikawa lab, unpublished). We plan to establish the success of direct cardiac AAV delivery in dogs utilizing a stop-flow technique with Impella pump support. Equipment: 1. Sterile surgical drapes, gowns, gloves, bowls, towels, gauze, scalpel, syringes, catheters 2. Echocardiograph/ Electrocardiograph machine (Veterinary Teaching Hospital) 3. LOGIQ e ultrasound machine (GE Healthcare) 4. 18 ga 2.75in Angiography introducer needle 5. 0.014" coronary guidewire. 6. Over-the-wire balloon dilator catheter (CS), Balloon wedge catheter 5Fr Swan-Ganz (CS), 5-7Fr introducer sheath + dilator (femoral artery), 6-7Fr deflectable sheath + dilator (jugular vein) 7. Fluoroscopy at NextGen: Artis Q Fluoroscopy with Volcano IVUS (performed by interventional cardiologist and/or Jan Ivey) 8. 3 Infusion pumps: Propofol, Saline, Nitroglycerin 9. DRE Bonair Electrical Ventilator with 800-3000ml Bellows (Avante Health Solutions) 10. 20-gauge butterfly catheter 11. Heating pad 12. Activated Clotting Time point of care testing (iStat) 13. Impella pump with Automated Impella Controller: 10Fr introducer and dilator sheath, 0.018" guidewire, 0.035" guidewire, 9Fr Impella pump, Purge Cassette Drugs: 1. Angiogram contrast agent: Iopamidol (Isovue-370) or Iodixanol (Visipaque) approximately 20-50ml as needed for catheter placement. 2. Acepromazine: 0.02mg/kg 3. Butorphanol: 0.4mg/kg 4. Propofol: 6mg/kg induction; 0.1-0.4mg/kg/min (maximal dosage of 1.2mg/kg/min) CRI for maintenance 5. Heparin: 100-300U/kg; hourly maintenance of 100U/kg 6. Nitroglycerin: 1mcg/kg/min infused for 15 mins prior to vector injection, continues for duration of injection, end after 10 mins elapsed from injection completion. 7. Protamine: 1 mg/100U of heparin to be inactivated, Dose decreased by 50% for every 30-60mins lapsed since heparin administered, given slowly. 8. Carprofen: 4.4mg/kg SQ after procedure, continue for up to 3 days following procedure. 9. Antibiotics (+/- cut down): veterinary recommendation 10. 70% alcohol and	Survival	[redacted]

Chlorhexidine 11. 0.9% NaCl: IV fluids 10ml/kg/hr 12. 5% Dextrose/water solution: used for Impella cartridge 13. Atropine: 0.02-0.04 mg/kg for cardiac emergency 14. Epinephrine: 0.05-0.5 mg for cardiac emergency 15. Calcium gluconate: 1-1.5ml/kg slowly for cardiac emergency 16. Lidocaine: 2-8mg/kg for cardiac emergency Procedure: The patient will have blood drawn to test complete blood count, serum maxi panel cardiac troponin and NT-proBNP the day of the procedure. They will then be premedicated using our approved pre-anesthesia protocol of Acepromazine (0.02mg/kg) and Butorphanol (0.4mg/kg) via intramuscular injection. An intravenous catheter will be placed in the cephalic vein to administer the induction dose of propofol (6mg/kg) and to maintain anesthesia using a constant rate infusion of propofol at a dosage of 0.1-0.4mg/kg/min (up to a maximal dosage of 1.2mg/kg/min). The contralateral cephalic vein will be catheterized for administration of the nitroglycerin via a syringe pump. A third catheter will be placed in one of the lateral saphenous veins for 0.9% NaCl fluid therapy. Nitroglycerin and fluid therapy will both be administered using syringe pumps. The patient will be intubated and mechanically ventilated with medical oxygen throughout the procedure. The patient will be placed in dorsal recumbency with hindlimbs secured to allow for femoral artery access and preparation. Vascular access locations will be shaved and pre-scrubbed using 70% alcohol and chlorhexidine following a surgical scrub pattern. The area will then again be scrubbed using sterile scrub technique using the same agents and pattern with sterile gloves. Percutaneous access locations may include the left and/or right femoral artery, the jugular vein, and the left common carotid artery (Holmberg and Pettifer, 1997;Goodman and Goodman, 2016;Komornik et al., 2020;Mavropoulos et al., 2022). Peripheral arteries will be accessed using the Seldinger method where a hollow needle is inserted then a guidewire is advanced through the needle. The left common carotid artery will be assessed via intravascular ultrasound and accessed either using the Seldinger method or a cut-down approach to easily visualize and cannulate the artery (Holmberg and Pettifer, 1997;Goodman and Goodman, 2016;Komornik et al., 2020). This decision will be made by the interventional cardiologist and/or surgeon. The arteries will be punctured using the appropriately sized introducer needle as determined by the interventional cardiologist. Once the needle is withdrawn, a sheath is slid over the guidewire. The patient will then receive heparin at a dosage of 300U/kg IV and nitroglycerin at a dosage of 1 mcg/kg/min. The nitroglycerin will begin 15 minutes before the start of the AAV vector injection and continue until 10 minutes past the completion of the AAV vector

