BMC Musculoskeletal Disorders

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Transcriptional changes of genes encoding sarcoplasmic reticulum calcium binding and up-taking proteins in normal and Duchenne muscular dystrophy dogs

Emily D. Morales[1†], Dongxin Wang[1†], Matthew J. Burke[1†], Jin Han[1], Drake D. Devine[1], Keqing Zhang[1] and Dongsheng Duan[1,2,3,4*]

Abstract

Background Cytosolic calcium overload contributes to muscle degradation in Duchenne muscular dystrophy (DMD). The sarcoplasmic reticulum (SR) is the primary calcium storage organelle in muscle. The sarco-endoplasmic reticulum ATPase (SERCA) pumps cytosolic calcium to the SR during muscle relaxation. Calcium is kept in the SR by calcium-binding proteins.

Methods Given the importance of the canine DMD model in translational studies, we examined transcriptional changes of SERCA (SERCA1 and SERCA2a), SERCA regulators (phospholamban, sarcolipin, myoregulin, and dwarf open reading frame), and SR calcium-binding proteins (calreticulin, calsequestrin 1, calsequestrin 2, and sarcalumenin) in skeletal muscle (diaphragm and extensor carpi ulnaris) and heart (left ventricle) in normal and affected male dogs by droplet digital PCR before the onset (≤ 2-m-old), at the active stage (8 to 16-m-old), and at the terminal stage (30 to 50-m-old) of the disease. Since many of these proteins are expressed in a fiber type-specific manner, we also evaluated fiber type composition in skeletal muscle.

Results In affected dog skeletal muscle, SERCA and its regulators were down-regulated at the active stage, but calcium-binding proteins (except for calsequestrin 1) were upregulated at the terminal stage. Surprisingly, nominal differences were detected in the heart. We also examined whether there exists sex-biased expression in 8 to 16-m-old dogs. Multiple transcripts were significantly downregulated in the heart and extensor carpi ulnaris muscle of female dogs. In fiber type analysis, we found significantly more type I fiber in the diaphragm of 8 to 16-m-old affected dogs, and significantly more type II fibers in the extensor carpi ulnaris of 30 to 50-m-old affected dogs. However, no difference was detected between male and female dogs.

Conclusions Our study adds new knowledge to the understanding of muscle calcium regulation in normal and dystrophic canines.

†Emily D. Morales, Dongxin Wang and Matthew J. Burke contributed equally to this work.

*Correspondence:
Dongsheng Duan
duand@missouri.edu
Full list of author information is available at the end of the article

Keywords Duchenne muscular dystrophy, DMD, Canine model, Calcium homeostasis, Gene expression, Sex, Fiber type, SERCA, Sarcoplasmic reticulum, ddPCR

Background

Duchenne muscular dystrophy (DMD) is the most common form of muscular dystrophy in children [1]. DMD is caused by the loss of dystrophin, a protein important for muscle integrity. Absence of dystrophin leads to muscle degeneration. Consequently, patients show progressive muscle weakness, losing the ability to walk in their early teenage years, and dying in early adulthood. It has long been recognized that aberrant cytosolic calcium elevation is a pivotal pathogenic mechanism underlying muscle degeneration in DMD [2, 3]. High levels of cytosolic calcium induce protein degradation by activating calcium-dependent proteases and induce lipid membrane degradation by activating calcium-dependent phospholipases [4, 5].

The sarcoplasmic reticulum (SR) is the primary calcium storage house in muscle [6]. It plays a critical role in regulating cytosolic calcium levels. During contraction, calcium is released from the SR. During relaxation, calcium is returned to the SR by the sarco-endoplasmic reticulum ATPase (SERCA) [7, 8]. The activity of SERCA is negatively regulated by inhibitory micropeptides including phospholamban (PLB), sarcolipin (SLN), and myoregulin [9]. SERCA function can also be enhanced by the activating micropeptide dwarf open reading frame (DWORF) [10]. Inside the SR, calcium is sequestered by calreticulin,

Table 1 Dog information

Dog ID#	Genotype	Sex	Age (m)	Used in transcript study	Used in fiber type study	
					Diaphragm	ECU
Dog #1	Normal	M	1.23	Yes	Yes	Yes
Dog #2	Normal	M	1.80	Yes	Yes	Yes
Dog #3	Normal	M	0.10	Yes	Yes	Yes
Dog #4	Normal	M	0.10	Yes	Yes	Yes
Dog #5	Affected	M	0.73	Yes	Yes	Yes
Dog #6	Affected	M	0.73	Yes	Yes	Yes
Dog #7	Affected	M	0.00	Yes	No	No
Dog #8	Affected	M	0.00	Yes	Yes	Yes
Dog #9	Normal	M	12.93	Yes	Yes	Yes
Dog #10	Normal	M	13.70	Yes	Yes	Yes
Dog #11	Normal	M	13.13	Yes	Yes	Yes
Dog #12	Normal	M	13.03	Yes	Yes	Yes
Dog #13	Affected	M	14.87	Yes	Yes	Yes
Dog #14	Affected	M	14.17	Yes	Yes	Yes
Dog #15	Affected	M	15.20	Yes	Yes	No
Dog #16	Affected	M	8.13	Yes	Yes	Yes
Dog #17	Normal	F	15.33	Yes	No	Yes
Dog #18	Normal	F	15.60	Yes	Yes	Yes
Dog #19	Normal	F	14.10	Yes	Yes	Yes
Dog #20	Affected	F	11.43	Yes	Yes	Yes
Dog #21	Affected	F	13.13	Yes	Yes	Yes
Dog #22	Affected	F	12.07	Yes	Yes	Yes
Dog #23	Normal	M	41.40	Yes	Yes	Yes
Dog #24	Normal	M	41.37	Yes	Yes	Yes
Dog #25	Normal	M	42.20	Yes	Yes	Yes
Dog #26	Normal	M	46.10	Yes	Yes	Yes
Dog #27	Affected	M	31.00	Yes	Yes	Yes
Dog #28	Affected	M	33.77	Yes	Yes	Yes
Dog #29	Affected	M	44.40	Yes	No	Yes
Dog #30	Affected	M	42.20	Yes	Yes	Yes

calsequestrin, and sarcalumenin. These calcium-binding proteins provide another layer of regulation.

In DMD, an increased amount of calcium is released from the SR while a decreased amount returns to the SR, leading to cytosolic calcium buildup [3]. Changes in the expression of SR calcium-binding proteins, SERCA, and SERCA regulators have been implicated in cytosolic calcium overloading in DMD [11–18].

