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## Measuring Tetanic Isometric Torque/Force at the Tibiotarsal Joint in Vivo

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## 1. OBJECTIVE

The measurement of muscle contractile function is an important end-point for assessing the efficacy of cell-, gene-, or pharmacologically-based therapies in DMD and the mdx mouse and GRMD dog models. Muscles in DMD and both the mdx mouse and GRMD dog are variably affected. In general, flexor muscles are affected initially, potentially due to their predominant role in movement early in life. Extensors are affected later due presumably to greater usage in weight bearing and the damaging effects of lengthening (eccentric) contractions. Thus, ideally, a technique that allows evaluation of both flexor and extensor muscles is needed. Moreover, results of force measurements must be interpreted in light of this differential muscle involvement, especially considering the animal's age and the natural history of the disease.

## 2. SCOPE AND APPLICABILITY

Disease severity in GRMD can vary markedly, with some dogs dying soon after birth due to extreme respiratory compromise and others demonstrating a remarkably mild phenotype.<sup>1,2</sup> This suggests that genetic factors, including epigenetics and modifier genes, may either lessen or exaggerate disease expression. Phenotypic variation can confound statistical analysis, necessitating larger group sizes to achieve significance. In the case of localized cell- or genetic treatments, the contralateral limb may serve as a control, with values compared between treated and untreated limbs. With systemic treatments, this problem can be at least partially offset if baseline measurements are done prior to the initiation of treatment. In this way, each dog serves as its own control. Accordingly, outcome is determined based on the difference in values at two or more time points in each treated animal, rather than at a single point in a group of animals.

With this background information in mind, we were motivated to develop an in vivo measurement of force that fulfilled the following two characteristics:

1. Minimally invasive, ideally not involving surgery, so that multiple measurements could be made over time.
2. Simultaneous evaluation of both extensor and flexor muscles to define the degree of differential muscle involvement. In the method described here, we measure isometric torque<sup>a</sup> generated by extension and flexion of the tibiotarsal (TTJ) subsequent to percutaneous stimulation of the tibial and common peroneal nerves, respectively.<sup>3,4</sup> Tests can be performed sequentially over time, thus allowing definition of the natural history of the disease. In the case of treatment trials, baseline measurements can be made at 2 to 3 months of age prior to the onset of treatment so that each dog serves as its own control. Tetanic values are selectively evaluated because they are more

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<sup>a</sup> Torque is the tendency of a force to rotate an object about a fulcrum or pivot; forces push or pull, while torque can be seen as twisting an object. Torque is the product of the force applied and the length of the moment arm (lever) acting at the pivot point. Units of measure are Newton-meters for torque and Newtons for force.

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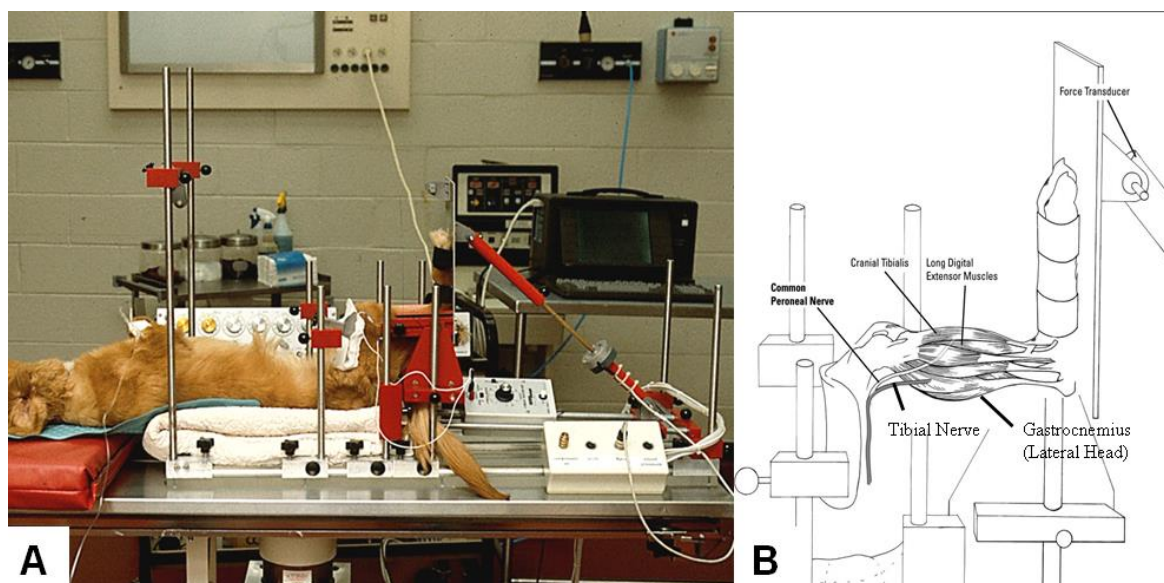
consistent than those for twitch. As with mdx mice, weakness is exaggerated by eccentric contractions<sup>5</sup> (see separate GRMD protocol).