injection. The heparin will be administered at a rate of 100U/kg/hr to achieve an activated clotting time of 250-300 seconds for Impella insertion. After insertion, the ACT will be maintained at 160-180 seconds. Activated clotting time will be monitored using an iStat point of care device. A 10Fr introducer/dilator is placed in the left common carotid artery for 9Fr Impella delivery. Using the 0.018" guide wire, the Impella is placed in the left ventricle and positioned using fluoroscopy. The Impella pump is then activated to support the blood flow out of the left ventricle, maintaining hemodynamics to the rest of the body during the procedure. The Impella is a device implanted in the left ventricle temporarily to maintain blood flow out of the heart during cardiac procedures. Using the pump, systemic circulation is maintained consistent while the cardiac blood flow is temporarily altered for viral injection (https://www.abiomed.com/products-and-services/impella/impella-25) (Glazier and Kaki, 2019). The Automated Impella Controller controls the flow of blood through the device's pump (https://www.abiomed.com/products-and-services/impella/impella-25). This device is used in human medicine for acute myocardial infarcts, high risk coronary angioplasty, and off-pump coronary bypass (Glazier and Kaki, 2019). Studies suggest a clear benefit to the use of an Impella device during coronary procedures. The jugular access will be used to cannulate the coronary sinus and advance an occlusion balloon into the great cardiac vein. The occlusion balloon is then inflated to confirm proper placement. The femoral artery is used to advance an angioplasty balloon into the coronary artery. An angiogram is performed, and the balloon is placed in the proximal aspect of the left anterior descending artery (LAD). The coronary balloon is then inflated 3 times for 15 seconds each to precondition the heart for ischemia. The purpose of the preconditioning is to prepare the heart for the ischemic conditions during vascular occlusion. This should not cause cardiac damage and will minimize the adverse effects possible during the viral injection. Kiyotake Ishikawa has reported this technique minimizes the incidence of ventricular infarcts and arrhythmias in pig subjects when Impella device is used. After preconditioning, the coronary sinus balloon is then inflated followed immediately by the coronary artery balloon for the duration of AAV vector injection, approximately 1 minute. This is repeated 3 times with the balloon being deflated between each injection. After three injections in the LAD, the coronary artery balloon is repositioned into the circumflex artery. The same preconditioning, inflation and injection procedure is followed. Upon the completion of the injection, protamine will be administered slowly, intravenously following the recommended antidote dosage. Catheters are removed following

Procedure	Description of procedure	Survival or Terminal	Building Name	Room Number or area
	completion of the viral injection. Hemostasis is achieved using manual pressure to the insertion site for several minutes in conjunction with the protamine. If necessary, sutures will be used to close the vessels to reduce bleeding before manual pressure. This will be under the guidance of the cardiologist(s) and/or surgeon present. The Impella device is weaned down over 15 minutes to prevent acute decompensation. The Impella device is removed, and hemostasis is achieved in a similar manner. The left common carotid artery may also be ligated to achieve hemostasis. Based on literary review and veterinary consultation (email with Dr. Jim Lattimer and Dr. Stacey Leach), this has been done successfully in canines with minimal observable adverse effects (Holmberg and Pettifer, 1997;Goodman and Goodman, 2016;Komornik et al., 2020). If the left common carotid artery was accessed using a cut-down method, the surgical site will be sutured closed.			

4. Surgery: Post-operative Care

Describe post-operative care (include **both short and long-term care**; monitoring, surgical wound care including suture removal, and list drugs and doses anticipated to be used).

Post-anesthesia dogs will recover in an appropriate environment with provided supplemental heat and oxygen as needed.

After surgical procedures, dogs will be carefully followed for their eating, drinking, activity, body weight, body temperature (and other vital signs) for a period of two days (twice a day). If anything abnormal is observed, we will consulate with OAR veterinarian(s) immediately and solve the issue on a case-by-case basis. Post-operative notes will be made by Duan personnel for all recovering dogs. Recovering animals are checked by personnel who are listed in the protocol including the people who did surgery. Student helpers may help with measuring body weight and monitoring eating post-surgery and throughout the experiments.

Dogs will be provided with soft food because affected dogs have swallowing problems due to the disease model. Carprofen and Ketoprofen provide excellent analgesia for the type of procedures we perform and does not result in the respiratory suppression seen with oxymorphone. Pain will be assessed daily while checking incisions (10-day post-op care). The first dose of analgesia will be given following anesthesia induction (carprofen) or immediately post-operatively (ketoprofen). We may also give carprofen twice a day or ketoprofen once per day for up to 3 days post-surgery to reduce post-operative pain.

Impella Supported Stop-Flow Method of Viral Delivery to the Heart:

Echocardiography and electrocardiography completed by the University of Missouri Veterinary Teaching Hospital Cardiology department will be performed and reviewed prior to the experiment date and prior to the termination date. During the procedure, the rate, rhythm, and peripheral blood pressure will be monitored and documented every 15 minutes. Immediately post-operatively, a Holter ECG will be placed on the dog for continuous monitoring for the first 24 hours. In the event of a cardiovascular incident, occlusion balloons will be deflated and the use of approved emergency drugs may be utilized in accordance with our approved dosages and routes. Emergency drug decisions will be made based on the situation and the

opinions of the interventional cardiologist and/or veterinary consult. Bloodwork (CBC, Maxi, cTn1, NT-proBNP) will be collected the day following the procedure and periodically throughout the study duration. Pain and discomfort will be assessed according to the same guidelines used for muscle biopsies. Carprofen will be administered immediately post-operatively and use of analgesics will be used based on veterinary guidance. Dogs will be closely monitored for at least 7 days following the procedure for signs of pain, cardiac abnormalities, and neurologic abnormalities. Veterinary staff will be consulted if any concerns arise during or following the procedure.

5. Surgery: Special Needs

   Special needs of the animals following surgery:

   N/A

6. Surgery: Length of Time Alive

   Length of time animals will be kept alive following surgery:

   For local muscle injection and for biopsy procedures, we will keep animals until the end of the experiment, up to 12 years of age.
   For breeders, they will be kept until the animal is no longer required for the breeding colony.
   After the Impella heart procedure the animal may be kept alive for up to 12 years of age based on the results of experiment and health status. (i.e., If the procedure shows cardiac improvement, we would like to demonstrate longevity of improvement.)

11. Potential Pain or Physical Stress

Potential Pain and/or Distress

Note: Animal Welfare Act regulations define a painful procedure as "any procedure that would reasonably be expected to cause more than slight or momentary pain ... in a human being to which that procedure was applied, that is, pain in excess of that caused by injections or other minor procedures." Procedures reasonably expected to cause pain in the absence of anesthetics or pain relieving drugs should be considered to have the potential to cause pain even with the use of such drugs.

1. Potential Side-Effects and Adverse Health Effects

   Describe any potential side-effects or anticipated adverse health effects of all procedures listed in the preceding sections: animal husbandry, description of non-surgical procedures, anesthetic procedures, and surgical procedures.

   Mild swelling and temporary discomfort associated with the surgery may be anticipated.

