

LONG-TERM EFFECTS OF SYSTEMIC GENE THERAPY IN A CANINE MODEL OF MYOTUBULAR MYOPATHY

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Accepted 21 March 2017

ABSTRACT: *Introduction:* X-linked myotubular myopathy (XLMTM), a devastating pediatric disease caused by the absence of the protein myotubularin, results from mutations in the *MTM1* gene. While there is no cure for XLMTM, we previously reported effects of *MTM1* gene therapy using adeno-associated virus (AAV) vector on muscle weakness and pathology in *MTM1*-mutant dogs. Here, we followed 2 AAV-infused dogs over 4 years. *Methods:* We evaluated gait, strength, respiration, neurological function, muscle pathology, AAV vector copy number (VCN), and transgene expression. *Results:* Four years following AAV-mediated gene therapy, gait, respiratory

performance, neurological function and pathology in AAV-infused XLMTM dogs remained comparable to their healthy littermate controls despite a decline in VCN and muscle strength. *Conclusions:* AAV-mediated gene transfer of *MTM1* in young XLMTM dogs results in long-term expression of myotubularin transgene with normal muscular performance and neurological function in the absence of muscle pathology. These findings support a clinical trial in patients.

Muscle Nerve 56: 943–953, 2017

Additional supporting information may be found in the online version of this article.

Abbreviations: AAV, adeno-associated virus; AAV8, AAV serotype 8; EM, electron microscopy; H&E, hematoxylin and eosin; NADH, reduced nicotinamide adenine dinucleotide; NAS, neurological assessment score; PIF, peak inspiratory flow; ROM, range of motion; T_{8w}, 8 weeks of age; T_{12w}, 12 weeks of age; T_{16w}, 16 weeks of age; T_{21w}, 21 weeks of age; T_{1y}, 1 year of age; T_{2y}, 2 years of age; T_{3y}, 3 years of age; T_{4y}, 4 years of age; VCN, vector copy number; WT, wild-type; XLMTM, X-linked myotubular myopathy

Key words: adeno-associated virus; canine; gait; gene therapy; muscle; neuromuscular disease; myotubular myopathy

M.E. and M.A.G. contributed equally to this work.

Funding: Association Française contre les Myopathies (France) to A.B.-B. and M.K.C.; Myotubular Trust (UK) to A.B.-B.; Genopole d'Evry (France) to A.B.-B.; INSERM (France) to A.B.-B.; U.S. NIH grants R21 AR064503 and R01 HL115001 to M.K.C.; R01AR044345 and HD075802 to A.H.B., and K08 AR059750 to M.W.L.; Audentes Therapeutics, Inc. to University of Washington, PI M.K.C. Muscular Dystrophy Association MDA383249 to A.H.B. and MDA/MVP to M.K.C.; the Joshua Frase Foundation and Where There's a Will There's a Cure to A.H.B. and M.K.C.; Peter Khuri Myopathy Research Foundation to M.K.C.; and Senator Paul D Wellstone Muscular Dystrophy Cooperative Research Center, Seattle (NIH U54AR065139).

Conflicts of Interest: A.H.B. has received support from, and/or has served as a paid consultant for Audentes Therapeutics. A.B.-B. has received support from, and/or has served as a paid consultant for Audentes Therapeutics. M.K.C. has received support from, and/or has served as a paid consultant for Audentes Therapeutics. M.W. L. has received support from, and/or has served as a paid consultant for Audentes Therapeutics. The remaining authors have no conflicts of interest.

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Published online 29 March 2017 in Wiley Online Library (wileyonlinelibrary.com). DOI 10.1002/mus.25658

X-linked myotubular myopathy (XLMTM) is the most common of the centronuclear myopathies; it is estimated to affect 1 in 50,000 male births.^{1–3} The disease is caused by mutation of the *MTM1* gene,^{4,5} and affected boys develop severe weakness of skeletal muscles due to a deficiency of the protein myotubularin.^{4,6} XLMTM presents at birth as marked hypotonia and respiratory impairment and is often fatal in the first year of life.^{7,8} This myopathy affects primarily males, except in cases where there is skewed X-inactivation.^{9,10} While there are instances of spontaneous occurrence, *MTM1* mutations are often inherited from asymptomatic carrier mothers.¹¹ The most severely affected boys become wheelchair dependent; however, a subset of patients with a milder form of the disease have been reported.^{12,13} These patients can survive until adulthood and have been known to remain ambulatory for a longer period of time.^{8,14}

In Labrador retriever and mixed breed dogs, a p.N155K missense mutation in canine *MTM1* results in profound skeletal muscle weakness in affected puppies analogous to the phenotype seen in boys with XLMTM.^{15,16} XLMTM dogs experience weakness and atrophy of skeletal muscles, causing difficulty with standing, eating, and respiratory function.^{17–20} The average lifespan of affected animals is 18–20 weeks of age.^{15,18,21}

Analogous to human patients, carrier female dogs are phenotypically normal.¹⁶

As XLMTM is caused by mutations in the *MTMI* gene, adeno-associated virus (AAV) -mediated gene replacement therapy is under intense investigation. We previously found that a direct intramuscular injection of AAV serotype 8 (AAV8) carrying a normal copy of the canine *MTMI* gene to the cranial tibialis muscle of *MTMI*-mutant dogs restored strength of the injected limb muscle to near-normal, but contiguous noninjected limb muscles remained weakened without a commensurate improvement in overall walking gait.²² Compared with normal dogs, XLMTM dogs averaged a 46% reduction in gait speed and a 68% decrease in stride length near the end of their lifespan at 18 weeks.²³

We concluded from these findings that intramuscular AAV resulted in marked strength improvement of a single paw flexor, the cranial tibialis, but targeted improvement in a single limb muscle alone is not sufficient to result in overall gait improvement.²⁴ In contrast, transvenous limb perfusion, where the limb is isolated before administration of high-pressure perfusion, allows for AAV infusion of the entire lower limb musculature.²⁵ Dogs treated this way demonstrated improved hind limb strength in the infused limb after treatment, but probably due to leak of AAV into the systemic circulation, effects were seen well beyond the isolated hindlimb muscles.²² This quasi-systemic delivery resulted in improved strength in contralateral untreated hindlimbs, improved respiratory function, and increased lifespan. These previous data suggested that, unlike a single targeted intramuscular injection, whole body transduction by AAV leads to overall improvement in walking gait in *MTMI* mutant dogs. To address the question of long-term duration of systemic AAV delivery, we performed a battery of physiological, clinical, and biochemical analysis over a period of 4 years in AAV-treated XLMTM dogs.

