

Therapeutic Potential of SAT-3247 in a Canine Model of Duchenne Muscular Dystrophy

Shanti Rayagiri

Ottawa Hospital Research Institute

Johnathan Smid

Ottawa Hospital Research Institute

Phil Lambert

Satellos Bioscience

James Jaquith

Satellos Bioscience

Tessa Murray

Satellos Bioscience

Ryan Mitchell

Satellos Bioscience

Frank Gleeson

Satellos Bioscience

Jacob Monast

Ottawa Hospital Research Institute

James Teixeira

University of Missouri

Yongping Yue

University of Missouri

Matthew Burke

University of Missouri

Gang Yao

University of Missouri

Dongsheng Duan

University of Missouri

Michael Rudnicki

`mrudnicki@ohri.ca`

Ottawa Hospital Research Institute <https://orcid.org/0000-0002-3866-5249>

Keywords: Duchenne muscular dystrophy (DMD), dystrophin, Canine DMD model, SAT-3247, Regenerative Index (RI), Regenerating myofibers, Necrotic myofibers, Tetanic force, Torque Force, Cardiac function

Posted Date: October 5th, 2026

DOI: <https://doi.org/10.21203/rs.3.rs-11220671/v1>

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Additional Declarations: The authors declare potential competing interests as follows: MAR is the Founding Scientist and Chief Discovery Officer of Satellos Bioscience and receives consulting remuneration and holds stocks and options. SSSR, JKS, PL, JJ, TM, RM and FG are employees of Satellos Bioscience and hold stock and/or options. The remaining authors declare no conflict of interest.

Abstract

Background

Duchenne muscular dystrophy (DMD) is an X-linked recessive disorder caused by the loss of functional dystrophin protein, resulting in progressive muscle degeneration in skeletal muscle due to myofiber fragility and muscle stem cell dysfunction. Satellos Biosciences developed an orally available small molecule SAT-3247 to promote repair and regeneration of injured skeletal muscle. SAT-3247 inhibits the kinase AAK1 to increase membrane localization of NUMB, resulting in enhanced polarity, increased numbers of asymmetric divisions, and increased generation of progenitors. Therefore, a pilot study was conducted with a mixed breed DMD canine model to explore the potential for SAT-3247 treatment to impact repair and regeneration of injured skeletal muscle.

Case presentation:

Two 9-month-old DMD dogs were treated for a period of 18 weeks, with oral delivery of SAT-3247 for 4 consecutive days per week (i.e., 3 days off) at 10 mg/kg dose. SAT-3247 treatment resulted in improved muscle morphology, decreased infiltration of inflammatory cells, increased regenerative index (measuring the ratio of regenerating to necrotic cells) in multiple muscles, improvement in locomotory parameters in both forelimb and hindlimbs including greater force generation, and stable cardiac function.

Conclusions

Overall, this proof-of-concept pilot study in a canine model highlights the therapeutic potential of SAT-3247 to repair and regenerate injured muscle and serve as a treatment for DMD. Together, these data provide a compelling preclinical groundwork for advancing SAT-3247 towards clinical evaluation in DMD patients.

Background

Duchenne muscular dystrophy (DMD) is an X-linked degenerative muscle disease with a prevalence of 4.8 in 100,000 males worldwide (1, 2). The DMD gene is the largest known human gene with 79 exons spanning 2.2 Mb (3). Mutations in the DMD gene result in loss-of-function mutations in dystrophin. Clinical delays in motor-milestones in DMD patients become notable between 2 to 5 years of age. Disease progression occurs rapidly, resulting in many patients becoming wheelchair-bound by 12 years of age. Typically, respiratory failure due to skeletal muscle weakness together with heart failure result in death in the second or third decade of life (4).

Dystrophin is a component of the large oligomeric dystrophin-glycoprotein complex (DGC) that spans the sarcolemma of myofibers in skeletal and cardiac muscle (5). The DGC acts to connect the actin/microtubule cytoskeleton of the myofiber to the surrounding extracellular matrix. Lack of functional dystrophin impairs DGC assembly making muscle prone to injury. In skeletal muscle, constant cycles of degeneration together with impaired regeneration lead to increasing levels of inflammation and fibrosis, progressive muscle weakening and loss of motor function (6).

Dystrophin is highly expressed in activated muscle stem cells where it localizes to the basal cortex and recruits the kinase Mark2, an important regulator of cell polarity. In the absence of dystrophin, cell polarity fails to be properly established, a component of the PAR-complex called Pard3 is no longer localized to the apical cortex, and the number of asymmetric divisions is significantly reduced. Many of these cells develop abnormal numbers of centrosomes, leading to elimination by programmed cell senescence (7, 8). Moreover, the loss of polarity establishment in dystrophin-deficient muscle stem cells results in altered epigenetic control of gene expression due to reduced nuclear translocation of the arginine methyltransferase Carm1(9). Consequently, the reduced generation of progenitors together with high levels of senescent cells results in impaired regeneration in adult skeletal muscle (10, 11) and impaired secondary myogenesis during fetal development (11) in *mdx* mice.

The current standard of care, corticosteroids, along with gene-targeting and exon skipping therapies, and other small molecule approaches for DMD may prolong ambulation and improve survival into adulthood. However, the disease remains relentlessly progressive, and most continue to experience life-limiting respiratory and/or cardiac complications preventing a normal lifespan (12, 13). Taken together, there is an urgent need for more effective and widely accessible treatments for DMD.

Satellos Bioscience developed SAT-3247, an orally available small molecule drug intended to treat DMD by promoting repair and regeneration of injured skeletal muscle. SAT-3247 restores polarity of satellite cells by inhibiting AAK1, a kinase that phosphorylates NUMB, resulting in its removal from the membrane. Notably, AAK1 deletion restores NUMB polarization and asymmetric division of DMD muscle stem cells (11). The therapeutic efficacy and safety profile of SAT-3247 are currently under Phase 2 clinical investigation in both pediatric (14) and adult cohorts of DMD patients (15). A phase 1 study (16) evaluated the safety and tolerability of SAT-3247 in healthy volunteers and adult DMD participants. In addition, over the 28-day evaluation period, DMD adult participants showed encouraging improvements in muscle strength. While active phase 2 clinical trials are now underway to assess the therapeutic efficacy of SAT-3247, this article will present the preclinical data in an outbred DMD canine model (17) where SAT-3247 was administered for a period of 18 weeks. Multiple parameters were evaluated including body weights, histological endpoints, motor and cardiac function functional assessments, and clinical pathology parameters.

