Supplementary Materials for

Assessment of systemic AAV-microdystrophin gene therapy in the GRMD model of Duchenne muscular dystrophy

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MATERIALS AND METHODS

Experimental design

Specific experimental procedures. The GRMD phenotype was initially determined based on elevation of serum CK (56) and confirmed by PCR (*72*). Each dog received a single intravenous dose of the rAAV9-CK8e-c-µDys5 canine-microdystrophin construct or vehicle (phosphate-buffered saline; PBS) (Fig. 1A). The AAV construct was suspended in ~ 40mls of PBS based on body mass and administered through an indwelling catheter in either the cephalic or saphenous vein over a period of 30 minutes.

The AAV-CK8e-c-μDys5 was administered on Day 0 when dogs were about 90±10 days of age. Prednisone (1 mg/kg) was given orally for 5 weeks without tapering the dose, starting on Day –7 and ending on Day 28 (Fig. 1C). Diphenhydramine was administered subcutaneously (2 mg/kg) 30 minutes before dosing to decrease the likelihood of a hypersensitivity response, as we have done previously when administering novel proteins to GRMD dogs (*28*). Various outcome parameters (below) were assessed at baseline (Day 0) within 5 days before μDys5 injection and then repeated on Days 45 and 90, each ± 5 days. The five-day range was necessary to accommodate scheduling the phenotypic tests.

For most of our previous GRMD preclinical studies, we have focused on the period from 3 to 6 months of age when the phenotype progresses particularly rapidly (*11,29*). Given the relative equivalency of the first year of a golden retriever's life to the initial 20 years for a human (*73*), the 3-to-6-month period for GRMD roughly corresponds to an analogous period of deterioration between 5-10 years in DMD (*74*). Dogs with GRMD and patients with DMD both demonstrate marked phenotypic variation, evidenced by a wide standard deviation in certain outcome parameters (*11, 74*). As detailed above, to counter this phenotypic variation, we first established baseline (pretreatment) values and then subsequently determined the fold change between these values and post-treatment test results at Days 45 and 90, each ± 5 days. All outcome data were collected and assessed in a blinded manner.

For follow up biopsies and functional studies requiring anesthesia, dogs were premedicated with acepromazine maleate (0.02 mg/kg), butorphanol (0.4

mg/kg), and atropine sulfate (0.04 mg/kg), masked, and then intubated and maintained with sevoflurane. Tibiotarsal joint torque and ECD studies were done on the left pelvic limb at baseline followed under the same anesthetic by open surgical biopsy of the left cranial sartorius and vastus lateralis muscles. At Day 45, the torque and ECD studies were repeated on the right limb and the left biceps femoris muscle was biopsied to gauge the potential for both dystrophin expression and immune response. Functional studies were repeated on the right pelvic limb under general anesthesia at Day 90 followed immediately by necropsy.

AAV distribution via quantitative PCR (qPCR).

Blood was assessed for AAV vector DNA in study dogs at baseline and Days 7, 16, 31, 61 and 90 after injection; stool was analyzed at baseline and Days 7, 45 and 90; nasal swabs were assessed at baseline and Days 7, 45 and 90; urine was analyzed only at Day 90. Here, and elsewhere, all assessments included 2-3 technical replicates per assay. Tissue samples assessed at necropsy on Day 90 included pancreas, lung, thyroid, spinal cord, brain (frontal cortex and cerebellum), gonad, adrenal, kidney, spleen, liver, heart (IVS, LV, and RV), diaphragm, and the left and right vastus lateralis, cranial sartorius, long digital extensor, triceps (lateral head), gastrocnemius, pectoralis, biceps femoris, semitendinosus, and biceps brachii muscles. The positive cut off was >100 vg copies/µg of genomic DNA (µg gDNA) in all samples.

AAV genome copies were measured using qPCR in tissues from study dogs injected with either vehicle or rAAV9-CK8e-c-µDys5 and subsequently harvested

at Texas A&M per established standard operating procedures in a manner that prevented cross contamination, snap frozen in liquid nitrogen, and stored at −80 °C until genomic DNA (gDNA) was extracted. gDNA from tissues, nasal swabs, and blood was isolated using a DNeasy blood and tissue kit (Qiagen), according to the manufacturer's instructions. Nasal swabs were first soaked in 1x phosphate buffered saline for 10 minutes at 56˚C before proceeding with the extraction process. gDNA was extracted from feces using a QIAamp DNA stool mini kit (Qiagen, Inc) according to manufacturer's instructions. gDNA was extracted from about one mL of urine at day 90 using a Zymo Quick-cfDNA Serum & Plasma Kit according to the manufacturer's protocol. gDNA concentrations were determined using the Nanodrop 1000 Spectrophotometer (Thermo Fisher Scientific) according to manufacturer's instructions. AAV genome copies present in gDNA were quantified by qPCR using the QuantStudio 3 Real-Time PCR System (Thermo Fisher Scientific) according to the manufacturer's instructions, and results were analyzed using the QuantStudio Design & Analysis v1.4.1 software. Briefly, primers and probe were designed to the codon-optimized canine dystrophin region of the AAV vector used. A standard curve was performed using plasmid DNA containing the same codon-optimized canine dystrophin target sequence. PCR reactions contained a total volume of 50 µl and were run at the following conditions: 50 °C for 2 minutes, 95 °C for 10 minutes, and 45 cycles of 95 °C for 15 seconds and 60 °C for 1 minute. DNA samples were assayed in triplicate. To assess PCR inhibition, the third replicate was spiked with plasmid DNA at a ratio of 10 copies/µg gDNA. If this replicate was greater than 40 copies/µg gDNA, the results were

considered acceptable. The cut-off for being positive versus negative for viral genomes was set at 100 copies/µg gDNA. Vector copy numbers reported were normalized per µg gDNA. Assay controls included a No Template Control (NTC), with acceptability criteria <15 copies and an established study specific standard curve slope range (+/- 3SD from three individual standard preparations and runs). Data were reported as AAV genome copies per µg total genomic DNA.

Immunological function

AAV antibody titers via ELISA. Anti-AAV9 circulating antibody levels were measured at the University of Florida for all dose groups at baseline (Day 0) and on Days 1, 3, 7, 16, 31, 61 and 90. Briefly, 96-well plates were coated with 1.2E9 AAV2 or AAV9 particles/well overnight at 4°C. A wash with PBS –Tween was followed by blocking for 2 hrs at 37°C with 10% fetal bovine serum (Cellgro; Mediatech). After a 1X wash with PBS–Tween, frozen serum samples received from Texas A&M and a known positive human standard were diluted between 1:10 and 1:10,240 and allowed to bind overnight at 4°C. Washing was followed by addition of the detection antibody at a dilution of 1:50,000 (for standard samples: goat anti-human immunoglobulin, conjugated with horseradish peroxidase [HRP]; Biosource International) and subject's samples at a 1:10,000 of rabbit anti-Canine IgG, HRP 50ul/well (Sigma, #a6792) for 2 hrs at 37°C. Finally, the plate was washed and exposed to tetramethylbenzidine (TMB) peroxidase detection substrate (KPL) and read at 450 nm with an enzyme-linked immunosorbent assay (ELISA) plate reader (Molecular Devices). Sample anti-AAV9 titers were then read

relative to a human standard curve derived from the same plate. The positive cutoff for this study was determined to be > 6,000 mU/mL). The mean and standard deviation for each data point included all values for study dogs at the corresponding dose and time point.

Dystrophin/μDys5 serum antibodies. Serum samples from vehicle and AAV-μDys5 treated dogs were evaluated for the presence of anti-dystrophin antibodies at day 90 post-administration by western blot. Replicates of protein extracts generated from normal dystrophin positive, GRMD dystrophin negative, and AAV-μDys5-treated microdystrophin positive GRMD muscle tissue samples were separated by SDS-PAGE, transferred to PVDF membranes, and probed using an anti-dystrophin positive control antibody or serum from animals in vehicle and AAV-μDys5-treated groups as the primary antibody. Presence of IgG antibodies towards the 427 kDa full-length dystrophin protein and 147 kDa microdystrophin protein were observed in a dose-dependent manner in dogs treated with AAV-μDys5.

Enzyme-linked immune absorbent spot (**ELISPOT) assay.** Methods for the ELISPOT assay were refined relatively late in the study. Data from only four dogs, one each of the three dose groups and controls, were reported.

Whole blood was collected from dogs at TAMU at baseline and ~ 31- and 91-days following vehicle (excipient) or rAAV9-CK8e-c-μDys5 intravenous administration. Blood was shipped to the University of Washington, where peripheral blood mononuclear cells (PBMCs) were isolated and their T-cell responses to cμD5 transgene were analyzed via IFNγ ELISPOT, generally

following methods used to detect the T-cell response to peptides from factor IX in hemophiliac dogs treated with an AAV-factor IX transgene construct (*75*).

A c-μD5 peptide library was synthesized as 15mers with an 11 amino acid overlap with the preceding peptides (Mimotopes). Peptides were resuspended in 1% acetic acid/50% acetonitrile in water and grouped in pools at a concentration of 0.5-0.84 μg/μL per individual peptide. Peptide pools were stored at -80^{o}C and used at a final concentration of ~ 2.5-4.2 μg/mL per individual peptide. All samples were run in duplicate.

Positive response criteria (must meet both criteria): Average value equal >50 SFU/1x10^{6} PBMCs and three times greater than the medium negative control value.

Molecular studies

Microdystrophin construct. Details on the construct, termed AAV9-CK8e-c-μDys5, have been published (*25*). The canine version codes for a 147 kDa versus normal 427 kDa protein with 5 instead of 24 spectrin repeats and 2 versus 4 major hinges in full-length dystrophin (Fig. 1A). Protein translated from μDys5 includes actin, nNOS, & dystroglycan binding and cysteine rich domains. The construct included the 438 bp CK8e Muscle-Specific Expression Cassette (MSEC) that contains highly modified sub-regions of the mouse muscle creatine kinase (CKM) gene 5'-enhancer and proximal promoter. Recombinant AAV-9 stock was produced by the University of Pennsylvania Vector Core (https://www.med.upenn.edu/gtp/vectorcore), using a research-grade transient

transfection manufacturing process (*76*). Release testing included SDS-PAGE purity assessment and endotoxin level measurement. As is typical of high-dose AAV large animal preclinical studies (*24*), multiple batches of vector were manufactured and used for dosing across the study. To limit the potential for batch-to-batch differences in factors such as impurity profiles or potency, batches were pooled for dosing.

Limb functional studies

Tibiotarsal joint (TTJ) torque and eccentric contraction decrement (ECD). Measurements of TTJ torque and ECD were made with a rapid-response servomotor/force transducer (model 310B LR, Aurora Scientific, Inc) controlled by a personal computer using custom LabView software (National Instruments) using established methods (*45, 47*) (Fig. 5A). Briefly, supramaximal 150 V, 100 μsec pulses were applied percutaneously (Model S48 Solid State Square Wave Stimulator, Grass Instruments) in a 1½ sec tetanic run of 75 pulses (50/sec) to the peroneal (flexion) and tibial (extension) nerves. The site of contact for the paw with the lever (moment arm) was estimated to be 75% of the distance between the point of the hock and the distal digit. Torque (Newton [N]-meters [m]) was divided by the moment arm (m) to convert to force (N) (data not shown).

