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Development of a next-generation multiplex ddPCR assay for measurement of in-frame dystrophin mRNA in subjects with Duchenne muscular dystrophy treated with BMN 351

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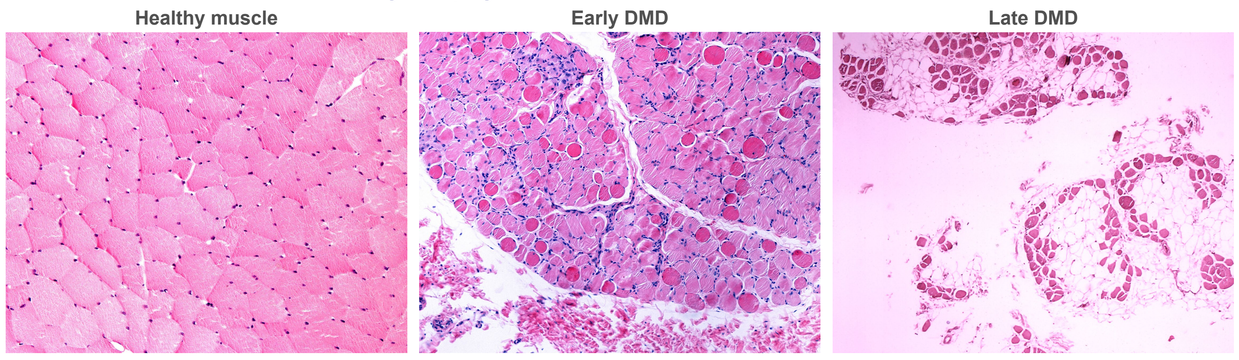
Background

- Duchenne muscular dystrophy is a severe neuromuscular disorder characterized by progressive muscle weakness and is frequently caused by frameshift mutations that produce a truncated, non-functional dystrophin protein (**Figure 1**)[1]

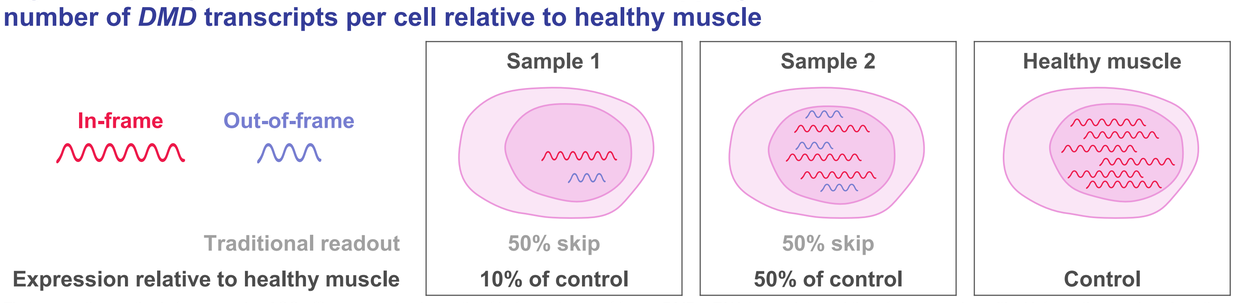
Figure 1. Muscle biopsy reveals infiltrating lymphocytes and replacement of myofibers with fatty tissue in Duchenne muscular dystrophy



A cross section of a skeletal muscle biopsy sample from a healthy individual highlights the centrally nucleated myofibers. Muscle histopathology from an individual with Duchenne muscular dystrophy in the early stages reveals the presence of infiltrating lymphocytes around myofibers, and at later stages of disease, the replacement of myofibers with fatty tissue. Images are from Shutterstock.
DMD, Duchenne muscular dystrophy.