Case presentation

Dog history

To better reflect the phenotypic heterogeneity of human DMD, the mixed breed canine DMD model comprising golden retriever, Labrador retriever, beagle, spaniel, and Welsh corgi breeds was used. Affected dogs carry the LRMD mutation in DMD, an intronic insertion in intron 19 (17,18). The DMD dogs exhibit a progressive and ultimately fatal course of disease (19). The course of the disease closely mimics human patients, showing variable fiber size, fatty and macrophage infiltration, central nucleation and fibrosis. Two 9-month-old DMD dogs (*Study DMD-1* and *Study DMD-2*) were used in this pilot study (Table 1). DMD dogs were dosed with SAT-3247 (10 mg/kg PO) for 18 weeks with 4 days on and 3 days off schedule. Schematic representation of the dosing schedule and sample collection is shown in Figure 1. In parallel, age-matched normal and dystrophic dogs/samples were evaluated where possible. Researchers were blinded to treatment status for histological analysis.

Table 1: Details of DMD dogs

Strain/Breed	Mixed breed DMD canine model with intronic insertion in intron 19 of DMD
<i>Study DMD-1</i> DOB	23 Feb 2023
<i>Study DMD-2</i> DOB	23 Feb 2023
Age at Initiation of Dosing	Approximately 9 months old
Body Weight at Initiation of Dosing	9.07 kg male - <i>Study DMD-1</i> 13.61 kg female - <i>Study DMD-2</i>
Initiation of Dosing	14 Nov 2023
Completion of In-life	19 Mar 2024

Body weights

Individual body weights of *Study DMD-1* and *Study DMD-2* were measured one week prior to dose initiation, then weekly during the treatment period to assess treatment-related body weight effects. Additionally, as part of the routine schedule at the testing site, body weights were collected for both dogs starting from the age of 6.2 months (Supplemental Table 1).

Study DMD-1 weighed 9.07 kg and *Study DMD-2* weighed 12.7 kg at 6.2 months of age. Before dosing, at 8.9 months of age *Study DMD-1* was 9.07 kg and *Study DMD-2* was 13.38 kg. From the age of 8.9 months to the completion of dosing at 13.0 months of age there was no loss of body weight indicating that SAT-3247 did not have any negative effects on the overall body weight of the experimental dogs tested. Conversely, both dogs had a 1.2-fold increase in body weight from dose initiation through to the end of the study at 13 months of age.

Histological analyses

All the muscle samples were received either in the form of OCT block or slides with muscle sectioned (10 mm thick). The muscles that were received as tissue blocks were sectioned on the cryostat (10 mm). For *biceps femoris* muscles, biopsies were collected immediately pre-treatment for *Study DMD-1* and *Study DMD-2* dogs. At necropsy, following 4-months of treatment with SAT-3247, tissue samples were collected from the *biceps femoris*, *diaphragm*, *vastus lateralis*, *gastrocnemius lateral* and *gastrocnemius medial* muscles of the DMD dogs. Tissue samples were also collected from untreated DMD dogs as a concurrent control. Pre-treatment and post-treatment *biceps femoris* samples, and untreated and treated *diaphragm*, *vastus lateralis*, *gastrocnemius lateral* and *gastrocnemius medial* samples were tested by immunofluorescence. For pre-treatment biopsy samples (start), five independent needle biopsies were performed. Biopsy and necropsy samples were snap-frozen in OCT embedding media by submersion in liquid nitrogen-cooled 2-methylbutane. Three biopsies were sectioned (10 mM) whereas necropsy samples were sectioned (10 mM) at three levels separated by a minimum of 100 mM thus providing three technical replicates. Muscle sections were incubated with primary antibodies Rabbit Anti-Laminin (Sigma L9393, Dilution -1:1000), Goat anti-dog IgG (Abcam AB112849, Dilution -1:200) and Mouse anti-eMHC IgG1 mAb (Santa Cruz sc-53091, Dilution -1:200) for 1 hr at RT followed by washes with PBS thrice, and incubated with species specific secondary antibodies for 30 min at RT. After incubation slides were washed thrice with PBS before adding DAPI for 5 min to visualize the nuclei. All slides were scanned on a Zeiss Axio Observer Z1/7 microscope. Image sizes were generally restricted to 7 x 7 fields of view, resulting in 49 tiles stitched together into a single large image for quantification.

Following the laminin trace as described in our previous study (20) minimum Feret's diameters of myofibers were determined. Healthy dogs exhibit a uniform, resting architecture with a mean minimum feret of 26.8 mm. Untreated DMD dogs exhibit enlarged myofibers with a mean of minimum feret of 35 mm (Figure 2). Accordingly, prior to the start of SAT-3247 treatment at 9 months of age *Study DMD-1* and *Study DMD-2* exhibited an elevated mean minimum feret of more than 30 mm. SAT-3247 treatment mitigated this structural expansion, yielding respective reductions of 15% and 9% in mean minimum feret in *Study DMD-1* (blue) and *Study DMD-2* (red) after treatment (Figure 2), as well as reductions compared to untreated DMD controls.

Regenerative Index

Muscle regeneration was assessed using the previously described Regenerative Index (RI), a method which determines the ratio of regenerative to necrotic activity as calculated by measuring both the number of embryonic myosin heavy chain (eMHC)-expressing regenerating myofibers and the number of immunoglobulin G (IgG)-infiltrated necrotic myofibers, and then dividing the former by the latter (21). The components of the RI were measured using Laminin, IgG and eMHC staining in *biceps femoris* (BF) muscles. No signal was observed in secondary only control (Supplemental Figure 1). To evaluate regenerative activity, newly formed myofibers were identified by immunostaining for expression of embryonic myosin heavy chain (eMHC). Necrotic myofibers were enumerated by immunofluorescent

detection of immunoglobulin G (IgG) infiltration (Figure 2a and Supplemental Figure 2). The RI was then calculated as the ratio of regenerating (eMHC⁺) myofibers to necrotic (IgG⁺) myofibers.