Spatiotemporal measures of gait using 2-dimensional video-based analysis are sensitive to changes caused by disease, as we published in XLMTM dogs.²³ Quantitative gait analysis established the effects of limb muscle impairment due to neuromuscular disease²⁶ in studies of canine models of Duchenne muscular dystrophy.^{27,28} Video-based kinematic analysis detects changes in range of motion (ROM) due to disease in canine models of neuromuscular disease.²⁷ However, kinematic analysis can be time intensive and requires extensive training for appropriate accuracy. Equipment set-up, including camera placement and frame rate, can also affect accuracy and precision.^{29,30} Given that measures of ROM are not

sufficiently sensitive to differentiate between XLMTM dogs and normal controls,²³ an instrumented carpet provides an alternate method for assessing gait function. The system has been validated for use in dogs, is relatively easy to use in a reproducible manner, and allows for quantification of some spatiotemporal measures unavailable with 2-dimensional analysis, such as step width.^{30,31}

In this study, our technical aim was to compare spatiotemporal measures from a video-based motion analysis with data collected using an instrumented carpet. We anticipated good agreement between methods, as both are validated approaches to quantify gait. Support of this idea would further justify the use of the more efficient instrumented walkway to quantify meaningful gait changes in the canine model of XLMTM. Our clinical aim was to compare gait between XLMTM dogs treated with a single intravenous infusion of AAV8-*MTMI* with control dogs over a 2-year time period to test the hypothesis that treatment of XLMTM dogs preserves gait function over time. This longitudinal assessment of function in the preclinical model of XLMTM is particularly informative, as gait function may indicate the overall duration of gene replacement therapy. This could inform expected patient outcomes and future treatment strategies upon translation to the clinic.

We report here the long-term follow-up of two dogs initially treated by loco-regional (quasi-systemic) perfusion with AAV8-*MTMI*.²² In addition to gait assessment using the instrumented carpet, we used assessment methods from previously published experiments to monitor the dogs' health 4 years postinfusion with AAV8-*MTMI*.³² These assessments included muscle strength, neurological function, respiratory effort, and histological and molecular examination of muscle biopsies.^{19,20}

MATERIALS AND METHODS

Dogs. Care and use of all laboratory animals was in keeping with the National Research Council guidelines and as approved by the Institutional Animal Care and Use Committee. XLMTM dogs ($N=2$) were identified after birth by Taqman genotyping assay.¹⁵ Two groups were evaluated. For short-term assessment, XLMTM dogs ($N=2$) named, "Pavlov" and "Turing," were measured before treatment at 8 weeks of age (T_{8w}) and then every 4 weeks posttreatment (T_{12w} and T_{16w}) until 21 weeks of age (T_{21w}) alongside wild-type (WT) male controls ($N=2$). For the long-term assessment, XLMTM dogs were measured 1 (T_{1y}), 2 (T_{2y}), 3 (T_{3y}), and 4 (T_{4y}) years after treatment as compared to normal carrier females ($N=3$ at 1 and 2 years; $N=2$ at 3 and 4 years). Data collected up to 1 year of age were taken from historical data sets, some of which were previously published in (20, 22). All dogs used in the study were true littermates. Hindlimb length was recorded in anesthetized dogs with the limb against a flat surface at all time points excluding the last at 2 years after treatment, where measures were

taken in awake, recumbent animals with the aid of a handler.

Treatment. XLMTM dogs were treated at 9 weeks of age by means of isolated limb perfusion as previously described.²² Briefly, a tourniquet was applied to the left hindlimb, and a single dose of AAV8-MTMI (2×10^{14} vg/kg, as determined by retrospective analysis of the administered product by an improved titration assay) was injected into the saphenous vein under high pressure.

Gait Assessment. Dogs walked on a 0.9×7 m instrumented carpet (GAITRite Electronic Walkway, CIR Systems Inc.) at 6 time points, 8, 12, 16, and 21 weeks of age (comparison with WT male controls), as well as at 1 and 2 years after treatment (comparison with carrier female controls). A trained handler walked alongside leashed dogs in alternating directions parallel to the instrumented carpet at the dog's self-selected pace. Food reward, praise, or squeaker toys were used as encouragement. Walks were selected based on consistency of gait pattern and speed and were analyzed using proprietary software (GAITFour version 4.1, CIR Systems Inc). Gait analysis with the instrumented carpet provided measures of gait speed, stride length, stride time, step length, step time, and stance time. These parameters were evaluated as outcome measures likely to display changes over time due to disease progression or subsequent recovery as a result of treatment.²³

Video motion capture data were simultaneously recorded in the middle 2.4 meters of the instrumented carpet in front of a dark cloth backdrop at 8, 12, and 16 weeks of age. As previously described,²³ retro-reflective tape was placed on the greater trochanter of the femur, a point equidistant between the lateral epicondyle of the femur and the fibular head, the lateral malleolus of the distal tibia, and the distolateral aspect of the fifth metatarsus. A spotlight was used during video capture for maximal marker brightness. Video was filmed with a single camera (DCR-SX63, Sony, Japan) at 30 Hz. The quality of videos was assessed based on consistency of the gait pattern and straightness of the head, body, and the walking path trajectory. Videos were digitized with MaxTRAQ software (Standard version 2.2.4.2) and exported to Visual 3D (C-Motion, Inc., Germantown, Maryland). Markers were filtered with a second-order bidirectional lowpass Butterworth filter with a cutoff frequency of 4 Hz, and heel-strike and toe-off events were identified for calculation of spatiotemporal measures across strides. Gait analysis with video motion capture provided measures of gait speed, stride length, and stride time.

Analysis. To compare spatiotemporal measures by the different gait analysis methods, the correlation between stride length and stride time using video motion capture and the instrumented carpet was calculated at the 16-week time point. This time point was selected due to the larger number of useful walks. At earlier time points, the young age of dogs, less experience with leash training, and smaller size of the dogs reduced the quality of video digitization. To compare gait data between XLMTM and control dogs, spatiotemporal parameters collected from the instrumented carpet were evaluated longitudinally and reported as means and SDs. Where relevant, measures outside of the 95% confidence intervals are indicated as deviations from control values in lieu of statistical comparisons with *P*-values.^{33,34}

Strength Assessment. We followed our published protocols for dogs.³⁵ Briefly, hindlimb torque values were measured in anesthetized dogs by placing the foot of the dog on a pedal that was attached to a motor that provides resistance to the extending or flexing muscle while also measuring the amount of torque produced by said muscle. The muscle was then stimulated using percutaneously inserted electrodes over a range of stimulation frequencies to determine torque-frequency relationships. Year 3 assessments were performed on the left limbs, and year 4 assessments were performed on the right.

