

INTRODUCTION

- Muscle stem cell dysfunction has become increasingly recognized as playing a key role in progressive muscular degeneration that occurs in Duchenne (DMD) and other muscular dystrophies¹⁻³.
 - Detection of embryonic myosin heavy chains (eMHC) has long been used to identify regeneration of newly forming myofibers, however evaluation of regeneration alone does not provide a complete representation as it comes in response to myonecrosis
 - Myonecrosis has been assessed by immunodetection of immunoglobulin G (IgG) infiltration. However, variability within and between muscles limits the utility of any determination of the rate of regeneration.
 - Given the close relationship between muscle regeneration and myonecrosis, accurately quantitating net effects, particularly at a fiber level, is a significant challenge.

- Here, we describe development and validation of an integrated histology-based regeneration index (RI) to assess and quantitate muscle regenerative capacity over time and to monitor potential therapeutic response to SAT-3247 in clinical trials.
- Forzapadin (SAT-3247) is an orally-administered small molecule designed to inhibit AAK1 (adaptor-associated kinase 1) re-establish polarity of muscle stem cells, muscle progenitor formation, and enhance muscle fiber regeneration (**Figure 1**). Forzapadin is currently under evaluation in two clinical trials in older boys and young adults (NCT06867107) and ambulatory children (NCT07287189) living with DMD and is being explored in other neuromuscular disorders (WMS Poster #3.31eP)

METHODS

- The research described here represents a collaborative effort between Satellos Bioscience, the Ottawa Hospital Research Institute, and the University of Iowa Department of Pathology.
- Muscle biopsy samples were obtained from Duchenne canines, boys with confirmed DMD ages 7-11 years, and age-matched controls. In preclinical experiments, DMD canines between 9-13 months of age were dosed with forzapadin for 4 months.

Determination of Regenerative Index (RI)

- Detailed methodology for immunofluorescence protocols, morphometric analyses, and determination of RI in DMD and non-DMD biopsy samples are described elsewhere* (QR code for access provided below). Regeneration of myofibers was determined via normalization to myonecrosis to reduce variability and assess net capacity of muscle:

Regenerative Index = newly forming eMHC⁺ fibers ÷ necrotic IgG⁺ fibers



RESULTS

Preclinical Assessments of Regenerative Capacity, Force, Function, and Forzapadin Response in DMD Canine Muscle

- Newly-forming eMHC⁺ and necrotic IgG⁺ fibers were identified by immunofluorescent staining of biceps femoris and diaphragm muscle biopsies from DMD and forzapadin-treated DMD canines. Following 4 months of forzapadin (SAT-3247) administration, RI was markedly increased in hindlimb and diaphragm muscle biopsy samples compared to DMD control (**Figure 2**).
- Along with increased RI, hindlimb contractile force and treadmill walking ability were both improved following forzapadin administration (**Figure 3**).

Assessment of Regenerative Capacity in Muscle from DMD Boys

- Consistent with results obtained in DMD canine muscle, newly-forming eMHC⁺ and necrotic IgG⁺ fibers, and DNA were successfully identified by immunofluorescent staining of muscle biopsy sections obtained from DMD boys (**Figure 4**).
- The percent of eMHC⁺ fibers decreased, whereas the percent IgG⁺ fibers was oppositely increased in DMD boys between the ages of 7-11 years (**Figure 4**).
- Calculation of RI which normalizes eMHC⁺ to IgG⁺ fibers resulted in a strong, significant negative correlation with age ($r = -0.888$) despite stable myofiber morphology and muscle stem cell pool (**Figure 5**).
- Direct comparison revealed that RI in older (9-11 years) DMD boys was markedly and significantly lower than in younger (7-8 years) boys ($p < 0.001$).

Figure 2: Preclinical immunofluorescence and RI

- Representative immunofluorescence stains of laminin (green), necrotic fibers (immunoglobulin G, red), and newly-forming fibers (embryonic myosin heavy chains, blue) in biceps femoris (top row) and diaphragm (bottom row) muscle cross-sections from a healthy dog, DMD control dog, and a forzapadin (SAT-3247)-treated DMD dog (left image panels).
- Following 4 months of forzapadin administration, regenerative index (RI) in hindlimb muscle and diaphragm was markedly higher than DMD controls (right figures).