- BMN 351 is an antisense oligonucleotide (ASO) intended for the treatment of Duchenne muscular dystrophy that alters the splicing of *DMD* pre-mRNA to exclude exon 51, thereby producing an in-frame *DMD* mRNA and near full-length dystrophin protein with an internal deletion that is anticipated to provide functional benefit
  - BMN 351 is currently being evaluated in an ongoing phase 1/2 dose-escalation clinical trial for individuals with Duchenne muscular dystrophy and exon 51 skip-amendable deletions (NCT06280209) that includes measurement of exon skipping
- Measurement of *DMD* mRNA can provide an early pharmacodynamic readout after ASO treatment, but first-generation methods may poorly predict dystrophin protein production and ASO efficacy (**Figure 2**)

Figure 2. First-generation methods to monitor exon skipping do not report differences in the total number of *DMD* transcripts per cell relative to healthy muscle



First-generation methods for assessing ASO efficacy report only a ratio of in-frame to out-of-frame *DMD* mRNA transcripts and therefore may miss critical information, such as how the quantity of in-frame *DMD* mRNA transcripts compare to levels in healthy muscle.
ASO, antisense oligonucleotide; *DMD*, mRNA encoding dystrophin protein.

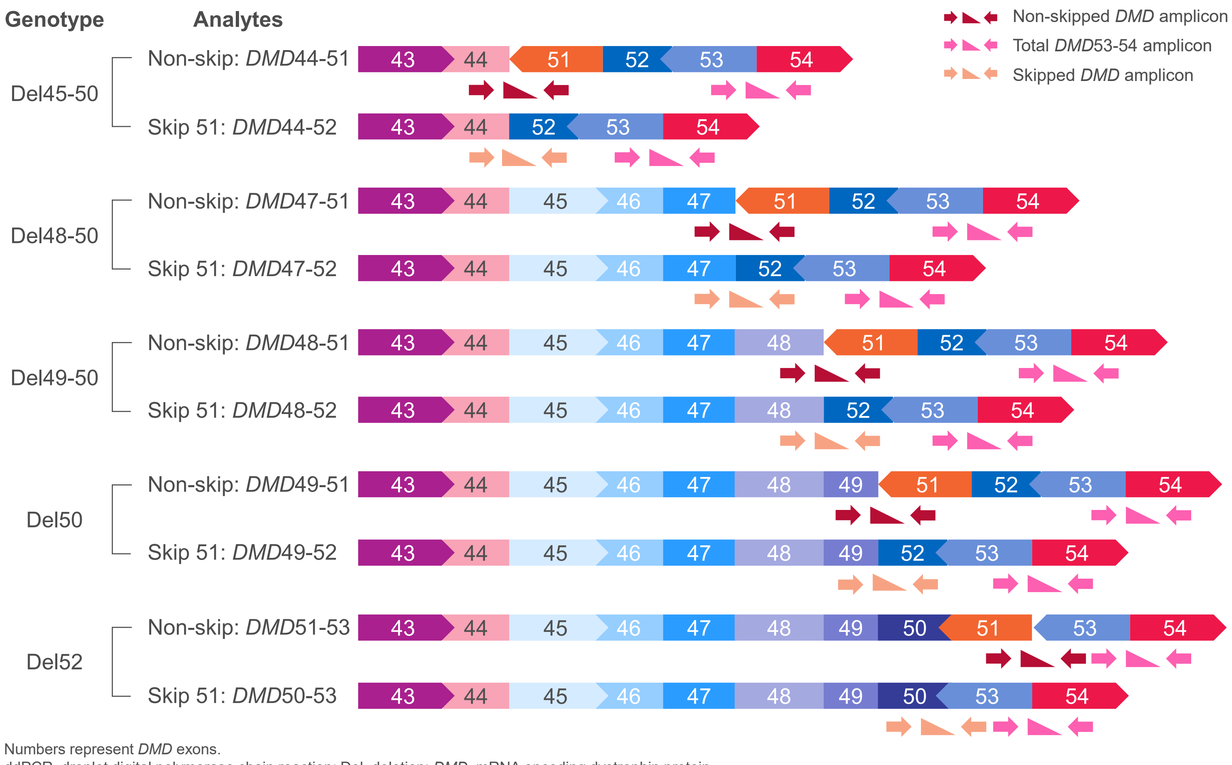
- Here, we describe a next-generation multiplexed method to measure exon skipping that is anticipated to correlate better with near full-length dystrophin protein expression induced by ASO treatment
  - This method is designed to measure both the ratio of in-frame to out-of-frame *DMD* mRNA transcripts and quantify the in-frame *DMD* mRNA transcripts relative to healthy muscle