### 3. CAUTIONS

Dogs must be anesthetized. A nerve stimulator and specialized, custom-made stereotactic frame are required. See METHODS and EVALUATION AND INTERPRETATION OF RESULTS (below) for guidance on interpretation.

### 4. MATERIALS

Separate personnel should be available for induction and maintenance of anesthesia and force measurement. Dogs are anesthetized and positioned in dorsal recumbency with one of the pelvic limbs placed in a custom-made stereotactic frame such that the hip (coxo-femoral), knee (stifle), and hock (tibiotarsal) joints are all at 90° angles (see discussion of optimal fiber length [ $L_0$ ] and **Figure 1**).<sup>3,4</sup> The TTJ is suspended in a stirrup and the paw is immobilized against a pedal interfaced with a force transducer (Aurora Scientific, Ontario,



**Figure 1.** Photograph (A) and drawing (B) of stereotactic frame used to immobilize dogs for torque measurements. Dogs are positioned in dorsal recumbency, such that the tibia is parallel to the table and forms approximately a 90° angle with the femur. Stimulation of the common peroneal and tibial nerves cause the distal pelvic limb to pull (flexion) or push (extension), respectively, against a lever interfaced with a transducer and ergometer.

Canada). Forces are measured by stimulating either the common peroneal (TTJ flexion) or tibial (TTJ extension) nerve using paired stimulating and reference 27-gauge monopolar needle electrodes placed just distal to the fibular head (common peroneal nerve) or within

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the gastrocnemius muscles (tibial nerve), respectively. As a result, the distal pelvic limb pulls (flexion) or pushes against (extension) the lever to which the paw is secured. The interface between the pedal and force transducer allows one to measure the isometric force generated by the paw. Supramaximal 150 V, 100  $\mu$ sec pulses are applied (Model S48 Solid State Square Wave Stimulator, Grass Instruments, Quincy, MA, USA) in a 1½ sec tetanic run of 75 pulses (50/sec). Passive torque is subtracted from total torque produced; only active torque generated by the muscles is measured.

## 5. METHODS

1. Anesthetic protocol (Note, in a preliminary study, mean alveolar concentration [MAC] values for isoflurane did not significantly affect force measurement values [Schueler RO, Koch J, Kornegay JN, unpublished data]).

*20-30 minutes prior to anesthesia induction:*

- Pre-anesthetic agents:
  - Atropine sulfate (0.04 mg/kg, IM)
  - Acepromazine maleate (0.02 mg/kg, IM) for dogs weighing greater than 5 kg
  - Butorphanol tartrate (0.4 mg/kg, IM)

*Anesthetic induction:*

- Anesthetic agents:
  - Propofol (up to 3 mg/kg, IV – slowly!) for dogs weighing greater than 5 kg
  - Isoflurane or sevoflurane (to effect, inhaled) (avoid masking down)

*Anesthetic monitoring:*

During anesthesia, ECG, heart and respiratory rate, blood pressure, end tidal (Et)CO<sub>2</sub>, and saturation of hemoglobin by peripheral oxygen (SpO<sub>2</sub>) are monitored continuously with a pulse oximeter (Cardell Multiparameter Monitor 9405, Minrad International, Inc, Orchard Park, NY). These values, as well as capillary refill time and anesthetic setting, are recorded every 15 minutes.

*Anesthetic recovery:*

Monitor affected and carrier dogs closely during anesthetic recovery until fully awake and in sternal recumbency.