Strikingly, following 4 months of SAT-3247 treatment of *Study DMD-1* and *Study DMD-2*, RIs were markedly elevated with values of 95.98 and 158.64, respectively, compared to 13.23 and 9.03 RI at the start of the study (Figure 2b). Untreated DMD dogs (n = 4) showed a mean RI of 6.64. In healthy dogs, no eMHC fibers were observed (as expected given their skeletal muscles were not injured) with only 0.03% of fibers being IgG positive (also as expected for the same reason); thus RI is negligible (Supplemental Figure 3).

When compared to age-matched untreated DMD dogs, other muscles also exhibited a similarly increased RIs following SAT-3247 treatment, including the *diaphragm* and the *vastus lateralis* (Figure 3a). The RIs of the *diaphragm* of treated *Study DMD-1* and *Study DMD-2* were both higher with values of 13.34 and 19.42, respectively, compared to a mean of 5.85 in untreated DMD dogs (n=3) (Figure 3b). The RIs of the *vastus lateralis* of treated *Study DMD-1* and *Study DMD-2* were higher with a value of 64.79 and 28.84, respectively compared to 13.39 RI in untreated DMD dogs (n=3) (Figure 3b). The RIs of the *gastrocnemius lateral* of treated *Study DMD-1* was higher at 22.93 and was 5.60 for *Study DMD-2* compared to a mean RI of 4.56 RI in untreated DMD (n=3) (Figure 3b). The RIs of the *gastrocnemius medial* of treated *Study DMD-1* and *Study DMD-2* were 6.65 and 20.07, respectively. Untreated DMD dogs have shown variable results for the *gastrocnemius medial* with a mean of 16.49 (n=3) (Figure 3b). Variable RI in untreated DMD may occur because the *gastrocnemius* group of muscles consistently reveal severe structural degeneration due to extreme compensatory load on ankle extensor (22).

Interestingly, in the *diaphragm* the proportion of myofibers exhibiting IgG infiltration is higher with 4.40% of total fibers IgG⁺ in untreated dogs; these levels declined in the SAT-3247 treated dogs to 1% and 0.65% of myofibers in the *diaphragm* being IgG⁺. The proportion of eMHC⁺ myofibers were variable in the *diaphragm* of untreated DMD dogs with a mean of 25.93%. By contrast, 13.34% and 19.42% of myofibers were eMHC⁺ in post-treatment *Study DMD-1* and *Study DMD-2 diaphragms*, respectively. This suggests that the increase in RI is primarily due to a reduction in necrotic myofibers.

Inflammatory response:

To assess the baseline inflammatory response, CD45, a pan-leukocyte marker staining was performed on *biceps femoris* (BF) muscles using Rat anti-dog CD45 (Biorad YKIX716.13, Dilution 1:100) along with Rabbit Anti-Laminin (Sigma L9393, Dilution -1:1000). Slides were incubated for 1 h followed by washes and incubation with secondary antibodies. No signal was observed in secondary only control (Supplemental Figure 4). Strikingly, following SAT-3247 treatment of *Study DMD-1* and *Study DMD-2*, the number of CD45⁺ cells exhibited an approximate 4-fold and 2.5-fold drop, respectively (Figure 4a and 4b). The pre-treatment number of CD45⁺ cells in *Study DMD-1* and *Study DMD-2* were 91 and 72 cells per mm², respectively, whereas post-treatment were 23 and 29 cells per mm², respectively. Untreated DMD dogs showed a mean of 61.5 (n = 4 dogs). In healthy dogs, only 4 CD45⁺ cells per mm² were observed

(Figure 4b). This is in line with the literature which indicates that less than 1-2% CD45⁺ cells are observed in healthy dogs, increasing 10-15% in DMD (23).

Whole limb function:

The contractile force of the flexor and extensor muscle groups was assessed as previously described (24). Twitch and Tetanic torque were measured for both the forelimbs and hindlimbs of *Study DMD-1* and *Study DMD-2* at the start of the study at 9 months of age (baseline) and after 4 months of treatment at 13 months of age, and expressed as N-cm/kg (Newton-centimeters per kilogram) to measure turning effect (torque) produced by a force applied at a distance and normalized to body weight, whereas the corresponding contraction force was expressed as N/kg (Newtons per kilogram) to represent force normalized to body weight (Figure 5). Prior to treatment, *Study DMD-1* and *Study DMD-2* exhibited reduced twitch and tetanic forces for forelimb flexion, hindlimb extension and hindlimb flexion, which is typical of DMD dogs (25). Following treatment with SAT-3247, *Study DMD-1* and *Study DMD-2* exhibited substantial increase of 51% and 39.3% respectively, in forelimb flexion tetanic force (Supplemental Figure 5a). Consistently, a 57.5% and 39.9% increase in torque was also observed for *Study DMD-1* and *Study DMD-2*, respectively. Forelimb flexion twitch force exhibited a slight increase in *Study DMD-1* and greater increase of 41.7% in *Study DMD-2* (Supplemental Figure 5a).

Following treatment with SAT-3247 between 9 months and 13 months of age, hindlimb flexion tetanic torque also increased substantially in *Study DMD-1* and *Study DMD-2* from 2.89 to 16.05 N-cm/kg (+455%) and from 3.06 to 13.99 N-cm/kg (+357%), respectively (Figure 5). Similarly, hindlimb flexion tetanic force also increased from 0.21 to 1.19 N/kg (+467%) and from 0.20 to 0.93 N/kg (+365%) (Supplemental Figure 5b). Hindlimb extension of *Study DMD-1* showed marked increases in normalized twitch force (0.57 to 1.05 N/kg, +84%) and tetanic torque (41.25 to 60.76 N-cm/kg, +47%) between 9 and 13 months of age, indicating preserved or improving muscle function (Figure 5). *Study DMD-2* demonstrated stable twitch force (0.39 to 0.37 N/kg) and modest increases in tetanic parameters, suggesting partial maintenance of strength, perhaps due to sex differences.