Methods

Multiplexed ddPCR assay design

- mRNA was isolated from 17 healthy human skeletal muscle samples obtained commercially
- mRNA quality was assessed by RNA integrity number equivalent values (RINe) measured on the Tapestation instrument from Agilent Technologies
- A QX ONE droplet digital PCR (ddPCR) instrument from BioRad was used for the multiplexed PCR and primer sets were designed to measure *DMD* mRNA (in-frame, out-of-frame, and total) to enable calculation of % skip for the 5 most common genotypes amenable to BMN 351 treatment (**Figure 3**)

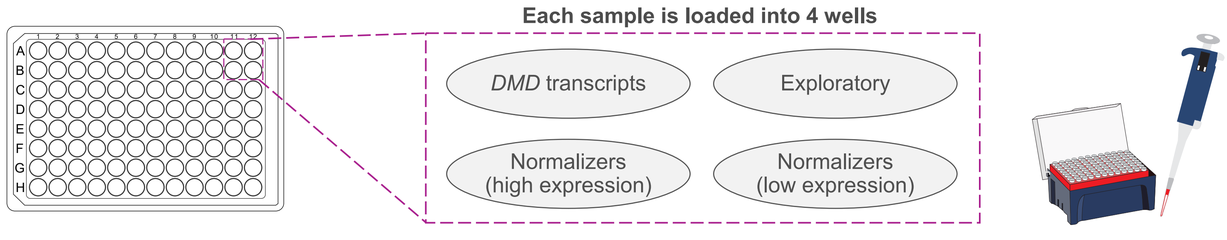
Figure 3. ddPCR primers targeting the 5 most common deletions amendable to BMN 351 treatment



Numbers represent *DMD* exons.
ddPCR, droplet digital polymerase chain reaction; Del, deletion; *DMD*, mRNA encoding dystrophin protein.

- ddPCR was selected because it offers a precise and multiplexed option for measurement of multiple mRNA transcripts per well (**Figure 4**)

Figure 4. Schematic of multiplexed ddPCR assay



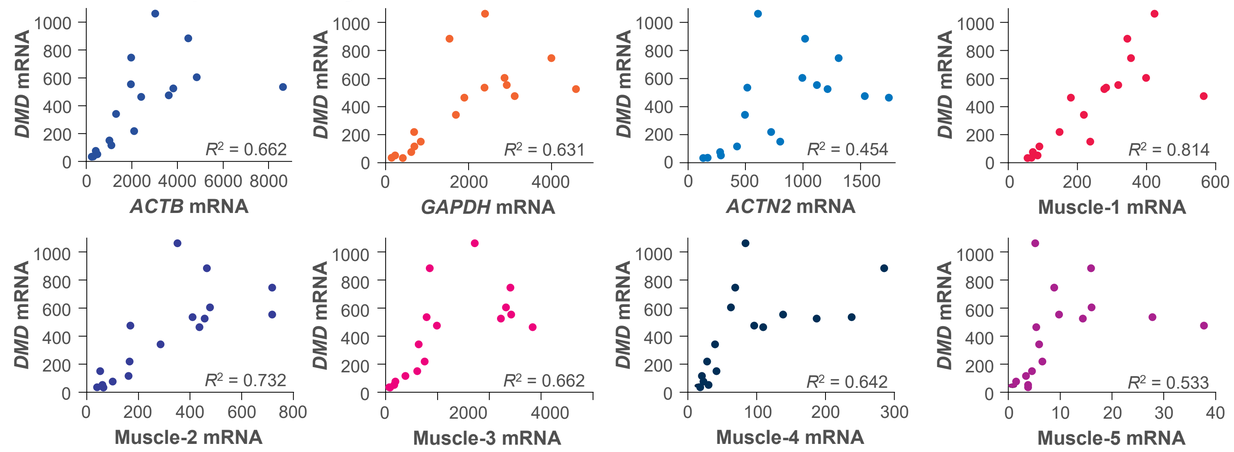
For each sample, material is loaded into 4 separate wells dedicated to measurement of multiple *DMD* mRNA transcripts, highly expressed normalizer genes, lowly expressed normalizer genes, and exploratory genes (including biomarkers of non-muscle cell content expected in muscle biopsies from individuals with Duchenne muscular dystrophy).
ddPCR, droplet digital polymerase chain reaction; *DMD*, mRNA encoding dystrophin protein.

