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Original Article

Genetic myostatin decrease in the golden retriever muscular dystrophy model does not significantly affect the ubiquitin proteasome system despite enhancing the severity of disease

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Abstract: Recent studies suggest that inhibiting the protein myostatin, a negative regulator of skeletal muscle mass, may improve outcomes in patients with Duchenne muscular dystrophy by enhancing muscle mass. When the dystrophin-deficient golden retriever muscular dystrophy (GRMD) dog was bred with whippets having a heterozygous mutation for the myostatin gene, affected GRMD dogs with decreased myostatin (GRippets) demonstrated an accelerated physical decline compared to related affected GRMD dogs with full myostatin. To examine the role of the ubiquitin proteasome and calpain systems in this accelerated decline, we determined the expression of the muscle ubiquitin ligases MuRF1, Atrogin-1, RNF25, RNF11, and CHIP: the proteasome subunits PSMA6, PSMB4, and PSME1: and calpain 1/2 by real time PCR in the cranial sartorius and vastus lateralis muscles in control, affected GRMD, and GRippet dogs. While individual affected GRMD and GRippet dogs contributed to an increased variability seen in ubiquitin ligase expression, neither group was significantly different from the control group. The affected GRMD dogs demonstrated significant increases in caspase-like and trypsin-like activity in the cranial sartorius; however, all three proteasome activities in the GRippet muscles did not differ from controls. Increased variability in calpain 1 and calpain 2 expression and activity in the affected GRMD and GRippet groups were identified, but no statistical differences from the control group were seen. These studies suggest a role of myostatin in the disease progression of GRMD, which does not significantly involve key components of the ubiquitin proteasome and calpain systems involved in the protein quality control of sarcomere and other structural skeletal muscle proteins.

Keywords: Ubiquitin, proteasome, calpain, myostatin, dystrophin, muscular dystrophy

Introduction

Duchenne muscular dystrophy (DMD) is an inherited X-linked recessive disorder characterized by progressive muscle wasting first appearing in early childhood. Muscle weakness occurs initially in the proximal limb muscles and subsequently affects more distal muscles [1]. A number of experimental therapies have been developed to stabilize the DMD myocyte by enhancing dystrophin expression; alternatively, therapies have been proposed to enhance the growth of muscle as well. Myostatin is a muscle protein that has inhibitory effects on muscle growth. Animals lacking myostatin have an enhanced musculature, including Belgian Blue cattle [2, 3] and the "bully" whippet canine which is associated with enhanced racing performance [4]. Given these findings, the inhibition of myostatin activity has been tested as a therapy in both mouse and dog models of DMD [5-8]. In contrast, human trials that have used myostatin

Table 1. GRMD-Myostatin Status

Dog Name	Gender	GRMD Status	Myostatin Status
F1 Generation (Mstn +/- Male x GRMD Carrier)			
Racer	Male	Normal	Normal
Dash	Male	Affected	Heterozygote
Flash	Male	Affected	Normal
Speedy	Female	Carrier	Heterozygote
Lightning	Female	Carrier	Normal
Zippy	Female	Normal	Heterozygote
F2 Generation (GRMD Male x Speedy)			
Endora	Female	Carrier	Normal
Esmerelda	Female	Carrier	Heterozygote
Samantha	Female	Affected	Normal
Tabitha	Female	Affected	Heterozygote
Hagatha	Female	Affected	Normal
Derrwood	Male	Affected	Heterozygote
Abner	Male	Affected	Heterozygote

inhibition (MYO-029, a neutralizing antibody) have not been clearly beneficial and resulted in a few side effects [9, 10]. Given the therapeutic potential of myostatin, we crossed the golden retriever muscular dystrophy (GRMD) dog model [11] with the "bully whippet" canine having a heterozygous mutation for myostatin [12] to elucidate the role of myostatin inhibition on the GRMD model. The resulting GRippets (Golden Retriever/Whippets) exhibited an accelerated decline in function, associated with differential muscle hypertrophy/atrophy, compared to muscular dystrophy littermates with full myostatin levels [13].

The primary phenotype of skeletal muscles in the GRMD model is skeletal muscle atrophy, with the exception of certain muscles such as the cranial sartorius muscle, which exhibits a true hypertrophy [14, 15]. A number of recent studies have identified a role for the ubiquitin proteasome and calpain systems in the role of mediating skeletal muscle atrophy [16-18]. To identify the role of the ubiquitin proteasome and calpain systems in the accelerated demise of dogs with decreased myostatin (GRMD/Mstn +/-), skeletal muscle was biopsied and analyzed for expression and activity of ubiquitin proteasome components. Surprisingly, myostatin inhibition had very limited effects on the proteasome and calpain systems in skeletal muscle, indicating other mechanisms may mediate differential muscle involvement and associated accelerated demise.