Spatiotemporal gait analysis

To evaluate functional locomotion, spatiotemporal parameters of the canine gait were assessed focusing on stride length and stride velocity. From baseline to 9.83 months of age, velocity was increased from 108.4 cm/s to 125.0 cm/s in *Study DMD-1* and from 93.5 cm/s to 136 cm/s in *Study DMD-2*. Velocity was maintained above 100 cm/s thereafter with a slight drop for *Study DMD-2* at 10.79 months. At the end of the study at 13 months of age, average velocity of *Study DMD-1* was 120 ±13 cm/s and *Study DMD-2* was 112 ±14 cm/s (Figure 5b). Stride length was measured for both *Study DMD-1* and *Study DMD-2* from 6.2 to 12.8 months of age. Following treatment with SAT-3247, there was an increase of stride length of at least 7 cm in *Study DMD-1* and 13 cm in *Study DMD-2*. At the end of the treatment period at 13 months of age *Study DMD-1* had an average stride length of 74 ± 5.7 cm and *Study DMD-2* had an average stride length of 77 ± 5.9 cm. Both *Study DMD-1* and *Study DMD-2* displayed highly

consistent stride lengths, with less than 0.5% bilateral variance across both forelimb and hindlimb pairs (Supplemental Figure 5c).

Cardiac function

To assess the heart function, an echocardiogram was performed for both *Study DMD-1* and *Study DMD-2* upon completion of treatment. All of the data collected and analyzed is based on the standard protocols documented in (24). Left ventricular ejection fraction (LVEF), which is the percentage of blood pumped out of the left ventricle with each heartbeat, was reported as 57.2% and 63.2% for *Study DMD-1* and *Study DMD-2*, respectively (Supplemental Table 2). Fractional shortening (FS), a measure of left ventricular systolic function, was found to be 37.70% and 30.63% in *Study DMD-1* and *Study DMD-2*, respectively. As reported in the literature, DMD dogs at 13 months of age, typically exhibit an LVEF of 43.3 ± 3.7 and a FS of $24.7 \pm 0.9\%$ (26). Heart rate was 120 bpm for *study DMD-1* and 160 bpm for *Study DMD-2*. Other parameters such as P wave and PR interval, QRS complex, QT interval and ST segment values were within the range of a normal dog with no signs of prolonged conduction interval (P-R, QRS, QT) changes that are usually detectable in DMD dogs at this age (27).

Serological analysis

Clinical Pathology (hematology and clinical chemistry) procedures were undertaken at the University of Missouri Veterinary Diagnostic Laboratory. During the period of the study, there were no alterations in platelet count, liver function (GLDH), and kidney function (BUN) (Supplementary Figure 6a). Other hematology and blood biochemistry biomarkers were also unremarkable. Creatine kinase (CK) levels were slightly reduced at 13 months following SAT-3247 treatment (Supplemental Figure 6b). However, CK remained elevated (<9000 U/L) compared to normal controls (<200 U/L), indicating ongoing muscle damage (28).

Serum cytokines were quantified by multiplex Luminex cytokine detection assay according to our published protocol (29). The concentration of various cytokines, including GM-CSF, IFN- γ , IL-2, IL-6, IL-7, IL-8, IL-15, IP-10, KC-Like, IL-10, IL-18 and MCP-1 (CCL2) were determined to assess SAT-3247 activity on immunomodulation in *Study DMD-1* and *Study DMD-2*. Following treatment of *Study DMD-1* with SAT-3247, a marked immunomodulatory response was observed, characterized by substantial increases in the anti-inflammatory cytokine IL-10 (15-fold) alongside significant reductions in pro-inflammatory mediators such as GM-CSF, IL-2, IL-6, IL-7, and IL-18 (Supplemental Figure 7a and 7b), with IFN- γ remaining elevated. However, in *Study DMD-2*, cytokine levels largely trended toward baseline, with IFN- γ remaining elevated but less dramatically, and no change in IL-10. Additionally, pro-inflammatory cytokine IL-18, showed consistent suppression in both dogs with a fold change of 0.29 and 0.48 in *Study DMD-1* and *Study DMD-2*, respectively. CCL2 levels before dosing were 463.34 pg/mL and 1,289.43 pg/mL in *Study DMD-1* and *Study DMD-2*, respectively. At the end of the study, the CCL2 levels were 471.81 pg/mL and 637.76 pg/mL in *Study DMD-1* and *Study DMD-2*, respectively. The CCL2 level did not change appreciably in *Study DMD-1* but decreased in *Study DMD-2*.

Discussion

Prolonged SAT-3247 treatment in DMD dogs appears safe, well-tolerated, and the phenotypic analysis is suggestive of therapeutic activity. Both DMD dogs dosed with SAT-3247 for 18 weeks exhibited substantial histological and functional amelioration, as evidenced by increased RI and increased muscle force generation. These findings in the treated DMD dogs are in contrast with the typical muscle and force generation observed in DMD-affected models at a similar age, where active regeneration markers decline with progressive and extensive fibrotic remodeling, and a marked decline in force generation (30,31). Morphometric assessment of myofibers of the *biceps femoris* muscle revealed a downward shift in minimum feret of myofibers in SAT-3247 treated DMD dogs suggesting a normalization of the muscle tissue due to potential therapeutic efficacy of the intervention.

The Regenerative Index (RI) was developed as a method to assess regeneration potential specifically for DMD (21). The RI is defined as the ratio of newly formed (eMHC⁺) myofibers to necrotic (IgG⁺) myofibers, thus normalizing the variation found between samples and between sections. Accordingly, the RI integrates both regenerative and degenerative states to assess the net regenerative potential of muscle. Importantly, RI was found to significantly decline by about 2.5-fold in paediatric DMD patients between 7 and 11 years of age (21). Notably, after treatment with SAT-3247 a substantial increase was observed in the RI in *biceps femoris*, *diaphragm*, and *vastus lateralis*. High variability among untreated DMD dogs of the *gastrocnemius lateral* and *gastrocnemius medial* muscle makes comparisons problematic. Overall, in untreated 13-month-old DMD dogs, the average RI value was approximately 6.6. Notably, following 18-weeks of SAT-3247 treatment in DMD dogs, RIs were 95.98 and 158.64 for the two treated animals with increases between 7.25-fold to 17.57-fold from baseline to the end of dosing. These data suggest that SAT-3247 is constructively impacting the repair and regeneration of injured skeletal muscle and supports the potential utility of RI determination as a method to assess the regenerative potential of candidate therapies.

Importantly, muscle force and torque of SAT-3247-treated dogs also showed marked increases between 9 and 13 months of age, contrasting with the progressive decline typically observed in DMD dogs. For example, forelimb flexion tetanic force and torque increased substantially by 40-50%. Hindlimb flexion tetanic force and torque showed dramatic increases of >300%. Hindlimb extension twitch/tetanic force and torque increased up to 84%. Thus SAT-3247-treated DMD dogs did not appear to exhibit the declines in force generation typically observed in DMD dogs, and were capable of generating force approaching that of healthy dogs (25,32).