Pulmonary Function. Respiratory assessment was carried out in anesthetized dogs before and after stimulation by a centrally acting stimulant, doxapram hydrochloride (1 mg/kg), as described.^{36–38} Briefly, data were collected in intubated dogs using a calibrated pneumotachometer, where changes in pressure across the device determined airflow. Flow-volume loops were created by replaying experimental data through the appropriate analyzer (iox 2.8.0.13, EMKA Technologies) and capturing 10 consecutive breaths during doxapram stimulation. Software (EMKA Technologies) calculated peak inspiratory flow (PIF), peak expiratory flow, inspired volume, expired volume, inspiratory time, and expiratory time.

Neurological Function. A neurological assessment score (NAS) was assigned to each dog by a board-certified veterinary neurologist (J.M.S.) as described.³⁹ Parameters assessed included: cranial nerve function, postural reactions, segmental spinal reflexes, gait stride length, ability to run and jump, and muscle atrophy. Dogs were observed for exercise intolerance or increased respiratory rate and effort following activity. The presence or absence of a dropped jaw (inability to hold the jaw in a closed position) was also noted. Following each examination, a neurological severity score was assigned on a scale of 10 to 1, with 10 indicating a normal examination and 1 indicating inability to maintain sternal recumbency. However, predetermined humane euthanasia criteria were usually met by a NAS score of 3. Results of neurological scoring for normal controls and for some untreated XLMTM dogs have been reported.³⁹

Histology. Muscle samples were collected and processed as described previously.⁴⁰ Tissues from the vastus lateralis, cranialis tibialis, and biceps brachii were collected from both WT control dogs and AAV8-MTMI infused dogs. Muscle histology was performed based on staining with hematoxylin and eosin (H&E), reduced nicotinamide adenine dinucleotide (NADH), and ATPase stain performed at pH 9.4. Slides were evaluated by a board-certified anatomic pathologist and neuropathologist (M.W.L.) with respect to the full range of possible pathologies. Muscle tissue (vastus lateralis) was fixed in 2.5% glutaraldehyde, processed at the Medical College of Wisconsin Electron Microscopy (EM) Core Facility and evaluated at each time point using a standard approach previously described.^{22,41} Comprehensive reports of pathological findings at the light and EM level were prepared using an adaptation of the National Institute of Neurological Disorders and Stroke Common Data Elements muscle biopsy reporting form.⁴²

Vector Copy Number and Transgene Expression. The number of vector genomes per diploid genome (vector copy number, VCN) was quantified from 32 ng of total DNA by Taqman real-time polymerase chain reaction using a

Table 1. Demographics for XLMTM dogs ($N = 2$), WT controls ($N = 2$), and carrier controls ($N = 3$ for years 1 and 2; $N = 2$ for years 3 and 4)*

Genotype	XLMTM		WT		Carrier			
	1	2	1	2	1	2	3	4
Dog identifier								
Gender	M	M	M	M	F	F	F	F
Body mass (kg)								
T_{8w}	4.5	4.9	6.0	6.7	--	--	--	--
T_{16w}	10.5	11.2	10.0	10.8	--	--	--	--
T_{1y}	12.7	14.5	--	--	15.1	12.7	14.5	--
T_{2y}	19.2	22.7	--	--	23.1	19.1	23.7	--
T_{3y}	18	18	--	--	--	16.7	--	13.3
T_{4y}	21.6	22.8	--	--	--	18	--	18
Limb length (cm)								
T_{8w}	12.3	13.2	13.3	13.7	--	--	--	--
T_{16w}	16.0	17.1	17.0	17.6	--	--	--	--
T_{1y}	17.4	18.4	--	--	17.6	16.1	18.2	--
T_{2y}	18.0	18.9	--	--	17.9	17.1	17.4	--
T_{3y}	18.0	18.9	--	--	--	17.5	--	16.5
T_{4y}	18.0	18.8	--	--	--	17.6	--	16.5

* T_{8w} , 8 weeks of age; T_{16w} , 16 weeks of age; T_{1y} , 1 year of age; T_{2y} , 2 years of age; T_{3y} , 3 years of age; T_{4y} , 4 years of age.

Light Cycler 480 (Roche). The canine β -glucuronidase gene was used for standardization. Primers used for vector genome amplification were: 5'-GCCTCGCCCGACTCTA-3' (forward), 5'-CTCAGGATCGGTGACCAGAGA-3' (reverse) and 5'-AGGATCCAGATCTAAGC-3' (probe). Primers and probe used for β -glucuronidase amplification were: 5'-ACGCTGATTGCTCACACCAA-3' (forward), 5'-CCCCAGG TCTGCTTCATAGTTG-3' (reverse) and 5'-CCCCGCCCC TGACCTTTGTGA-3' (probe) (Applied Biosystems).

Immunoblotting. Proteins were extracted from tissues using a lysis buffer containing 10 mM Tris HCl pH 7.4, 150 mM NaCl, 1 mM ethylenediaminetetraacetic acid, 1 mM ethyleneglycoltetraacetic acid, 2 mM Na orthovanadate, 100 mM NaF, 4 mM sodium pyrophosphate, 1% Triton X-100, 0.5% IGEPAL (octylphenoxypolyethoxyethanol), and a Protease Inhibitor Cocktail (Roche Applied Science), and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (NuPage Novex 4–12% BisTris gels, Invitrogen) and Western blotting as previously described (6). Membranes were probed with a polyclonal antibody against the C-terminus of canine myotubularin (R1040, Genethon) and a mouse monoclonal antibody against glyceraldehyde-3-phosphate dehydrogenase (MAB374, Millipore) as internal control. Detection was performed with a secondary antibody coupled to IRDye 680 (LI-COR) and the Odyssey infrared imaging system (LI-COR Biotechnology Inc.).