Materials and methods

Creation of golden retriever muscular dystrophy / myostatin-deficient whippet (GRippet) dogs

The University of North Carolina-Chapel Hill GRMD colony derived from the original founder [19] was used in these studies. Dogs were cared for according to the principles outlined in the National Research Council Guide for the Care and Use of Laboratory Animals. The GRMD status was identified based on the elevation of serum creatinine kinase (CK) and characteristic clinical signs. The genotype was confirmed by PCR analysis if the CK results were ambiguous. These studies were approved by the University of North Carolnia Institutional Animal Care and Use Committee (IACUC #11-110, "Cross Breeding of Muscular Dystrophy and Myostatin-Null Dogs").

Whippet dogs homozygous for the myostatin-null allele ($Mstn^{-/-}$) have gross muscle enlargement, while Mstn heterozygotes (+/-) have intermediate muscle mass [4]. Heterozygous myostatin (Mstn +/-) semen was used to artificially inseminate an obligate GRMD carrier to generate an F1 generation (**Table 1**). Of the resulting offspring, Speedy, a GRMD and Mstn +/- carrier was then bred to a GRMD male to produce an F2 generation. DNA was isolated from buccal swab samples to assign genotypes for myostatin status. Genotyping confirmed the two base pair deletion at nucleotides 939 and 940 previously reported [4].

Muscle biopsies

Dogs were anesthetized using conventional preanesthetic drugs, propofol (normal dogs only), and sevoflurane. The muscle(s) were exposed sharply at surgery to allow removal of a sample of approximately 1 X 0.5 X 0.5 cm, snap frozen in liquid nitrogen, and stored at -80°C for further processing.

Gene expression analyses

Frozen tissue was homogenized using a glass homogenizer (Kontes, USA) in 600 µl of RTL Buffer from the Qiagen RNeasy Kit (Valencia, CA) and RNA purified according to manufacturers' instructions. Purified RNA was eluted in 50 µl of RNase free water and the concentration measured using a Nanodrop Spectrophotometer

(Thermo Fisher Scientific, Inc., Waltham, MA). Fifty ng of RNA was used for cDNA synthesis using the Life Technologies High Capacity cDNA Reverse Transcription kit (Carlsbad, CA). Fifty μl cDNA synthesis reactions were performed for each RNA template (1X RT Buffer, 4 mM dNTPs, 1X random primers, 250 units RNAse inhibitor, 125 units of Multiscribe reverse transcriptase). The reactions were cycled for 25°C for 10 minutes, 37°C for 120 minutes, 85°C for 5 seconds, then cooled to 4°C. Gene expression was evaluated using TaqMan Real Time PCR Master Mix using canine specific probes for calpain 1 (Cf02704115_m1), calpain 2 (Cf02645870_m1), STUB1 (aka CHIP) (Cf02644017_m1), muscle atrophy F-box (aka Atrogin-1) (FBXo32; Cf02667148_mi), MDM2 (Cf026759237_m1), muscle ring finger protein 1 (MuRF1; Cf026-49993_mi), proteasome subunit alpha type 6 (PSMA6; Cf02666165_g1), proteasome subunit beta type 4 (PSMB4; Cf01123846_m1), proteasome subunit activator type 1 (PSME1; Cf02646187_g1), Ubiquitin (UbB, Mm0162-2233_g1), UNC4/5 homolog E2 (UBE2D1, Cfr02657121_m1), UBC9 homolog E2 (UBE2I), dystrophin (DMD, Cf02623221_m1), myostatin (MSTN Cf02704228_m1), ring finger 11 (RNF-11, Cf02708288_s1), ring finger 25 (RNF25, Cf02713677_m1), ring finger 115 (RNF115, Cf02658284_m1), and analyzed using an Applied Biosystems 7500 series real time PCR machine (Applied Biosystems, Inc., Carlsbard, CA). The relative expression of the genes were normalized to 18S using the delta-delta C_t ($\Delta\Delta C_t$) method.