Determination of LVEF and FS represent the primary hallmarks to measure onset and progression of DMD-associated cardiomyopathy. In canine DMD, cardiac function remains stable for the first 6 months; followed by a pronounced decline in LVEF and FS thereafter and the development of dilated cardiomyopathy after 30 months (27,33,34). A decrease in FS below 27% is a critical clinical cutoff below which indicates transition from compensation to systolic dysfunction (33), while healthy dogs maintain an FS of 30-45%. Notably, following SAT-3247 treatment, there was no observed marked decline in

cardiac function. Instead, both LVEF and FS remained within the normal range. These data suggest that SAT-3247 is not detrimental to cardiac function and may even be protective. Further studies will be required to investigate these phenomena and to understand the mechanism of action.

Another hallmark of DMD is chronic, muscle-wide inflammation wherein a massive immune response recruits inflammatory cells and loads the muscle tissue with a distinct profile of cytokines and chemokines (35). In the absence of dystrophin, DGC assembly is impaired which renders muscle fibers susceptible to injury. Constant contraction-induced stress results in continuous cycles of degeneration and regeneration. Subsequent accumulation of inflammation lead to progressive muscle weakening (36). Thus, DMD creates an inflammatory environment and at the same satellite cells are recruited to repair muscle, however, the chronic environment plus intrinsic dystrophin-related defects of satellite cells makes them less effective at producing functional new muscle (37).

Cytokine profiling revealed an increase in anti-inflammatory cytokines IL-10 (15-fold) in *Study DMD-1* coupled with decreases in pro-inflammatory cytokines including GM-CSF, IL-2, IL-6, IL-7, and IL-18 in both *Study DMD-1* and *Study DMD-2*. This suggests a shift toward a more regenerative immune environment, contrasting with the heightened inflammation typically seen in DMD dogs, in alignment with observations made in *mdx* mice and human tissues (38). IL-10 forces transition of M1 to M2 macrophages (39) and is considered to act as a double-edged sword, as at later stages of DMD it may over-activate fibro-adipogenic progenitors (FAPs).

Interestingly, the number of CD45⁺ cells decreased with the treatment. Further studies will be required to better understand the effects long-term treatment with SAT-3247 has on inflammation.

Creatine kinase (CK) levels remained elevated as untreated DMD compared to normal controls, indicating ongoing muscle damage (28), consistent with the expectation that SAT-3247 treatment does not prevent damage. The ongoing muscle-fiber damage initiates a regenerative response, with satellite cells responding to that damage to repair and regenerate muscle. However, this response may not be sufficient to prevent ongoing muscle injury or reduce CK levels, particularly in the context of persistent dystrophin deficiency and chronic inflammation.

The variation in response between *Study DMD-1* and *Study DMD-2* may reflect sex differences. *Study DMD-1* is a male dog whereas *Study DMD-2* is female. Female DMD dogs generally express a milder clinical disease course (40,41). At the molecular level, females show a significant downregulation of calcium-binding transcripts in heart relative to males (42). Nevertheless, this study has shown an increase or improvement in RI, hindlimb function, length of stride, heart fractional shortening with a reduction in inflammation in both the dogs that were tested.

Conclusion

We present a case report of two DMD dogs treated for 18 weeks with SAT-3247, an investigational new drug designed to promote polarity and restore muscle stem cell function by promoting asymmetric cell

divisions, thus stimulating regeneration. Results are consistent with the proposed mechanism of action and support the assertion that SAT-3247 enhances skeletal muscle regeneration. The treated DMD dogs show an overall improvement in force measurements, increased RI, improved muscle architecture, and possess a cytokine profile consistent with decreased inflammation. However, a limitation of this study is that no statistical analysis can be performed due to the small number of dogs available for drug testing together with an absence of a placebo control group. Nevertheless, this proof-of-concept pilot study in a canine DMD model highlights the therapeutic potential of SAT-3247 as a treatment for DMD. Together, these data provide a compelling preclinical groundwork for advancing SAT-3247 towards clinical evaluation in DMD patients.

Abbreviations

AAK-1: Adapter-associated kinase-1; BUN: Blood urea nitrogen; CD45: Cluster of differentiation; DMD: Duchenne Muscular Dystrophy; eMHC: Embryonic Myosin Heavy Chain; FS: Fractional shortening; GLDH: Glutamate dehydrogenase; IgG: Immunoglobulin G; LVEF: Left ventricular ejection fraction; MFF: minimal fiber Feret (minimal Feret's diameter); PBS: phosphate-buffered saline; RI: Regenerative Index; RT: room temperature; DAPI: 4',6-diamidino-2-phenylindole

Declarations

Ethics approval

All animal experiments were conducted at the University of Missouri and approved by the Animal Care and Use Committee of the University of Missouri, as per all relevant ethical regulations for animal testing.

Availability of data and materials

All data needed to evaluate the conclusions in the paper are present in the paper. Any additional information required to reanalyze the data reported in this work paper is available from the lead contact (mrudnicki@ohri.ca).

Competing interests

MAR is the Founding Scientist and Chief Discovery Officer of Satellos Bioscience and receives consulting remuneration and holds stocks and options. SSSR, JKS, PL, JJ, TM, RM and FG are employees of Satellos Bioscience and hold stock and/or options. The remaining authors declare no conflict of interest.

Funding

This study was supported by research contracts from Satellos Bioscience to DD and MAR.

Authors' contributions

SSSR, JKS, PL, RM, TM, GY, DD, FG and MAR made contributions to the conception, data analysis and analyzed data. JJ designed and developed the drug. SSSR wrote the paper. JAT, YY, MJB, GY, JGM performed experiments. MAR, SSSR, PL, RM, TM and JKS designed the experiments and edited the manuscript.

Acknowledgments

The authors thank the University of Ottawa Imaging Core for use of microscopes and training required for scanning of entire muscle biopsy sections. We also thank Jin Han, Keqing Zhang and Charis McPherson for expert technical assistance. We extend our thanks to Dr. Wildon Farewell for careful reading of the manuscript.