Dystrophin-deficient canines are excellent models for translational studies. Unlike the mouse model, the canine model has a similar dystrophic phenotype to human patients [19–21]. Unfortunately, little is known about transcriptional changes of the genes encoding SR calcium handling proteins in normal and affected dogs. To address this issue, we quantified transcript levels of four calcium-binding proteins (calreticulin, calsequestrin 1, calsequestrin 2, and sarcalumenin), two SERCA isoforms (SERCA1 and SERCA2a), and four SERCA regulators (PLB, SLN, myoregulin, and DWORF) using droplet digital PCR (ddPCR) in the heart (left ventricle), diaphragm, and extensor carpi ulnaris (ECU) muscle of age-matched male normal and affected dogs before the onset of muscle disease (≤2-m-old) [19, 21], at the symptomatic stage (8 to 16-m-old), and the terminal stage (30 to 50-m-old). Significant differences were detected in skeletal muscles but not in the heart. We further compared 8 to 16-m-old male and female dogs and found significant differences in the heart and ECU muscle but not the diaphragm. Interestingly, we did not detect a correlation between mRNA expression and skeletal muscle fiber type composition.

Methods

Tissues

This study used curated tissues previously collected at the University of Missouri. Tissue collection was approved by the Animal Care and Use Committee of the University of Missouri and was performed in accordance with National Institutes of Health guidelines. Specifically, tissues were from 12 normal males, 3 normal females, 12 affected males, and 3 affected females. In males, one-third were less than 2 months, one-third were 8 to 16 months, and one-third were 30 to 50 months. All females were 8 to 16 months. Only male dogs were used to evaluate disease-stage (pre-onset, symptomatic, and terminal) associated changes. Dog information and sample size are provided in Table 1. No animals were euthanized for the purpose of this study.

Myofiber type evaluation

Fiber type was examined by myosin heavy-chain isoform immunofluorescence staining on ten-micron cryosections using our published protocol [22, 23]. Fiber type-specific monoclonal antibodies were purchased from the University of Iowa Hybridoma Bank (https://dshb.biology.uiowa.edu/monoclonal/mouse). Specifically, type I fibers were detected with antibody BA-D5 (1:20 dilution). Type IIa/IIx fibers were detected with antibody SC-71 (1:100 dilution). Type IIb fibers were detected with antibody BF-F3 (1:40 dilution). Embryonic myosin heavy chain was detected with antibody F1.652. Myofiber outlines were recognized using a goat anti-rabbit antibody against laminin (1:200 dilution; Sigma-Aldrich, St. Louis, MO). Myofiber type composition was quantified from 6 to 9 random 20× field images for each muscle.

Table 2 TaqMan PCR primers and probes

Calreticulin	**Forward Primer**	**5′-GACTGGGATGAAGAGATGGATG-3′**
	Probe	**5′-CTGAGTACAAGGGCGAGTGGAAGC-3′**
	Reverse Primer	**5′-GCCCTTGTAATCTGGGTTGT-3′**
CSQ1	Forward Primer	5′-CGTGGAATTGATCGAAGG-3′
	Probe	5′-TCTGAATCCTTGTTCTTGAAGTAGCCA-3′
	Reverse Primer	5′-CTCGAAGGCCTTGTAATG-3′
CSQ2	Forward Primer	5′-CCATCCCTGACAAACCTTACA-3′
	Probe	5′-CGTAGGGTGGGTCTTTGGTGTTCC-3′
	Reverse Primer	5′-GGATGCCGTTCAAATCATCTTC-3′
Sarcalumenin	Forward Primer	5′-CTGAATGAGGACAAGCCAGT-3′
	Probe	5′-TGCAGCACCACAGAGTAGTCATCC-3′
	Reverse Primer	5′-GCTTGATGGAGGAATGGTAGAT-3′
SERCA1	Forward Primer	5′-CCTCCACTTCCTCATCCTCTA-3′
	Probe	5′-ACCCTCTGCCGATGATCTTCAAGC-3′
	Reverse Primer	5′-CGAGCCCAATGACTGGAAA-3′
SERCA2a	Forward Primer	5′-GCTTGGTTTGAAGAAGGTGAAG-3′
	Probe	5′-TGGGTGTATGGCAGGAGAGAAATGC-3′
	Reverse Primer	5′-CTTAAGTGCTTCGATCGCATTC-3′
PLB	Forward Primer	5′-ACTTGAGACTTCCTGCTTTCC-3′
	Probe	5′-AGTCCAATACCTCACTCGCTCTGCTA-3′
	Reverse Primer	5′-CGTGCTTGTTGAGGCATTTC-3′
SLN	Forward Primer	5′-CCTGAGTTGGAGAGAGAGAGAA-3′
	Probe	5′-CTACTGCAGCCAGGTGAGGACAAG-3′
	Reverse Primer	5′-TGAGGGCACACCCAAGA-3′
Myoregulin	Forward Primer	5′-GCAAGAGCATGTTGGATATGAT-3′
	Probe	5′-TCTACTACGATTCCCACAAGTCCA-3′
	Reverse Primer	5′-GAAGTCTTCCAACAATTTCATCTTC-3′
DWORF	Forward Primer	5′-CCTTCTGGTCCCTATTCTTCTC-3′
	Probe	5′-TCTTCTCCTAGAAAGGCAAGAAGACCT-3′
	Reverse Primer	5′-AATCTGTCACGTTCATGCTTT-3′

Transcript quantification

Tissue collection, RNA extraction and quantification, cDNA preparation and quantification, and ddPCR were performed according to our published protocols [24]. Briefly, the fresh left ventricle, diaphragm, and ECU samples were snap-frozen in liquid nitrogen. Approximately 30 mg tissues were used for RNA extraction using the RNeasy Fibrous Tissue Mini kit (Qiagen, Hilder, Germany; Cat No: 74704). Reverse transcription was performed using the SuperScript IV VILO Master Mix with ezDNase Enzyme (Thermo Fisher Scientific, Hampton, NH, USA; Cat No: 11766050). cDNA concentrations were determined using the Qubit ssDNA assay kit (Thermo Fisher Scientific, Cat No: Q10212). cDNA was diluted in the range of 0.5 to 50 ng/ul for ddPCR. Loading mass (ng) per reaction for detection of each transcript was empirically determined. ddPCR was performed using the QX200 ddPCR system (Bio-Rad Laboratories, Inc., Hercules, CA, USA) using ddPCR supermix for probes (no dUTP) (Bio-Rad, Cat No: 186–3024). Primers and probes used in the study are listed in Table 2. All probes contained a 5′ 6-carboxyfluorescein, an internal ZEN quencher, and a 3' Iowa black quencher. Results are presented as absolute copies of transcript per ng of cDNA used in the reaction.