Proteasome activity determination using fluorogenic substrates

Chymotrypsin-like, Trypsin-like, and Caspase-like proteasome activities were determined by measuring the rate by which activity-specific fluorogenic substrates were cleaved. Briefly, tissue was homogenized in lysis buffer (250 mM Sucrose, 50 mM Tris pH 7.5, 5 mM $MgCl_2$, 0.5 mM EDTA, 1 mM DTT, 2 mM ATP, 0.03% Digitonin) using a glass homogenizer (Kontes, USA). The lysate was centrifuged at 12,000 rpm and the supernatant stored at -80°C. 25 μg of lysate was then assayed in triplicate for the rate of cleavage of peptide substrates for Chymotrypsin-like (Suc-Leu-Leu-Val-Tyr-AMC), Trypsin-like (Ac-Arg-Leu-Arg-AMC), and Capase-like (Z-Leu-Leu-Glu-AMC) activities using commercially available substrates (Enzo Life Sciences, Farmingdale, NY). Twenty-five μg of lysate was combined with 50 μl of 2X proteasome assay buffer plus 150 μM of peptide substrate. Reactions were incubated at 37°C and fluorescence measured (excitation 360 nm emission 465 nm) every 2 minutes for 80 minutes in a Wallac Victor 2 spectrophotometer (excitation 355 nm, emission 460 nm). Parallel samples were pre-incubated with the proteasome inhibitor epoxomicin (20 μM) for 30 min at 37°C, to determine the non-specific substrate hydrolysis. These fluorescence units were then subtracted from each measurement.

Calpain 1 / Calpain 2 activity assay

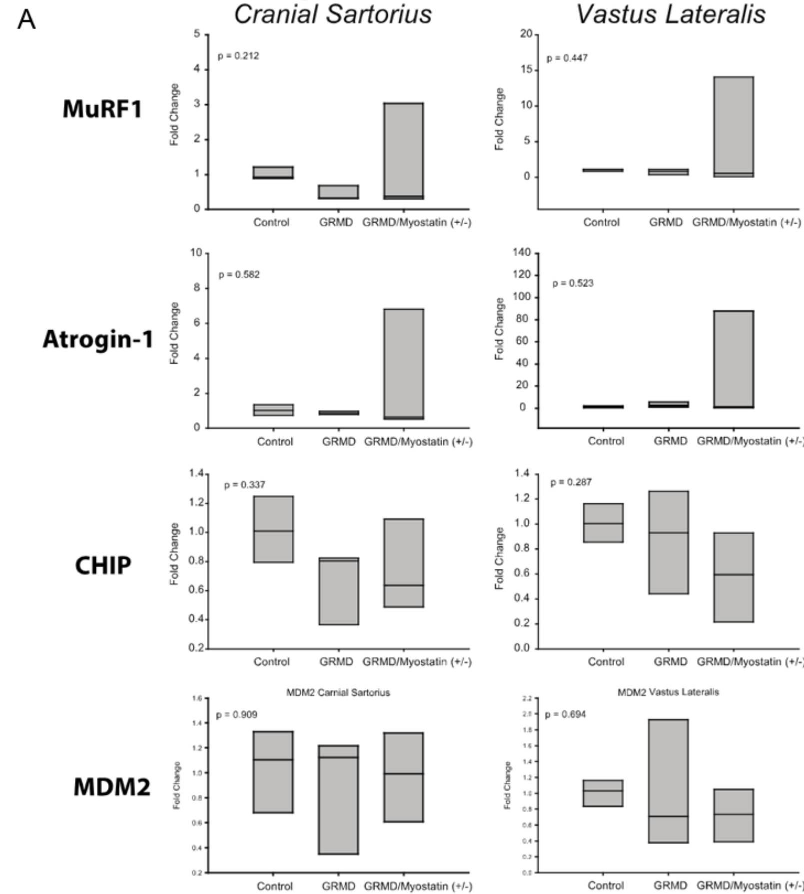
Calpain 1 / Calpain 2 activities were determined using the Sensolyte ® AMC Calpain Activity Fluorometric Assay kit according to the manufacturer's instructions (Sensolyte AMC Calpain Assay Component C, Freemont, CA). The Calpain 1 / Calpain 2 activity was determined by the hydrolysis of the fluorogenic peptide substrate succinyl-leucine-leucine-valine-tyrosine-4-methyl-7-courmarylamide (Suc-LLVY-AMC). Briefly, tissues were homogenized in lysis buffer using a glass homogenizer (Kontes, USA), the lysate centrifuged at 12,000 rpm for 10 minutes, and the supernatant stored at -80°C. 25 μg of lysate was incubated with the fluorogenic peptide substrate succinyl-leucine-leucine-valine-tyrosine-4-methyl-7-courmarylamide (Suc-LLVY-AMC) for 1 hour at 37°C. Fluorescence was measured using a Perkin Elmer Wallac Victor II Multi-label Microplate reader (excitation 350 nm, emission 460 nm, Waltham, MA).