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Figures

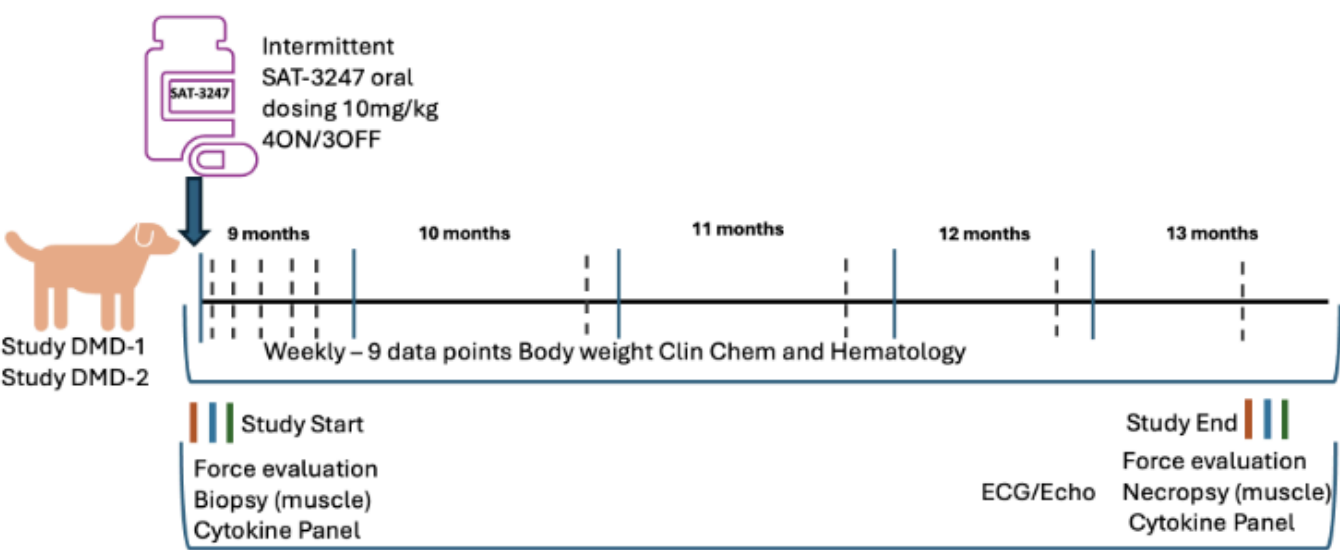


Figure 1

Schematic representation of the dosing schedule, sample collection. SAT-3247 dosing strategy with sample acquisition timelines are depicted. Body weight and diagnostic assessment measurements were taken on weekly basis. Samples for cytokine profiling collected before the start of the study and end of the study. For muscle regeneration assessments, biopsies were collected before the start of the study and necropsies were collected at the end of the study. Muscles collected include *biceps femoris*, *vastus lateralis*, *diaphragm*, *gastrocnemius medial* and *gastrocnemius lateral*. Force measurements were also performed before and after dosing SAT-3247.