Statistical analysis

Data are presented as mean ± standard error of the mean. The normality of the data was determined by the Shapiro–Wilk test. Parametric data were analyzed by the unpaired Student t-test between two groups. Non-parametric data were analyzed by the Mann-Whitey test between the two groups. Analyses were performed using GraphPad PRISM software version 9.1.2 (GraphPad Software, La Jolla, California). A $p < 0.05$ was considered statistically significant.

Results

Characterization of fiber type composition of the diaphragm and ECU muscle

In this study, we evaluated the diaphragm and ECU muscle. We chose the diaphragm because it is the most severely affected muscle in DMD [1]. We chose the ECU

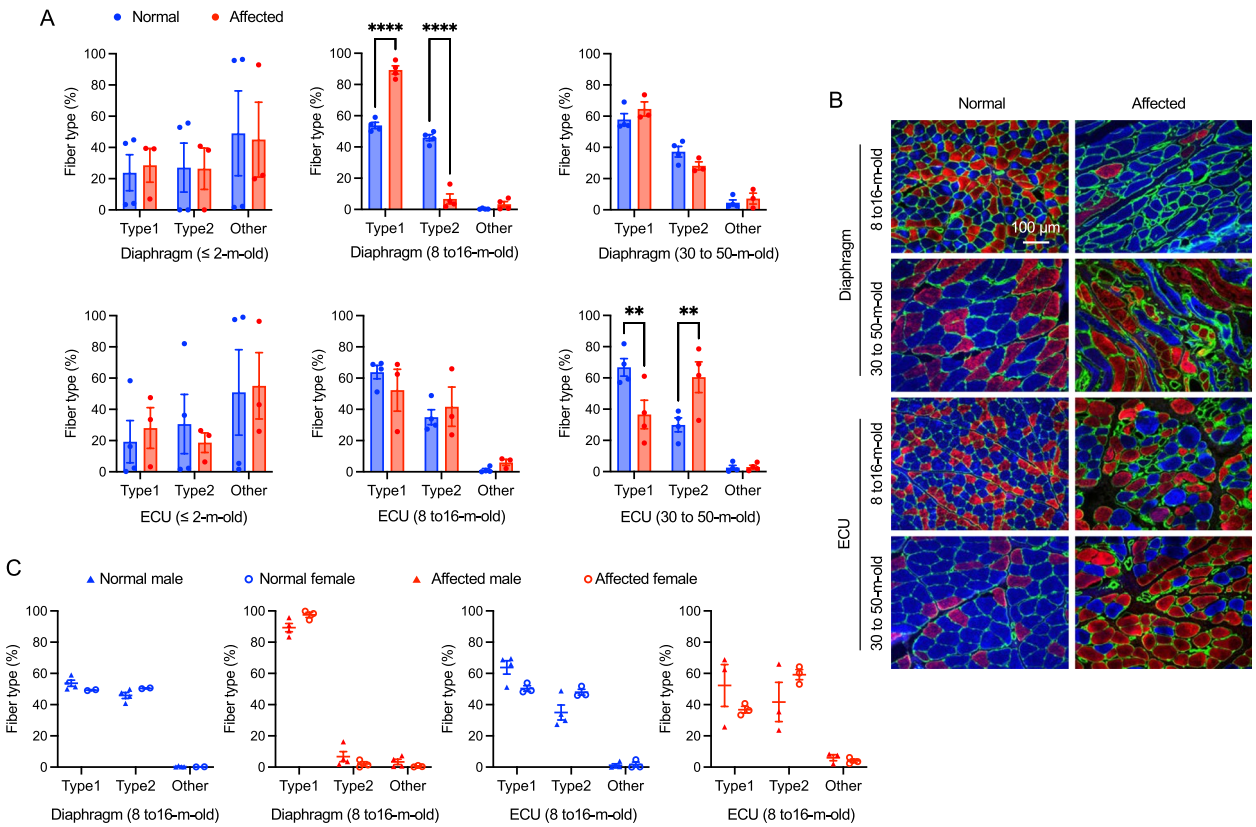

Fig. 1 Evaluation of myofiber type composition. **A** Fiber type composition in the diaphragm (top panels) and the extensor carpi ulnaris (ECU) muscle of ≤ 2-m-old, 8 to 16-m-old, and 30 to 50-m-old normal and affected dogs. Fiber types are classified as type I (slow), type II (fast), and other. Other refers to hybrid myofibers that expressed both type I and type II myosin heavy chain and myofibers that expressed embryonic myosin heavy chain. **B** Representative photomicrographs of fiber type immunofluorescence staining. Type I fibers are in blue. Type II fibers are in red. Myofibers are outlined by laminin (green) **C** Comparison of fiber type composition of 8 to 16-m-old male and female normal and affected dogs

muscle because its histopathology and function have been extensively characterized [22, 25], and this muscle has also been used to test genetic therapeutics in the canine model [26–28]. We quantified the fiber type distribution (Fig. 1). In the diaphragm, statistically significant differences were detected in 8 to 16-m-old dogs. In this age range, affected dogs had significantly more type I fibers, while normal dogs had significantly more type II fibers [29]. In the ECU muscle, statistically significant differences were detected in 30 to 50-m-old dogs. In this age range, affected dogs had significantly more type II fibers, while normal dogs had significantly more type I fibers [22]. We also compared the fiber type composition of 8 to 16-m-old male and female dogs. No significant difference was detected (Fig. 1B).

Before the onset of muscle disease

Dystrophin-deficient dogs show no dystrophic symptoms before weaning (≤2-m-old) [19, 21]. At this stage, no significant differences were detected between normal and affected puppies in the transcript levels of SR calcium-binding proteins (calreticulin, calsequestrin 1, calsequestrin 2, and sarcalumenin) or SERCAs and their regulators (SERCA1, SERCA2a, PLB, SLN, myoregulin, and DWORF) (Fig. 2). Interestingly, the transcript levels of SERCA1, SLN, myoregulin, and DWORF in the left ventricles were several thousand times lower than those in the diaphragm and ECU muscle (Fig. 2).