RESULTS

Dog Demographics. XLMTM dogs had lower average body weights before treatment at 8 weeks of age (4.7 kg) as compared to their WT littermates (6.4 kg), despite being close in size as measured by hindlimb length (XLMTM, 12.6 cm; WT controls, 13.5 cm; Table 1). By 16 weeks of age, XLMTM and WT control dogs were of similar mass and sizes, with mean weight of 10.9 kg and limb length of 16.6 cm for XLMTM dogs, and mean weight of 10.4 kg and limb length of 17.3 cm for WT control animals. XLMTM and carrier control dogs also

demonstrated comparable weights and hindlimb lengths at 1 and 2 years of age (Table 1). At 1 year, mean weight was 13.6 kg for XLMTM dogs and 14.1 (1.2) kg for carrier controls, and mean hindlimb length was 17.9 cm for XLMTM dogs and 17.3 (1.1) cm for carrier controls. At 2 years, mean weight was 21.0 kg for XLMTM dogs and 22.0 (2.5) kg for carrier controls, and mean hindlimb lengths were 18.5 cm for XLMTM dogs and 17.5 (0.4) cm for carrier controls. At 3 years, the mean weight was 18 kg for XLMTM dogs and 15 kg for carrier controls, and mean hindlimb lengths were 18 cm for XLMTM dogs and 15 cm for carrier controls. By 4 years, XLMTM dogs demonstrated greater body weight (22 vs. 18 kg) with longer hindlimb lengths (18.4 vs. 17 cm) compared with their age-matched carrier controls (Table 1).

Comparison of Longitudinal Gait Changes in AAV-Infused XLMTM and Control Dogs over 4 Years. To evaluate effects of AAV on limb function and mobility, gait was evaluated while dogs walked over an instrumented carpet to capture spatiotemporal data. Four years after infusion with AAV8-MTMI, XLMTM dogs demonstrated walking gait speed and stride lengths comparable to age-matched control dogs (Fig. 1). Before infusion, gait speed in XLMTM dogs was slightly reduced at 8 weeks of age, where XLMTM dogs walked 7% more slowly than control dogs (Table 2). However, following AAV infusion XLMTM dogs walked at speeds comparable to controls at 12, 16, and 21 weeks of age. Gait speed was maintained in XLMTM dogs over 4 years after treatment. In normal carriers, gait speed was 20% faster than XLMTM dogs at 1 year, but XLMTM gait speed was comparable to controls

XLMTM dog gait parameters over 4 years

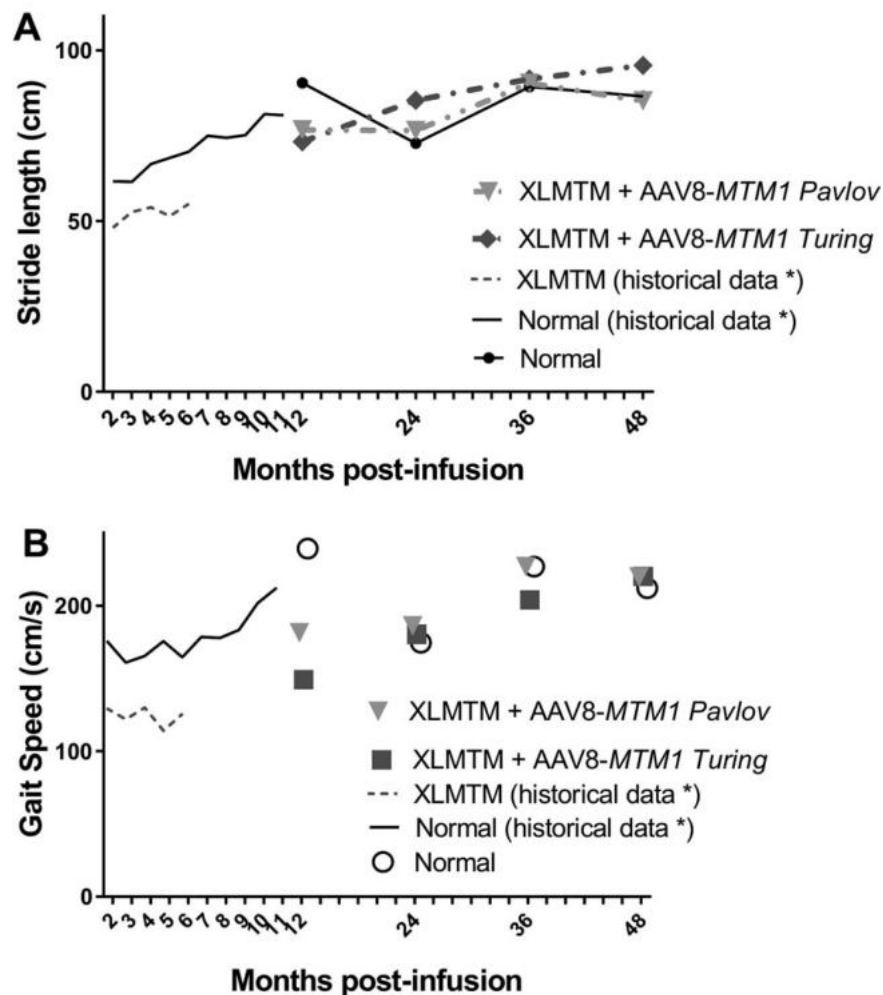


FIGURE 1. Gait speed over a 4-year period in XLMTM dogs (Pavlov and Turing) infused with AAV8-MTM1 vector remains comparable with age-matched WT controls (A). Stride length over a 4-year period in XLMTM dogs infused with AAV8-MTM1 vector remains comparable with age-matched WT controls (B). Data points represent mean values.

at 2, 3, and 4 years after treatment (Fig. 1A; Table 2). After 3 years postinfusion, XLMTM dogs exhibited 10% and 0.03% slower gait speeds, respectively, compared with age-matched controls. Four years postinfusion, both XLMTM dogs demonstrated 4% faster gait speeds when compared with their age-matched controls.

Stride lengths in XLMTM dogs were 16% shorter than controls at 10 weeks of age but were comparable to controls at all other time points as measured by the instrumented carpet (Table 2). Two years after treatment, XLMTM dogs demonstrated 10% longer strides than control dogs (Table 2). At 3 years after treatment, XLMTM

Table 2. Gait speed, and stride length over time for XLMTM dogs compared to normal WT and carrier controls*

	T _{8w}	T _{12w}	T _{16w}	T _{21w}	T _{1y}	T _{2y}	T _{3y}	T _{4y}
Gait speed (cm/s)								
XLMTM	110.9 (39.0)	172.4 (15.1)	187.5 (7.7)	185.2 (6.6)	186.1 (21.8)	183.7 (6.6)	215.5 (15.9)	219.9 (.3)
WT	119.3 (10.3)	175.4 (3.0)	192.5 (2.4)	--	--	--	--	--
Carrier	--	--	--	--	233.4 (12.1)	174.7 (11.6)	226.9 (7.3)	211.9 (46)
Stride length (cm)								
XLMTM	42.3 (3.1)	64.5 (1.5)	71.5 (2.8)	78.1 (0.9)	84.1 (0.4)	79.9 (5.5)	90.9 (.8)	90.4 (7.4)
WT	50.5 (1.1)	67.8 (2.8)	74.5 (3.5)	--	--	--	--	--
Carrier	--	--	--	--	90.1 (10.9)	72.8 (8.7)	89.3 (1.1)	86.5 (12.6)

*Data indicate mean and SD.