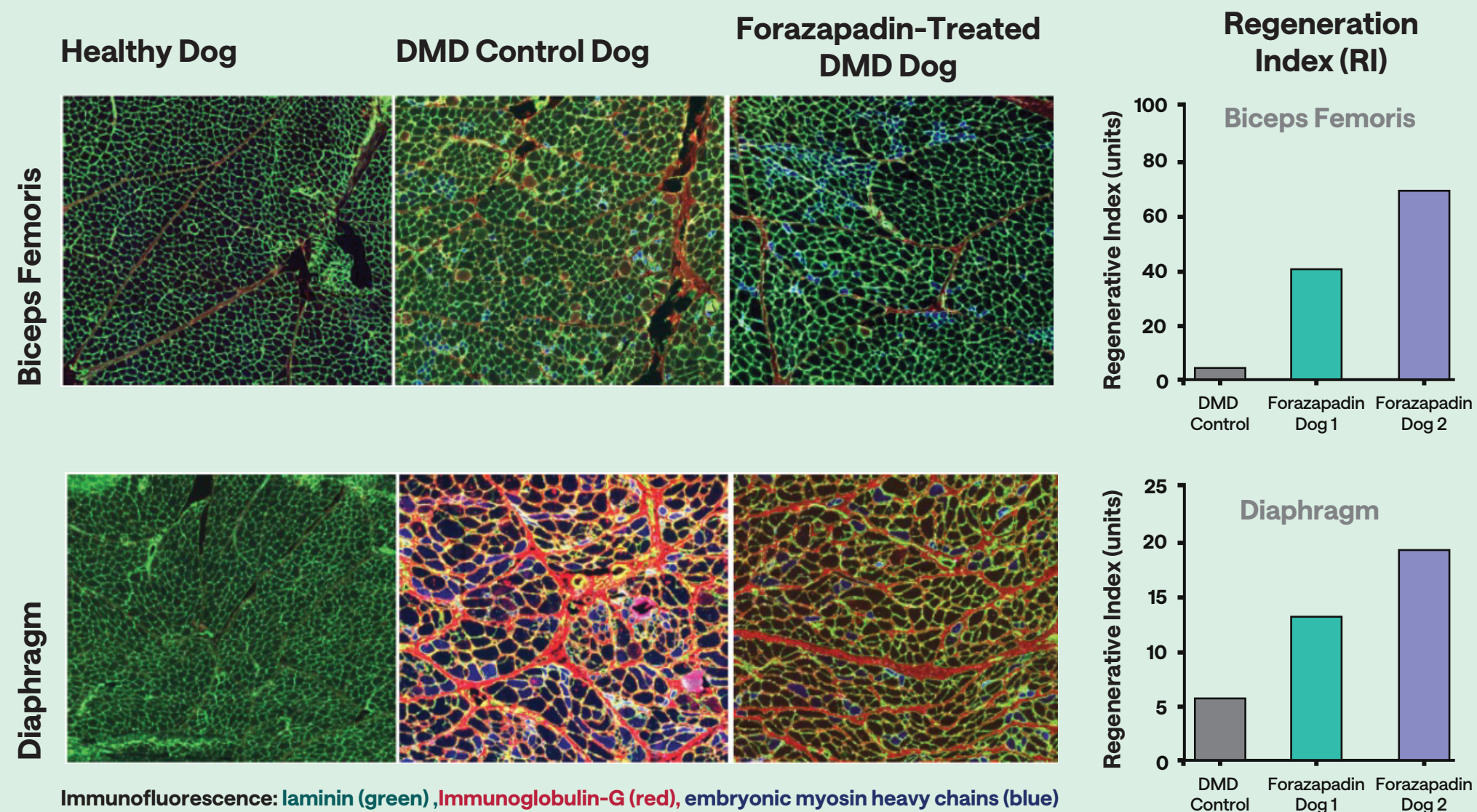


Figure 3: Preclinical muscle strength and gait

- Concomitant with increased muscle regenerative capacity, contractile force in isolated hindlimb muscles (biceps femoris shown) precipitously increased during 4 months of forzapadin (SAT-3247) administration in DMD canines, with forces reaching levels that were consistent with historical healthy control canines. Untreated DMD canines are also provided for context (left panel).
- Forzapadin-treated DMD canines demonstrated improved stride velocity during treadmill walking (right panel). Of note, stride velocity is being evaluated in the BASECAMP Phase 2 study of forzapadin in boys with DMD.