At the symptomatic stage

Between 8 and 16 months, affected dogs display clinical signs and symptoms of muscular dystrophy. In the diaphragm of affected dogs, the transcript levels of SERCA1 and DWORF were significantly reduced (Fig. 3A). In the ECU muscle of affected dogs, the transcript levels of sarcalumenin, SERCA1, SERCA2a, PLB, myoregulin, and DWORF were significantly reduced (Fig. 3B). No statistically significant differences were detected between normal and affected dogs for any transcripts in the heart (Fig. 3C). The transcript levels of SERCA1, SLN, myoregulin, and DWORF remained drastically reduced in cardiac muscle compared to skeletal muscle (Fig. 3).

At the terminal stage

The lifespan of affected dogs is approximately 3 years [19]. To examine transcript changes at the end stage, we

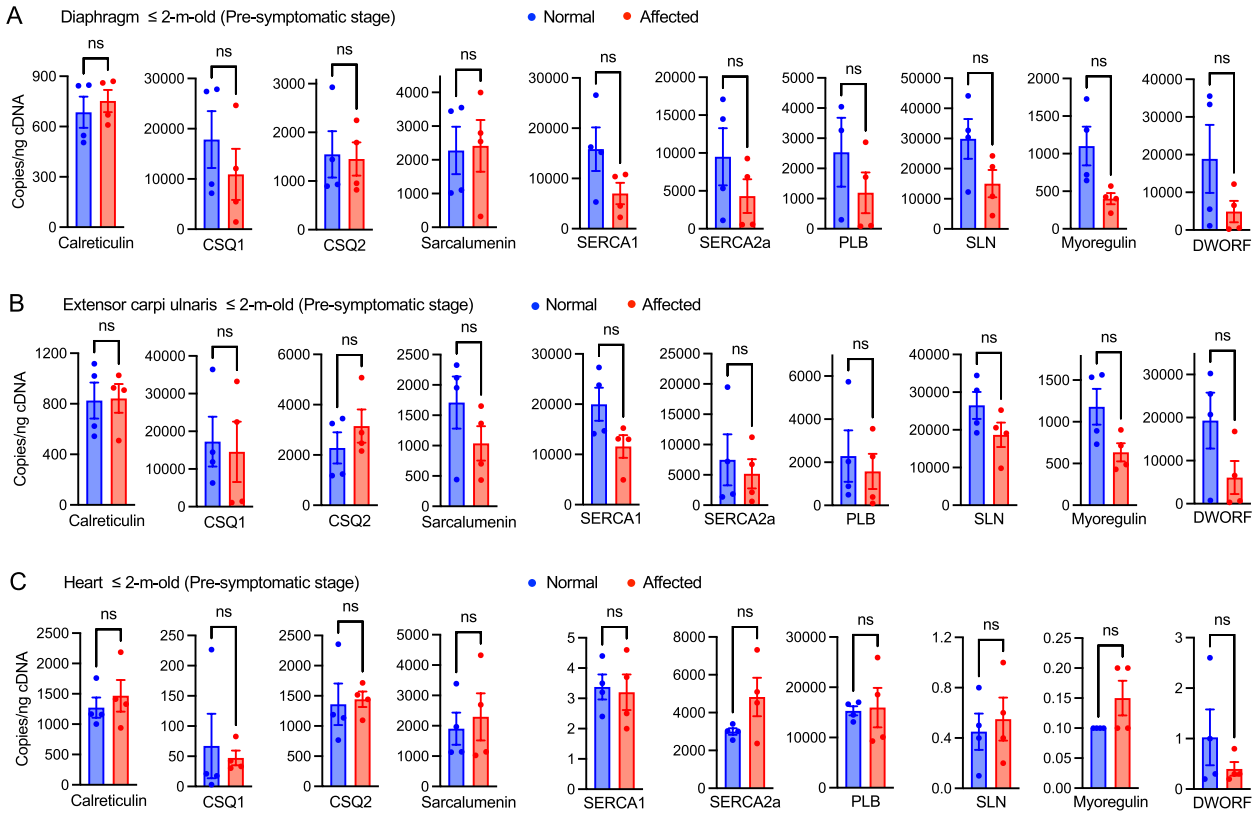

Fig. 2 Quantification of the cDNA copy number in genes encoding SR calcium-binding protein, SERCAs, and SERCA regulators in pre-symptomatic dogs. **A** Diaphragm. **B** extensor carpi ulnaris. **C** Heart. CSQ1, calsequestrin 1; CSQ2, calsequestrin 2; DWORF, dwarf open reading frame; ns, not statistically significant; PLB, phospholamban; SERCA, sarcoendoplasmic reticulum calcium ATPase; SLN, sarcolipin; SR, sarcoplasmic reticulum