Statistical analyses

Statistical analyses were performed using SigmaPlot 11 (Systat Software Inc, San Jose California). A Rank-Sum test was performed to compare the three groups as the data were determined to be non-parametric using a normality test and (when appropriate) an equal variance. Statistical significance was determined if $p \leq 0.05$.

Results

Semen collected from a myostatin heterozygous whippet sire was used to artificially inseminate an obligate GRMD carrier, producing an initial litter that included Racer, Dash, and



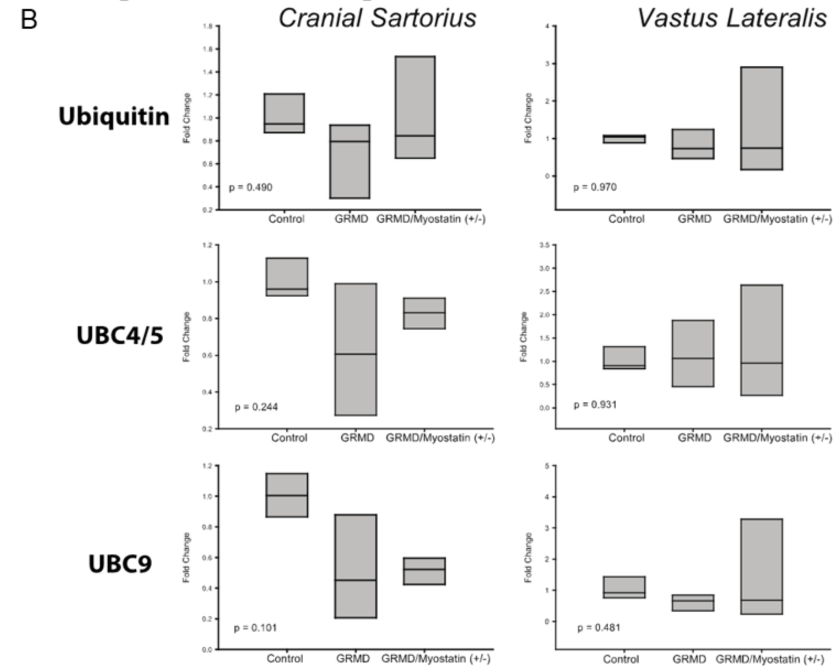


Figure 1. Ubiquitin ligases and ubiquitin components expression in skeletal muscle from control, affected GRMD, and GRMD/Myostatin +/- (GRippet) dogs. A. Ubiquitin ligases implicated in skeletal muscle mass regulation and cell survival. B. Complementing ubiquitin and ubiquitin conjugating enzymes (E2) involved in protein quality control systems in striated muscle.