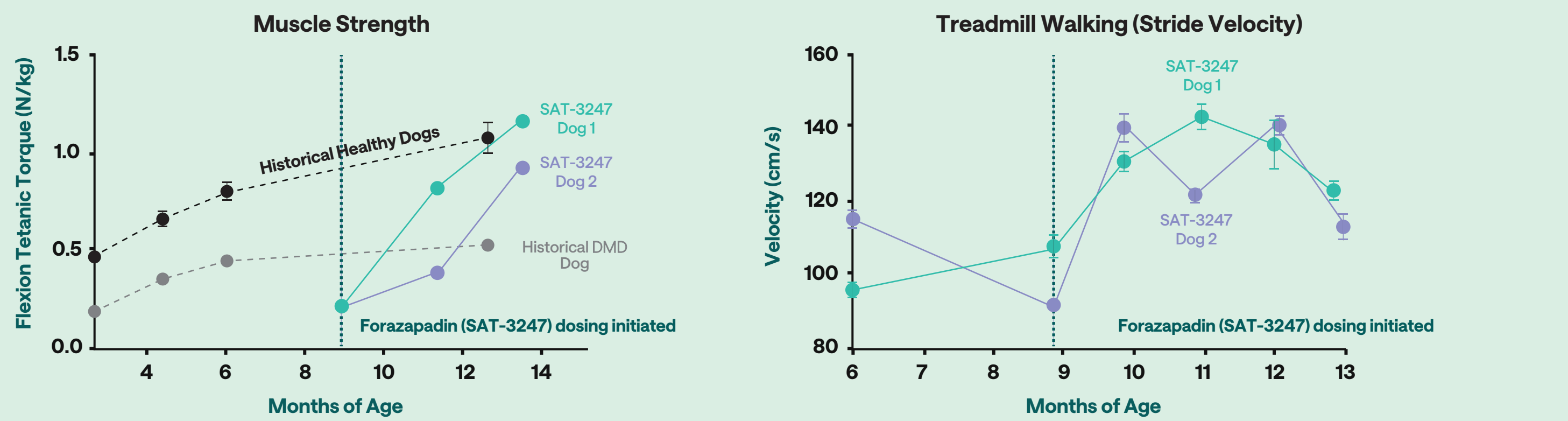


Figure 4: Immunofluorescence and age-related changes in DMD boys

- Representative immunofluorescence images of biopsies obtained from DMD boys aged 7-11 years, stained for immunoglobulin G (IgG) and embryonic myosin heavy chains (eMHC), laminin, DNA by DAPI, and automated outlining of individual fibers in yellow (top panels). Presence or absence of either eMHC or IgG is denoted by white arrows.
- Correlations between either percent of eMHC⁺ (bottom left) or IgG⁺ fibers (bottom right), along with direct comparisons of each parameter in younger (7-8 years) and older (9-11 years). Overall, muscle biopsies from older boys with DMD demonstrated numerically lower newly-forming (eMHC⁺) fibers and numerically higher numbers of necrotic (IgG⁺) fibers. Between 689-6771 fibers were assessed per muscle biopsy sample.