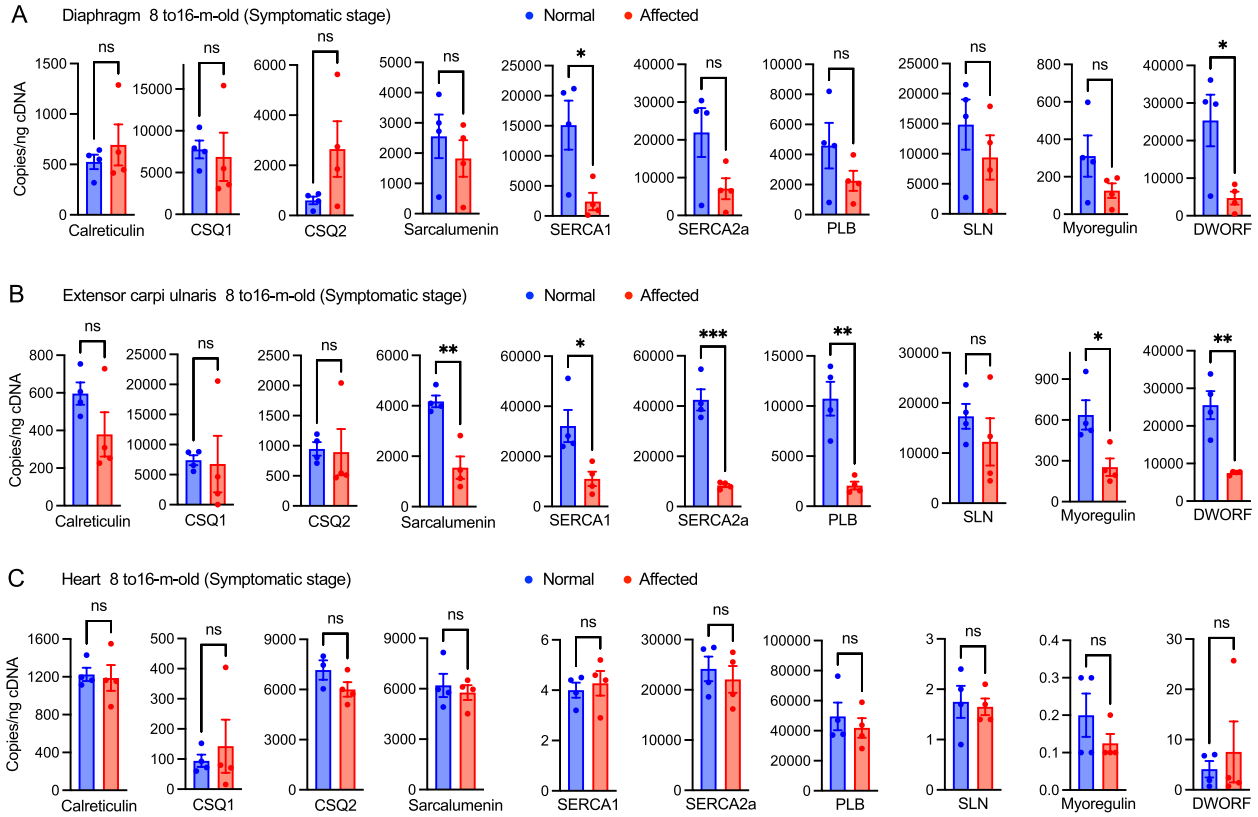

Fig. 3 Quantification of the cDNA copy number in genes encoding SR calcium-binding protein, SERCAs, and SERCA regulators in 8 to 16-m-old dogs. **A** Diaphragm. **B** Extensor carpi ulnaris. **C** Heart. CSQ1, calsequestrin 1; CSQ2, calsequestrin 2; DWORF, dwarf open reading frame; ns, not statistically significant; PLB, phospholamban; SERCA, sarcoendoplasmic reticulum calcium ATPase; SLN, sarcolipin; SR, sarcoplasmic reticulum. *, $p < 0.05$; **, $P < 0.01$; ***, $p < 0.001$

collected tissues from 30 to 50-m-old dogs. In the diaphragm of affected dogs, the transcript level of calsequestrin 2 was significantly upregulated but the transcript levels of calsequestrin 1 and SERCA1 and DWORF were significantly downregulated at this stage (Fig. 4A). In the ECU muscle of affected dogs, the transcript levels of calreticulin, sarcalumenin, and myoregulin were significantly upregulated (Fig. 4B). In the heart of affected dogs, the transcript level of sarcalumenin was significantly upregulated (Fig. 4C).

Differences between male and female dogs

We compared transcript levels in 8 to 16-m-old male and female dogs. In the diaphragm, the only significant change is the upregulation of calsequestrin 1 in normal female dogs (Figs. 5A and 6A). In the ECU muscle, the transcript levels of calreticulin, calsequestrin 2, sarcalumenin, PLB, and DWORF were significantly higher in normal males (Fig. 5B). The transcript level of SERCA2a was significantly upregulated in both normal and affected males (Figs. 5B and 6B). In the heart, the transcript levels of calreticulin, calsequestrin 2, sarcalumenin, and SERCA2a were significantly higher in male dogs irrespective of muscle disease (Figs. 5C and 6C). The PLB transcript was only significantly upregulated in affected male dogs although a trend was detected in normal male dogs (Fig. 6C).

Discussion

In this study, we examined the expression of genes encoding SR calcium-binding proteins (calreticulin, calsequestrin 1, calsequestrin 2, and sarcalumenin), SR calcium pumps (SERCA1 and SERCA2a), and SERCA regulators (PLB, SLN, myoregulin, and DWORF) in the heart, diaphragm, and limb muscle of the canine DMD model and age/sex-matched normal dogs by ddPCR. No statistically significant differences between normal and affected dogs were detected in the heart (Figs. 2, 3, and 4). However, disease stage-associated changes were observed in the diaphragm and extensor carpi ulnaris muscle (Figs. 2, 3, and 4). We also compared 8 to 16-m-old male and female dogs and found significant downregulation of multiple transcripts in the heart and extensor carpi ulnaris muscle in female dogs (Figs. 5, and 6). Since many of these