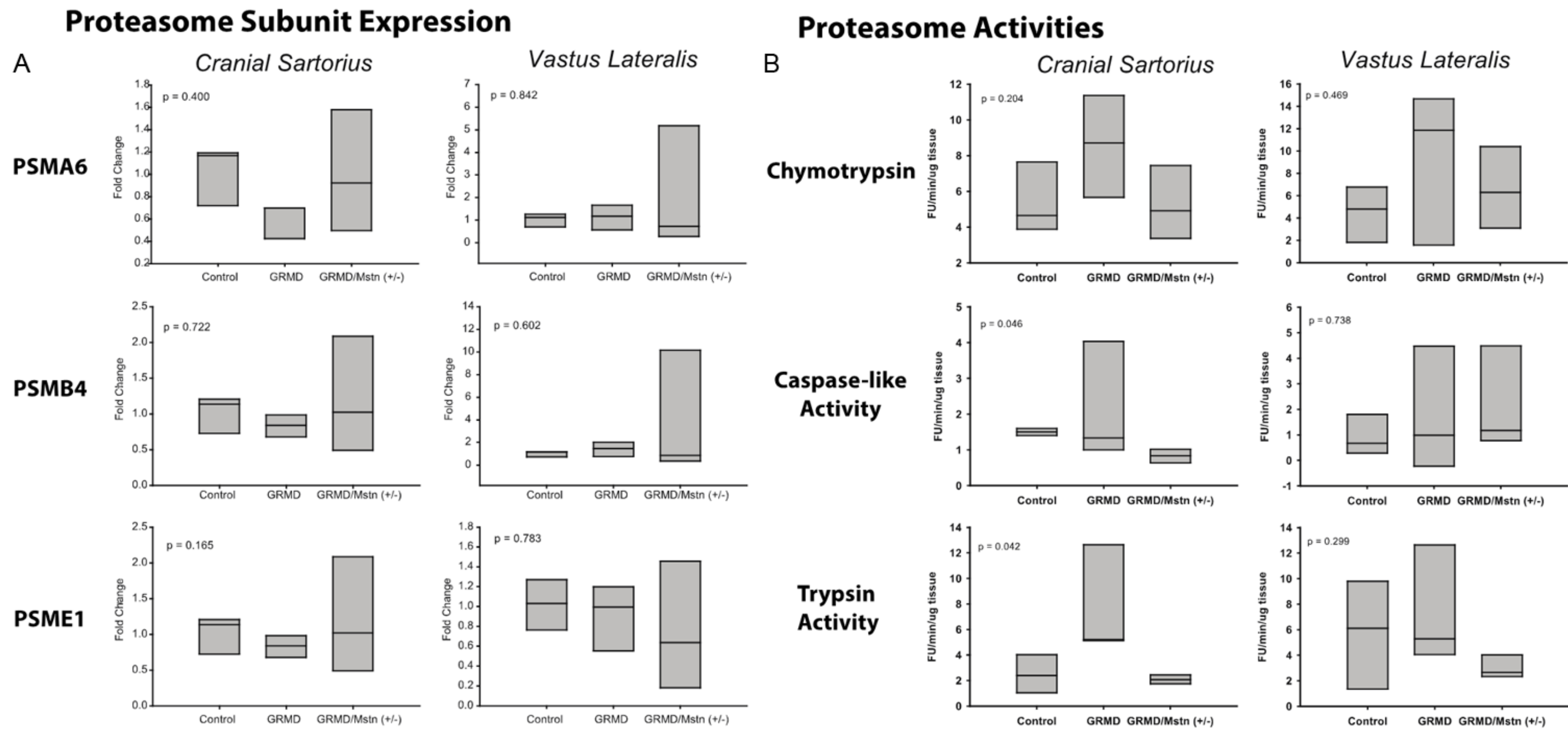


Figure 2. Analysis of proteasome subunit expression and activities in skeletal muscle from control, affected GRMD, and GRMD/Myostatin +/- (GRippet) dogs. A. mRNA expression of proteasome subunits PSMA6, PSMB4, and PSME1 and B. parallel proteasome activities: chymotrypsin, caspase-like activity, and trypsin activity.

Flash (**Table 1**). A second litter was generated using semen from a GRMD male and a female GRMD carrier/myostatin heterozygote (Mstn +/-) from litter 1 (Speedy) generating a second litter. The resulting dogs were classified as non-dystrophic controls (either normal, GRMD carriers, or Mstn heterogygous), GRMD affected-Mstn-myostatin homozygote (GRMD) and GRMD affected/Myostatin deficient (GRMD/Myostatin +/-; GRippets) based on their genotyping (**Table 1**). For sake of data analysis, four GRippets (Dash, Derrwood, Abner, Tabitha), three GRMD dogs (Flash, Hagatha, Samantha), and three controls (Racer, Esmerelda, Endora) were assessed. Previous studies from our group have identified that the cranial sartorius (CS) undergoes true hypertrophy, while most other muscles such as the vastus lateralis (VL) atrophy [14, 15]. The CS is a hip flexor, while the VL extends the stifle (knee). Therefore, we investigated the ubiquitin proteasome and calpain systems in the CS and VL from these three phenotypically distinct muscles.

GRMD status and myostatin expression does not affect the expression of the ubiquitin ligases MuRF1, Atrogin-1, CHIP, and MDM2 or other ubiquitin components

Real time PCR analysis of muscle specific ubiquitin ligase expression was performed on CS and VL in control, GRMD affected, and GRMD/Myostatin +/- GRippet dogs. Using non-parametric statistical analyses, no significant differences in MuRF1, Atrogin-1, CHIP, and MDM2 expression were identified among the three groups (**Figure 1A**) in either CS or VL muscles. However, 1 of 4 dogs (Dash) in the GRMD/Myostatin +/- group had highly increased MuRF1 and Atrogin-1 expression, which accounted for variability seen in that group (**Figure 1A**). The ubiquitin, UBC 4/5, and UBC9 mRNA were also not significantly different among the three groups (**Figure 1B**). However, the median UBC9 was decreased in both GRMD and GRMD/Myostatin +/- groups.