   Neonatal systemic injection will be an important regimen to test the early life AAV vector efficacy. It has the potential to cause distress to the animal due to the volumes (depend on the titer of AAV vectors) of fluid injected. Yue et al. (2008 Molecular Therapy) has characterized some of the potential distress responses and complications, including lethargy, unresponsiveness to feeding or other stimulations, lung murmurs likely due to fluid build-up, and cardiovascular changes. Post-injection, animals will be monitored for 1 month for signs of decreased nursing (suckling) response, lung sounds/function and cardiovascular function. Potential complications are managed by care and stabilization feeding, including tube feeding and administration of subcutaneous fluids. If the condition does not improve, it will suggest that we have approached the maximum volume the dog can tolerate, and we will euthanize the individual. All dogs will be closely monitored post AAV vector IV delivery for signs of fluid overload. We will also contact with the veterinarian(s) regarding any complications and treatments.

   Following open muscle biopsies, there is a potential for individual animals to experience transient mobility

problems. However, in the >100 such biopsies performed to date on such animals, no such mobility decrease has been observed. In the event of a mobility difficulty in these experiments, especially in affected dogs, animals will be carefully monitored and will not be exposed to undue exercise or training. Occasionally side effects include lack of appetite, vomiting, soft or mucoid stool and diarrhea. Staff veterinarians will be consulted to treat these adverse effects.

Cyclosporine may cause some gastrointestinal upset. We combined with metoclopramide which will enhance the stomach movement reducing the time of cyclosporine in the stomach and hopefully reducing the GI symptoms. Prolonged use of cyclosporine can result in bacterial or fungal infection related to suppression of the immune system. Long-term use can also promote the development of cancers, such as a cancer of the lymph glands (lymphoma). However, only transient immune suppression will be applied in our protocol. We do not expect to see these long-term complications in our experimental dogs. As an effort to reduce cyclosporine dose, we have proposed co-administration of ketoconazole. This drug has been shown to effectively reduce CSP dose by half in dogs. However, ketoconazole itself also has its own side effects. Common side effects include pruritus, nausea, rash, abdominal pain, fatigue. Ketoconazole also has hepatotoxicity. Mild case may include liver enzyme elevation. In severe case, it may lead to liver failure. So, we will carefully monitor liver function when using this drug and we will consult with OAR doctors if we notice any abnormality. Based on OAR doctors' suggestion, we will decide whether to stop the drug or reduce the dose. We will also follow OAR doctors' suggestion to treat any side effects.

Corticosteroids may cause an increase in thirst and hunger. Steroids also can lead to low levels of steroid hormones if stopped too quickly, thus we utilize a tapering method to prevent sudden level changes. Only transient immune suppression will be applied to this protocol thus long-term adverse effects associated with corticosteroids are not expected. Common side effects include increased thirst and hunger, panting, GI upset, potential for skin infections or worsening of current infections. All dogs undergoing immune suppression will be closely monitored for weight loss/gain, food consumption and possible skin irritations/infections.

Prolonged use of combinational therapy such as prednisolone, cyclosporine and MMF may result in other adverse effects. Combinational therapy can lower the immune system significantly making secondary infections more common. With long term treatment dogs are at risk of opportunistic infections due to the destruction of beneficial microflora. Metoclopramide increases cyclosporine bioavailability which may increase serum concentration and therefore the risk of toxicity. Similarly, there is interaction between cyclosporine and prednisolone which may increase the concentration of steroid in the blood. This could lead to signs of hyperadrenocorticism such as polyuria, polydipsia, alopecia, abdominal enlargement, and seborrhea. Kidney and liver function may also be altered due to the drugs. Following the completion of immune suppression there is the risk of hypoadrenocorticism due to the cessation of corticosteroids. Signs of hypoadrenocorticism include electrolyte disturbances, gastrointestinal upset, dehydration, inappetence and weight loss. (For detailed drug interaction information see attachments in Section 1.)

If experimental dogs show signs of sickness (for example lethargic, not responsive, rapid loss of body weight (>2 lb/day) or fever etc), we will contact OAR veterinarian(s) to determine if the dog is treatable. If yes, we will follow treatment prescription from the veterinarian(s). If not, we will euthanize the dog.

(1) Side effect from non-surgical procedures:

A.) Heart function assay: ECG and echo - no side effect.

B.) Dobutamine assay - Dobutamine has been shown to precipitate arrhythmia. We will stop the test if one of the following conditions appears during the assay: decrease in ST value (ST value < (baseline ST value - 0.2 mY]), hypotension (SBP < {baseline SBP - 20 mm Hg]), hypertension (SBP > 240 mm Hg), sinus tachycardia (heart rate> 240 beats/min), ventricular tachycardia, or uncontrollable excitement of the dog.

C.) MRI/Respiratory function assay/Kornegay limb force protocol/surgery - side effects from anesthesia include respiratory and cardiac complications. Emergency equipment and supplies will be readily available in the event of cardiac or respiratory complications. Our anesthesia machine is equipped with a ventilator should we need one. Supplies may include epinephrine (0.05-0.5 mg IV, sublingual, IT), doxapram (5-10mg/kg IV, SQ, IM or sublingual), or other potential live saving measures as deemed necessary by the veterinarian present or James Teixeira. No side effects have been observed following the Kornegay limb force assay, nor have any been reported in the literature. The Kornegary limb force assay induces tetany under anesthesia. The dogs will be observed for discomfort in the days following the assay. This discomfort should be similar to the discomfort felt several days after a hard workout. The OAR veterinary staff will be consulted if dogs appear to be uncomfortable in the days following the assay. Analgesics may be used to alleviate discomfort if recommended by the OAR veterinary staff.

D.) EIM - no side effect.

E.) Obedient training/activity monitoring: when a dog cannot physically afford the training and testing, we will stop training and testing. Occasionally, the dog may suffer from a medical condition. We will consult the veterinary doctor to decide whether we should stop training.

(2) Muscle biopsy:

An OAR veterinarian will be contacted if signs of infection (pain, swelling, redness, heat, or discharge) are noted or if the wound begins to dehisce. An OAR veterinarian will also be contacted if any abnormal gait/ physical presentation is noted post-procedure. During the procedure, complications associated with the sedation/anesthesia may be observed including apnea, cardiac and respiratory depression and rhythm alterations, and generally depressed mentation.

(3) Blood draw:
Pain, hematoma, and bleeding at the site of needle penetration may occur. An OAR veterinarian will be contacted if any of the above conditions happen for treatment suggestions.