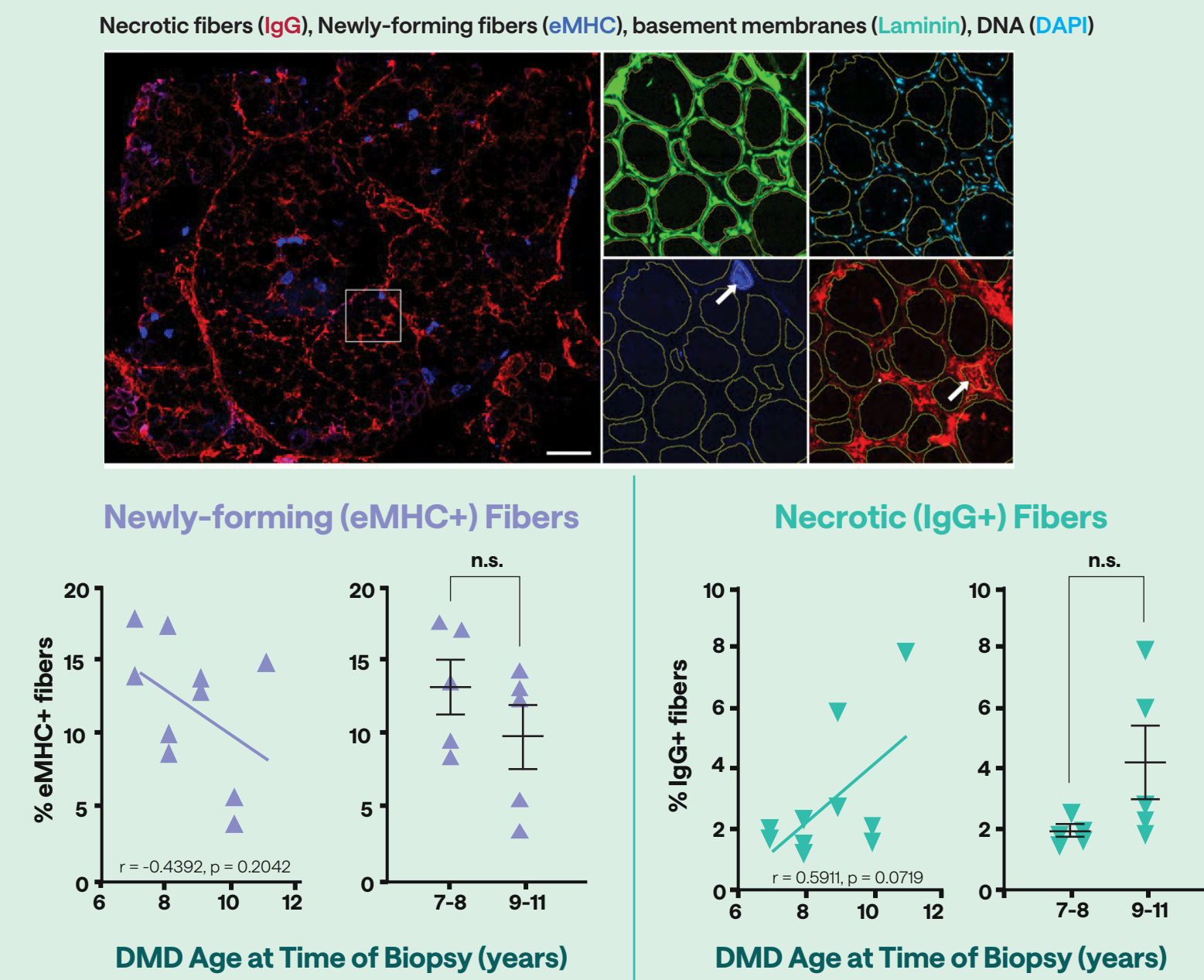
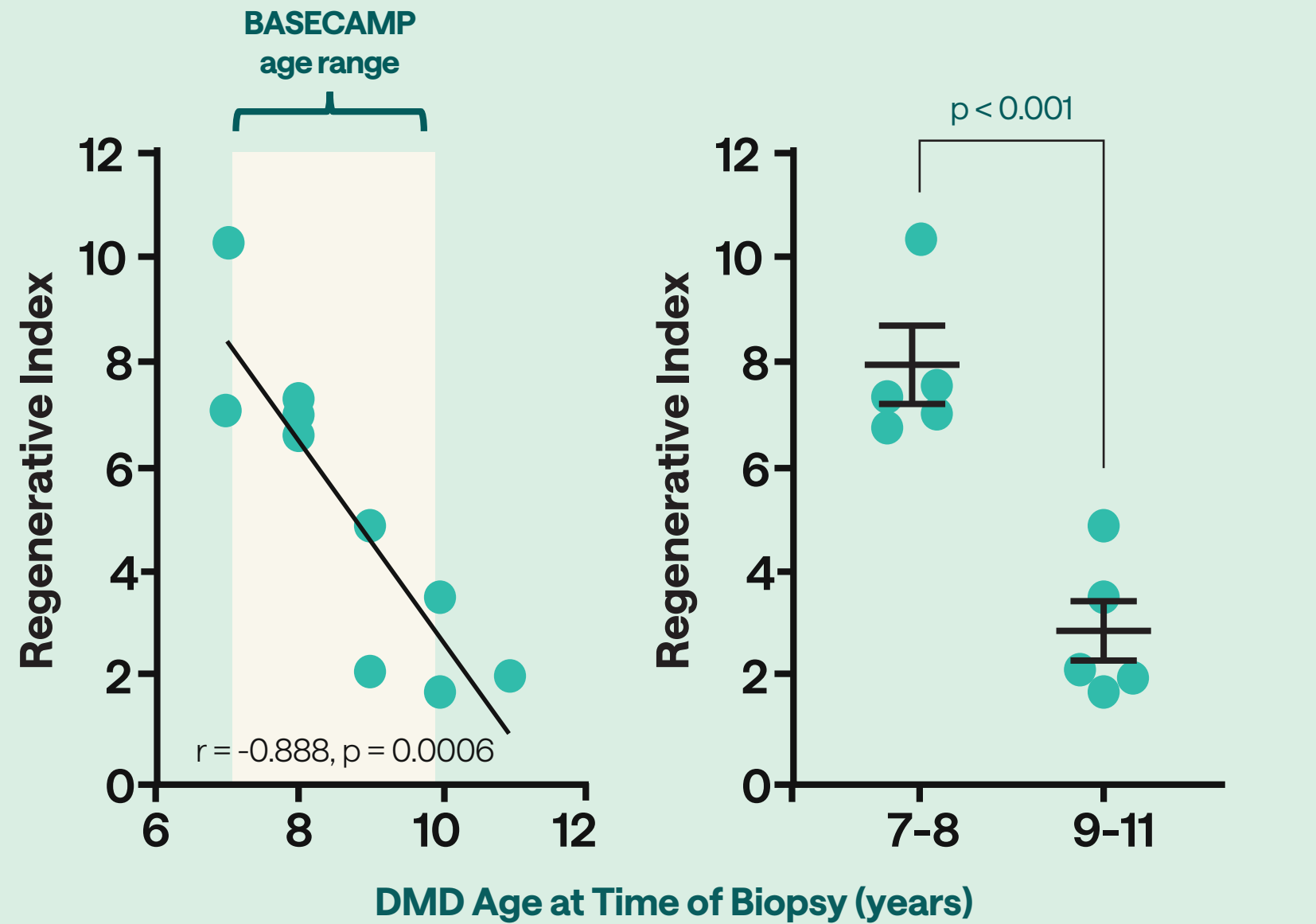