Eccentric contractions were induced by percutaneously stimulating the peroneal nerve using square wave pulses of 100 μsec duration in a tetanic run for 1 sec at a frequency of 50 Hz while simultaneously extending the TTJ with a servomotor (Aurora Scientific) (*47*). Thus, the muscles of the cranial tibial

compartment were repeatedly stretched to induce mechanical damage. Three sets of 10 stretches for a total of 30, each set separated by 4 min, were performed. Contraction-induced injury was quantified by the force (torque) deficit (Fd) using the following equation: Fd = (Maximal isometric tetanic force [Po] before stretch - Po after stretch / Po before stretch) X 100. While the level of ECD is significantly higher in GRMD versus normal dogs, neither group shows evidence of pain, lameness, or other clinical effects upon recovery from anesthesia.

6-minute-walk test (6MWT). Dogs were assessed using a published protocol (*46*). Briefly, a 6MWT course was laid out in the hallway of a building used for veterinary care. Testing was done at a consistent time during the day when there was minimal activity that could be a distraction. Cones were placed 8.8 meters apart, such that the dog would repeatedly circle them over a period of 6 minutes. Body mass, height at the withers, length from the occiput to the rump, and heart and respiratory rate were recorded before and/or after each test.

Two leashes of equal length were crossed around the neck and one each per thoracic limb to create a body harness. Dogs were leash-walked by the same technician, while another technician sat at the far end of the course. A timer was set to six minutes and the number of laps completed was recorded. Dogs were encouraged to walk by verbal prompts and given food treats. A slight leash tug was applied if the dog stopped. The number of times a dog rested was recorded, while time continued to elapse on the timer. If the dog stopped to urinate or defecate, the timer was stopped and resumed when the dog continued to walk.

Respiratory studies

We employed the same procedures for RIP reported previously in adult dogs, unless otherwise noted (*50*). Dogs were instrumented with a commercially available, telemetric, jacketed system (Data Sciences International). Rib cage and abdominal elastic RIP bands were incorporated within a spandex shirt (Fig. 6A). A loose-fitting outer mesh jacket held wires and a telemetric device that transmitted data to the processing computer. RIP data were collected from un-sedated dogs in lateral recumbency for ~ 5 minutes. Voltage signals from the abdominal and rib cage bands were stored and analyzed using a commercial physiology software platform (Ponemah Physiology Platform 4.90 – SP2, Data Sciences International). Quantitative values from a minimum of 8 acceptable breaths were averaged for each dog. Breaths were eliminated from analysis based on the following criteria: presence of motion artifacts, difference between inspiratory and expiratory tidal volume exceeding 5%, or respiratory rate greater than 55 breaths/minute. We focused on calculation of the PEF:PIF ratio, given that it distinguished GRMD and phenotypically normal carrier dogs in our prior study (50).

Cardiac studies

The cardiac evaluations were performed at pre-treatment (Day 0) and on Days 45 and 90 using established methods (*52*). At each time point, dogs were transported and rested in a quiet room at least 10 minutes before the procedure. Physical exams were performed to assess health status and resting heart rate, respiratory

rate, and blood pressure were evaluated. All cardiac studies were performed in a blinded manner by the same veterinarian (L.-J.G.).

Electrocardiography. Dogs were premedicated and placed under general anesthesia using the same anesthetic protocol detailed above. Electrocardiography (ECG) was recorded using an established protocol with dogs in a right lateral recumbent position (https://www.parentprojectmd.org/wp-content/uploads/2018/04/3.1.Dog_ECG_42815.DF_.DDa_.pdf). The 6-lead ECG was collected on a Philips PageWriter Trim III ECG machine (Philips Medical System) for clinical assessment. The ECG data were later sent to a contracted veterinary cardiologist for analysis. Heart rate, RR interval, PR interval, QRS duration, QT interval, and QTc interval were measured.

Echocardiography. Echocardiography was performed immediately after the ECG recording, with dogs still under general anesthesia. A GE Vivid E9 (GE Healthcare) ultrasound machine was used, and echocardiographic scans were performed using an established protocol (52). All echocardiographic recordings were later imported into a GE EchoPAC workstation (GE Healthcare) for data analysis. We performed comprehensive left heart assessments, including left ventricular (LV) chamber size, systolic and diastolic function, Doppler flow, and myocardial circumferential strain. The LV end-diastolic volume (LVEDV), end-systolic volume (LVESV), and ejection fraction (EF) were measured and calculated from the right parasternal long-axis view using the modified single-plane Simpson's method. Fractional shortening (FS) was obtained using M-mode analysis from the right parasternal short-axis view at the level of the papillary muscles. Myocardial

circumferential strain was measured from the same short-axis view using speckle tracking. The ratio of the mitral valve E wave to early diastolic myocardial velocity in tissue Doppler imaging (E/Em ratio) was used to assess diastolic function. All measurements were obtained from an average of at least 3 to 5 cardiac cycles.

After echocardiography, anesthetized dogs were assessed in a separate cardiac stress study (unpublished). Dobutamine was administered intravenously in 5 μg/kg/min increments until a total dosage of 25 μg/kg/min was reached. For this study, FS value (FS_{10}) and the change of FS ($\Delta FS_{0\text{-}10}$) at 10 μg/kg/min dobutamine infusion were used as markers of cardiac stress response. After the cardiac stress study, dogs were recovered from anesthesia.

Cardiac magnetic resonance (CMR) imaging. The CMR scans were performed using a previously published protocol (*52*) at least 24 hours apart from echocardiographic scans. Dogs were placed under general anesthesia using the same anesthetic protocol, positioned in a dorsal recumbent position, and scanned on a 3-Tesla magnetic resonance imaging machine (Siemens 3T Magnetom Verio, Siemens Medical Solutions) at the Texas A&M Institute for Preclinical Studies (TIPS). A cardiac dedicated surface coil was used with an ECG gating system. Three long-axis (two, four, and three chamber) views and a series of short-axis views, from the LV base to apex, were scanned using fast low angle shot imaging (FLASH) sequence to generate the cine images. All CMR images were imported to a Siemens Syngo workstation (Siemens Medical Solutions) and Siemens Argus software (Siemens Medical Solutions) was used for analysis. The LV function

analysis included LVEDV, LVESV, EF, stroke volume, cardiac output, and myocardial mass at end-diastole.

Histopathology, IF staining, western blot, and mass spectrometry

Histopathology. For toxicity studies, a comprehensive set of tissues taken at necropsy at Texas A&M on Day 90, were fixed in formalin and shipped to the Medical College of Wisconsin, where they were processed for paraffin embedment. Tissue blocks were shipped from Wisconsin to SNBL USA Ltd. for sectioning, staining, and microscopic evaluation. Slides were blindly examined using a light microscope. Muscle samples to determine efficacy were taken from the mid-muscle belly by biopsy at the baseline (Day 0) and interim (Day 45) time points and at necropsy terminally (Day 90) at Texas A&M, frozen, shipped to the Medical College of Wisconsin, and processed for histopathologic and IF analysis using published methods (*77*). Laboratory personnel performing the tissue sections, staining, analysis, and interpretation of the results were blinded to the treatment status of the samples under analysis.

Briefly, frozen tissue specimens were sectioned at 8 microns in thickness and stained with H&E, DYS2 (to detect full-length dystrophin), or MANEX44A (to detect μDys5). For all dogs, H&E slides were qualitatively evaluated in a blinded manner for degree and type of inflammation plus dystrophic and regenerative changes, prior to evaluating percent of sample positive for IF expression of each protein.

Pathologic changes were subdivided into six grades based on the nature and severity of lesions (Fig. 4C): *Normal*. No dystrophic pathology or chronic regenerative changes; *Not active*. Chronic regenerative changes; no active dystrophic pathology; *Very mild dystrophic changes*. Myofiber/myocyte degeneration and possibly active/recent regeneration is present, but only seen in very rare fibers/myocytes **OR** Active/recent and chronic regeneration, without evidence of degeneration; *Mild dystrophic changes*. Rare foci of myofiber/myocyte degeneration and active/recent regeneration, typically involving 20% or less of the sampled tissue. *Moderate dystrophic changes*. Multiple small to medium-sized foci of myofiber/myocyte degeneration and active/recent regeneration, involving ~ 30-50% of the sampled area. A sample with a single large focus of degeneration might also be placed in this category. *Severe dystrophic changes*. Multiple foci of small, medium, and large areas of myofiber/myocyte degeneration and active/recent regeneration, involving >60% of the sampled area. In many cases, the inflammatory response was no longer restricted to the area of myofiber/myocyte degeneration. For the purposes of comparing results across groups, categories of pathologic changes were assigned numerical values (scores): 0 - Normal; 1 - Not active; 1.5 - Very mild; 2 - Mild; 2.5 Mild/Moderate; 3 - Moderate; 3.5 - Moderate/Severe; 4 – Severe.

Immunofluorescence (IF). We used two dystrophin antibodies, MANEX44A (Developmental Studies Hybridoma Bank, University of Iowa) (*34*) and DYS2 (C-terminus; Novocastra, Leica Biosystems) to distinguish μDys5 microdystrophin from full length dystrophin, respectively (fig. S4A). Other proteins and antibodies

included ß-sarcoglycan (43-DAG) (Novocastra; BETASARC1/5B1), MHCd (Leica, NCL-MHCd; mouse IgG1), utrophin (Leica, NCL-DRP2; mouse IgG1), and nNOS (Sigma-Aldrich, N7280; rabbit IgG).

For IF studies, the mean percent of cross-sectional area positive for each of full-length dystrophin, μDys5, and β-sarcoglycan was estimated in entire muscle sections on microscope slides prepared from a group of individual muscles taken at necropsy followed by assessment of developmental myosin heavy chain (MHCd), β-sarcoglycan, utrophin, and nNOS in slides from terminal biceps femoris samples. Cross sectional area of positive fibers was estimated in 5% increments. Estimated levels between 0 and 5% were further specified, e.g., 1-4%. These were large necropsy muscle sections estimated to contain 3-10,000 fibers.

Estimates of the percent cross sectional area of fibers positive for DYS2, ß-sarcoglycan, and the other proteins were made based on visual inspection using a 100x objective, whereas MANEX44A myofibers/myocytes were assessed using a 200x objective. Different objectives were necessary because of variations in fluorescence intensity when comparing the red and green channels. Estimates of the entire sample on the microscope slide were made (not just the photographed region) based on visual inspection by a board-certified anatomic- and neuropathologist (M. W. L).

We used different criteria for judging myofibers to be positive for the respective proteins based on their expected patterns of expression. Because of the patchy staining of DYS2 and MANEX44A, any myofiber or myocyte showing expression was counted as positive. This included partial or full circumferential

membrane expression, and all expression intensity levels. With ß-sarcoglycan and utrophin staining, only myofibers exhibiting complete circumferential membrane expression at high intensity levels (indicative of appropriate levels of β-sarcoglycan expression) (*78*) were counted as positive. Notably, dystrophic animals may express β-sarcoglycan in the absence of dystrophin, but the level of expression would be expected to increase in correlation to the stabilizing presence of μDys5. For MHCd staining, only myofibers exhibiting internal expression at high intensity levels were counted as positive.