Muscle specific expression of proteasome components PSMA6, PSMB4, PSME1 and proteasome activities are largely unaffected by GRMD and myostatin levels

Real time PCR analysis of the mRNA expression of MSMA6, PSMB4, and PSME1 in CS and VL in control, GRMD affected, and GRMD/Myostatin +/- dogs demonstrated no significant differences (**Figure 2A**). There were consistent increases in all three proteasome components in individual GRMD/Myostatin +/- dogs, but only in 1-2 of the four dogs in this group, so these changes did not reach statistical significance. We next assayed the chymotrypsin, caspase-like, and trypsin proteasome activities in the same muscle biopsies and identified a general increase in all three activities in both CS and VL in the GRMD group (**Figure 2B**). The GRMD group with decreased myostatin (Myo +/-) also generally had a decrease in all three activities; the only significant changes that were identified were in the CS caspase-like and trypsin activities (**Figure 2B**, bottom left 2 panels).

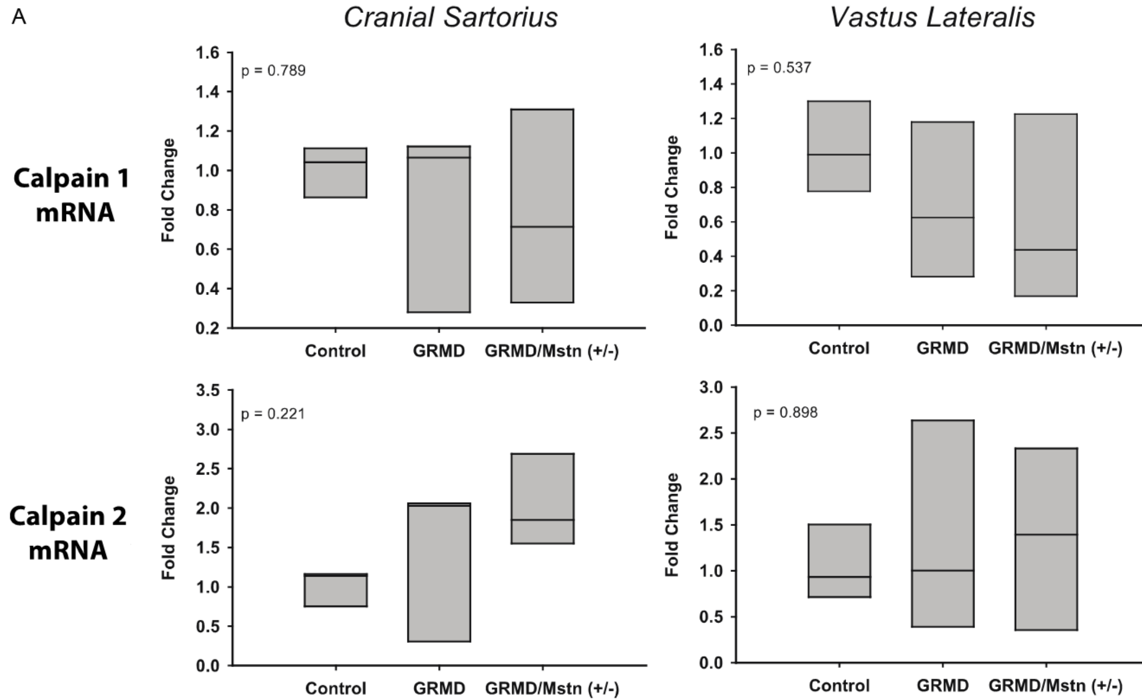
Muscle specific calpain 1 and 2 expression and calpain activity

No differences in calpain 1 or calpain 2 mRNA expressions were identified between GRMD and GRMD/Myostatin +/- groups in either the CS or VL (**Figure 3A**). Increased variability in both of these enzymes was seen in the GRMD and GRMD/myostatin +/- groups. The calpain 1 and calpain 2 activities were not significantly different between groups either, although the GRMD/Myostatin +/- group had more variability due to a subset of dogs.

Discussion

Experimentally, dystrophin-deficient mdx mice lacking myostatin or with myostatin inhibited post-natally appear to have a less severe phenotype [5, 20]. This has led to the interest in therapies that inhibit myostatin to promote muscle grown and possibly improve function in muscular dystrophy [21, 22]. In other dystrophic mouse models lacking myostatin, the results have varied with some mice having increased morbidities [23], including abnormalities in muscle tendons from $Mstn^{-/-}$ mice [24]. In the present study, the phenotype of GRMD dogs with decreased myostatin (Myostatin +/- GRippets) have disproportionate muscle effects and increased phenotype severity [13], consistent with some murine studies.