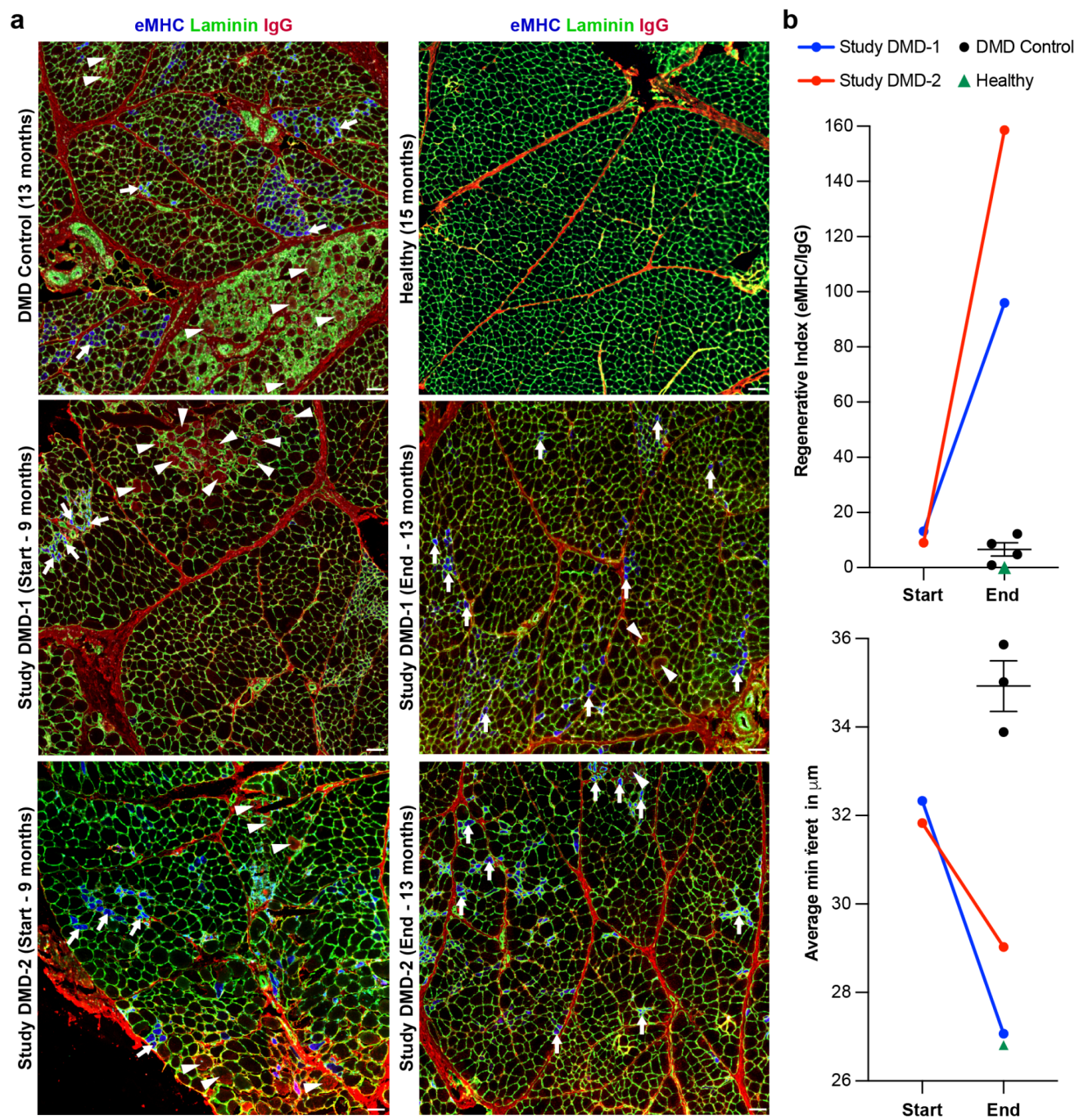


Figure 2

SAT-3247 treatment enhances increased Regenerative Index (RI) and decreased minimal fiber feret. (a) Representative immunofluorescence image of *biceps femoris* muscle sections of DMD (untreated), healthy, *Study DMD-1* and *Study DMD-2* stained for IgG (red, indicated with arrowheads) and embryonic myosin heavy chain (eMHC, blue, indicated with arrows) (b) Graph shows the regenerative index of all the four sample groups. *Study DMD-1* (blue) and *Study DMD-2* (red) are higher at the end of the study compared to pretreatment with a fold change of 6 and 10, respectively, untreated DMD dogs (n=4) healthy dog. Average minimum feret is plotted for all 4 groups. *Study DMD-1*(blue) and *Study DMD-2* (red) show reduction in minimum feret, respectively after treatment with SAT-3247. Scale bar is 100 mm. Error bars represent SEM.

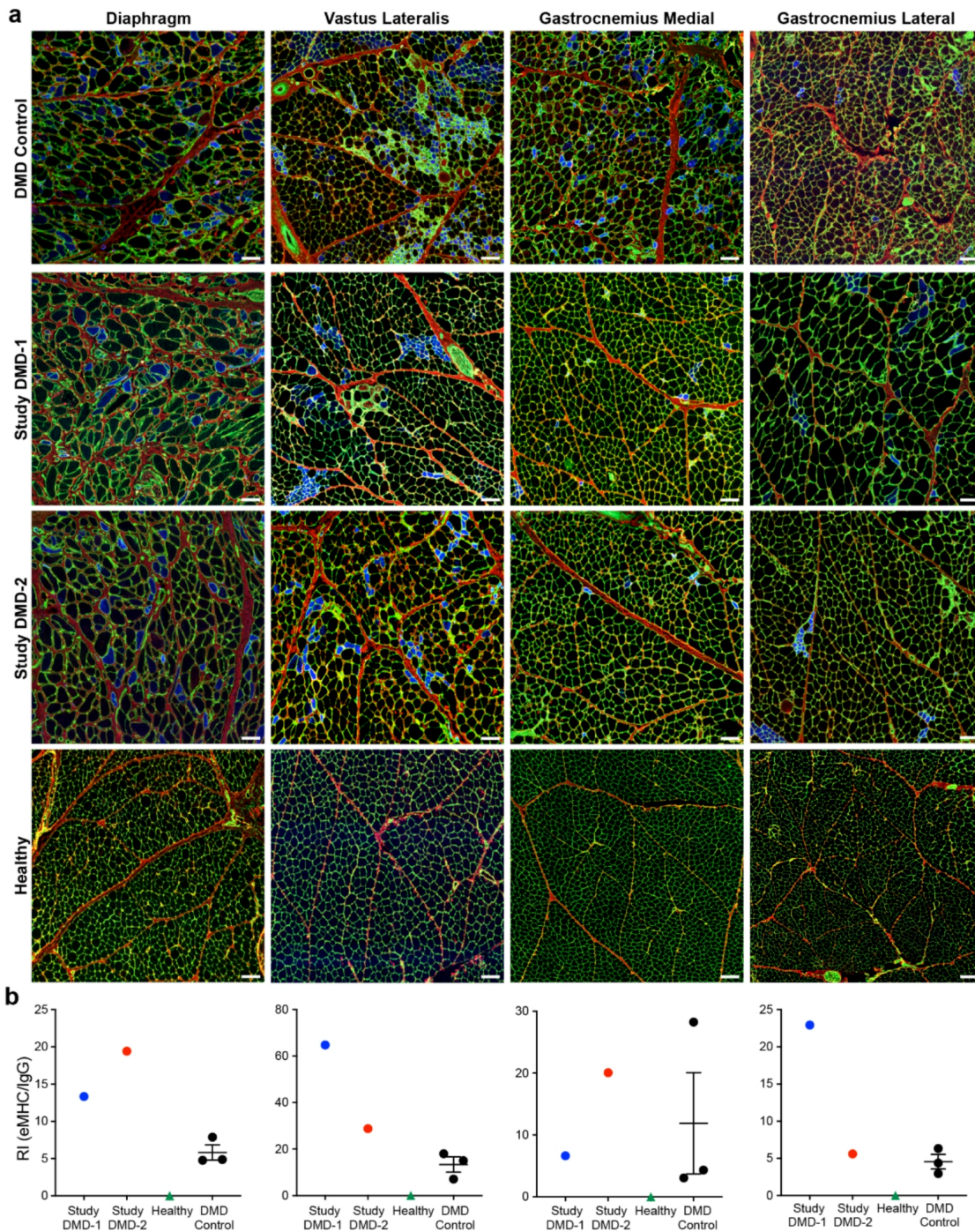


Figure 3

RI measured for four different muscles: *Diaphragm*, *vastus lateralis*, *gastrocnemius lateral* and *gastrocnemius medial*. (a) Representative immunofluorescence image of four different necropsy muscles stained for IgG (red) and embryonic myosin heavy chain (eMHC, blue) and Laminin (green) of DMD (untreated), healthy, *Study DMD-1* and *Study DMD-2* (b) Graphs show the regenerative index of all the four sample groups. In both *diaphragm* and *vastus lateralis* muscles RI in *Study DMD-1* (blue) and

Study DMD-2 (red) is higher compared to untreated DMD controls (n=3) with zero RI for healthy dog. Scale bar is 200 mm. Error bars represent SEM.