Western blot. Following necropsy and tissue freezing on about Day 90, five muscle tissues (diaphragm, vastus lateralis, cranial sartorius, left ventricle, and biceps brachii) and the liver from all dogs and dose groups were processed using a western blot method to quantify μDys5 protein. Quantification was reported as a percentage of normal dystrophin based on a 5-point standard curve included on every blot derived from a combination of protein from WT and dystrophin deficient canine tissue using regression analysis. Both dystrophin and μDys5 were detected with MANEX44A antibody.

Frozen tissue specimens were sectioned and placed into frozen polypropylene tubes followed by protein extraction and western blot analysis. Quantified μDys5 protein was reported as a percentage of normal dystrophin based on a regression slope of a 5-point standard curve included on every blot derived from a combination of protein from WT and dystrophic deficient canine tissue at 100%, 50%, 25%, 10% and 0% percent of normal. Dystrophin and μDys5 proteins were detected using MANEX44A antibody. MANEX44A antibody is

selective for spectrin-like repeat R17 of human dystrophin, and has cross-species compatibility for canine dystrophin and rAAV9-CK8e-c-μDys5. Densitometry was recorded for a single band at ~ 427 kDa for all five calibration curve bands for dystrophin and a single band at ~ 147 kDa for each μDys5 sample. All values below 10% are reported as below the limit of quantification (BLOQ). Only results from gels passing predetermined acceptance criteria were reported. Gels that did not pass the acceptance criteria were repeated.

Each gel featured a calibration curve made using a combination of protein from WT and dystrophin deficient canine tissue, with a constant total protein amount and yielding conditions of 100%, 50%, 25%, 10% and 0% of WT dystrophin levels in lanes 2 to 6. Each gel contained up to a maximum of 11 study samples per gel (lanes 7-17).

Mass spectrometry. Following necropsy and tissue freezing on about Day 90, samples from the diaphragm, vastus lateralis, cranial sartorius, and biceps brachii muscles and the left ventricle from all dogs and dose groups were analyzed to quantify relative μDys5 protein using methods based on those previously published (*37, 38*). Proteins were extracted from each tissue, spiked with a stable isotope labeled internal standard, separated by gel electrophoresis, and in-gel digested. Resulting peptides were subjected to time-targeted parallel reaction monitoring nano-LC-MS/MS. Quantification was reported as a percentage of normal dystrophin based on a 5-point standard curve derived from a combination of protein from WT and dystrophin deficient canine tissue using regression analysis. Both full length dystrophin and μDys5 samples were spiked with the same

stable isotope internal standard prior to enzymatic digestion and mass spectrometry analysis.

Clinical pathology

Blood was collected from nonfasted dogs for hematology and serum chemistry analysis during acclimation (Weeks -4 and -2) and prior to immunosuppression with prednisone and during Weeks 1, 3, 5, and 9. Additional samples were collected during Weeks 9, 11, 13, and/or 14 from a few dogs to monitor health. A small amount of urine was collected from the control Group 4 male on Days 60 and 63 for a health assessment. The clinical pathology data were sent to SNBL USA (now Altasciences Preclinical Seattle LLC) for the preparation of tables and interpretation of the results. An animal number was assigned to each dog. The collection dates of some dogs were adjusted to allow for comparative statistics within timepoints. The test time points were not altered for these dogs.

Skeletal muscle magnetic resonance imaging (MRI)

Dogs were anesthetized (as above) and scanned with the same 3T Siemens MRI unit used for CMR (above) that is highly compatible with the Siemens 3T Tim Trio that had previously been used by our group at the University of North Carolina-Chapel Hill. We generally followed our previously published protocols for image acquisition and analysis (*28*, *44*). Each MRI study included T_2-weighted images with and without fat suppression using a turbo spin echo (TSE) sequence and a multi-slice, five-echo T_2 Carr-Purcell-Meiboom-Gill (CPMG) sequence (T_2FIT for

T_2 mapping). The biomarker analysis included muscle volumes, T2 values, fat and water maps and several texture analysis features, including a first-order intensity histogram texture feature (entropy) and two high order run length matrix features (small lesion index [SL] and heterogeneity index [HI]) that are markers of patchy lesions such as necrosis.

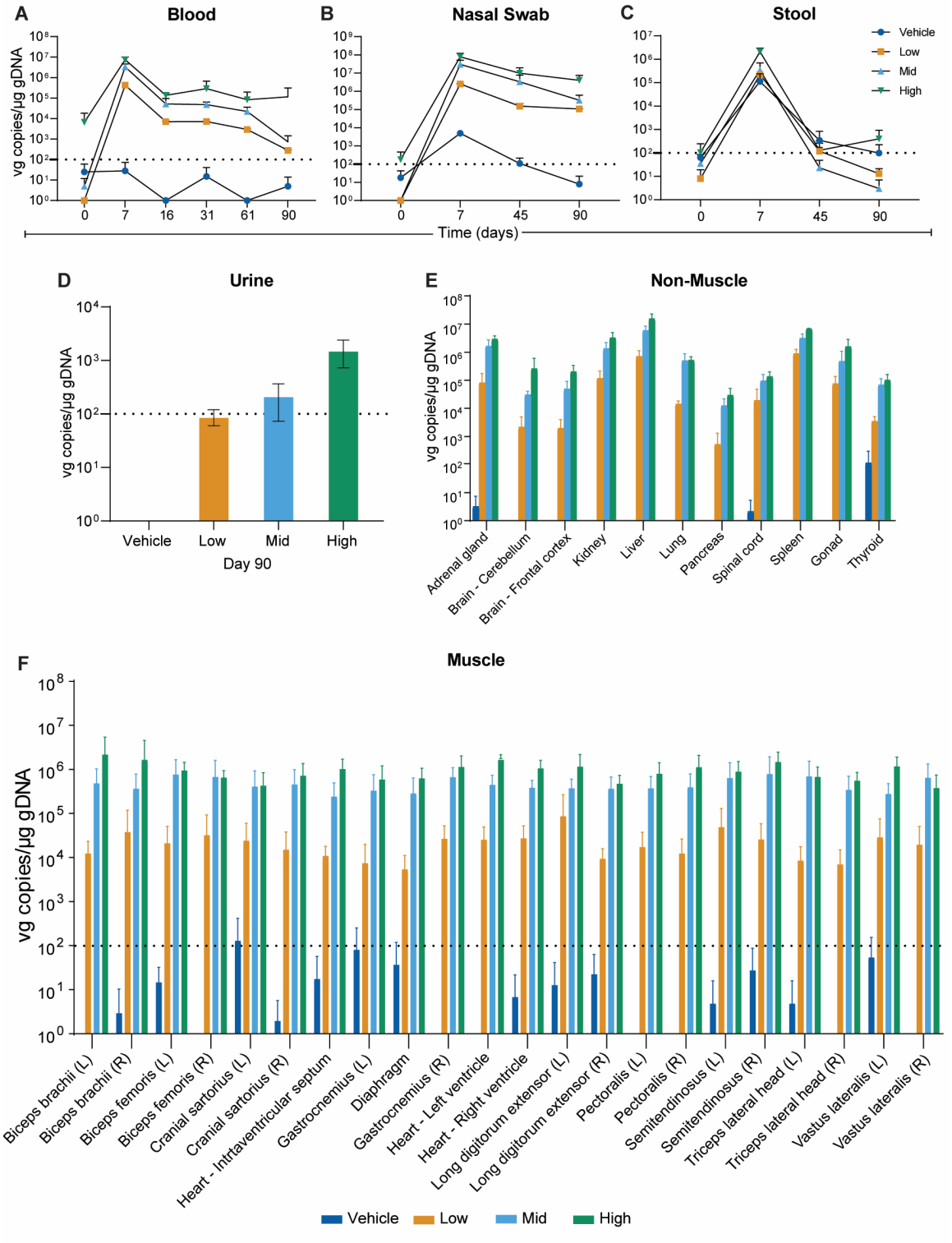

A
Blood
B
Nasal Swab
C
Stool
vg copies/µg gDNA
Vehicle
Low
Mid
High
Time (days)
D
Urine
Day 90
E
Non-Muscle
Adrenal gland
Brain - Cerebellum
Brain - Frontal cortex
Kidney
Liver
Lung
Pancreas
Spinal cord
Spleen
Gonad
Thyroid
F
Muscle
Biceps brachii (L)
Biceps brachii (R)
Biceps femoris (L)
Biceps femoris (R)
Cranial sartorius (L)
Cranial sartorius (R)
Heart - Intraventricular septum
Gastrocnemius (L)
Diaphragm
Gastrocnemius (R)
Heart - Left ventricle
Heart - Right ventricle
Long digitorum extensor (L)
Long digitorum extensor (R)
Pectoralis (L)
Pectoralis (R)
Semitendinosus (L)
Semitendinosus (R)
Triceps lateral head (L)
Triceps lateral head (R)
Vastus lateralis (L)
Vastus lateralis (R)

Fig. S1. AAV vector DNA levels. Measurements were made by qPCR in blood (**A**), nasal swabs (**B**), stool (**C**), and urine (**D**) at multiple time points and in non-muscle (E) and muscle (F) tissues terminally at Day 90. Means and standard deviations for all study subjects within a dose group and time point are plotted. Positive values in some control samples presumably occurred because of sharing of body fluids with treated dogs. Positivity set at >100 vg copies/μg gDNA.