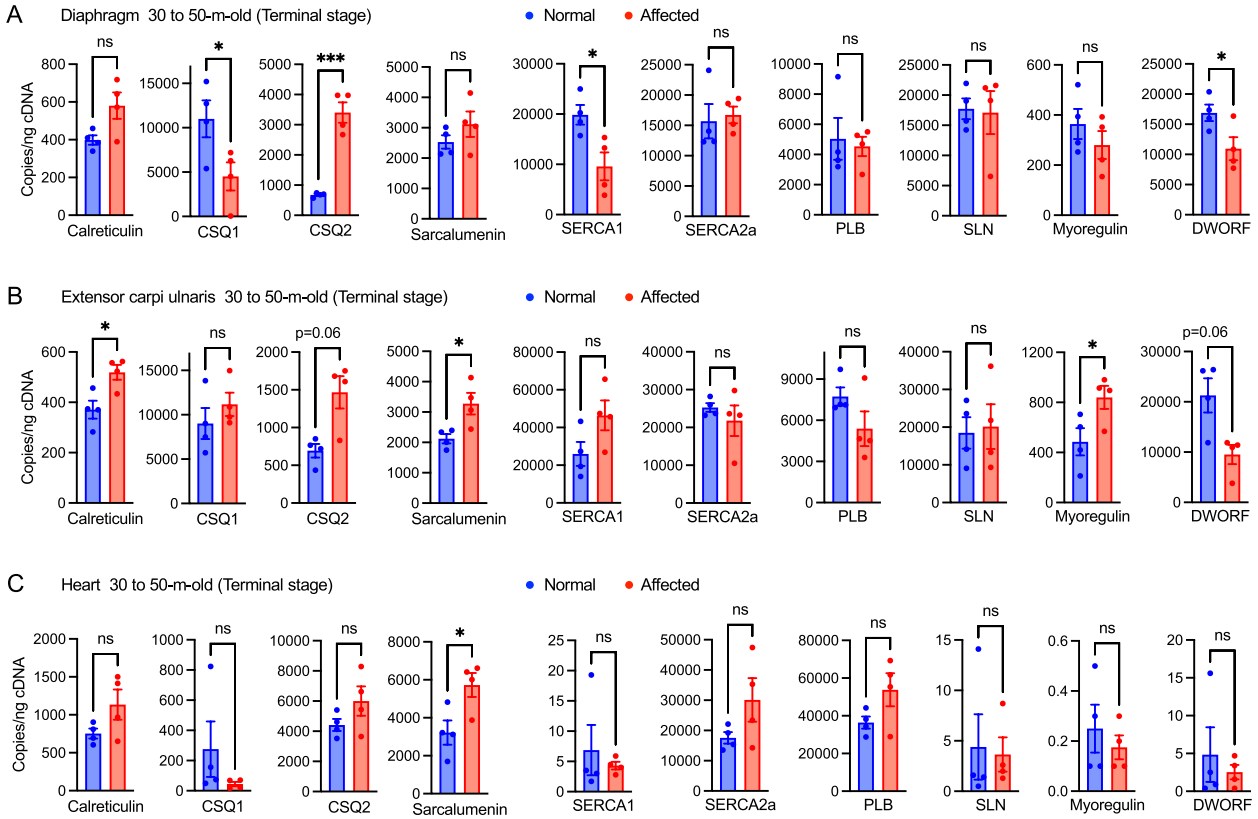

Fig. 4 Quantification of the cDNA copy number in genes encoding SR calcium-binding protein, SERCAs, and SERCA regulators in 30 to 50-m-old dogs. **A** Diaphragm. **B** Extensor carpi ulnaris. **C** Heart. CSQ1, calsequestrin 1; CSQ2, calsequestrin 2; DWORF, dwarf open reading frame; ns, not statistically significant; PLB, phospholamban; SERCA, sarcoendoplasmic reticulum calcium ATPase; SLN, sarcolipin; SR, sarcoplasmic reticulum. *, $p < 0.05$

proteins are expressed in a fiber type-specific manner in skeletal muscle, we also evaluated fiber type composition (Fig. 1). Interestingly, we did not observe a consistent correlation between the transcript level and fiber type composition.

Calcium homeostasis is pivotal to muscle health and function. Calcium mishandling in DMD leads to pathological calcium accumulation in the cytosol [3]. Aberrant cytosolic calcium overloading is in part due to changes in SR calcium-binding proteins, SERCA, and SERCA regulators. These changes may occur at the mRNA level, the protein level, or through post-translational protein modifications (such as nitrosylation and phosphorylation). Here, we focused on transcript changes in the clinically relevant canine model. The most striking observation is the differences between cardiac and skeletal muscles. Nominal changes were detected between normal and affected dogs in the heart (Figs. 2, 3, and 4), suggesting transcription is not a primary regulatory mechanism in the cardiac muscle of the canine DMD model. Interestingly, an earlier Northern blot study showed a down-regulation of SERCA2a mRNA in the heart of mdx mice [30]. We recently found that DWORF transcript is significantly reduced in the heart of mdx mice [18]. These results suggest the existence of important differences in transcriptional regulation between canine and murine models.

Another interesting difference between cardiac and skeletal muscle is the extremely low transcription of SERCA1, sarcolipin, myoregulin, and DWORF in the left ventricle. The copy numbers of these transcripts were hundred times lower than those in skeletal muscle in both normal and affected dogs (Figs. 2, 3, and 4). These results highlight tissue-specific calcium regulation at the transcriptional level. It is also worth noting that the RNA levels of SERCA1, sarcolipin, and myoregulin were also very low in the mouse ventricles [31, 32]. However, the DWORF RNA is highly expressed in the mouse ventricles, suggesting a species-specific regulation mechanism for DWORF RNA expression [10, 18].

In contrast to the heart, skeletal muscle showed distinctive gene expression patterns at different stages of the disease course. Before the onset of muscle disease, there was no difference between dystrophin-null puppies

and normal puppies (Fig. 2). This is consistent with the notion that calcium mishandling is a secondary, rather than the primary, pathogenic mechanism in DMD. In other words, dystrophin deficiency alone cannot induce transcriptional changes in the absence of muscle damage. Our results are different from a microarray study in pre-symptomatic DMD patients [33]. Pescatori et al. examined gene expression profile of the quadriceps from 1.5 to 22-month-old DMD patients using the Affymetrix technology. The authors found a significant down-regulation of PLB and up-regulation of calreticulin. PLB is a negative regulator of SERCA. Reduction in PLB expression would increase SERCA activity. Calreticulin is a calcium-binding protein in the SR. Increased calreticulin expression would favor calcium retention in the SR. Collectively, these changes would prevent, rather than induce cytosolic calcium overloading. We do not have a good explanation for why the muscles of pre-symptomatic DMD patients, but not affected puppies, show counterintuitive changes in PLB and calreticulin expression.

At the active stage of the disease, the most prominent changes are the reduced expression of SERCA and DWORF (Fig. 3). Statistically significant differences were detected in the diaphragm and ECU muscle for SERCA1 and DWORF, and the ECU muscle for SERCA2a. DWORF is a positive regulator of SERCA [10]. Downregulation of SERCA and DWORF would reduce the active transportation of cytosolic calcium to the SR and contribute to cytosolic calcium overload. DWORF is a recently discovered protein. DWORF gene expression has not been studied in dystrophin-deficient skeletal muscle. However, SERCA gene expression has been extensively profiled in transcriptomic studies in rodent DMD models, patient-derived pluripotent stem cells, and patient muscles. SERCA1 mRNA is significantly reduced in 2 to 4-m-old mdx mouse limb muscle [34]. SERCA1 mRNA and SERCA2a mRNA are significantly reduced in the tibialis anterior muscle of the rat model at 9 months of age [35]. Morera et al. and Mournetas et al. showed significant reduction of SERCA1 mRNA and SERCA2a mRNA, respectively, in patient cell-derived cellular models [36, 37]. Haslett et al. and Nieves-Rodriguez et al. found significant reduction of SERCA2a mRNA in the quadriceps of 5 to 7-year-old patients and the tibialis anterior muscle of 2 to 7-year-old patients, respectively [38, 39]. In summary, our ddPCR results from the canine