- Naloxone (up to 0.4 mg/kg, SQ) for rapid recovery; given in ½ dose increments (1<sup>st</sup> dose given while the dog is still intubated and breathing O<sub>2</sub>; 2<sup>nd</sup> dose, if necessary, after extubated and/or if respiration drops below 7 breaths per minute).
2. Position the dog in dorsal recumbency.
  3. Measure joint angles (see separate GRMD protocol).
  4. Calibrate the force/torque transducer. Note, depending on their range, different force/torque transducers may have to be used for the flexion and extension recordings. Calibration should be repeated before each set of measurements.
  5. Place one of the pelvic limbs in a stereotactic frame such that the hip (coxofemoral), knee (stifle), and hock (tibiotarsal) joints are all at 90° angles (**Figure 1**). The tibiotarsal joint is suspended in a stirrup and the paw is immobilized against a pedal using tape. The angle at which maximal joint torque is generated during isometric

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contractions has been termed the optimal joint angle,<sup>6</sup> which is analogous to the optimal fiber length ( $L_0$ ) for individual muscle force measurements. We have previously shown that force values vary depending on the angle formed by the TTJ. In a preliminary study of two normal dogs, values measured at 90° exceeded those made at 45° and 135° for both flexion and extension. Accordingly, the TTJ angle was positioned at a 90° angle for subsequent studies. To more critically evaluate the TTJ angle at which  $L_0$  would be achieved, measurements were subsequently evaluated at 10° increments between 60° and 120° in seven GRMD and four normal dogs. For both groups, flexion plateaued at approximately 100°, while extension was minimal at about 70° (Childers MK, Kornegay JN, unpublished data). The length-tension relationship was not shifted for normal versus GRMD dogs.

6. The common peroneal nerve (TTJ flexion) can be palpated just distal to the head of the fibula as it courses across the bone. Paired stimulating and reference 27-gauge monopolar needle electrodes are placed subcutaneously on either side of the nerve. Single, supramaximal (150 V, 100  $\mu$ sec) pulses are applied to produce twitch mechanical potentials (i.e., torque responses). Needles are repositioned several times to obtain the maximal single twitch potential. An electromyography (EMG) unit (TE42, Teca Corp, Pleasantville, NY, USA) can be used to record electrical activity in TTJ flexor muscles (cranial tibialis, peroneus longus, and long digital extensor) to ensure proper needle placement. Once the maximal single twitch potential has been obtained, the nerve is stimulated with a 1½ sec tetanic run of 75 pulses (50/sec) to obtain a tetanic mechanical potential. The potential should plateau, indicating that summation has occurred (i.e., maximal torque production).
7. Force generated by TTJ extension is measured using the same basic procedure. The tibial nerve is stimulated as it courses caudally and distally within the gastrocnemius muscle bellies. Paired stimulating and reference 27-gauge monopolar needle electrodes are placed deep within the musculature caudal and just proximal to the stifle. The approximate point of placement is along a line that bisects the 90° angle formed by the stifle (knee). Single, supramaximal (150 V, 100  $\mu$ sec) pulses are applied to produce twitch mechanical potentials. Needles are repositioned several times to obtain the maximal single twitch potential. An electromyography (EMG) unit (TE42, Teca Corp, Pleasantville, NY, USA) can be used to record electrical activity in the TTJ extensor muscles (gastrocnemius and superficial digital flexor muscles) to ensure proper needle placement. Once the maximal single twitch potential has been obtained, the nerve is stimulated with a 1½ sec tetanic run of 75 pulses (50/sec) to obtain a tetanic mechanical potential. The potential should exhibit a plateau indicating that summation has occurred.
8. Torque is recorded by the computer software. For both flexion and extension measurements, passive torque is subtracted from total torque produced; only active torque generated by muscles is measured.
9. The torque value can be divided by the length of the metatarsus pivot arm to convert to force. Measure the length of the metatarsus from the distal tip of the digits to the