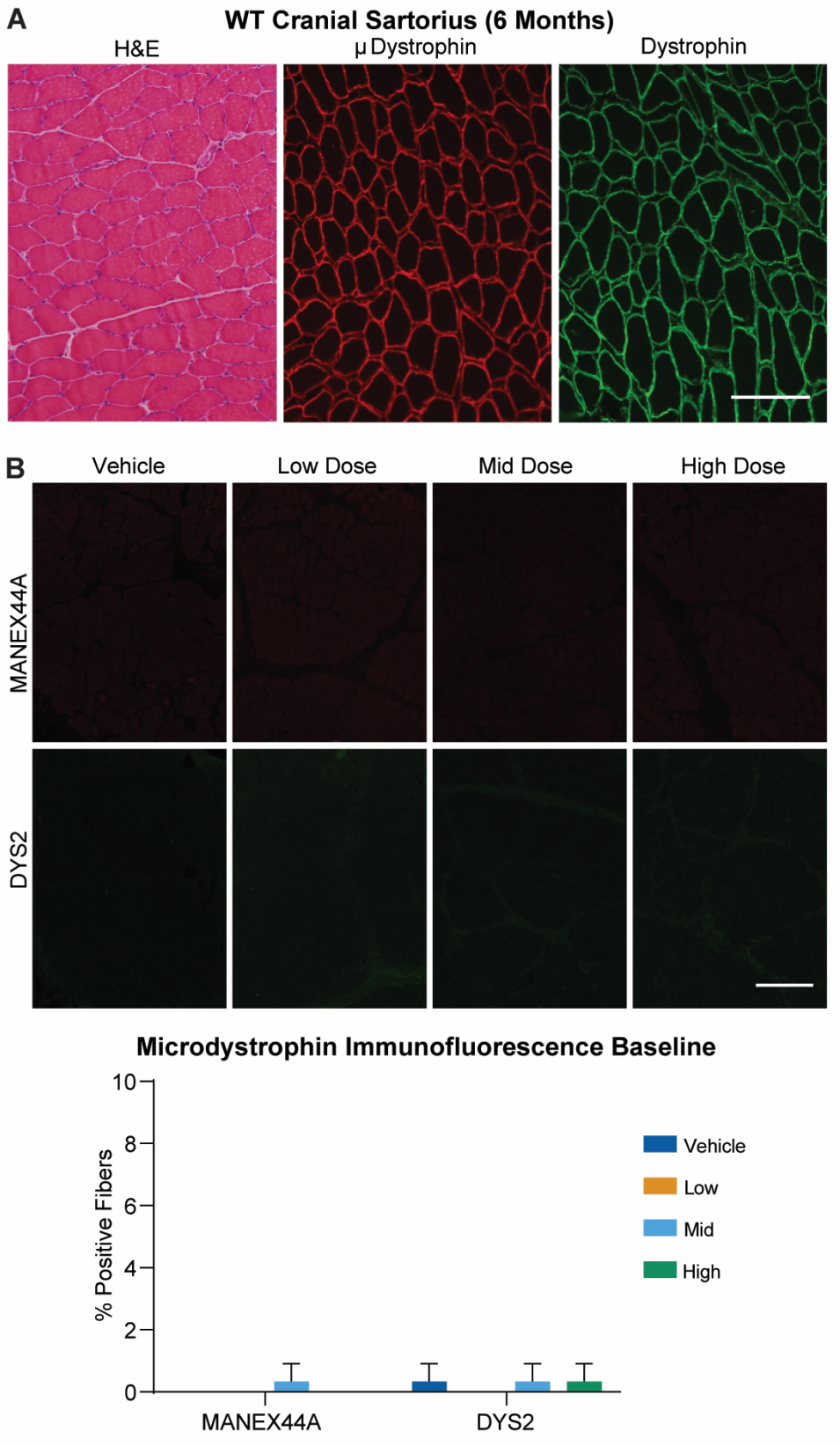

A
WT Cranial Sartorius (6 Months)
H&E
µ Dystrophin
Dystrophin
B
Vehicle
Low Dose
Mid Dose
High Dose
MANEX44A
DYS2
Microdystrophin Immunofluorescence Baseline
% Positive Fibers
10
8
6
4
2
0
Vehicle
Low
Mid
High
MANEX44A
DYS2

Fig. S2. Immunofluorescence of muscle using antibodies reactive to dystrophin and microdystrophin. (**A**) Hematoxylin and eosin (H&E) (left) and immunofluorescence (IF) (middle and right) images of cranial sartorius muscle at Day 90 from a wild type (WT) dog. (**B**). Immunofluorescence evaluation of baseline dystrophin protein in biopsies of the cranial sartorius muscle of GRMD dogs prior to treatment (top) and percent positive fibers depicted with a histogram (bottom). Antibodies reacted to regions in full length dystrophin (DYS2; Leica, C-terminus) (green) or microdystrophin (MANEX44A, DSHB, R16/17) (red) (bar = 100μm).

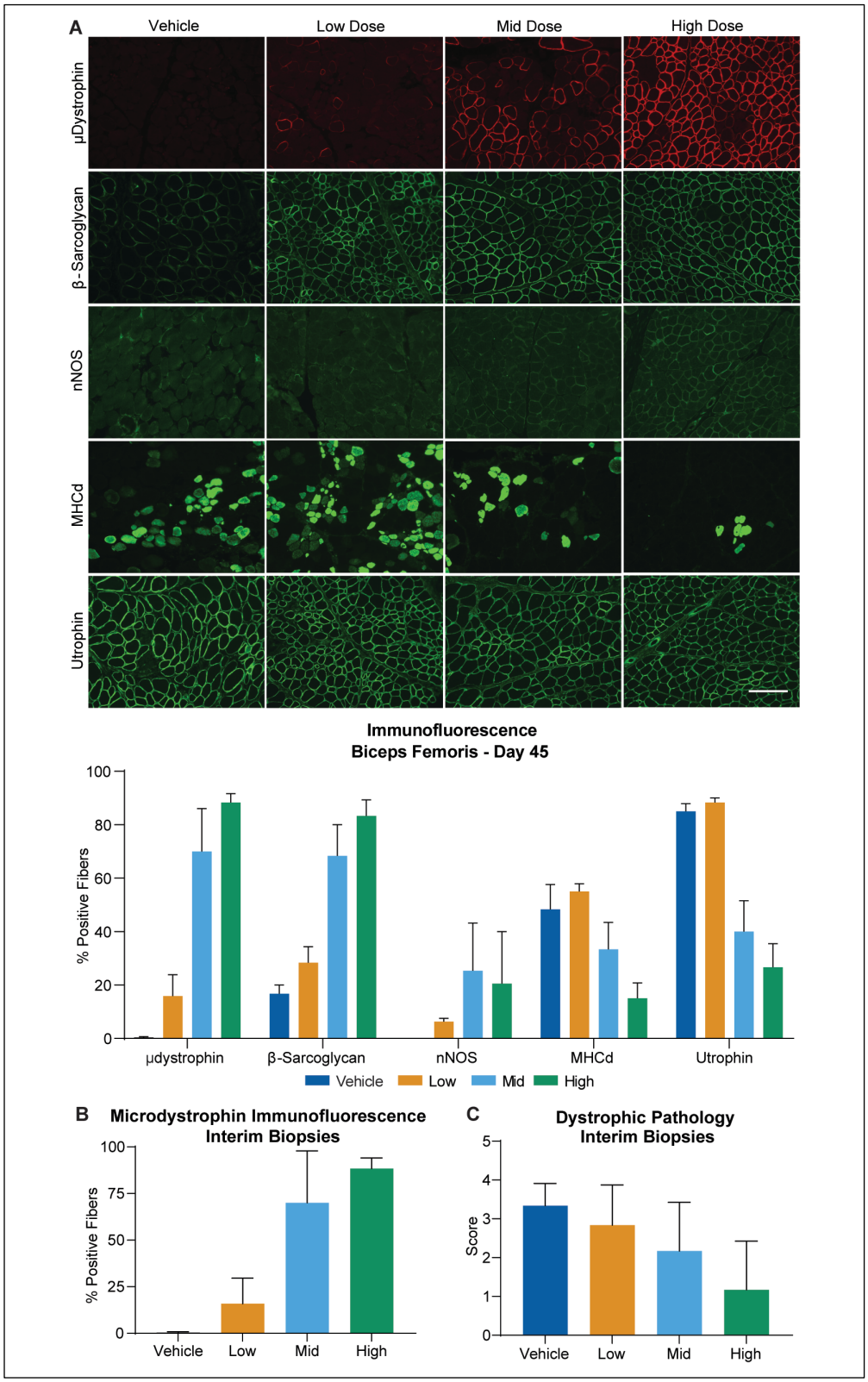

A
Vehicle
Low Dose
Mid Dose
High Dose
µDystrophin
β-Sarcoglycan
nNOS
MHCd
Utrophin
Immunofluorescence
Biceps Femoris - Day 45
% Positive Fibers
100
80
60
40
20
0
µdystrophin
β-Sarcoglycan
nNOS
MHCd
Utrophin
Vehicle
Low
Mid
High
B
Microdystrophin Immunofluorescence
Interim Biopsies
% Positive Fibers
100
75
50
25
0
Vehicle
Low
Mid
High
C
Dystrophic Pathology
Interim Biopsies
Score
5
4
3
2
1
0
Vehicle
Low
Mid
High

Fig. S3. Protein expression quantified by immunofluorescence (IF) and histopathologic lesion scores in interim biopsy (Day 45) muscle samples. (**A**) Antibody-mediated IF stains (top) include microdystrophin (row 1, red), the dystrophin glycoprotein complex (DGC) members β-sarcoglycan and nNOS (rows 2 and 3; both green), MHCd (row 4, green), and utrophin (row 5; green) (bar = 100μm). A histogram (bottom) depicts group means of the percent cross sectional area containing positive fibers. (**B**) Histogram showing mean percent cross sectional area positive for microdystrophin. (**C**) Mean histopathologic lesion scores as defined under "Histopathology." Note these scores tend to track inversely with the percent cross sectional area positive for microdystrophin in B.

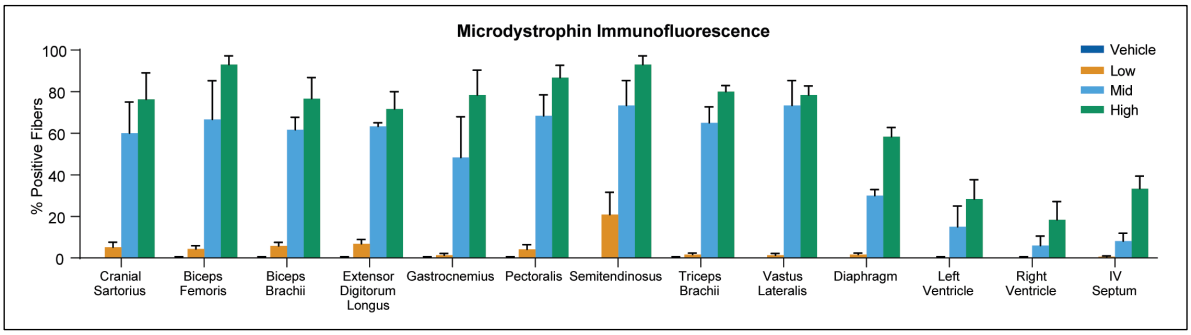
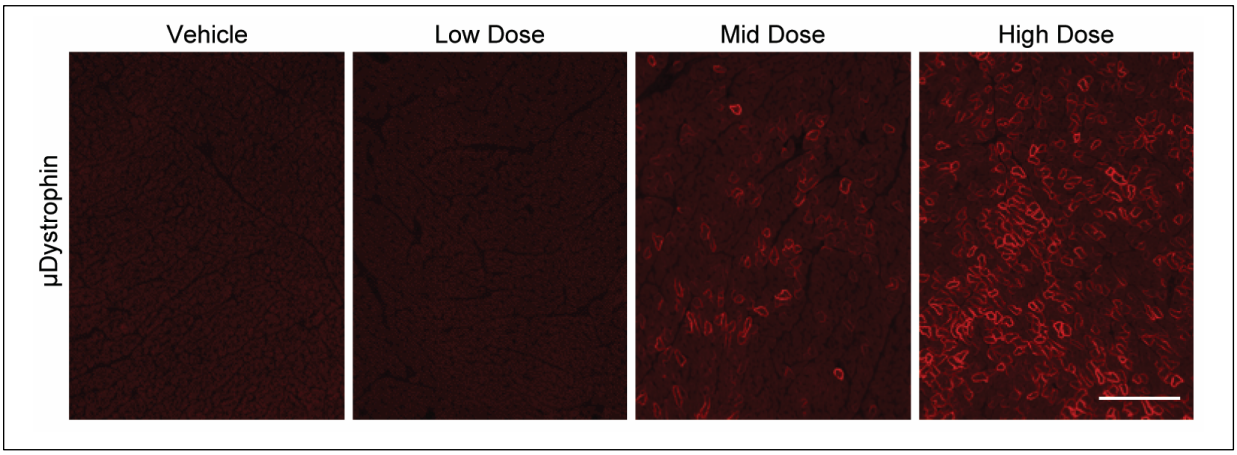


Fig. S4. Microdystrophin positivity across skeletal muscles and the heart. Mean percent cross sectional area positive for microdystrophin on immunofluorescence shows a general dose-related increase in skeletal muscles and the heart. IV = interventricular.