T_{8w}, 8 weeks of age; T_{12w}, 12 weeks of age; T_{16w}, 16 weeks of age; T_{1y}, 1 year of age; T_{2y}, 2 years of age; T_{3y}, 3 years of age; T_{4y}, 4 years of age.

Peak inspiratory flow velocities in AAV infused dogs over 4 years

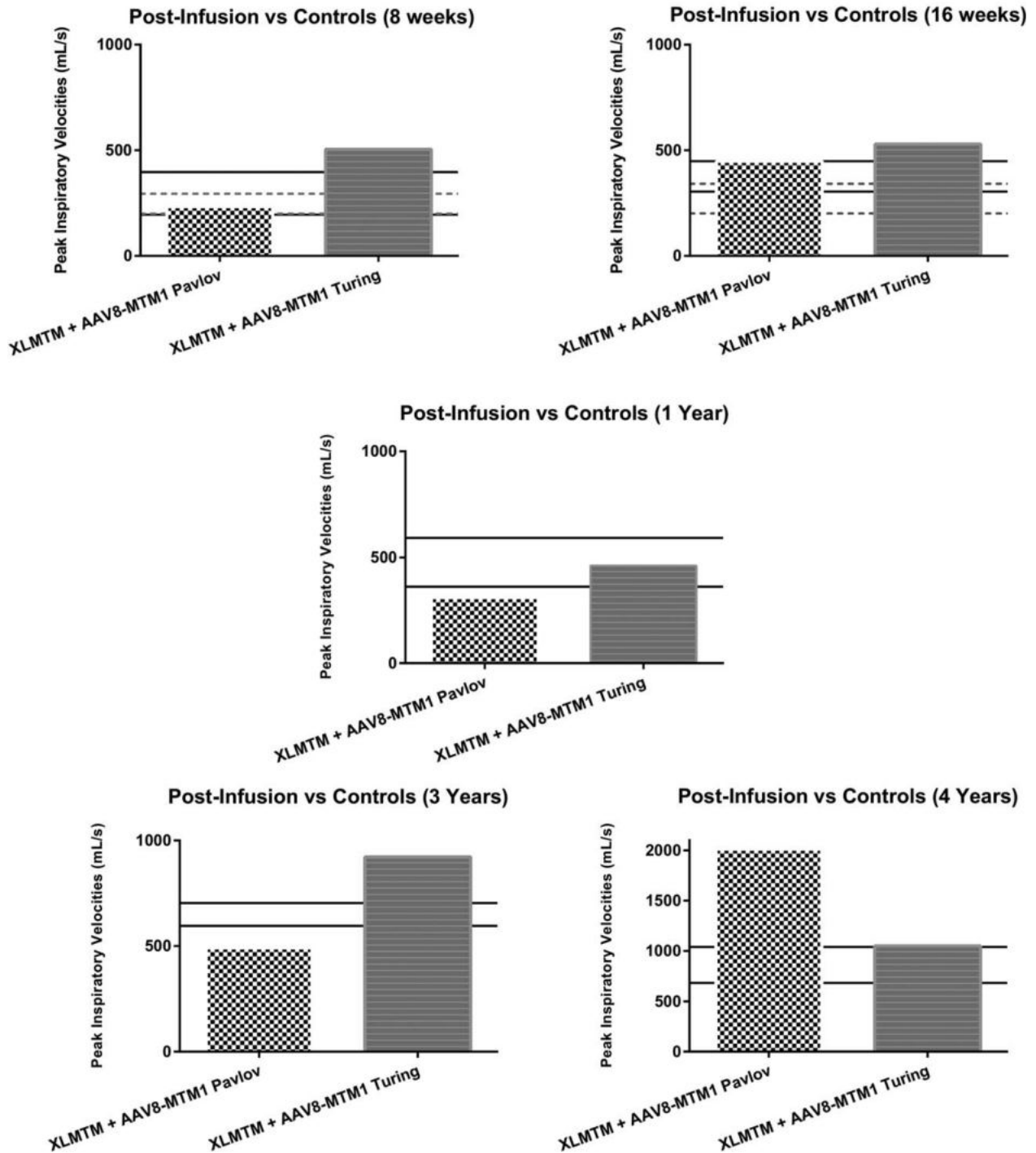


FIGURE 2. PIF velocities in AAV infused dogs over 4 years. PIF velocities after a single infusion of AAV8-MTM1 in XLMTM dogs measured after 2 and 4 months, and 1, 3, and 4 years compared with age-matched controls. Solid lines represents WT control SD values; dotted line represents the XLMTM untreated dogs' SD values.

dogs had 3% and 1% longer strides, respectively, than control dogs. At 4 years after treatment, 1 XLMTM dog had 2% shorter strides, while the other demonstrated 10% longer strides compared with controls (Table 2).

Respiratory Function in AAV-Infused XLMTM Dogs Compared with Controls. To test for effects of AAV on respiratory function, we evaluated airflow exchange in anesthetized dogs. PIF velocity appears comparable between AAV-treated XLMTM

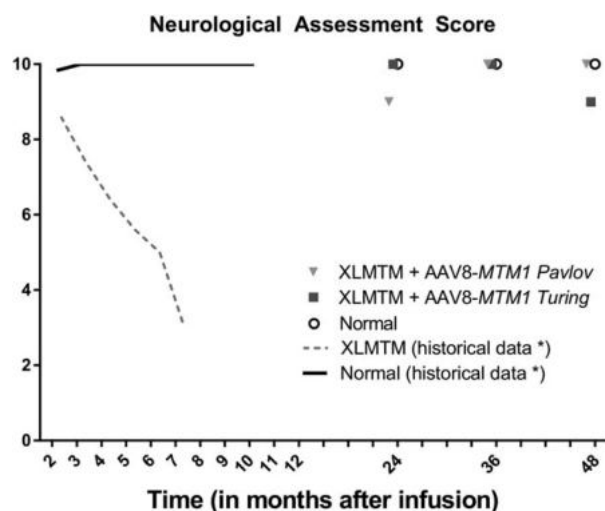


FIGURE 3. Neurological assessment score in XLMTM dogs over 4 years. Neurological assessment score in XLMTM dogs infused with AAV8-MTM1 remain comparable with age-matched WT controls. Solid line represents WT data; dotted line represents XLMTM untreated data. Data shown are mean values.