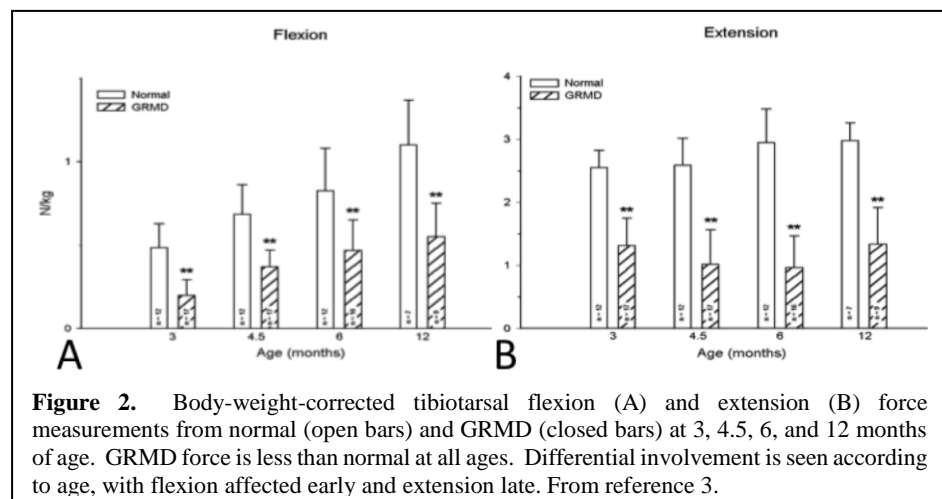
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point of the hock. We estimate that the moment arm is approximately 75% of this value (see 11 below).

10. Ideally, multiple tetanic recordings should be made, allowing 5 minutes for recovery between measurements. However, we have found that the second value often is lower than the first. We now typically record only a single value. Serial measurements made at weekly/monthly intervals would likely provide ensure greater consistency.
11. Because GRMD dogs vary in weight, we have historically divided absolute tetanic torque by the body weight (kg) to obtain a weight-corrected value (**Figure 2**). With this said, because loss of muscle mass (and strength) contributes to the overall decrease in GRMD body weight, body-weight-correction likely artificially lowers relative force in dystrophic dogs. Body height or bony length would probably be a better means to correct for size effect. Moreover, torque values generated with this method are expressed in Newton-meters and must be divided by the length of the moment arm (estimated at 75% of the length of the metatarsus; see 9 above) to provide a force value (Newtons).

## 6. EVALUATION AND INTERPRETATION OF RESULTS

We have evaluated torque generated by TTJ flexion and extension to determine both the natural history of GRMD<sup>3</sup> and the response of GRMD dogs to treatment.<sup>7-9</sup> In our initial study, force values were



measured at 3, 4.5, 6, and 12 months of age. Absolute and body-weight-corrected GRMD twitch and tetanic force values were lower than normal at all ages ( $P < 0.01$  for most). However, tarsal flexion and extension were differentially affected (**Figure 2**). Flexion values were especially low at 3 months, whereas extension was affected more at later ages. Several other GRMD findings differed from normal. The twitch/tetany ratio was generally lower; post-tetanic potentiation for flexion values was less marked; and extension relaxation and contraction times were longer. The consistency of GRMD values was studied to determine which measurements would be most useful in evaluating treatment outcome. Standard deviation was proportionally greater for GRMD versus normal recordings. More consistent values were seen for tetany versus twitch and for flexion versus extension. Left and right limb tetanic flexion values did not differ in GRMD; extension values were more variable. These results suggested that measurement of tarsal tetanic flexion force should be most useful to

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document therapeutic benefit in GRMD dogs (note, see discussion of the paradoxical decrease of tibiotarsal flexion values seen in GRMD dogs treated with prednisone below). Groups of 15 and five would be necessary to demonstrate differences of 0.2 and 0.4 in the means of treated and untreated GRMD dogs at 6 months of age, with associated powers of 0.824 and 0.856, respectively (Sigma Stat, Jandel Scientific, 2591 Kerner Blvd., San Rafael, CA, USA).<sup>3</sup> Results from functional tests tend to correlate with one another and with other clinicopathologic features. In one study, tibiotarsal force was correlated with TTJ angle (see separate GRMD protocol) in 51 dogs.<sup>10,11</sup> Correlation coefficients were obtained using a simple coefficient equation. There was a strong correlation between TTJ isometric tetanic force and angle. Values (mean  $\pm$  SD) for extension ( $2.138 \pm 0.915$  N/kg) correlated directly ( $r = 0.54$ ;  $p < 0.0001$ ; power = 0.987), while those for flexion ( $0.443 \pm 0.132$  N/kg) correlated inversely ( $r = -0.70$ ;  $p < 0.0001$ ; power = 1.00) with joint angle ( $148.08 \pm 12.84^\circ$ ). Dogs with weak extension and strong flexion force values tended to have TTJ flexor contractures. Values in heterozygous males and homozygous females do not differ statistically, so both genders can be assessed in parallel during treatment trials.<sup>12</sup> By comparing serial measurements from treated and untreated groups, one can document improvement or delayed progression of disease.<sup>7,8</sup>