S5. Immunofluorescence evaluation of microdystrophin protein in heart. Images of immunofluorescence staining with the MANEX44A antibody shows dose-related expression of microdystrophin in the interventricular septum collected at necropsy. Bar = 100µm.

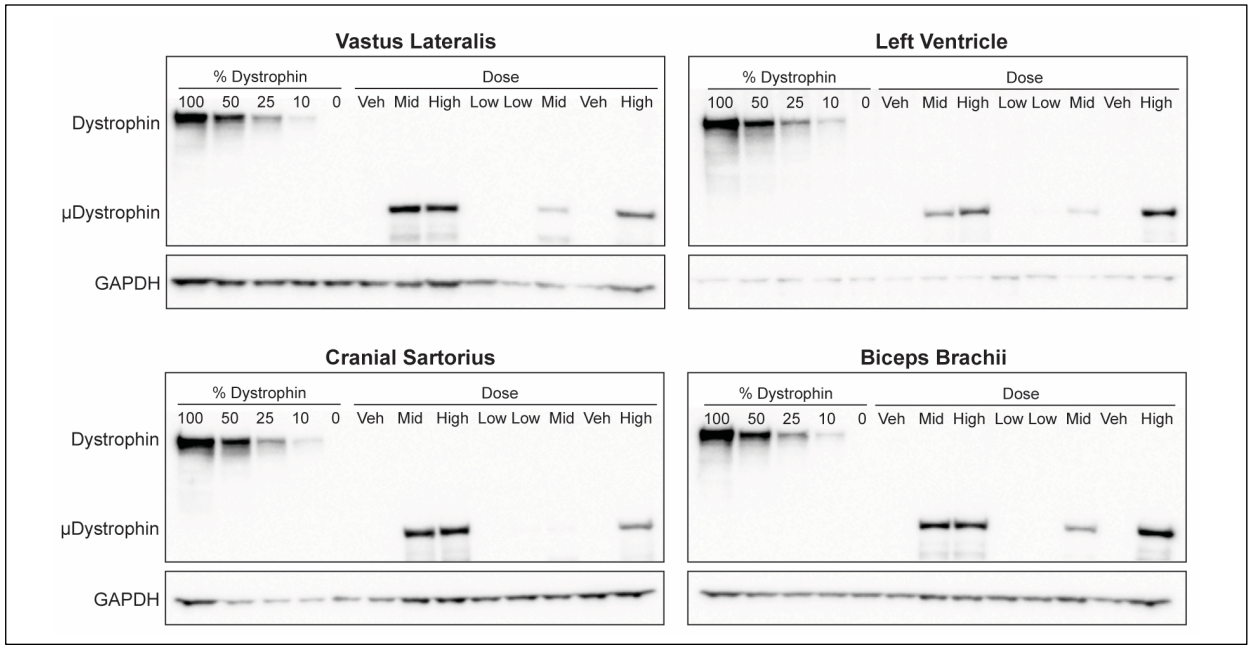


Fig. S6. Western blot images of necropsy tissues evaluated for microdystrophin expression. Lanes were loaded as in Figure 3. GAPDH was used to confirm sample loading.

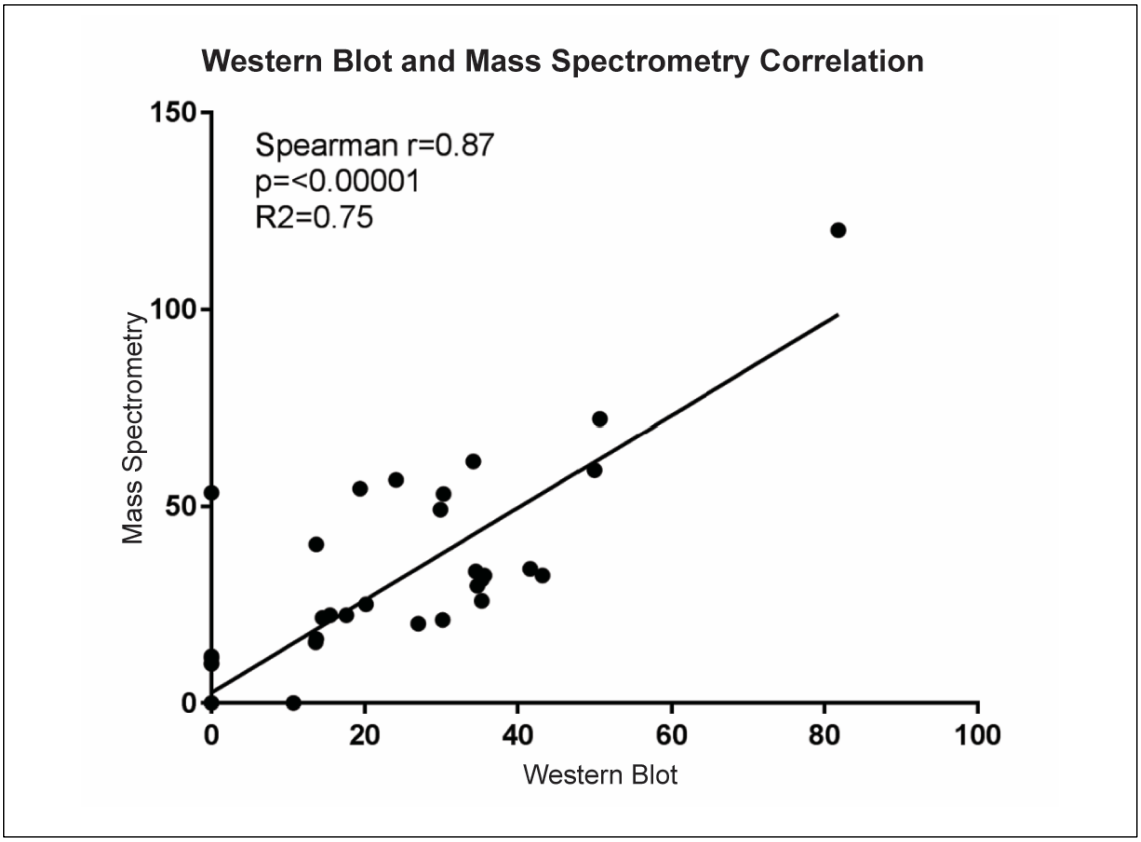


Fig. S7. Correlation of wWestern blot (WB) and mass spectrometry (MS) values. A Spearman-rank order test showed strong correlation between WB and MS values.

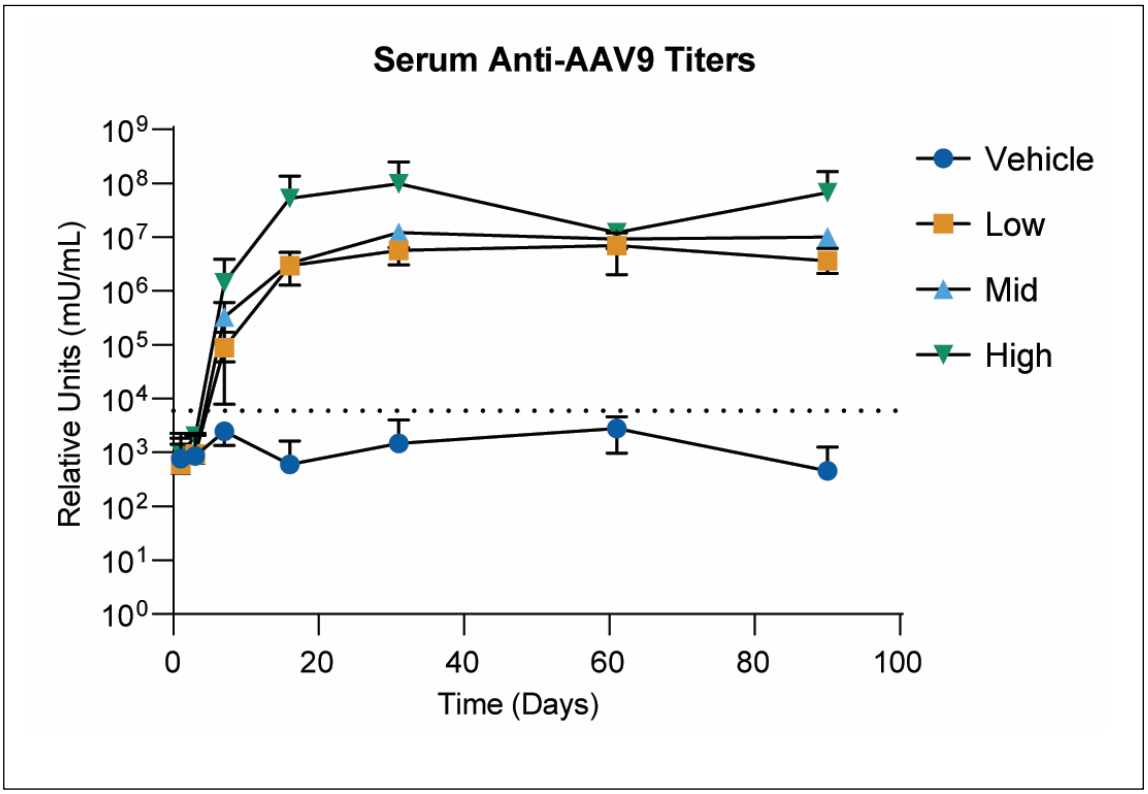


Fig. S8. Anti-AAV9 circulating antibodies. Measurements were made for all dose groups at baseline (Day 0) and on Days 1, 3, 7, 16, 31, 61 and 90. Concentrations increased after dosing and remained significantly above baseline by one-way ANOVA ($P < 0.05$). Values did not differ across dose groups at Day 90 (P=0.37).

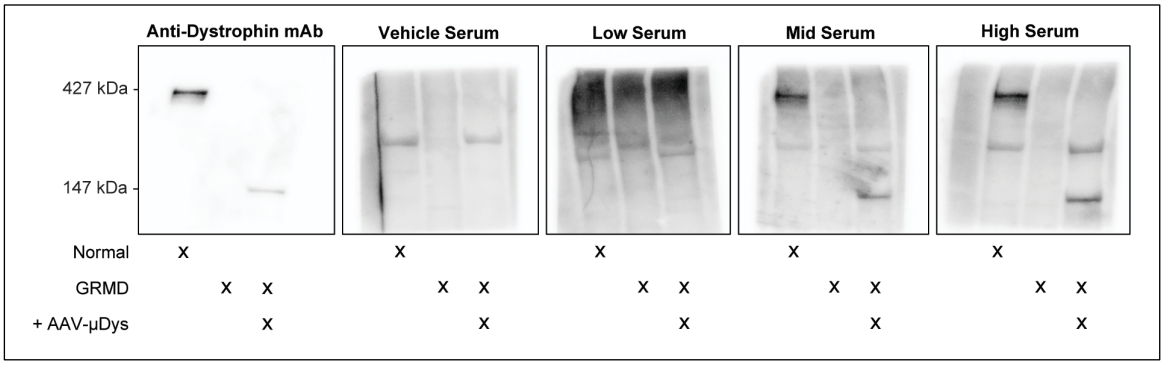


Fig. S9. Anti-dystrophin antibodies. Serum samples from control and treated dogs were evaluated by western blot for the presence of anti-dystrophin antibodies at Day 90 post-administration. Replicates of protein extracts generated from normal dystrophin positive, GRMD dystrophin negative, and AAV-µDys5-treated microdystrophin positive GRMD muscle tissue samples were separated by SDS-PAGE, transferred to PVDF membranes, and probed using an anti-dystrophin positive control antibody or serum from animals in vehicle and AAV-µDys5-treated groups as the primary antibody. The two “X’s” in the third lanes indicate the samples were from GRMD dogs treated with the AAV-µDys5 construct. Presence of IgG antibodies towards the 427 kDa full-length dystrophin protein and 147 kDa microdystrophin protein were observed in a dose-dependent manner in animals treated with AAV-µDys5.