(4) Muscle injection and tattoo:
Complications of intramuscular injection and tattoo include:
a.) abscess
b.) hematoma
c.) injury to blood vessels and peripheral nerves
d.) pain at the injection site
e.) tingling or numbness
f.) infection
g.) bleeding
h.) allergic reaction

(5) Cystocentesis:
Complications of cystocentesis may include scant to mild hemorrhage originating from the skin, subcutaneous tissues, and/or muscle at the site of needle placement, and rarely, in accidental laceration of the bladder or intra-abdominal vasculature. Hemostasis at the site of needle placement will be accomplished with gauze and digital pressure. OAR veterinary staff will be contacted immediately if laceration of the bladder or intra-abdominal vasculature is suspected. An OAR veterinarian will be contacted if any of the above condition happens to get advice for therapy.

(6) Inherited conditions in the affected dogs:
These dogs suffer similar disease as human patients. They may have rapid weight loss, difficult to swallow, muscle atrophy, respiratory and cardiac complications (such as aspiratory pneumonia and arrythmia), dermatitis, fever, hernia, bacterial infection, bone fracture, lymphoma and sarcoma. An OAR veterinarian will be contacted if any of the above conditions happen to get advice for therapy.

Muscular Dystrophy also has the potential to cause pain and distress. Potential side effects of MD include muscle wasting and weakness, abnormal ambulation, cardiomyopathy (usually dilated), diaphragmatic hernias, and mega-esophagus. All affected animals showing signs of poor-doing (weight loss, lethargy, anorexia, regurgitation/vomiting) will be evaluated by an OAR veterinarian. Treatment of the affected animal will be based upon clinical evaluation by a veterinarian. The published neonatal mortality for affected dog is 28% (Valentine 1988) and 32% (Shimatsu 2005). The published neonatal normal dog mortality is 13.3% (Shimatsu 2005). Our mortality rate is below the published percentages for the entire colony.

(7) Impella Supported Stop-Flow Method of Viral Delivery to the Heart:
Complications of this procedure would likely occur during the preparation of the catheters. Even with a board-certified interventional cardiologist, there is the potential of irritating the vascular lumen during guidewire, sheath, or catheter placement and direction. The equipment is designed for this purpose thus the risk is likely local inflammation that will resolve with time and post no threat to the dog. During the process of pre-conditioning the heart for ischemia, there is the potential for ventricular arrhythmias to occur (as reported in some swine procedures). The incidence of arrhythmias is reduced with the insertion of the Impella pump, hence the inclusion of that in our procedure. Based on the extensive collateralization between coronary arteries in canines and the Impella support, the risk of spontaneous arrhythmias is low (Piktel JS and Wilson LD 2019). Lastly, since anticoagulants (heparin CRI) are necessary for the procedure there is the potential for delayed clotting time. The use of protamine should reverse the heparin's anticoagulant effect before the animal is recovered. Ligation of the common carotid may also be necessary to achieve hemostasis in the larger vessel. Side effects from anesthesia include respiratory and cardiac complications. Emergency equipment and supplies will be readily available in the event of cardiac or respiratory complications.

(8) Subcutaneously injected antisense oligonucleotides:
Subcutaneous injections may cause localized irritation and inflammation that should resolve quickly. When properly administered, the injection may cause minimal to no pain to the puppy. Injection location will be slightly different (in varying locations in the scruff) to minimize skin damage and underlying tissue irritation. The blood collection will follow our approved method to reduce the incidence of hematomas and localized irritation. Overall, this procedure will be minimally invasive and have limited pain. Based on the current information there should be not severe adverse reactions to this treatment.

(9) Oral administration of SAT-3247:
Our dogs handle oral medicating well; thus, we do not anticipate additional stress with this treatment. Vomiting/nausea may occur which we will administer maropitant and/or consult with veterinary staff regarding the specifics. Overall health will be evaluated daily prior to administration and periodically throughout the study timeline to evaluate any clinical changes. Overall, this treatment will be minimally invasive and have limited discomfort associated with the study.

(10) A pilot study to evaluate the safety of systemic AAV delivery in adult dogs.
We anticipate pain/discomfort from the initial IV injection to be minimized to local irritation and similar signs associated with blood draw listed above. Days following the injection there is the potential for the development of peripheral edema, pericardial effusion and acute respiratory stress. Dogs will be closely monitored for these signs and veterinary guidance sought to best maintain quality of life and/or humane euthanasia to minimize the discomfort and stress. The most likely discomfort will be increased respiratory effort due to fluid effusions.

2. Assurance of Limited Discomfort and Pain

Describe how it is assured that discomfort and pain are limited to that which is unavoidable for the conduct of this experimentation.

Dogs will be anesthetized during invasive procedures. Dogs do not have significant short-term or long-term pain or dysfunction subsequent to biopsy. We are not adding additional discomfort or pain to the affected dogs.

Analgesics may be used following non-surgical procedures (such as MRI, respiratory function assay, Kornegay limb force protocol) upon veterinary consultation. If any adverse events are noted, as a result of experimental procedure or as a consequence of the model itself, an OAR veterinarian will be notified, and animals will be treated per veterinary recommendations.

The cardiac stop flow vector delivery procedure is not expected to cause additional discomfort or pain. The dog will be anesthetized before any invasive manipulation or cannulation. The discomfort is expected to be no more than that of a muscle biopsy. We are not adding additional discomfort or pain to the affected dogs. Pain management will be provided as needed based on veterinary advice.

The pilot study using the systemic AAV delivery in adult dogs may potentially cause some respiratory stress as a result of fluid extravasation. Close monitoring will ensure identification of early increased respiratory effort and resulting further testing, such as radiographs and cardiac assessments, to minimize the extent. Veterinary guidance will always be consulted when there is a concern for animal quality of life and/or a medical emergency that does not require immediate euthanasia. If the adverse effects compromise the quality of life and/or pose an immediate concern for life, humane euthanasia will be performed.

3. Pain and Distress Form

Is there a Pain and Distress form associated with this protocol?

See: Painful or Distressful Procedures

○ Yes ● No

12. Disposition of Animals

1. Animal Disposition

Check all that apply

- [x] Adoption (See MU adoption policy)
- [ ] Market
- [x] Euthanasia
- [x] Transfer to different project, PI, or another institution
- [ ] Returns to breeding colony, herd, source, owner, or wildlife site
- [ ] Other

2. Euthanasia

Euthanasia Statement

As noted in the Guide, "Euthanizing animals is psychologically difficult for some animal care, veterinary, and research personnel, particularly if they perform euthanasia repetitively or are emotionally attached

to the animals being euthanized (Arluke 1990; NRC 2008; Rollin 1986; Wolfle 1985). When delegating euthanasia responsibilities, supervisors should be sensitive to this issue."