and normal controls over time (Fig. 2). PIF velocity for the AAV-infused dog, Pavlov, remained lower than both the controls and the AAV-infused dog, Turing. Year 1 assessments show that Pavlov's PIF value was approximately 35% less than the average PIF for the normal controls; this difference decreased to 22% at the 3-year assessment. During the 4-year assessment, Pavlov's PIF was 133% greater than the controls and increased 286% when compared with his year 3 average PIF. Turing's PIF values at the 1-year assessment were approximately 3% less than the controls, however, his values surpassed the controls at years 3 and 4.

Neurological Function in AAV-Infused XLMTM Dogs Remain at Near-Normal Levels after 4 Years. To measure changes in neurological function, we used a validated clinical scoring instrument developed for dogs, the NAS. Results indicate that 2 and 3 years postinfusion, both XLMTM treated dogs achieved scores identical to controls; at 4 years one of the AAV-infused dogs (Turing) declined by 1 point (Fig. 3).

Limb Strength in AAV-Infused XLMTM Dogs Approaches Normal Function 4 Years Postinfusion. To measure strength, *in vivo* isometric flexion torque was repeatedly measured in the hind limbs of anesthetized dogs (Fig. 4). Hindlimb flexion torque values for AAV-treated XLMTM dogs were greater than saline-infused dogs at 9 and 17 weeks, and generally comparable with WT controls at most time points up to 3 years. At the 4-year assessment, torque values of AAV-treated XLMTM dogs dropped to approximately half values of WT controls. Overall, hindlimb flexion torque data

indicate that following AAV infusion, strength in XLMTM dogs was maintained comparable to normal age-matched controls for 3 years then began to decline.

Muscle Pathology in AAV-Infused XLMTM Dogs Is Absent 4 Years Following Treatment. To examine effects of AAV on muscle pathology, a blinded neuropathologist examined biopsies from the vastus lateralis, cranial tibialis, and biceps brachii muscles. H&E staining of XLMTM dogs treated with AAV could not be distinguished from normal controls in almost all sampled muscles (Fig. 5), with the exception of the 4 year, biceps brachii muscle of Turing. This specimen displayed myofiber smallness, internal nucleation, and organelle mislocalization consistent with XLMTM in a subset of fibers, and these changes were not apparent in the 3-year biopsy from the same muscle.⁴³ In other sampled muscles from WT and XLMTM dogs, there was no evidence of organelle mislocalization or pathological myofiber smallness or internal nucleation. There were also occasional small fibers present in all dogs, consistent with our observation of the skeletal muscle histology of nondiseased dogs in this colony in prior studies.^{22,43} There was no evidence of inflammation, myonecrosis, myophagocytosis, myofiber regeneration, endomysial fibrosis, or fatty infiltration. EM of sampled muscles revealed easily identifiable triad structures in both WT and XLMTM dog muscles, and animal genotype could not be distinguished on the basis of EM findings (not shown). Overall, the findings are consistent with a near-total reversal of XLMTM pathology at 3 and 4 years posttreatment, with a possible waning of pathological recovery in the biceps brachii from Turing at 4 years posttreatment.

AAV Vector and Transgene Expression. We analyzed VCN from muscle biopsies taken in anesthetized XLMTM dogs. Data over 4 years show an overall consistent decrease of VCN (Fig. 6). The control group remained below the limit of detection at all time points. Myotubularin transgene expression (Supplementary Fig. S1, which is available online) generally reflected VCN in immunoblot images. Whereas no detectable myotubularin expression bands could be seen in any XLMTM dog infused with saline, clearly defined bands could be seen in muscle lysates taken from biceps, cranial tibialis, and vastus muscles 4 years postinfusion with AAV.

DISCUSSION

Our findings indicate that a single infusion of recombinant AAV8 carrying a canine *MTM1* gene driven by a desmin promoter can preserve and maintain limb function over 4 years in a large animal model of a congenital myopathy. Our findings