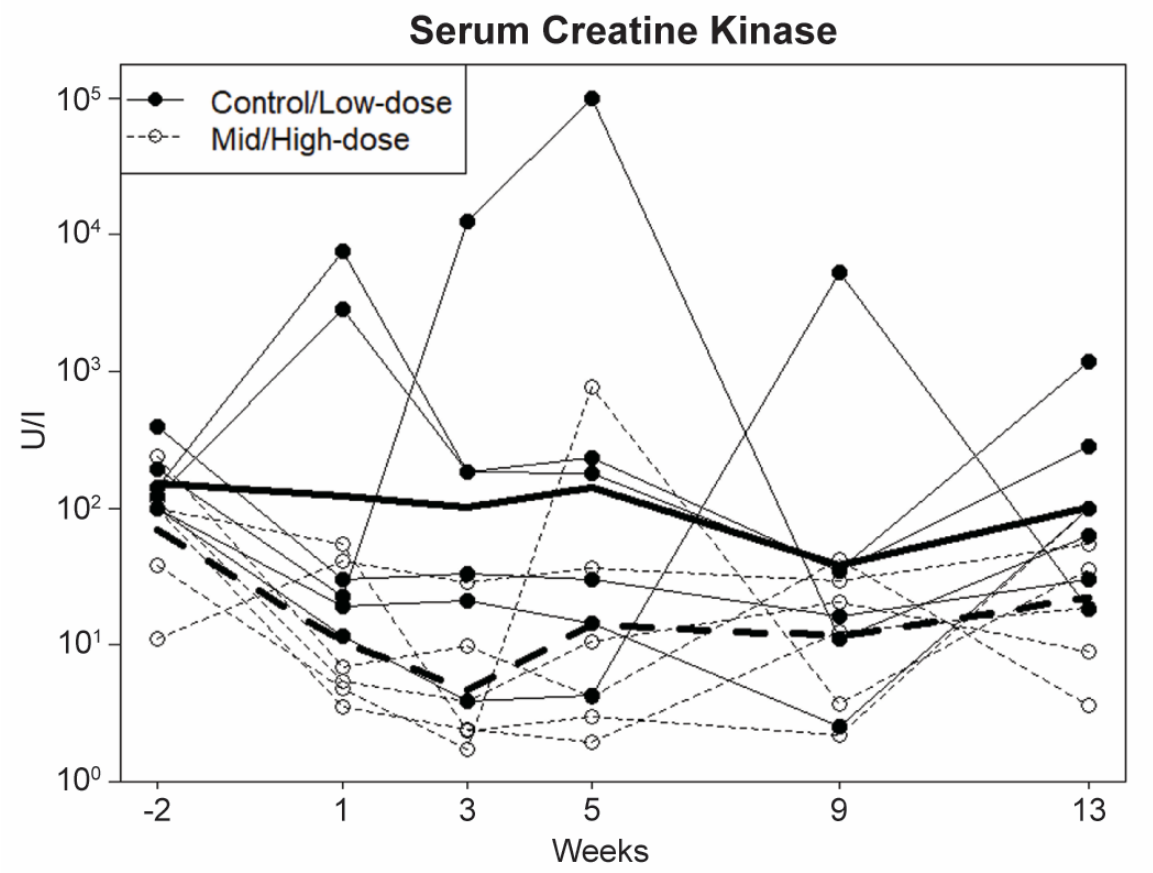


Fig. S10. Serum creatine kinase (CK) values over the treatment period. Data (U/l) are plotted for individual dogs at the different time points and mean values from the combined control/low (bold solid line) and mid/high (bold hatched line) dose groups. Low dog numbers and phenotypic variation precluded significance, with the most prominent trend occurring for the control/low versus mid/high dose dogs at Week 3 ($p = 0.16$). Normal CK values for dogs range from ~ 150-350 U/l (*56*).

Table S1. Blood collection time points.

Table S1. Blood Collection Time Points			
Time Point	CBC/Chemistry	Anti-AAV9 Antibodies	ELISpot
Pre-dose 2[a,b]	X		
Pre-dose 1[a]	X	X	X
Time Point	CBC/Chemistry	Anti-AAV9 Antibodies	ELISpot
Day 0 (infusion day)			
1 day post-infusion		X	
Day 3 post-infusion		X	
Day 7 post-infusion	X	X	
Day 16 post-infusion	X	X	
Day 31 post-infusion	X	X	X
Day 61 post-infusion	X	X	
Day 90 post-infusion	X	X	X

[a] Pre-dose blood draws were performed prior to initiation of prednisone treatment as well as prior to vector administration.

[b] A sample from pre-dose 2 was sent to the University of Florida immediately to test for antibodies prior to the infusion date so dogs with antibodies to AAV9 could be identified and removed from the study.

Table S2. Group means of percent cross sectional area positive for µDys5 (MANEX44A) in terminal samples.

Table S2. Group Means of Percent Cross Sectional Area Positive for µDys5 (MANEX44A) in Terminal Samples

Muscle	Dose							
	0 vg/kg (n=3)		1E13 vg/kg (n=3)		1E14 vg/kg (n=3)		2E14 vg/kg (n=3)	
	Mean	StDev	Mean	StDev	Mean	StDev	Mean	StDev
Cranial Sartorius	0.0	0.0	5.2	4.2	60.0	26.0	76.3	22.0
Biceps Femoris	0.3	0.6	4.3	2.8	66.7	32.1	93.0	7.2
Biceps Brachii	0.3	0.6	5.8	2.9	61.7	10.4	76.7	17.6
Extensor Digitorum Longus	0.3	0.6	6.8	3.5	63.3	2.9	71.7	14.4
Gastrocnemius	0.3	0.6	1.3	1.5	48.3	34.0	78.3	20.8
Pectoralis	0.3	0.6	4.2	3.8	68.3	17.6	86.7	10.4
Semitendinosus	0.0	0.0	20.8	18.8	73.3	20.8	93.0	7.2
Triceps Brachii	0.3	0.6	1.7	1.2	65.0	13.2	80.0	5.0
Vastus Lateralis	0.0	0.0	1.3	1.5	73.3	20.8	78.3	7.6
Diaphragm	0.0	0.0	1.7	1.2	30.0	5.0	58.3	7.6
Left Ventricle	0.0	0.0	0.3	0.6	15.0	17.3	28.3	16.1
Right Ventricle	0.0	0.0	0.3	0.6	6.0	7.8	18.3	15.3
Interventricular Septum	0.0	0.0	0.7	0.6	8.2	6.5	33.3	10.4

StDev: Standard Deviation

Table S3. Group means of percent cross-sectional area positive for proteins in terminal biceps femoris samples.

Table S3. Group Means of Percent Cross-Sectional Area Positive for Proteins in Terminal Biceps Femoris Samples

Protein	Dose							
	0 vg/kg (n=3)		1E13 vg/kg (n=3)		1E14 vg/kg (n=3)		2E14 vg/kg (n=3)	
Protein	Mean	StDev	Mean	StDev	Mean	StDev	Mean	StDev
MHCd	45.0	5.0	51.7	10.4	21.7	5.8	13.3	14.4
Utrophin	53.3	30.6	95.0	8.7	31.7	7.5	26.7	20.2
β-Sarcoglycan	36.7	11.5	60.0	36.1	76.7	10.4	80.0	18.0
nNOS	0.3	0.6	1.3	0.6	46.7	15.3	70.0*	N/A

StDev: Standard Deviation; *Data available from n=1 due to tissue quality issues impacting scoring of additional tissues.

Table S4. Percent cross-sectional area positive for proteins in biceps femoris interim biopsy samples.

Table S4. Percent Cross-Sectional Area Positive for Proteins in Biceps Femoris Interim Biopsy Samples

Protein	Dose							
	0 vg/kg (n=3)		1E13 vg/kg (n=3)		1E14 vg/kg (n=3)		2E14 vg/kg (n=3)	
	Mean	StDev	Mean	StDev	Mean	StDev	Mean	StDev
Microdystrophin	0.3	0.6	15.8	13.8	70.0	27.8	88.3	5.8
MHCd	48.3	16.1	55.0	5.0	33.3	17.6	15.0	10.0
Utrophin	85.0	5.0	88.3	2.9	40.0	20.0	26.7	15.3
β-Sarcoglycan	16.7	5.8	28.3	10.4	68.3	20.2	83.3	10.4
nNOS	0.0	0.0	6.3*	1.8*	25.3	30.8	20.5*	27.6*

*Data available from n=2 due to tissue quality issues impacting scoring of additional tissues

Table S5. Microdystrophin expression as percent of normal dystrophin measured by Western blot.

Table S5. Microdystrophin Expression as Percent of Normal Dystrophin Measured by Western Blot								
Dose	0 vg/kg (n=3)		1E13 vg/kg (n=3)		1E14 vg/kg (n=3)		2E14 vg/kg (n=3)	
Tissue	Mean	StDev	Mean	StDev	Mean	StDev	Mean	StDev
Diaphragm	BLOQ	N/A	BLOQ	N/A	13.7*	0.0	38.2	10.4
Vastus Lateralis	BLOQ	N/A	BLOQ	N/A	30.5*	15.7	28.8	5.8
Cranial Sartorius	BLOQ	N/A	BLOQ	N/A	22.0*	11.8	34.1	16.5
Left Ventricle	BLOQ	N/A	BLOQ	N/A	12.6*	2.7	28.3	7.3
Biceps Brachii	BLOQ	N/A	BLOQ	N/A	25.6*	14.2	53.4	24.9
Liver	BLOQ	N/A	BLOQ	N/A	BLOQ	N/A	BLOQ	N/A

BLOQ: Below Limit of Quantification; StDev: Standard Deviation; *n of 2 based on 2 samples out of 3 above BLOQ

Table S6. Microdystrophin protein expression fold change high/mid (2E14/1E14 vg/kg) by ~~W~~western blot.

Table S6. Microdystrophin Protein Expression Fold Change High/Mid (2E14/1E14 vg/kg) by Western Blot					
Fold Change	**Diaphragm**	**Vastus Lateralis**	**Cranial Sartorius**	**Left Ventricle**	**Biceps Brachii**
2E14 / 1E14	2.8	0.9	1.6	2.2	2.1

Table S7. Microdystrophin expression as percent of normal dystrophin measured by mass spectrometry.