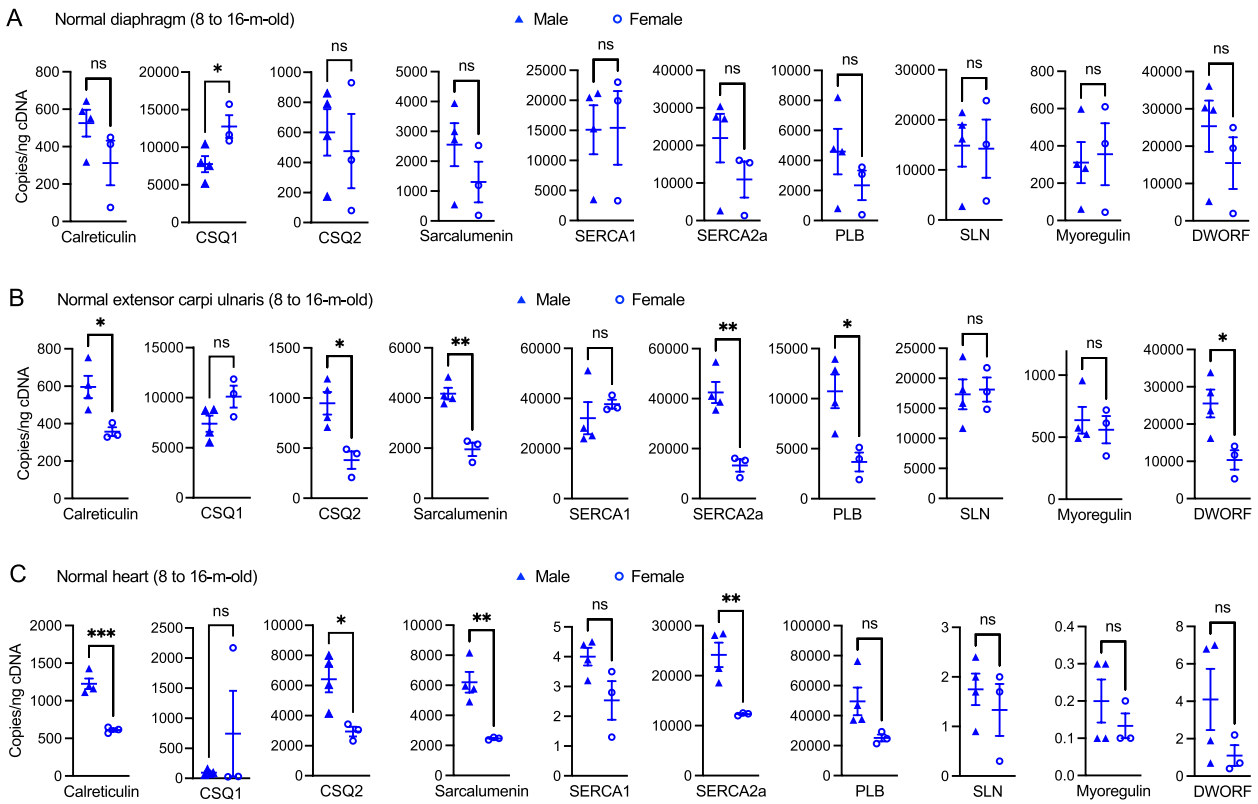

Fig. 5 Quantification of the cDNA copy number in genes encoding SR calcium-binding protein, SERCAs, and SERCA regulators in 8 to 16-m-old normal dogs. **A** Diaphragm. **B** Extensor carpi ulnaris. **C** Heart. CSQ1, calsequestrin 1; CSQ2, calsequestrin 2; DWORF, dwarf open reading frame; ns, not statistically significant; PLB, phospholamban; SERCA, sarcoendoplasmic reticulum calcium ATPase; SLN, sarcolipin; SR, sarcoplasmic reticulum. *, $p < 0.05$; **, $P < 0.01$; ***, $p < 0.001$

model and findings from transcriptomic studies in rodent models, patient cells, and patient muscles suggest that transcriptional downregulation of SERCA and DWORF contributes to calcium mishandling in DMD skeletal muscle.

At the terminal stage of the disease, the most consistent finding is the upregulation of genes encoding SR calcium-binding proteins calreticulin, calsequestrin-2, and sarcalumenin. Statistical significance was reached for calsequestrin-2 in the diaphragm and for calreticulin and sarcalumenin in the ECU muscle (Fig. 4). Increased calcium binding in the SR would reduce free calcium for cytosolic release. Consequently, this would help to reduce the cytosolic calcium level. We speculate that the transcriptional changes in SR calcium-binding proteins may reflect a compensatory mechanism in the advanced stage of DMD. Future studies are needed to determine whether this is unique to the canine DMD model or a common finding in rodent models and human patients.

Given sex is a biological variable, we compared male and female dogs (Figs. 5 and 6). Most transcriptional differences were observed in the heart. Female hearts exhibited significantly reduced transcript levels of calreticulin, calsequestrin 2, sarcalumenin, and SERCA2a, regardless of whether the canines were normal or affected (Figs. 5C and 6C). These findings suggest major differences between male and female calcium regulation in the heart. Statistically significant differences between male and female dogs were also detected in the ECU muscle of normal dogs. Specifically, the female ECU muscle exhibited significantly reduced transcript levels of calreticulin, calsequestrin 2, sarcalumenin, SERCA2a, PLB, and DWORF (Fig. 5B). Interestingly, except for SERCA2a, these differences were lost in the affected ECU muscle, suggesting muscle disease may differentially impact transcription in male and female dogs (Fig. 6B). Except for the upregulation of calsequestrin 1 in normal female dogs, nominal transcriptional differences were found in the diaphragm (Figs. 5A and 6A). This is unexpected given sex is an important regulator of gene expression in mammalian organ development and evolution [40]. It would be interesting to examine gene expression in other cellular processes in the diaphragm of male and female dogs to determine whether only calcium-handling proteins are the exceptions to sex-biased expression.