Results

Selection of candidate normalizer genes that correlate with *DMD* mRNA transcripts

- From public gene and protein-expression databases, we identified ubiquitously expressed and muscle-specific transcripts that correlated with *DMD* mRNA transcripts
- The muscle-specific transcript *ACTN2* demonstrated the lowest correlation with *DMD* mRNA (**Figure 5**)
- Ubiquitously expressed transcripts *ACTB* and *GAPDH* correlated with *DMD* mRNA in healthy muscle
- Muscle-specific normalizers may better account for varying muscle cell content in Duchenne samples

Figure 5. Correlation of ubiquitously expressed and muscle-specific normalizer genes with *DMD* mRNA transcripts in healthy muscle

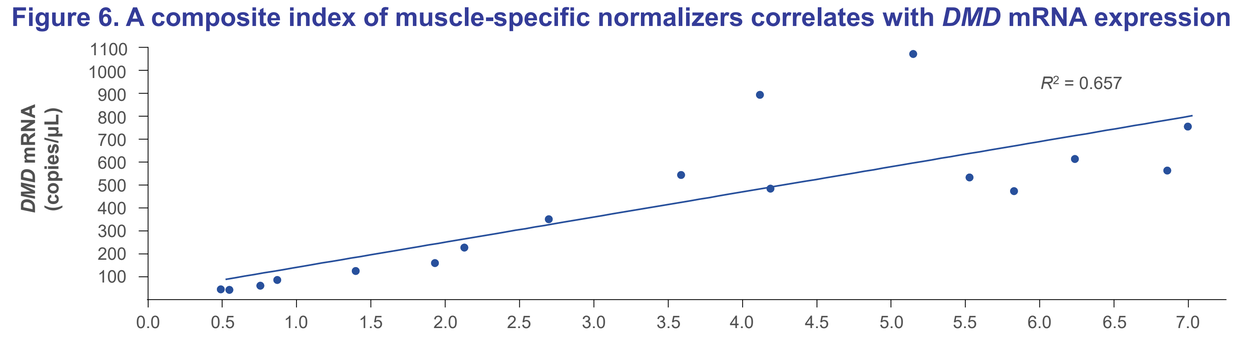


The Spearman rank correlation coefficient was calculated for each candidate normalizer gene. Axes show copies per microliters measured for each transcript.
ACTB, beta-actin; *ACTN2*, alpha-actinin; *DMD*, mRNA encoding dystrophin protein; *GAPDH*, glyceraldehyde 3-phosphate dehydrogenase.

A composite index of muscle-specific mRNA transcripts correlates with *DMD* mRNA expression in healthy muscle samples

- Total *DMD* mRNA transcripts across 17 healthy muscle samples correlated with a composite index of 3 candidate muscle-specific normalizer genes (**Figure 6**)

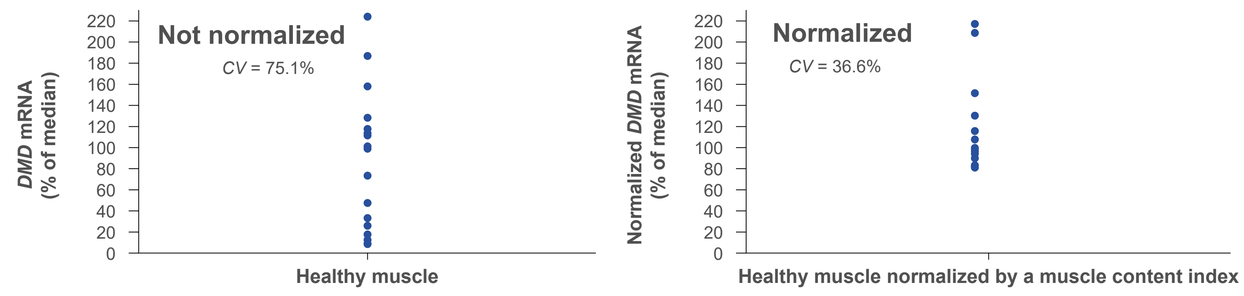
Figure 6. A composite index of muscle-specific normalizers correlates with *DMD* mRNA expression



To give equal weight to muscle-specific normalizers with high and low expression, the number of copies per microliter of each gene in each sample was divided by the median expression for that gene across the 17 healthy muscle samples analyzed. The formula for the muscle-specific mRNA normalizer index for each sample was as follows: Muscle Content Index = (gene 1/median of gene 1) + (gene 2/median of gene 2) + (gene 3/median of gene 3). The simple linear regression and R^2 value are presented.
DMD, mRNA encoding dystrophin protein.