Table S7. Microdystrophin Expression as Percent of Normal Dystrophin Measured by Mass Spectrometry								
Dose	0 vg/kg (n=3)		1E13 vg/kg (n=3)		1E14 vg/kg (n=3)		2E14 vg/kg (n=3)	
Tissue	Mean	StDev	Mean	StDev	Mean	StDev	Mean	StDev
Diaphragm	BLOQ	N/A	BLOQ	N/A	14.6	2.3	46.0	14.9
Vastus Lateralis	BLOQ	N/A	BLOQ	N/A	32.9	22.2	34.3	19.6
Cranial Sartorius	BLOQ	N/A	BLOQ	N/A	46.7*	9.0*	52.0	26.3
Left Ventricle	BLOQ	N/A	BLOQ	N/A	16.5*	7.2*	26.6	6.3
Biceps Brachii	BLOQ	N/A	10.0#	N/A	36.0	15.8	61.3	51.0

BLOQ: Below Limit of Quantification; StDev: Standard Deviation; #: n of 1 based on 1 sample out of 3 above BLOQ; *: n of 2 based on 2 samples out of 3 above BLOQ

Table S8. Microdystrophin protein expression fold change high/mid (2E14/1E14 vg/kg) by mass spectrometry.

Table S8. Microdystrophin Protein Expression Fold Change High/Mid (2E14/1E14 vg/kg)* by Mass Spectrometry					
Fold Change	Diaphragm	Vastus Lateralis	Cranial Sartorius	Left Ventricle	Biceps Brachii
2E14 / 1E14	3.2	1.0	1.1	1.6	1.7

*Fold change was calculated by dividing the high dose value by the mid dose value.

Table S9. Group means for anti-AAV9 circulating antibodies by day and dose group.

Table S9. Group Means of Anti-AAV9 Circulating Antibody Levels by Day and Dose Group								
Day	Dose							
	0 vg/kg (n=3)		1E13 vg/kg (n=3)		1E14 vg/kg (n=3)		2E14 vg/kg (n=3)	
	Mean	STD	Mean	STD	Mean	STD	Mean	STD
Baseline	1	0	1	-	1	-	1	-
Day 1	769	1.09E3	602	850	593	838	939	1.33E3
Day 3	869	1.23E3	947	1.34E3	907	1.28E3	2.00E3	567
Day 7	2.47E3	1.12E3	8.91E4	8.12E4	3.31E5	2.82E5	1.43E6	2.44E6
Day 16	600	1.04E3	2.95E6	3.62E5	3.25E6	1.96E6	5.22E7	8.37E7
Day 31	1.47E3	2.54E3	5.65E6	2.61E6	1.21E7	5.71E6	9.83E7	1.51E8
Day 61	2.81E3	1.84E3	6.99E6	4.97E6	9.14E6	3.02E6	1.22E7*	2.74E6
Day 90	459	793	3.59E6	1.47E6	1.01E7	3.86E6	6.71E7	9.70E7

1 = Result was less than the lower limit of detection at dilutions performed for all group samples; *A blood sample from the highest responder in the high dose group was inadvertently not collected at day 61, potentially causing this value to be artifactually lower.

Table S10. Group means (*n* =3) for baseline, interim (Day 45), and terminal (Day 90) biopsy lesion scores.

Table S10. Group Means (n =3) for Baseline, Interim (Day 45), and Terminal (Day 90) Biopsy Lesion Scores				
	Vehicle	**1E13 vg/kg**	**1E14 vg/kg**	**2E14/kg**
Cranial Sartorius				
Baseline	Mild/Moderate	Mild/Moderate	Mild	Mild
Day 90	Moderate	Moderate	Moderate	Mild/Moderate
Vastus Lateralis				
Baseline	Moderate	Mild/Moderate	Moderate	Moderate
Day 90	Moderate	Moderate	Moderate	Mild
Biceps Femoris				
Day 45	Moderate/Severe	Moderate	Mild	Very Mild
Day 90	Moderate	Mild/Moderate	Mild/Moderate	Very Mild

Table S11. MRI data from AAV9-μDys5-treated dogs.

Table S11. MRI Data from AAV9-μDys5-Treated Dogs

Age (~ days)	Muscle Volume			T2 Value			SLI			HI		
	BF	ST	CS	BF	ST	CS	BF	ST	CS	BF	ST	CS
Low Dose												
0	13.5 ±13	7.83 ±2.4	2.35 ±0.96	57.1 ±5.9	37.5 ±28.1	37.2 ±32.4	0.99 ±0.01	0.99 ±0.01	0.89 ±0.10	2120 ±620	1460 ±950	659 ±270
90	47.7 ±13	19.1 ±7.7	5.55 ±0.98	54.4 ±6.5	36.2 ±21	50.4 ±1.0	1.00 ±0.00	0.99 ±0.01	0.98 ±0.00	3770 ±1800	2620 ±1200	1180 ±290
% Change (0-90)	272 ±83	137 ±32	177 ±120	-3.66 ±18	37.1±82	-9.34 ±8.8	0.46 ±0.70	0.33 ±0.33	16.0 ±16	90.4 ±100	109 ±130	132 ±34
Mid Dose												
0	14.4 ±4.3	8.18 ±1.3	1.41 ±0.54	49.4 ±0.9	50.4 ±2.8	54.2 ±8.8	0.99 ±0.00	0.99 ±0.00	0.80 ±0.09	3060 ±240	1860 ±200	359 ±98
90	63.5 ±13	27.5 ±2.7	6.73 ±1.2	42.6 ±7.9	37.9 ±0.73	52.7 ±9.3	1.00 ±0.00	1.00 ±0.00	0.97 ±0.02	5070 ±2700	3800 ±1100	1570 ±480
% Change (0-90)	359 ±120	245 ±89	415 ±160	-20.4 ±5.0	-24.7 ±4.9	-3.01 ±6.1	0.46 ±0.33	0.76 ±0.41	22.5 ±14	70.8 ±100	110 ±82	364 ±200
High Dose												
0	14.3 ±0.20	8.16 ±0.55	2.04 ±0.55	48.5 ±0.68	48.1 ±0.31	48.6 ±0.85	0.99 ±0.00	0.99 ±0.01	0.93 ±0.02	2690 ±410	1780 ±240	819 ±82
90	64.7 ±8.5	24.7 ±4.3	4.91 ±12.6	35.2 ±17.9	29.8 ±28.9	33.4 ±29	1.00 ±0.00	1.00 ±0.00	0.97 ±0.02	5720 ±2100	3540± 670	1130 ±790
% Change (0-90)	353 ±58	201 ±33	133 ±120	-27.4 ±26	-37.9 ±37	-31.1 ±60	0.35 ±0.16	0.82 ±0.75	4.72 ±1.3	108 ±60	102 ±55	32.5 ±84
Controls												
0	19.1 ±2.8	10.2 ±0.88	2.20 ±0.57	46.7 ±1.3	43.8 ±4.2	46.8 ±1.4	1.00 ±0.00	0.99 ±0.00	0.96 ±0.01	3640 ±430	2370 ±340	1100 ±470
90	58.3 ±7.1	24.9 ±4.7	6.43 ±2.4	47.8 ±0.80	45.9 ±5.5	46.7 ±3.8	1.00 ±0.00	1.00 ±0.00	0.98 ±0.00	5690 ±1200	4510 ±1700	1490 ±420
% Change (0-90)	215 ±99	169 ±54	148 ±120	1.42 ±3.2	0.12 ±5.0	4.46 ±3.3	0.23 ±0.14	0.33 ±0.31	2.57 ±1.5	56.2 ±23	86.4 ±46	51.0 ±76
Combined Control/Low Dose												
0	15.7 ±5.0	8.79 ±2.2	2.29 ±0.74	52.9 ±7.1	40.0 ±20.3	41.1 ±23.5	0.99 ±0.00	0.99 ±0.00	0.92 ±0.07	2880 ±960	1910 ±810	879 ±420
90	53.0 ±11	22.0 ±6.5	6.08 ±1.8	51.1 ±5.5	41.0 ±14.7	48.2 ±3.4	1.00 ±0.00	0.99 ±0.00	0.98 ±0.00	4730 ±1700	3560 ±1700	1370 ±370
% Change (0-90)	249 ±83	150 ±40	162 ±99	-1.63 ±13	22.3 ±61	-2.44 ±9.6	0.35 ±0.47	0.33 ±0.29	7.94 ±11	73.3 ±68	97.7 ±86	83.4 ±72
Combined Mid/High Dose												
0	14.3 ±2.7	8.17 ±0.88	1.73 ±0.59	49.0 ±2.7	49.3 ±2.2	51.4 ±6.4	0.99 ±0.00	0.99 ±0.01	0.86 ±0.09	2880 ±360	1820 ±200	589 ±260
90	64.1 ±9.9	2.61 ±3.6	5.82 ±2.2	38.9 ±9.2	33.9 ±12.2	43.1 ±21.9	1.00 ±0.00	1.00 ±0.01	0.97 ±0.02	5400 ±2200	3670 ±840	1350 ±630
% Change (0-90)	356 ±84	223 ±65	274 ±200	-20.1 ±18	-31.3 ±25	-17.1 ±41	0.40 ±0.24	0.79 ±0.54	13.6 ±13	90±79	106 ±63	198 ±230

SLI: small lesion index; HI: heterogeneity index

Table S12. Joint angle data from AAV9-µDys5-treated dogs.

Table S12. Joint Angle Data from AAV9-µDys5-Treated Dogs					
Group ~ Study Day	TTJ	Hip			Pelvic
		Rest	Flex	ROM	
Low Dose					
0	160±6.6	104±5.5	42.7±3.1	112±5.3	36.0±4.4
45	158±10	108±6.8	54.3±5.7	96.3±1.5	39.3±8.4*
% Change (0-45)	-1.07±4.1	4.78±10	27.2±6.7	-13.8±5.2	44.6±17
90	140±18	107±11	53.0±17	98.0±30	36.0±5.3
% Change (0-90)	-12.5±8.9	4.07±16	24.3±39	-13.1±23	2.34±29
Mid Dose					
0	164±1.5	100±3.0	38.7±4.2	114±5.2	38.3±2.5
45	166±2.9*	115±7.0	50.0±8.5	105±11	39.3±2.5*
% Change (0-45)	1.64±2.6	14.9±3.6	31.9±37	-7.52±13	3.60±14
90	158±4.4	108±7.1	54.0±11	110±22	39.3±6.4
% Change (0-90)	-3.44±3.5	8.26±3.9	42.8±44	-2.72±22	3.60±23
High Dose					
0	157±7.8	99.0±6.1	39.0±8.7	110±22	33.7±2.9
45	156±1.0	97.0±1.0*	45.3±4.5	110±21	35.3±3.5*
% Change (0-45)	-0.48±4.8	-1.78±6.0	21.0±32	5.65±44	8.00±19
90	156±5.1	103±10	50.7±5.9	109±14	35.3±2.1
% Change (0-90)	-0.72±5.0	3.88±4.0	32.9±25	1.17±44	5.77±19
Controls					
0	156±1.5	99.0±2.7	44.3±8.4	113±12	35.7±2.5
45	146±6.8	114±9.5	62.7±9.0	98.3±17	51.3±3.5
% Change (0-45)	-6.39±5.2	14.9±11	45.4±38	-11.3±22	44.6±16
90	152±6.7	108±2.0	57.3±5.1	97.7±8.1	45.3±7.5
% Change (0-90)	-2.56±4.49	9.14±3.18	32.37±27.07	-13.07±5.01	28.19±28.80
Combined Control/Low Dose					
0	158±4.9	101±4.6	43.5±5.7	112±38	35.8±3.2
45	152±10	111±8.0	58.5±8.1	97.3±11	35.8±8.7
% Change (0-45)	-3.73±5.1	9.84±11	36.3±26	-12.6±14	26.8±24
90	146±14	108±7.1	55.2±11	97.8±20	40.7±8.7
% Change (0-90)	-7.50±5.0	6.60±10	28.3±31	-13.1±15	15.3±29
Combined Mid/High Dose					
0	160±6.2	99.5±4.6	38.8±6.1	112±14	36.0±3.5
45	161±6.0	106±11	47.7±8.1	107±15	37.7±4.1
% Change (0-45)	0.58±3.6	6.58±10	26.4±32	-.094±30	5.43±15
90	157±4.5	106±8.5	52.3±8.2	110±16	37.3±4.8
% Change (0-90)	-2.08±4.2	6.07±4.3	37.8±33	-0.78±16	4.69±17

*Significantly different versus control $p < 0.05$; TTJ = Tibiotarsal joint; ROM = Range of motion

Table S13. Electrocardiography results.