A. Primary Method of Euthanasia

Methods that do not require ACUC proficiency verification

- ☐ Inhalant agent
- ☑ Physical Method with Anesthesia
- ☑ Noninhalent Pharmaceutical Agent

B. Primary Method of Euthanasia (ACUC proficiency verification required unless performed on rodents <7 days old)

Methods requiring ACUC proficiency verification

- ☐ Cervical Dislocation without Anesthesia
- ☐ Decapitation without Anesthesia

C. Euthanasia Descriptions

	Species	Agent/Method	Dose/Volume	Route
1	Dog	over 10 lb: Sleepaway, Beuthanasia, Euthasol	2 ml for first 10lb, 1 ml/10lbs thereafter	IV
2	Dog	under 10 lb: Under Xylazine IM sedation, Sleepaway, Beuthanasia, Euthasol	2 ml for all animals under 10lb	IV
3	Dog	under 10 lb: Sleepaway, Beuthanasia, Euthasol	2 ml for all animals under 10lb	IV
4	Dog	Adult: Under deep anesthesia; exsanguination and heart removal	under 5% Isoflurane anesthesia	will be used specifically for terminal assay under deep general anesthesia

D. Additional Explanation of Euthanasia Procedures

Include any additional explanation of euthanasia procedures here.

The exsanguination method is only used for dogs that will undergo terminal experiment. Euthanasia will be accomplished by exsanguination until the heart stops beating (confirmed by ECG monitoring), and following by bilateral pneumothorax, and removal of the heart. Death will be confirmed in Sleepaway method by bilateral pneumothoraces. URL: http://www.avma.org/resources/euthansia.pdf Exsanguination may also be performed under general anesthesia in the same manner as with the terminal function if/when pentobarbitol is on backorder (such was the case in June 2021). We will continue to follow up regarding the status of pentobarbital products' availability.

Young puppies may receive Xylazine at either 3 mg/kg SQ (primary) or 2 mg/kg IM (secondary) for sedation before administration of euthanasia drug intravenously. We have experienced that it is difficult to place an IV line in young dogs while they are fully aware and active. The use of Xylazine will be more humane way to euthanize them as it reduces undue stress and relaxes them. There are other investigators who also suggest this practice: http://www.petmd.com/blogs/fullyvetted/2008/september/killing-me-softly-chemical-drugeuthanasiapets-101-5780, http://avetsguidetolife.blogspot.com/2011/10/

is-euthanasia-painful.html. If IV line is able to be placed, young puppies may be euthanized without the use of Xylazine.

E. Scientific Justification for Use

- [x] AVMA Approved Method
- [ ] Not AVMA Approved Method

F. Secondary (Physical) Means of Assuring Euthanasia

- [x] Bilateral pneumothorax
- [ ] Cervical dislocation
- [ ] Decapitation
- [x] Exsanguination
- [x] Removal of vital organs
- [x] Other

i. Please specify:

Dogs will undergo necropsy. Heart will be opened, and blood bled; Bilateral pneumothorax followed by exsanguination with removal of the heart following invasive cardiac catheter assay. If the dog is

euthanized with Sleepaway with barbiturate anesthesia, bilateral pneumothoraxes will be conducted. URL: http://www.avma.org/resources/euthansia.pdf

13. Project Information

1.

Associate	Role	Responsibilities	Animal Care & Use	OHSP Training	P&D Training	Survival Surgery
Duan, Dongsheng [redacted]	Principal Investigator Authorized to order animals Access to view cages Editor	Surgery Euthanasia P&D assessment	☑ Dec 5, 2022	☑ Oct 28, 2022	☑ Nov 5, 2021	☑ Apr 18, 2008
Burke, Matthew James mjb4n8@missouri.edu	Co-Investigator Access to view cages Editor		☑ Dec 27, 2022	☑ Oct 12, 2023	⊖	☑ Dec 27, 2019
[redacted]	Co-Investigator Editor		☑ Apr 3, 2023	☑ Mar 20, 2023	☑ Nov 5, 2021	☑ Nov 15, 2013
[redacted]	Co-Investigator Authorized to order animals Access to view cages Editor	Surgery Euthanasia	☑ Oct 19, 2022	☑ Jan 16, 2023	⊖	☑ Oct 19, 2022
[redacted]	Co-Investigator Authorized to order animals Access to view cages Editor	Surgery Euthanasia	☑ May 31, 2023	☑ Apr 19, 2023	☑ Nov 5, 2021	☑ Nov 1, 2002
[redacted]	Key Personnel	Surgery	☑ Dec 8, 2022	☑ Sep 26, 2022	⊖	☑ Dec 8, 2022
	Key Personnel		☑ May 20, 2024	☑ May 20, 2024	⊖	☑ May 20, 2024
	Key Personnel		☑ Feb 27, 2023	☑ Feb 27, 2023	⊖	☑ Feb 27, 2023
	Key Personnel		⊖	⊖	⊖	☑ Oct 14, 2014
	Key Personnel Access to view cages	Surgery Euthanasia P&D assessment	☑ Nov 20, 2024	☑ Jun 21, 2024	☑ Jun 27, 2024	☑ Jun 25, 2024
	Key Personnel		☑ Mar 20, 2023	☑ Aug 25, 2023	⊖	☑ Mar 20, 2023

Associate	Role	Responsibilities	Animal Care & Use	OHSP Training	P&D Training	Survival Surgery
[redacted]	Key Personnel		☑ Mar 20, 2023	☑ Feb 28, 2022	⊖	☑ Mar 21, 2023
[redacted]	Key Personnel		☑ Dec 4, 2023	☑ Dec 4, 2023	⊖	☑ Dec 4, 2023
[redacted]	Key Personnel	Surgery	☑ Feb 2, 2025	☑ Feb 2, 2025	⊖	☑ Feb 2, 2025
[redacted]	Key Personnel		⊖	☑ Jun 30, 2023	⊖	⊖
[redacted]	Key Personnel	Surgery	☑ Jun 7, 2023	☑ Jun 1, 2023	⊖	⊖
OAR, Staff acuc+staff@missouri.edu	Key Personnel		⊖	⊖	⊖	⊖
[redacted]	Key Personnel		⊖	⊖	⊖	☑ Feb 9, 2021
	Key Personnel Access to view cages		☑ Oct 15, 2024	☑ Sep 3, 2024	⊖	⊖
	Key Personnel		☑ Mar 19, 2023	☑ Mar 19, 2023	⊖	☑ Mar 19, 2023
	Key Personnel Authorized to order animals		☑ Mar 8, 2024	☑ Aug 20, 2023	☑ Nov 5, 2021	☑ Jan 31, 2011
	Protocol Creator Editor		☑ Apr 17, 2024	☑ Oct 16, 2023	☑ Nov 5, 2021	☑ Mar 16, 2022