- Without normalization, the level of *DMD* mRNA transcripts measured across samples with equal RNA input was highly variable, but after normalization with a composite index, most samples fell within 80% to 160% of the median level with 2 high outliers (**Figure 7**)

Figure 7. Muscle-specific normalizer genes provide a median and range of *DMD* mRNA transcripts in healthy muscle

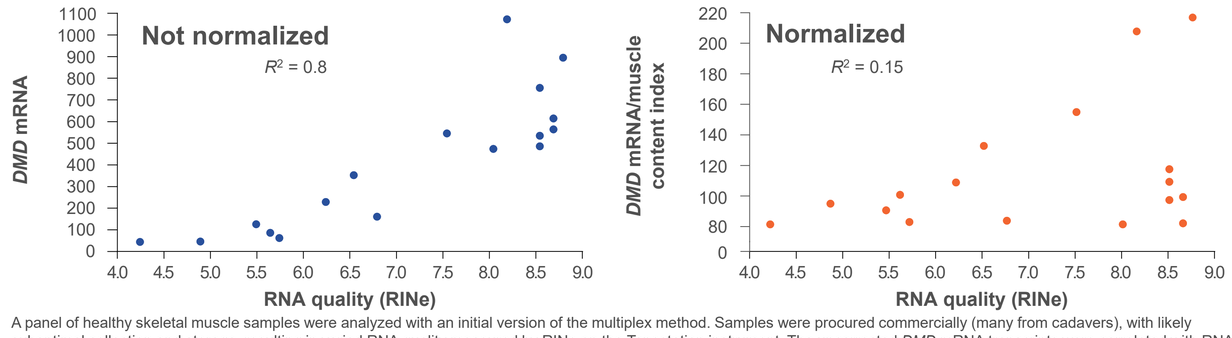


The measurement of muscle-specific normalizer genes in a broad panel of healthy muscle samples may enable determination of a median and range for *DMD* mRNA transcripts in healthy muscle. This can then serve as a reference for in-frame *DMD* mRNA transcripts induced by BMN 351 treatment in Duchenne muscular dystrophy.
CV, coefficient of variation; *DMD*, mRNA encoding dystrophin protein.

Normalization compensates for differences in RNA quality

- Total *DMD* mRNA transcripts correlated with RNA quality, but *DMD* mRNA transcripts normalized by the muscle content index were not significantly correlated with RNA quality (**Figure 8**)

Figure 8. After normalization with a muscle content index, the impact of RNA quality on *DMD* mRNA quantitation is reduced



A panel of healthy skeletal muscle samples were analyzed with an initial version of the multiplex method. Samples were procured commercially (many from cadavers), with likely suboptimal collection and storage, resulting in varied RNA quality measured by RINe on the Tapestation instrument. The uncorrected *DMD* mRNA transcripts were correlated with RNA quality as assessed by RINe (Spearman rank correlation was $R^2 = 0.8$, $P = 1.35 \times 10^{-6}$), but the *DMD* mRNA transcripts were not correlated when normalized by the muscle content index (Spearman rank correlation was $R^2 = 0.15$, $P = 0.131$).
DMD, mRNA encoding dystrophin protein; RINe, RNA integrity number equivalent.

Conclusions

- This multiplexed ddPCR method enables measurement of in-frame *DMD* mRNA expression relative to healthy muscle in addition to exon skip efficiency (% skip)
- In-frame *DMD* mRNA expression reported as a percentage of healthy control may better predict efficacy compared with % skip
- Muscle-specific normalization has the potential to reduce variability across healthy tissues and enable measurement of in-frame *DMD* mRNA adjusted for muscle content in dystrophic biopsies

References

1. Duan D, et al. *Nat Rev Dis Primers.* 2021;7(1):13.

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Disclosures

All authors are current or former employees and shareholders of BioMarin Pharmaceutical Inc.

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