Since a number of recent studies have suggested that myostatin regulates various components of the ubiquitin proteasome [25-27] and calpain activity has been linked to muscular dystrophy severity [28, 29], we investigated



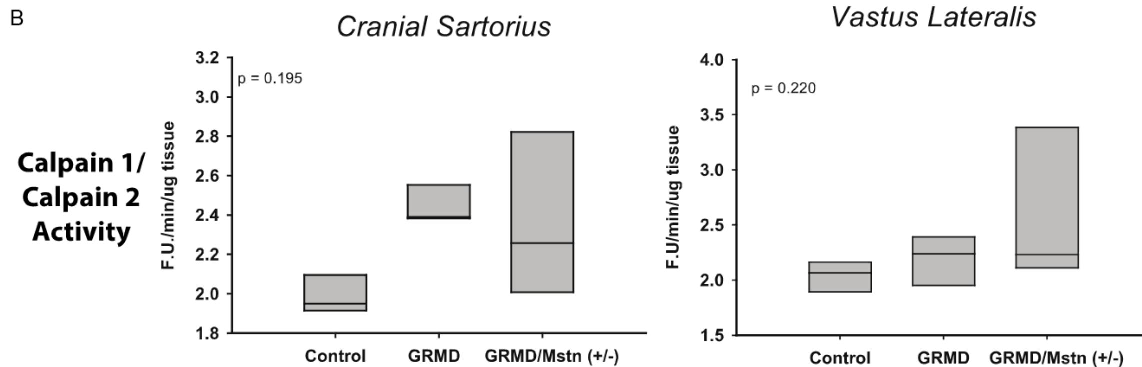


Figure 3. Analysis of the calpain enzymatic system expression and activities in skeletal muscle from control, affected GRMD, and GRMD/Myostatin +/- (GRippet) dogs. A. mRNA expression of calpain 1 and calpain 2 and B. Calpain 1 / Calpain 2 combined activities.

both systems in the GRippet model. While recent studies have identified that some GRMD muscle types (e.g. gastrocnemius, anterior tibialis) have increased proteasome and calpain activity, these changes were much less uniform than that previously identified in mouse studies [30]. When we investigated how decreased myostatin affected these same ubiquitin proteasome system and calpain expression and activity, we identified fewer changes in the GRMD with decreased myostatin despite the worse phenotype [13]. The results largely indicate that the major components of the ubiquitin proteasome system, including the ubiquitin ligases MuRF1, Atrogin-1, CHIP, and MDM2 found in skeletal muscle are not consistently affected in the cranial sartorius and vastus lateralis in dogs with GRMD and GRMD with myostatin deficiency compared to controls. While the expression of the proteasome components PSMA6, PSMB4, and PSME1 in the CS and VL muscles did not differ among groups,