Toque-frequency relationship of torque hindlimb flexion over time

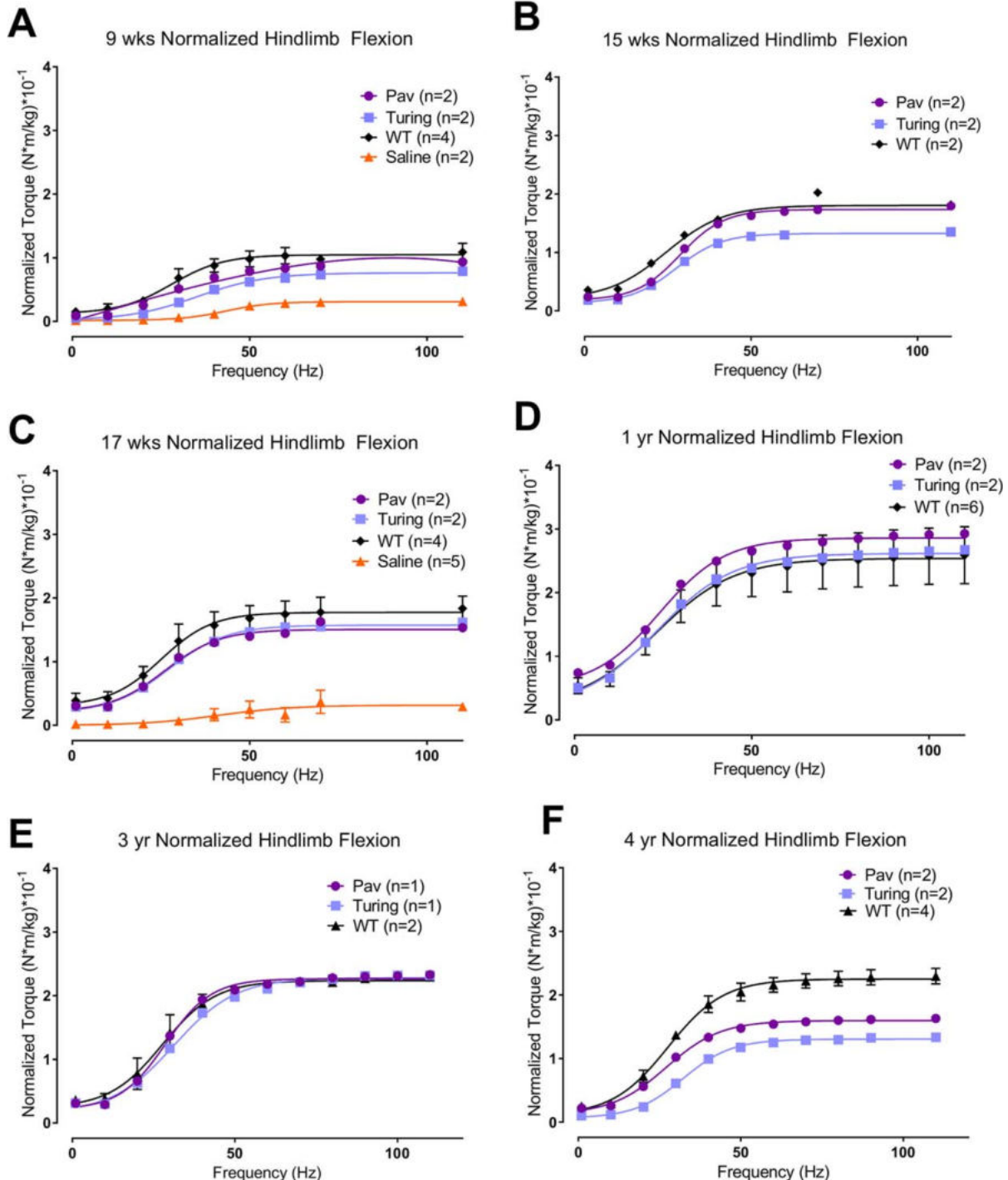


FIGURE 4. Torque-frequency relationship of torque hindlimb flexion over time. Hindlimb flexion torque values of XLMTM dogs treated with a single dose of AAV8-MTM1 and age-matched WT controls, over time: 9 week flexion torque (A); 15 week flexion torque (B); 17 week flexion torque (C); 1 year flexion torque (D); 3 year flexion torque (E); 4 year flexion torque (F). Data shown are mean and SEM.

in the p.N155K canine XLMTM model further suggest that *MTM1* gene replacement using AAV8 in young patients might similarly lead to sustained preservation of muscle strength and neurologic function. Our recent report of AAV8 experiments

in this canine model should inform future clinical trials with respect to dosing and timing of AAV infusion.³²

The technical aim of our study addressed the comparison of spatiotemporal measures with the

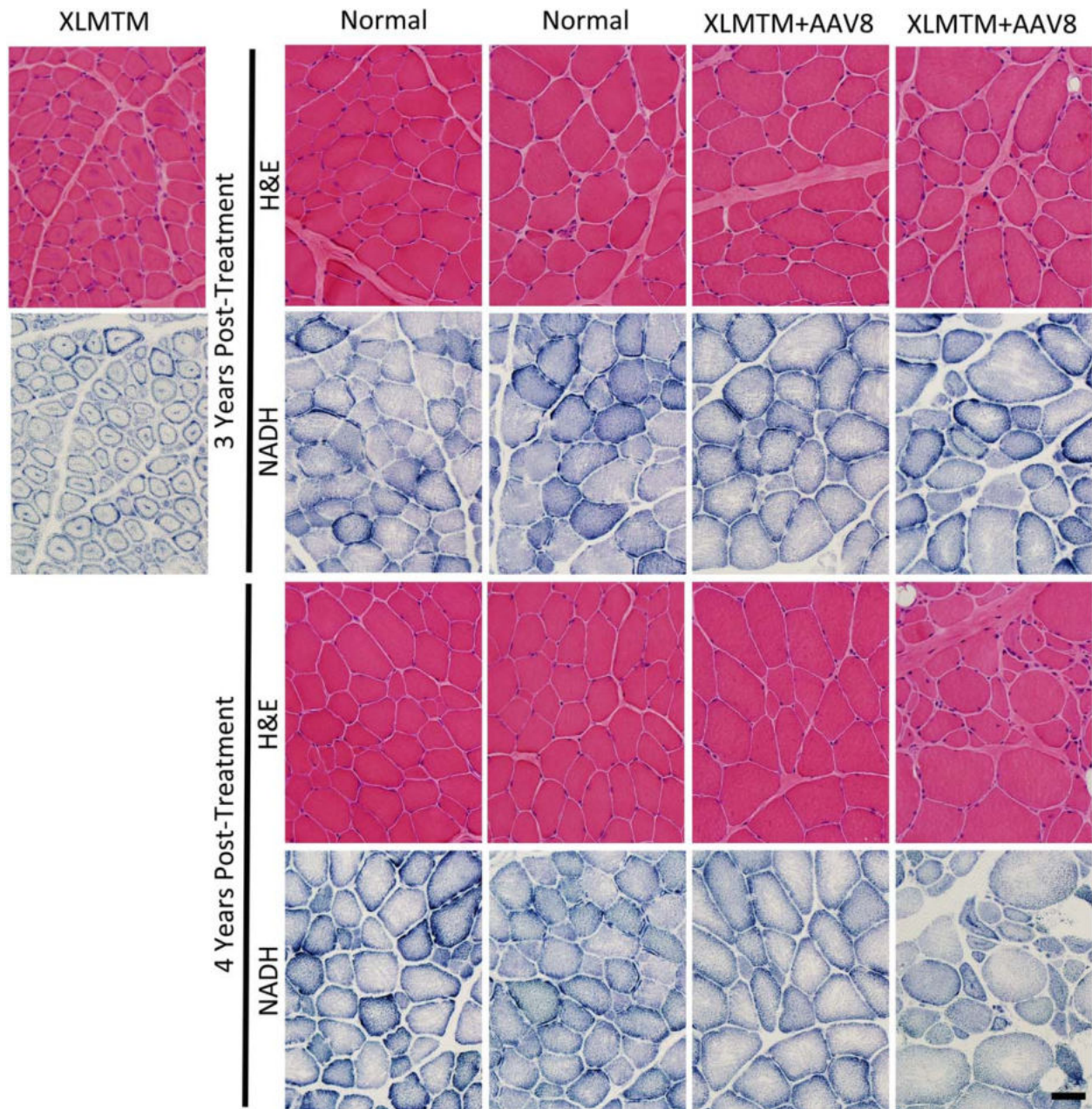


FIGURE 5. Pathological features of XLMTM muscle. Pathological features of XLMTM muscle remain dramatically improved in AAV8-MTM1 treated canines at 1, 3, and 4 years posttreatment. Features of XLMTM muscle pathology in untreated XLMTM canines at 22 weeks are shown (XLMTM, top left). The WT condition illustrates normal histopathological features, including appropriate fiber size and peripherally located nuclei on H&E staining and an even distribution of organelles on NADH staining. The XLMTM Untreated condition illustrates histopathological findings typical for canine XLMTM, including myofiber hypertrophy, internally nucleated myofibers, and organelle mislocalization (clustering of organelles at the center and periphery of myofibers on NADH staining). A single infusion of AAV8-MTM1 resulted in correction of fiber size, nuclear placement, and organelle localization at 1, 3, and 4 years posttreatment. Black bar = 40 microns.