Table S13. Electrocardiography Results

	Baseline			Day 45			Day 90		
	Control/Low Dose	Mid/High Dose	P Value	Control/Low Dose	Mid/High Dose	P Value	Control/Low Dose	Mid/High Dose	P Value
HR (bpm)	156±18	164±11	0.52	157±18	157±25	1.00	160±16	148±17	0.18
RR (msec)	388±49	366±23	0.58	386±49	392±67	1.00	378±36	409±51	0.18
PR (msec)	72±10	78±6	0.09	74±6	84±8	0.04*	75±7	85±6	0.049*
QRS (msec)	39±3	42±5	0.46	43±2	42±4	0.69	42±3	43±4	0.95
QT (msec)	209±8	210±9	0.70	213±26	216±23	0.98	202±14	211±15	0.33
QTc (msec)	262±5	265±8	0.48	266±22	268±18	0.81	256±12	262±12	0.27
% Change of HR 0-45				2.36±21.83	-4.63±13.36	0.82			
% Change of HR 0-90							3.57±15.79	-9.57±11.67	0.39
% Change of PR 0-45				3.77±10.79	8.14±11.55	0.59			
% Change of PR 0-90							5.28±10.60	9.90±10.71	0.78

Table S13. Electrocardiographic Results

	Baseline			Day 45			Day 90		
	Control/Low Dose	Mid/High Dose	P Value	Control/Low Dose	Mid/High Dose	P Value	Control/Low Dose	Mid/High Dose	P Value
HR (bpm)	156±18	164±11	0.52	157±18	157±25	1.00	160±16	148±17	0.18

Table S14. Echocardiography, stress echocardiography, and cardiac magnetic resonance imaging results.

Table S14. Echocardiography, Stress Echocardiography, and Cardiac Magnetic Resonance Results

	Baseline			Day 45			Day 90		
	Control/Low Dose	Mid/High Dose	P Value	Control/Low Dose	Mid/High Dose	P Value	Control/Low Dose	Mid/High Dose	P Value
Echocardiography									
IVSd (cm)	0.57±0.05	0.52±0.04	0.24	0.68±0.08	0.7±0.09	0.99	0.75±0.08	0.72±0.08	0.73
LVIDd (cm)	1.97±0.27	1.98±0.20	0.92	2.50±0.28	2.58±0.17	0.42	2.58±0.33	2.72±0.12	0.12
LVPWd (cm)	0.50±0.09	0.48±0.08	0.99	0.57±0.10	0.68±0.08	0.10	0.68±0.08	0.72±0.08	0.71
IVSs (cm)	0.67±0.10	0.63±0.05	0.51	0.80±0.09	0.85±0.10	0.56	0.87±0.12	0.85±0.05	1.00
LVIDs (cm)	1.52±0.21	1.50±0.18	1.00	1.95±0.27	1.97±0.24	1.00	2.00±0.30	2.10±0.24	0.42
LVPWs (cm)	0.62±0.12	0.65±0.08	0.56	0.77±0.10	0.83±0.08	0.32	0.88±0.12	0.93±0.10	0.63
FS (%)	23.33±4.84	25.00±2.83	0.41	22.17±2.40	23.83±5.34	0.67	22.83±2.56	22.33±5.89	0.73
LVEDV (mL)	6.63±1.79	7.13±2.49	0.95	13.71±2.64	15.88±2.01	0.14	15.67±2.23	16.63±3.50	0.79
LVESV (mL)	3.67±1.16	4.00±1.26	1.00	7.67±1.26	9.42±2.65	0.29	8.54±2.28	9.54±3.30	0.66
EF (%)	45.71±5.26	46.25±3.50	0.73	43.04±7.52	42.13±11.99	0.82	45.17±8.57	43.67±8.72	0.97
MV E/A Ratio	2.09±0.24	1.58±0.22	0.008*	1.79±0.36	1.65±0.18	0.37	1.65±0.24	1.63±0.17	0.67
Circ Strain (%)	-9.63±2.78	-11.67±1.40	0.31	-9.73±2.20	-10.72±3.06	0.59	-10.81±2.94	-10.06±2.35	0.82
E/Em	11.69±2.65	12.88±3.96	0.70	11.37±2.84	10.57±3.06	0.46	11.84±3.84	15.78±3.36	0.13
% Change FS 0-45				-1.60±23.22	-4.02±22.97	0.82			
% Change FS 0-90							0.22±17.54	-10.58±22.25	0.31
% Change EF 0-45				-5.03±18.16	-8.32±27.50	1.00			
% Change EF 0-90							-1.02±16.48	-5.23±19.80	0.56

Table S14 (continued). Echocardiography, Stress Echocardiography, and Cardiac Magnetic Resonance Results

	Baseline			Day 45			Day 90		
Stress Echocardiography									
FS_{10} (%)	42.50±6.95	43.00±6.32	0.94	45.00±8.71	50.50±10.84	0.22	49.33±11.17	51.17±4.58	0.73
ΔFS_{0-10} (%)	18.67±4.72	18.83±5.04	0.90	21.33±7.99	26.17±7.68	0.48	26.17±10.26	29.33±6.77	0.73
Cardiac Magnetic Resonance									
LVEDV (mL)	10.78±2.52	10.20±2.18	0.70				23.00±6.74	22.90±5.45	0.48
LVESV (mL)	6.47±2.18	5.85±1.74	0.48				13.73±5.82	13.53±4.86	0.82
SV (mL)	4.32±0.55	4.33±1.41	0.97				9.27±2.21	9.38±2.28	1.00
EF (%)	41.18±7.60	42.45±10.27	0.94				41.50±9.79	42.37±12.09	0.82
CO (L/min)	0.54±0.10	0.56±0.17	0.79				1.17±0.36	1.13±0.30	1.00
Myo Mass (g)	16.48±3.54	14.82±2.03	0.42				37.68±7.52	40.48±3.35	0.33
% Change EF 0-90							1.28±18.47	4.11±37.73	1.00

Values are shown as Mean±SD. Six dogs were in each of the combined control/low and mid/high dose groups. Anesthesia caused lower FS and EF values. Baseline differences of mitral valve E/A ratio likely related to biological variability or measuring artifacts. IVSd, interventricular septum in diastole; LVIDd, left ventricular internal diameter in diastole; LVPWd, left ventricular posterior wall in diastole; IVSs, interventricular septum in systole; LVIDs, left ventricular internal diameter in systole; LVPWs, left ventricular posterior wall in systole; FS, fractional shortening; LVEDV, left ventricular end-diastolic volume; LVESV, left ventricular end-systolic volume; EF, ejection fraction; MV E/A Ratio, ratio of mitral valve E wave to A wave; Circ Strain, circumferential strain; E/Em, ratio of the mitral valve E wave to early diastolic myocardial velocity in tissue Doppler imaging; % Change, percentage change; FS_{10}, FS value under dobutamine 10 µg/kg/min stress; ΔFS_{0-10}, change of FS value during dobutamine 10 µg/kg/min stress; SV, stroke volume; CO, cardiac output; Myo mass, myocardial mass at end-diastole.

Table S15. Serum creatine kinase (U/l) means and standard deviations at various time points for the different groups.

Table S15. Serum Creatine Kinase (U/l)*#						
Dose Group	Week -2^ (Day -8 to -13)	Week 1 (Day 7)	Week 3 (Day 16)	Week 5 (Day 30 to 34)	Week 9 (Day 60 to 63)	Week 13 (Day 91 to 99)
Control	4.29E4±2.9E3	4.34E4±3.0E4	5.13E4±2.8E4	5.68E4±3.9E4	2.00E4±1.1E4	4.58E4±1.4E4
Low (1E13)	4.46E4±6.5E3	4.02E4±2.6E4	2.92E4±1.7E4	2.92E4±1.6E4	4.32E4±2.7E4	3.46E4±1.3E4
Control-Low	4.38E4±4.6E3	4.17E4±2.5E4	4.02E4±2.4E4	4.30E4±3.1E4	3.16E4±2.3E4	4.02E4±1.3E4
Mid (1E14)	3.40E4±1.1E4	1.93E4±1.1E4	1.62E4±1.1E4	1.92E4±1.3E4	2.58E4±3.7E3	2.65E4±7.9E3
High (2E14)	3.98E4±8.0E3	2.18E4±1.1E4	1.07E4±8.2E3	2.66E4±2.7E4	1.69E4±1.4E4	2.74E4±1.5E4
Mid-High	3.69E4±9.3E3	2.05E4±1.0E4	1.34E4±9.3E3	2.29E4±1.9E4	2.14E4±1.0E4	2.69E4±1.1E4

*Median value for normal dogs 159-341 (~~9~~569~~9~~). #n=3 for all groups at each age. ^Blood draws were done within 7 days before or after the target date; in most cases, this was on the same day or within 2-3 days.

Table S16. Serum Alanine Aminotransferase (U/L) values.

Table S16. Serum Alanine Aminotransferase (ALT) (U/L) Values*								
	Dose							
	0 vg/kg (n=3)		1E13 vg/kg (n=3)		1E14 vg/kg (n=3)		2E14 vg/kg (n=3)	
Day Relative to Dosing^	Mean	StDev	Mean	StDev	Mean	StDev	Mean	StDev
-8 to -13	375	92	296	40	353	98	346	62
7	327	72	294	56	267	68	243	69
16	391	122	252	59	199	81	118	24
31	377	126	275	108	230	105	162	52
61	339	108	263	63	309	94	212	24
90*	352	N/A	303	N/A	397	N/A	320	N/A

*Median value for normal dogs 14-40 (5699). ^Blood draws were done within 7 days before or after the target date; in most cases, this was on the same day or within 2-3 days. StDev = Standard deviation.

Table S17. Serum Aspartate Aminotransferase (AST) (U/L) values.

Table S17. Serum Aspartate Aminotransferase (AST) (U/L) Values*								
Day Relative to Dosing^	0 vg/kg (n=3)		1E13 vg/kg (n=3)		1E14 vg/kg (n=3)		2E14 vg/kg (n=3)	
	Mean	StDev	Mean	StDev	Mean	StDev	Mean	StDev
-8 to -13	647	153	660	201	539	239	735	389
7	613	432	632	293	276	164	339	210
16	649	268	330	254	227	149	114	30
31	850	539	449	245	328	196	403	331
61	400	64	702	433	427	39	289	164
90*	804	N/A	400	N/A	351	N/A	551	N/A

*Median value for normal dogs 23-30 (5699). ^Blood draws were done within 7 days before or after the target date; in most cases, this was on the same day or within 2-3 days. StDev = Standard deviation.