Figure 5: Age-related change in net muscle regenerative capacity

- Muscle net regenerative capacity (RI), directly assessed in muscle biopsies from boys with DMD was sharply decreased with increasing age over the range of 7-11 years, evidenced by a negative correlation between RI (ratio of newly-forming eMHC⁺ to necrotic IgG⁺ myofibers) and increasing age was observed (left). The boys in this analysis represent a similar age range to those being evaluated in the BASECAMP Phase 2 study of forzapadin in DMD (WMS Poster 2.74P).
- Direct comparison of RI in muscle biopsies obtained from DMD boys revealed a significantly lower regenerative capacity in older boys (9-11 years) compared to younger boys (7-8 years).
- Normalization of eMHC⁺ to IgG⁺ fibers during calculation of RI reduced variability obtained by assessment of eMHC⁺ alone, resulting in a stronger correlation coefficient and greater ability to discriminate between age groups.



CONCLUSIONS & NEXT STEPS

- **Preclinical determination of regenerative capacity:** immunofluorescent detection of newly-forming (eMHC⁺) and necrotic (IgG⁺) fibers in biopsy samples from DMD canines, and subsequent calculation of RI, enabled quantitation of net regenerative capacity in hindlimb (biceps femoris) and diaphragm muscles and, determination that regenerative capacity in muscle from forzapadin (SAT-3247)-treated DMD canines was markedly increased compared to untreated controls.
 - Improved muscle strength and respiratory function have been observed in older boys with advanced DMD after 28 days of forzapadin dosing^{5,6}
- **Determination of regenerative capacity in human muscle:** a sharp decline in muscle regenerative capacity was determined in muscle biopsy samples obtained from DMD boys 7-11 years, an age range associated with significant clinical decline.

- **Collectively, these results demonstrate that the RI methodology:**
 1. Enables direct quantitation of net muscle regenerative capacity
 2. Is suitable to discriminate between healthy and DMD muscle
 3. Is translatable from preclinical models to humans
 4. Correlates with DMD progression over time, and
 5. Is a potential endpoint to determine response to therapies that are designed to enhance muscle regeneration.
- Future longitudinal studies that correlate RI with functional outcomes in DMD, examining RI in other muscle diseases and age groups, as well as integrating transcriptomic and metabolic profiling will be important to further validate and refine the use of RI in research and clinical settings.
- As such, the RI approach is currently being employed to assess net muscle regenerative capacity in the Phase 2 BASECAMP study of forzapadin, which is enrolling boys similar in age to those evaluated in this research (WMS poster 2.74P).

REFERENCES

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