2. Training and Qualifications

Provide a description of the training and qualifications for each individual listed above under Protocol Associates. Provide adequate detail to allow the ACUC to determine if the individual has adequate training and experience with the species and procedures to perform their role proficiently. If they do not have prior training or experience, how will this be obtained?

	Associate	Experience with research animals:	Which procedures will this person perform?	Experience with each procedure:	Employment Status
1	Duan, Dongsheng	25 years	Principle Investigator overseeing the whole project. Perform and assist in every experimental procedure mentioned in this protocol.	Approximately 25 years' experience in animal breeding, animal surgery involved in this and other animal models of human diseases. He has been involved in the development of the animal surgery procedures listed in this protocol. Dr. Duan has extensive experience working with small animal models including the mouse, rat and ferret. He has also worked with the canine muscular dystrophy model for more than 15 years. Duan has had operated in human patients for six years after graduating from medical school. He has done mouse surgery for 20 years and dog surgery in last 15 years.	Full-time employee
2	[redacted]	Graduate student working in Dr. Duan's lab as a research assistant for more than 5 years	Assist in every experimental procedure mentioned in this protocol	Graduate student working in Dr. Duan's lab as a research assistant for more than 5 years working with dogs. During that time, he has gained significant experience in caring for DMD dogs.	Grad student/ Professional student
3	[redacted]	DVM is a board certified veterinary anesthesiologist	Oversee the anesthesia and sedation procedure when needed. Assist in every experimental procedure mentioned in this protocol.	[redacted] is faculty at the Veterinary Medical Teaching Hospital and clinically works with dogs with respiratory disease. They also both perform comparative and translational pulmonary mechanics research using a feline asthma model. They have extensive experience with ventilator acquired pulmonary mechanics using the Engstrom Carestation.	Full-time employee
4	[redacted]	[redacted]as worked in the Guo Lab for 6 years and is now a research technician in the Duan Lab. She will assist with all aspects of dog experimentaion and dog care.	Assist in every experimental procedure mentioned in this protocol.	[redacted]has worked in the Guo Lab for 6 years and is now a research technician in the Duan Lab. She will assist with all aspects of dog experimentaion and dog care.	Full-time employee

	Associate	Experience with research animals:	Which procedures will this person perform?	Experience with each procedure:	Employment Status
5	[redacted]	veterinary cardiologist in the Veterinary Teaching hospital. He will perform ECG and echocardiogram study for our dogs. He will sedate the dog only if necessary.	Perform and assist non-invasive dog heart ECG and Echocardiography	veterinary cardiologist in the Veterinary Teaching hospital. He will perform ECG and echocardiogram study for our dogs. He will sedate the dog only if necessary.	Full-time employee
6	OAR, Staff	ACUC training, lab training	Assist in every experimental procedure mentioned in this protocol.	ACUC training	Full-time employee, Part-time employee, and Undergraduate student
7	[redacted]	PhD, Post-doc fellow. He has worked with different animal models (mice, zebrafish, dog etc.) since 1996. [redacted] has participated in all dog-related procedures for more than 6 years at my lab. His main project is on dog gene therapy (mice will also be used for confirmative purpose). He has six years' experience in dog surgery and post-op care.	Perform and assist in every experimental procedure mentioned in this protocol.	PhD, Post-doc fellow. He has worked with different animal models (mice, zebrafish, dog etc.) since 1996. [redacted] as participated in all dog-related procedures for more than 6 years at my lab. His main project is on dog gene therapy (mice will also be used for confirmative purpose). He has six years' experience in dog surgery and post-op care.	Full-time employee and Postdoc fellow/ Resident

	Associate	Experience with research animals:	Which procedures will this person perform?	Experience with each procedure:	Employment Status
8	[redacted]	[redacted] holds a PhD in Functional Anatomy and Evolution from [redacted] research focuses on the links between diet and mechanical loading on the morphology of the mammalian chewing system (particularly the jaws, masticatory muscles, and teeth). She investigates this relationship using biomechanical modelling and data from gross anatomy, muscle fiber architecture, and 3D models generated from microCT scans.	Perform and assist in every experimental procedure mentioned in this protocol	[redacted] holds a PhD in Functional Anatomy and Evolution from [redacted] research focuses on the links between diet and mechanical loading on the morphology of the mammalian chewing system (particularly the jaws, masticatory muscles, and teeth). She investigates this relationship using biomechanical modelling and data from gross anatomy, muscle fiber architecture, and 3D models generated from microCT scans.	Full-time employee and Volunteer/non-employee
9	[redacted]	Dog colony manager. [redacted] received his AS in Veterinary Technology and BS in veterinary sciences; conc. Clinical Medicine. He has over 10 years experience in a vet clinic setting. He has completely all required training of our institute.	Perform and assist in every experimental procedure mentioned in this protocol.	Dog colony manager. [redacted] received his AS in Veterinary Technology and BS in veterinary sciences, conc. Clinical Medicine. He has over 10 years' experience in a vet clinic setting. He has completely all required training of our institute.	Part-time employee