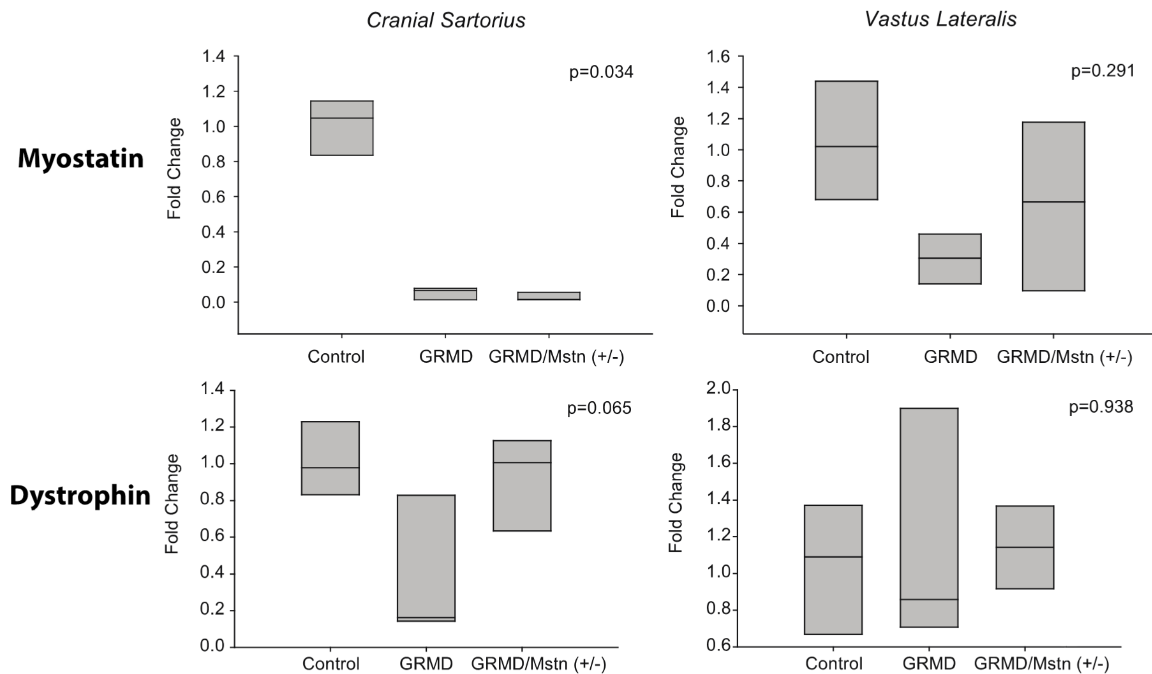


Figure 4. mRNA expression of myostatin and dystrophin from control, affected GRMD, and GRMD/Myostatin +/- (GRippet) dogs.

the GRMD group with decreased myostatin (Myostatin +/-) generally had a decrease in all three activities, which were significant for the CS caspase-like and trypsin activity. Changes in the UPS and calpain systems were minor at most. Therefore, our data do not support a role for these systems in differential muscle involvement in the GRippet model.

Multiple studies have reported that reducing myostatin activity has apparent therapeutic benefit in both mouse and dog models of DMD [5-8]. However, phase II human clinical trials for ACE-031, a humanized antibody mimicking the myostatin receptor ActRIIB to inhibit myostatin activity were stopped due to unfavorable safety profiles [31]. Additionally, previous human clinical trials of adult muscular dystrophy patients using myostatin neutralizing antibodies also demonstrated no beneficial outcomes for muscle strength, function, or growth [10]. Recent studies have also demonstrated that the elimination of myostatin in other muscular dystrophy models (laminin-deficiency) has proven to increase postnatal lethality [23]. The GRippet model similarly appears to be made worse when myostatin is reduced [13]. This clinical severity may result from both direct and indirect effects on the adult skeletal muscles investigated. Despite myostatin's possible regulation of the ubiquitin proteasome systems [25-27] and the link of calpain activity to muscular dystrophy severity [28, 29], these systems do not appear to be dysregulated extensively or parallel the severe differences in phenotype that are observed with GRMD with myostatin haploinsufficiency.

Recent studies have identified that myostatin mRNA is reduced in DMD patients [32], with the processing and maturation of myostatin protein inhibited in some patients [33]. Decreased myostatin in the mdx mouse model has also been reported [34]. In the present study, we similarly identified that myostatin was decreased in both the GRMD and GRMD/Mstn (+/-) groups significantly in the CS (p=0.034), but not in the VL (p=0.291), indicating that these changes may be muscle specific (**Figure 4**). Unlike myostatin mRNA levels, dystrophin levels in the CS and VL were not significantly different between the dystrophic and control dog groups. This is inconsistent with the generally accepted concept that mRNA levels are reduced in DMD due to of nonsense mediated decay [35, 36]. Dystrophin mRNA levels were

also reduced in initial studies of muscle from mdx mice [37] and GRMD dogs [38]. However, a recent RT-qPCR study showed that dystrophin mRNA levels in mdx muscles were comparable, if not even higher, than those in wild type mice [39]. This study also showed that mRNA expression was greater at the 5' versus 3' end and that this imbalance played a major role in dystrophin expression. For sake of canine data presented here, the dystrophin probe used identified a region spanning exon 32-33, which would be relatively more 5', which may explain the unexpected DMD mRNA levels detected.

We used the GRMD model of DMD as its severity and variability parallels the human disease to a much greater extent than mouse models [14, 19, 30, 38]. Despite the proposed utility of proteasome inhibitors in DMD, we identified that less than ½ of the muscles assayed had increases in proteasome activity and only ½ had increased calpain activity [30]. Similarly, the transcriptional regulation of the ubiquitin proteasome system in skeletal muscle was largely unaffected [30]. Since both the ubiquitin proteasome and calpain systems are crucial mediators of skeletal muscle atrophy, we anticipated that the GRMD/Myo +/- group would have an enhanced UPS and calpain activities. However, there were no clear consistent increases in the regulation and activity of the UPS and calpain systems, indicating that other mechanisms were likely involved in the disproportionate muscle involvement and associated accelerated decline seen in the GRippet group.

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Disclosure of conflict of interest

The authors do not have any conflicts of interest to disclose.

Abbreviations

AAV, adeno-associated virus; CS, Cranial sartorius; DMD, Duchenne muscular dystrophy; GRMD, golden retriever muscular dystrophy; MDM2, murine double mutant 2; MuRF1, muscle ring finger-1; VL, vastus lateralis.

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