Functional outcome values have varied considerably, even among dogs within the same litter, suggesting that modifier genes significantly influence the phenotype.<sup>1,2</sup> Importantly, phenotypic variation confounds data analysis, requiring larger group sizes to demonstrate significance. Establishing baseline outcome values prior to treatment so that each dog serves as its own control can offset the effects of phenotypic variation on statistical analysis. With localized treatments, the effect of phenotypic variation is less of a concern because the untreated opposite limb can serve as the control. Evaluation of the force decrement incurred due to eccentric muscle contraction offers another option by which each dog can be used as its own control (see separate GRMD eccentric contraction protocol).

We have utilized tetanic TTJ force measurements to evaluate effects of prednisone given to GRMD dogs for a 4-month period beginning at 2 months of age.<sup>7</sup> Extension forces in GRMD dogs treated daily with 1 and 2 mg/kg of prednisone measured  $2.349 \pm 0.92$  and  $3.486 \pm 0.67$  N/kg, respectively, compared to  $1.927 \pm 0.63$  N/kg in untreated GRMD controls ( $p < 0.05$  for 2 mg/kg group); GRMD flexion forces measured  $0.435 \pm 0.13$  and  $0.303 \pm 0.08$  N/kg, respectively, compared to  $0.527 \pm 0.01$  N/kg in untreated GRMD controls ( $p < 0.05$  for both groups). The paradoxical decline in flexor force measurements was attributed to the fact that some GRMD flexor muscles undergo necrosis early in life with subsequent functional hypertrophy.<sup>13</sup> Treatment with prednisone could have attenuated this early necrosis and functional hypertrophy. This finding is compatible with the inverse correlation seen between TTJ angle and flexion force (see above) and suggests that increased flexor force could actually predict a deleterious outcome. In keeping with our results, others have subsequently shown that TTJ flexion *decreases* in GRMD dogs treated with a daily regimen of prednisone (2 mg/kg) and cyclosporine (20 mg/kg).<sup>14</sup> We have also shown an analogous treatment effect with NBD, a NF- $\kappa$ B inhibitor.<sup>9</sup> Providing further validation for force/torque measurements as biomarkers,



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values have tended to track with other outcome parameters, such as MRI, and with disease mechanisms.<sup>15,16</sup>

### **Potential Advantages/Disadvantages of the Methodology**

#### **Advantages**

As discussed under “Principle” above, measurement of TTJ force allows non-invasive, serial evaluation of muscle function in GRMD dogs to establish both the natural history of the disease and response of affected dogs to treatment. Results correlate with the severity of TTJ contractures (see separate GRMD protocol), suggesting a potential cause and effect relationship between TTJ contractures and the relative disease involvement of flexors and extensors. Values in the left and right limbs are consistent, suggesting that this test can be used to accurately predict the benefit of localized treatments by comparing results in treated and untreated limbs.

#### **Disadvantages**

With systemic treatments, phenotypic variation confounds statistical analysis in GRMD, necessitating larger group sizes to achieve significance. This problem can be at least partially offset if baseline measurements are done early in life prior to the initiation of treatment. In this way, each dog serves as its own control. Accordingly, outcome is determined based on the difference in values at two or more time points in each treated animal, rather than at a single time point in a group of animals. Values for extension vary more than those for flexion, necessitating larger group sizes and, potentially, limiting the use of this end-point in treatment trials. However, with more recent studies, the degree of variation for extension values has been less pronounced, and we were able to demonstrate improvement with prednisone in a group of six treated GRMD dogs.<sup>7</sup>

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