	Associate	Experience with research animals:	Which procedures will this person perform?	Experience with each procedure:	Employment Status
10	[redacted]	DVM, Ph.D, DACT, MS	Supervise and assist with all aspects of dog reproduction and perform surgical AI.	DVM, ACT performs all of these duties routinely in the Veterinary Medical Teaching Hospital for outside clients. She is a licensed veterinarian, completed a residency in clinical reproduction, and obtained board certification as a theriogenologist in 2018. She also has a Masters in Large Animal Reproduction, and a Doctorate in Reproductive Physiology. She may perform surgical AI if needed and may be responsible for post-surgical AI care and monitoring.	Full-time employee
11	[redacted]	Senior Research Specialist/Duan lab manager: More than 10 years' experience in animal surgery,	Perform and assist in every experimental procedure mentioned in this protocol.	B.A., Senior Research Specialist/Duan lab manager: More than 10 years' experience in animal surgery, 9 years' experience in breeding transgenic, knock-out and other strains of mice. She will be involved in producing high quality AAV stock and performing muscle tissue analysis in this protocol. [redacted] has extensive experience working with small animal models including the mouse, rat and ferret. She has also worked with the canine muscular dystrophy model for more than 10 years. She has more than 15 years' experience in animal (mouse, rat, ferret, dog) surgery and post-op care.	Full-time employee
12	[redacted]	BS degree in biology. Has worked on animal for more than 8 years. Has experience in whelping and caring puppies and adult dogs.	Perform and assist in every experimental procedure mentioned in this protocol.	BS degree in biology. Has worked on animal for more than 8 years. Has experience in whelping and caring puppies and adult dogs.	Full-time employee

	Associate	Experience with research animals:	Which procedures will this person perform?	Experience with each procedure:	Employment Status
13		None	Assistance with basic puppy care as needed under supervision of [redacted] until confident in the role. This will include weighing, general assessment of puppies and tube feeding as needed.	Experience will be gained from in person trainings and shadowing of [redacted]. Student has experience with human patient care already.	Undergraduate student
14	[redacted]	Porcine	Arterial and venous catheterization for introducer sheath placement in the coronary arteries and heart. Implanting Impella device in the Left Ventricle for cardiovascular hemodynamic support during procedure.	[redacted] received his MD in 2002 from the [redacted]. He then completed a Cardiovascular Medicine Fellowship at [redacted] in 2008 and an Interventional Fellowship in 2009. Since then he has been an attending interventional cardiologist physician in the Division of Cardiology at [redacted] He has been an Associate Professor of Clinical Medicine at University Hospital since 2016. [redacted] holds certification in the American Board of Internal Medicine, Interventional Cardiology and American Board of Internal Medicine, Cardiovascular Medicine. [redacted] has been using the Impella device and placing it since 2009 in human patients. His work with porcine included coronary angiography and stem cell injections. He has authored many papers in the field of Cardiology and Interventional Cardiology.	Full-time employee
15	[redacted]	[redacted] has worked with mice for 3 years. His experience includes blood draw, tail vein injection, dissection, and general care (changing food, water, treatments, etc).	Perform and assist in every experimental procedure mentioned in this protocol.	[redacted] holds a PhD. of Biomedical Engineering and a MS in Cell Biology. He may work alongside other lab members during canine functions and care.	Postdoc fellow/ Resident

	Associate	Experience with research animals:	Which procedures will this person perform?	Experience with each procedure:	Employment Status
16	[redacted]	DVM, Ph.D, MS	Supervise and assist with all aspects of dog reproduction and perform surgical AI.	[redacted] received his Masters in 2012 and his Ph.D in 2016. He was an assistant teaching professor at [redacted] for Theriogenology before joining the University of Missouri's theriogenology service early 2023.	Full-time employee
17	[redacted]	In training, some previous experience with farm animals	Assistance with basic puppy care and animal handling as needed under supervision of [redacted] until confident in the role. This will include handling, weighing, general assessment of dogs/puppies and tube feeding as needed.	Experience will be gained from in-person trainings and shadowing of [redacted] Student has some previous experience with animal care.	Undergraduate student
18	[redacted]	Research Swine and Mice at MU [redacted]	Assistance with basic puppy care and animal handling as needed under supervision of [redacted] or other lab members until confident in the role. This will include handling, weighing, general assessment of dogs/puppies.	Experience will be gained from in-person trainings and shadowing of [redacted] and other lab members. Student has some previous experience with animal care.	Grad student/ Professional student
19	[redacted]	None	Assistance with basic puppy/dog care as needed under supervision of [redacted] and other lab members until confident in the role. This will include weighing and general assessment of puppies.	Experience will be gained from in-person trainings and shadowing of [redacted] and other lab members. Student has been in the lab >6mo working with mouse tissues and AAV/DNA experiments.	Undergraduate student
20	[redacted]	None yet. Will receive training on animal handling from [redacted] and [redacted]	Basic animal handling (ie. body weight, feeding, etc).	None yet	Part-time employee and Undergraduate student

	Associate	Experience with research animals:	Which procedures will this person perform?	Experience with each procedure:	Employment Status
21	[redacted]	One year	Perform and assist in every experimental procedure mentioned in this protocol.	[redacted] has a Bachelor's degree in veterinary science and animal husbandry. He joined my lab as a graduate student. He will gain experience from in-person training and shadowing [redacted] and other lab members on procedures related to this protocol.	Grad student/ Professional student

Training Requirements

Note: The ACUC required Basic Training can be found at: https://research.missouri.edu/acqa/. This training must be updated every three years in order to receive protocol approval.

Note: It is the Principal Investigator's responsibility to ensure that all persons listed in Protocol Associates above participate in the MU Occupational Health and Safety Program. See Section 7:020 MU Business Policy and Procedures Manual for details. For enrollment procedures visit the OHSP website.

3. Funding Source

What is the funding source for this project? (Note: If funded internally or by a non-peer-reviewing agency, a peer review of scientific merit may be required.)

- [x] PHS (NIH, CDC, FDA, NSF, NASA)
- [x] DoD
- [ ] VA
- [ ] AHA
- [ ] USDA
- [x] Foundation/Industry
  - i. Name of foundation or industry:

    Foundation: Jackson Freel DMD Research Fund, Jett Foundation, Hope for Javier, PPMD, MDA. Industry: TBD
- [ ] Internal
- [x] Other
  - i. Specify funding source:

NIH, DOD, Jesse's, Duchenne Alliance etc.

14. Refinements or Literature Search

Attach relevant files in the attached files section.

1. Painful Procedures

Any procedure that may potentially cause more than momentary or slight pain or distress requires a literature search for animal alternatives.

Are you performing any procedures that may potentially cause more than momentary or slight pain or distress?

● Yes ○ No

2. USDA Covered Species

Does this protocol utilize animals covered by the Animal Welfare Act or assigned to Category E? (AWA covered species include all warm blooded animals except birds, rats of the genus Rattus, and mice of the genus Mus, bred for use in research, horses not used for research purposes, and other farm animals.)