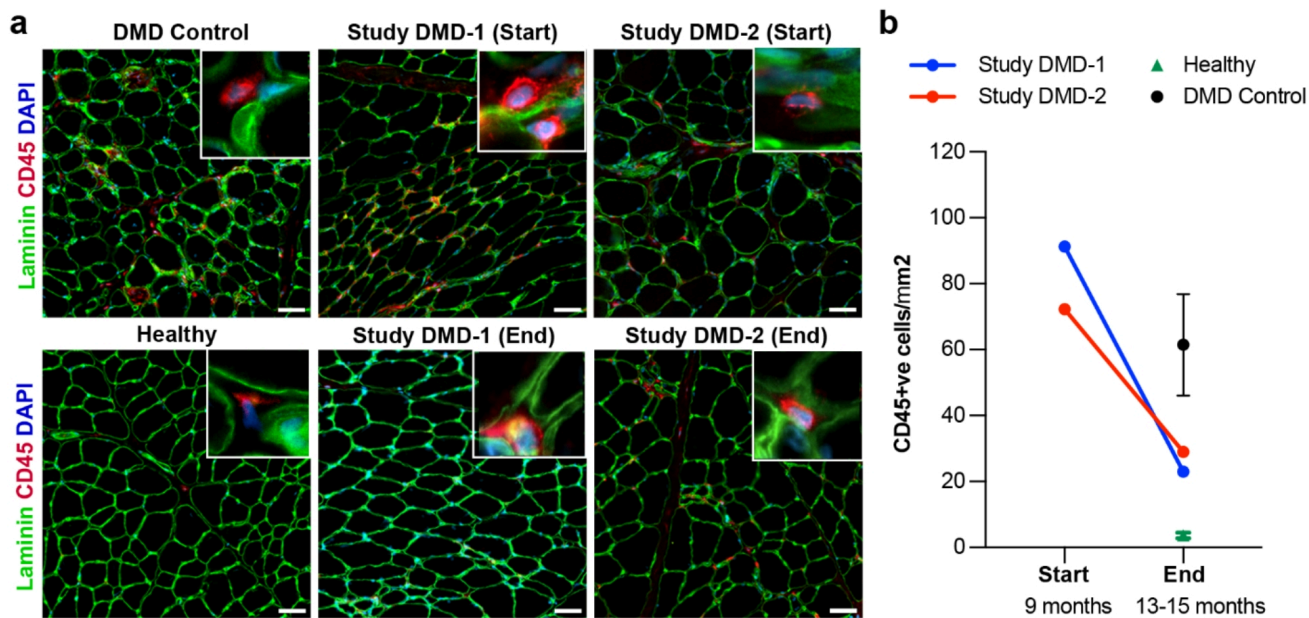


Figure 4

Marked reduction in leukocyte infiltration in SAT-3247 treated canine muscle. (a) Representative immunofluorescent cross-sections of *biceps femoris* muscle stained for CD45 shown in red, Laminin in green and counterstained with DAPI in blue. (b) CD45⁺ cells were markedly reduced with SAT-3247 treatment. Scale bar is 50 mm. Inserts show magnified image of 28 mm x 28 mm. Error bars represent SEM.

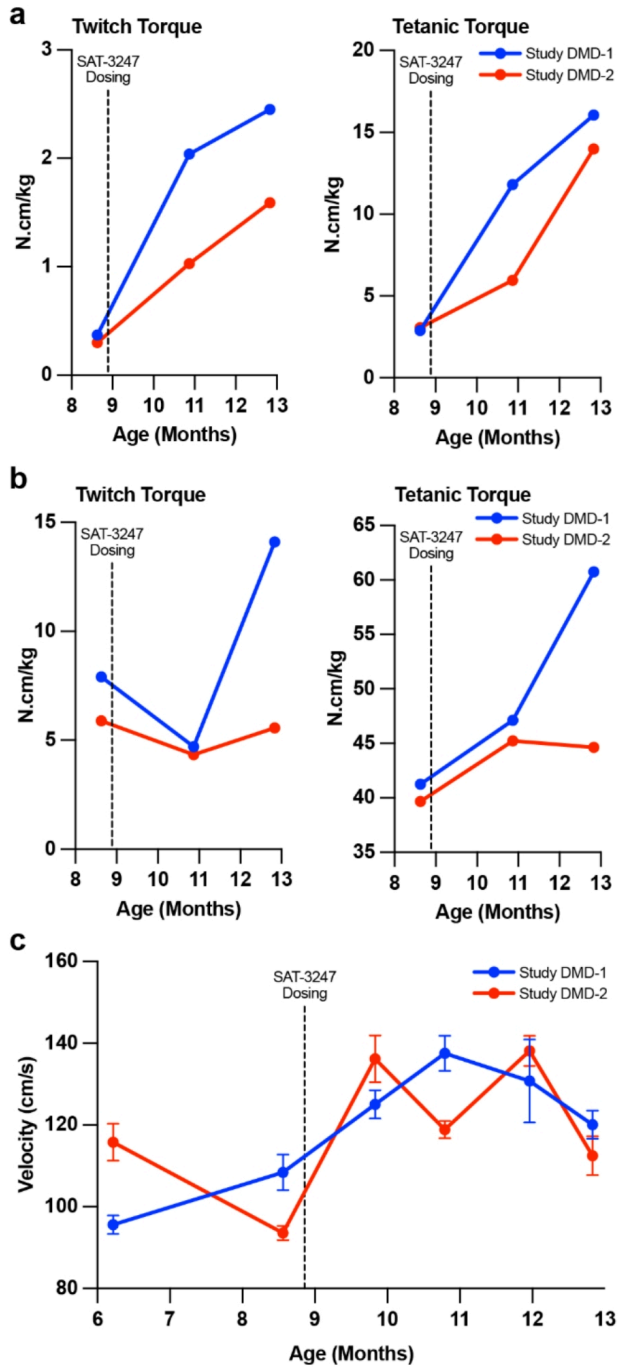


Figure 5

Whole-limb functional assessment: (a) Hindlimb flexion contractile properties increased in *Study DMD-1* (blue) and *Study DMD-2* (red) after dosing with SAT-3247 indicating an increase in muscle function after SAT-3247 treatment. (b) **Hindlimb extension** contractile properties increased in *Study DMD-1*(blue) and *Study DMD-2* (red) after dosing with SAT-3247 indicating an increase in muscle function after SAT-3247 treatment.

(c) Velocity (cm/s) of *Study DMD-1* (blue) and *Study DMD-2* (red) was above 100 cm/s at the study end, indicating SAT-3247 may be increasing their velocity and slowing down disease progression.

Supplementary Files

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