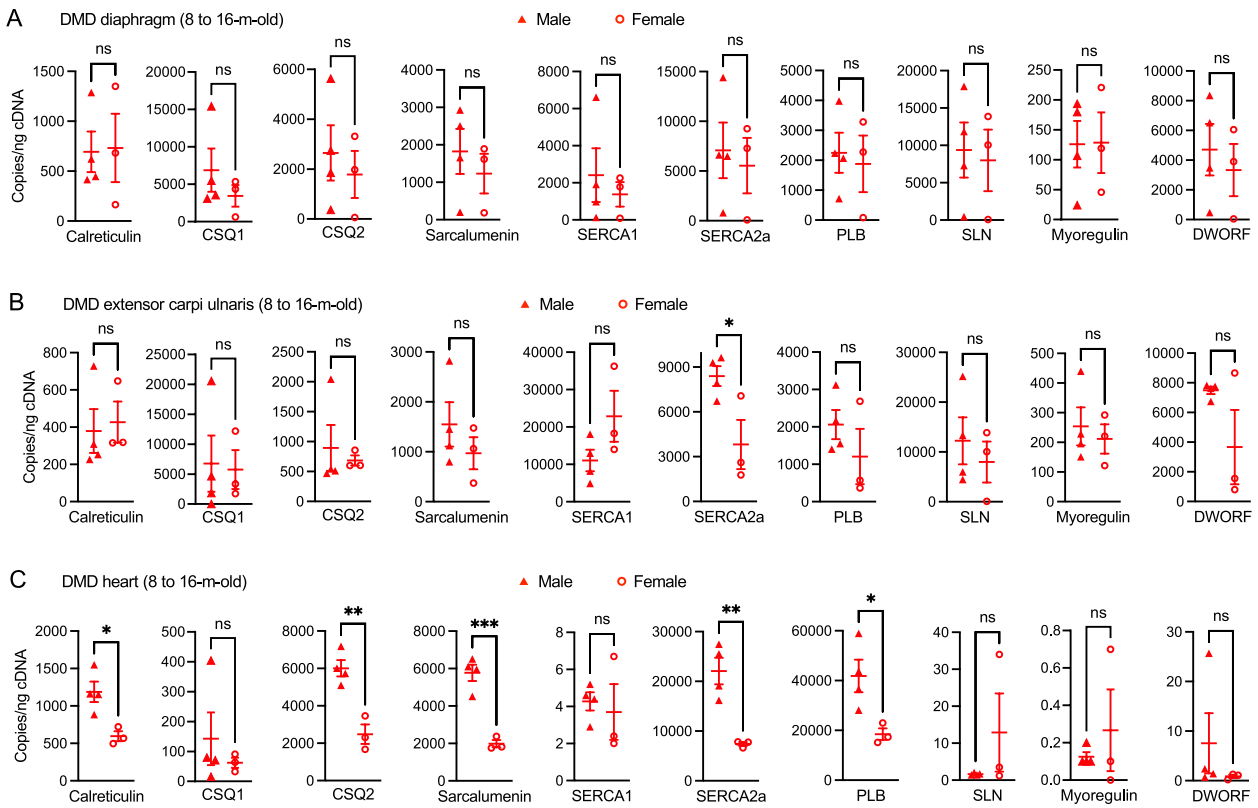

Fig. 6 Quantification of the cDNA copy number in genes encoding SR calcium-binding protein, SERCAs, and SERCA regulators in 8 to 16-m-old affected dogs. **A** Diaphragm. **B** Extensor carpi ulnaris. **C** Heart. CSQ1, calsequestrin 1; CSQ2, calsequestrin 2; DWORF, dwarf open reading frame; ns, not statistically significant; PLB, phospholamban; SERCA, sarcoendoplasmic reticulum calcium ATPase; SLN, sarcolipin; SR, sarcoplasmic reticulum. *, $p < 0.05$; **, $P < 0.01$; ***, $p < 0.001$

Many proteins involved in SR calcium binding and uptake show fiber type-specific expression. For example, SERCA1 and calsequestrin 1 are mainly expressed in type II fibers, while SERCA2a, calsequestrin 2, and DWORF are highly expressed in type I fibers [3, 10, 32, 41, 42]. In the diaphragm of 8 to 16-m-old affected dogs, there were significantly more type I fibers (Fig. 1A) [29]. However, in transcript analysis, not only the SERCA1 level was significantly reduced, but the DWORF level was also significantly reduced (Fig. 3A). In the ECU muscle of 8 to 16-m-old affected dogs, we detected significant downregulation of sarcalumenin, SERCA1, SERCA2a, PLB, myoregulin, and DWORF (Fig. 3B). However, there were no significant changes in the fiber type composition (Fig. 1A). Collectively, our data suggest that changes in the transcription level of calcium-handling proteins did not directly correlate with fiber type composition in the diaphragm and ECU muscle of the canine DMD model.

Conclusions

Calcium mishandling is an important pathogenic mechanism and therapeutic target in DMD. Most of the knowledge on calcium handling comes from studies conducted in mice. Given the importance of the canine model in translational studies, we quantified transcriptional changes of genes encoding SR calcium-binding proteins, SERCAs, and SERCA regulators in normal and dystrophin-deficient dogs at different stages of the disease. Consistent with transcriptomic studies performed in rodents and patients, we found mRNA expression represents an important mechanism in regulating cytosolic calcium levels in skeletal (but not cardiac) muscle of the canine DMD model. We also compared expression in male and female dogs and found sex-biased downregulation in female dogs. We further showed no direct correlation between fiber type composition and mRNA level changes. These findings expand knowledge on calcium regulation in canine muscles and muscular dystrophy.

Abbreviations

CSQ1	Calsequestrin 1
CSQ2	Calsequestrin 2
ddPCR	Droplet digital PCR
DMD	Duchenne muscular dystrophy
DWORF	Dwarf open reading frame
ECU	Extensor carpi ulnaris
PLB	Phospholamban
SERCA	Sarcoendoplasmic reticulum calcium ATPase
SLN	Sarcolipin
SR	Sarcoplasmic reticulum

Authors' contributions

Design the study (EDM, DW, MJB, and DD), data acquisition (EDM, DW, MJB, JH, DDD, and KZ), data analysis (EDM, DW, MJB, and DD), data interpretation (EDM, DW, MJB, and DD), funding acquisition (DD), supervision (DW, MJB, and DD), writing and editing (EDM, DW, MJB, and DD).

Funding

This study was supported by grants from the National Institutes of Health (AR70517, to DD), Department of Defense (MD210064, to DD), The Jesse Davidson Foundation-Defeat Duchenne Canada (to DD), Jackson Freel DMD Research Fund (to DD), the University of Missouri Life Science Fellowship (to EDM), and the University of Missouri Research Excellence Program (to DW).

Data availability

The dataset analyzed in the current study is available from the corresponding author upon reasonable request.

Declarations

Ethics approval and consent to participate

This study used curated tissues previously collected at the University of Missouri. Tissue collection was approved by the Animal Care and Use Committee of the University of Missouri. No animals were euthanized for the purpose of this study. All the study protocols were followed according to ARRIVE guidelines.

Consent for publication

Not applicable.

Competing interests

DD is a member of the scientific advisory board for Solid Biosciences and an equity holder of Solid Biosciences, a member of the scientific advisory board for Sardocor Corp, and an inventor of several issued and filed patents on DMD gene therapy and AAV vectors. All other authors have nothing to disclose.

Author details

[1]Department of Molecular Microbiology and Immunology, School of Medicine, The University of Missouri, Columbia, MO 65212, USA. [2]Department of Neurology, School of Medicine, The University of Missouri, Columbia, MO 65212, USA. [3]Department of Chemical and Biomedical Engineering, College of Engineering, The University of Missouri, Columbia, MO 65212, USA. [4]Department of Biomedical Sciences, College of Veterinary Medicine, The University of Missouri, Columbia, MO 65212, USA.

Received: 12 April 2024 Accepted: 7 October 2024
Published online: 14 October 2024

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