● Yes, includes USDA covered species or Category E ○ No

3. Includes USDA covered species or Category E

Search for Animal Alternatives

In the literature search and in the written narrative, replacement by non-animal systems, reduction in numbers of animals and refinement of experimental methods (the three R's) must be addressed.

Provide at least two sources of information: one of these sources must be a scientific literature database; documented expert consultation may be used as one source of information.

If you are in the School of Medicine and need assistance with this item, please contact Rachel Alexander, HSL Research Support Librarian, at AlexanderRL@health.missouri.edu. Others can contact the Zalk Veterinary Medical Library, at MU CVM VetMed Library for help.

See also:

https://www.nal.usda.gov/awic/sample-searches
https://library.missouri.edu
Literature Search Help

A. Source 1: Literature Database

Complete the information below:

	Date of Search	Name of Database	Years Covered by Search	Keywords and Search Strategy
1	12/07/2022	Ovid MEDLINE	2019-2022	Replacement/reduction search: (exp Muscular Dystrophies/ OR muscular dystrophy) AND (exp Gene Therapy/ OR adeno-associated virus) AND (Animal Experimentation/ OR Animal Welfare/ OR exp Models, Animal/ OR exp Animal testing alternatives/ OR Computer Simulation/) Refinement/reduction search: (Dogs/) AND ("adeno-associated virus" OR AAV OR transgene OR microgene OR minigene OR "micro-dystrophin gene" OR "mini-dystrophin gene" OR "muscle force" OR "limb force" OR "muscle biopsy" OR dobutamine OR "tidal breathing flow-volume loop" OR "cardiac catheter assay" OR "electrical impedance myography" OR exsanguination/ OR exsanguin* OR "bilateral pneumothorax" OR "diaphragm function assay") AND (Animal Welfare/ OR refine* OR enrich* OR distress OR Pain/ OR reduc*)

B. Source 2: Literature Database

For the second source you may use a literature database search or an expert consultation (see following question).

	Date of Search	Name of Database	Years Covered by Search	Keywords and Search Strategy
1	12/07/2022	CAB	2019-2022	Replacement/reduction search: (Dog OR dogs OR canine) AND muscular dystrophy AND (Gene Therapy OR adeno-associated virus) AND (Animal Experimentation OR Animal Welfare OR Animal Models OR Animal testing alternatives OR Computer Simulation) Refinement search: (Dog OR Dogs OR Canine) AND (adeno-associated virus OR AAV OR transgene OR microgene OR minigene OR micro-dystrophin gene OR mini-dystrophin gene OR muscle force OR limb force OR muscle biopsy OR dobutamine OR tidal breathing flow-volume loop OR electrical impedance myography OR cardiac catheter assay OR exsanguin* bilateral pneumothorax) AND (welfare OR refine* OR enrich* OR pain OR distress OR toxicity OR adverse effects OR enrichment)
2	12/07/2022	PubMed	2010-2022	Alternatives for surgical AI in canines; reduction and refinement of canine breeding strategies.

C. Source 2: Expert Consultation (alternative)

For the second source you may use a literature database search or an expert consultation. Documented expert consultation may be used as one source of information.

No Sources...

D. Animal Alternatives Narrative

Based on the information from the sources above, provide a written narrative of alternatives to procedures that may potentially cause more than momentary or slight pain or distress. The narrative should be such that the ACUC can readily assess whether the search topics were appropriate and whether the search was sufficiently thorough.

If a possible alternative was identified or is known, but will not be employed, discuss why.

Our goal is to develop gene therapy for human patients, and to test the efficacy of gene therapy in a large animal model is a critical procedure to accomplish the goal. The literature searches show that there are no adequate alternatives to conducting studies in animal models.

We use multiple procedural refinements in the current protocol. An example of refinements included is that we will use analgesia provided prior to recovery from anesthesia so that pain management is ongoing as the animal regains consciousness. Another example of refinement is the measurement of muscle function will be accomplished using the limb muscle force assay, which is a non-surgical, non-terminal technique. The limb muscle force assay has been performed successfully in affected muscular dystrophy dogs including repeated measurements in the same dog. This could lead to a reduction in the number of animals needed to answer muscle function questions. Our search did reveal that using dobutamine to assess heart function is a valid alternative to using exercise testing in animals, such as our dogs, and human patients that have difficulty exercising. We also searched for alternatives to surgically taking muscle biopsies. This search resulted in 70 articles from the period of 2019 to 2022 Dec. None of these articles offered an alternative to the method we propose that would reduce animal numbers, replace using animals, or refine our current technique, or result in reduced pain or distress. No further refinements were found from our searches, and no similar studies were found in the literature search. See attached file.

We did a search for alternative approaches for surgical artificial insemination on 12/07/2022 on two searching database, and cover 2010 to 2022 publications, and could not find alternative approaches to replace the current used surgical AI. We covered years 2010 to 2019 publications and could not find alternative approaches to replace the current used surgical AI.

15. Investigator Assurances

1. ABSL-2 Assurance

I will provide training to the husbandry/veterinary staff at least 48 hours prior to exposing animals to a biohazard regarding (but not limited to): the health hazards and symptoms of the biohazard(s) being used; husbandry related research specific SOP's (e.g. handling live exposed animals and contaminated cages); and animal/carcass disposition.

- ● Yes, I will meet the requirements of this statement.
- ○ No, I will not meet the requirements of this statement.
- ○ Not Applicable

2. Investigator Assurances

- ☑ 1. The information provided herein is accurate to the best of my knowledge.
- ☑ 2. Procedures involving vertebrate animals will be performed only by trained or experienced personnel, or under the direct supervision of trained or experienced persons.
- ☑ 3. Any change in the care and use of vertebrate animals involved in this protocol, will be promptly forwarded to the MU ACUC for review; such changes will not be implemented until the committee's approval is obtained.
- ☑ 4. The number of animals proposed is the minimum necessary to conduct valid experimentation.
- ☑ 5. I assure that I am not unnecessarily duplicating previous experiments.
- ☑ 6. I have considered alternative methods to using animals.

- [x] 7. I understand that animal housing must be coordinated with the facility veterinarian and/or facility manager and that approval of this protocol does not guarantee space to house animals.

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