more time-intensive video-based motion capture analysis. Our results indicate a high correlation between measures of stride length from both video-based motion analysis simultaneous assessment with an instrumented carpet. Correlations were lower for stride time, despite similar values. This may be due to the small sample size as well as technical aspects of the video motion capture. The

video motion capture in this case had relatively low temporal resolution, with a low frame rate for video capture. As stride time values are relatively small, even slight differences will reduce correlation. Both the instrumented carpet and 2-dimensional video motion capture are validated methods of quantifying spatiotemporal parameters of gait in canines.^{30,44,45} Unlike the instrumented

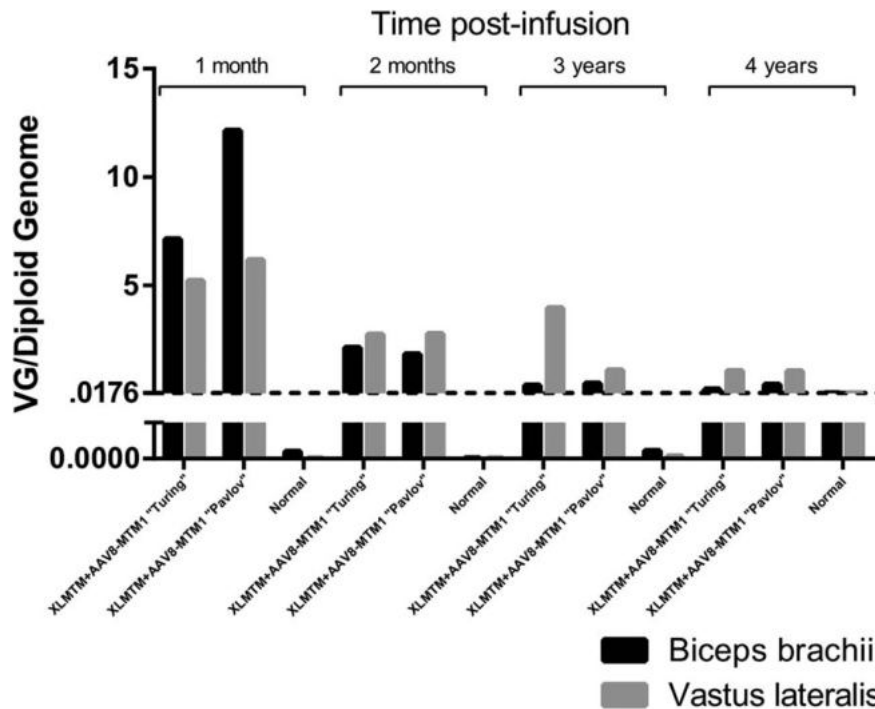


FIGURE 6. AAV vector copy number over time. XLMTM dogs show steady decreases in vector genome (VG)/diploid genome values over 4 years after a single infusion of AAV8-MTM1 vector. Normal indicates WT (noninfused) control samples. The dashed horizontal line indicates the lowest standard recorded (0.0176VG/diploid genome); Values beneath the dashed line are below the lowest standard. dark black bars, biceps brachii muscle; gray bars, vastus lateralis muscle.

carpet, video motion capture can also be used to assess kinematic or angular joint movements. With modification, video motion capture can be expanded to assess bilateral movements in 3-dimensional space to provide additional information on spatiotemporal and kinematic aspects of gait.

However, video capture requires a large space to accommodate and correctly position the multiple cameras needed for appropriate capture volumes. Set up and analysis of the carpet is simpler, requires less training, and is largely automated, which allows rapid and reliable assessment of several animals with fewer resources. In contrast, multiple types of software may be necessary to convert, digitize, and analyze video capture data, resulting in more time-intensive data processing and increased potential for loss of data integrity. As there is limited additional value of hindlimb angular kinematic measurements in the XLMTM dog,²³ the instrumented carpet is our preferred method. However, for canine models where changes in joint angles are observed due to disease, such as the golden retriever muscular dystrophy model,²⁷ kinematic analysis at a higher resolution using motion capture methods may be preferable.

We previously reported that XLMTM dogs walk more slowly than their normal control littermates. They experienced a 13% decrease in speed at 10 weeks of age, and a decrease of 46% of normal by

the time of humane euthanasia at 17 weeks of age.²³ Strides were also reduced to 86% of normal at 10 weeks of age and 68% of normal by the terminal endpoint.²³ However, normal gait involves the concerted effort of several limb muscles to maintain stability and support as the disease advances. In this study, we found that, while XLMTM dogs walk more slowly and with shorter strides before receiving AAV8-MTM1, whole body gene replacement therapy is associated with improved and maintained speed and stride length over 2 years, indicating preserved ambulation and limb function. This is in keeping with increased limb muscle strength and improvements in myofiber size, myotubularin expression, and in histology, particularly the sarcomeric organization in hindlimb muscles, such as the quadriceps and biceps.²² As upper limb function is of particular concern to patients, similar changes in the forelimbs are also encouraging,²² and strength and functional assessment separate from the hindlimb in the future may be useful.

In summary, our findings indicate that AAV-mediated gene replacement can preserve normal muscle and neurological function in a large animal model of XLMTM, a congenital myopathy for which there is currently no effective clinical therapy. An instrumented carpet can be useful to monitor effectiveness of investigational gene replacement on limb function in this model.

We thank K. Williams, A. Spangler Dean, Mandy Lockard, Emily Burlingame, MacKenzie Johnson, S. Browne, Shelby Hamm, Kate Bukovec, and Emily Troiano for their contribution to these experiments. Ethical Publication Statement: We confirm that we have read the Journal's position on issues involved in ethical publication and affirm that this report is consistent with those guidelines.

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